

Mitogenic Effects of Urokinase on Melanoma Cells Are Independent of High Affinity Binding to the Urokinase Receptor*

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Jaap L. Koopman, Jennichjen Slomp, Anton C. W. de Bart, Paul H. A. Quax,
and Jan H. Verheijen‡

From the Gaubius Laboratory, TNO Prevention and Health, 2301 CE Leiden, The Netherlands

The structural and functional properties of the urokinase-type plasminogen activator (u-PA) that are involved in the mitogenic effect of this proteolytic enzyme on human melanoma cells M14 and IF6 and the role of the u-PA receptor (u-PAR) in transducing this signal were analyzed. Native u-PA purified from urine induced a mitogenic response in quiescent IF6 and M14 cells that ranged from 25 to 40% of the mitogenic response obtained by fetal calf serum. The half-maximum response in M14 and IF6 cells was reached at u-PA concentrations of approximately 35 and 60 nM, respectively. Blocking the proteolytic activity of u-PA resulted in a 30% decrease of the mitogenic effect, whereas inhibition of plasmin activity did not alter the mitogenic effect. No mitogenic response was elicited by low molecular weight u-PA, lacking the growth factor domain and the kringle domain. The ATF domain of u-PA induced a mitogenic response that was similar to complete u-PA. Defucosylated ATF and recombinant u-PA purified from *Escherichia coli* lacking all post-translational modifications did not induce a mitogenic response. Blocking the interaction of u-PA with u-PAR, using a specific monoclonal antibody, did not alter the mitogenic effect induced by u-PA. The binding of radiolabeled u-PA to M14 and IF6 cells was characterized by high affinity binding mediated by u-PAR and low affinity binding to an unknown binding site. These results demonstrate that proteolytically inactive u-PA is able to induce a mitogenic response in quiescent melanoma cells *in vitro* by a mechanism that involves the ATF domain but is independent of high affinity binding to u-PAR. Furthermore, it suggests that u-PA is able to bind with low affinity to a hitherto unidentified membrane associated protein that could be involved in u-PA-induced signal transduction.

Plasminogen activators are multi-domain serine proteases that are involved in tumor invasion and metastasis (1–4). Two types of plasminogen activators have been described, tissue-type (t-PA)¹ and urokinase-type (u-PA) plasminogen activator.

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‡ To whom correspondence should be addressed: Gaubius Laboratory, TNO Prevention and Health, P.O. Box 2215, 2301 CE Leiden, The Netherlands. Tel.: 31-71-5181505; Fax: 31-71-5181904; E-mail: jh.verheijen@pg.tno.nl.

¹ The abbreviations used are: t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; u-PAR, u-PA receptor; ATF, amino-terminal fragment; LMW u-PA, low molecular weight form of u-PA; DFP, diisopropylfluorophosphate; DMEM, Dulbecco's modified

Both t-PA and u-PA contain a serine-protease catalytic domain and are able to activate plasminogen into plasmin by proteolytic cleavage and are secreted from various cell types in the single chain form. Whereas single chain t-PA is an active enzyme, single chain u-PA is a pro-enzyme and is activated by plasmin cleavage resulting in two-chain u-PA. Further limited plasmic degradation of two-chain u-PA results in the release of the amino-terminal fragment (ATF) of u-PA and the formation of a low molecular weight form of u-PA (LMW u-PA) that contains the fully active catalytic domain.

u-PA and t-PA both have a growth factor domain in the amino-terminal part of the molecule. This growth factor domain is structurally similar to the receptor binding region of epidermal growth factor and is involved in the binding of u-PA to a high affinity cell surface receptor (5). This u-PA receptor (u-PAR) was cloned (6) from the monocyte-like cell line U937 and was found to be a glycosyl-phosphatidylinositol-linked membrane protein (7).

In addition to its proteolytic activity evidence is accumulating that u-PA also has signal transduction properties (8). This signal transduction can lead to a change in the adhesive (9, 10), chemotactic (11, 12), and mitogenic (13–17) response of various cell types. In a number of studies the mitogenic response depends on both u-PA activity and interaction with a cell surface receptor mediated by the ATF domain (16, 18–20). Catalytically inactive u-PA has also been reported to induce mitogenic effects (15) in osteosarcoma cells by a mechanism that involved the interaction of u-PA with the high affinity binding site of u-PAR. However, u-PAR has no trans-membrane nor cytoplasmic domain, and therefore the assistance of an adaptor protein to transduce the signal seems necessary (21). Recently it was reported that active site-inhibited u-PA was able to elicit a mitogenic response in smooth muscle cells independent of high affinity binding to u-PAR (17).

Previously it was demonstrated that u-PA bound to its cellular receptor u-PAR could contribute to the metastatic phenotype of human melanoma cells (22–24). In this study we focus on the signal transduction properties of u-PA on melanoma cells and the possible involvement of u-PAR. We demonstrate that u-PA has a mitogenic effect on two human melanoma cell lines and that this effect is independent of binding to u-PAR.

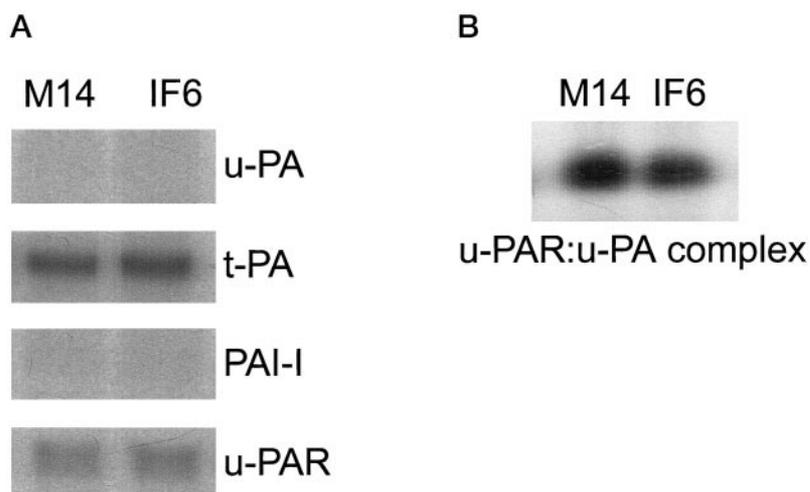
EXPERIMENTAL PROCEDURES

Determination of u-PA, t-PA, PAI-1, and u-PAR Protein Expression—Antigen levels of u-PA, t-PA, and PAI-1 were determined in culture medium by enzyme immunoassays (22) and the presence of u-PAR on the cell membrane of M14 and IF6 cells was determined by cross-linking experiments (22, 25).

Inactivation of u-PA by Diisopropylfluorophosphate—Native u-PA purified from urine (Serono, Coinsins, Switzerland), recombinant u-PA purified from *Escherichia coli* (a gift from Dr. Günzler, Grünenthal,

Eagle's culture medium; FCS, fetal calf serum; mAb, monoclonal antibody; PAI-1, plasminogen activator inhibitor type-1.

FIG. 1. A, Northern blot analysis of RNA extracted from M14 and IF6 cells. The filters were hybridized with cDNA fragments encoding human t-PA, u-PA, PAI-1, and u-PAR. B, cross-linking analysis of functional u-PAR expression on the cell membrane of M14 and IF6 cells. DFP-treated u-PA labeled with ^{125}I was added to cell lysates of M14 and IF6 cells. After cross-linking with disuccinimidylsuberate, samples were analyzed using SDS-polyacrylamide gel electrophoresis and visualized using autoradiography.



Germany) and LMW u-PA containing amino acids 136–411 (Abbott Laboratories, Abbott Park, IL) were dissolved in phosphate-buffered saline, pH 7.4, to a concentration of 1 mg/ml. DFP (Sigma) dissolved in dry isopropanol (0.1 M) was added to a final concentration of 1 mM and incubated for 1 h at 4 °C. The u-PA solutions were dialyzed against phosphate-buffered saline for 24 h at 4 °C, and the u-PA activity was measured using the synthetic substrate S-2444 (Chromogenix, Mölndal, Sweden) as described by the manufacturers. The catalytic activity of DFP-treated native u-PA and recombinant u-PA preparations was less than 0.1% of the original activity, whereas DFP-treated LMW u-PA demonstrated a residual activity of approximately 5%.

Mitogenic Experiments—Human M14 (26) and IF6 (27) melanoma cells were routinely grown in Dulbecco's modified Eagle's culture medium (DMEM) containing 4.5 g/liter glucose and Glutamax supplemented with 10% fetal calf serum (FCS) (Life Technologies, Inc.), 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Biowhittaker, Verviers, Belgium) at 37 °C, 5% CO_2 and 95% air for 72–96 h. Prior to mitogenic experiments, cells were detached from the culture flasks (Costar, Cambridge, MA) by incubation with 0.05% (w/v) EDTA followed by the addition of 5 volumes of DMEM/FCS. The cells were centrifuged for 10 min at $250 \times g$ and subsequently suspended in DMEM/FCS to a concentration of 10^5 cells/ml.

One ml of these suspensions was added to 24-well tissue culture plates (Costar) and incubated for 5 h at 37 °C, 5% CO_2 . The wells were washed twice with serum-free DMEM medium and incubated for 20 h in serum-free medium at 37 °C, 5% CO_2 . Cells were stimulated by replacing the serum-free medium by 1.0 ml of serum-free DMEM containing urine-derived u-PA, recombinant u-PA, t-PA purified from melanoma cells (28), LMW u-PA, or fucosylated and defucosylated ATF domain of u-PA (29) (a gift from Drs. A.P. Mazar and J. Henkin, Abbott Laboratories, Abbott Park, IL). Maximum mitogenic stimulation was achieved by adding DMEM containing 5% FCS to the cells, whereas the basal level of [^3H]thymidine incorporation was determined by incubation with serum-free DMEM medium.

The effect of 100 kallikrein inhibitor units/ml Trasylol (Bayer AG, Leverkusen, Germany), 10 $\mu\text{g}/\text{ml}$ anti u-PAR monoclonal antibody H2 (a gift from Dr. U. Weidle, Boehringer Mannheim, Penzberg, Germany), and 1 $\mu\text{g}/\text{ml}$ pertussis toxin (Sigma) was measured by preincubation of the cells with these components for 30 min before u-PA was added. Cells were stimulated with the mitogenic agonists for 22 h at 37 °C and labeled with 0.5 μCi of [^3H]thymidine (Amersham Pharmacia Biotech) well for the last 5 h. After stimulation the cells were washed twice with serum-free DMEM and precipitated with 10% (w/v) trichloroacetic acid. The precipitate was washed with ice-cold phosphate-buffered saline and dissolved in 1.0 ml of 1 M NaOH, and the [^3H]thymidine incorporation was measured. The mitogenic effect of the different agonists was expressed as a percentage of the mitogenic effect that was induced by 5% FCS and was calculated as described earlier (15).

u-PA Binding Experiments—Urine-derived u-PA and recombinant u-PA were radiolabeled with Na^{125}I (Amersham Pharmacia Biotech) by the iodogen method (Pierce) resulting in a specific activity of 0.77 and 1.9 mCi/nmol, respectively. Binding was performed on 70–90% confluent cells in 24-well tissue culture plates that were cultured in serum-free DMEM medium containing 0.05% human serum albumin (Bio Products Laboratory, Elstree, UK) for 16 h at 37 °C. Cells were incubated on ice for 1 h in serum free-medium or serum-free medium

supplemented with anti u-PAR mAb H2 at a concentration of 10 $\mu\text{g}/\text{ml}$. ^{125}I -Labeled u-PA was mixed with different amounts of the corresponding unlabeled u-PA and added to the cells to give final concentrations that ranged from 0.3 to 200 nM. The cells were incubated on ice for 2 h and subsequently washed two times with serum-free culture medium containing 0.05% human serum albumin and twice with phosphate-buffered saline. Cells were dissolved in 0.2 M NaOH, and the radioactivity bound to the cells was measured. Specific binding to the cells was calculated by subtracting the nonspecific adsorption of radiolabeled u-PA to the tissue culture plates, which was measured for each u-PA concentration used.

Northern Blot Analysis—The mRNA expression levels of t-PA, u-PA, PAI-1, and u-PAR were determined in M14 and IF6 cells, which were cultured under serum-free conditions for 24 h. The mRNA levels of the proliferation markers *c-fos* and *c-myc* were determined in M14 and IF6 cells that were cultured as described under “mitogenic experiments” except that no [^3H]thymidine was added. Total RNA was extracted as described (30), and 10 μg was fractionated by electrophoresis on a 1.2% (w/v) denaturing agarose gel containing 0.75% (w/v) formaldehyde and transferred to a nylon membrane (Hybond, Amersham) using a Vacu-gene system (Pharmacia Biotech Inc.). The cDNA fragments were labeled with [^{32}P]dCTP (Amersham) using the random primer method (Multiprime, Amersham), and membranes were hybridized with 1 ng of ^{32}P -labeled cDNA/ml in 0.5 M sodium phosphate buffer (pH 7.2) containing 7% (w/v) SDS and 10 mM EDTA at 65 °C and subsequently washed twice with $2 \times \text{SSC}$ containing 1% (w/v) SDS at 65 °C.

The membranes were exposed to Fuji Phosphor-imager screens for 16–48 h, and relative intensities of the bands were quantified by a Fuji Bas 1000 Phosphor-imager.

RESULTS

Analysis of t-PA, u-PA, u-PAR, and PAI-1 Expression in M14 and IF6 Cells—Neither cell line demonstrated detectable expression of u-PA and PAI-1 mRNA in the cells (Fig. 1A), and no u-PA or PAI-1 antigen was found in the culture medium after 24 h (Table I). In contrast both cell lines showed high expression of t-PA mRNA (Fig. 1A), and t-PA antigen amounted to 600–700 ng in culture medium after 24 h of 10^6 M14 cells and IF6 cells. Northern blotting and cross-linking experiments demonstrated that both M14 and IF6 cells expressed similar amounts of u-PAR mRNA (Fig. 1A) and u-PAR antigen on the cell surface (Fig. 1B).

Characteristics of the Mitogenic Effect of u-PA on M14 and IF6 Melanoma Cells—The mitogenic effect in response to increasing u-PA concentrations on M14 and IF6 cells was determined by measuring the increase in [^3H]thymidine incorporation after a 22-h incubation period using u-PA purified from urine. The mitogenic effect of u-PA was expressed as a percentage of the effect obtained by incubation with 5% v/v FCS. The increase in [^3H]thymidine incorporation after stimulation of the cells with 5% FCS was approximately 4–6-fold as compared with unstimulated cells. Native u-PA elicited a mitogenic

TABLE I

Antigen levels of u-PA, t-PA, and PAI-1 in M14 and IF6 cells

u-PA, t-PA, and PAI-1 antigen were measured by enzyme-linked immunosorbent assay in the 24-h culture medium of M14 and IF6 cells. The results are expressed as ng of protein synthesized in 24 h by 10^6 cells.

	M14	IF6
	<i>ng</i>	
u-PA	<0.1	<0.1
t-PA	677	623
PAI-1	<0.1	<0.1

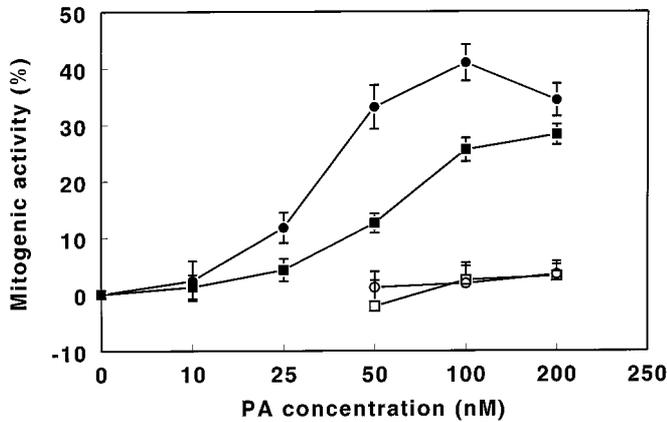


FIG. 2. Dose-dependent mitogenic effect of native HMW u-PA (■, ●) and t-PA (□, ○) on M14 (○, ●) and IF6 cells (□, ■). The mitogenic activity was measured and calculated as described under "Experimental Procedures." Each point represents the mean of quadruplicate measurements, and the error bars represent the S.E.

response in both M14 and IF6 cells reaching half-maximum stimulation of DNA synthesis at u-PA concentrations of approximately 35 and 60 nM, respectively (Fig. 2). The maximum response in M14 was 30–40% of the mitogenic response obtained with FCS, whereas the maximum response in IF6 cells was 25–30% of the FCS response.

To determine whether plasmin activity was involved in the mitogenic effect observed with u-PA, the plasmin inhibitor Trasylol® was added 30 min prior to the addition of u-PA and was present in the medium during the 16-h incubation period with u-PA. The addition of Trasylol® had no effect on the mitogenic stimulus of u-PA (Fig. 3), demonstrating that plasmin activity did not contribute to the mitogenic effect induced by u-PA on M14 and IF6 cells. These results suggest that the growth factor-like properties of u-PA are independent of its plasminogen activating properties. To confirm this, t-PA was also tested for its ability to elicit a mitogenic response in the M14 and IF6 cells. In contrast to u-PA, the addition of up to 200 nM of t-PA did not show any mitogenic effect on these cells (Fig. 1). This indicates that the mitogenic effect on M14 and IF6 cells is specific for u-PA and is independent of plasminogen activation. To determine whether u-PA activity *per se* was involved in the mitogenic effect we used DFP-treated u-PA that had less than 0.1% of its original enzymatic activity. The mitogenic signal in M14 and IF6 cells decreased approximately 31 and 36%, respectively (Fig. 3), indicating that a part of the mitogenic signal was dependent on u-PA activity, whereas the major part of the mitogenic effect was independent of the enzymatic activity of u-PA.

LMW u-PA that lacks the growth factor domain and the Kringle domain but contains the full proteolytic activity of u-PA had no mitogenic effect at all (Fig. 4). Purified ATF domain of u-PA, containing the growth factor domain and Kringle domain but lacking any proteolytic activity, induced a mi-

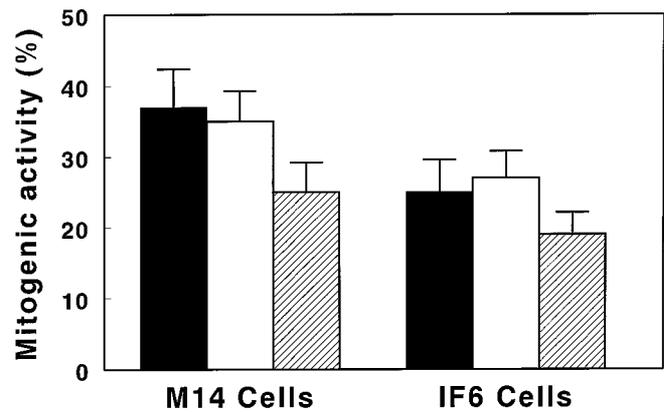


FIG. 3. Influence of the catalytic properties of u-PA on the mitogenic activity. Quiescent M14 and IF6 cells were incubated with 100 nM HMW u-PA in the absence (closed bars) and presence of 100 kallikrein inhibitor units/ml Trasylol (open bars) or with 100 nM of DFP-treated u-PA (hatched bars). Each point represents the mean of quadruplicate measurements, and the error bars represent the S.E.

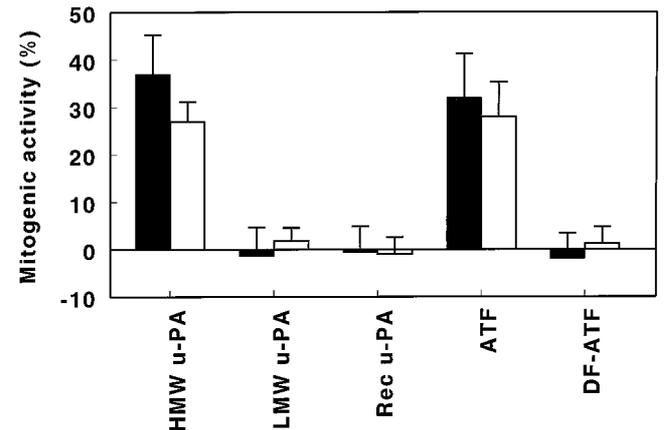


FIG. 4. The mitogenic activity of u-PA analogs on M14 and IF6 cells. Quiescent M14 cells (closed bars) and IF6 cells (open bars) were incubated with 100 nM of HMW u-PA, LMW u-PA lacking the ATF domain, recombinant u-PA produced in *E. coli* lacking all post-translational modifications, and 150 nM ATF and defucosylated ATF (DF-ATF). All bars represent the mean of quadruplicate measurements, and the error bars represent the S.E.

toxic response that was similar to that of intact native u-PA.

To determine whether post-translational modifications were involved in the mitogenic effects observed in the M14 and IF6 cells, a defucosylated form of ATF and a recombinant form of u-PA lacking all post-translational modifications were used. Both had no mitogenic effect on M14 and IF6 cells (Fig. 4), indicating that post-translational processing of u-PA was essential to the mitogenic effect observed in M14 and IF6 cells. The addition of a 5-fold molar excess of recombinant u-PA over native u-PA resulted in a decrease of the mitogenic effect of native u-PA in both M14 and IF6 cells (Fig. 5). A 5-fold molar excess of DFP-treated LMW u-PA did not influence the mitogenic effect of native u-PA (Fig. 5). This indicates that recombinant u-PA is capable of competing with native u-PA for the interactions, mediated by the ATF domain, that are involved in the signal transduction pathways.

Because the mitogenic effect of u-PA on M14 and IF6 cells is largely independent of its enzymatic activity, is mediated by the ATF domain, and therefore most likely involves the interaction with a cell surface receptor, we determined whether the high affinity binding of u-PA to u-PAR was pivotal to mitogenesis induced by u-PA. The mitogenic effect of u-PA on M14 and IF6 cells was measured in the presence of the anti-u-PAR mAb

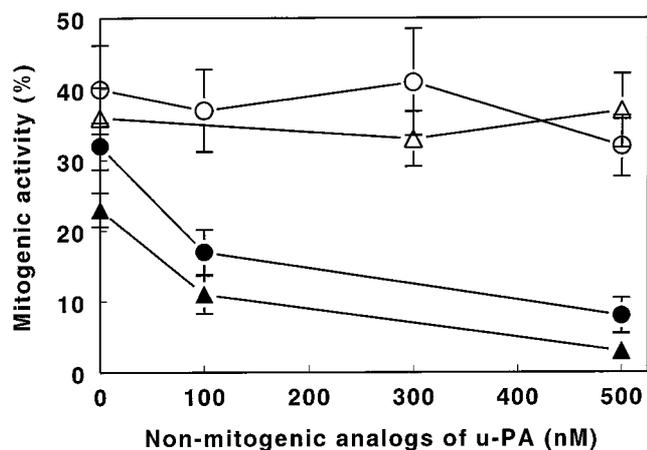


FIG. 5. Inhibition by recombinant u-PA (●, ▲) and LMW u-PA (○, △) of the mitogenic response induced by 100 nM native u-PA in quiescent M14 cells (●, ○) and IF6 cells (▲, △). Each point represents the mean of quadruplicate measurements, and error bars represent the S.E.

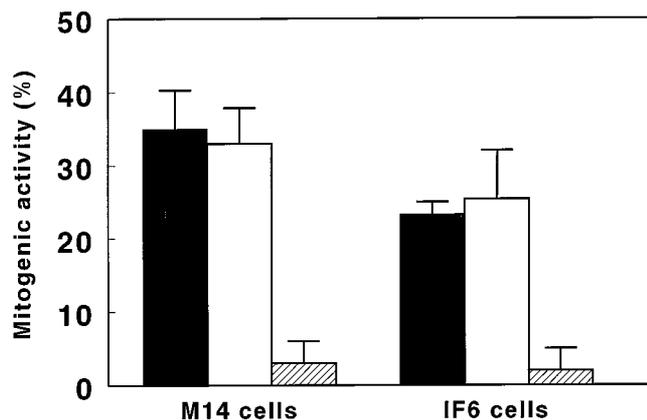


FIG. 6. The role of u-PA: u-PAR interaction in the mitogenic effect of u-PA. Quiescent M14 and IF6 cells were pre-incubated with culture medium (closed bars) or with H2 mAb (open bars) that blocks the binding of u-PA to u-PAR after which HMW u-PA was added to a final concentration of 100 nM. The mitogenic effect of the H2 mAb in the absence of u-PA was also determined (hatched bars). Each bar represents the mean of quadruplicate measurements, and the error bars represents the S.E.

H2 that specifically blocks binding of u-PA to u-PAR. Surprisingly, the results of these experiments show that the mitogenic effect of u-PA on the M14 and IF6 cells was not affected by the presence of mAb H2 (Fig. 6), indicating that the mitogenic effect is not mediated by u-PA-u-PAR interaction. Binding of the mAb H2 antibody to u-PAR in the absence of u-PA did not induce any mitogenic signal.

Characterization of the Binding of u-PA to M14 and IF6 Cells—The binding to M14 and IF6 melanoma cells of DFP-treated u-PA was measured in the presence and absence of the anti-u-PAR mAb H2 to confirm that this antibody completely blocked the high affinity binding to u-PAR. Scatchard analysis of the binding data showed that the binding of DFP-treated u-PA to M14 and IF6 cells in the absence of anti-u-PAR mAb H2 was characterized by a biphasic Scatchard plot (Fig. 7). This is in agreement with the presence of two different classes of binding sites on the M14 and IF6 cells. The high affinity binding site on M14 cells has a K_d of ~ 1.5 nM and approximately 5×10^4 binding sites/cell, and the low affinity binding site is characterized by a K_d of ~ 90 nM and 3×10^5 binding sites/cell. The binding to IF6 cells was very similar to that of M14 cells with a high affinity K_d of ~ 1 nM and $\sim 6 \times 10^4$ binding sites/

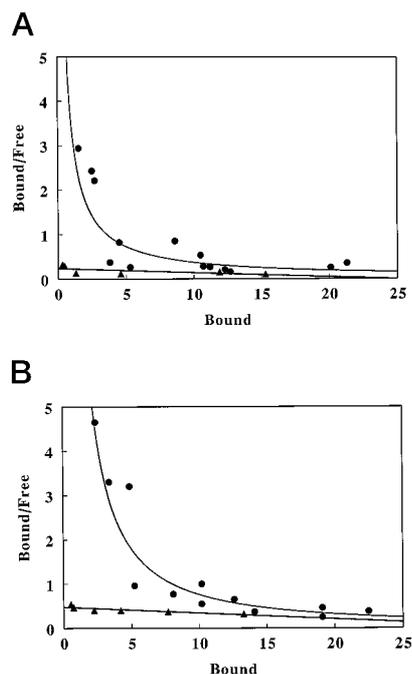


FIG. 7. Scatchard analysis of u-PA binding to M14 and IF6 cells. Radiolabeled u-PA was added to M14 cells (A) and IF6 cells (B) in concentrations that ranged from 0.3 to 200 nM. Cells were pre-incubated for 1 h with culture medium (●) or with anti-u-PAR H2 mAb (▲). Nonspecific binding at each u-PA concentration was subtracted.

cell, whereas the low affinity binding site on IF6 cells had a K_d of ~ 70 nM with $\sim 4 \times 10^5$ binding sites/cell. Scatchard analysis of binding data in the presence of the anti-u-PAR mAb H2 demonstrated that the high affinity binding on both M14 and IF6 cells was completely blocked (Fig. 7), whereas the low affinity binding of u-PA to these cells is due to interaction with u-PAR and that the low affinity binding is not mediated by u-PAR. The binding of recombinant u-PA treated with DFP on M14 and IF6 cells was similar to the binding of DFP-treated native u-PA to these cells (data not shown) and was characterized by a high affinity K_d of ~ 1.8 nM and a low affinity K_d of ~ 70 nM in M14 cells, whereas for IF6 cells K_d values were ~ 1.0 nM and ~ 50 nM for high and low affinity binding, respectively. This indicates that the post-translational modifications in native u-PA did not influence the binding of u-PA to both cell types.

Signal Transduction-related Events Induced by u-PA in M14 and IF6 Cells—Previously it was shown that mRNA levels of the proliferation markers *c-fos* and *c-myc* are induced in OC-7 cells in response to u-PA (17, 31). To determine whether this was also the case in M14 and IF6 cells, mRNA was analyzed by Northern blotting after different periods of incubation with u-PA. The addition of u-PA to both M14 and IF6 cells did not increase *c-fos* mRNA levels. In these experiments we found that *c-fos* mRNA expression is extremely sensitive to changes in the composition of the culture medium, e.g. the addition of as little as 10% fresh medium without any mitogenic agonist increased the *c-fos* mRNA approximately 3-fold in M14 and IF6 cells. *c-myc* mRNA was increased approximately 2-fold in M14 cells and 1.2-fold in IF6 cells by the addition of u-PA.

To determine the involvement of G-proteins in the signal transduction pathway induced by u-PA, pertussis toxin was added to M14 and IF6 cells prior to stimulation by u-PA. The mitogenic effect of DFP-treated u-PA on both M14 and IF6 cells was completely blocked by pertussis toxin (Fig. 8), indicating that a cell surface receptor linked to G-proteins was involved.

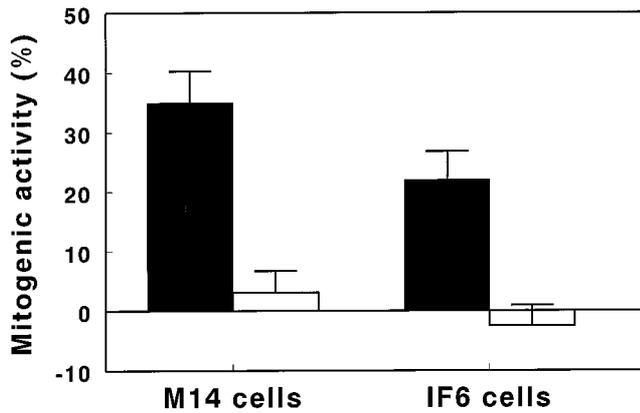


FIG. 8. Effect of pertussis toxin on mitogenic activity of u-PA. Quiescent M14 and IF6 cells were preincubated for 30 min with culture medium (closed bars) or 1 µg/ml pertussis toxin (open bars) before 100 nM u-PA was added. Each bar represents the mean of quadruplicate measurements, and the error bars represent the S.E.

DISCUSSION

Using [³H]thymidine incorporation as a measure for DNA synthesis, we demonstrated that the multi-domain serine protease u-PA has a mitogenic effect on two human melanoma cells *in vitro*. These results are in agreement with reports that demonstrated the induction of mitogenesis by u-PA in neural cells (32), fibroblasts (16) smooth muscle cells (17), and different tumor cells (13–15, 19, 20). The structural and functional characteristics of u-PA that are pivotal to the mitogenic properties of this protease vary between the different studies that have been reported and are most likely related to the different cell types studied. Our results demonstrate that the mitogenic effect of u-PA on the melanoma cells M14 and IF6 is not mediated by plasmin formation. Blocking u-PA activity *per se* resulted in a 30–35% decrease of the mitogenic effect, which might be related to the proteolytic activation of inactive growth factors as transforming growth factor-β (33) or hepatocyte growth factor (34) by u-PA. However, the major pathway that leads to induction of DNA synthesis by u-PA in M14 and IF6 cells is independent of u-PA activity. Both the activity-dependent and -independent mitogenic effect are mediated through the ATF domain, suggesting that the binding of u-PA to u-PAR is involved. Moreover, the involvement of u-PAR in signal transduction by u-PA leading to a mitogenic effect has been either proven (16) or suggested (15) previously. Surprisingly, blocking of the u-PA high affinity binding site on u-PAR, with a specific monoclonal antibody, did not change the mitogenic response induced by u-PA in M14 and IF6 cells. This indicates that the mitogenic effect of u-PA in these cells is not mediated by high affinity binding to u-PAR.

The fact that the mitogenic effect of u-PA on M14 and IF6 cells is independent of both u-PA activity and binding to the high affinity binding site on u-PAR suggests that u-PA is able to interact with the cell surface of these cells by an alternative mechanism. Binding experiments demonstrated that the binding of u-PA to the melanoma cells M14 and IF6 was characterized by the presence of a high affinity binding site and a low affinity binding site. The binding of u-PA to M14 and IF6 cells, with a K_d of 1–1.5 nM, was mediated by the high affinity binding site on u-PAR, whereas the low affinity binding with a K_d of 70–90 nM was not. This suggests that the low affinity binding of u-PA to the cell surface could be involved in the mitogenic effect instead of the high affinity binding mediated by u-PAR. This hypothesis is supported by the fact that half-maximum stimulation of DNA synthesis was observed at u-PA concentrations of approximately 35 and 60 nM, values that are

compatible with a K_d of 70–90 nM as measured for the low affinity binding on M14 and IF6 cells. The low affinity u-PA binding site on M14 and IF6 cells has not yet been characterized and could consist of a novel membrane protein or of a complex between u-PAR and additional proteins creating a secondary low affinity binding site on u-PAR. The characteristics of the low affinity binding of u-PA to M14 and IF6 cells are very similar to the ones reported for binding to platelets (35, 36). Recently smooth muscle cells were found to have a mitogenic response to u-PA by a mechanism presumably independent of u-PAR (17), and the presence of an unidentified u-PA binding protein on the cell surface of smooth muscle cells was suggested. The maximum stimulation on smooth muscle cells was reached at concentrations of u-PA in the same order of magnitude as observed in the melanoma cells. This indicates that the K_d of the binding that is responsible for the mitogenic effect is similar in smooth muscle cells and in M14 and IF6 cells. Whether low affinity u-PA binding to platelets, smooth muscle cells, and melanoma cells is mediated by the same protein is still unknown.

The fucosyl group linked to threonine 18 in the ATF domain of u-PA is needed to elicit mitogenesis in the M14 and IF6 cells but is not involved in the binding of u-PA to these cells. These results are compatible with the findings that in SaOs2 cells the mitogenic effect of u-PA was also dependent on the fucosyl group (15), whereas the binding of u-PA was independent of this post-translational modification. The possibility exists that the fucosyl group by itself is able to induce a mitogenic effect and that u-PA functions merely as a carrier or presenter of the fucosyl group. However, t-PA, carrying an identical fucosyl group attached to a threonine residue (37) that is surrounded by amino acids similar to the ones that surround the fucosylated threonine residue in u-PA, does not induce any mitogenic effect in M14 and IF6 cells at a concentration up to 200 nM. This indicates that both the u-PA protein moiety and the fucosyl group are essential for the induction of mitogenic stimuli in M14 and IF6 cells.

The pathway of signal transduction that is induced by u-PA and the changes in cellular phenotype or response to these signals show a high degree of diversity in the different cell types. In M14 and IF6 cells the pathway of signal transduction induced by u-PA involves G-proteins, indicating that the unknown binding protein on the cell surface could be a G-protein-linked receptor. No changes in the mRNA levels of *c-fos* were observed in M14 and IF6 cells after incubation with u-PA, which is different from the results obtained with OC-7 cells (31) and smooth muscle cells (17).

In conclusion, proteolytic inactive u-PA is able to induce a mitogenic response in quiescent human melanoma cells *in vitro*, which is independent of u-PA binding to the classical u-PA receptor. Low affinity binding of u-PA to the cell membrane of the melanoma cells suggests that u-PA-induced signal transduction could be mediated by a hitherto unidentified membrane-associated protein. Studies are in progress to establish the identity of this new u-PA binding protein and to determine whether these mitogenic properties of u-PA contribute to the aggressive phenotype of melanoma cells *in vivo*.

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Mitogenic Effects of Urokinase on Melanoma Cells Are Independent of High Affinity Binding to the Urokinase Receptor

Jaap L. Koopman, Jennichjen Slomp, Anton C. W. de Bart, Paul H. A. Quax and Jan H. Verheijen

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