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13. ABSTRACT *(Maximum 200 words)*

It has been recognized for many years that cancer invasion and metastasis are promoted by a cascade of extra-cellular proteinases which is organized on the external cell surface. This cascade is initiated by urokinase-type plasminogen activator (uPA) which binds to its cell surface receptor, uPAR, and then activates cell-associated plasminogen. The resulting plasmin may digest extracellular matrix proteins or promote the activation of other proteinases. The major goal of this research program was to characterize the role of cytokeratin 8 (CK8) as a newly-discovered cell-surface binding site for plasmin(ogen). Our results documented for the first time that CK8, an intracellular cytoskeletal protein, penetrates to the external cell surface in breast cancer cells. We characterized the biochemistry of CK-proteinase interactions, determined that CK8 is the principal plasminogen binding protein in certain breast cancer cell lines, and completed a molecular analysis of the binding sites for plasminogen and tissue-type plasminogen activator in CK. Importantly, CK8-associated plasminogen was not activated by uPAR-associated uPA. This led us to reassess the role of uPAR in breast cancer cell physiology. In new studies, we demonstrated a novel linkage between uPAR and breast cancer cell physiology, in which uPA triggers a signal transduction cascade which activates H-Ras, MAP kinase, and myosin light chain kinase to promote motility. Our studies contribute to a model in which proteinases regulate breast cancer cell physiology by multiple mechanisms.

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Steven L. Gonias
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Introduction :

Cancer invasion and metastasis are promoted by proteinases which are organized on the cell surface by a series of cellular receptors. These proteinases form a cascade which culminates in the digestion of basement membrane and extracellular matrix proteins, allowing tumor cells to penetrate through tissue (1, 2). At the top of the tumor invasion cascade is urokinase-type plasminogen activator (uPA), a plasminogen activator which binds to a specific cell-surface receptor called uPAR (2,3). Studies of human breast cancers have shown that tumors which contain high levels of extractable uPA and/or uPAR are typically aggressive and tend to metastasize (4,5).

When uPA is bound to uPAR, the proteinase effectively activates cell-associated plasminogen to generate plasmin. Plasmin, in turn, digests glycoprotein components of the extracellular matrix and may activate other proteinases (6,7). A second plasminogen activator, tissue-type plasminogen activator (tPA), also binds to tumor cell surfaces and may substitute for uPA in certain circumstances (8).

Migrating cells show the capacity to polarize the tumor invasion proteinase cascade to the leading edge of cellular migration (1). Thus, the cell may selectively digest structural proteins in the direction of migration. When this research program was initiated, the nature of the cellular binding sites for plasminogen in breast cancer cells was unknown. Plasminogen binding sites had been described in other cell types, but in most cases, subsequent studies demonstrated that these binding sites are not responsible for substantial fraction of the plasminogen binding capacity (9-12). Nevertheless, the importance of plasminogen binding to the cell surface, in cancer metastasis, had been supported (13).

The major goal of this research program was to identify and characterize plasminogen binding sites in breast cancer cells. We considered this binding site to be an exciting and unexplored target for potential disruption of the tumor invasion proteinase cascade. In the section to follow, I will describe our results implicating cytokeratin 8 as a significant binding site for plasminogen and breast cancer cells. I will then describe our experimental results that led us to partially shift our emphasis, upstream in the tumor invasion proteinase cascade, to the cell surface receptor for urokinase. This slight change in orientation has been extremely successful, resulting in one paper published in *J. Biol. Chem.* and a second which is currently submitted for publication to *J. Cell Biol.*

Body:

Our original grant application and the first three years of our research focused on an intracellular cytoskeleton protein, cytokeratin 8 (CK8). Our studies demonstrated for the first time that CK8 penetrates through the plasma membrane to the external surfaces of breast cancer cells, where it functions as a receptor for plasminogen. Using an antibody which was developed in our laboratory, we demonstrated that the plasminogen binding site is localized to the C terminus of CK8. We also demonstrated that CK8 is responsible for the majority of the binding of plasminogen to the

surfaces of various breast cancer cell lines. In MCF-7 cells, CK8 accounted for 60-80 % of the total plasminogen-binding capacity. Our antibody, 1E8, which specifically targeted the plasminogen-binding site in CK8, represented a possible reagent for disrupting the tumor invasion proteinase cascade. These studies were published in papers that were described in full in previous grant reports (14, 15).

Our first accomplishment in Grant Year #4 was to complete a study which was initiated in the prior year. That study is now in press in *J. Protein Chem.* The goal of that study was to elucidate the molecular nature of the complex involving cytokeratins which resulted in an enhanced rate of plasminogen activation by tPA. We chose to prepare genetic constructs since purified cytokeratins are highly insoluble and thus subject to only limited biochemical analysis. Our first construct encoded a GST-fusion protein containing 174 amino acids from the C terminus of CK8. The second construct contained 134 amino acids from the C terminus of wild type CK18. We also prepared a third construct which was identical to the first, except that the C-terminal lysine in CK8 was mutated to glutamine. As expected, this mutation substantially reduced plasminogen binding to the CK8 fusion protein; however, some residual binding still remained. Using these genetic constructs, we demonstrated that while plasminogen binds only to CK8, tPA may bind with equal affinity to CK8 or CK18. Furthermore, we demonstrated that plasminogen can completely inhibit tPA binding to CK8 but has no effect on tPA binding to CK18. Thus, a single CK8 monomer cannot independently support the formation of an enzyme (tPA)-substrate (plasminogen) complex. Instead, the true co-factor was a cytokeratin dimer in which at least one monomer was CK8. The other monomer may be CK8 or CK18.

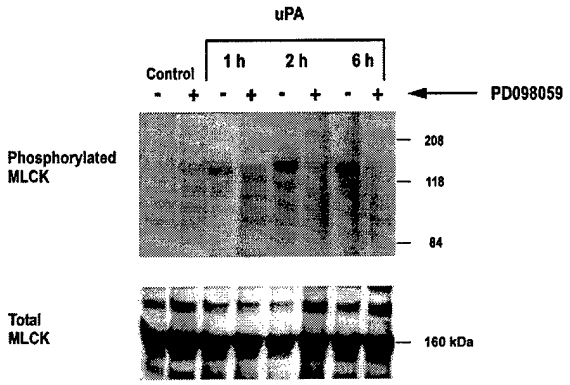
Since uPA and uPAR have been strongly implicated in the promotion of breast cancer, we undertook studies to determine whether CK8-associated plasminogen serves as a substrate for uPAR-associated uPA. As reported in our last progress report, our studies with antibody 1E8, which inhibits binding of plasminogen to CK8, led us to conclude that CK8-associated plasminogen is not a substrate for uPAR-associated uPA. These results led us to consider whether uPAR might function in other capacities to promote breast cancer invasion or metastasis. Recent studies have demonstrated that in addition to its ability to localize uPA at the cell surface, uPAR expresses diverse activities including: direct binding to vitronectin; association with the extracellular domains of integrins; and the ability to initiate signal transduction responses (16). Ossowski and colleagues (17) demonstrated that cytokeratins may become phosphorylated when uPA binds to uPAR. Thus, we undertook studies to determine whether uPAR-initiated signaling may influence breast cancer cell physiology.

Our first study successfully demonstrated that uPA promotes the migration of MCF-7 cells and that this response is entirely dependent on the activation of the MAP kinases, extracellular signal-regulated kinase 1 and 2. I will briefly describe the results of these studies; however, the entire study has been published and a reprint is included with this report. The paper which appeared in *J. Biol. Chem.* acknowledges this grant for support.

In our study, we demonstrated that ERK1 and ERK2 are phosphorylated within one minute of adding uPA to cultures of MCF-7 cells. The amino terminal fragment (ATF) of uPA which binds to uPAR but lacks proteinase activity, also activated ERK1 and ERK2. The responses to uPA and

ATF were eliminated when the cells were treated with the specific MEK inhibitor, PD098059.

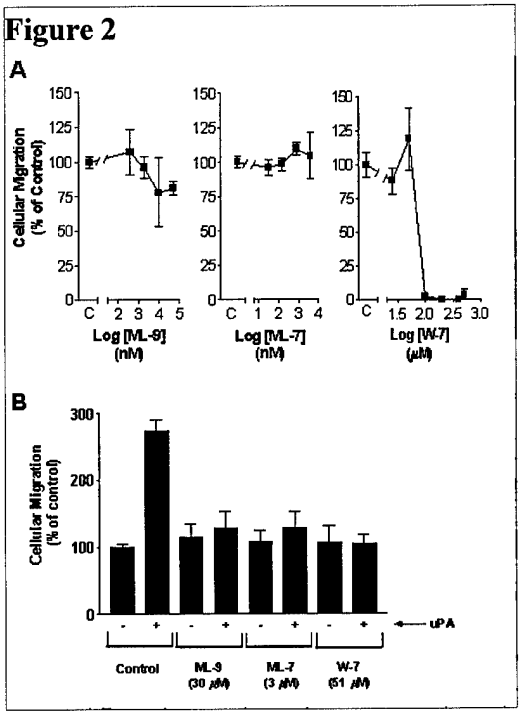
Figure 1



We subsequently demonstrated that uPA promotes the migration of MCF-7 cells across Transwell membranes coated with serum. Migration was increased 7.7 ± 1-fold when uPA was added to both the bottom and top chambers at a concentration of 10 nM. Importantly, when PD098059 was added to the culture chambers, in order prevent the activation of ERK1 and ERK2, the migration-promoting activity of uPA was completely neutralized. These studies provided the first evidence that signal transduction through uPAR is entirely responsible for the ability of uPA to promote motility in a specific experimental system.

In new studies that have been submitted for publication, we have confirmed that activation of ERK1 and ERK2 are essential for uPA promoted cellular migration by transfecting MCF-7 cells with dominant-negative H-Ras or MEK1 mutants. These signal transduction proteins function upstream of ERK1 and ERK2. When the dominant negative mutants were expressed in MCF-7 cells, the baseline motility of the cells was not affected; however, the response to uPA was entirely eliminated. We have also demonstrated for the first time that uPA treatment of MCF-7 cells results in the phosphorylation of myosin light chain kinase (MLCK). MLCK was immunoprecipitated from MCF-7 cells that were metabolically labeled with [^{32}P]orthophosphate and then treated with uPA. Figure 1 shows that MLCK was phosphorylated within one hour of exposure to uPA; however, unlike ERK1/2, MLCK phosphorylation was sustained for at least six hours. When the cells were pre-treated with the MEK inhibitor, PD098059, prior to uPA exposure, MLCK phosphorylation was entirely inhibited. These studies demonstrate that, in the presence of uPA, MLCK is phosphorylated via a MEK-dependent pathway.

Figure 2



Since uPA increased the level of MLCK phosphorylation in MCF-7 cells, studies were undertaken to determine whether MLCK activity is

required for uPA-promoted cellular migration. Initially, we studied MCF-7 cell migration in the absence of uPA. Figure 2, Panel A, shows that the specific MLCK inhibitors, ML-7 and ML-9, have little or no effect on MCF-7 cell migration at concentrations up to 3 μM and 30 μM, respectively. W-7 was also inactive at concentrations of 50 μM; however, nearly complete inhibition of migration was observed with W-7 at concentrations ≥ 0.1 mM. The activity of W-7, at high concentrations, may reflect its less specific mechanism of action.

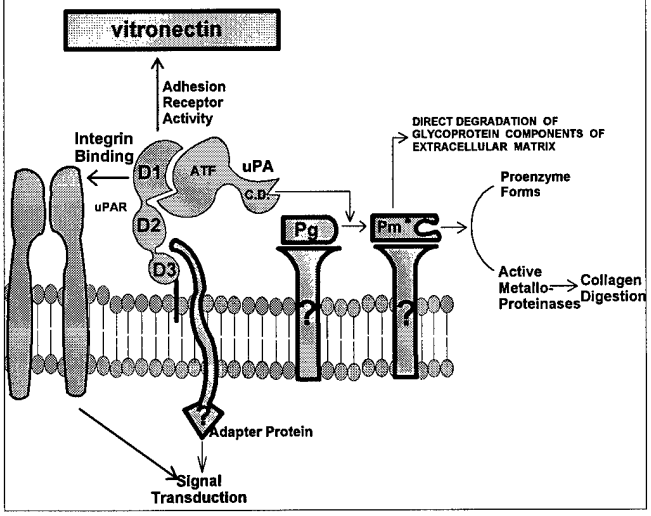
To determine whether MLCK is essential for uPA-promoted cellular migration, we pretreated MCF-7 cells with all three MLCK inhibitors, at concentrations that did not affect basal migration and were consistent with reported K_i values for MLCK (18). The cells were then treated with 10 nM uPA. As shown in Figure 2, Panel B, the MLCK inhibitors completely prevented the uPA-induced increase in MCF-7 cell migration thereby demonstrating a critical role for MLCK activity in uPA promoted MCF-7 cell migration.

Thus, we have identified a signal transduction cascade which is initiated when uPA binds to uPAR. Although others have published studies regarding uPAR initiated cell signaling, our studies are the first to link a specific signal transduction pathway to cellular motility. We think these studies may provide new insight into the potential mechanism whereby uPA and uPAR may influence breast cancer cell physiology.

Conclusions:

Based on our work, we have gained new insight into how proteinases may affect breast cancer cell physiology. Our studies identified cell-surface cytokeratin 8 as a major plasminogen receptor in certain breast cancer cells. However, the CK8-associated pool of cell-surface plasminogen was not activated at an increased rate by uPAR-associated uPA. Thus, binding of plasminogen to CK8 on the cell surface may not promote the generation of proteinase activity through a uPA-initiated mechanism.

Figure 3



In addition to its ability to affect cell surface proteinase activity, we have shown that uPAR, in a breast cancer cell line, can initiate a signal transduction cascade which increases the ability of the breast cancer cells to migrate. This signal transduction cascade is dependent on H-Ras, ERK1 and ERK2, and MLCK. These results suggest that uPA and uPAR may influence breast cancer physiology by mechanisms that are independent of uPA proteolytic activity. The various activities of uPA and uPAR are shown in figure form in Figure 3.

References:

1. Liotta, L.A., Steeg, P.S., and Stetler-Stevenson, W. (1991) *Cell* **64**, 327-336
2. Roldan, A.L., Chubellis, M.V., Masucci, M.T., Behrendt, N., Lund, L.R., Dano, K., Apella, E., and Blasi, F. (1990) *EMBO* **9**, 467-474
3. Behrendt, N., Ronne, E., Ploug, M., Petri, T.I., Lober, D., Nielsen, L.S., Schleuning, W.-D., Blasi, F., Apella, E., and Dano, K. (1990) *J. Biol. Chem.* **265**, 6453-6460
4. Duffy, M.J., Reilly, D., O'Sull9ivan, C., O'Higgens, N., Fennelly, J.J., and Andreasen, P. (1990) *Cancer Res.* **50**, 6827-6829
5. Bianchi, E., Cohen, R.L., Thor, A.T., Todd, R.L., Mizukami, I.F., Lawrence, D.A., Ljung, B.M., Shuman, M.A., and Smith, H.S. (1994) *Cancer Res.* **54**, 861-866
6. Liotta, L.A., Goldfarb, R.H., Brundage, R., Siegal, G.P., Teranova, V., and Garbrisa, S. (1981) *Cancer Res.* **41**, 4629-4635
7. Mignatti, P., Robbins, E., and Rifkin, D.B. (1986) *Cell* **47**, 487-498
8. Alizadeh, H., Ma, D., Berman, M., Bellingham, D., Comerford, S.A., Gething, M.J.H., Sambrook, J.F., and Niederkorn, J.Y. (1995) *Curr. Eye Res.* **14**, 449-458
9. Miles, L.A., Dahlberg, C.M., Plescia, J., Felez, J., Kato, K., and Plow, E.F. (1991) *Biochemistry* **30**, 1682-1691
10. Hajjar, K.A., Jacovina, A.T., and Chacko, J. (1994) *J. Biol. Chem.* **269**, 21191-21197
11. Cesarman, G.M., Guevera, C.A., and Hajjar, K.A. (1994) *J. Biol. Chem.* **269**,21298-21303
12. Redlizt, A., Fowler, B.J., Plow, E.F., and Miles, L.A. (1995) *Eur. J. Biochem.* **227**, 407-415
13. Kobayashi, H., Shinohara, H., Takeuchi, K.I., Itoh, M., Fujie, M., Saitoh, M., and Terao, T. (1994) *Cancer Res.* **54**, 844-849
14. Hembrough, T.A., Li, L., and Gonias, S.L. (1996) *J. Biol. Chem.* **271**, 25684-25691
15. Hembrough, T.A., Vasudevan, J., Allietta, M.M., Glass II, W.F., and Gonias, S.L. (1995) *J. Cell Sci.* **108**, 1071-1082
16. Andreasen, P.A., Kjøller, L., Christensen, L., and Duffy, M.J. (1997) *Int. J. Cancer* **72**, 1-22
17. Busso, N., Masur, S.K., Lazega, D., Waxman, S., and Ossowski, L. (1994) *J. Cell Biol.* **126**, 259-270

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Binding of Urokinase-type Plasminogen Activator to Its Receptor in MCF-7 Cells Activates Extracellular Signal-regulated Kinase 1 and 2 Which Is Required for Increased Cellular Motility*

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Binding of urokinase-type plasminogen activator (uPA) to its receptor, uPAR, regulates cellular adhesion, migration, and tumor cell invasion. Some of these activities may reflect the ability of uPAR to initiate signal transduction even though this receptor is linked to the plasma membrane only by a glycosylphosphatidylinositol anchor. In this study, we demonstrated that single-chain uPA activates extracellular signal-regulated kinase 1 (ERK1) and ERK2 in MCF-7 breast cancer cells. Phosphorylation of ERK1 and ERK2 was increased 1 min after adding uPA and returned to baseline levels by 5 min. The amino-terminal fragment (ATF) of uPA, which binds to uPAR but lacks proteinase activity, also activated ERK1 and ERK2. Responses to uPA and ATF were eliminated when the cells were pretreated with PD098059, an inhibitor of mitogen-activated protein kinase kinase. uPA and ATF promoted the migration of MCF-7 cells across serum-coated Transwell membranes *in vitro*. Migration was increased 2.1 ± 0.4-fold when uPA was added to the top chamber, 4.8 ± 0.8-fold when uPA was added to the bottom chamber, and 7.7 ± 1.0-fold when uPA was added to both chambers. MCF-7 cells that were pulse-exposed to uPA for 30 min, and then washed to remove unbound ligand, demonstrated increased motility even though migration was allowed to occur for 24 h. PD098059 completely neutralized the effects of uPA on MCF-7 cellular motility, irrespective of whether the uPA was present for the entire motility assay or administered by pulse-exposure. These results demonstrate a novel, receptor-dependent signaling activity which is required for uPA-stimulated breast cancer cell migration.

Glycosylphosphatidylinositols (GPI)[1] are complex glycolipids that anchor a variety of proteins to the external surfaces of eukaryotic cells (1). GPI-anchored proteins do not contain transmembrane or intracellular domains. Nevertheless, recent studies suggest that ligand binding to many GPI-anchored proteins initiates signal transduction responses (2–4). Stefanova *et al.* (3) isolated *src* family tyrosine kinases in immunoprecipitates of various GPI-anchored proteins, including CD59, CD55, CD48, CD24, CD14, Thy-1, and Ly-6. The same investigators also showed that antibodies which bind GPI-anchored proteins induce tyrosine phosphorylation of cellular proteins. Jurkat cells that are treated with a monoclonal antibody specific for the GPI-anchored protein, CD59, demonstrate not only increased intracellular $[Ca^{2+}]$, but also increased interleukin-2 expression and increased proliferation (4). These studies suggest that GPI-anchored proteins may be components of multiprotein complexes which transmit signals across the plasma membrane.

The urokinase-type plasminogen activator receptor (uPAR) is a highly glycosylated 55–65-kDa GPI-anchored protein (5). uPAR binds the single-chain form of urokinase-type plasminogen activator (scuPA), which lacks enzymatic activity, and the fully active two-chain form of uPA (tcuPA) (6, 7). Under some conditions, scuPA that binds to uPAR may acquire enzymatic activity in its single-chain form (8, 9); however, uPAR-associated scuPA is also rapidly converted into tcuPA by cell-associated plasmin and by other cell surface proteinases (10, 11). uPAR-associated tcuPA activates a cascade of proteinases, culminating in the effective digestion of structural proteins and the disruption of tissue barriers, including basement membranes (12). By this mechanism, uPA and uPAR function to promote diverse processes that require cellular migration *in vivo*, including cancer invasion and metastasis.

Recent studies have demonstrated that uPA and uPAR express activities that do not depend on the function of uPA as a proteinase. For example, uPAR may regulate cellular adhesion and migration by associating with the extracellular domains of integrins or by binding directly to vitronectin (13, 14). The interaction of uPAR with integrins and vitronectin may be modified when uPAR binds uPA (15–17). Like other GPI-anchored proteins, uPAR also initiates signal transduction responses and thereby alters gene expression. Binding of uPA to uPAR activates cellular protein tyrosine kinases in a variety of cell types (18–21). In ovarian carcinoma cells, ligation of uPAR with uPA induces expression of c-*fos* and this response is blocked by herbimycin, a protein tyrosine kinase inhibitor (20). Furthermore, in WISH cells, uPA activates protein kinase Cε (22). Whether these signal transduction responses are responsible for some of the observed effects of uPA and uPAR on cellular adhesion and motility remains unclear.

Klemke *et al.* (23) proposed that activation of the mitogen-activated protein (MAP) kinases, extracellular signal-regulated kinase 1 (ERK1) and ERK2, may represent an essential

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[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; uPAR, urokinase-type plasminogen activator receptor; scuPA, single-chain urokinase-type plasminogen activator; tcuPA, two-chain urokinase-type plasminogen activator; ERK, extracellular signal-regulated kinase; ATF, amino-terminal fragment; EGF, epidermal growth factor; FBS, fetal bovine serum; MAP, mitogen-activated protein; MEK, MAP kinase kinase; PAGE, polyacrylamide gel electrophoresis; DIP, diisopropyl phospho.

step in the pathway by which cytokines and integrins promote cellular motility. In the present investigation, we studied the effects of uPA on the activation of ERK1 and ERK2 in MCF-7 breast cancer cells. Our results demonstrate that both MAP kinases are activated by uPA. Activation of ERK1 and ERK2 depended on uPA binding to its receptor and did not require uPA proteolytic activity. uPA also promoted the migration of MCF-7 cells across serum-coated Transwell membranes *in vitro*. The motility stimulating activity of uPA was neutralized by an inhibitor of MAP kinase kinase (MEK). These results suggest that uPA promotes breast cancer cell migration by a mechanism that is dependent on uPAR-initiated signal transduction.

MATERIALS AND METHODS

Proteins and Reagents—Leupeptin was from Boehringer Mannheim. Sodium orthovanadate, aprotinin, sodium fluoride, dithiothreitol, and bovine serum albumin were from Sigma. The MEK inhibitor, PD098059, was from Calbiochem. IODO-BEADS were from Pierce. $Na^{125}I$ was from Amersham. Earle's balanced salt solution was from Life Technologies, Inc. ScuPA, tcuPA, and the amino-terminal fragment of scuPA (ATF) were provided by Drs. Jack Henkin and Andrew Mazar of Abbott Laboratories (Abbott Park, IL). ATF is a 135-amino acid fragment of scuPA which contains the binding site for uPAR but lacks proteinase activity (24). Recombinant human epidermal growth factor (EGF) was from R&D Systems (Minneapolis, MN). Anti-active MAP kinase polyclonal antibody, which recognizes the phosphorylated forms of ERK1 and ERK2 (p44/42), was kindly provided by Dr. Michael Weber (University of Virginia, Charlottesville, VA). Polyclonal antibody, which recognizes total ERK1 and ERK2 antigen, was supplied by Zymed (San Francisco, CA). Anti-rabbit IgG peroxidase-conjugated antibody was from Sigma.

Cell Culture—MCF-7 human breast cancer cells were obtained from the ATCC. The cells were cultured in RPMI supplemented with 10% fetal bovine serum (FBS) (Hyclone), 100 units/ml penicillin (Life Technologies, Inc.), and 100 μg/ml streptomycin (Life Technologies, Inc.). MCF-7 cells were passaged with Cell Dissociation Buffer (Enzyme Free, Hank's based, Life Technologies, Inc.). After passaging, all cultures were maintained at 37 °C for 48 h before conducting experiments.

Inactivation of tcuPA—TcuPA was incubated with 20 mM diisopropyl fluorophosphate (Sigma) in 0.1 M sodium phosphate, pH 7.4, for 2 h at room temperature. Greater than 95% of the enzymatic activity of tcuPA was eliminated, as determined by the velocity of hydrolysis of the uPA-specific chromogenic substrate, L-pyroglutamyl-glycyl-arginine-*p*-nitroanilide HCl. Final preparations of DIP-uPA were dialyzed extensively against 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (phosphate-buffered saline).

Urokinase Binding to MCF-7 Cells—To quantitate uPAR expression by MCF-7 cells, DIP-uPA was radioiodinated to a specific activity of 1–2 μCi/μg using IODO-BEADS. Increasing concentrations of ^{125}I-DIP-uPA were incubated with MCF-7 cells in Earle's balanced salt solution supplemented with 10 mM HEPES, pH 7.4, and 10 mg/ml bovine serum albumin (EHB medium) for 4 h at 4 °C. A 50-fold molar excess of nonradiolabeled DIP-uPA was added to some cultures to inhibit specific binding. Binding was terminated by washing the cultures three times with ice-cold EHB and once with Earle's balanced salt solution, 10 mM HEPES, pH 7.4 (no bovine serum albumin). Cell associated radioactivity was recovered in 0.1 M NaOH, 1% (w/v) SDS. Radioactivity was quantitated in a γ-counter. Cellular protein was determined by bicinchoninic acid assay (Sigma).

Analysis of MAP Kinase Activation—MCF-7 cells were cultured in 6-well plates (Costar). When the cultures were 80% confluent, the cells were transferred to serum-free medium for 12 h and then treated with different concentrations of scuPA, 10 nM ATF, or 25 ng/ml EGF. Control cultures were treated with an identical volume of vehicle. After incubation for the indicated times at 37 °C, reactions were terminated by aspirating the medium and washing the cells with ice-cold phosphate-buffered saline, containing 1 mg/ml sodium orthovanadate. The cells were then extracted at 4 °C with 1.0% Nonidet P-40, 50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 0.4 mg/ml sodium orthovanadate, 0.4 mg/ml sodium fluoride, and 5 mg/ml dithiothreitol, pH 7.4. The extracts were boiled for 5 min and subjected to SDS-polyacrylamide gel electrophoresis using 12% acrylamide slabs. After electrotransferring the proteins to nitrocellulose membranes (Micron Separations Inc.), Western blot analysis was performed using primary antibodies that recognize phosphorylated ERK1 and ERK2 (1:1,000 dilution) or total ERK1 and ERK2 (1:10,000 dilution). Primary antibodies were detected by incubation with peroxidase-conjugated anti-rabbit IgG (1:3,000 dilution) followed by enhanced chemiluminescence (Amersham).

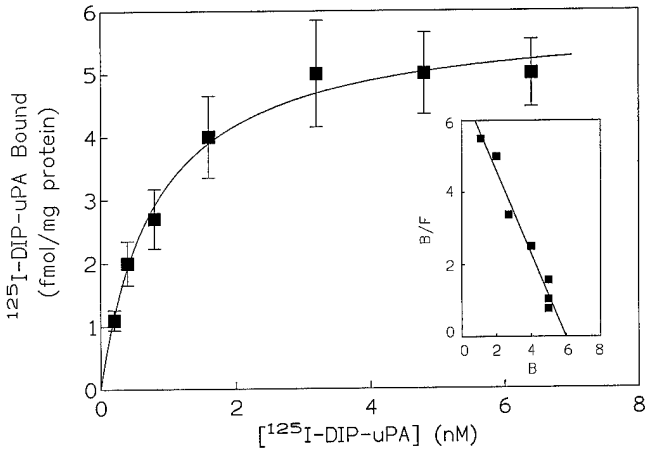

FIG. 1. **Equilibrium binding of ^{125}I-DIP-uPA to MCF-7 cells.** MCF-7 cells were incubated with increasing concentrations of ^{125}I-DIP-uPA for 4 h at 4 °C. The specific binding isotherm is shown. Each point represents the mean of results from four separate experiments, each with triplicate determinations. The Scatchard transformation of the same data is shown in the *inset*. *B/F*, bound/free.

Cell Migration—Cell migration assays were performed using tissue culture-treated 6.5-mm Transwell chambers with 8.0-μm pore membranes (Costar). For most experiments, both sides of each membrane were coated with 20% FBS in RPMI by incubation for 2 h at 37 °C. Under these conditions, the Transwells become coated primarily with vitronectin, which serves as the major attachment and spreading factor (25). In some experiments, only the bottom surfaces of the membranes were coated with FBS. The membranes were then washed with serum-free RPMI. Monolayer cultures of MCF-7 cells were dissociated by incubation with Cell Dissociation Buffer for 10 min at 37 °C and added to the top chamber of each Transwell apparatus at a density of 10^6 cells/ml (100 μl per chamber) in serum-free medium. The bottom chambers contained 800 μl of RPMI supplemented with 10% FBS. ScuPA or ATF (10 nM) was added to the top chamber, bottom chamber, or both chambers. In some experiments, the MCF-7 cells were pulse-exposed to uPA in suspension for 30 min or 1 h, washed, and then added to Transwell chambers that did not contain uPA or ATF. Cells were allowed to migrate for 24 h at 37 °C. After removing the cells that remained in the top chamber, the top surface of each membrane was cleared of cells with a cotton swab. Cells that had penetrated to the bottom side of the membrane were then fixed in buffered formalin, stained using a Diff-Quik Stain Set (Dade Diagnostics of P.R. Inc.), and counted. Each reported value represents the mean of results obtained in four separate experiments, each with triplicate determinations.

RESULTS

uPAR Expression by MCF-7 Cells—Previous studies have reported that MCF-7 cells express low levels of uPAR (26, 27); however, the actual number of uPA-binding sites per MCF-7 cell has not been determined. In our studies, ^{125}I-DIP-uPA bound in a specific and saturable manner to MCF-7 cells at 4 °C (Fig. 1). The K_D was 0.9 ± 0.1 nM which is in good agreement with K_D values determined for uPA binding to uPAR in other cell types. The B_{max} was 5.5 ± 1.3 fmol/mg of cell protein (n = 4), which corresponds to 3,300 ± 800 receptors/cell, assuming an average cellular mass of 1.0 ng/cell.

Activation of ERK1 and ERK2 by ScuPA—When scuPA was added to MCF-7 cell cultures, ERK1 and ERK2 were rapidly activated, as determined by the extent of phosphorylation of these MAP kinases (Fig. 2). In five separate experiments, phosphorylation of ERK1 and ERK2 maximized within 1–2 min after adding 10 nM scuPA and returned to near baseline levels by 5 min. The extent of phosphorylation of ERK1 and ERK2

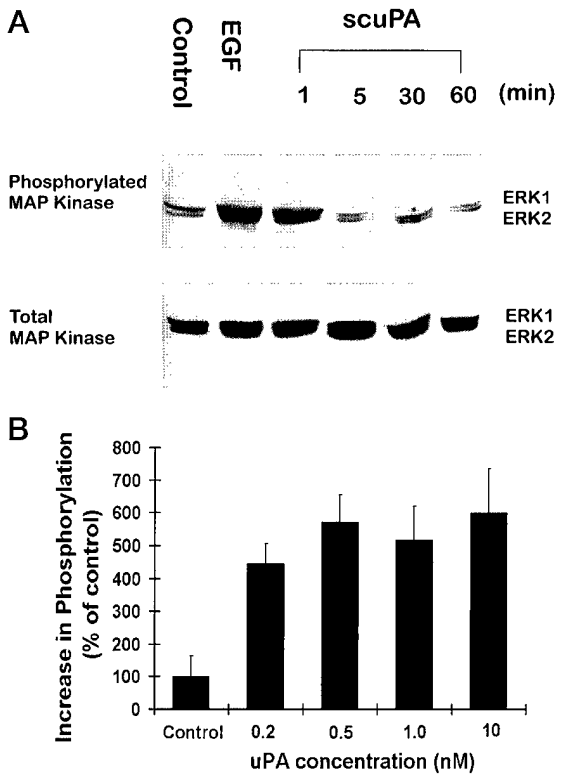

FIG. 2. **Phosphorylation of ERK1 and ERK2 in MCF-7 cells treated with scuPA.** *Panel A*, Western blot analysis detecting phosphorylated ERK1 and ERK2 in MCF-7 cells treated with scuPA for the indicated times, or with EGF or vehicle. The identical blot was probed with an antibody to detect total ERK1 and ERK2. *Panel B*, MCF-7 cells were treated with increasing concentrations of scuPA or with vehicle for 1 min. Phosphorylated ERK1 and ERK2 were then detected by Western blot analysis. Levels of phosphorylated ERK1 and ERK2 were compared by densitometry of autoradiography bands, using Image Quant by Molecular Dynamics. Each level of phosphorylated MAP kinase was internally standardized by comparison with the corresponding level of total ERK1/ERK2 and then compared with the level of phosphorylated ERK1/ERK2 detected in cells which were treated only with vehicle (n = 3).

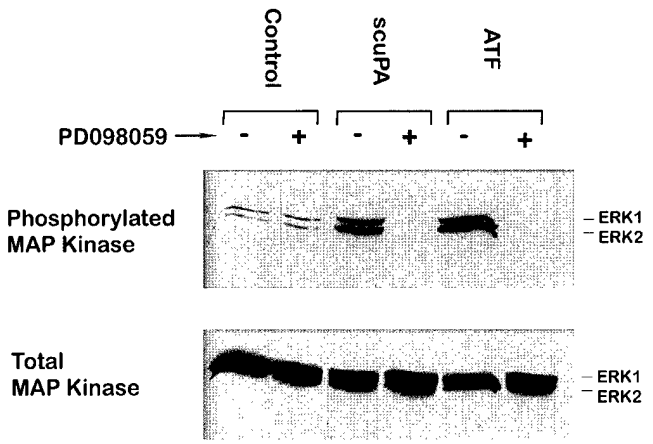

FIG. 3. **Phosphorylation of ERK1 and ERK2 in response to ATF and the effects of PD098059.** *Upper*, Western blot analysis showing phosphorylated ERK1 and ERK2 in MCF-7 cells treated with scuPA or ATF for 1 min. Some MCF-7 cells were pretreated with the MEK inhibitor, PD098059 (50 μM) for 15 min prior to stimulation with scuPA or ATF (shown with "+"). Other cultures were not treated with PD098059 (shown with "−"). *Lower*, the identical blot was probed with an antibody to detect total ERK1 and ERK2.

was increased 4.5–6-fold, compared with the baseline level, in cells treated with 0.2–10 nM scuPA. Thus, scuPA activated ERK1 and ERK2 at concentrations that approximated the K_D for uPA binding to uPAR. The increase in phosphorylation of ERK1 and ERK2, induced by scuPA, was similar in magnitude to that observed with 25 ng/ml EGF; however, the EGF response was sustained for longer periods of time (up to 30 min, results not shown). Although both ERK1 and ERK2 were phosphorylated in response to scuPA, preferential phosphorylation of ERK2 was occasionally observed.

Activation of ERK1 and ERK2 by ATF—ScuPA is a zymogen which may acquire activity when bound to uPAR, as a direct result of receptor binding (8, 9) or due to cleavage by cell surface-associated proteinases (10, 11). The rapidity with which scuPA activated ERK1 and ERK2 suggested that this response does not require proteolytic activity. To confirm this hypothesis, we examined the MCF-7 cell response to ATF, a uPA derivative which lacks proteinase activity but retains receptor binding activity (24). As shown in Fig. 3, ATF (10 nM) caused the rapid activation of ERK1 and ERK2, mimicking the activity of scuPA. Within 5 min after adding ATF, the level activated ERK1 and ERK2 returned to baseline levels (n = 4).

Activation of ERK1 and ERK2, in response to scuPA or ATF, was neutralized when the MEK inhibitor, PD098059 (50 μM), was preincubated with the MCF-7 cells for 15 min. PD098059 did not alter the total level of ERK1 and ERK2, as determined by Western blot analysis. In control experiments, Me_2SO (1:1,000 v/v), which was used to dissolve the PD098059, did not affect basal levels of phosphorylated ERK1 and ERK2 or the increase in phosphorylation of ERK1 and ERK2 in response to scuPA or ATF (results not shown).

Effects of uPA on MCF-7 Cell Migration—MCF-7 cell migration was initially studied using Transwell chambers, in which the membranes were precoated on both sides with FBS. The major protein which adsorbs to the membrane is vitronectin (25). In the absence of uPA, minimal migration was observed, despite the presence of 10% FBS in the lower chamber; the number of cells penetrating through the membrane to the lower surface was 70 ± 25 per membrane in 24 h (n = 5). Addition of 10 nM scuPA to the top chamber increased migration by 2.1 ± 0.4-fold (Fig. 4). Cellular migration increased by 4.8 ± 0.8-fold when scuPA was added to the bottom chamber and by 7.7 ± 1.0-fold when scuPA was added to both chambers. The effects of uPA on cellular motility were statistically significant ($p < 0.05$ when scuPA was added to one chamber and <0.01 when scuPA was added to both chambers).

In one set of experiments, cellular migration was studied, in the presence and absence of scuPA, for 6, 12, or 18 h, rather than 24 h. Differences in migration were still observed under the various conditions (scuPA in the bottom chamber, scuPA in the top chamber, or scuPA in both chambers); however, the number of cells penetrating through the membranes was decreased proportionately to the decrease in assay time (results not shown). These results demonstrated that cellular migration occurred during the entire course of the motility assay and not only during the time period immediately following introduction of the cells into the upper chamber.

Haptotactic Migration Toward Vitronectin—When FBS was used to coat only the underside of the Transwell membranes, MCF-7 cell migration was greatly increased under all conditions; however, a significant effect of scuPA was still observed ($p < 0.01$). In the absence of scuPA, 851 ± 122 cells penetrated through the membrane to the lower surface in 24 h (n = 3). When 10 nM scuPA was added to both chambers, 1,908 ± 204 cells penetrated to the underside surface (n = 3); these cells formed relatively dense monolayers on the lower surfaces of the membranes. Thus, the increase in cellular migration caused by scuPA, in these experiments, may have been underestimated due to partial obstruction of the pores with cells and/or release

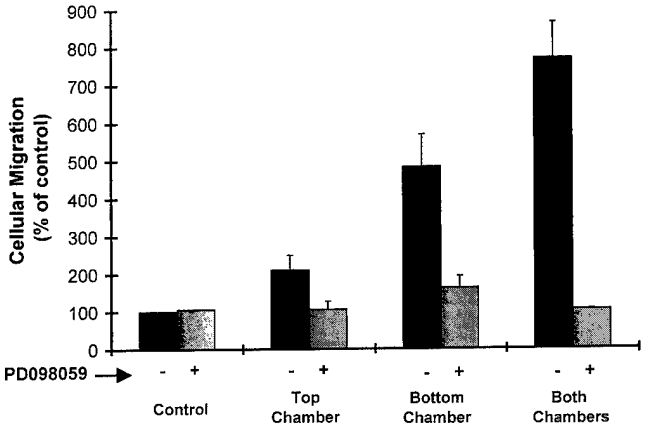

FIG. 4. **Migration of MCF-7 cells through serum-coated Transwell membranes.** MCF-7 cells were allowed to migrate for 24 h through Transwell membranes that were precoated with serum. ScuPA was added to the *top* and/or *bottom* chambers as shown. In some Transwells, the MEK inhibitor, PD098059, was added to the top chamber after pretreating the MCF-7 cells with the drug for 15 min (marked "+"). Migration of MCF-7 cells to the underside surfaces of the membranes was quantitated as a percentage of that observed in control Transwells that were not supplemented with scuPA or PD098059.

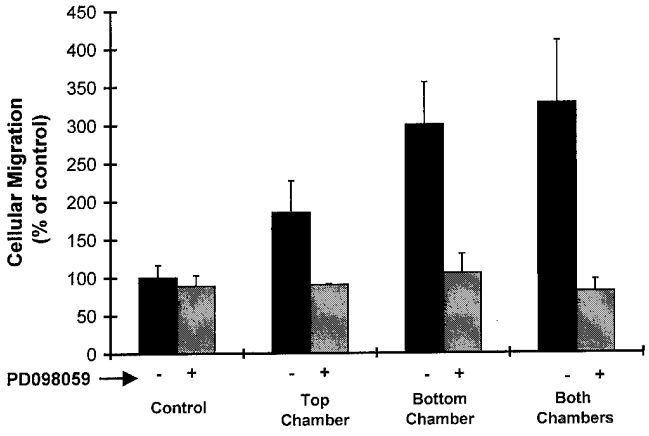

FIG. 5. **Migration of MCF-7 cells in response to ATF.** MCF-7 cells were allowed to migrate for 24 h through Transwell membranes that were precoated with serum. ATF was added to the *top* and/or *bottom* chambers as shown. In some Transwells, the MEK inhibitor, PD098059, was added to the top chamber after pretreating the MCF-7 cells with the drug for 15 min. Migration of MCF-7 cells to the underside surfaces of the membranes was quantitated as a percentage of that observed in control Transwells that were not supplemented with ATF or PD098059.

of cells from the underside of the membranes into the bottom chambers.

Effects of MEK Inhibitor on MCF-7 Cell Migration—MCF-7 cells were pretreated with PD098059 (50 μM) for 15 min and then added to Transwells in the presence of the drug. As shown in Fig. 4, PD098059 had no effect on cellular migration in the absence of uPA. By contrast, PD098059 substantially inhibited the motility stimulating activity of scuPA under all experimental conditions. These results suggest that activation of ERK1 and/or ERK2 is required for uPA-promoted MCF-7 cell migration.

ATF (10 nM) promoted MCF-7 cell migration, indicating an essential role for uPA-receptor binding in the migration response (Fig. 5). When ATF was present in the top chamber, migration was increased by 1.9 ± 0.2-fold (n = 4). Migration was increased by 3.0 ± 0.5-fold when ATF was present in the bottom chamber and by 3.3 ± 0.8-fold when ATF was present in both chambers. The effects of ATF on cellular motility were statistically significant when compared with control cultures that were not ATF-treated ($p < 0.1$, 0.05, and 0.01 for studies in which ATF was added to top chamber, bottom chamber, and both chambers, respectively). PD098059 completely neutralized the motility promoting activity of ATF, again indicating an essential role for ERK1 and/or ERK2.

To determine whether scuPA affects MCF-7 cell proliferation and thereby alters the number of cells available to migrate across Transwell membranes, MCF-7 cells were incubated with [^{3}H]thymidine for 24 h, in the presence or absence of scuPA. [^{3}H]Thymidine incorporation was not affected by the scuPA (results not shown).

Pulse-exposure of MCF-7 Cells to uPA—ScuPA and ATF increased the motility of MCF-7 cells during a 24-h assay even though the effects of these agents on the activation of ERK1 and ERK2 were transient. Thus, we hypothesized that the transient activation of ERK1 and ERK2 was followed by the more stable modification of downstream substrates, so that changes in cellular physiology were maintained during the 24-h motility assay. To test this hypothesis, we pulse-exposed MCF-7 cells to scuPA for 30 min or 1 h in suspension and then washed the cells before adding them to Transwell chambers. Migration was then allowed to occur in the absence of uPA. As shown in Fig. 6, pulse-exposure to scuPA for as little as 30 min was sufficient to induce an increase in motility which was

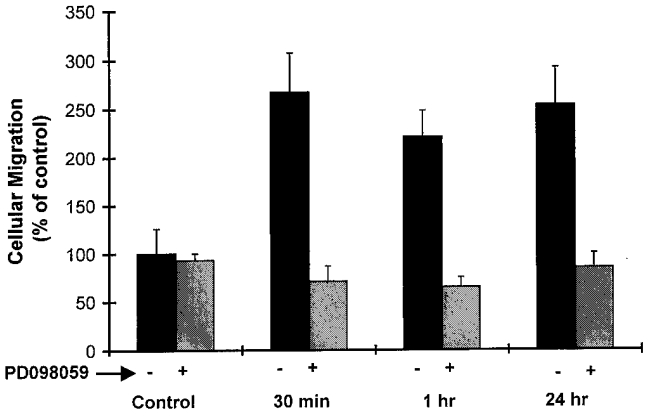

FIG. 6. **Migration of MCF-7 cells after pulse-exposure to scuPA.** MCF-7 cells in suspension were pulse-exposed to 10 nM scuPA for the indicated times. Control cells were treated with an identical volume of vehicle. Cultures which are marked "+" were treated with PD098059, beginning 15 min prior to adding scuPA. Before adding cells to the Transwell chambers, the cells were washed to remove free scuPA and drug (if present). Migration was allowed to occur in the absence of uPA for 24 h. As a positive control, MCF-7 cells were allowed to migrate in Transwells that contained scuPA in the upper chamber (labeled "24 h"). Migration of MCF-7 cells to the underside surfaces of the membranes was quantitated as a percentage of that observed in control Transwells (cells that were not exposed to scuPA).

comparable to that observed when scuPA was present in the upper Transwell chamber for the entire 24-h assay. The increase in cellular motility caused by pulse-exposure to scuPA was statistically significant ($p < 0.05$).

PD098059 was added to some cultures 15 min prior to pulse-exposure with scuPA. The drug was then removed together with the scuPA by washing the cells before the cells were added to the Transwell chambers. As shown in Fig. 6, the MEK inhibitor neutralized the motility promoting activity of scuPA in the pulse-exposure experiments.

DISCUSSION

Numerous clinical studies have demonstrated a strong correlation between cellular expression of uPA and aggressive behavior of breast cancers. Malignant tumors almost always express increased levels of uPA compared with benign tumors (26–29). Among malignant tumors, those that express the highest levels of uPA have the greatest tendency to metasta-

size, recur after resection, and limit life expectancy (26, 27, 30, 31). uPAR expression has also been identified as a negative prognostic factor in breast cancer (29, 32). These clinical studies may be explained by the ability of uPAR-associated uPA to initiate an extracellular proteolytic cascade that leads to the digestion of tissue barriers, such as basement membranes (12). However, the effects of uPA and uPAR on cellular signaling, adhesion, and motility may also be involved.

To study the response of breast cancer cells to uPA, we chose the MCF-7 cell line as a model system. MCF-7 cells are estrogen-dependent for growth. In nude mice, MCF-7 cells grow locally but do not metastasize (33). Compared with the more aggressive breast cancer cell lines, MDA-MB-231 and MDA-MB-435, MCF-7 cells express lower levels of uPAR and uPA (34). Our binding studies demonstrated about 3,000 copies of uPAR per MCF-7 cell, which is apparently sufficient to mediate the activation of ERK1 and ERK2. We did not independently measure uPA synthesis by MCF-7 cells; however, we failed to detect significant levels of uPA mRNA by Northern blot analysis (results not shown). The low level of uPA synthesis and the slow rate of MCF-7 cell growth (34, 35) may have aided in the use of this cell line as a model system, since baseline levels of MCF-7 cell ERK1 and ERK2 activation were typically low. In studies that are not shown, we demonstrated higher levels of phosphorylated ERK1 and ERK2, under basal conditions, in MDA-MB-231 and MDA-MB-435 cells.

Our studies demonstrated that the activation of ERK1 and ERK2 by scuPA, in MCF-7 cells, depends on receptor binding and does not require uPA proteolytic activity. To our knowledge, the activation of ERK1 and ERK2 by scuPA has not been previously reported. By contrast, uPA has been shown to promote the motility of various cell types including monocytoid cells, smooth muscle cells, endothelial cells, and cells transfected with uPAR expression constructs (12, 18, 22, 36). Waltz *et al.* (37) recently reported that ATF can inhibit cellular motility; however, for cellular migration to occur in their experimental system, it was necessary for the cells to detach from vitronectin, which was coated only on the top surfaces of Transwell chambers, and migrate over a surface which was not vitronectin-coated. Thus, the inhibitory activity of ATF likely reflected the ability of this reagent to strengthen cellular adhesion to vitronectin.

We considered two possible mechanisms to explain the motility promoting activity of uPA. First, the activity may have resulted from the ability of uPA to bind to uPAR and regulate the association of uPAR with vitronectin and/or integrins (13, 14). Second, the motility promoting activity may have resulted from uPAR-dependent uPA signal transduction. Our studies demonstrating activation of ERK1 and ERK2 by scuPA and ATF support the hypothesis that uPAR signaling is at least partially responsible for the motility promoting activity of uPA. Diverse signals that activate ERK1 and ERK2 have been previously shown to promote cellular motility; in this pathway, myosin light chain kinase serves as a critical downstream substrate for the MAP kinases (23). The ability of PD098059 to neutralize the motility promoting activities of scuPA and ATF strongly supports our hypothesis regarding the importance of uPAR signaling. Since PD098059 was active in the scuPA pulse-exposure experiments, which required contact of the cells with the drug for only 45 or 75 min, it is unlikely that the MEK inhibitor has other unrecognized activities that contributed to the observed results.

Since serum was used to coat the Transwell membranes, we assumed that vitronectin was the major protein which adsorbed to the membrane surfaces (25). In MCF-7 cells, the major vitronectin receptor is $\alpha_V\beta_5$; these cells express little or no $\alpha_V\beta_3$ (38). Yebra *et al.* (39) studied pancreatic carcinoma cells and demonstrated $\alpha_V\beta_5$-dependent motility only after the cells were treated with reagents that induce expression of uPA and uPAR. When the pancreatic carcinoma cells were transfected to express $\alpha_V\beta_3$, the dependence on the uPA/uPAR system for cellular motility was lost. In the same cell type, activation of protein tyrosine kinases or protein kinase C also allowed $\alpha_V\beta_5$-directed motility (40). These studies suggest that the integrin expression pattern of various cells may be important in determining the effects of uPA and uPAR on cellular motility. Expression of $\alpha_V\beta_5$ by the MCF-7 cells may have been important in the relationship between uPA signaling, MAP kinase activation, and cellular motility.

The ability of scuPA to activate ERK1 and ERK2 raises new questions regarding the gene regulatory activities of uPAR. For example, since uPAR expression is increased in response to multiple factors that activate ERK1 and ERK2 (41), might uPA regulate expression of its own receptor? Furthermore, activation of ERK1 and ERK2 may lead to either increased expression of uPA or cathepsin L, depending on whether or not there is concomitant activation of c-Jun NH_2-terminal kinase (42). Thus, uPAR ligation may alter the levels of extracellular proteinases in the microenvironment of cancer cells. Our findings and these previous reports suggest an important activity for uPA and uPAR as an integrator of various cellular functions required for cancer progression *in vivo*.

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REFERENCES

1. Yeh, E., Kamitani, T., and Chang, M. (1994) *Semin. Immunol.* **6,** 73–80
2. Anderson, R. (1994) *Semin. Immunol.* **6,** 89–95
3. Stefanova, I., Horejsi, V., Ansotegui, I., Knapp, W., and Stockinger, H. (1991) *Nature* **254,** 1016–1019
4. Korty, P., Brando, C., and Shevach, E. (1991) *J. Immunol.* **146,** 4092–4098
5. Ploug, M., Ronne, E., Behrendt, N., Jensen, A. L., Blasi, F., and Dano, K. (1991) *J. Biol. Chem.* **266,** 1926–1933
6. Vassalli, J.-D., Baccino, D., and Belin, D. (1985) *J. Cell Biol.* **100,** 86–92
7. Cubellis, M. V., Nolli, M. L., Cassani, G., and Blasi, F. (1986) *J. Biol. Chem.* **261,** 15819–15822
8. Manchanda, N., and Schwartz, B. S. (1991) *J. Biol. Chem.* **266,** 14580–14584
9. Wang, J., Mazar, A., Quan, N., Schneider, A., and Henkin, J. (1997) *Eur. J. Biochem.* **247,** 256–261
10. de Munk, G. A. W., and Rijken, D. C. (1990) *Fibrinolysis* **4,** 1–9
11. Berkenpas, M. B., and Quigley, J. P. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7768–7772
12. Andreasen, P. A., Kjoller, L., Christensen, L., and Duffy, M. J. (1997) *Int. J. Cancer* **72,** 1–22
13. Wei, Y., Waltz, D. A., Rao, N., Drummond, R. J., Rosenberg, S., and Chapman, H. A. (1994) *J. Biol. Chem.* **269,** 32380–32388
14. Wei, Y., Lukashev, M., Simon, D. I., Bodary, S. C., Rosenberg, S., Doyle, M. V., and Chapman, H. A. (1996) *Science* **273,** 1551–1555
15. Nusrat, A. R., and Chapman, H. A. (1991) *J. Clin. Invest.* **87,** 1091–1097
16. Waltz, D. A., and Chapman, H. A. (1994) *J. Biol. Chem.* **269,** 14746–14750
17. Higazi, A. A-R., Upson, R. H., Cohen, R. L., Manuppello, J., Bognacki, J., Henkin, J., McCrae, K. R., Kounnas, M. Z., Strickland, D. K., Preissner, K. T., Lawler, J., and Cines, D. B. (1996) *Blood* **88,** 542–551
18. Resnati, M., Guttinger, M., Valcomonica, S., Sidenius, N., Blasi, F., and Fazioli, F. (1996) *EMBO J.* **15,** 1572–1582
19. Bohuslav, J., Horejsi, V., Hansmann, C., Stockl, J., Weidle, U. H., Majdic, O., Bartke, I., Knapp, W., and Stockinger, H. (1995) *J. Exp. Med.* **181,** 1381–1390
20. Dumler, I., Petri, T., and Schleuning, W.-D. (1993) *FEBS Lett.* **322,** 37–40
21. Mirshahi, S. S., Lounes, K. C., Lu, H., Pujade-Lauraine, E., Mishal, Z., Benard, J., Bernadou, A., Soria, C., and Soria, J. (1997) *FEBS Lett.* **411,** 322–326
22. Busso, N., Masur, S. K., Lazega, D., Waxman, S., and Ossowski, L. (1994) *J. Cell Biol.* **126,** 259–270
23. Klemke, R. L., Cai, S., Giannini, A. L., Gallagher, P. J., de Lanerolle, P., and Cheresh, D. (1997) *J. Cell Biol.* **137,** 481–492
24. Appella, E., Robinson, E. A., Ullrich, S. J., Stoppelli, M. P., Corti, A., Cassani, G., and Blasi, F. (1987) *J. Biol. Chem.* **262,** 4437–4440
25. Haymen, E. G., Pierschbacher, M. D., Ohgren, Y., and Ruoslahti, E. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 4003–4007
26. Sumiyoshi, K., Serizawa, K., Urano, T., Takada, Y., Takada, A., and Baba, S. (1992) *Int. J. Cancer* **50,** 345–348
27. Sumiyoshi, K., Baba, S., Sakaguchi, S., Urano, T., Takada, Y., and Takada, A. (1991) *Thrombos. Res.* **63,** 59–71
28. O'Grady, P., Lijnen, H. R., and Duffy, M. J. (1985) *Cancer Res.* **45,** 6216–6218
29. Costantini, V., Sidoni, A., Deveglia, R., Cazzato, O. A., Bellezza, G., Ferri, I.,

Bucciarelli, E., and Nenci, G. G. (1996) *Cancer* **77,** 1079–1088
30. Duffy, M. J., Reilly, D., O'Sullivan, C., O'Higgins, N., Fennelly, J. J., and Andreasen, P. (1990) *Cancer Res.* **50,** 6827–6829
31. Schmitt, M., Goretzki, L., Janicke, F., Calvete, J., Eulitz, M., Kobayashi, H., Chucholowski, N., and Graeff, H. (1991) *Biomed. Biochim. Acta* **50,** 731–741
32. Duggan, C., Maguire, T., McDermott, E., O'Higgins, N., Fennelly, J. J., and Duffy, M. J. (1995) *Int. J. Cancer* **61,** 597–600
33. Thompson, E. W., Paik, S., Brunner, N., Sommers, C. L., Zugmaier, G., Clarke, R., Shima, T. B., Torri, J., Donahue, S., Lippman, M. E., Martin, G. B., and Dickson, R. B. (1992) *J. Cell. Physiol.* **150,** 534–544
34. Holst-Hansen, C., Johannessen, B., Hoyer-Hansen, G., Romer, J., Ellis, V., and Brunner, N. (1996) *Clin. Exp. Metastasis* **14,** 297–307
35. Sutherland, R. L., Hall, R. E., and Taylor, I. W. (1983) *Cancer Res.* **43,** 3998–4006
36. Odekon, L. E., Sato, Y., and Rifkin, D. B. (1992) *J. Cell. Physiol.* **150,** 258–263
37. Waltz, D. A., Natkin, L. R., Fujita, R. M., Wei, Y., and Chapman, H. A. (1997) *J. Clin. Invest.* **100,** 58–67
38. Heegaard, C. W., Simonsen, A. C. W., Oka, K., Kjoller, L., Christensen, A., Madsen, B., Ellgaard, L., Chan, L., and Andreasen, P. (1995) *J. Biol. Chem.* **270,** 20855–20861
39. Yebra, M., Parry, G. C. N., Stromblad, S., Mackman, N., Rosenberg, S., Mueller, B. M., and Cheresh, D. A. (1996) *J. Biol. Chem.* **271,** 29393–29399
40. Klemke, R. L., Yebra, M., Bayna, E. M., and Cheresh, D. A. (1994) *J. Cell Biol.* **127,** 859–866
41. Lengyel, E., Wang, H., Gum, R., Simon, C., Wang, Y., and Boyd, D. (1997) *Oncogene* **14,** 2563–2573
42. Silberman, S., Janulis, M., and Schultz, R. M. (1997) *J. Biol. Chem.* **272,** 5927–5935