Overexpression of Urokinase Receptor Increases Matrix Invasion without Altering Cell Migration in a Human Osteosarcoma Cell Line

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ABSTRACT

Proteolysis triggered by receptor-bound urokinase-type plasminogen activator (uPA) involves a cascade of species-specific molecular interactions. To study the role of the uPA receptor (uPAR) in such interactions, a human osteosarcoma cell line (HOS), which normally expresses low levels of uPAR, was transfected with human uPAR complementary DNA. One of several stably transformed clonal cell lines, designated 2A2, was characterized and compared to the parental HOS, revealing the following: (a) stable incorporation of uPAR complementary DNA into the genome demonstrated by Southern blot analysis; (b) a 10-fold increase in steady state mRNA levels of uPAR assessed by Northern blot analysis; (c) a 2-fold increase in the surface expression of glycosylphosphatidylinositol anchored uPAR protein determined by enzyme-linked immunosorbent assay and by the specific binding of radiolabeled single chain uPA; (d) a 2-fold increase in internalization and degradation of radiolabeled uPA/PAl-1 complexes; and (e) a 2-fold increase in receptor-bound uPAR-mediated plasmin generation measured by the cleavage of a chromogenic substrate and degradation of 125I-labeled laminin. The involvement of uPAR in cellular processes was determined by comparing 2A2 and HOS cells in vitro migration and invasion assays. The migration of 2A2 cells was slower on fibronectin-coated surfaces in a linear under-agarose assay, but both cell lines migrated at the same rate on uncoated polycarbonate filters in Boyden chamber assays. In the invasion experiments, 4 times more 2A2 than HOS cells penetrated through the barrier of reconstituted basement membrane Matrigel. These data suggest that uPAR does not potentiate random cell migration but facilitates matrix degradation and subsequent cell invasion.

INTRODUCTION

Cell migration and invasion are complex processes involving sequential, regulated interactions between cells and extracellular substratum. Studies on a variety of cells have revealed that proteolysis is part of these complex processes and uPA3 was shown to be one of the most frequently implicated enzymes. The serine protease uPA promotes matrix degradation by generating plasmin from the abundantzymogen plasminogen. Plasmin can digest the major components of the extracellular substratum either directly or by activating procollogenase. Plasmin generation is controlled to a large extent by plasminogen activators and inhibitors, while plasmin activity is regulated primarily by \( \alpha_2 \)-antiplasmin (for a review, see Ref. 1).

The involvement of uPA in a series of normal and pathological processes of migration and invasion including wound healing, inflammation, and tumor cell metastasis has been supported by numerous findings (for a review, see Ref. 2). Recent studies have suggested that the effect of urokinase is not mediated solely by its actions as a soluble enzyme (3, 4). Rather, cell migration and invasion require localized and directional proteolysis which may be aided by the activity and localization of specific cell surface receptors.

In recent years such a receptor for uPA has been extensively characterized on many cell types (for a review, see Ref. 5). The uPAR is a highly glycosylated GPI-anchored protein (6), with a relative molecular mass ranging from 46 kDa on umbilical vein endothelial cells (7) to ~55–60 kDa on U937 cells (8). Deglycosylation of uPAR from both sources yields a single protein with a molecular mass of ~35–36 kDa. The uPAR binds both scuPA and tcuPA in a species-specific fashion (9–11). Plasmin can readily convert receptor-bound zymogen scuPA to active tcuPA which remains receptor associated, thereby promoting formation of additional plasmin on the cell surface. In the presence of plasminogen, the receptor-bound scuPA accelerates plasin generation with a higher rate than does the fluid phase scuPA or tcuPA (12, 13), suggesting that adjacent localization of uPA and plasmin/plasminogen on the cell surface may enhance pericellular proteolysis.

Recently, direct involvement of uPAR in the invasion process has been demonstrated by an in vivo model of invasion in which transformed mouse fibroblasts expressing human uPAR were cocultured with cells secreting human uPA (4). Neither cell type alone was capable of penetrating into the chick embryo chorioallantoic membrane, while upon coculture, the human proenzyme secreted by one cell line enabled the human receptor bearing mouse cells to invade into the membrane. One limitation of this study is that it involved interactions between proteins and cells originating from three different species. In addition, these experiments did not directly analyze the effects of uPAR on cell migration as distinct from cell invasion, a process which requires both migration and matrix degradation.

To address these two issues, a human cell line that overexpresses the human uPAR has been established and characterized. HOS cell line, which is neither tumorigenic nor metastatic in nude mice (14) and expresses little uPA or uPAR, was transfected with human uPAR. We compared the behavior of this transfected cell with its parent to delineate the contribution of uPAR to the processes of cell migration and invasion. Our studies demonstrate that overexpression of functionally active uPAR does not increase cell migration per se but enhances the capacity of HOS to traverse through an extracellular matrix barrier.

MATERIALS AND METHODS

Cloning and Subcloning of uPAR. Human uPAR-specific cDNA was RT-PCR amplified as we previously described (7). The PCR fragment was inserted into pGEM-3z (Promega, Madison, WI) to yield a vector designated pGEMuPAR. The nucleotide sequence and the orientation of the uPAR insert were confirmed by Sequenase (USB, Cleveland, OH). The uPAR insert was subcloned into pBC12-Bl (kind gift of Dr. B. Cullen, Roche Institute, Nutley, NJ) and into pMAMneo-BLUE (Clontech Laboratories, Palo Alto, CA) to yield mammalian expression vectors (pBC12 uPAR and pMAMuPAR, respectively). Plasmids were purified on Qiagen columns (Qiagen, Chatsworth, CA).
Transcription and Translation of uPAR. Plasmid pGEMuPAR (1 μg) was linearized and transcribed to capped mRNA (50 μg) using an in vitro transcription kit (Stratagene, La Jolla, CA) and SP6 RNA polymerase (BRL, Rockville, MD). The quality of synthesized RNA was tested by Northern blot analysis. The RNA (5 μg) was translated using rabbit reticulocyte lysates (Promega) in the presence of [35S]cytysteine (Amersham, Arlington Heights, IL). To test posttranslational processing such as glycosylation, the lysates were supplemented with canine pancreatic microsomal membranes (Promega). The in vitro synthesized protein products and 14C-labeled protein molecular weight markers (Amersham) were separated on SDS-polyacrylamide gel electrophoresis (10%) and autoradiographed.

The in vitro transcribed uPAR mRNA (0.3 μg in 20 μl DME) was microinjected into frog oocytes. Control oocytes were given injections of DME alone. Cells were cultured at 22°C for 2.5 h. Specific binding of 125I-scuPA to oocytes was determined as described (13). Four eggs were used for each condition and the experiments were performed in duplicates. The viability of injected oocytes was determined by trypan blue exclusion immediately after the binding assay was performed.

Immunoprecipitation of uPAR. Metabolically labeled, in vitro translated uPAR (37 and 46 kDa) and Brome Mosaic Virus proteins (110, 97, 35, 20, and 15 kDa) were mixed (1:50 molar ratio) in Staph-A buffer (100 mM NaCl-10 mM phosphate buffer (pH 7.4)-1% Triton X-100-0.1% SDS-0.5% sodium deoxycholate-0.1% NaN3) in a 200-μl final volume. The mixture was aliquoted and immunoprecipitation was performed using Protein A-Sepharose (Pharmacia) and preimmune or anti-uPAR murine serum generated against oligopeptide corresponding to amino acids 130-147 of the mature human uPAR (16). Proteins were separated on SDS-polyacrylamide gel electrophoresis (10%) under reducing condition and the gels were analyzed using autoradiography.

Transfection of HOS Cells. HOS cells (2 × 10^6) were transfected with 0.4 μg of pMAMuPAR using Lipofectin (BRL) according to the manufacturer. Approximately 48 h after the transfection, cells were washed and grown in a G418 (GIBCO) (400 μg/ml) supplemented culture medium. The selective medium was changed twice a week for three weeks until all nontransformed cells died on the control plate. On some plates, neo" colonies were stained and counted. Ultimately, 31 neo" colonies were isolated and characterized further.

Southern and Northern Blots. Genomic DNA was isolated from the parental HOS cells and their transformed variants using a desalting procedure as described (17). The DNA concentration was estimated on a 0.6% agarose gel. The genomic DNA (~20 μg) was digested with Hind III (Boehringer Mannheim, Indianapolis, IN) and separated on 1% agarose gel in Tris-Borate-EDTA (TBE) buffer (15). After UV-irradiation of the gel, DNA fragments were transferred and UV-cross-linked to a Nylon filter (Schleicher and Schuell, Keene, NH). The filter was probed for uPAR as described below and analyzed using autoradiography.

Parental and transformed HOS cells were lysed with a buffer containing NP40 and RNAs were isolated from the cytoplasmic fractions (15). Ten μg of RNA was separated and analyzed on Northern blot as described (7). For detection of uPAR mRNA, the filters were probed with a ~1.0-kilobase purified uPAR insert. The GAPD and uPA transcripts were detected using the GAPD and uPA transcripts were detected using the Type Culture Collection (Rockville, MD). To detect α-enolase mRNA, an RT-PCR-amplified product was used. The α-enolase specific primers were designed based on the cDNA sequence obtained from the GenBank. The 5'-primer (5' GTCTCTTCAGGCTGCTG'3') corresponds to nucleotides 180-197; the 3'-primer (5' CAAACAGGTCAGCGATGAAG'3') corresponds to nucleotides 1250-1229. The PCR was performed as described (7) and the amplified product specificity was confirmed by comparing with restriction enzymes (Bal I, Stu I, and Pvu II).

To probe the Northern or Southern blots, approximately 25 ng DNA were labeled using a random prime labeling kit (Boehringer Mannheim). The filters were hybridized at 42°C for 12-16 h with the prehybridization solution containing the labeled and denatured probe as described (7). The filters were washed and exposed to Kodak XAR film using intensifier screen, at -70°C for 4-48 h. Before reprobing, the filters were stripped from the old probe and reexposed to confirm successful stripping. Autoradiograms were quantified by densitometric scanning. mRNA levels were normalized for minor differences in loading based on the value for GAPD mRNA.

Radioligand Binding Assays and ELISA. Purified single-chain urokinase-type plasminogen activator (scuPA) (kind gift of Dr. Jack Henkin, Abbott Laboratories, Abbott Park, IL) was radiolabeled as described (13). Radiolabeled scuPA (4 nM; specific activity ~2-5 × 10^6 dpm/μg) was incubated at 4°C for 3 h with the parental and transformed HOS cells in 96-well plates (5 × 10^4 cells/well) in the absence or presence of 50-fold molar excess unlabeled scuPA as a measure of total and nonspecific binding, respectively. The cells were then washed 5 times with PBS-BSA and removed from the plate with trypsin/EDTA, and the cell-associated radioactivity counted. Specific binding was defined as a difference between total binding and nonspecific binding. All experiments were performed in triplicate, with 4 independent experiments carried out.

As an independent measure of uPAR protein, we performed a solid phase ELISA and radioligand binding in replicate microtiter wells as previously described (16). Briefly, cells were washed in PBS-BSA buffer, preincubated with or without 0.5 units/ml PI-PLC (kind gift of Dr. Martin Low, Columbia University) for 45 min at 37°C, and then incubated with a monoclonal anti-uPAR antibody #3936 (kind gift of Dr. Richard Hart, American Diagnostica, Greenwich, CT) or with an isotype control. Antibody binding was detected using a peroxidase-conjugated goat antimurine Ig (Organon-Teknika, West Chester, PA) and the absorbance was determined in an ELISA plate reader (Dynatech Laboratories Inc. Chantilly, VA). Specific binding was defined as the difference between the binding of anti-uPAR antibody and the isotype control that was PI-PLC cleavable (see below). Radioligand binding was performed as noted above. In separate experiments, radiolabeled scuPA was prebound to the transformed and control cell lines. The susceptibility of the bound 125I-scuPA to removal by PI-PLC was measured.

Internalization and Degradation Assays. Two-chain uPA was generated from radiolabeled scuPA by limited exposure to plasmin (10 μg/ml) for 30 min, and the reaction was stopped with aprotinin (100 μg/ml). The labeled tucPA was incubated with an excess of guanidine- HCL reactivated recombinant PAI-1 (kind gift of Dr. Christopher Reilly of Merck, Sharpe and Dohme, West Point, PA) to form labeled tucPA/PAI-1 complexes. HOS and 2A2 cells were plated in 24 well plates (2.5 × 10^5 cells/well) for 24 h, the cells were washed with PBS-BSA 3 times, and radiolabeled tucPA/PAI-1 complexes (3.6 nM) were added for 3 h at 4°C in the presence or absence of 50-fold molar excess ligand. The cells were washed again and incubated in 50 μs 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, containing 1% BSA for an additional 3 h at 37°C. Total, nonspecific, and specific binding were defined as above. The cells and medium were then separated. The amount of radioligand specifically remaining on the cell surface was determined by acid elution (incubation with 0.1 mM NaCl and 50 mM glycine-HCl, pH 3.0, for 5 min). The amount of radioactivity released into the supernatant was fractionated into the portion that remained soluble in trichloroacetic acid (20% trichloroacetic acid on ice for 30 min) and that which was trichloroacetic acid precipitable. The radioactivity in each fraction was presented as a percentage of the amount specifically bound initially to that cell type. In addition, the mean values of absolute counts in each fraction for the two cell types were compared using an unpaired t-test with a P value <0.05 considered significant.

Plasminogen Activation. The ability of cell-bound uPA to generate plasmin from plasminogen was assessed indirectly using a soluble chromogenic plasmin substrate S-2251 (Kabi Vitrum) as previously reported (13). We also determined the ability of plasmin, generated by cell-associated uPA, to degrade 125I-labeled laminin located beneath the cell (18). Laminin was iodinated using chloramine T (19). Free iodine was removed by dialysis and the labeled laminin was aliquoted and stored in 12-well plates at 4°C until use. Each well usually contained 2 μg laminin (specific activity ~5 × 10^6 dpm/μg). Cells were harvested with 5 mM EDTA in PBS, washed twice in DME, and then resuspended and incubated with or without additional scuPA (50 nM) at 4°C for 45 min. The cells were then washed twice with DME and plated into 12-well plates (10^6 cells/well) which had previously been coated with 125I-laminin. The cells were incubated for an additional 2 h at 37°C to permit the cells to attach and form a confluent layer. Plasminogen (300 nM) was then added to some of the wells and the cells were incubated at 37°C for 1 h. The radioactivity in aliquots from the medium was measured. Specific release was determined by subtracting the amount of radioactivity measured in the absence of cells.

Migration in Under-Agarose Assays. The rate of cell migration was determined using a linear under-agarose assay by measuring the distance taken by
RESULTS

Transcription and Translation of uPAR cDNA. A cDNA comprising the coding region of uPAR was cloned from PMA-treated U937 cells using RT-PCR and ligated into the plasmid pGEM-3z. The nucleotide sequence of the uPAR insert was identical to that reported previously (22). uPAR-specific mRNA was transcribed from the linearized pGEMuPAR construct in vitro. Translation of this mRNA using rabbit reticulocyte lysates generated a ~37-kDa protein product in close agreement with the 36.9-kDa value predicted for uPAR based on the putative amino acid sequence. When the mRNA was translated using rabbit reticulocyte lysates supplemented with canine microsomes, uPAR migrated as a ~46-kDa protein, presumably due to glycosylation. Both the 37- and 46-kDa proteins were specifically identified by immunoprecipitation using a sequence-specific polyclonal anti-uPAR antibody (16). Proteins of these sizes are consistent with the size of the predominant intracellular and glycosylated cell surface form of uPAR expressed by human umbilical vein endothelial cells (7).

To test whether the cloned uPAR would be transcribed, translated, and processed into a functioning protein in vivo, uPAR-specific products were tested in frog oocytes and COS cells. Oocytes injected with uPAR mRNA specifically bound ~20-fold more scuPA than control oocytes (4.3 ± 0.1 versus 0.2 ± 0.4 fmol/oocyte). In addition, COS cells transiently transfected with pBCuPAR-bound ~3-fold more 125I-scuPA than the mock transfected cells (4.1 ± 1.8 versus 1.2 ± 0.8 fmol/10^5 cells).

Stable Transfection of HOS Cells with uPAR. The human uPAR cDNA was then subcloned into a mammalian expression vector pMAMneo-BLUE. The cDNA was inserted downstream from a retroviral enhancer and promoter elements and upstream from an SV40 REP sequence.

UROKINASE RECEPTOR-MEDIATED TUMOR CELL INVASION

Migration and Invasion in Boyden Chambers. The capacity of 2A2 and HOS cells to migrate or invade was tested in Boyden chambers (Neuro Probe, Inc., Cabin John, MD). In each assay, the upper and lower compartments of the chamber were separated by polycarbonate filters (Nucleopor, Pleasanton, CA) containing 8-μm pores. The filters were uncoated or coated with Matrigel (Collaborative Research) by the manufacturer or by adding 135 μl Matrigel (1 mg/ml)/filter as described (21). The filters were placed into the chambers, the lower compartments of which were filled with tumor cell conditioned medium which served as a chemotaxtractant. This conditioned medium was made by incubating DU-145 prostate cancer cells for 72 h in 2% fetal calf serum-supplemented medium. In each experiment, cells (3 × 10^5) harvested with 10 mM EDTA in PBS were suspended in 400 μl DME and seeded into the upper compartment. The cells were then incubated in the absence or presence of either scuPA (25 μM) or plasminogen (300 μM) or in the presence of both for 5 or 21 h at 37°C in a sealed chamber under 5% CO₂. After the incubation the cells were fixed and stained with Diff-Quick (Scientific Products, Columbia, MD). Stained cells attached to the upper surface were first photomicrographed to assess uniformity of cell adhesion and viability and then removed with a cotton swab. Stained cells accumulated on the lower aspects of the filters were quantitated.

To quantify invasiveness, cells were counted on photomicrographs (×10) comprising ~75-90% of the area of the filter by computer imaging depending on the filter size (Image 1.41; NIH). The computer-generated numbers were confirmed by manual counting of the invasive cells in representative experiments. To quantify migration, filters were scanned optically and analyzed by densitometry (Image 1.41) because of the high cell number/unit area. Filters without cells were used as a background and the values subtracted. The absorbances obtained correlated linearly with cell counts performed in parallel. Statistical comparisons between HOS and 2A2 were made using an unpaired two-tailed t test. A P value of <0.05 was considered to be significant.

Fig. 1. Northern blot analysis of pMAMuPAR-transformed HOS cells. Cytoplasmic RNA was isolated from HOS cells, from pMAMuPAR-transformed uPAR neo− (2A2;1D4) and uPAR neo+ (3D1;1B4) variants and from vascular smooth muscle cells. The RNA was separated in agarose (1.49H formaldehyde gel and transferred to a nitrocellulose filter and hybridized with random labeled uPAR DNA purified from pGEMuPAR. Additional uPAR specific bands are present in samples of 2A2 and 1D4.

Fig. 2. Northern blot analysis of pMAMuPAR-transformed HOS cells. Cytoplasmic RNA was isolated from HOS cells, from pMAMuPAR-transformed uPAR neo− (2A2;1D4) and uPAR neo+ (3D1;1B4) variants and from vascular smooth muscle cells. The RNA was separated in agarose (1.49H formaldehyde gel and transferred to a nitrocellulose filter and hybridized with specific DNA probes.
The Northern blots were also tested for the presence of other relevant mRNAs such as α-enolase, a putative plasminogen receptor (23), uPA. neo+ clones expressed very low steady state levels of uPAR mRNA. One of the neo+ clones, designated ID4, contained the highest level of uPAR mRNA. The uPAR gene was detectable in all cell lines and was used to correct for minor differences in loading of the gel (Fig. 2). Densitometric quantitation of the Northern blots is summarized in Table 1.

Characterization of uPAR on 2A2 Cells. In order to test whether the 10-fold increase in uPAR mRNA expressed by 2A2 cells was associated with a comparable increase in uPAR protein on the cell surface, we measured the binding of labeled scuPA to 2A2 and HOS. 2A2 expressed approximately 3-fold more binding sites for scuPA on its surface than did any of the other variants or the parental HOS cells (Fig. 3). The ligand binding experiments repeated on acid washed cells revealed no significant difference in binding, suggesting <5% occupancy with endogenous uPA (data not shown). In separate experiments the binding of 125I-scuPA was directly compared in replicate wells with binding of a monoclonal anti-uPAR antibody. Binding of scuPA was increased 1.9 ± 0.1-fold in 2A2 compared to HOS, whereas the specific binding of anti-uPAR antibody was increased 2.0 ± 0.3-fold. The increase in the number of binding sites was confirmed by PI-PLC-mediated release from 2A2 cells, with approximately 40% of the isolated neo+ clones being uPAR+ (Fig. 5). The ligand binding experiments repeated on acid washed cells revealed no significant difference in binding, suggesting <5% occupancy with endogenous uPA (data not shown).

Table 1. Densitometric analysis of Northern blots

<table>
<thead>
<tr>
<th>mRNA</th>
<th>HOS</th>
<th>2A2</th>
<th>1D4</th>
<th>3D1</th>
<th>1B4</th>
<th>SMC</th>
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<tbody>
<tr>
<td>uPAR</td>
<td>1.0</td>
<td>10.2</td>
<td>2.2</td>
<td>0.6</td>
<td>2.1</td>
<td>5.2</td>
</tr>
<tr>
<td>α-enolase</td>
<td>1.0</td>
<td>1.6</td>
<td>1.4</td>
<td>1.1</td>
<td>0.9</td>
<td>0.3</td>
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</table>

Fig. 3. Specific binding of scuPA to HOS cells transformed with pMAMuPAR. Cells were incubated with 4.0 nM 125I-labeled scuPA at 4°C for 3 h and the cell associated radioactivity was determined. Specific binding was calculated by subtracting the counts not competed by 50-fold molar excess unlabeled scuPA and based on the specific activity of labeled scuPA. Labeling for uPAR and neo+ was based on evaluation in Southern blot analysis. The data shown represent the mean ± SD of four independent experiments performed in triplicates.

Fig. 4. Scatchard analysis of equilibrium binding data. HOS and 2A2 cells were incubated with 125I-labeled scuPA at 4°C for 3 h and the specific cell-associated radioactivity was determined. Each point was performed in triplicate with the values shown from a representative experiment performed in parallel.

Fig. 5. Release of uPAR from 2A2 cell by PI-PLC treatment. Cells were incubated with 125I-labeled scuPA for 3 h at 4°C. Unbound scuPA was removed and washed cells were incubated with PI-PLC for 20 min at 37°C. Released and cell-associated radioactivity was determined. Specific PI-PLC-releasable receptor numbers were calculated by subtracting the counts not competed by 50-fold molar excess unlabeled scuPA and based on the specific activity of labeled scuPA, assuming a 1:1 stoichiometry of scuPA binding to uPAR.
Cells were incubated with 125I-tcPA/PAI-1 complexes (5 nM) with or without 50-fold molar excess of unlabeled complex at 4°C for 3 h. After washing, cells were incubated with PBS-BSA at 37°C for 3 h. Then cells were chilled rapidly and buffers were collected to determine cell-dissociated (TCA-precipitable) and internalized/degraded (TCA-soluble) fractions. Cells were analyzed for cell surface-associated (HCl-releasable) and for intracellular radioactivity. Distribution of 125I-labeled tcPA/PAI-1 was measured in three independent experiments performed in triplicate.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Internalized (Acid nonreleasable)</th>
<th>Degraded (TCA-soluble)</th>
<th>Cell-dissociated (TCA-precipitable)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOS</td>
<td>688 ± 484b</td>
<td>496 ± 27b</td>
<td>262 ± 119b</td>
</tr>
<tr>
<td>HOS</td>
<td>232b</td>
<td>32b</td>
<td>13b</td>
</tr>
<tr>
<td>2A2</td>
<td>826 ± 155b</td>
<td>1017 ± 288b</td>
<td>447 ± 218b</td>
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<tr>
<td>2A2</td>
<td>32b</td>
<td>37b</td>
<td>12b</td>
</tr>
<tr>
<td>2A2/HOS</td>
<td>1.2d</td>
<td>2.1d</td>
<td>1.7d</td>
</tr>
</tbody>
</table>

*a* TCA, trichloroacetic acid.

*b* Mean absolute value in cpm ± SEM.

* Percentage of the amount recovered from the initially bound complex for that cell type.

* Ratio of mean absolute values in each fraction.

* P < 0.05.

Fig. 6. Laminin solubilization by cell associated scuPA. Cells collected with EDTA were incubated in the presence or absence of 50 nM scuPA at 4°C for 45 min. Then cells were seeded on plates precoated with 125I-labeled laminin. Cells, forming confluent layer, were further incubated at 37°C for 1 h with or without plasminogen (300 nM) supplementation. Aliquots were taken and counted for radioactivity. The data were calculated from three independent experiments performed in duplicate. Data were corrected for measurements obtained in presence of the various additives but in the absence of cells. Values were normalized to control cells incubated in the absence of scuPA and plasminogen.

by Scatchard analysis of equilibrium binding data (Fig. 4). The B_max was 2.3 ± 0.4 × 10^5 sites/cell (n = 6) for 2A2 and 1.4 ± 0.3 × 10^5 sites/cell (n = 5) for HOS cells (P < 0.05). The K_d of scuPA for 2A2 and for the parental HOS line (1.1 ± 0.3 nM and 0.6 ± 0.2 nM, respectively) was not statistically different. These data demonstrate that the excess uPAR presented on the transfected cells had the same affinity for scuPA binding as the native receptors, in contrast to the results reported when the human receptor was transfected into murine cells (22).

We then examined whether the uPAR expressed by the transformed 2A2 cells was anchored by GPI as reported for authentic human and murine receptors (6, 24), of which 20-80% is released by PI-PLC. PI-PLC (1 unit/ml) treatment released 5.9 fmol of cell-associated scuPA from 1 × 10^5 2A2 compared with 2.0 fmol from 1 × 10^5 parental HOS cells (Fig. 5), equivalent to 71 and 50% of the total bound scuPA, respectively. The concentrations required for one-half maximal cleavage of uPAR from both cell types were similar. These data indicate that the excess uPAR on the 2A2 cells is bound to the plasma membrane by a GPI anchor.

We then compared the capacity of 2A2 to mediate the internalization and degradation of cell-associated tcuPA/PAI-1 complexes, which were reported to be internalized by uPAR of U937 and keratinocytes (25, 26). The 2A2 cells bound, internalized, and degraded more complex than did HOS. The amount of internalized and degraded ligand complex was proportional to the number of uPARs expressed by the cells (Table 2).

**Generation of Plasmin by 2A2 Cells.** To know whether the receptor-bound uPA on 2A2 cells functions as a plasminogen activator, we compared 2A2 and HOS cells in two indirect assays, both of which measure the plasmin generation. In a chromogenic assay, the receptor-
UROKINASE RECEPTOR-MEDIATED TUMOR CELL INVASION

HOS 2A2

Polycarbonate filters with 8-μm pores were placed into Boyden chambers of which lower compartments were filled with chemoattractants. Cells were placed into the upper compartments of the chambers and incubated for 5 h. Then filters were stained and cells migrated to the lower aspects of the filters were photographed. Representative photomicrographs (× 25) for the various conditions for both HOS and 2A2 cells; final concentrations: scuPA (25 nM); plasminogen (300 nM).

Fig. 8. Migration assay in Boyden chamber. Polycarbonate filters with 8-μm pores were placed into Boyden chambers of which lower compartments were filled with chemoattractants. Cells were placed into the upper compartments of the chambers and incubated for 5 h. Then filters were stained and cells migrated to the lower aspects of the filters were photographed. Representative photomicrographs (×25) for the various conditions for both HOS and 2A2 cells; final concentrations: scuPA (25 nM); plasminogen (300 nM).

Fig. 9. Quantitation of cell migration measured in Boyden chamber assays. Cells migrating through the pores of the uncoated polycarbonate filters were performed as in Fig. 8. Data were quantified using optical densitometry of the stained filters over a user defined region of interest that encompassed the entire filter area exposed to cells. The densities were corrected for measurements made on filters without cells. The data represent the mean ± SEM from 9 experiments. The range of values in arbitrary density units were as follows for HOS: control (34-45); scuPA (28-47); scuPA/plasminogen (18-51).

For 2A2: control (34-73); scuPA (31-75); scuPA/plasminogen (29-67).

bound scuPA generated 3-fold more plasmin activity on 2A2 than it could on HOS cells (data not shown). This increase was proportional to the increase in scuPA binding, performed in parallel. In these tests, the plasmin activity was assayed using a soluble substrate. As a second measure, we compared the ability of 2A2 and HOS to degrade an immobilized, 125I-labeled laminin substrate. Laminin degradation initiated by receptor-bound scuPA was increased 2-fold for 2A2 relative to HOS in the presence of plasminogen (Fig. 6).

Migration Potentials of 2A2 Cells. To test the involvement of uPAR in cell migration, we compared the rate of movement by HOS and 2A2 cells on a fibronectin-coated surface using a linear under agarose assay. In this assay, 2A2 moved slightly slower than HOS in three independent experiments (Fig. 7). Since the rate of migration is relatively low in this assay, we were concerned that differences in the rate of proliferation of the cells could partially explain these results. Therefore, we repeated these studies over 7 h, monitoring individual cell motion using time lapse videomicroscopy. Over this time period ~5% of the monitored cell divided and the total distance that the cellular front had moved in 7 h was the same for 2A2 and HOS. We noted a more prominent undulating lamellae at the leading cell edge during HOS movement, whereas 2A2 were more rigid and tended to move out in a linear sheet.

To measure migration rate over a shorter time period (5 h), we utilized Boyden chambers and monitored cell migrated through the pores of uncoated polycarbonate filters (Fig. 8). As shown, there were no readily discernible differences in the migration rates of HOS and 2A2, nor was migration altered by the addition of scuPA alone or scuPA added together with plasminogen. Contrary to the linear under agarose assay performed on coated surfaces, there was a tendency for the 2A2 cells to move slightly faster on the uncoated polycarbonate surface in each condition, although none of these differences reached statistical significance (Fig. 9).

Invasion by 2A2 Cells. Finally, our last series of experiments was designed to test whether the 2A2 cells would penetrate a Matrigel barrier more readily than HOS when each was permitted to form plasmin by addition of scuPA and plasminogen. We used a relatively...
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HOS 2A2

Fig. 10. Invasion assay in Boyden chamber. Polycarbonate filters (8-μm pores) were precoated with Matrigel (Collaborative Research) and placed into Boyden chambers of which lower compartments were filled with chemoattractants. Suspension of HOS and 2A2 were layered on the top of solidified Matrigel. After a 21-h incubation, the filters were stained and cells that invaded the Matrigel barrier and accumulated on the lower aspects of the filters were photographed. Depicted here are higher power photomicrographs (X 25) from an experiment representative of three so performed in the presence of scuPA (25 nM) alone or with plasminogen (300 nM), while neither protein was added to control cells.

Fig. 11. Quantitation of cell invasion measured in Boyden chamber assays. Cells invading the Matrigel barrier were assayed in three experiments performed as in Fig. 10. Data were quantitated using individual cell counting of the major central portion of the filter (~75% of the area) using low power (X 10). The data represent the mean of three experiments ± SEM. The range of values for HOS were control (59-243), scuPA (61-297), and scuPA/plasminogen (75-258); for 2A2 the range of values were: control (193-457), scuPA (96-473), and scuPA/plasminogen (488-1186). The only difference that was statistically significant was when HOS and 2A2 were compared in the presence of scuPA/plasminogen (P < 0.05) (Fig. 11).

DISCUSSION

A receptor for urokinase-type plasminogen activator has been characterized on many cell types, and it has been implicated in a variety of biological processes (reviewed in Ref. 27). To delineate the role of uPAR in cell migration, Blasi and coworkers (4, 22, 28) established, characterized, and examined the capacity of a murine cell line (LB-6) that overexpresses human uPAR to degrade and invade extracellular matrix. These studies indicated that the capacity of cells to localize uPA to the cell surface facilitates both processes. However, these investigators noted a significant decrease in the affinity of scuPA for its receptor in a murine background, which may alter its ability to generate plasmin, internalize uPA/PAI-1 complexes, or recycle receptor to the cell surface. Since the interactions between uPA and its receptor is species specific, we established an experimental system where the human uPAR was overexpressed in a human cell type.

We found that the steady state level of uPAR mRNA was approximately 10-fold higher in an uPAR-transformed HOS cell line (2A2) than its parental line (Fig. 2; Table 1). However, when the same cell lines were analyzed for the expression of uPAR antigen and scuPA binding (Fig. 3) only a 2-3-fold increase in receptor number was detected. It is possible that in 2A2 cells the translation of mRNA, which is transcribed from the incorporated uPAR cDNA, is less efficient. Our construct does not contain noncoding sequences of the authentic uPAR, which may have impaired ribosomal binding and translation. We cannot rule out other mechanisms such as differences in thick coating for these experiments, such that an overnight incubation was required for adequate numbers of cells to invade the solidified layer of Matrigel. When thin coatings of Matrigel were used, invasion occurred in 5 h, and there was no demonstrable difference in invasion between HOS and 2A2 (data not shown). However, when thick coatings of Matrigel were used only a few cells could penetrate through the Matrigel barrier over 21 h (Fig. 10). However, when scuPA and plasminogen were added to the cells, we observed a 4-fold increase in the number of 2A2 relative to HOS cells which penetrated through the matrix barrier and accumulated on the lower aspects of the filters (P < 0.05) (Fig. 11).
in posttranslational modifications that make transfected uPAR less stable or less efficient in membrane insertion. However, we did not detect the accumulation of unglycosylated 37-kDa uPAR or other intermediates in immunoprecipitation assays (data not shown).

Several laboratories, including our own, have observed that increased expression of uPAR may be associated with a decrease in the affinity for uPA as reflected in a higher dissociation constant (29–31). For example, we reported that incubation of human umbilical vein endothelial cells with PMA resulted in a 3–4-fold increase in number of uPAR/cell and 4-fold decrease in affinity, without obvious changes in the apparent molecular mass of the uPAR (16). However, PMA may also affect posttranslational modification (8) in addition to transcription (9, 32). As a second example, the affinity of the human uPAR expressed on transformed mouse LB6 cells for uPA was 10 times lower than that on human cells (22). It was proposed that for maximal binding potential the receptor might require species-specific interactions with other molecules (22). Our results support this hypothesis in that the transformed 2A2 cell line expressed 2–3-fold more receptors/cell (Fig. 3) with no significant alteration in receptor affinity (Fig. 4).

The 2A2 cell line also internalized and degraded cell-bound uPA/PAI-1 complexes to a greater degree than the parental lines suggesting that the process is mediated by the additional uPAR expressed on the transfected cell. The proportion of radioactivity found in each fraction was the same, suggesting that the uPAR derived from the inserted cDNA functioned in a similar fashion as native uPAR. It has recently been demonstrated that the presence of the α1-macroglobulin receptor/low density lipoprotein receptor-related protein, which has been implicated in the removal of uPA/PAI-1 complexes from the cell surface, is required for blastocyst invasion in vivo (33). This process has been thought to be mediated by uPA (34). Internalization of the complexes from surface receptors may permit them to dissociate from uPAR in an acidic intracellular compartment. Unoccupied uPAR may then recycle to the cell surface where it can again bind functionally active uPA (33).

Cell migration is a complex process involving the continuous reconstitution of the cytoskeleton by assembling and disassembling actin filaments and the synchronized detachment from and attachment to the extracellular substrate (35). Extracellular proteases such as metalloproteinases and plasmin have been implicated in the migration of endothelial cells (36, 37). It has been proposed that receptor-bound uPA activity at the cell-substratum contact site can trigger pericellular matrix degradation enabling cells to migrate (2, 27, 37). In agreement with this concept, colocalization of uPA and uPAR in migratory cells and the absence of uPA in nonmigratory, quiescent fibroblasts have been reported (38). However, it has not been clear whether uPAR-dependent plasmin formation alters cell attachment and migration or solely modulates invasion. We have found that uPAR-transformed cells migrate slower than parental HOS cells on a fibronectin-coated surface but not on uncoated polycarbonate surfaces. Using time lapse videomicroscopy, the 2A2 cells appeared to have a decrease in undulating lamellae at the leading edge of cells, and tended to move in a more rigid, adherent fashion on the fibronectin-coated surface. Whether uPAR are directly or indirectly responsible for these effects is unclear. Nevertheless, it is possible that similar to the PMA-treated U937 cells, uPA bound to uPAR may actually increase cell adhesion to the extracellular substratum (39) which could result in decreased random motility. An extensive evaluation of the role of uPA and uPAR in various extracellular matrix interactions is beyond the scope of this investigation but might yield important information involving mechanisms of migration and invasion.

A growing body of evidence supports the idea that receptor-bound uPA plays a central role in localized plasmin generation and subsequently cell invasion. Recently it was shown that the invasive potential of uPAR producer cells can be increased by uPA provided by surrounding secretory cells (4, 40, 41). However, the possible contribution of species-specific interactions with ligands, receptors, substrates, cofactors, and inhibitors cannot be adequately assessed in such systems. In experiments described here, we observed that saturation of uPAR with scuPA and addition of plasminogen resulted in a 4.3-fold increase in invasion of a Matrigel barrier in a totally human system. When thin coatings or no coatings were used, no difference between cell types was observed and no effect was detected upon the addition of scuPA or plasminogen. These data demonstrate a potential limitation of this and similar studies, in that the final results may be dramatically affected by the specific model used. Taken together, these data provide supportive evidence in a human cell system that overexpression of the human uPAR may enhance invasion of extracellular matrix barriers through an effect on matrix degradation. This model system may be useful in studying the role of novel inhibitors of individual components of the plasminogen activation system on such invasion.

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Overexpression of Urokinase Receptor Increases Matrix Invasion without Altering Cell Migration in a Human Osteosarcoma Cell Line

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