The Very Low Density Lipoprotein Receptor Regulates Urokinase Receptor Catabolism and Breast Cancer Cell Motility in Vitro*

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The very low density lipoprotein receptor (VLDLr) binds diverse ligands, including urokinase-type plasminogen activator (uPA) and uPA-plasminogen activator inhibitor-1 (PAI-1) complex. In this study, we characterized the effects of the VLDLr on the internalization, catabolism, and function of the uPA receptor (uPAR) in MCF-7 and MDA-MB-435 breast cancer cells. When challenged with uPA-PAI-1 complex, MDA-MB-435 cells internalized uPAR; this process was inhibited by 80% when the activity of the VLDLr was neutralized with receptor-associated protein (RAP). To determine whether internalized uPAR is degraded, we studied the catabolism of [3H]methionine-labeled uPAR. In the absence of exogenous agents, the uPAR catabolism $t_{1/2}$ was 8.2 h. uPA-PAI-1 complex accelerated uPAR catabolism ($t_{1/2}$ to 1.8 h), while RAP inhibited uPAR catabolism in the presence ($t_{1/2}$ of 7.8 h) and absence ($t_{1/2}$ of 16.9 h) of uPA-PAI-1 complex, demonstrating a critical role for the VLDLr. When MCF-7 cells were cultured in RAP, cell surface uPAR levels increased gradually, reaching a new steady-state in 3 days. The amount of uPA which accumulated in the medium also increased. Culturing in RAP for 3 days increased MCF-7 cell motility by 2.2 ± 0.1-fold and by 4.4 ± 0.3-fold when 1.0 nm uPA was added. The effects of RAP on MCF-7 cell motility were entirely abrogated by an antibody which binds uPA and prevents uPA binding to uPAR. MCF-7 cells that were cultured in RAP demonstrated increased levels of activated mitogen-activated protein kinases. Furthermore, the MEK inhibitor, PD098059, decreased the motility of RAP-treated cells without affecting control cultures. These studies suggest a model in which the VLDLr regulates autocrine uPAR-initiated signaling and thereby regulates cellular motility.

The very low density lipoprotein receptor (VLDLr)$^1$ is a member of the LDL receptor family, which includes the LDL receptor-related protein (LRP) and gp330/megalin (1, 2). These receptors have equivalent structural motifs and bind many of the same ligands, including apolipoprotein E-enriched chylomicron remnants, lipoprotein lipase, thrombospondin I, urokinase-type plasminogen activator (uPA), uPA-plasminogen activator inhibitor-1 (PAI-1) complex, and receptor-associated protein (RAP) (3–10). RAP is a 39-kDa protein chaperone which normally remains entirely intracellular (11, 12); however, when incubated with cells in culture, RAP blocks the binding of all known ligands to the VLDLr, LRP, and gp330/megalin (1, 13–15). Some ligands do not bind interchangeably to different members of the LDL receptor family. For example, activated $\alpha_2$-macroglobulin and Pseudomonas exotoxin A bind only to LRP (16, 17).

In normal mouse development, LDL receptor homologues play distinct roles. Homozygous LRP deficiency is embryonic lethal (18), gp330/megalin-deficient mice survive gestation but die shortly thereafter due to abnormal lung development (19), whereas VLDLr-deficient mice survive but demonstrate decreased body weight, body mass index, and adipose tissue mass (20). These diverse phenotypes may reflect differences in the cells or tissues that express the various LDL receptor homologues. Alternatively, uncharacterized differences in receptor function may be involved.

Our laboratory recently identified a possible role for LRP as a regulator of cellular motility (21). We studied murine embryonic fibroblasts (MEFs) that are LRP-deficient and wild-type MEFs from the same mouse strain. These cells do not express the VLDLr or gp330/megalin (22). When allowed to migrate into denuded areas of vitronectin-coated cell culture wells, the LRP-deficient MEFs migrated almost twice as rapidly as wild-type cells (21). The increased motility of the LRP-deficient MEFs was at least partially explained by an increase in the level of cell surface uPAR and by an increase in the amount of uPA which accumulated in the conditioned medium of these cells (21). In diverse systems, uPA binding to uPAR promotes cellular migration by localizing cell surface proteinase activity, initiating signal transduction, and/or by regulating cellular adhesion (reviewed in Refs. 23 and 24).

Unlike LRP-deficient MEFs, vascular smooth muscle cells (VSMCs), which are treated with RAP to deactivate LDL receptor homologues, demonstrate decreased motility (25, 26). Interestingly, when MEFs are treated with RAP, while the migration assay is underway, no change in motility is observed (21). Although it was suggested that the uPA/uPAR system may be responsible for the changes in VSMC motility which accompany RAP treatment (25, 26), experiments were not performed to address this possibility. Other LRP ligands also may be involved. For example, thrombospondin 1 has been shown to inhibit the motility of VSMCs but not fibroblasts (27). It is also...
possible that RAP affects VSMCs differently than MEFs since VSMCs express VLDLr in addition to LRP (25, 28).

The hypothesis that LRP regulates cellular motility by altering the activity of the uPA/uPAR system is supported by recent studies demonstrating a role for LRP in uPAR endocytosis. uPA-PAI-1 complex, which is bound to uPAR, still binds to LRP (29). This interaction not only results in the internalization of uPA-PAI-1 complex, but promotes uPAR internalization as well (30). Thus, it has been proposed that uPA-PAI-1 complex bridges uPAR to LRP by forming a tetramolecular complex that undergoes endocytosis as an intact unit (18, 31). Internalized uPAR recycles back to the cell surface (31); however, the efficiency of recycling remains unclear. If the efficiency is less than 100%, then LRP may promote uPAR degradation in lysosomes, explaining why LRP-deficient MEFs have increased levels of cell surface uPAR (21).

The goal of the present investigation was to characterize the role of the VLDLr in the regulation of cell surface uPAR expression and cellular motility. Our studies were performed using breast cancer cell lines which express VLDLr but do not express LRP or gp330/megalin. When the VLDLr was neutralized, by culturing these cells in the presence of RAP, the level of cell surface uPAR increased gradually, reaching a new steady-state. The increase in cell surface uPAR was explained by a decrease in the rate of uPAR catabolism. Neutralizing the VLDLr also increased cellular motility. The increase in motility was entirely counteracted by an antibody which binds endogenously produced uPA and prevents uPAR ligation. We have previously shown that uPA promotes MCF-7 cell motility by activating the MAP kinases, extracellular signal-regulated kinase (ERK) 1 and ERK2 (32). In RAP-treated breast cancer cells, the levels of activated ERK1 and ERK2 were increased. Furthermore, the motility of RAP-treated cells was selectively inhibited by an antagonist of ERK-dependent signaling. These newly identified activities of the VLDLr indicate a potentially important role for this receptor as a regulator of cancer cell physiology.

MATERIALS AND METHODS

Proteins and Reagents—Single-chain uPA (scuPA), two-chain uPA (tcuPA), and a polyclonal antibody which specifically recognizes human uPAR were provided by Drs. Jack Henkin and Andrew Mazar (Abbott Laboratories). tcuPA was inactivated with diisopropyl fluorophosphate to form DIP-uPA, as described previously (32). A monoclonal antibody specific for the amino-terminal fragment of human uPAR was from American Diagnostica. This antibody prevents the binding of uPA to cell surface uPAR (33). Polyclonal antibody 399R, which recognizes human uPAR, was also from American Diagnostica. PAI-1 was provided by Drs. Keith McCrae and Mats Gåvfr. LRP heavy chain was detected by monoclonal antibody 8G1, provided by Dr. Dudley Strickland (American Red Cross, Rockville, MD).

Cellular Degradation of 125I-GST-RAP—GST-RAP was radioiodinated, using Iodo-Beads (Pierce), to a specific activity of 1–2 μCi/μg. MCF-7 cells were washed with EBSS, 25 mM Hepes, pH 7.4, and 5 mg/ml bovine serum albumin (EBH medium). 125I-GST-RAP (10 nm) was then added to the cultures. A 100-fold molar excess of nonradioiodinated GST-RAP was added to some cultures so that specific RAP degradation could be determined. The cells were allowed to incubate for various periods of time at 37 °C. Cellular degradation of 125I-GST-RAP was detected by measuring the increase in trichloroacetic acid soluble radioactivity in the medium.

Binding of DIP-uPA to MCF-7 Cells—MCF-7 cells were cultured in the presence or absence of GST-RAP (200 nm) for up to 5 days. The medium and GST-RAP were replaced daily. Our analysis of RAP catabolism by MCF-7 cells demonstrated that the concentration of GST-RAP in the medium decreased by less than 1% in each 24-h culturing period. Specific binding of DIP-uPA to RAP-treated and control MCF-7 cells was compared. DIP-uPA was radioiodinated with Iodo-Beads to a specific activity of 2–4 μCi/μg. The cultures were washed three times and then incubated with 125I-DIP-uPA (0.15–10 nm) in EHB for 4 h at 4 °C. In this uPA concentration range, high affinity binding to uPAR is selectively detected and low affinity interactions, such as those that might occur with the VLDLr, do not contribute significantly (3, 32, 37, 39). A specific immunoprecipitate of a 50-fold molar excess of excess radiolabeled DIP-uPA was added to some cultures. At the end of each binding experiment, the cultures were washed four times at 4 °C; cell associated radioactivity was recovered in 1 M NaOH and quantitated in a γ-counter. Cellular protein was determined by the bichrominic acid assay (Sigma). To calculate the number of specific uPA-binding sites per cell, the average mass of the MCF-7 cell was determined. Suspended cells were counted using a Coulter counter (yielding equivalent results) and then extracted for protein determination. The mass was 0.94 ± 0.07 ng/cell (n = 5).

uPA Accumulation in Conditioned Medium—MCF-7 cells were incubated for 24 h in RPMI, without serum, in the presence or absence of 0.1 μM GST-RAP. Conditioned medium (CM) was recovered and concentrated 30-fold using Centricon concentrators with 10-kDa exclusion filters (Amicon). To detect plasminogen activator, concentrated CM was diluted 1:10 into solutions that contained 1.0 μM Glu1-iplasminogen and 0.5 mM Val-Leu-Lys-7-amido-4-methylcoumarin (VLK-AMC). Fluorescence emission at 460 nm (excitation at 380 nm) was monitored for 1 h at 25 °C. These tracings were converted using a first derivative function so that the resulting plots showed relative plasmin concentration at 25 °C. These tracings were converted using a first derivative function so that the resulting plots showed relative plasmin concentration at 25 °C. These tracings were converted using a first derivative function so that the resulting plots showed relative plasmin concentration at 25 °C.
at 37 °C. uPA-PAl-1 complex (10 nM), which was pre-formed by reacting tcuPA with PAI-1 at a 1:1 molar ratio, or DIP-tcuPA (10 nM) was added to the medium and incubation was allowed to proceed for 20 min at 37 °C. The cultures were then placed on ice and washed 3 times with ice-cold EBSS, 10 mM HEPES, pH 7.4. A mild acid wash was then performed to dissociate uPA-PAl-1 complex or DIP-tcuPA (21, 31). The acid wash sequence was: 50 mM glycine-HCl, 100 mM NaCl, pH 3.0, for 10 min; 0.5 mM NaCl, pH 7.5; and then three washes with ice-cold EBSS, 10 mM HEPES, pH 7.4. Cell surface uPAR was quantitated by measuring specific binding of 125I-DIP-uPA (10 nM).

**Kinetics of uPAR Catabolism and the Role of the VLDLr—**

MCF-7 cells were cultured for 12 h in methionine-free Dulbecco’s modified Eagle’s medium and then for 24 h in methionine-free Dulbecco’s modified Eagle’s medium supplemented with [35S]methionine (10 μCi/ml). The cultures were chased for 1 h with methionine-containing complete medium, washed, and incubated in fresh medium, in the presence of either uPA from human plasma or uPA-PAl-1 complex (5 mM) and GST-RAP (200 nM). After various times, the cells were solubilized in 10 mM HEPES, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 100 mM N-octyl glucoside, 10 μg/ml E-64, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, pH 7.4. [35S]Methionine-labeled uPAR was recovered from equal amounts of each cell extract by immunoprecipitation with uPAR-specific antibody (42 μg/ml) and GST-RAP (200 nM). Anti-body-antigen complexes were isolated with Protein A-agarose (Sigma). In control experiments, glycoprotein CD44 was recovered from the same cell extracts by immunoprecipitation using an antibody from Endogen (41) and rabbit anti-mouse IgG (Jackson Immunoresearch Laboratories). Immunoprecipitated proteins were subjected to SDS-PAGE and electrotransferred to nitrocellulose. [35S]Methionine-labeled uPAR or CD44 was quantitated by PhosphorImager analysis. Western blot analysis was performed to confirm the identity of the immunoprecipitated proteins.

**Analysis of MAP Kinase Activation—**

MCF-7 cells were cultured for 3 days in the presence of 200 nM GST-RAP or vehicle. Activation of ERK1 and ERK2 was then determined as described previously (32). Briefly, the medium was aspirated and replaced with cold phosphate-buffered saline containing 1 mg/ml sodium orthovanadate. The cells were extracted at 4 °C with 1% (v/w) vialbumin and 10 mM EDTA. Antibody-antigen complexes were isolated with Protein G-agarose (Sigma). In control experiments, glycoprotein CD44 was recovered from the same cell extracts by immunoprecipitation using an antibody from Endogen (41) and rabbit anti-mouse IgG (Jackson Immunoresearch Laboratories). Immunoprecipitated proteins were subjected to SDS-PAGE and electrotransferred to nitrocellulose. [35S]Methionine-labeled uPAR or CD44 was quantitated by PhosphorImager analysis. Western blot analysis was performed to confirm the identity of the immunoprecipitated proteins.

**Degradation of GST-RAP by MCF-7 Cells—**

Studies analyzing the binding of 125I-GST-RAP to MCF-7 cells are shown in Fig. 2. Binding was specific and saturable; the Scatchard transformation (not shown) was linear ($r^2 = 0.94$), suggesting that a single class of binding sites was detected. The $K_d$ was 6 nM and the $B_{max}$ was 110 fmol/mg of cell protein (28). Assuming that RAP binds exclusively to the VLDLr, in MCF-7 cells, and that there is one RAP-binding site per VLDLr, then the $B_{max}$ corresponds to 60,000 copies of cell surface VLDLr/cell. RAP binding studies should be interpreted with caution since RAP has been reported to bind to cell surface sites that are independent of the LDL receptor family (45). To study MCF-7 cell VLDLr function, we examined the kinetics of RAP degradation, using a nearly saturating concentration of 125I-GST-RAP (10 nM). RAP degradation is mediated only by receptors in the LDL receptor family (1, 13). Fig. 2, panel B, shows that after an anticipated lag phase, specific RAP degradation occurred at a nearly constant rate ($1.4 ± 0.2 \times 10^4$ molecules/cell-h) for at least 6 h. Non-radiolabeled RAP (200 nM) inhibited 125I-GST-RAP degradation by 95 ± 3%.

**RESULTS**

**VLDLr Expression in Breast Cancer Cell Lines—**

Previous studies have demonstrated that MCF-7 cells express VLDLr, but not LRP or gp330/megalin (3, 44). Our ligand blot analyses confirmed this result (Fig. 1). When GST-RAP was incubated with proteins that were extracted from MCF-7 cells and immobilized on nitrocellulose, a single band with an apparent mass of 105 kDa was detected. The mobility of this band was identical to that of the VLDLr, as determined by immunoblot analysis. MDA-MB-435 cells, which express increased levels of uPAR compared with MCF-7 cells (40), also expressed VLDLr but no other members of the LDL receptor family, as determined by RAP ligand blotting. As a control, we prepared extracts of human embryonic fibroblasts. GST-RAP bound to a single high molecular mass band in the human embryonic fibroblast extracts. The mobility of this band was identical to that of the LRP heavy chain, as determined by immunoblot analysis. VLDLr was not detected in the human embryonic fibroblast extracts.
Regulation of Cell Surface uPAR Expression by the VLDLr—Evidence for VLDLr-mediated uPAR Endocytosis—To determine whether the VLDLr mediates uPAR endocytosis, MDA-MB-435 cells were pretreated with RAP or vehicle for 15 min and then challenged with DIP-uPA or uPA-PAI-1 complex for 20 min at 37 °C. After acid washing the cells to remove uPAR-associated ligands, the level of cell surface uPAR was determined by measuring the binding of 125I-DIP-uPA (10 nM). Without prior ligand challenge, MDA-MB-435 cells bound 115 ± 5 fmol of DIP-uPA per mg of cell protein (Fig. 4). Cells that were treated with RAP for 20 min demonstrated unchanged specific 125I-DIP-uPA binding, as expected. 125I-DIP-uPA binding was also unchanged when cells were pretreated with nonradiolabeled DIP-uPA. This result confirms that DIP-uPA does not promote rapid uPAR internalization (30). By contrast, MDA-MB-435 cells, which were pretreated with uPA-PAI-1 complex, demonstrated a 90% decrease in 125I-DIP-uPA binding. Thus, uPA-PAI-1 complex promoted uPAR endocytosis in MDA-MB-435 cells. When the cells were pretreated with RAP and then exposed to uPA-PAI-1 complex, uPAR endocytosis was blocked by 80%. These results suggest that the VLDLr is required for uPA-PAI-1 complex-mediated uPAR endocytosis in MDA-MB-435 cells.

The VLDLr Promotes uPAR Catabolism—For VLDLr-mediated uPAR endocytosis to decrease the steady-state level of cell surface uPAR, the VLDLr must promote uPAR catabolism. To determine whether the VLDLr mediates uPAR endocytosis, MDA-MB-435 cells were pretreated with RAP or vehicle for 15 min and then challenged with DIP-uPA or uPA-PAI-1 complex for 20 min. As shown in Fig. 3, panel A, specific binding of 125I-DIP-uPA (10 nM) to RAP-treated MCF-7 cells (●) and vehicle-treated MCF-7 cells (○) was determined. Panel B, MCF-7 cells were cultured in GST-RAP or in vehicle for 3 days. Specific binding of 125I-DIP-uPA was then studied. Specific binding isotherms are shown for RAP-treated MCF-7 cells (●) and vehicle-treated MCF-7 cells (○). Each point represents the mean of results from four separate experiments, each with duplicate determinations.

FIG. 2. Binding and cellular degradation of GST-RAP by MCF-7 cells. In panel A, MCF-7 cells were incubated with increasing concentrations of 125I-GST-RAP for 4 h at 4 °C. The specific binding isotherm is shown. In panel B, degradation of GST-RAP by MCF-7 cells was determined by measuring trichloroacetic acid soluble radioactivity in the medium. Specific GST-RAP degradation is plotted as a function of time. Each point represents the mean of results from four separate experiments, each with duplicate determinations.

FIG. 3. Binding of DIP-uPA to MCF-7 cells cultured in RAP. Panel A, MCF-7 cells were cultured in the presence or absence of GST-RAP for 1–5 days. Specific binding of 125I-DIP-uPA (10 nM) to RAP-treated MCF-7 cells (●) and vehicle-treated MCF-7 cells (○) was determined. Panel B, MCF-7 cells were cultured in GST-RAP or in vehicle for 3 days. Specific binding of 125I-DIP-uPA was then studied. Specific binding isotherms are shown for RAP-treated MCF-7 cells (●) and vehicle-treated MCF-7 cells (○). Each point represents the mean of results from four separate experiments, each with duplicate determinations.
surface uPAR, either the distribution of uPAR between cell surface and intracellular pools must be shifted or a fraction of the internalized uPAR must be catabolized. To study uPAR catabolism, MDA-MB-435 cells were metabolically labeled with $^{[35S]}$methionine. The cells were then cultured in the presence or absence of RAP. At various times, uPAR was recovered by immunoprecipitation. Representative immunoprecipitates are shown in Fig. 5, panel A. We confirmed that the major band was uPAR by immunoblot analysis (results not shown). The minor bands are probably proteins which co-immunoprecipitate with uPAR, as previously demonstrated by others (46–49).

The kinetics of uPAR catabolism are shown in Fig. 5, panel B. With both RAP-treated and control cells, linear graphs were obtained when the amount of labeled uPAR was plotted against time, according to the equation: $\log \left(\frac{a_{t=0}}{a_{t=t_1}}\right) = \frac{t}{t_1}$ (23). This result suggests that uPAR catabolism follows first-order kinetics. RAP significantly decreased the rate of uPAR catabolism. In the absence of RAP, the rate constant for uPAR catabolism was $8.5 \times 10^{-2}\text{h}^{-1}$, corresponding to a uPAR survival $t_1$ of 8.2 h. In the presence of RAP, the uPAR catabolism rate constant was $4.1 \times 10^{-2}\text{h}^{-1}$, corresponding to a $t_1$ of 16.9 h. Thus, RAP treatment caused an approximate doubling of the uPAR survival $t_1$.

As a control, we studied the catabolism of $^{[35S]}$methionine-labeled CD44. CD44 is a glycoprotein receptor which is expressed by MDA-MB-435 cells (50). When cells were cultured for 24 h in the absence of RAP, 70 ± 5% of the $^{[35S]}$methionine-labeled CD44 remained ($n = 4$). In the presence of RAP, CD44 survival was unchanged; after 24 h, 68 ± 4% of the labeled CD44 remained. Thus, the effects of RAP on uPAR survival in MDA-MB-435 cells are specific.

Our results demonstrated that uPA-PAI-1 complex promotes uPAR internalization. To determine whether uPA-PAI-1 complex accelerates uPAR catabolism, we cultured metabolically labeled MDA-MB-435 cells in the presence of 0.5 nM uPA-PAI-1 complex. As shown in Fig. 5, panel C, the rate of uPAR catabolism was substantially increased; the first-order rate constant was $3.8 \times 10^{-1}\text{h}^{-1}$, which corresponds to a $t_1$ of 1.8 h. When RAP was added to the culture medium with uPA-PAI-1 complex, uPAR catabolism was inhibited; the rate constant was $8.9 \times 10^{-2}\text{h}^{-1}$ and the $t_1$ was 7.8 h. Thus, recycling of internalized uPAR is not 100% efficient. Instead, a significant fraction of the uPAR, which is internalized via a pathway that requires both uPA-PAI-1 complex and the VLDLr, is targeted for degradation.

**Regulation of MCF-7 Cell Motility by the VLDLr and the Role of the uPA/uPA System—Cellular migration was studied using serum-coated Transwell membranes (Fig. 6, panel A). When MCF-7 cells were not pre-cultured in RAP or treated with uPA, 80 ± 18% cells penetrated the membranes within 6 h. ScuPA (1 nM) promoted MCF-7 cell migration, as previously reported (32). Pre-culturing in RAP for 3 days also increased MCF-7 cell motility (2.2 ± 0.1-fold, $n = 8$, $p < 0.001$); however, when RAP was incubated with the cells only while the migration assay was underway (no pre-culturing), cellular motility was unchanged (results not shown). ScuPA (1 nM) increased the motility of MCF-7 cells that had been pre-cultured in RAP still further (4.4 ± 0.3-fold, $n = 8$). When the Transwell membranes were coated with purified vitronectin instead of serum, identical results were obtained (results not shown). In control experiments, we demonstrated that GST-RAP does not affect MCF-7 cell proliferation.**

In our previous study (21), results were presented to suggest...
that autocrine activation of uPAR by endogenously produced uPA may be responsible for the increased motility of LRP-deficient MEFs. To determine whether the uPA/uPAR system is responsible for the increased motility of RAP-treated MCF-7 cells, we performed migration assays in the presence of a uPA-specific antibody that blocks uPA binding to uPAR (25 μg/ml). As shown in Fig. 6, the antibody had no effect on the motility of control cells, suggesting that autocrine activation of uPAR is not significant when the VLDLr is active. By contrast, the antibody completely neutralized the activity of exogenously added uPA, confirming the effectiveness of the antibody in this system. uPA-specific antibody also inhibited the motility of RAP-treated cells; these cells migrated comparably to cells that had not been precultured in RAP. In control experiments, non-immune mouse IgG (25 μg/ml) did not affect the migration of control MCF-7 cells or cells that had been cultured in RAP. Furthermore, non-immune IgG did not inhibit the response to exogenously added uPA. These results suggest that the increase in MCF-7 cell motility, which is induced by culturing in RAP, results from the activity of endogenously produced uPA.

Previous studies have either failed to detect uPA expression by MCF-7 cells or have detected very low levels (32, 40). Thus, new experiments were performed to determine whether exogenously added uPA, at very low concentrations, promotes MCF-7 cell motility. Fig. 7 shows that 50 pM uPA induced a statistically significant increase in MCF-7 cell motility (p < 0.0001). The activity of 50 pM uPA was entirely neutralized by uPA-specific antibody and by uPAR-specific antibody 399R. In separate control experiments, we confirmed that antibody 399R completely inhibits the specific binding of 125I-DIP-uPA to MCF-7 cells. We have also shown that antibody 399R inhibits MAP kinase activation in response to uPA. Thus, these experiments demonstrate that uPA, at low concentrations, increases MCF-7 cell motility by a mechanism that requires binding to uPAR.

MAP Kinase Is Activated in RAP-treated MCF-7 Cells—uPA activates ERK1 and ERK2 in MCF-7 cells and this response is necessary for uPA-promoted migration (32). Since RAP-treated MCF-7 cells have increased levels of cell surface uPAR and accumulate increased amounts of uPA, we undertook experiments to determine whether the extent of activation of ERK1 and/or ERK2 is increased in these cells as well. MCF-7 cells were cultured in standard FBS-supplemented medium, in the presence or absence of 200 nM GST-RAP for 3 days, and isolated without adding exogenous stimuli 12 h after the last change in medium. Activated ERK1 and ERK2 were detected by im-

\[ \text{Fig. 6. Migration of RAP-treated MCF-7 cells and the role of the uPA/uPAR system. Panel A, MCF-7 cells that were precultured in RAP for 3 days and control cells that were cultured in vehicle (C) were allowed to migrate in serum-coated Transwell chambers in the presence or absence of scuPA, as shown. uPA-specific antibody (uPA-Ab) or non-immune IgG were also added as indicated. The number of cells migrating across the membrane is expressed as a percentage of that observed with control cells (no RAP pre-treatment or scuPA exposure). Each bar represents the results of four separate experiments with triplicate determinations. Panel B, to study uPA secretion by MCF-7 cells, cultures were incubated in serum-free medium for 24 h, in the presence or absence of RAP. CM was collected and concentrated. The concentrated CM was incubated with plasminogen and VLLK-AMC. Substrate hydrolysis is shown. "Background" shows the results obtained when plasminogen was activated using un-conditioned medium.} \]
munoblot analysis. A single experiment, in which six separate cultures were analyzed, is shown in Fig. 8, panel A. Culturing in RAP increased the levels of activated ERK1 and ERK2 by $2.8 \pm 0.5$- and $2.6 \pm 0.6$-fold, respectively ($n = 6$). Furthermore, PD098059, a selective inhibitor of MAP kinase kinase (MEK), inhibited the migration of RAP-treated cells without affecting the migration of control cells (Fig. 8, panel B). These studies suggest that a MAP kinase-dependent signaling pathway may be selectively activated in RAP-treated MCF-7 cells and that this pathway is required for enhanced motility on serum-coated surfaces.

DISCUSSION

Previous studies have shown that LRP and the VLDLr express similar activities as receptors for free uPA and uPAR-associated uPA-PAI-1 complex (3, 7, 18, 29). In this study, we demonstrated that the VLDLr, like LRP (30), mediates the endocytosis of uPAR through an indirect mechanism that depends on uPA-PAI-1 complex. When MDA-MB-435 cells were treated with uPA-PAI-1 complex at 37 °C, uPAR was rapidly internalized by a RAP-inhibited pathway. The most likely explanation for this result is that uPA-PAI-1 complex bridges the VLDLr and uPAR, on the cell surface, so that uPAR is internalized in clathrin-coated pits with the VLDLr. uPA binding to uPAR causes conformational changes in the receptor which could also be involved in the VLDLr interaction (51). In our 20-min endocytosis assays, DIP-uPA did not promote uPAR internalization, consistent with previous studies demonstrating that uPA-uPAR complex is stable on the cell surface (52, 53). However, we cannot rule out the possibility that uPA affects the rate of internalization of uPAR over a period of days.

In cells that express LRP, internalized uPAR is transferred to acidified endosomes where uPA-PAI complex is dissociated before the uPAR recycles back to the cell surface (18, 31, 54). If recycling is 100% efficient, the expected outcome of this pathway is to re-generate un-liganded uPAR on the cell surface, which is available to bind free uPA (1, 18, 31). In MCF-7 cells that were cultured for at least 3 days in RAP, the level of cell surface uPAR was increased. To explain these results, we studied the catabolism of metabolically labeled uPAR. When cultured in the presence of uPA-PAI-1 complex, MDA-MB-435 cells rapidly digested uPAR ($t_1/2$ of 1.8 h versus 8.2 h) and this process was inhibited by RAP, indicating that at least a fraction of the uPAR, which is internalized in association with the VLDLr, is degraded. RAP also prolonged the survival of uPAR under standard cell culturing conditions, in the absence of
exogenously added uPA-PAI-1 complex. At this time, we do not know whether this process depended on the formation of uPA-PAI-1 complex from endogenously produced uPA and PAI-1.

uPAR was still catabolized, albeit at a slower rate, when MDA-MB-435 cells were cultured in the presence of RAP. The residual catabolism may reflect bulk plasma membrane turnover, enzymatic release of uPAR from the cell surface, or the function of receptors outside the LDL receptor family. Nykjaer et al. (49) recently demonstrated that the mannose 6-phosphate/insulin-like growth factor-II receptor interacts with cell surface uPAR and targets uPAR for catabolism in lysosomes. Thus, cell surface uPAR levels may be controlled by diverse plasma-membrane interactions.

MCF-7 cells that were cultured in the presence of RAP for 3 days demonstrated increased motility on serum- or vitronectin-coated surfaces. When the cells were not precultured in RAP and allowed to migrate in Transwell chambers in the presence of RAP, motility was unchanged. Thus, the mechanism by which RAP promotes MCF-7 cell motility probably requires a change in the phenotype of the cell, which occurs slowly, as opposed to the more simple mechanism in which motility is influenced entirely by agents that accumulate at increased levels in solution when the VLDLr is blocked. Importantly, uPA-specific antibody inhibited the migration of MCF-7 cells that were exposed to exogenous uPA or precultured in RAP. The same antibody had no effect on the motility of control MCF-7 cells. These results provide evidence that autocrine activation of uPAR is responsible for the increase in the motility of RAP-treated cells. Apparently, in control cells, the level of cell surface uPAR and/or the amount of uPAR which accumulates in the medium are too low to significantly affect motility.

The ability of the VLDLr to regulate the uPA/uPAR system may be considered in relation to multicellular tissues such as intact breast cancers. If the VLDLr is expressed by malignant epithelial cells, as previously demonstrated (3, 44), it should regulate pericellular uPA levels, irrespective of whether the uPA is synthesized by cancer cells or benign stromal cells. Alternatively, our results suggest that uPAR regulation depends on co-expression of uPAR and the VLDLr by the same cell type. LRP, which is expressed by macrophages and fibroblasts, may also regulate uPA levels in the microenvironment of the cancer but will not regulate cell surface uPAR levels in the malignant cells, if these cells are LRP negative. Thus, members of the LDL receptor family may regulate the activity of the uPA/uPAR system within cancers by both autocrine and paracrine mechanisms.

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