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Extracellular signal-regulated kinase functions in the urokinase receptor-dependent pathway by which neutralization of low density lipoprotein receptor-related protein promotes fibrosarcoma cell migration and Matrigel invasion

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SUMMARY

The low density lipoprotein receptor-related protein (LRP) has been reported to regulate cellular migration. In this study, an antisense RNA expression strategy was used to reduce LRP to undetectable levels in HT 1080 fibrosarcoma cells. The LRP-deficient cells demonstrated increased levels of cell-surface uPAR, higher levels of uPA in conditioned medium, increased migration on vitronectin-coated surfaces, and increased invasion of Matrigel. LRP-deficient cells also demonstrated increased levels of phosphorylated extracellular signal-regulated kinase (ERK) in the absence of exogenous stimulants. Antibodies which block binding of endogenously produced uPA to uPAR reduced ERK phosphorylation and migration of LRP-deficient cells to the levels observed with control cells. Inhibitors of ERK activation, including PD098059 and dominant-negative MEK1, also decreased the migration of LRP-deficient but not control cells. By contrast, constitutively active MEK1 stimulated the migration of control but not LRP-deficient cells. Although Matrigel invasion by LRP-deficient cells was inhibited by the proteinase inhibitor, aprotinin, PD098059 in combination with aprotinin was necessary for an optimal effect. Expression of the VLDL receptor in LRP-deficient cells reversed the changes in cellular migration and invasion. These studies demonstrate that binding of endogenously produced uPA to uPAR may serve as a major determinant of basal levels of activated ERK and, by this mechanism, regulate cellular migration and invasion. By regulating the uPA/uPAR system, LRP may also regulate ERK activation, cellular migration, and invasion.

Key words: Low density lipoprotein receptor-related protein, Urokinase, MAP kinase, VLDL receptor

INTRODUCTION

The low density lipoprotein receptor-related protein (LRP) is a member of the LDL receptor family, which also includes the very low density lipoprotein receptor (VLDLr), gp330/megalin, and apoER2 (Krieger and Herz, 1994; Strickland et al., 1995; Stockinger et al., 1998; Gliemann, 1998). These receptors localize to clathrin-coated pits and undergo rapid constitutive endocytosis and recycling (Anderson et al., 1978; Goldstein et al., 1979; Krieger and Herz, 1994). Associated ligands are also internalized and most often targeted for degradation in lysosomes (Strickland et al., 1995; Gliemann, 1998). Since LDL receptor homologues, including LRP, bind numerous ligands that are both structurally and functionally diverse, it has been difficult to assess how these receptors affect cell physiology.

Some proteins which bind to LRP also bind to distinct signaling receptors. For example, urokinase-type plasminogen activator (uPA) is a LRP ligand (Kounnas et al., 1993) which also binds to the glycosyl phosphatidylinositol-anchored receptor, uPAR, and has been reported to activate diverse cell-signaling effectors including the Src family tyrosine kinase, p56/p59*hck*, focal adhesion kinase, JAK/STAT, and the MAP kinases, extracellular signal-regulated kinase 1 (ERK1) and ERK2 (Busso et al., 1994; Resnati et al., 1996; Koshelnick et al., 1997; Nguyen et al., 1998; Konakova et al., 1998; Tang et al., 1998; Dumler et al., 1999). Thrombospondin-1 is another LRP ligand (Godyna et al., 1995) which binds to integrin-associated protein/$\alpha_v\beta_3$ complex, thereby activating protein tyrosine kinases, PI-3 kinase, and protein kinase C (Gao et al., 1996). uPA- and thrombospondin-initiated cell signaling may regulate cellular adhesion, spreading, and migration.

In addition to binding soluble ligands, LDL receptor homologues may regulate levels of other plasma membrane receptors. When cells are treated with uPA-plasminogen activator inhibitor-1 (PAI-1)-complex, LRP and the VLDLr

internalize not only the uPA-PAI-1 complex, but also uPAR (Conese et al., 1995; Webb et al., 1999). uPAR recycles back to the cell surface; however, the efficiency of recycling is less than 100% and a fraction of the internalized uPAR is catabolized (Nykjaer et al., 1997; Webb et al., 1999). Increased levels of cell-surface uPAR have been observed in LRP-deficient murine embryonic fibroblasts (MEFs) and in MCF-7 cells that are treated with receptor-associated protein (RAP) to block the activity of the VLDLr (Weaver et al., 1997; Webb et al., 1999). These increases in cell-surface uPAR apparently reflect the decreased rate of uPAR catabolism.

LRP-deficient MEFs and RAP-treated MCF-7 cells demonstrate increased migration on vitronectin-coated surfaces (Weaver et al., 1997; Webb et al., 1999); however, neutralizing the function of LRP and the VLDLr in vascular smooth muscle cells either inhibits (Okada et al., 1996; Wijnberg et al., 1997) or has no effect (Degryse et al., 1999) on cellular migration. Differences in the response of various cells to LDL receptor homologue-neutralization may reflect diverse characteristics of the experimental system, including the LDL receptor homologues which are expressed by the specific cell type, the biochemical nature of the substratum, and the function of distinct signaling receptors which may be differentially activated in the presence or absence of LDL receptor homologues. We have demonstrated that the effects of RAP on cellular phenotype also depend on the length of time that the cells are exposed to RAP (Weaver et al., 1997; Webb et al., 1999). Furthermore, some of the cell type-specific activities of LDL receptor homologues may reflect the ability of these receptors to directly associate with the adaptor proteins, mDab1 and FE65 (Trommsdorff et al., 1998, 1999).

To assess the function of LRP as a regulator of cellular signaling, migration, and invasion, HT 1080 fibrosarcoma cells were transfected to express LRP antisense RNA. New cell lines were cloned in which LRP expression was undetectable. The LRP-deficient cells demonstrated increased ERK activation and this change in cell-signaling was entirely attributable to an autocrine pathway in which increased levels of endogenously produced uPA bound to cell-surface uPAR. ERK activation was necessary for the increase in cellular migration on vitronectin and contributed to an increase in Matrigel invasion. These changes in cellular phenotype were reversed when the LRP antisense RNA-expressing cells were transfected to express the VLDLr. Thus, we propose that LRP may regulate the activity of other signaling receptors and by this mechanism, regulate cellular migration and invasion.

MATERIALS AND METHODS

Reagents and proteins

ScuPA and two-chain uPA (tcuPA) were provided by Drs Jack Henkin and Andrew Mazar (Abbott Laboratories). tcuPA was inactivated with diisopropyl fluorophosphate to form DIP-uPA, as previously described (Nguyen et al., 1998). Vitronectin was purified by the method of Yatohgo et al. (1988), [Glu[1]]plasminogen by the method of Deutsch and Mertz (1970), and α_2M by the method of Imber and Pizzo (1981). α_2M was converted into the 'activated' (receptor-recognized) conformation by reaction with methylamine-HCl. uPA-specific antibody, which recognizes the amino terminus of uPA (#3471), and uPAR-specific antibody (399R) were from American Diagnostica. LRP-specific monoclonal antibody 8G1 was provided by Dr Dudley Strickland (American Red Cross, Rockville, MD). Glutathione-S-transferase (GST)-RAP was expressed and purified as previously described (Webb et al., 1995) using a construct obtained from Dr Joachim Herz (Texas Southwestern Medical Center). The VLDLr cDNA was provided by Drs Mats Gåfvels and Keith McCrae (Case Western Reserve University). The expression constructs encoding constitutively active and dominant-negative MEK1 were from Dr Michael Weber (University of Virginia). Hemagglutinin (HA)-specific antibody 12CA5 was from Babco (Berkeley, CA). PD098059 and phosphorylated ERK-specific antibody were from Promega (Madison, WI). Total ERK was detected using an antibody from Zymed (San Francisco, CA). c-Src-specific monoclonal antibody 2-17 was from Dr Sarah Parsons (University of Virginia). Raf-1-specific antibody C-12 was from Santa Cruz Biotechnology. Myosin light chain kinase (MLCK) specific monoclonal antibody K36 and myosin II regulatory light chain-specific-antibody MY21 were from Sigma.

Cell culture and transfection methods

HT 1080 fibrosarcoma cells were obtained from the ATCC and cultured in MEM (Gibco BRL) supplemented with 10% FBS and penicillin/streptomycin. The LRP antisense RNA expression construct pBK-CMV-Lα is described in detail by Hussaini et al. (1999). This construct contains a 608 bp *Sac*I/*Xho*I restriction fragment of the LRP cDNA, inserted in reverse orientation into the multiple cloning site of pBK-CMV. To generate LRP antisense RNA-expressing cell lines, 2×10^5 HT 1080 cells were transfected with pBK-CMV-Lα (2.5 μg) using 10 μl of Superfect (Qiagen, Santa Clarita, CA). Cultures were selected in G418 (1 mg/ml) for 3 weeks and demonstrated 40-50% decreases in cell-surface LRP expression, as determined by binding of the LRP-selective ligand, activated α_2M. Single-cell cloning by serial dilution yielded 60 separate clones which were screened for LRP expression by determining susceptibility to *Pseudomonas* exotoxin A (PEA; 400 ng/ml for 48 hours). PEA is internalized by LRP and thus selectively toxic towards LRP-expressing cells (Kounnas et al., 1992). As a control, HT 1080 cells were transfected with the empty vector, pBK-CMV. The cells were selected in G418 for 3 weeks. Single-cell cloning was performed and a single clone (BK cells) was selected for further study.

In some experiments, HT 1080 cells were cultured in medium supplemented with GST-RAP (200 nM) for up to 5 days. The medium and GST-RAP were replaced daily. The concentration of RAP decreased by less than 1% in each 24 hour period, as determined by analyzing rates of ^{125}I-RAP catabolism. RAP had no effect on HT 1080 cell viability, as determined by trypan blue exclusion or cellular proliferation in 12 hours, which was the time of our standard cell migration assay.

Ligand blot and immunoblot analysis

HT 1080, BK, and LRP antisense RNA-expressing cells were solubilized in 50 mM HEPES, 0.5 M NaCl, 0.05% Tween-20, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml E-64, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Equal amounts of cellular protein (50 μg) were subjected to SDS-PAGE on 5% slabs and electrotransferred to nitrocellulose membranes (Millipore). The membranes were blocked with 5% milk. Ligand blot analysis was then performed to detect membrane-associated RAP-binding proteins. The membranes were incubated with 200 nM GST-RAP for 1 hour at 25°C and then probed with GST-specific monoclonal antibody, followed by goat anti-mouse IgG-peroxidase conjugate (Sigma). Secondary antibody was detected by enhanced chemiluminescence. The identical cell extracts were assessed for LRP heavy chain by immunoblot analysis, using monoclonal antibody 8G1. To determine levels of c-Src, Raf-1, ERK, MLCK and myosin II regulatory light chain, AS-58, BK, and HT 1080 cells were extracted with 2.0% SDS in the presence of proteinase inhibitors and subjected to immunoblot analysis.

Analysis of LRP function in HT 1080 cells

Activated α_2M binds to LRP and not to most other members of the LDL receptor family, including the VLDLr, gp330/megalin, and the LDL receptor (Strickland et al., 1995). To compare levels of cell-surface LRP in control and antisense RNA-expressing HT 1080 cells, specific binding of activated α_2M was determined at 4°C, as previously described (Hussaini et al., 1999). To assess the function of LRP at 37°C, cellular degradation of ^{125}I-GST-RAP was studied. Cells were incubated with 10 nM GST-RAP for up to 6 hours. Accumulation of trichloroacetic acid (TCA)-soluble radioactivity in the medium was determined. A 100-fold molar excess of nonradiolabeled GST-RAP was added to some cultures to determine specific ^{125}I-GST-RAP degradation.

DIP-uPA binding to HT 1080 cells

Specific binding of DIP-uPA was determined as previously described (Webb et al., 1999). Prior to performing binding studies, confluent cultures were subjected to a mild acid wash to dissociate uPAR-associated ligands (Weaver et al., 1997; Nykjaer et al., 1997). The cells were then incubated with ^{125}I-DIP-uPA (0.15-20 nM) for 4 hours at 4°C. In the specified uPA concentration range, high-affinity binding to uPAR is selectively detected and low-affinity interactions, such as those that might occur with LRP, do not significantly affect the results (Vassalli et al., 1985; Stoppelli et al., 1985; Cubellis et al., 1986; Kounnas et al., 1993).

uPA accumulation in conditioned medium

HT 1080, BK, and LRP antisense RNA-expressing cells were incubated in serum-free medium without phenol red for 24 hours. Conditioned medium (CM) was recovered and concentrated using Centricon concentrators (Amicon). Plasminogen activator in the CM was determined by a coupled-substrate activity assay, using purified plasminogen and the plasmin-specific substrate, val-leu-lys-p-nitroanilide (VLK-pNA; Weaver et al., 1997). This assay detects scuPA and tcuPA but not uPA-PAI-1 complex. uPA in the CM was also detected by immunoblot analysis.

Analysis of ERK phosphorylation

Phosphorylated and total ERK were detected by immunoblot analysis. The medium was aspirated from HT 1080 cell cultures and replaced with ice-cold PBS containing 1 mg/ml sodium vanadate. The cells were then extracted with 1.0% Nonidet P-40, 50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1 µg/ml leupeptin, 2 µg/ml aprotinin, 0.4 mg/ml sodium vanadate, 0.4 mg/ml sodium fluoride, and 5 mg/ml dithiothreitol, pH 7.4. The extracts were subjected to SDS-PAGE on 12% slabs. Proteins were transferred to nitrocellulose membranes and probed with antibodies that detect phosphorylated and total ERK.

To assess ERK phosphorylation in mutant MEK1-expressing cells, 2×10^6 AS-58 or BK cells were co-transfected with 5 µg of the construct encoding constitutively active MEK1 and with 1.25 µg of an HA-tagged ERK1 expression construct (Chu et al., 1996). The cells were maintained in serum-supplemented medium for 36 hours and then extracted in 0.1% SDS, 1% deoxycholate, 1% NP-40, 10 mM sodium phosphate, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, 0.1 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, 0.1 µg/ml leupeptin, and 100 KIU/ml aprotinin. HA-ERK1 was immunoprecipitated with antibody 12CA5. Samples were then subjected to SDS-PAGE and transferred to nitrocellulose. Phosphorylated HA-ERK1 was detected by immunoblot analysis.

Transwell cellular migration assays

Migration of LRP antisense RNA-expressing and control cells was studied using tissue culture-treated 6.5 mm Transwell chambers with 8.0 µm pore membranes (Costar). Both surfaces of each membrane were coated with 5 µg/ml purified vitronectin for 2 hours at 37°C and then blocked with 0.5% BSA for 2 hours at 37°C. The membranes were washed with serum-free MEM. Cells were dissociated from monolayer culture, washed with serum-free medium, and transferred to the top chamber of each Transwell at a density of 5×10^5 cells/ml (100 µl). Upper and lower chambers both contained serum-free MEM. Migration was allowed to proceed for 12 hours at 37°C. At the conclusion of each experiment, non-migrating cells were removed from the top surface of each membrane with a cotton swab. The membranes were then fixed in methanol and stained with 0.1% Crystal Violet. The dye was eluted with 10% acetic acid and the absorbance at 600 nm was determined. Standard curves were prepared by determining the Crystal Violet absorbance of pre-counted populations of HT 1080 cells; these graphs were linear.

Migration of mutant MEK1-expressing cells

BK and AS-58 cells were co-transfected to express green fluorescent protein (GFP) and mutant forms of MEK1. The cells were then washed and cultured in MEM supplemented with 10% FBS for 36 hours before analysis. Transfection efficiencies were determined by fluorescence microscopy to detect GFP and were typically 25-35%. Co-transfection efficiencies were determined as described by Nguyen et al. (1999) and were always greater than 90%. Thus, transfected cells could be selectively analyzed in heterogeneous populations based on GFP fluorescence. The mutant MEK1-transfected cells were added to translucent Biocoat Cell Culture Inserts, which were coated with vitronectin and blocked with BSA. Cells which migrated to the underside of each membrane were fixed with 4% paraformaldehyde and counted by fluorescence microscopy. To standardize the number of migrating fluorescent cells, cell counts were divided by the transfection efficiency, which was determined in each experiment. Migration of cells transfected to express the VLDLr was studied using the same procedure except that the cells were cultured in serum-supplemented MEM for 96 hours prior to analysis.

Cellular invasion through Matrigel

Matrigel was reconstituted on the top surfaces of Transwell membranes at 100 µg protein/ cm^2 of surface area. BK or AS-58 cells (5×10^4 in 100 µl) were added to the upper chamber in SFM supplemented with 0.5 µM plasminogen. The bottom chamber contained MEM supplemented with 10% FBS. The cells were allowed to invade for 24 hours at 37°C, at which time the Matrigel and cells that were associated with the top surfaces of the membranes were removed with cotton swabs. Cells that penetrated through the Matrigel to the underside surfaces of the membranes were fixed and stained with 0.1% Crystal Violet. The number of migrating cells was determined as described in the migration assays. To study the invasion of BK and AS-58 cells which were co-transfected to express GFP and VLDLr, Biocoat Cell Culture Inserts were used instead of Transwells. Other modifications to the protocol were equivalent to those used in the migration assays.

RESULTS

Antisense RNA expression in HT 1080 cells neutralizes LRP

To study the function of LRP, it was necessary to identify cells lines which do not express other LDL receptor homologues with redundant activities. RAP ligand-blotting experiments detect multiple LDL receptor homologues, including LRP and the VLDLr (Webb et al., 1999). However, in HT 1080 cells, a single RAP-binding protein was identified (Fig. 1A). This protein was identical in molecular mass to the LRP heavy chain, as determined by immunoblot analysis.

HT 1080 cells were transfected to express LRP antisense RNA and screened for residual LRP expression by determining

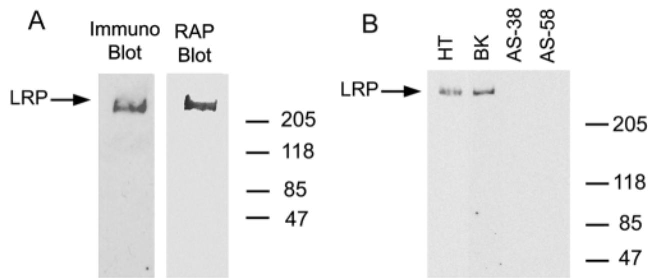

Fig. 1. LRP levels in control and antisense RNA-expressing HT 1080 fibrosarcoma cells. (A) Cell extracts were prepared from HT 1080 cells, subjected to SDS-PAGE on 5% slabs and transferred to nitrocellulose. In the RAP-ligand blot, membranes were incubated with GST-RAP and then probed with GST-specific monoclonal antibody followed by goat anti-mouse IgG-peroxidase conjugate. The secondary antibody was detected by enhanced chemiluminescence. In the immunoblot, LRP heavy chain was detected on the same membranes using monoclonal antibody 8G1. (B) Immunoblot analysis comparing LRP expression in HT 1080 cells (HT), BK cells, and two LRP antisense RNA-expressing cell lines (AS-38 and AS-58).

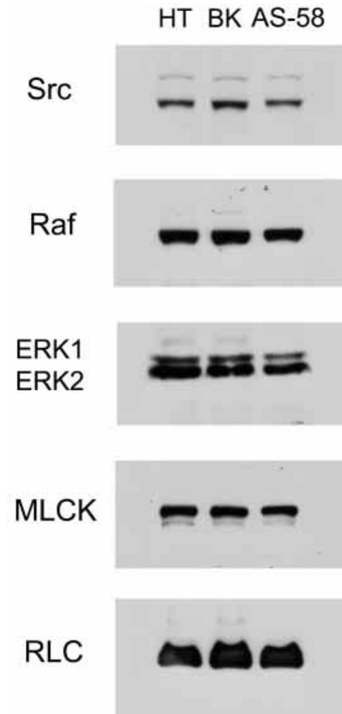

Fig. 2. Immunoblot analysis of signaling protein expression in LRP-deficient and control cells. HT 1080 (HT), BK, and AS-58 cells were solubilized in 2.0% SDS supplemented with proteinase inhibitors. Cellular protein concentrations were determined using bicinchoninic acid. Equal amounts of cellular protein were loaded in each lane, subjected to SDS-PAGE, and electrotransfered to nitrocellulose for detection with specific antibodies (RLC, myosin II regulatory light chain).

sensitivity to PEA, which is selectively internalized by LRP-expressing cells (Kounnas et al., 1992). PEA-resistance was interpreted as evidence for decreased LRP expression and was observed in more than twenty separate clones. To more quantitatively characterize residual LRP activity in PEA-resistant clones, ^{125}I-RAP degradation was studied. Two cell lines, AS-38 and AS-58, demonstrated rates of ^{125}I-RAP degradation that were decreased by 94% and 96%, respectively, compared with the parental HT 1080 cells (results not shown). RAP degradation is an exclusive function of LDL receptor homologues (Strickland et al., 1995). Failure of the AS-38 and AS-58 cells to degrade RAP not only suggests substantial neutralization of LRP expression, but also confirms that these cells do not express other RAP-degrading LDL receptor homologues.

BK cells were single-cell cloned from HT 1080 cells that were transfected with the empty vector, pBK-CMV. Northern blot analysis demonstrated neomycin resistance gene mRNA in the cloned BK cells, indicating stable integration of the vector (results not shown). When screened in the PEA-toxicity assay, a decrease in the sensitivity of BK cells to PEA was not observed. Furthermore, ^{125}I-RAP was degraded by BK cells at an unchanged rate compared with HT 1080 cells.

As shown in Fig. 1B, LRP heavy chain was not detected in extracts of AS-38 and AS-58 cells, by immunoblot analysis, but was easily identified in equivalent amounts of cellular protein obtained from BK and HT 1080 cells. In separate studies, AS-38 and AS-58 cells failed to bind significant levels of activated ^{125}I-α_2M (results not shown). By contrast, HT 1080 and BK cells demonstrated specific and saturable α_2M-binding. The K_D values were 1.5±0.5 and 1.4±0.4 nM, respectively; the B_{max} values were 13±1 and 11±1 fmol/mg cell protein, respectively (n=4). Thus, we demonstrated by multiple methods that LRP expression is neutralized in AS-38 and AS-58 cells.

LRP antisense RNA expression does not alter levels of representative signaling proteins

To examine the specificity of the LRP antisense RNA expression construct, we determined levels of representative cell-signaling proteins in equivalent amounts of protein extracted from AS-58, BK, and HT 1080 cells. Some of the proteins which were evaluated are essential members of a signaling pathway which may promote cellular migration in uPA-stimulated cells (Nguyen et al., 1999). Fig. 2 shows that total antigenic levels of c-Src, Raf-1, ERK, MLCK, and myosin II regulatory light chain were equivalent in the BK, AS-58, and HT 1080 cells, as determined by immunoblot analysis. These results demonstrate that the LRP antisense RNA did not non-specifically inhibit cellular expression of other proteins which may impact on signaling and cellular migration.

Effects of LRP on the uPA/uPAR system in HT 1080 cells

LRP-deficient MEFs and RAP-treated MCF-7 cells demonstrate increased levels of cell-surface uPAR and accumulate increased amounts of uPA in CM, apparently reflecting decreased rates of uPA and uPAR catabolism (Weaver et al., 1997; Webb et al., 1999). To compare the levels of cell-surface uPAR in LRP-deficient and control HT 1080 cells, DIP-uPA-binding experiments were performed. AS-58, AS-38, BK, and HT 1080 cells all bound ^{125}I-DIP-uPA in a specific and saturable manner (Fig. 3A). The Scatchard transformation for each individual experiment (not shown) was linear (r^2= 0.90-0.97), suggesting that a single class of uPA-

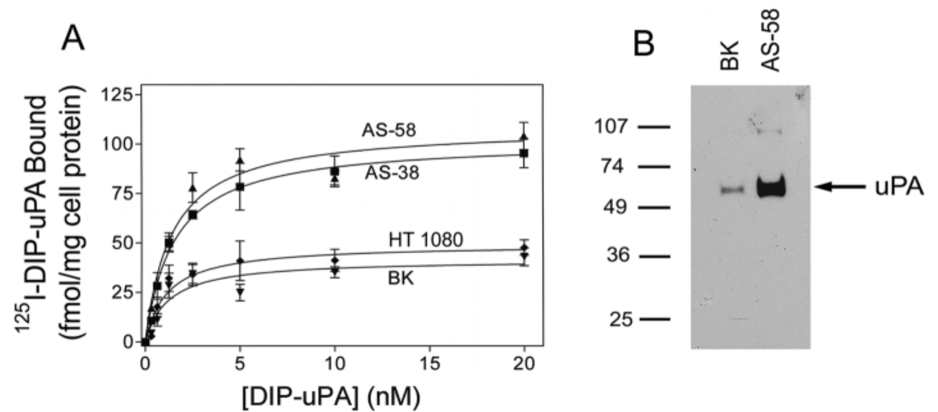

Fig. 3. uPA and cell-surface uPAR in LRP antisense RNA-expressing cells. (A) Specific binding of DIP-uPA to HT 1080 cells (◆), BK cells (▼), AS-38 cells (■), and AS-58 cells (▲) was determined by incubating increasing concentrations of ^{125}I-DIP-uPA with each cell type (in the presence and absence of a 50-fold molar excess of nonradiolabeled DIP-uPA) for 4 hours at 4°C. (B) Immunoblot analysis comparing uPA levels in medium which was conditioned by BK and AS-58 cells for 24 hours. The CM was concentrated 10-fold prior to electrophoresis.

binding sites was detected. Equivalent K_D values were determined for all four cell lines (1.1-1.5 nM). These K_D values are consistent with the known binding-affinity of uPA for uPAR (Stopelli et al., 1985; Vassalli et al., 1985; Cubellis et al., 1986). By contrast, the maximum uPA-binding capacity (B_{max}) was increased from 49±4 and 42±4 fmol/mg cell protein, in the HT 1080 and BK cells, respectively, to 102±6 and 109±5 fmol/mg cell protein, in the AS-38 and AS-58 cells, respectively (n=4, P<0.01). The mass of an HT 1080 cell was determined as previously described (Nguyen et al., 1999) and was 0.9±0.1 ng (n=5). Based on this value, we estimated that each AS-58 cell expresses 59,000 copies of cell-surface uPAR, compared with 26,000 copies/HT 1080 cell.

To confirm that AS-58 cells have increased levels of cell-surface uPAR, we used an assay which detects uPAR-associated uPA activity, as previously described (Weaver et al., 1997). ScuPA (1 nM) was incubated with AS-58 and BK cells for 4 hours at 4°C. The cultures were then washed and re-equilibrated in fresh serum-free medium containing [Glu[1]]plasminogen (1.0 μM) and the plasmin-specific substrate, VLK-pNA. Substrate hydrolysis was measured and was increased 2.9±0.6-fold in wells that contained AS-58 cells, suggesting an increased capacity of these cells to bind uPA (results not shown).

To determine whether LRP-deficient HT 1080 cells accumulate increased levels of uPA, 1×10^5 AS-58 or BK cells were incubated in serum-free medium for 24 hours. uPA in the conditioned medium (CM) was determined by immunoblot analysis using antibody #3471. Fig. 3B shows that uPA was greatly increased in CM from the AS-58 cells. The faint band with an apparent mass of 105-kDa probably represents uPA-PAI-1 complex. We also measured uPA in CM by activity assay (Weaver et al., 1997). CM was collected from 1×10^5 AS-38, AS-58, BK, and HT 1080 cells. All four CM samples contained plasminogen activator which was inhibited by at least 90% when amiloride was added, suggesting that the plasminogen activator was uPA. The concentration of uPA in CM was determined as previously described (Weaver et al., 1997) and was increased about 8-fold in CM from AS-38 (170±10 pM) and AS-58 (190±20 pM) cells, compared with CM from HT 1080 (25±5 pM) and BK (20±4 pM) cells. Thus, LRP-deficient HT 1080 cells have increased levels of cell-surface uPAR and accumulate increased amounts of uPA in CM.

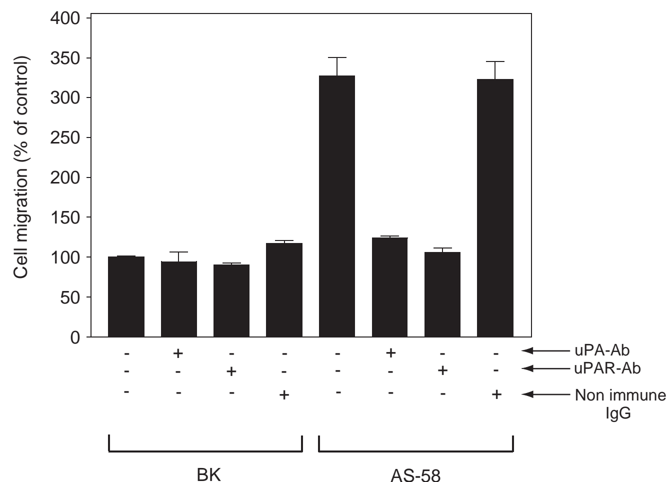

Fig. 4. LRP antisense RNA-expressing cells demonstrate increased migration on vitronectin. Transwell chambers were coated on both surfaces with purified vitronectin. BK and AS-58 cells were allowed to migrate for 12 hours, in the presence (marked '+') or absence (marked '-') of uPA-specific antibody (uPA-Ab), uPAR-specific antibody (uPAR-Ab), or non-immune IgG. Cellular migration is expressed as a percentage of that observed with BK cells in the absence of antibodies.

The effects of LRP on HT 1080 cell migration

Cellular migration was studied using Transwell culture chambers in which the membranes were pre-coated with purified vitronectin. In this system, BK and HT 1080 cells migrated equivalently; the number of migrating BK cells in each individual experiment (1170±90 cells/membrane) was arbitrarily set at 100%. Fig. 4 shows that migration of AS-58 cells was increased 3.2±0.2-fold, compared with BK cells. AS-38 cells also demonstrated increased migration (2.5±0.2-fold). Interestingly, when Transwell membranes were coated with type I collagen instead of vitronectin, BK, AS-58, and AS-38 cells migrated equivalently (results not shown), suggesting that the effects of LRP-deficiency on cellular migration may be substratum-selective. A similar dependency on substratum was previously observed in the MEF system (Weaver et al., 1997). Furthermore, we have demonstrated that the ability of exogenously added uPA to promote cellular migration by activating ERK may be vitronectin-selective (Nguyen et al., 1999). In control experiments, we demonstrated that adhesion of AS-58 and AS-38 cells to vitronectin was not altered

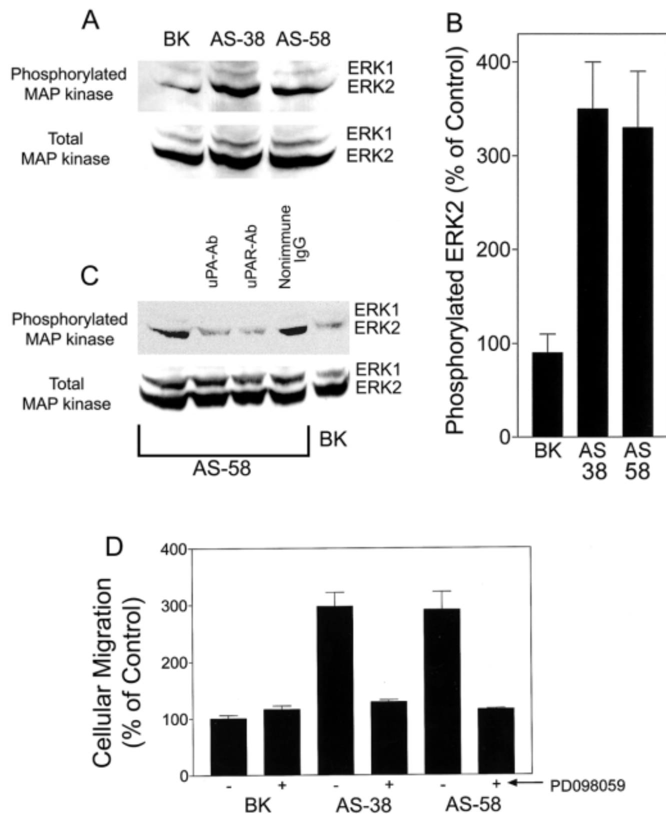

Fig. 5. LRP antisense RNA-expressing cells demonstrate increased levels of activated ERK2. (A) Immunoblot analysis to detect phosphorylated and total ERK1 and ERK2 in BK, AS-38, and AS-58 cells. All cells were maintained in standard serum-supplemented culture medium and extracted when 90% confluent. (B) Levels of phosphorylated ERK2 were standardized to the level detected in HT 1080 cells in individual experiments (n=4). (C) AS-58 cells were cultured in the presence of uPA-specific antibody, uPAR-specific antibody, or non-immune IgG for 12 hours and then extracted to assess ERK phosphorylation. AS-58 and BK cells which were cultured in the absence of antibody are shown as a control. (D) BK, AS-38, and AS-58 cells were allowed to migrate through vitronectin-coated Transwell membranes for 12 hours in serum-free medium. The MEK inhibitor, PD098059, was added to the top chamber as indicated by '+'. Cellular migration was standardized to that observed with BK cells which were not treated with drug.

compared with BK and HT 1080 cells. Thus, the Transwell migration results reflect differences in the ability of LRP-deficient cells to migrate once they have adhered to the vitronectin-coated Transwell membranes.

To determine whether binding of endogenously produced uPA to uPAR is necessary for the increase in AS-58 cell migration, Transwell migration assays were performed in the presence of antibodies (25 μg/ml) that bind to uPA or uPAR and block uPA-uPAR interactions. In control experiments, we demonstrated that both antibodies decrease specific-binding of ^{125}I-DIP-uPA to HT 1080 cells by greater than 90%. Furthermore, the uPAR-specific antibody did not independently promote ERK phosphorylation in BK cells. However, when the antibodies were added to the Transwell chambers, AS-58 cell migration was reduced to the level observed with control cells. By contrast, neither antibody

Table 1. The effects of dominant-negative MEK1 on cellular motility

GFP-expressing cells	Cellular migration (% of control)
BK cells	100±6
DN MEK1-expressing BK cells	101±5
AS-58 cells	303±21
DN MEK1-expressing AS-58 cells	105±7

BK cells and AS-58 cells were transfected to express GFP or co-transfected to express GFP and dominant-negative (DN) MEK1. After 36 hours, cellular migration assays were performed. Cellular migration was determined by fluorescence microscopy and standardized to the level observed with GFP-expressing BK cells.

inhibited BK cell migration. In control experiments, non-immune IgG did not affect the migration of AS-58 or BK cells. Thus, binding of endogenously produced uPA to uPAR is required for the increase in migration of LRP-deficient HT 1080 cells. The uPA/uPAR system apparently does not play a significant role in the regulation of HT 1080 cell migration when LRP is active, as previously reported (Nguyen et al., 1999).

As a second method to block LRP function, HT 1080 cells were cultured in the presence of RAP for 5 days. Prolonged incubation with RAP is necessary to allow re-equilibration of cell-surface uPAR at an increased level (Webb et al., 1999). The RAP-treated cells demonstrated 2.8±0.4-fold increased migration on vitronectin in Transwell assays (results not shown). Similar results were obtained when human embryonic fibroblasts were cultured in the presence of RAP and then studied in denudation migration assays, as previously described (Weaver et al., 1997). Thus, neutralizing LRP in fibroblasts, at the level of translation or receptor activity, promotes cellular migration on vitronectin.

Phosphorylated ERK is increased in LRP-deficient HT 1080 cells

In MCF-7 and HT 1080 cells, exogenously added uPA promotes cellular migration by activating an ERK-dependent signaling pathway (Nguyen et al., 1998, 1999). Since our results suggested that binding of endogenously produced uPA to uPAR is required for increased migration in LRP-deficient cells, studies were undertaken to determine whether the level of ERK phosphorylation is increased in these cells, in the absence of exogenous stimulants. As shown in Fig. 5A, AS-38, AS-58, and BK cells express primarily ERK2 and some ERK1, as has been previously reported for HT 1080 cells (Konakova et al., 1998). The baseline level of phosphorylated ERK was increased in LRP-deficient cells. To accurately quantitate the increase in phosphorylated ERK, low-exposure autoradiography films from four separate experiments were subjected to densitometry analysis. Fig. 5B shows that the level of phosphorylated ERK was increased more than 3-fold in the AS-38 and AS-58 cells, compared with the level observed in HT 1080 cells. By contrast, the level of phosphorylated ERK was unchanged in the BK cells.

In order to determine whether the endogenous uPA/uPAR system is necessary for the increase in ERK phosphorylation in AS-58 cells, we cultured these cells in the presence of uPA- or uPAR-specific antibody for 12 hours. In the presence of either antibody, the level of ERK phosphorylation was decreased back to the level observed with BK cells (Fig. 5C),

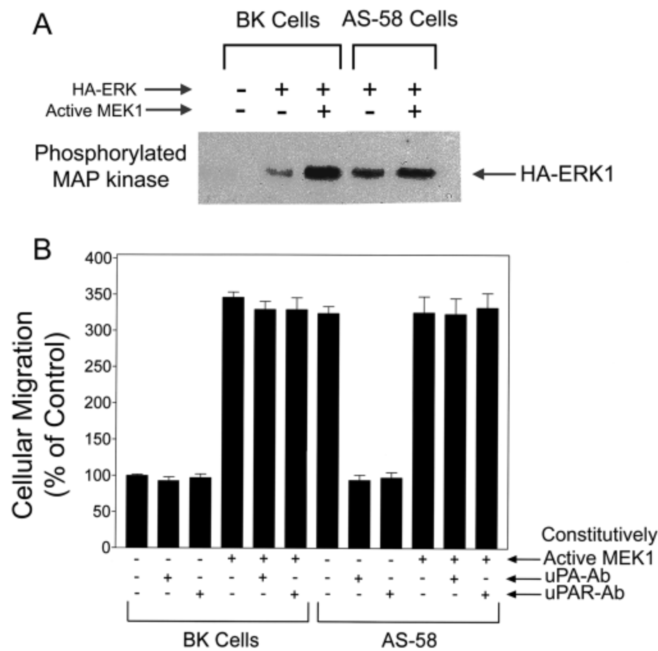

Fig. 6. Expression of constitutively active MEK1 increases migration of BK but not AS-58 cells. (A) BK and AS-58 cells were transfected to express constitutively active MEK1 and HA-tagged ERK1, as indicated by '+'. HA-ERK1 was immunoprecipitated from cell extracts and studied by immunoblot analysis to assess phosphorylation. (B) BK and AS-58 cells were transfected to express GFP and constitutively active MEK1, as indicated by '+'. Cells were allowed to migrate for 12 hours in the presence of uPA-specific or uPAR-specific antibody (marked '+') or vehicle (marked '-'). Cellular migration was determined by fluorescence microscopy and standardized to the level observed with GFP-expressing BK cells in the absence of antibodies.

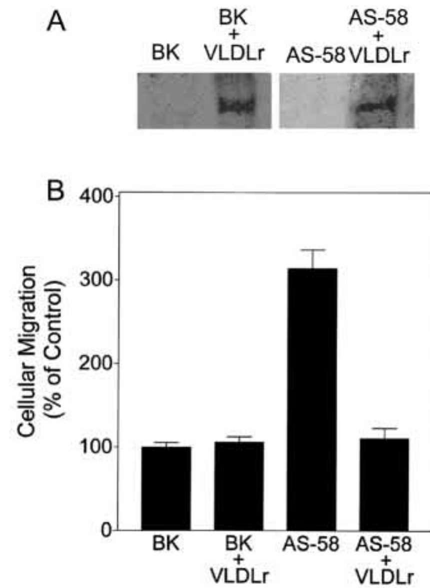

Fig. 7. Expression of VLDLr in LRP antisense RNA-expressing cells reverses the increase in migration which is associated with LRP neutralization. (A) BK and AS-58 cells were transfected to express GFP and the VLDLr. After 96 hours, cell extracts were analyzed by immunoblot analysis using a polyclonal anti-human VLDLr antibody. Transfected cells are labeled '+VLDLr'. Cells that were transfected to express GFP but not VLDLr are labeled 'BK' and 'AS-58'. (B) Migration assays were performed to compare VLDLr-expressing and BK cells. Cellular migration was detected by fluorescence microscopy and standardized to the level observed with GFP-expressing BK cells.

suggesting that autocrine activation of uPAR by endogenously produced uPA is necessary for the increase in baseline ERK activity. In control experiments, we demonstrated that the uPA- and uPAR-specific antibodies do not inhibit ERK phosphorylation in HT 1080 cells that are serum-starved for 12 hours and then re-stimulated with 10% FBS. Thus, the mechanism of action of these antibodies appears to be specific.

In order to determine whether the increase in ERK activation is linked to the increase in the migration of LRP-deficient cells, we assessed the effects of the selective MEK inhibitor, PD098059 (50 μM), on the migration of AS-58, AS-38, and BK cells. As shown in Fig. 5D, PD098059 did not inhibit the migration of BK cells, suggesting that MEK activity is not required for this process. By contrast, PD098059 blocked the increase in migration which was associated with LRP-deficiency in AS-58 and AS-38 cells. In control experiments, we demonstrated that the PD098059 vehicle, DMSO, does not affect HT 1080 or AS-58 cell migration. Although PD098059 may down-regulate uPA expression in some cell lines (Simon et al., 1996), uPA accumulation in CM from PD098059-treated AS-58 cells was decreased by only 8±5% (results not shown). These results provide evidence that ERK activation is required for the increased migration of AS-58 and AS-38 cells.

To confirm the results of the PD098059 experiments, we co-transfected AS-58 and BK cells to express GFP and dominant-negative MEK1 (S217→A). In control experiments, we demonstrated that GFP alone does not alter the migration of BK or AS-58 cells. BK cell migration was not significantly affected by the mutant MEK1, whereas AS-58 cell migration was decreased to the level observed with BK cells (Table 1). Thus, MEK activity is necessary in the pathway by which LRP neutralization stimulates cellular migration. If other signaling pathways are activated in the AS-58 cells, apparently these pathways do not promote migration independently of the ERK/MAP kinase pathway. The unchanged rate of migration of BK cells, following dominant-negative MEK1 expression, confirms that the ERK/MAP kinase pathway is not essential for HT 1080 cell migration when LRP is active.

ERK is optimally activated to promote migration of AS-58 cells

To further explore the role of ERK in the mechanism by which LRP deficiency stimulates HT 1080 cell migration, AS-58 and BK cells were transfected to express constitutively active MEK1 (S218→D/S222→D). The activity of the mutant MEK1 was demonstrated by co-transfecting cells to express HA-tagged ERK1. Whole-cell extracts of the transfected AS-58 and BK cells demonstrated equivalent levels of total HA-ERK1 in immunoblotting experiments with antibody 12CA5. As shown in Fig. 6A, constitutively active MEK1 induced a substantial increase in the level of phosphorylated HA-ERK1 in BK cells, as expected (Fig. 6A). In AS-58 cells,

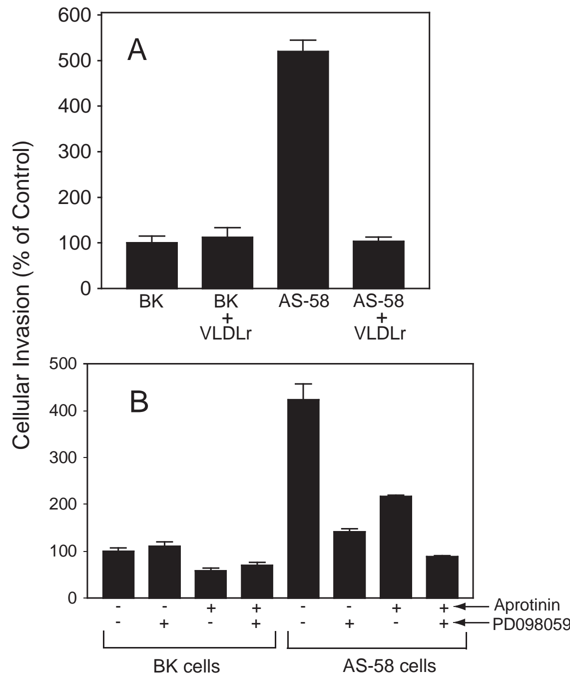

Fig. 8. LRP regulates HT 1080 cell invasion through Matrigel in an ERK-dependent manner. Matrigel matrices (100 μg protein/cm^2) were reconstituted in Transwell chambers. (A) BK and AS-58 cells were transfected to express GFP and VLDLr or GFP alone. The cells were then added to Transwell chambers (top) in serum-free medium supplemented with 0.5 μM plasminogen. The bottom chamber contained MEM supplemented with 10% FBS. The cells were allowed to invade for 24 hours at 37°C. Cells which invaded through the Matrigel and penetrated the membrane were detected by fluorescence microscopy. (B) BK and AS-58 cells were added to the top chambers in serum-free medium supplemented with 0.5 μM plasminogen. The MEK inhibitor, PD098059, and/or aprotinin were added to the top chamber as indicated by '+'. The cells were allowed to invade for 24 hours at 37°C. Cells that penetrated to the underside of the membranes were fixed and stained with Crystal Violet.

phosphorylation of HA-ERK1 was increased in the absence of the mutant MEK1, confirming the endogenous activation of the ERK/MAP kinase pathway in these cells. However, when AS-58 cells were transfected to express constitutively active MEK1, only a slight, additional increase in the level of phosphorylated HA-ERK1 was observed.

To study the effects of constitutively active MEK1 on cellular migration, cells were co-transfected to express GFP. In BK cells, expression of constitutively active MEK1 increased cellular migration to the level observed with AS-58 cells (Fig. 6B); however, uPA- and uPAR-specific antibodies did not affect the migration of these cells, suggesting that activation of the endogenous uPA/uPAR system was not involved. AS-58 cells that were transfected to express constitutively active MEK1 demonstrated unchanged migration; however, these cells were no longer susceptible to the effects of uPA- or uPAR-specific antibodies. Thus, MEK1 functions downstream of uPAR in the signaling pathway which promotes migration of LRP-deficient HT 1080 cells. Furthermore, the extent of ERK activation, which is induced by the endogenous uPA/uPAR system in AS-58 cells, is apparently sufficient to stimulate migration to the level observed with constitutively active MEK1-expressing cells.

Although our antibody studies demonstrated that uPA and uPAR are not necessary for the increase in migration of constitutively active MEK1-expressing BK cells, experiments were performed to assess the uPA/uPAR system in these cells. BK cells were transfected to express constitutively active MEK1 and GFP or GFP alone. uPA accumulation in medium conditioned by both cell preparations was equivalent, as determined by immunoblot analysis. Furthermore, levels of cell-surface uPAR were equivalent, as determined by specific binding of ^{125}I-DIP-uPA. These results suggest that in HT 1080 cells, constitutively active MEK1 does not induce a substantial change in the steady-state levels of cell-surface uPAR or available uPA. However, steady-state protein levels may be influenced by catabolism as well as synthesis. Thus, we cannot rule out a change in uPA or uPAR expression, as has been previously reported in other cell lines (Simon et al., 1996; Lengyel et al., 1997). Furthermore, since our experiments were performed with unsorted cell preparations (transfection efficiency of 35%), small changes in uPA or uPAR levels may not have been detected.

VLDLr reverses the increase in migration associated with LRP deficiency

In MCF-7 breast cancer cells, the VLDLr suppresses the activity of the uPA/uPAR system and inhibits cellular migration, similarly to LRP (Webb et al., 1999). To determine whether VLDLr expression reverses the phenotypic changes associated with LRP neutralization in HT 1080 cells, we transfected BK and AS-58 cells to express the VLDLr. Expression of the VLDLr was confirmed in both cell lines by immunoblot analysis (Fig. 7A). As shown in Fig. 7B, VLDLr-expressing BK cells migrated at an unchanged rate compared with control cells. By contrast, VLDLr-expressing AS-58 cells migrated at a decreased rate which was equivalent to that observed with BK cells.

LRP regulates HT 1080 cell invasion through Matrigel

Although the mechanism by which the uPA/uPAR system stimulates AS-58 cell migration requires ERK activation, uPA may also function in vivo to promote proteolysis of extracellular matrix and thereby allow cellular invasion through tissues (Saksela and Rifkin, 1988; Andreasen et al., 1997). To model invasion in vitro, we studied cellular penetration of reconstituted Matrigel matrices (100 μg protein/cm^2). Invasion of AS-58 cells was increased four- to fivefold compared with BK cells when determined by Crystal Violet-staining (results not shown) or by GFP-fluorescence (Fig. 8A). Expression of the VLDLr in AS-58 cells reduced Matrigel invasion back to the level observed with BK cells. Thus, LRP and the VLDLr regulate cellular invasion of Matrigel as well as migration.

To determine whether uPAR-initiated signaling contributes to Matrigel invasion, BK and AS-58 cells were allowed to invade Matrigel in the presence of the plasmin inhibitor, aprotinin (25 μM), the MEK inhibitor PD098059 (50 μM), or both inhibitors simultaneously. As shown in Fig. 8B, aprotinin inhibited the migration of BK and AS-58 cells, as anticipated. PD098059 significantly inhibited AS-58 cell invasion, but did

not affect BK cell invasion. The combination of aprotinin and PD098059 reduced AS-58 cell invasion most effectively. These studies demonstrate that the uPA/uPAR system may promote Matrigel invasion by more than one mechanism. By regulating uPAR-initiated cell signaling and other activities of the uPA/uPAR system, LDL receptor family members may affect multiple properties of cancer cells which are relevant to cancer progression.

DISCUSSION

The ability of LRP to bind and internalize multiple ligands that are both structurally and functionally diverse has made it difficult to understand the function of this receptor on a cellular level and in animal model systems (Krieger and Herz, 1994; Strickland et al., 1995). Redundancies in the ligand specificities of LRP and other LDL receptor homologues have further complicated interpretation of experimental results. Furthermore, association of cytoplasmic adaptor proteins, such as mDab and FE65, with members of the LDL receptor family (Trommsdorff et al., 1998, 1999), may be responsible for activities that are cell-type specific. In this study, we define an LRP-regulated pathway which controls fibrosarcoma cell migration and Matrigel invasion. The pathway is dependent on the activity of the endogenous uPA/uPAR system, which we have defined as an important regulator of baseline ERK phosphorylation. LRP deficiency increased uPAR levels slightly more than 2-fold and available levels of endogenously produced uPA by about 8-fold. These changes resulted in a threefold increase in ERK phosphorylation.

Although we previously demonstrated a relationship between LRP expression, the uPA/uPAR system, and cellular migration in MEFs (Weaver et al., 1997), we were not able to demonstrate that the uPA/uPAR system is necessary for the increase in migration of LRP-deficient MEFs due to a lack of appropriate murine-specific reagents. Thus, the antisense RNA-expressing HT 1080 cells represented an important new model system for our continued work in this area. In these cells, the increase in cellular migration, which resulted from LRP neutralization, was completely reversed by two separate antibodies that block binding of endogenously produced uPA to uPAR. The same antibodies decreased the level of phosphorylated ERK, in LRP-deficient cells, which was a critical determinant of cellular migration. Two separate methods were used to inactivate the ERK/MAP kinase pathway in LRP-deficient cells; in both cases, the decrease in cellular migration was equivalent to that observed when the autocrine uPA/uPAR system was disrupted. These results provide strong evidence that the uPA/uPAR system and uPAR-initiated cell-signaling play a pivotal role in the pathway by which LRP neutralization stimulates the migration of fibroblast-like cells.

The importance of ERK in regulating cellular migration has been previously demonstrated. Activated ERK phosphorylates and thereby activates myosin light chain kinase, increasing cytoskeletal contractility which may promote cellular migration (Klemke et al., 1997; Nguyen et al., 1999). Activated ERK also regulates integrin function, possibly deactivating certain integrins and promoting the disassembly of focal adhesions (Hughes et al., 1997; Xie et al., 1998). Thus, regulating ERK activity may have profound effects on various physiologic processes that involve cellular migration. Although previous studies have shown that exogenously added uPA activates ERK in diverse cell types, including HT 1080 cells, MCF-7 cells, and endothelial cells (Nguyen et al., 1998; Konakova et al., 1998; Tang et al., 1998), the function of the endogenous uPA/uPAR system as a regulator of baseline levels of activated ERK has not been described and may have far-reaching implications. In AS-58 cells, constitutively active MEK1 had only a minimal effect on the level of phosphorylated HA-ERK1 and did not promote cellular migration. We interpret these results to mean that the extent of ERK activation, induced by the endogenous uPA/uPAR system, may be substantial when LDL receptor homologues are either not expressed or neutralized in activity. In multicellular tissues, LDL receptor homologues may regulate ERK activation by regulating cell-surface uPAR levels in the same cell or by regulating the level of uPA available to ligate uPAR in the same or adjacent cells.

Results obtained previously with HT 1080 cells (Nguyen et al., 1999) and with BK cells, in the present study, suggest that autocrine activation of uPAR by endogenously produced uPA is insufficient to stimulate cellular migration when LRP is expressed. This conclusion was not entirely anticipated since others have demonstrated that HT 1080 cells express significant levels of uPA (Tsuboi and Rifkin, 1990; Laug et al., 1992). Calculation of the expected fractional occupancy (y) of uPAR by uPA in BK cells, according to the equation: $y=[L]/(K_D+[L])$, using a K_D of 1.2 nM and the concentration of uPA which accumulates in BK cell-conditioned medium in 24 hours ($[L]$), yields a value that is less than 2%. The very low y is consistent with our previous results demonstrating that in HT 1080 cells, the majority of the cell-surface uPAR is free and available to bind exogenously added uPA (Nguyen et al., 1999). In AS-58 cells, we estimate that uPAR ligation by endogenously produced uPA is increased more than 30-fold (based on a y of 14% and the increase in B_{max}). Of course these calculations assume reversible binding of uPA to uPAR and do not account for uPA-PAI-1 complex which may accumulate to increased levels on the surfaces of LRP-deficient cells. At the present time, the effects of uPA-PAI-1 complex on uPAR signaling and cellular migration remain unknown.

Although LRP-deficient HT 1080 cells migrated more rapidly than control cells on vitronectin-coated surfaces, no difference in migration was observed on type I collagen. The equivalent substratum dependency was previously demonstrated with MEFs (Weaver et al., 1997). These results are intriguing since MCF-7 cells that are treated with exogenous uPA migrate more rapidly on vitronectin but not type I collagen (Nguyen et al., 1999). The increase in migration in response to exogenously added uPA depends on activation of ERK and myosin light chain kinase (Nguyen et al., 1999). Furthermore, exogenously added uPA promotes MCF-7 cell migration on vitronectin under the control of the naturally occurring integrins ($\alpha_V\beta_5$ and $\alpha_V\beta_1$) but not when the cells are transfected to express $\alpha_V\beta_3$. These results may be explained if uPA-induced ERK activation promotes localized contraction of the actin cytoskeleton and, as a result, supports the function of select integrins in mediating cellular migration. Cells which express $\alpha_V\beta_5$ frequently require stimulation with agonists that activate ERK in order to migrate on vitronectin (Klemke et al., 1994; Brooks et al., 1997) and indeed, HT 1080 cells express

$\alpha_v\beta_5$ (Conforti et al., 1994). However, in COS-7 cells, activation of ERK and MLCK promotes migration on collagen as well as vitronectin (Klemke et al., 1997). Further studies will be necessary to sort out the substratum-selectivity of agents which activate ERK and thereby promote cellular migration.

Cellular migration in vivo differs from migration in vitro in that tissue barriers, such as basement membranes, must be penetrated (Saksela and Rifkin, 1988; Andreasen et al., 1997). The importance of this process is well recognized in cancer invasion and metastasis; however other processes also require boundary penetration, including angiogenesis, neointima formation, and inflammatory cell recruitment. Although uPA and uPAR gene knock-out mice develop normally, recent studies have demonstrated abnormal leukocyte recruitment to sites of inflammation in animals that are deficient in uPA or uPAR (Gyetko et al., 1996; Shapiro et al., 1997; May et al., 1998). Thus, the uPA/uPAR system may play a critical role in cellular migration/invasion in vivo under specific conditions. We hypothesized that under the same conditions, LRP and other LDL receptor homologues may indirectly regulate cellular invasion. To model this process, we studied HT 1080 cell invasion of Matrigel and demonstrated that LRP functions as an inhibitor of this process. This result is intriguing since LRP expression correlates with a less aggressive phenotype in some cancers (reviewed by Gonias et al., 1994).

The activity of the uPA/uPAR system in promoting Matrigel invasion by tumor cells is well documented (Saksela and Rifkin, 1988; Cajot et al., 1989; Mohanam et al., 1993; Stahl et al., 1994). For the most part, it has been assumed that uPA promotes Matrigel invasion by activating a cascade of proteinases which digest extracellular matrix proteins in Matrigel, such as laminin and type IV collagen. The plasmin inhibitor, aprotinin, and other reagents which neutralize plasmin activity decrease Matrigel invasion (Cajot et al., 1989; Meissauer et al., 1991, 1992). However, recent studies indicate that the uPA/uPAR system expresses multiple activities in addition to localizing proteinase activity to the cell-surface, including direct binding of uPAR to vitronectin, signal transduction, and modulation of integrin function (Chapman, 1997). To explore the possible involvement of uPAR-initiated signaling in Matrigel invasion, we compared AS-58 and BK cell invasion of Matrigel in the presence of aprotinin and PD098059. Aprotinin inhibited the invasion of both cell lines, as anticipated; however, PD098059 inhibited only AS-58 cell invasion. This was an important observation since our data demonstrated significant ERK activation in the AS-58 cells but not in the BK cells. Previous studies have suggested a role for the Ras-dependent MAP kinase pathway in tumor cell invasion (Jeffers et al., 1998). The results presented here demonstrate that the uPA/uPAR system may regulate cellular invasion by multiple mechanisms, including controlling basal levels of activated ERK.

In a recent study, we demonstrated that the VLDLr regulates cell-surface uPAR, uPA accumulation in conditioned medium, and cellular migration, similarly to LRP (Webb et al., 1999). We have now shown that the VLDLr reverses the changes in cellular migration and Matrigel invasion which are associated with LRP deficiency. Since we did not generate stable VLDLr-expressing AS-58 cell lines, we were unable to determine whether VLDLr expression reverses the changes in cell-surface uPAR expression and uPA accumulation in conditioned medium. However, our new results coupled with the known similarities in the function of LRP and the VLDLr in mediating the internalization of uPA-PAI-1 complex and uPAR (Conese et al., 1995; Webb et al., 1999) suggest that LRP and other LDL receptor homologues may function as interchangeable regulators of cellular migration and invasion.

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