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.

Glycosylphosphatidylinositol (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+]i, 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. The MEK inhibitor, PD098059, was from Calbiochem. IODO-BEADS were from Pierce. Na125I was from Amersham. Hank’s based, Life Technologies, Inc.). After passaging, all cultures were maintained at 37 °C for 48 h before conducting experiments.

Inactivation of uPA—uPA 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 uPA was inactivated, as determined by the loss of activity of hydrolysis of the uPA-specific chromogenic substrate, 1-pyroglutamyl-glycine-2-p-nitroanilide-4-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 125I-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 (EHBM medium) for 4 h at 4 °C. A 50-fold molar excess of nonradioabeled DIP-uPA was added to some cultures to inhibit specific binding. Binding was terminated by washing the cultures three times with ice-cold EBH 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% (v/v) SDS. Radioactivity was quantitated in a γ-counter. Cellular protein was determined by bicionchinic 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 mM ATF, or 25 mM/ECF. 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 lysed with 1% Triton X-100. 50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1 μg/ml leupeptin, 2 μg/ml aprotonin, 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 subject 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 (1:000 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).

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 106 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 mM) 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 scraped off 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 uPAR-binding sites per MCF-7 cell has not been determined. In our studies, 125I-DIP-uPA bound in a specific and saturable manner to MCF-7 cells at 4 °C (Fig. 1). The Kd was 0.9 ± 0.1 nM which is in good agreement with Kp values determined for uPA binding to uPAR in other cell types. The Bmax 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 cells, 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...
ERK1 and ERK2 at concentrations that approximated the KD
was neutralized when the MEK inhibitor, PD098059 (50 M),
activated ERK1 and ERK2 returned to baseline levels (Fig.
caused the rapid activation of ERK1 and ERK2, mimicking the
(ERK1/ERK2 detected in cells which were treated only with vehicle
and then compared with the level of phosphorylated
molecular dynamics. Each level of phosphorylated MAP kinase was inter-
blot analysis. Levels of phosphorylated ERK1 and ERK2 were compared
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 inter-
ally standardized by comparison with the corresponding level of total
ERK1/ERK2 detected in cells which were treated only with vehicle
(n = 3). 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 Kp
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 re-
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)
casted 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 M),
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, Me2SO (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 migra-
tion was initially studied using Transwell chambers, in which
the membranes were precoated on both sides with FBS. The
major protein which adsors 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 migra-
tion 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 signif-
icant (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 de-
creased 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 condi-
tions; 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
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 ATP 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 ATP was present in the bottom chamber and by 3.3 ± 0.8-fold when ATP was present in both chambers. The effects of ATP 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 ATP was added to top chamber, bottom chamber, and both chambers, respectively). PD098059 completely neutralized the motility promoting activity of ATP, 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 [3H]thymidine for 24 h, in the presence or absence of scuPA. [3H]Thymidine incorporation was not affected by the scuPA (results not shown).

**Pulse-exposure of MCF-7 Cells to uPA**—ScuPA and ATP 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 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 metastas-
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 monocytes, MDCK 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 demonstrated activation of ERK1 and ERK2 by scuPA and ATF support the hypothesis that uPA 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 uPA 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 $\alpha_v\beta_3$-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 $\alpha_v\beta_3$-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 $\alpha_v\beta_3$ 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 uPA. For example, since uPA 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 NH2-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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