[CANCER RESEARCH 54, 3066–3071, June 1, 1994]

Binding of Urokinase to Its Receptor Promotes Migration and Invasion of Human Melanoma Cells *in Vitro*[1]

Andreas Stahl[2] and Barbara M. Mueller[3]

Department of Immunology, The Scripps Research Institute, La Jolla, California 92037

ABSTRACT

Previously, we reported that urokinase-type plasminogen activator (uPA) plays a pivotal role in extracellular matrix dissolution by malignant melanoma cells. Here, we demonstrate that a highly metastatic melanoma cell line (M24met) that secretes uPA expresses high levels of the uPA receptor (uPAR), 2.4×10^6 binding sites/cell with a K_D of 5.67×10^{-10} M. The receptor was identified as a 55,000–60,000 kDa cell surface protein. Although M24met cells secrete uPA, they are unable to efficiently utilize this enzyme for invasion, unless it is bound to its receptor. This contention is based on the finding that an antibody against uPAR (monoclonal antibody 3936) inhibited invasion of M24met cells through a reconstituted basement membrane (Matrigel) up to 33%, while a reduction of uPA catalytic activity by its plasminogen activator inhibitor-2 resulted in 46% inhibition of invasion. Furthermore, uPAR is involved in signal transduction events in M24met cells, since both uPA and its amino-terminal fragment stimulated the migration of melanoma cells toward Matrigel, resulting in maximal increases of 32 and 72%, respectively. Our results indicate that both uPA and uPAR are involved in melanoma metastasis and that uPAR contributes to at least two important steps in this process, matrix dissolution and migration.

INTRODUCTION

During the complex process of tumor cell invasion and metastasis, cells have to leave the primary tumor site, migrate through the ECM[4] of the surrounding tissue, penetrate the basement membrane of a blood vessel, and extravasate into a target organ. These processes require tumor cells to secrete a number of different proteases, including uPA and various metalloproteases (1).

The serine protease uPA catalyzes the activation of plasmin from the abundant zymogen plasminogen. Plasmin, a potent protease with broad substrate specificity, can initiate a proteolytic cascade that leads to the degradation of multiple elements of the ECM, including fibronectin, laminin, and type IV collagen (2, 3). Several studies (4, 5) provided evidence that the expression of catalytically active uPA by malignant cells correlates with their invasive potential in animal models. Further evidence for the importance of uPA catalytic activity came from our recent study demonstrating that overexpression of PAI-2 in human melanoma cells inhibits their ability to spontaneously metastasize in SCID mice.[5]

A cell surface receptor for uPA has been identified and characterized at the molecular level (6), indicating that this receptor is an M_r 55,000–60,000 glycoprotein (7) anchored in the cell membrane via a glycosylphosphatidylinositol moiety (8). The binding of uPA to its receptor not only increases its enzymatic activity (9) but also allows a focal and directional proteolysis of the ECM (10). It is through this same receptor that uPA can act like a growth factor (11, 12) and stimulate cellular proliferation and migration (13, 14). Several lines of evidence demonstrated the importance of uPAR during the process of metastasis: (*a*) malignant transformation of human fibroblasts correlated with increased levels of receptor-bound uPA activity (15); (*b*) only LB6 mouse cells expressing both uPA and uPAR were efficient in invading experimentally modified chorioallantoic membranes of chick embryos (16); (*c*) *in vitro* invasion by human glioblastoma cells could be modulated with an anti-uPAR antibody, blocking uPA binding to the receptor (17). In the tumor progression of human melanoma, neither uPA nor uPAR are detectable in benign or early stages but appear frequently in advanced primary melanoma and melanoma metastatic lesions (18).

We have previously shown that M24met human melanoma cells express uPA and that its secretion plays a central role in ECM dissolution by these cells (19). Here, we demonstrate that our highly metastatic melanoma cell line expresses uPAR and that this receptor plays a role in at least two cellular functions that have been implicated in tumor cell metastasis: (*a*) uPAR is involved in melanoma cell invasion through reconstituted basement membrane (Matrigel) and (*b*) uPA binding to its receptor induces cell migration toward a mixture of ECM components. These data strongly support the idea that melanoma cells have to express both uPA and uPAR in order to establish an aggressive metastatic phenotype.

MATERIALS AND METHODS

Antibodies and Enzymes. The mouse anti-human uPAR mAb 3936 (IgG2a) and the polyclonal rabbit anti-human uPAR antibody, 399R, were kindly provided by Dr. Richard Hart (American Diagnostica, Greenwich, CT). The purified murine monoclonal antibody KS1/4 (IgG2a) directed against a human carcinoma antigen (20) was produced in this laboratory and used as an isotype control. Human high molecular weight urokinase was purchased from Calbiochem (San Diego, CA). Recombinant human prourokinase was originally provided by Dr. Stephen Gillies (Damon Biotech, Needham Heights, MA) and later by Dr. Jack Henkin (Abbott Laboratories, Abbott Park, IL). The purified amino-terminal fragment of human urokinase also was a kind gift of Dr. Henkin. Recombinant human PAI-2 was generously provided by Dr. Gregor Schulz (Behring Werke, Marburg, Germany).

Tumor Cell Line. The human melanoma cell line M24 was originally supplied by Dr. D. L. Morton (UCLA, Los Angeles, CA). M24met cells were derived from the lymph node of a nude mouse in which M24 cells were injected s.c. (21). M24met cells were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM glutamine (Whittaker Bioproducts, Walkersville, MD). Cells were passaged using versene-0.5 mM EDTA-0.15 M NaCl-0.02 M HEPES.

Received 12/13/93; accepted 4/5/94.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Data presented in this publication were obtained by A.S. as part of his doctoral thesis to be submitted to the Faculty of Biology at the University of Hamburg. This is The Scripps Research Institute manuscript 8370-IMM.

[2] Recipient of a fellowship from the Studienstiftung des Deutschen Volkes.

[3] To whom requests for reprints should be addressed, at Department of Immunology, The Scripps Research Institute, 10666 N. Torrey Pines Road, La Jolla, CA 92037.

[4] The abbreviations used are: ECM, extracellular matrix; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; PAI-2, plasminogen activator inhibitor-2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, 137 mM sodium chloride-2.7 mM potassium chloride-4.3 mM sodium phosphate dibasic-1.4 mM potassium phosphate monobasic; PBS-T, PBS plus 0.1% Triton X-100; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; DSS, disuccinimidyl suberate; BSA, bovine serum albumin; ATF, amino-terminal fragment of urokinase-type plasminogen activator; PAGE, polyacrylamide gel electrophoresis.

[5] B. M. Mueller, J. B. Yu, and W. E. Laug. Overexpression of plasminogen activator inhibitor 2 in human melanoma cells inhibits spontaneous metastasis in SCID mice, manuscript submitted, 1993.

Binding Assays. Pro-uPA was iodinated for saturation binding assays with Iodo-Gen (Pierce Chemical Co., Rockford, IL), as described previously (21). ^{125}I-pro-uPA was used at a specific activity of 3700 cpm/ng protein. M24met cells (3×10^4/well) in serum-free RPMI were plated into 24-well plates precoated with collagen type I (Collaborative Research, Bedford, MA) and allowed to attach overnight. To strip receptor-bound endogenous uPA, cells were washed once with 0.9% NaCl, then incubated with an acidic buffer (50 mM glycine-HCl-0.1 M NaCl, pH 3.0) for 5 min, and immediately neutralized by adding 2 volumes of neutralizing buffer (0.4 M HEPES-0.1 M NaCl, pH 7.5). For binding assays, plates containing acid-treated or -untreated cells were placed on ice, and their wells were washed twice with ice-cold binding buffer (RPMI 1640 containing 0.1% BSA-0.02% NaN_3-20 mM HEPES, pH 7.5). Cells suspended in binding buffer were incubated with ^{125}I-pro-uPA (3–300 ng/ml). Nonspecific binding was determined in the presence of a 200-fold excess of unlabeled pro-uPA and was generally found to be <15% of the total bound radiolabel. After 2 h incubation on ice, the plates were washed with PBS containing 0.1% BSA-0.02% NaN_3. After this step, control wells from each plate were trypsinized to assess the number of cells remaining, and this cell count was used to calculate the number of binding sites/cell. Cell-bound radiolabel in duplicate samples was determined in a γ-scintillation counter, and the data were analyzed by the method of Scatchard (22). To test whether mAb 3936 can inhibit the binding of pro-uPA to the M24met cells, binding assays similar to those described above were performed. In this case, cells were preincubated for 60 min on ice with unlabeled pro-uPA or mAb 3936 at 10, 100, and 1000 nM and subsequently incubated for 2 h with 1 nM ^{125}I-pro-uPA.

Immunoprecipitation and Western Blotting. Approximately 5×10^6 cells were removed from the culture flasks with versene and then homogenized in 5 ml lysis buffer (10 mM Tris, pH 7.4-150 mM sodium chloride-1 mM EDTA-1% Nonidet P-40-1 mM phenylmethylsulfonyl fluoride). Lysates were precleared with protein-A Sepharose and incubated for 1 h with 10 μg mAb 3936 bound to a 50-μl slurry of protein-A. Immunoprecipitates were washed 4 times with washing buffer (50 mM Tris, pH 8.1-10 mM EDTA-150 mM sodium chloride-0.1%-1% desoxycholic acid sodium salt-1% Triton X-100). Proteins were boiled for 3 min in Laemmli buffer under reducing conditions and analyzed on a 5–15% denaturing polyacrylamide (Protogel; National Diagnostics, Atlanta, GA) gradient gel. Gels were silver stained (described in Ref. 23) or proteins were transferred to a nitrocellulose membrane (BioBlot-NC; Costar, Cambridge, MA) using the semidry approach (0.8 mA/cm^2 membrane; transfer buffer containing 20 mM Tris-150 mM glycine-0.1% SDS-20% methanol). Membranes were blocked overnight in PBS-T and 5% nonfat dry milk powder. Primary antibody (399R) was diluted 1:1000 in PBS-T containing 1% non-fat dry milk powder and incubated for 1 h with the membrane. Nitrocellulose membranes were washed three times in PBS-T and incubated for 1 h with a 1:3000 diluted goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad, Richmond, CA). Membranes were washed 5 times in PBS-T, and specifically bound secondary antibody was visualized with 3,3'-diaminobenzidine tetrahydrochloride (ICN, Cleveland, OH).

Cross-linking. M24met cells (5×10^5) were acid treated as described previously (19) and incubated in 1 ml serum-free medium containing 200 ng ^{125}I-pro uPA (16,000 cpm/ng). These cells were then washed 4 times with PBS. Cross-linking was done with 1 mM DSS (Pierce) for 15 min, and this reaction was stopped by addition of ammonium acetate to a final concentration of 10 mM. After the cross-linking process, the cells were directly lysed in Laemmli buffer and separated on a 5–15% gradient SDS-PAGE under reducing conditions. The gel was dried and exposed to X-ray film (X-OMAT AR; Kodak, Rochester, NY).

Invasion Assays. Invasion assays were performed as described previously (24). Briefly, cells were incubated for 12 h in RPMI supplemented with 10% FBS containing 2 μCi [^{3}H]thymidine/1000 cells and acid treated as described above. Matrigel (Collaborative Research) was diluted in ice-cold RPMI to a final concentration of 1 mg/ml. Aliquots (135 μl) were added to the upper chambers of 12-mm Transwell plates with 12-μm pore polycarbonate membranes (Costar) and incubated for 1 h at 37°C. [^{3}H]Thymidine-labeled cells (1.5×10^4) in a volume of 500 μl supplemented RPMI were placed in the upper compartment of these Transwell chambers, and 1 ml tissue culture medium was placed in the lower compartment. The cells were incubated for 3 days at 37°C in the presence of mAb 3936, KS1/4, or PAI-2 with the indicated concentrations or remained untreated. The medium from upper and lower chamber were separately collected and pooled with the cells from the upper and lower sides of the membrane which were harvested by repeated trypsin washes. Cells were pelleted by centrifugation at 800 × g for 15 min, and 3 ml scintillation cocktail (Ecolume; ICN, Irvine, CA) was added to each pellet. Relative cell numbers were obtained by β-scintillation counting (LS 5000 TD; Beckman, Fullerton, CA), and the percentage of invasion was calculated by the ratio of total counts to counts obtained from the lower chamber of each well.

Migration Assays. Migration assays were performed as previously described (25). Briefly, the lower chamber and the lower side of a 8-μm pore size polycarbonate membrane in a 6.5-mm Transwell plate (Costar) were coated with 0.5% BSA or Matrigel (20 μg/ml) in migration buffer (RPMI containing 2 mM $CaCl_2$-2 mM $MnCl_2$-2 mM $MgCl_2$). The plates were incubated for 1 h at 37°C. The Matrigel and BSA solutions were exchanged for migration buffer supplemented with 0.5% BSA. Acid-treated cells were preincubated for 1 h with varying concentrations of uPA or ATF. M24met cells (1×10^5 cells in a volume of 100 μl migration buffer) were added to the upper compartment of the Transwell plates and incubated at 37°C for 8 h. Cells from the inside of the upper chamber were removed with a cotton-tipped swab. The membrane inserts were placed in 250 μl freshly prepared crystal violet solution (1% crystal violet in 50 mM borate buffer, pH 9.0, with 2% ethanol), and cells were stained for 20 min. Inserts were washed thoroughly in water and drained, and the upper chamber was wiped dry with a cotton swab. Cells were counted with an inverted microscope (×40) in one microscopic field in the center of each membrane.

RESULTS

M24met Cells Bind Pro-uPA in a Specific and Saturable Manner. The established human melanoma cell line M24met expressed uPAR on the cell surface, as determined by preliminary indirect immunofluoresence analysis (data not shown). To determine the numbers of these receptors and their binding affinity, saturation binding assays were done with ^{125}I-pro-uPA (Fig. 1). Prior to these binding studies, cells were acid treated to eliminate the effects of previously bound uPA. Alternatively, the cells were left untreated in order to estimate the number of occupied receptors under cell culture conditions. Scatchard analysis of these binding data indicated the existence of a single class of receptor molecules on the surface of the cells with a K_D of $5.67 \pm 1.9 \times 10^{-10}$ M (mean ± SEM), obtained in 3 independent binding experiments. The total receptor number on acid-treated cells was $2.47 \pm 0.4 \times 10^6$ binding sites/cell. Experiments with untreated cells showed $1.99 \pm 0.4 \times 10^6$ receptors/cell, indicat-

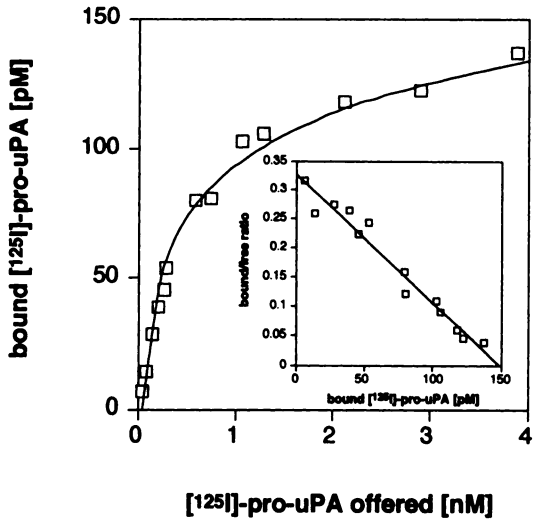

Fig. 1. Scatchard analysis of ^{125}I-pro-uPA binding to M24met melanoma cells. M24met cells were acid treated and incubated with ^{125}I-pro-uPA. Specific binding of ^{125}I-pro-uPA was determined by subtracting the activity bound in the presence of a 200-fold excess of unlabeled pro-uPA from the total. *Inset*, Scatchard analysis of the ^{125}I-pro-uPA-binding data.

ing that only 20% of the receptors were occupied under tissue culture conditions (data not shown).

In our studies, we used a monoclonal antibody (mAb 3936) that specifically recognizes the uPA-binding site of uPAR. To evaluate the ability of this antibody to block ligand binding, we performed competition binding assays. Thus, M24met cells were preincubated either with a 10, 100, or 1000 nM solution of unlabeled pro-uPA or with mAb 3936, and then the inhibition of binding of 1 nM ^{125}I-pro-uPA was determined (Fig. 2). A 1000-fold excess of unlabeled pro-uPA resulted in a 98.9% inhibition of ^{125}I-pro-uPA binding, while mAb 3936 at the same molar concentration inhibited binding by 67%. The inability of mAb 3936 to achieve a complete inhibition at high molar excess suggests that it has a lower affinity for uPAR than uPA has for its receptor.

uPA Receptor on Melanoma Cells Is a 55,000–60,000 kDa Surface Protein. To characterize the uPA-binding molecule on M24met melanoma cells, intact cells were incubated with radiolabeled pro-uPA, and the bound urokinase was cross-linked with DSS. Autoradiography of cell lysates that were subjected to SDS-PAGE demonstrated, in addition to the uPA band (M_r 55,000), a second component with an apparent molecular mass of 100,000–110,000, indicating a close association of uPA with an M_r 55,000 molecule (Fig. 3*A*). Binding of uPA to this receptor was specific, since the high molecular weight component could be greatly reduced with a 50-fold excess of unlabeled pro-uPA. The receptor was further characterized by immunoprecipitation with mAb 3936 (Fig. 3*B*). In certain experiments immunoprecipitates were subjected to subsequent Western blotting with the polyclonal serum 399R raised against the human uPAR (Fig. 3*C*). Both experiments revealed three distinct components in the molecular mass range of 55–60 kDa. The results from cross-linking, immunoprecipitation, and Western blotting agree with previous reports describing the human uPA receptor (7).

Enzymatic Activity and Receptor Binding of uPA Are Required for Optimal Invasion of Melanoma Cells *in Vitro*. It has been reported that expression of uPAR by tumor cells can play an important role in contributing to their metastatic phenotype (5, 26). We assessed the contribution to the effective invasion of melanoma cells of the catalytic activity of uPA and the binding of uPA to its receptor. To this end, uPA activity was inhibited by its physiological inhibitor, while binding of uPA to its receptor was blocked by mAb 3936. On average, 18% of M24met melanoma cells invaded through the reconstituted basement membrane in a 3-day assay. PAI-2 treatment of the melanoma cells reduced their invasiveness in a dose-dependent manner (Fig. 4). Thus, 100 μg/ml PAI-2 inhibited the invasiveness of the melanoma cells by 46%. Reduction of uPA binding to its receptor by mAb 3936 (100 μg/ml) also inhibited the invasiveness of these cells by 33% (Fig. 5). Inhibition of invasion by mAb 3936 (33 ± 3%) and PAI-2 (46 ± 3%) was highly significant in a *t* test for both ($P < 0.005$) compared to an isotype-matched control monoclonal antibody (KS1/4) that had no effect on the invasiveness of the cells. These experiments were each done in quadruplicate, and similar results were obtained in several independent experiments.

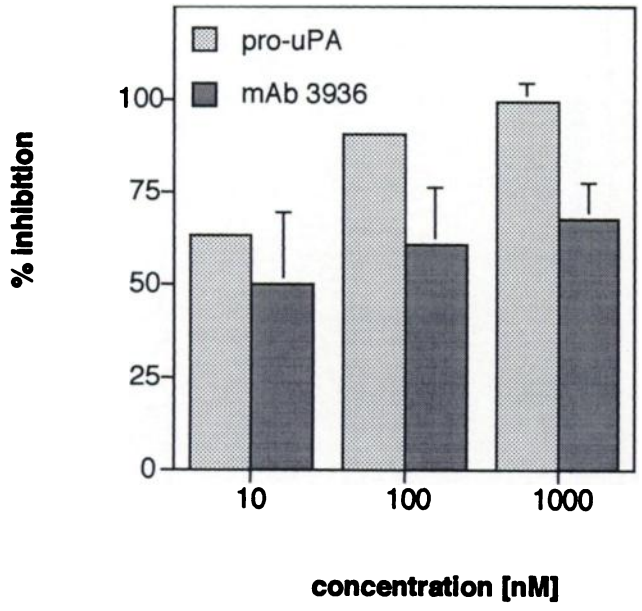

Fig. 2. Inhibition of ^{125}I-pro-uPA binding to M24met cells. M24met melanoma cells were preincubated with 10, 100, and 1000 nM pro-uPA or mAb 3936, followed by incubation with 1 nM ^{125}I-pro-uPA. *Columns* (*bars*), means (±SEM) of quadruplicates.

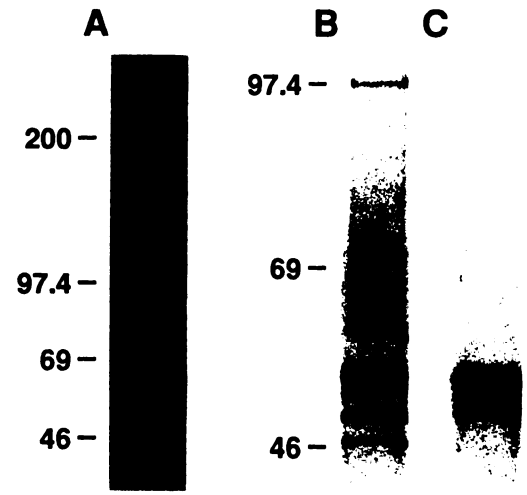

Fig. 3. Characterization of the uPAR on human melanoma cells. *A*, cross-linking of ^{125}I-pro-uPA with DSS to its receptor on M24met cells. *B*, immunoprecipitation of M24met cell lysates with mAb 3936 and subsequent silver staining. *C*, immunoprecipitation of M24met cell lysates with mAb 3936 and subsequent Western blotting with 399R. Bound antibodies were visualized by 3,3'-diaminobenzidine tetrahydrochloride staining. All proteins were separated on a reducing gradient SDS-PAGE (5–15%). *Ordinates*, relative molecular mass in kDa.

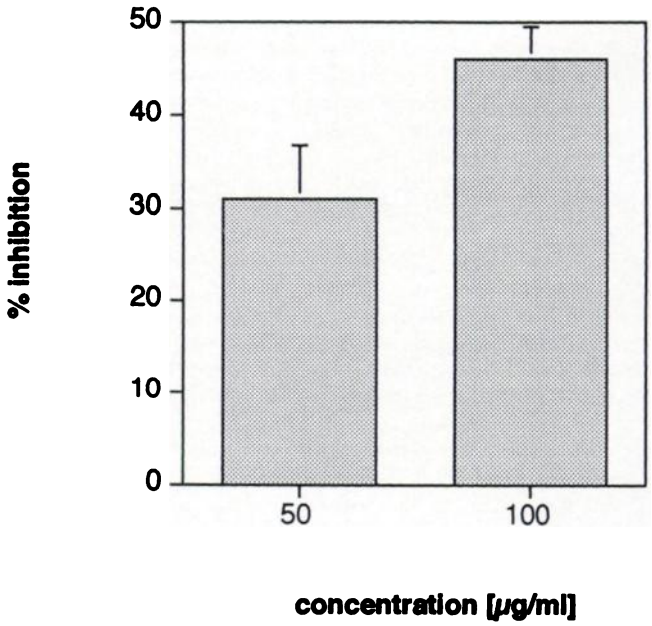

Fig. 4. Dose-dependent inhibition of M24met cell invasion by PAI-2. The melanoma cells were treated in a 72-h invasion assay with 50 or 100 μg/ml PAI-2, and the invasion was compared with those of untreated cells. *Columns* (*bars*), means (±SEM) of quadruplicates.

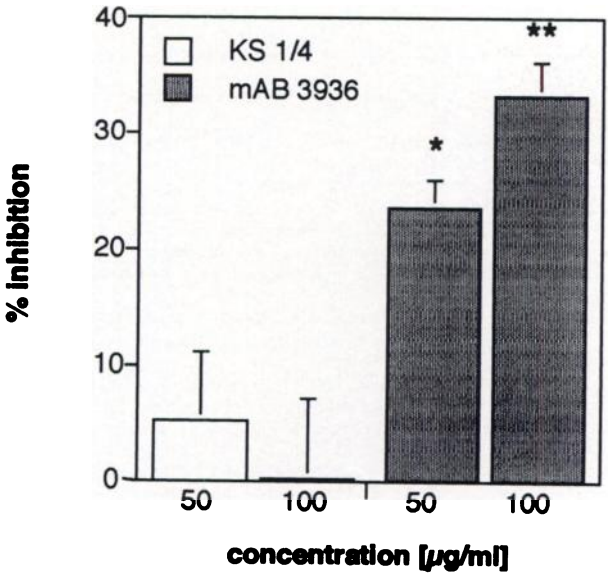

Fig. 5. Inhibition of M24met invasion by mAb 3936. The melanoma cells were incubated during the 72-h period of the invasion assay with mAb 3936 or an isotype-matched control antibody (KS1/4) in the indicated concentrations. The inhibition caused by mAb 3936 was significantly different from the control (*P = 0.03; **P = 0.005). *Columns* (*bars*), means (±SEM) of quadruplicates. Similar results were obtained in 3 independent experiments.

uPA Receptor Is Involved in Melanoma Cell Migration. Additional experiments were performed to assess whether uPAR is only important for melanoma cell invasion because of its enhancement of matrix degradation subsequent to uPA binding or whether other processes independently of proteolytic activity may also be of importance. Since uPA is known to stimulate migration of human keratinocytes (12), we determined the effects of uPA and its enzymatically inactive ATF on the haptotaxis of melanoma cells. In this regard, M24met cells were observed to migrate toward Matrigel in a modified Boyden chamber assay under serum-free conditions in a time-dependent manner (data not shown). After 8 h, an average of 64 melanoma cells were found in one microscope field (×40). Thus, M24met cells were incubated with different concentrations of uPA and ATF for 8 h, and the migration of these cells were compared with those of untreated cells or cells migrating toward a control protein (BSA). Cells did not migrate in an 8-h assay to the lower side of the Transwell plate when BSA was used as a chemoattractant; however, both uPA and ATF stimulated migration toward Matrigel in a dose-dependent fashion (Fig. 6). Maximal stimulation of migration was achieved at 0.25 nM uPA or ATF. At this concentration, ATF produced an increase in migration of 72% compared with untreated cells, while uPA increased cell migration through the membrane by 31%. These results indicate that uPA can stimulate melanoma cell migration independently of its catalytic activity.

DISCUSSION

In this report, we demonstrate that uPAR is expressed on a highly metastatic melanoma cell line (M24met) and that uPAR is functionally involved in invasion and migration of these cells. uPAR on M24met cells is an M_r 55,000–60,000 surface protein that was identified by a monoclonal antibody and a polyclonal serum that were both raised against human uPAR expressed on the monocytic cell line U937. These results indicate immunological similarity of uPAR on M24met melanoma cells with the previously described human uPAR on monocytes (7). Analysis of the ^{125}I-pro-uPA-binding data indicated 2.5×10^6 binding sites/M24met cell with a K_D of 5.67×10^{-10} M. This binding constant is similar to that previously published for uPAR on U937 cells (27) and human keratinocytes (13). However, M24met melanoma cells express an unusually high level of uPAR, which is comparable to that found on phorbol-12-myristate-13-acetate-stimulated U937 cells, *i.e.*, 1.2×10^6 receptors/cell as estimated from previous reports (28, 29). The finding of 2.5×10^6 receptors on the aggressively metastasizing M24met cells supports also the contention that uPAR expression correlates with the degree of malignancy exhibited by tumor cells (30, 31). A comparison of the binding data obtained from untreated M24met cells with those that were acid treated revealed that under tissue culture conditions only 20% of uPAR sites were occupied by endogenous uPA, even though excess urokinase is secreted by these cells (19). Previous reports indicated that the percentage of uPAR occupied by endogenously secreted urokinase may vary from 0–100% (32–34); however, factor(s) that influence the degree of autocrine binding of uPA to its receptor remain to be determined.

We studied the role of uPAR in melanoma cell invasion with an *in vitro* assay that uses Matrigel as a reconstituted basement membrane. Matrigel, a mixture of extracellular matrix proteins secreted by EHS sarcoma cells, is composed mainly of type IV procollagen, laminin, and heparan sulfate proteoglycans (35). Matrigel was chosen for these invasion assays, since it can be dried and reconstituted on membrane filters, forming a uniform basement membrane-like barrier that was demonstrated to be of use for quantifying the degree of tumor cell invasion (24).

In order to assess the contribution of uPA enzymatic activity to matrix degradation in this assay, we inhibited uPA with PAI-2 and found that this could decrease invasion of melanoma cells up to 46%. This finding is in accordance with our recent data indicating that PAI-2 inhibits M24met from degrading matrix glycoprotein *in vitro* (19) and that overexpression of PAI-2 in human M24met melanoma cells inhibits their spontaneous metastasis in SCID mice.[5] To investigate whether the secretion of enzymatically active uPA is not only necessary but also sufficient for matrix degradation during *in vitro*

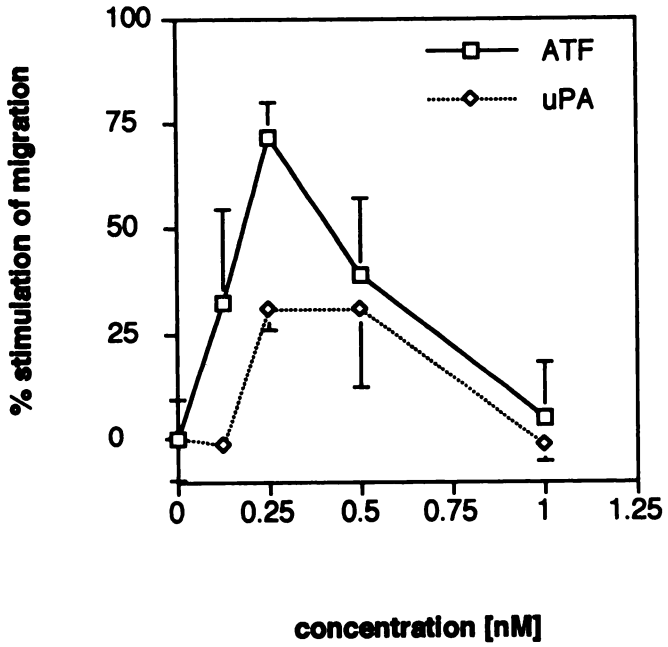

Fig. 6. Concentration-dependent effects of uPA and ATF on M24met haptotaxis toward Matrigel. Migration experiments were performed over an 8-h time period. The migration was specific toward Matrigel, since none of the cells migrated toward BSA. *Points* (*bars*), means (±SD) of triplicates. Similar results were obtained in 4 independent experiments.

invasion, we used an experimental approach to distinguish between receptor-bound and soluble uPA activity. To this end, the uPA-binding capacity of uPAR was reduced to different degrees by incubating M24met melanoma cells with varying concentrations of the antagonistic mAb 3936 during the invasion assay. In competition assays, mAb 3936 inhibited pro-uPA binding to uPAR up to 67%. This antibody was also reported to partially block binding of uPA to uPAR on human glioblastoma cells (17). The mAb 3936 could reduce invasion of M24met cells in a dose-dependent manner. Thus, incubation of these cells with mAb 3936 (100 μg/ml) led to a 33% inhibition of invasiveness when compared with untreated cells. This inhibition of invasion caused by mAb 3936 was specific, since an isotype-matched control mAb KS1/4 directed against a carcinoma antigen had no effect on invasion when used at an equal concentration. The inhibition of invasiveness obtained, *i.e.*, 33%, is remarkably high, since our binding studies demonstrate that mAb 3936 only partially blocks binding of pro-uPA to its receptor. We conclude from these data that, although melanoma cells secrete proteolytic active uPA, they cannot effectively utilize this enzyme for invasion unless it is bound to its receptor. A possible explanation for this phenomenon could be the increase of local concentration of uPA, as well as the enhancement of its proteolytic activity upon receptor binding. These processes would promote the generation of plasmin and, hence, result in a more rapid ECM dissolution (9). Furthermore, the direction of uPA activity to focal contact sites and the leading edge of migrating cells could promote the invasiveness of tumor cells (10). In addition to its function in enhancing ECM degradation, uPA-uPAR interactions can promote other processes involved in melanoma cell invasion independently of the catalytic activity of uPA. We based this conclusion on the results obtained from migration experiments performed with M24met cells. Establishment of a gradient of matrix components that can serve as an immobilized chemoattractant, as well as an adhesion substrate for cells in a Transwell chamber, has been demonstrated to induce haptotactic migration of melanoma cells (25). Specifically, M24met cells migrated in this assay system toward Matrigel components in a time-dependent manner, while none of these cells migrated toward the control protein BSA. Migration of melanoma cells could be enhanced in a dose-dependent manner by incubating them with uPA or ATF. Both uPA and ATF showed a maximal stimulation of migration, 31 and 72%, respectively, at 0.25 nM. Since ATF lacks enzymatic activity, its stimulation of migration is not due to activation of latent growth factors that may be present in a matrix-bound state but is more likely attributable to signal transduction by uPAR. The observation that stimulation of melanoma cell migration with uPA or ATF occurred in a relatively narrow range of nanomolar concentrations, while higher concentrations had no effect on the migration of these cells is in accordance with data derived from the migration of keratinocytes (13) and endothelial cells (36) following stimulation by uPA or ATF.

In conclusion, our findings demonstrate that uPA and its receptor are involved in at least two independent processes, matrix dissolution and migration, both of which are of potential importance for metastasis of human melanoma cells *in vivo*.

ACKNOWLEDGMENTS

The authors thank Dr. Ralph A. Reisfeld for his support and encouragement. They also acknowledge the expert technical assistance of Dorothy Markowitz and helpful discussion by Dr. John Bognacki. The authors are grateful to Dr. Anthony Montgomery for reading this manuscript.

REFERENCES

1. Duffy, M. J. Plasminogen activators and cancer. Blood Coagul. Fibrinolysis, *1:* 681–687, 1990.
2. Kwaan, H. C. The plasminogen-plasmin system in malignancy. Cancer Metastasis Rev., *11:* 291–311, 1992.
3. Mackay, A. R., Corbit, R. H., Hartzler, J. L., and Thorgeirsson, U. P. Basement membrane type IV collagen degradation: evidence for the involvement of a proteolytic cascade independent of metalloproteinases. Cancer Res., *50:* 5997–6001, 1990.
4. Quax, P. H., van Muijen, G. N. P., Weening-Verhoeff, E. J. D., Lund, L. R., Danø, K., Ruiter, D. J., and Verheijen, J. H. Metastatic behavior of human melanoma cell lines in nude mice correlates with urokinase-type plasminogen activator, its type-1 inhibitor, and urokinase-mediated matrix degradation. J. Cell Biol., *115:* 191–199, 1991.
5. Crowley, C. W., Cohen, R. L., Lucas, B. K., Liu, G., Shuman, M. A., and Levinson, A. D. Prevention of metastasis by inhibition of the urokinase receptor. Proc. Natl. Acad. Sci. USA, *90:* 5021–5025, 1993.
6. Roldan, A. L., Cubellis, M. V., Masucci, M. T., Behrendt, N., Lund, L. R., Danø, K., Appella, E., and Blasi, F. Cloning and expression of the receptor for human urokinase plasminogen activator, a central molecule in cell surface, plasmin dependent proteolysis. EMBO J., *9:* 467–474, 1990.
7. Nielsen, L. S., Kellerman, G. M., Behrendt, N., R., P., Danø, K., and Blasi, F. A 55,000–60,000 M_r receptor protein for urokinase-type plasminogen activator. J. Biol. Chem., *236:* 2358–2363, 1988.
8. Ploug, M., Rønne, E., Behrendt, N., Jensen, A. L., Blasi, F., and Danø, K. Cellular receptor for urokinase plasminogen activator. Carboxyl-terminal processing and membrane anchoring by glycosyl-phosphatidylinositol. J. Biol. Chem., *266:* 1926–1933, 1991.
9. Rønne, E., Behrendt, N., Ellis, V., Ploug, M., Danø, K., and Høyer-Hansen, G. Cell-induced potentiation of the plasminogen activation system is abolished by a monoclonal antibody that recognizes the NH_2-terminal domain of the urokinase receptor. FEBS Lett., *288:* 233–236, 1991.
10. Takahashi, K., Ikeo, K., Gojobori, T., and Tanifuji, M. Local function of urokinase receptor at the adhesion contact sites of a metastatic tumor cell. Thromb. Res., *10*(Suppl.)*:* 55–61, 1990.
11. He, C. J., Rebibou, J. M., Peraldi, M. N., Meulders, Q., and Rondeau, E. Growth factor-like effect of urokinase type plasminogen activator in human renal cells. Biochem. Biophys. Res. Commun., *176:* 1408–1416, 1991.
12. Rabbani, S. A., Mazar, A., Bernier, S., Haq, M., Bolivar, I., Henkin, J., and Goltman, D. Structural requirements for the growth factor activity of the amino-terminal domain of urokinase. J. Biol. Chem., *267:* 14151–14156, 1992.
13. Del Rosso, M., Fibbi, G., Dini, G., Grappone, C., Pucci, M., Caldini, R., Magnelli, L., Fimiani, M., Lotti, T., and Panconesi, E. Role of specific membrane receptors in urokinase-dependent migration of human keratinocytes. J. Invest. Dermatol., *94:* 310–316, 1990.
14. McNeill, H., and Jensen, P. J. A high-affinity receptor for urokinase plasminogen activator on human keratinocytes: characterization and potential modulation during migration. Cell Regul., *1:* 843–852, 1990.
15. Jankun, J., Maher, V. M., and McCormick, J. J. Malignant transformation of human fibroblasts correlates with increased activity of receptor-bound plasminogen activator. Cancer Res., *51:* 1221–1226, 1991.
16. Ossowski, L., Clunie, G., Masucci, M. T., and Blasi, F. *In vivo* paracrine interaction between urokinase and its receptor: effect on tumor cell invasion. J. Cell Biol., *115:* 1107–1112, 1991.
17. Mohanam, S., Sawaya, R., McCutcheon, I., Ali, O. F., Boyd, D., and Rao, J. S. Modulation of *in vitro* invasion of human glioblastoma cells by urokinase-type plasminogen activator receptor antibody. Cancer Res., *53:* 4143–4147, 1993.
18. DeVries, T. J., Quax, P. H. A., Denijn, M., Verrijp, K. N., Verheijen, J. H., Verspaget, H. W., Weidle, U. H., Ruiter, D. J., and van Muijen, G. N. P. Plasminogen activators, their inhibitors and urokinase receptor emerge in late stage of melanocytic tumor progression. Am. J. Pathol., *144:* 70–81, 1994.
19. Montgomery, A. M., De Clerck, Y. A., Langley, K. E., Reisfeld, R. A., and Mueller, B. M. Melanoma-mediated dissolution of extracellular matrix: contribution of urokinase-dependent and metalloproteinase-dependent proteolytic pathways. Cancer Res., *53:* 693–700, 1993.
20. Varki, N. M., Reisfeld, R. A., and Walker, L. E. Antigens associated with a human lung adenocarcinoma defined by monoclonal antibodies. Cancer Res., *44:* 681–687, 1984.
21. Mueller, B. M., Romerdahl, C. A., Trent, J. M., and Reisfeld, R. A. Suppression of spontaneous melanoma metastasis in SCID mice with an antibody to the epidermal growth factor receptor. Cancer Res., *51:* 2193–2198, 1991.
22. Scatchard, D. The attraction of proteins for small molecules and ions. NY Acad. Sci., *51:* 660–665, 1949.
23. Oakley, B. R., Kirsch, D. R., and Morris, N. R. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem., *105:* 361–363, 1980.
24. Repesh, L. A. A new *in vitro* assay for quantitating tumor cell invasion. Invasion Metastasis, *9:* 192–208, 1989.
25. Leavesley, D. I., Schwartz, M. A., Rosenfeld, M., and Cheresh, D. A. Integrin beta 1- and beta 3-mediated endothelial cell migration is triggered through distinct signaling mechanisms. J. Cell Biol., *121:* 163–170, 1993.
26. Ossowski, L. Invasion of connective tissue by human carcinoma cell lines. Cancer Res., *52:* 6754–6760, 1992.
27. Vassalli, J. D., Baccino, D., and Belin, D. A cellular binding site for the Mr 55,000 form of the human plasminogen activator, urokinase. J. Cell Biol., *100:* 86–92, 1985.
28. Stoppelli, M. P., Corti, A., Soffientini, A., Cassani, G., Blasi, F., and Assoian, R. K.

Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. Proc. Natl. Acad. Sci. USA, *82:* 4939–4943, 1985.

29. Vassalli, J. D., Wohlwend, A., and Belin, D. Urokinase-catalyzed plasminogen activation at the monocyte/macrophage cell surface: a localized and regulated proteolytic system. Curr. Top. Microbiol. Immunol., *181:* 65–86, 1992.
30. Boyd, D., Florent, G., Kim, P., and Brattain, M. Determination of the levels of urokinase and its receptor in human colon carcinoma cell lines. Cancer Res., *48:* 3112–3116, 1988.
31. Casslén, B., Gustavsson, B., and Astedt, B. Cell membrane receptors for urokinase plasminogen activator are increased in malignant ovarian tumours. Eur. J. Cancer, *27:* 1445–1448, 1991.
32. Reiter, L. S., Kruithof, E. K., Cajot, J. F., and Sordat, B. The role of the urokinase receptor in extracellular matrix degradation by HT29 human colon carcinoma cells. Int. J. Cancer, *53:* 444–450, 1993.
33. Schlechte, W., Brattain, M., and Boyd, D. Invasion of extracellular matrix by cultured colon cancer cells: dependence on urokinase receptor display. Cancer Commun., *2:* 173–179, 1990.
34. Stoppelli, M. P., Tacchetti, C., Cubellis, M. V., Corti, A., Hearing, V. J., Cassani, G., Appella, E., and Blasi, F. Autocrine saturation of pro-urokinase receptors on human A431 cells. Cell, *45:* 675–684, 1986.
35. Kleinman, H. K., McGarvey, M. L., Liotta, L. A., Robey, P. G., Tryggvason, K., and Martin, G. R. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry, *21:* 6188–6193, 1982.
36. Odekon, L. E., Sato, Y., and Rifkin, D. B. Urokinase-type plasminogen activator mediates basic fibroblast growth factor-induced bovine endothelial cell migration independent of its proteolytic activity. J. Cell Physiol., *150:* 258–263, 1992.