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Embryonic Fibroblasts That Are Genetically Deficient in Low Density Lipoprotein Receptor-related Protein Demonstrate Increased Activity of the Urokinase Receptor System and Accelerated Migration on Vitronectin*

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Low density lipoprotein receptor-related protein (LRP) mediates the endocytosis of diverse ligands, including urokinase plasminogen activator (uPA) and its receptor, uPAR, which have been implicated in cellular migration. The purpose of this study was to determine whether LRP affects cellular migration. Murine embryonic fibroblasts (MEF) that are LRP-deficient due to targeted gene disruption and exotoxin selection (MEF-2), heterozygous fibroblasts (PEA-10), and wild-type fibroblasts (MEF-1) were compared. When cultures were denuded of cells in a 1-mm-wide strip, all three cell types migrated into the denuded area. The MEF-2 cells migrated nearly twice as rapidly as the MEF-1 cells or PEA-10 cells. The difference in migration velocity was duplicated in culture wells that were precoated with serum or vitronectin and partially duplicated in wells coated with fibronectin but not in wells coated with type I collagen or Matrigel. uPA was detected in MEF-2 conditioned medium (CM) at a concentration of 0.30 ± 0.02 nM, which was 13-fold higher than the level detected in MEF-1 CM or PEA-10 CM, suggesting one potential mechanism for the enhanced migration of MEF-2 cells. uPAR was also increased on MEF-2 cells by 4–5-fold, as determined by PI-PLC release, and by 2.5-fold, as determined by a uPA/uPAR activity assay. Mannosamine treatment, which down-regulates cell-surface uPAR, decreased MEF-2 migration by 40% without significantly affecting MEF-1 migration. MEF-2 CM, which is uPA-rich, increased the rate of MEF-1 migration, and MEF-1 CM did not. These studies demonstrate alterations in cellular migration and in the activity of the uPA/uPAR system which accompany complete deficiency of LRP expression in fibroblasts. We propose that uPA and uPAR form an autocrine loop for promoting fibroblast migration and that LRP counteracts the activity of this system.

Cellular migration occurs in many normal and pathological processes, including bone remodeling, embryogenesis, angiogenesis, invasion and metastasis of cancer cells, and neointimal formation in atherosclerosis (1). In wound healing, fibroblasts, endothelial cells, and tissue macrophages migrate into the site of injury (2). Likewise, extravasation and migration of leukocytes into tissue spaces is critical for defense against microbial challenge (1, 2). The process of migration involves a cycle of regulated adhesion and disadhesion events, involving cell-surface receptors and the extracellular matrix (ECM)[1] (1, 3–5). High affinity binding of cells to ECM is mediated by integrins, a class of heterodimeric transmembrane proteins that link the cytoskeleton to the extracellular environment (6, 7). Integrins have been strongly implicated in cellular migration (1, 2, 5). For example, expression of $\alpha_v\beta_3$ is correlated with angiogenesis and migration of vascular smooth muscle cells (8, 9). Embryonic stem cells that are β_1-deficient fail to migrate (10), and the absence of β_2 subunits in the disease, leukocyte adhesion deficiency syndrome, results in defective migration of neutrophils across the endothelium and into the tissue spaces (11).

A second set of proteins involved in the regulation of cellular migration includes the serine proteinase, urokinase plasminogen activator (uPA), and its receptor (uPAR). uPAR is a 55–65-kDa glycosylphosphatidylinositol (GPI)-anchored glycoprotein (12). uPA that is bound to uPAR activates cell-associated plasminogen and thereby initiates a proteolytic cascade that causes localized digestion of ECM (13). This process is important for the movement of cells through tissue boundaries and has been shown to play a critical role in the migration of neoplastic and non-neoplastic cells (13–17). uPAR is localized in focal contacts in stationary cells (18), whereas in migrating cells, uPAR localizes primarily to the leading edge (19, 20). This shift in uPAR subcellular distribution, in migrating cells, allows focused digestion of ECM only where it is required for cellular penetration of tissue boundaries.

In addition to promoting activation of cell-surface proteinases, uPAR expresses activities that are independent of uPA proteolytic activity. Ligation of uPAR with uPA or with uPA derivatives that lack enzyme activity promotes protein kinase activity (21–23), c-*fos* gene expression (22), macrophage cysteine- and metalloproteinase activity (24, 25), monocyte adhesion (24, 26), and cellular migration in the absence of ECM barriers (21). uPAR may also affect cellular adhesion and migration independently of ligand (20, 27), and this is explained,

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[1] The abbreviations used are: ECM, extracellular matrix; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; GPI, glycosylphosphatidylinositol; LRP, low density lipoprotein receptor-related protein; PAI-1, plasminogen activator inhibitor-1; MEF, murine embryonic fibroblasts; scuPA, single-chain uPA; tcuPA, two-chain uPA; VLK-*p*NA, H-D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide; PI-PLC, phosphatidylinositol-specific phospholipase C; RAP, receptor-associated protein; PGAD, 3-phosphoglyceraldehyde dehydrogenase; CM, conditioned medium; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

at least in part, by the fact that uPAR functions as a receptor for vitronectin (28–30). uPAR has been recovered from cells in multimeric complexes with protein kinases and integrins (21, 23). Furthermore, physical association of uPAR with integrins regulates integrin function and apparently promotes cellular adhesion to vitronectin (27). The uPA inhibitor, plasminogen activator inhibitor-1 (PAI-1), binds to vitronectin and inhibits binding of uPAR (29) and $\alpha_V\beta_3$ (31) to vitronectin. Thus, uPA/uPAR and the integrins may form an interdependent system for the regulation of cellular adhesion and migration.

Low density lipoprotein receptor-related protein (LRP) is a multifunctional receptor that mediates binding and endocytosis of free uPA (30, 32) and uPAR-associated uPA·PAI-1 complex (33, 34). When uPAR-associated uPA·PAI-1 complex undergoes LRP-mediated endocytosis, the uPAR may be internalized as well (35). Thus, LRP may shuttle large macromolecular complexes from the cell surface into intracytoplasmic pools. The goal of the present investigation was to determine whether LRP regulates cellular migration. We hypothesized that LRP might regulate migration based on its function as an endocytic receptor for uPA/uPAR; however, other LRP ligands, including thrombospondin, activated α_2-macroglobulin, and various Serpin-proteinase complexes (36) could be also be involved. We chose, for these experiments, to examine murine embryonic fibroblasts that are genetically deficient in LRP (MEF-2). Willnow and Herz (37) initially isolated these cells from embryos lacking one functional LRP allele due to targeted gene disruption. The second LRP allele was then eliminated by a gene conversion event under *Pseudomonas* exotoxin A selection.

The results of our studies demonstrate that LRP-deficient fibroblasts migrate approximately 2-fold faster than wild-type murine embryonic fibroblasts (MEF-1), when the migration surface is vitronectin-coated. The MEF-2 cells migrated about 50% faster than MEF-1 cells on fibronectin-coated plates; however, the two cell types migrated equivalently on Matrigel- and type I collagen-coated plates. The LRP-deficient fibroblasts accumulated greatly increased levels of single chain uPA (scuPA) in conditioned medium and expressed increased levels of cell-surface uPAR. Evidence is presented to suggest that the enhanced activity of the uPA/uPAR system, in the MEF-2 cells, is responsible for the increased rate of migration of these cells on vitronectin-coated surfaces. We propose that LRP plays a critical role as a regulator of uPA/uPAR-enhanced cellular migration.

MATERIALS AND METHODS

Proteins and Reagents—[Glu^1]Plasminogen was purified from human plasma by the method of Deutsch and Mertz (38). Vitronectin was purified from human plasma by the method of Yatohgo *et al.* (39). Mannosamine, amiloride, *p*-nitrophenyl *p*′-guanidinobenzoate HCl, and type I collagen were from Sigma. Fibronectin was from Promega and the ECM preparation from EH Sarcoma cells, Matrigel®, was from Collaborative Research. H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide (VLK-*p*NA) was from Chromogenix, and H-Glu-Gly-Arg-chloromethyl ketone was from Bachem. Phosphatidylinositol-specific phospholipase C (PI-PLC) was from Boehringer Mannheim. Rat receptor-associated protein (RAP) was expressed as a glutathione *S*-transferase fusion protein and purified as described previously (40). The glutathione *S*-transferase-RAP expression construct was kindly provided by Dr. Joachim Herz (Southwestern Medical Center, Dallas, TX). RAP binds to LRP and inhibits binding of all other known LRP ligands (36). The K_D for RAP binding to LRP is about 5–20 nM (37, 40, 41); intact glutathione *S*-transferase-rat RAP binds to murine LRP without loss of affinity (37). Polyclonal antibody specific for uPAR, antibody αD2+3, was raised in rabbits that were immunized with a purified chymotryptic fragment of soluble human uPAR that contains domains 2 and 3 of the receptor.

Cell Lines—MEF-1 and MEF-2 cells were obtained as a gift from Drs. Thomas Willnow and Joachim Herz; however, the MEF-2 cells are now available from the ATCC (Rockville, MD). MEF-1 cells are wild-type murine embryonic fibroblasts derived from the same mouse strain as the MEF-2 cells. PEA-10 cells were obtained from the ATCC. PEA-10 cells are also embryonic fibroblasts obtained from the same mouse strain as the MEF-2 cells but containing a single disrupted LRP allele (37). LRP expression by PEA-10 cells is decreased by 50% compared with wild-type MEF-1 cells, as determined by the binding and degradation of various LRP ligands (37). All cell cultures were maintained in DMEM with 10% fetal bovine serum (FBS).

Cellular Migration Assays—Migration of MEF-1, MEF-2, and PEA-10 cells was studied according to the method of Glass *et al.* (42). Cells were plated in 35-mm culture wells (Amersham Life Sciences, Inc.) and grown until 95% confluent. Since the medium contained serum, the cell culture wells became coated with vitronectin, which served as the major attachment and spreading factor (43). To initiate an experiment, a 0.8–1.0-mm-wide band down the center of the culture was denuded of cells using a rubber policeman. The cells were then washed with DMEM, 10% FBS and returned to the incubator, at 37 °C, in fresh medium. The external surface of each well was marked with a pen so that the identical field of cells could be viewed by phase contrast microscopy and photographed at different times. Photographs were taken immediately after denuding the cultures and at various times thereafter. In each photograph, distances between the two opposing lead edges of cellular migration (at opposite sides of the denudation area) were measured at eight evenly spaced intervals, using a MicroPlan II Image Analyzer (Computer Systems). Photographs taken at different times were matched using the ink marks. Migration distance (M) was then calculated as shown in Equation 1.

$$M = c(D_{t=x} - D_{t=0})/2 \quad \text{(Eq. 1)}$$

$D_{t=x}$ is the average distance between the lead edges of migration at a specific time; $D_{t=0}$ is the average distance immediately after denudation. c is a constant used to convert migration distances into actual units (microns), based on the power of microscopic magnification and the enlargement used to generate the prints.

Some migration experiments were performed in 35-mm bacterial culture dishes (Falcon) precoated with various extracellular matrix proteins, including vitronectin (20 μg/cm^2), type I collagen (20 μg/cm^2), fibronectin (5 μg/cm^2), a 1:20 dilution of Matrigel, or 10% FBS. Precoating was allowed to proceed for 2 h at 37 °C. The wells were then blocked with bovine serum albumin (10 mg/ml) for 2 h at 37 °C and sterilized overnight under UV light. Cells were added to the wells and grown to 95% confluence in DMEM with 10% FBS. Migration was then studied as described above except for the use of serum-free medium (DMEM with 20 ng/ml platelet-derived growth factor-BB) after denudation. The bacterial culture dishes promote binding of most proteins in general, whereas tissue culture-treated plastic preferentially adsorbs vitronectin.

To test for the contribution of uPAR to MEF migration, cultures were preincubated with 10 mM mannosamine (Sigma) for 6 h at 37 °C. Denudation was then performed, and migration was studied in DMEM with 10% FBS + 10 mM mannosamine.

To test for the contribution of uPA to MEF migration, conditioned medium (CM) from MEF-1 cells or from MEF-2 cells was concentrated 20-fold, using a Centricon concentrator with a 10-kDa exclusion filter (Amicon), and then diluted 1:10 into MEF-1 cultures. In a second protocol, MEF-1 and MEF-2 cells were washed with mild acid to dissociate uPAR-associated uPA prior to performing migration assays. The acid-washing protocol involved incubating the cultures with 50 mM glycine HCl, 100 mM NaCl, pH 3.0, for 3 min at 25 °C. The medium was rapidly neutralized by diluting a solution of 0.5 M HEPES, 0.1 M NaCl, pH 7.5, 1:5, directly into the acid-containing culture wells. The cells were then washed and migration assays were performed under serum-free conditions (DMEM + 20 ng/ml platelet-derived growth factor-BB).

Northern Blot Analyses—The 2-kilobase pair complete coding sequence of the mouse uPA cDNA (obtained from ATCC) was excised with *Xba*I/*Sma*I to generate the probe for Northern blots. The probe for PAI-1 was a 2-kilobase pair *Sac*I/*Eco*RI fragment of the mouse PAI-1 cDNA, kindly provided by Dr. Michael Cole (Princeton University). The probe for mouse uPAR was generated by reverse transcription and the polymerase chain reaction using total cellular RNA from the mouse macrophage-like cell line, RAW 264.7, as a template and the primers designed by Hayden and Seeds (44). Reverse transcription was carried out using the GeneAmp RNA polymerase chain reaction kit (Perkin-Elmer). Total RNA (1 μg), RNasin (1.0 unit), antisense uPAR primer (0.25 μM), reverse transcriptase (2.5 units), and dNTPs (each at 1 mM) were reacted at 42 °C for 15 min, at 99 °C for 5 min, and then on ice for 5 min. The polymerase chain reaction amplification reaction, containing reverse transcription products, 2.5 units of *Taq* polymerase, and 1.5 mM $MgCl_2$, pH 8.5, was carried out for 30 cycles at 94 °C for 1 min, 55 °C for

1 min, and 72 °C for 3 min.

Total RNA was isolated from MEF-1 and MEF-2 cells using Trizol reagent (Life Technologies, Inc.), according to the manufacturer's instructions. Equal amounts of RNA (20 μg) were denatured with glyoxal, subjected to electrophoresis in 1% (w/v) agarose gels, and electrotransferred to Zeta-Probe membranes (Bio-Rad). Following electroblotting, the RNA was cross-linked by UV irradiation and prehybridized in 50% formamide, 5 × SSPE, 2 × Denhardt's reagent, 0.5% SDS, and 1.0% salmon testes DNA, at 42 °C for 18–24 h. The cDNA probes were labeled with [α-^{32}P]dCTP (DuPont NEN), using the Random Primers Labeling System (Life Technologies, Inc.), and hybridized with membrane-immobilized RNA for 24 h at 42 °C. As a control for load, the membranes were also hybridized with [α-^{32}P]dCTP-labeled 3-phosphoglyceraldehyde dehydrogenase (PGAD) cDNA (45). After hybridization, membranes were washed with 0.1 × SSPE, 1.0% SDS at 65 °C. Specific hybridization of probes was analyzed by PhosphorImager analysis or by exposure to x-ray film, followed by densitometry.

Determination of Active uPA in Fibroblast-conditioned Medium—MEF-1, MEF-2, and PEA-10 cells were incubated for 24 h in DMEM without serum at 37 °C. The conditioned medium (CM) was then recovered and concentrated 20-fold using Centricon concentrators with 10-kDa exclusion filters (Amicon). To determine uPA activity, CM was diluted 1:15 into a spectrophotometer cuvette containing 0.94 μM [Glu^1]plasminogen and 0.65 mM VLK-*p*NA in 20 mM sodium phosphate, 150 mM NaCl, pH 7.4. Amiloride (1 mM) was also present in solution with the plasminogen and VLK-*p*NA in some experiments. Amiloride is a selective inhibitor of uPA that does not inhibit tissue-type plasminogen activator (46).

Immediately after adding CM, the absorbance at 406 nm was measured. Subsequent measurements were made at 15-s intervals for up to 2000 s. The temperature was maintained at 22 °C throughout the study. Absorbance measurements were plotted as a function of time and then transformed using a first-derivative function to yield plots of $dA_{406\ nm}/dt$ against time (t). Kinetic constants for the hydrolysis of VLK-*p*NA by plasmin (k_{cat} of 12 s^{-1}; K_m of 180 μM) were used to convert y axes from $dA_{406\ nm}/dt$ to plasmin concentration (47). Maximum velocities of plasmin formation were measured only after an initial lag-phase of 1000 s, the cause of which is described under "Results." To assess the state of the plasminogen in the activation mixture at 1000 s, samples of the activation solution were treated with *p*-nitrophenyl *p′*-guanidinobenzoate HCl and H-Glu-Gly-Arg-chloromethyl ketone to inactivate any plasmin and uPA. The samples were then subjected to SDS-polyacrylamide gel electrophoresis under reducing and nonreducing conditions. This analysis demonstrated that the fraction of the plasminogen, which had been activated by CM at 1000 s, was undetectable by Coomassie-staining (results not shown). Conversion of [Glu^1]plasminogen into the more readily activated derivative, [Lys^{78}]plasminogen, was also undetectable. Thus, we used kinetic constants for the activation of [Glu^1]plasminogen by two-chain uPA (tcuPA) (k_{cat} of 3.6 s^{-1}; K_m of 78 μM) to convert our measured velocities of plasminogen activation into actual tcuPA concentrations (48). The presented values are estimates since the original kinetic parameters (48) were determined with recombinant human tcuPA and the CM samples contain murine tcuPA. Also, in continuous assays, such as that performed here, plasmin substrates can affect velocities of plasminogen activation (49); however, these potential sources of error were identical for the analysis of each CM sample. Therefore, the relative amounts of uPA in the CM samples should not have been affected. In control experiments, medium which had not been exposed to cells (DMEM) did not activate [Glu^1]plasminogen. When [Glu^1]plasminogen was omitted from the CM-containing activation solutions, VLK-*p*NA hydrolysis was not observed.

Analysis of Cell-surface uPAR by PI-PLC Treatment—MEF-1 and MEF-2 cells were treated with 0.5 units/ml PI-PLC in Earle's balanced salt solution, 25 mM HEPES, pH 7.4, for 2 h at 37 °C. The medium was then recovered and concentrated 7-fold in a Centricon concentrator with a 10-kDa exclusion filter (Amicon). Equal amounts of PI-PLC-conditioned medium (corrected for differences in total cellular protein per well) were subjected to SDS-polyacrylamide gel electrophoresis on 10% slabs and electrotransferred to nitrocellulose membranes (Millipore). Blots were probed with uPAR-specific polyclonal antibody. Antibody binding was detected by enhanced chemiluminescence (Amersham Corp.).

Analysis of Cell-surface uPAR by uPA Activity—MEF-1 and MEF-2 cells in 35-mm wells were washed in dilute acid to dissociate uPA·uPAR complex and/or uPA-PAI-1·uPAR complex. Bovine serum albumin-coated culture wells without cells were treated identically. All of the wells were then washed with Earle's balanced salt solution, 25 mM HEPES, 1 mg/ml bovine serum albumin, pH 7.4 (EHB buffer). MEF-2 CM, which had been concentrated 20-fold, was diluted 1:6 into EHB and incubated in one-half of the cultures for 3 h at 4 °C. As will be described under "Results," MEF-2 CM is a rich source of mouse uPA. The other half of the cultures were incubated, for the identical period of time, in EHB without MEF-2 CM. After completing the incubation, cultures were washed three times with ice-cold EHB and then incubated with [Glu^1]plasminogen (0.8 μM) and VLK-*p*NA (0.65 mM) in EHB (total volume, 1.2 ml) for 20 min at 37 °C. Media samples were recovered, and VLK-*p*NA hydrolysis was determined by measuring the absorbance at 406 nm.

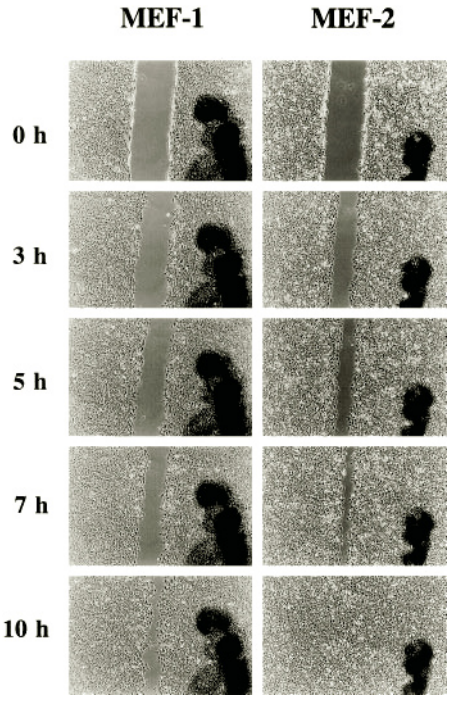

FIG. 1. **Representative study showing migration of MEF-1 and MEF-2 cells into an area of denudation.** Monolayer cultures were wounded, and migration was studied as a function of time. The medium was DMEM with 10% fetal bovine serum. The *dark ink marks* on each photograph are used to ensure that measurements are taken at the identical positions at each time point.

RESULTS

Migration of MEF-1 and MEF-2 Cells in Serum-supplemented Medium—MEF-1 and MEF-2 cells migrated rapidly into an area of total denudation when the cells were cultured in serum-supplemented medium. Fig. 1 shows that the MEF-2 fibroblasts completely filled a gap measuring more than 10 times the average diameter of the cell in less than 10 h. The MEF-1 cells failed to fill an equivalently sized gap in the same period. Fig. 2 presents the results of three representative experiments in which photographs were taken at 0, 3, 5, and 7 h. The graphs were nearly linear, indicating that the rates of cellular migration were invariable during the entire 7-h incubation. The absolute velocities of MEF-1 migration and MEF-2 migration were fairly consistent from experiment to experiment.

In the studies shown in Fig. 2, the MEF-2 cells migrated 1.7–2.0-fold faster than the MEF-1 cells. Equivalent results were obtained in three additional experiments in which photographs were taken and measured at 8 or 9 h (results not presented). In the three experiments that included an 8–9-h incubation protocol, the MEF-2 cells migrated 1.9 ± 0.1-fold faster than the MEF-1 cells (mean ± S.E.). These experiments suggest that LRP deficiency is associated with an increased rate of cellular migration.

Migration of MEF-1 and MEF-2 Cells in Serum-free Medium—To determine whether the effects of LRP expression on

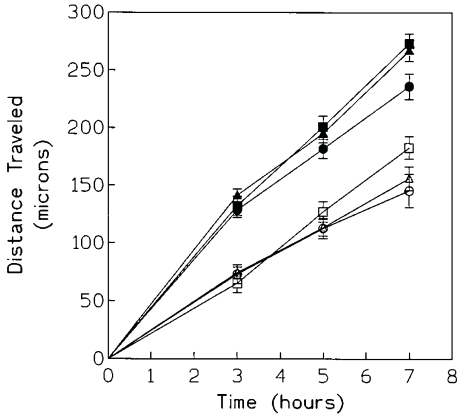

FIG. 2. **Migration of MEF-1 and MEF-2 cells in serum-supplemented medium.** Migration of MEF-1 cells (*open symbols*) and MEF-2 cells (*solid symbols*) was studied as a function of time. The results of three separate experiments are shown. Experiments with MEF-1 and MEF-2 cells, which were performed simultaneously, are shown by using the equivalent symbol (*squares, triangles*, or *circles*).

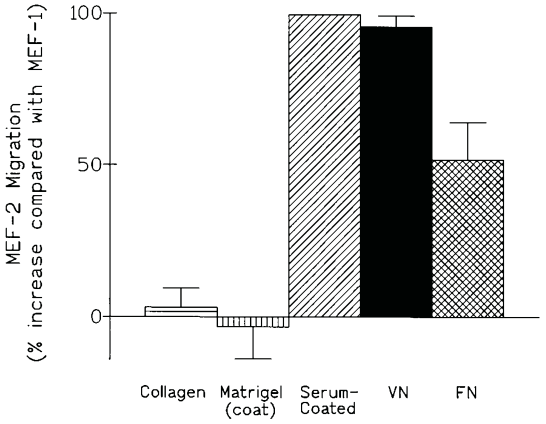

FIG. 3. **MEF migration in culture wells precoated with specific ECM protein preparations.** Cells were cultured in wells coated with the specified ECM proteins or mixed protein preparations. *VN* is vitronectin, and *FN* is fibronectin. The cultures were then denuded, and migration was allowed to proceed in DMEM supplemented with 20 ng/ml platelet-derived growth factor-BB. Migration velocities were determined from the slopes of the migration curves (distance *versus* time). The velocity of MEF-2 migration is compared with the velocity of MEF-1 migration so that a 100% increase indicates that the MEF-2 cells migrated twice as fast as the MEF-1 cells (mean ± S.E., $n = 3$).

fibroblast migration depend on the adhesion substrate, cultures were established in wells precoated with specific preparations of ECM proteins. The cells were then allowed to migrate into a denuded area, in serum-free medium, to avoid surface contamination with vitronectin. As shown in Fig. 3, the MEF-2 cells migrated nearly twice as fast as the MEF-1 cells when the adhesion substrate was vitronectin. The absolute rates of MEF-1 and MEF-2 cell migration, on vitronectin, in serum-free medium, were 20 ± 3 and 40 ± 7 microns/h, respectively. These rates were comparable with those observed in serum-containing medium. The increased rate of migration of MEF-2 cells, compared with MEF-1 cells, on purified vitronectin was anticipated since the major adhesion protein, which is adsorbed onto cell culture plates from serum, is vitronectin (43). Equivalent results were obtained when cell culture wells were precoated with serum and migration assays were performed in serum-free medium.

When the adhesion substrate was changed so that vitronectin was not the predominant component, MEF-1 cell migration velocity was not significantly affected. By contrast, the rate of MEF-2 cell migration was decreased. For example, in culture wells precoated with type I collagen, the velocity of MEF-2 cell migration was 23 ± 2 microns/h, which was not significantly different than the velocity of MEF-1 cell migration. There was also no difference in the migration of MEF-1 and MEF-2 cells in wells coated with Matrigel. Matrigel is a complex mixture of basement membrane proteins; however, the major components are laminin and type IV collagen, with minimal vitronectin (50). In wells that were precoated with purified fibronectin, MEF-2 cells migrated at a significantly increased velocity compared with MEF-1 cells ($p < 0.01$); however, the magnitude of the effect was only 50% of that observed with vitronectin. Thus, the effects of LRP deficiency on cellular migration rate are dependent on the adhesion substrate; of the substrates studied, vitronectin was optimal in promoting migration of the LRP-deficient cell line.

Northern Blot Analysis of LRP Ligand mRNA Levels—The next goal of this investigation was to determine the mechanism by which LRP deficiency accelerates the MEF-2 cellular migration rate. We began by analyzing mRNA levels of LRP ligands implicated in cellular migration. As shown in Fig. 4, MEF-1 and MEF-2 cells expressed equivalent levels of uPAR and PAI-1 mRNA. The level of uPA mRNA was slightly increased in MEF-2 cells, compared with MEF-1 cells, in each of five separate experiments (standardized by PGAD mRNA level). The mean increase (± S.E.) was 58 ± 12%.

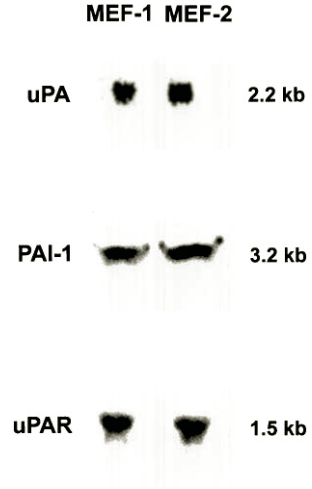

FIG. 4. **Northern blot analysis of uPA, uPAR, and PAI-1 mRNA.** Total cellular RNA was harvested from MEF-1 and MEF-2 cells. Northern blots were probed for the presence of the specified mRNAs. For each of the presented experiments, RNA load was equivalent, as determined by hybridization with a probe for PGAD mRNA.

Analysis of Plasminogen Activator Activity in CM—Since uPA is an LRP ligand that may serve as a regulator of uPAR- and/or integrin-mediated cellular adhesion and migration, experiments were performed to quantitate uPA in CM from MEF-1 and MEF-2 cells. Initially, we studied the activation of plasminogen by the CM preparations. Plasmin was detected as a function of time based on the velocity of VLK-*p*NA hydrolysis. As shown in Fig. 5, substantial amounts of plasminogen activator were detected in MEF-2 CM; however, the velocity of plasminogen activation did not maximize until 1000 s after initiating the incubation. When the uPA-specific inhibitor, amiloride, was added to the activation solution, the maximum velocity of plasminogen activation was reduced by greater than 90%. Thus, it is highly likely that the principal plasminogen activator in MEF-2 CM was uPA and not tissue-type plasminogen activator.

The lag phase in the MEF-2 plasminogen activation curve probably represented the time required to convert scuPA into

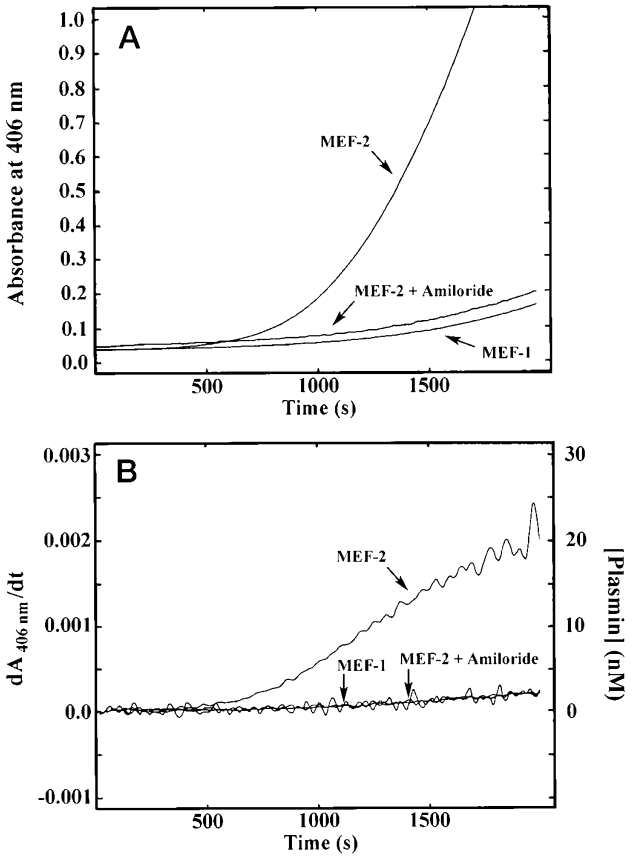

FIG. 5. **Kinetic analysis of plasminogen activator activity in MEF-conditioned medium.** CM was diluted 1:15 into "activation solution" that contained [Glu[1]]plasminogen and VLK-*p*NA. As shown in *A*, substrate hydrolysis was monitored continuously by determining the absorbance at 406 nm every 15 s with 3-s integration periods. *B* shows the data from *A* transformed using a first derivative function. The *left-* and *right-hand axes* show $dA_{406\ nm}/dt$ and the corresponding plasmin concentrations, respectively. The presented experiments with both MEF-1 and MEF-2 cells are representative of four replicates performed with different CM preparations.

the active two-chain structure and/or the time required to overcome any plasminogen activator inhibitor present in the CM. The lag phase could also have reflected conversion of [Glu[1]]plasminogen into the more readily activated form, [Lys[78]]plasminogen; however, our SDS-polyacrylamide gel electrophoresis analyses demonstrated that [Glu[1]]plasminogen was completely intact (within the limits of detection of the Coomassie Blue staining method), after incubation with MEF-2 CM for 1000 s (results not shown). Using the kinetic parameters listed under "Experimental Procedures," we calculated the concentration of active tcuPA in the MEF-2 activation solution at 1000 s. We then calculated the concentration of uPA in the original MEF-2 CM preparation, assuming that all of the scuPA was converted to tcuPA in the activation solution, at 1000 s, and that no plasminogen activator inhibitor was present. The uPA concentration in MEF-2 CM was 0.30 ± 0.02 nM ($n = 4$). This concentration is significant since it is close to the published K_D values (about 0.1–1.0 nM) for the binding of uPA to uPAR (51). If substantial amounts of plasminogen activator inhibitor were present in MEF-2 CM, then the concentration of uPA may have been higher than 0.3 nM.

MEF-1 CM was ineffective in plasminogen activation studies. When the scale used in Fig. 5 was expanded, a 1000-s lag phase was apparent, as had been the case in experiments with MEF-2 CM. Based on the active tcuPA detected at 1000 s, we calculated that the concentration of uPA in MEF-1 CM was 23 ± 3 pM ($n = 4$), near the sensitivity limit of the method. Again, this value may have been underestimated if plasminogen activator inhibitor was present in the CM. Nevertheless, the plasminogen activation analysis suggested that the level of uPA in MEF-2 CM was substantially greater than the level present in MEF-1 CM.

Analysis of Cell-surface uPAR—PI-PLC releases uPAR from viable cells by cleaving the GPI anchor (12). To compare levels of cell-surface uPAR, MEF-1 and MEF-2 cells were subjected to PI-PLC treatment under equivalent conditions. The PI-PLC-conditioned medium was then subjected to Western blot analysis with antibody αD2+3. A single species with an apparent mass slightly less than 70-kDa was detected in the PI-PLC-conditioned medium from MEF-1 cells and MEF-2 cells (Fig. 6*A*). In two separate experiments, the amount of uPAR released from the MEF-2 cells was 4–5-fold higher than that released from MEF-1 cells.

Expression of increased amounts of cell-surface uPAR by MEF-2 cells is consistent with the studies of Conese *et al.* (35), which demonstrated a role for LRP in mediating the internalization of cell-surface uPAR. We hypothesized that LRP deficiency, in the MEF-2 cells, allowed these cells to establish a higher equilibrium level of cell-surface uPAR. Since uPAR promotes the rapid activation of plasminogen by uPA at the cell surface (51), we compared uPAR levels in MEF-1 and MEF-2 cells based on the ability of these cells to bind uPA and then activate plasminogen. MEF-1 and MEF-2 cells were acid-washed and then incubated with MEF-2 CM (a source of murine scuPA) for 3 h at 4 °C. The cells were then washed at neutral pH and incubated with [Glu[1]]plasminogen and VLK-*p*NA for 20 min at 37 °C. Fig. 6*B* shows that the MEF-2 cells generated increased amounts of plasmin compared with MEF-1 cells. The change in absorbance at 406 nm was 2.5-fold greater in the MEF-2 cultures.

The Role of the uPA/uPAR System in MEF Migration—MEF-1 and MEF-2 cells were treated with 10 mM mannosamine for 6 h. Mannosamine is an inhibitor of α1,2-mannosyltransferase, which is required for the formation of functional glycoinositol phospholipid anchors (52). In mannosamine-treated cells, GPI-anchored proteins, including uPAR, are secreted, and the level of cell-surface uPAR is substantially down-regulated (25, 52). After incubation with mannosamine, cultures of MEF-1 and MEF-2 cells were denuded, and migration was allowed to occur for 3 h in serum-supplemented, mannosamine-containing medium. Phase contrast microscopy revealed no change in MEF morphology; however, as shown in Fig. 7*A*, mannosamine treatment significantly decreased the rate of MEF-2 migration ($p < 0.001$). The rate of MEF-1 migration was decreased, but the magnitude of the effect was small and not statistically significant ($p = 0.3$). Overall, the effect of mannosamine was to greatly diminish the difference in the migration velocities of MEF-1 and MEF-2 cells. Although we cannot rule out a contribution by other unknown GPI-anchored proteins, these mannosamine treatment experiments suggest that MEF-2 uPAR plays an important role in the accelerated migration of the LRP-deficient cell line.

To explore the function of uPA in MEF migration, cultures of MEF-1 and MEF-2 cells were subjected to a mild acid wash immediately prior to beginning a migration assay. In control experiments, the acid wash protocol dissociated at least 80% of the uPA from MEF-2 cells, as determined using the plasminogen/VLK-*p*NA hydrolysis assay shown in Fig. 6*B* (results not shown). Fig. 7*B* shows that acid-washed MEF-1 cells migrated at a slower rate compared with control cultures that were not acid-washed (14 ± 4% decrease, $n = 3$). Acid-washed MEF-2 cells also migrated more slowly compared with control MEF-2

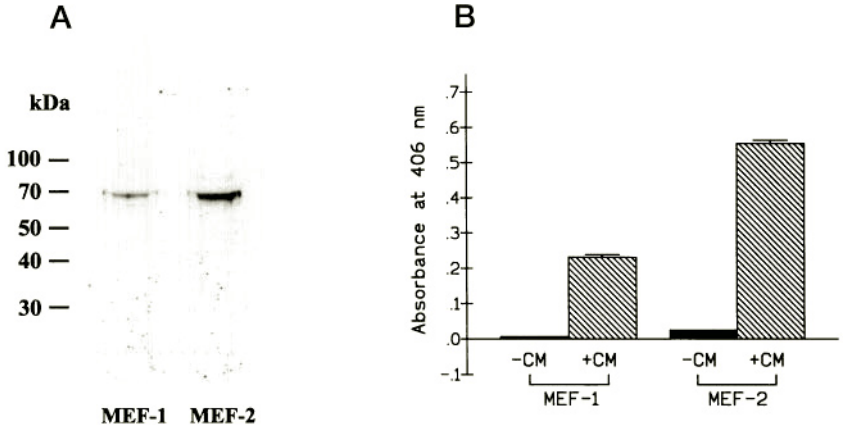

FIG. 6. **Cell-surface expression of uPAR in MEF-1 and MEF-2 cells.** *A,* cultures were incubated with PI-PLC for 2 h at 37 °C. Conditioned medium was then recovered and subjected to Western blot analysis using antibody αD2+3. *B,* acid-washed MEF-1 and MEF-2 cells were incubated in EHB with MEF-2 CM (+*CM*), a rich source of mouse uPA, or in EHB without CM (−*CM*) for 3 h at 4 °C. The cultures were then washed to remove unbound uPA and incubated with plasminogen and VLK-*p*NA for 20 min at 37 °C. Activation of plasminogen by cell-associated uPA was detected by hydrolysis of the VLK-*p*NA (absorbance at 406 nm). The identical procedure was executed using bovine serum albumin-coated wells; the absorbance at 406 nm was less than 0.020, irrespective of whether MEF-2 CM had been added. The presented results show the mean ± S.E. of four separate replicates in a single experiment. In a separate experiment, executed identically, the relative activities observed under the various conditions were equivalent.

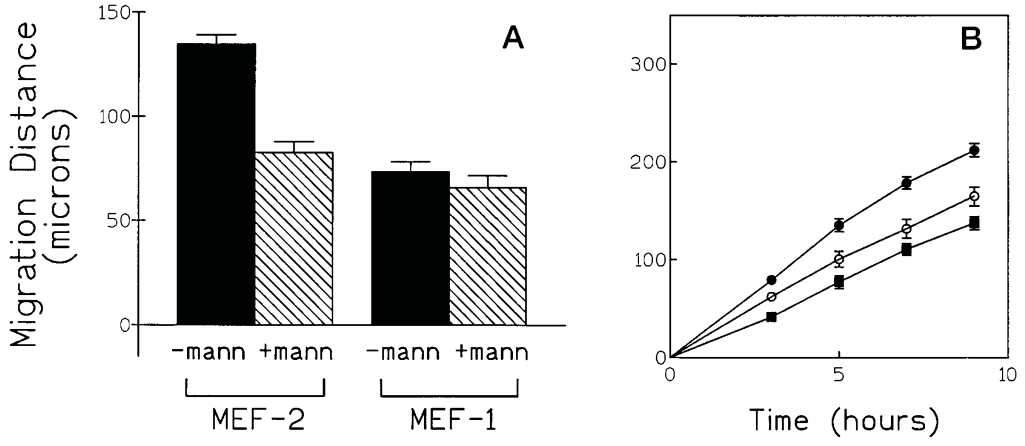

FIG. 7. **Function of the uPA/uPAR system in MEF migration.** *A,* cultures were pretreated for 6 h with mannosamine (+*mann*) or not treated (−*mann*). Migration was then allowed to proceed for 3 h ($n = 4$). *B,* migration of MEF-1 cells was studied in serum-free medium (○) or in serum-free medium supplemented with MEF-2 CM (●). MEF-1 cells were also subjected to mild acid washing prior to studying migration in serum-free medium (■).

cells; the decrease in migration velocity induced by acid washing the MEF-2 cells was 29 ± 3% ($n = 3$, results not shown). These studies suggest that uPA which is endogenously associated with uPAR may promote cell migration. The larger percent decrease in the migration rate of acid-washed MEF-2 cells suggests the presence of more cell-associated uPA or a greater role for uPA in this cell line.

The role of uPA in MEF migration was further probed by studying the migration of MEF-1 cells in the presence of MEF-2 CM, which was concentrated 2-fold so as to saturate a significant percentage of the MEF-1 uPAR. As shown in Fig. 7, MEF-2 CM significantly increased the migration velocity of the MEF-1 cells, compared with untreated MEF-1 cells ($p < 0.01$). In control experiments, MEF-1 CM, which was added to MEF-1 cultures, had no effect on migration ($n = 3$, results not shown). Thus, MEF-2 cells produce a migration-promoting factor that can be transferred to another culture. In the presence of the transferable factor, MEF-1 cells still do not migrate as rapidly as MEF-2 cells, suggesting the involvement of other factors as well (such as altered uPAR expression). Although we cannot absolutely rule out the possibility that MEF-2 CM contains other migration modulators, the documented high level of uPA in MEF-2 CM and the inability of MEF-1 CM to reproduce the activity of MEF-2 CM strongly suggests that the transferable, migration-promoting factor was uPA.

Migration and uPA Secretion by PEA-10 Cells—Expression of LRP is decreased by 50% in PEA-10 cells compared with MEF-1 cells (37). In two separate experiments, the migration velocity of PEA-10 cells in serum-supplemented medium was 23 and 24 microns/h, which is essentially identical to the migration velocity of MEF-1 cells. Thus, partial deficiency in LRP is not sufficient to induce a significant change in cellular migration rate. We next examined the level of uPA in PEA-10 CM, using the plasminogen/VLK-*p*NA-coupled enzyme assay. The concentration of uPA in PEA-10 CM was 25 pM, which is equivalent to the level found in MEF-1 CM. Thus, the amount of LRP expressed by PEA-10 cells is sufficient to prevent accumulation of uPA in the medium. Furthermore, the correlation between uPA accumulation in CM and cellular migration velocity is upheld.

MEF-1 Migration in the Presence of RAP—RAP inhibits binding and endocytosis of all known LRP ligands (36). Thus, we performed experiments to determine whether RAP accelerates the migration of LRP-positive MEF-1 cells. After denuding MEF-1 cultures, the cells were incubated in serum-containing medium supplemented with 180 nM RAP. The RAP concentra-

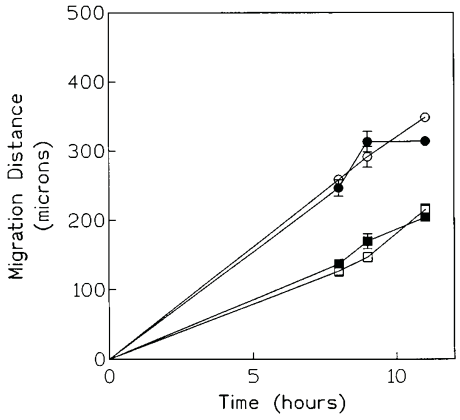

FIG. 8. **Effects of RAP on cellular migration.** MEF-1 (*squares*) and MEF-2 (*circles*) cultures were denuded, and migration was allowed to occur in serum-containing medium with 180 nM RAP (*solid symbols*) and without RAP (*open symbols*). The averaged results of two separate experiments are shown.

tion was sufficient to almost entirely saturate the available LRP; however, as shown in Fig. 8, the MEF-1 cell migration rate was unchanged. MEF-2 cells also migrated at an unchanged rate in RAP-supplemented medium, as expected. Thus, neutralizing the endocytic activity of LRP, at the time of cellular migration, does not affect migration velocity. Although these data could be explained if LRP affects cellular migration by a mechanism that is independent of its endocytic activity, we propose that LRP deficiency induces a stable change in the phenotype of MEF-2 cells that is not mimicked by neutralizing MEF-1 LRP endocytic activity during the course of the migration assay (see "Discussion").

DISCUSSION

In this study, we demonstrated that fibroblasts, which are deficient in LRP, migrate at an accelerated rate compared with wild-type fibroblasts, in serum-supplemented medium and in serum-free medium, when the culture wells are precoated with serum. The increase in cellular migration rate associated with LRP deficiency was substrate-specific. MEF-2 cells migrated 100% faster than MEF-1 cells on vitronectin-coated surfaces and 50% faster on fibronectin-coated surfaces; however, there was no difference in the rate of migration of the two cell lines on type I collagen- or Matrigel-coated surfaces. In addition, we demonstrated changes in the uPA/uPAR system which are associated with LRP deficiency. The MEF-2 cultures accumulated increased amounts of uPA in the medium, and the cells expressed increased amounts of cell-surface uPAR. These findings are consistent with previous studies demonstrating binding of free uPA (not uPAR-associated) to LRP (30, 32) and endocytosis of uPAR in complex with LRP under certain conditions (35).

A number of the experiments presented here indicate that increased activity of the uPA/uPAR system, in the LRP-deficient fibroblasts, is responsible for the increased rate of migration of these cells. First, we demonstrated that MEF-2 CM, a rich source of mouse uPA, increases the rate of migration of MEF-1 cells. Addition of MEF-1 CM to the MEF-1 cultures had no effect on migration rate, as expected. Second, acid-washing the MEF cultures decreased the rate of cellular migration, and the decrease was larger with the MEF-2 cells, as anticipated, since we predicted that these cells would have more uPAR-associated uPA prior to initiating the migration assays. Third, PEA-10 cells, which are 50% deficient in LRP (37), failed to accumulate increased amounts of uPA in CM and also migrated at an unchanged rate compared with wild-type cells. Finally, treatment with mannosamine, to down-regulate cell-surface uPAR, decreased the rate of MEF-2 migration by 40% while having an insignificant effect on MEF-1 migration. As a result, mannosamine eliminated most of the difference in the migration rates of the MEF-1 and MEF-2 cell lines. Although we cannot rule out the possibility that other GPI-anchored proteins contributed to the decrease in MEF-2 cell migration velocity, the mannosamine experiments and our studies demonstrating increased cell-surface uPAR expression in MEF-2 cells strongly implicate uPAR in the mechanism for accelerated migration of the LRP-deficient cells. In addition to down-regulating cell-surface uPAR, mannosamine may also have decreased the percent saturation of residual cellular uPAR with uPA by causing the secretion of soluble receptor (25, 52).

The MEF-2 cell line was generated by selection with *Pseudomonas* exotoxin A (37). LRP is the major receptor that mediates endocytosis of this toxin, accounting for the toxin resistance of LRP-deficient cells (53). Willnow and Herz (37) performed experiments showing that loss of the second LRP allele in the MEF-2 cells was due to gene conversion as opposed to partial chromosomal deletion. Thus, we considered it unlikely that genes other than LRP, which might affect cellular migration, would be deleted in the MEF-2 cell line. This assumption was supported by our early observation that MEF-2 cell migration is ECM substrate-dependent and then by our studies implicating the uPA/uPAR system. Northern blot analyses showed that uPAR and PAI-1 mRNA levels were unchanged in MEF-2 cells. Interestingly, in five separate experiments, a slight increase in MEF-2 steady-state uPA mRNA was observed. This increase was insufficient to account for the vast majority of the uPA in MEF-2 CM, suggesting that decreased catabolism was primarily responsible for the accumulation of this ligand. Nevertheless, the Northern blot analyses of uPA mRNA levels suggest that LRP deficiency may be associated with a slight increase in uPA gene transcription or mRNA stability, probably through an indirect mechanism involving uPA itself or some other LRP ligand.

The ability of vitronectin to enhance migration of LRP-deficient cells with some degree of specificity may be related to the ability of this protein to bind uPAR (28–30). uPA regulates adhesion of cells to vitronectin by at least two mechanisms, by increasing the affinity of uPAR for vitronectin (28) and/or by reacting with vitronectin-associated PAI-1 (29, 31). The latter reaction promotes dissociation of the PAI-1 and apparently reveals the binding site in vitronectin for $\alpha_V\beta_3$ and uPAR. uPAR has been shown to specifically interact with various integrins, including Mac1/CR3, β_1-integrin subunit, and β_2-integrin subunit (23, 27, 54–56). Furthermore, transfection of 293 cells with GPI-uPAR promotes the migration of these cells on vitronectin, apparently due to an interaction of the uPAR with integrins (27). Thus, we propose that LRP deficiency facilitates interactions involving uPAR, vitronectin, and potentially integrins and that these enhanced interactions are at least partially responsible for the increase in MEF-2 migration rate. Our acid-wash and CM transfer experiments (Fig. 7) provide evidence that uPA modulates vitronectin-dependent migration of MEF cells. Since the uPA is synthesized by the fibroblasts themselves, uPA may form an autocrine loop responsible for promoting cellular migration. LRP has the role of limiting the activity of this autocrine pathway.

Addition of RAP after culture denudation had no effect on the rate of migration of MEF-1 cells. The most likely explanation for this result is that LRP deficiency in MEF-2 cells induces a long term, stable change in cellular phenotype, which cannot be rapidly duplicated by RAP in MEF-1 cultures. In the absence of PAI-1, uPAR-associated uPA has a $t_{1/2}$ of >5 h (57, 58), and cell-surface uPAR is internalized extremely slowly (59). Thus,

at the beginning of a migration assay, MEF-2 cells already have the advantage of an up-regulated uPAR level and probably a substantially increased fractional occupancy of uPAR with uPA. If these conditions can be induced by RAP, in the MEF-1 cultures, we would predict that re-equilibration of the system would occur slowly and that the migration assays would be complete by the time that re-equilibration had occurred. Our hypothesis regarding the inactivity of RAP could be tested by analyzing the kinetics of uPA catabolism and uPAR endocytosis in MEF-1 cells. Such studies represent a topic for future investigation.

In experiments to quantitate cell-surface uPAR, slightly different results were obtained depending on whether we used the PI-PLC release method (MEF-2 uPAR increased 4–5-fold compared with MEF-1 uPAR) or the cell-surface plasminogen activation assay (MEF-2 uPAR increased 2.5-fold compared with MEF-1 uPAR). We are not certain of the reason for this; however, the results of the plasminogen activation assays might have been slightly affected by indeterminate variables such as plasminogen binding to the cell surface or the amount of matrix-associated PAI-1. Alternatively, the PI-PLC release assay may have overestimated the difference in cell-surface uPAR if this receptor is weakly associated with LRP in the MEF-1 cells and thus less accessible to PI-PLC. Irrespective of which assay provides the more accurate results, both techniques confirm the presence of increased uPAR levels on the surfaces of the MEF-2 cells.

PEA-10 cells, which have one functional LRP allele, express 50% of the LRP found in wild-type MEF-1 cells; however, these cells regulate extracellular uPA and migrate equivalently to MEF-1 cells. These results suggest that substantial changes in the level of cell-surface LRP are necessary to affect cellular migration. Recent studies have demonstrated that LRP expression may be regulated in various cell types by cytokines. In vascular smooth muscle cells, epidermal growth factor and platelet-derived growth factor-BB increase expression of cell-surface LRP by 2–3-fold (60). Although this increase in LRP expression may be critical for the metabolism of other LRP ligands, based on the PEA-10 results, we would predict that these cytokines would have little effect on the ability of smooth muscle cell LRP to regulate migration. By contrast, macrophages that are activated with IFN-γ or endotoxin show profound decreases in cell-surface LRP, approaching 100% (45, 61). Thus, the change in LRP expression induced by IFN-γ in macrophages may be of sufficient magnitude to have an impact on the migration of these cells.

In summary, we have demonstrated a substantial change in the activity of the uPA/uPAR system and a change in cellular migration rate that are associated with LRP deficiency. The increase in cell-surface uPAR and its saturation with uPA are apparently sufficient to explain the increased rate of migration of LRP-deficient cells. In tissues that are composed of different cell types LRP expression may influence not only the mobility of the cell type that expresses it but also the mobility of neighboring cells.

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