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Lysosomal degradation of receptor-bound urokinase-type plasminogen activator is enhanced by its inhibitors in human trophoblastic choriocarcinoma cells

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We have studied the effect of plasminogen activator inhibitors PAI-1[1] and PAI-2 on the binding of urokinase-type plasminogen activator (u-PA) to its receptor in the human choriocarcinoma cell line JAR. With ^{125}I-labeled ligands in whole-cell binding assays, both uncomplexed u-PA and u-PA-inhibitor complexes bound to the receptor with a K_d of ~100 pM at 4°C. Transferring the cells to 37°C led to degradation to amino acids of up to 50% of the cell-bound u-PA-inhibitor complexes, whereas the degradation of uncomplexed u-PA was 15%; the remaining ligand was recovered in an apparently intact form in the medium or was still cell associated. The degradation could be inhibited by inhibitors of vesicle transport and lysosomal hydrolases. By electron microscopic autoradiography, both ^{125}I-u-PA and ^{125}I-u-PA-inhibitor complexes were located over the cell membrane at 4°C, with the highest density of grains over the membrane at cell-cell interphases, but, after incubation at 37°C, 17 and 27% of the grains for u-PA and u-PA-PAI-1 complexes, respectively, appeared over lysosomal-like bodies. These findings suggest that the u-PA receptor possesses a clearance function for the removal of u-PA after its complex formation with a specific inhibitor. The data suggest a novel mechanism by which receptor-mediated endocytosis is initiated by the binding of a secondary ligand.

Introduction

Invasion as a biological phenomenon is a hallmark of malignant cancerous diseases but occurs also in normal physiological processes. During invasion, the degradation of the surrounding tissue requires the action of proteolytic enzymes. The metastatic potential of cancer cells seeded onto a chorioallantoic membrane was abolished by antibodies against the urokinase-type plasminogen activator (u-PA), emphasizing the importance of this serine proteinase in invasion (Ossowski and Reich, 1983). The biochemical function of u-PA is to convert the latent plasminogen to the active proteinase plasmin, which, besides working directly on the extracellular matrix, is capable of activating other latent extracellular proteinases, e.g., procollagenases (Werb *et al.,* 1977; He *et al.,* 1989). A crucial point of control of the catalytic activity of u-PA is the conversion of latent single-chain pro-u-PA to active two-chain u-PA (Ellis *et al.,* 1989). Additional modulation of u-PA's enzymatic activity is contributed by the M_r 55 000 u-PA receptor (Stoppelli *et al.,* 1985; Vassalli *et al.,* 1985; Nielsen *et al.,* 1988; Estreicher *et al.,* 1989; Roldan *et al.,* 1990), which focuses the catalytic activity near the plasma membrane, and the fast-acting plasminogen activator inhibitors type 1 (PAI-1) and type 2 (PAI-2) (for a review, see Andreasen *et al.,* 1990). In addition, each of these components may be regulated by hormones, cytokines, and growth factors (for a review, see Saksela and Rifkin, 1988).

Mammalian trophoblasts are transiently endowed with invasive and migratory properties

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[1] Abbreviations: BSA, bovine serum albumin; DFP, diisopropylfluorophosphate; LMW-u-PA, M_r 33 000 catalytic fragment of u-PA; PAI-1, plasminogen activator inhibitor type 1; PAI-2, plasminogen activator inhibitor type 2; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; t-PA, tissue-type plasminogen activator; Tris, tris(hydroxymethyl)aminomethane; u-PA, urokinase-type plasminogen activator.

during embryonic implantation into the uterine mucosa (Kirby, 1965). This invasion also involves the plasminogen activation system. In mice, invasiveness is correlated to the plasminogen activator production (Strickland *et al.*, 1976) and the presence of u-PA mRNA (Sappino *et al.*, 1989). The in vitro invasiveness of human trophoblasts (Fisher *et al.*, 1985, 1989; Yagel *et al.*, 1988; Kliman and Feinberg, 1990), which are capable of secreting u-PA (Queenann *et al.*, 1987), was suppressed by antisera against u-PA (Yagel *et al.*, 1988). By the use of immunohistochemistry, PAI-1 and PAI-2 have been localized to both first- and third-trimester trophoblastic cells (Åstedt *et al.*, 1986; Feinberg *et al.*, 1989). We have found recently that syncytiotrophoblast microvillous membranes from third-trimester placentas do not contain u-PA receptors. In contrast, they contain binding sites forming covalent bonds with catalytically active u-PA; the binding could be inhibited with antibodies against PAI-2 (Jensen *et al.*, 1989b).

The purpose of the present work was to see whether u-PA is bound to the JAR cell line, which is derived from a trophoblastic tumor of placenta (Patillo *et al.*, 1971). This cell line has, with respect to invasiveness in vitro, been found to behave as first-trimester trophoblasts (Yagel *et al.*, 1988). It turned out that JAR cells have u-PA receptors, but not PAI-2 related binding sites. These receptors bound u-PA as well as complexes between u-PA and PAI-1 or PAI-2 with equal high affinity, but mediated internalization and degradation of u-PA-inhibitor complexes to a much higher extent than u-PA alone. This is similar to a recent finding of Cubellis *et al.* (1990) with the monocytoid cell line U-937. By electron microscopy, we found that u-PA receptors are located at distinct parts of the plasma membrane, and that the degradation takes place, at least partially, in lysosome-like vesicles.

Results

Binding of u-PA at 4°C

Table 1 shows a saturable binding of 5 pM ^{125}I-u-PA to JAR choriocarcinoma cells. The binding is independent of the active site, because 90% of the binding of tracer was inhibited by an excess of unlabeled ligands with a blocked active site (diisopropylfluorophosphate inactivated u-PA [DFP-u-PA] and pro-u-PA). The binding was not inhibited by the M_r 33 000 catalytic fragment of u-PA (LMW u-PA), which contains the active site, but not the amino-terminal receptor binding part, of the molecule. Large concentrations of single chain and two chain tissue-type plasminogen activator (t-PA) or antibodies against PAI-1 and PAI-2 did not inhibit the binding. Receptor sites did not seem to be occupied by endogenous u-PA, because the binding of tracer was not increased by pretreatment of the cells with a pH 3 buffer (data not shown) as described by Stoppelli *et al.* (1985). Studies of the time course of association showed that the binding reached a plateau after 6–8 h at 4°C (data not shown), and a 16-h incubation period was chosen for all 4°C experiments.

Table 1. Specificity of ^{125}I-u-PA binding to JAR cells

Nonradioactive competitors	% Bound	SD
None	17.0	0.7
DFP-u-PA (100 nM)	0.8	0.3
DFP-u-PA (1 nM)	1.8	0.7
Pro-u-PA (10 nM)	2.0	0.5
LMW-u-PA (1 mM)	21.9	0.8
Single-chain t-PA (15 nM)	17.1	0.7
Two-chain t-PA (15 nM)	16.8	0.6
Monoclonal anti-PAI-1 (0.1 mg/ml)	16.8	0.3
Goat anti-PAI-2 (1.0 mg/ml)	18.2	0.8

The cells were incubated with 5 pM ^{125}I-u-PA at 4°C for 16 h, a time sufficient to obtain a plateau of binding. Nonradioactive competitors were added with the tracer, except the antibodies, which were added 1 h before to the addition of tracer. The results are the mean values ± 1 SD of three experiments, each with three replicate incubations.

Figure 1 shows characterization of bound ^{125}I-u-PA by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Cell-bound ^{125}I-u-PA and ^{125}I-DFP-u-PA comigrated in SDS-PAGE with nonincubated M_r 54 000 ^{125}I-u-PA (lanes 1–3), with no appearance of larger SDS-resistant species that could be u-PA-inhibitor complexes. The absence from the cell extract of the M_r 33 000 catalytic fragment of u-PA, present as a contaminant in the added tracer, further emphasizes the absence of any binding requiring the active site of u-PA. Cross-linking of ^{125}I-u-PA to a JAR cell extract results in a band corresponding to M_r 105 000–110 000 (lane 5).

Conclusively, u-PA binds via its N-terminal part to an M_r 60 000 receptor in trophoblast-derived JAR choriocarcinoma cells. The receptor of JAR cells is, by the criteria usually employed, indistinguishable from the u-PA receptor described in other cell lines (Blasi, 1988).

Binding of u-PA-inhibitor complexes at 4°C

Table 2 shows that the u-PA-displaceable binding of 5 pM labeled u-PA, u-PA-PAI-1 com-

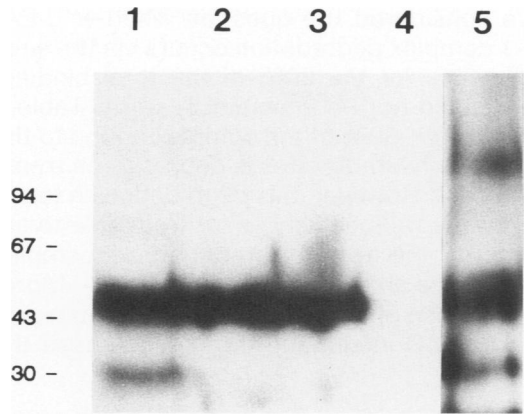


***Figure 1.* Cross-linking of ^{125}I-labeled u-PA to JAR choriocarcinoma cells.** Cells were incubated for 16 h at 4°C with 60 pM tracer followed by direct extraction or cross-linking. The samples were subjected to 8–16% nonreducing SDS-PAGE and autoradiography. (Lane 1) ^{125}I-u-PA tracer. (Lane 2) Cell-bound ^{125}I-u-PA. (Lane 3) Cell-bound ^{125}I-DFP-u-PA. (Lane 4) Cell-bound ^{125}I-u-PA in the presence of 100 nM DFP-u-PA. (Lane 5) ^{125}I-u-PA cross-linked to cells.

plexes, and u-PA-PAI-2 complexes to JAR cells is approximately the same. Figure 2 shows that the competition curves with unlabeled u-PA are superimposable (apparent $K_d \sim 90$ pM) with ^{125}I-u-PA and ^{125}I-u-PA-PAI-1 complex as the tracer, provided that the data are corrected for the u-PA-nondisplaceable binding. Thus, u-PA and u-PA-inhibitor complexes bind to the u-PA receptor with indistinguishable affinities. To verify the identity of the bound ligand after incubation with ^{125}I-u-PA-PAI-1 complex, we extracted cell-associated radioactivity and subjected it to SDS-PAGE; the extracted radioactivity comigrates almost exclusively with nonincubated complex as an M_r 94 000 band (data not shown). Binding of ^{125}I-u-PA-PAI-1 complex, in contrast to ^{125}I-u-PA and ^{125}I-u-PA-PAI-2 complex, could not be inhibited completely by 100 nM unlabeled u-PA; ~20% of the total binding was nondisplaceable (Table 2). This observation was further elaborated by experiments in which the binding of labeled u-PA and u-PA-PAI-1 complex was compared in suspended cells and the incubation terminated by pelleting the cells through silicone oil. The binding of 5 pM ^{125}I-u-PA-PAI-1 to suspended cells (2×10^6/ml) in the presence of 100 nM unlabeled u-PA was similar to that for adherent cells given as in Table 2, being 20.3% ± 3.7% (mean ± SD of 3 experiments) of the binding in the absence of unlabeled u-PA. This experiment shows that the u-PA-PAI-1 complex binding, which is nondisplaceable by unlabeled u-PA, is binding to the cells, not merely binding to plastic. Thus, JAR cells bind u-PA-PAI-1 complexes both via u-PA receptors and, to a minor extent, to as-yet-undefined sites.

Dissociation and degradation of receptor-bound ligands at 37°C

Figure 3A shows that labeled ligands prebound at 4°C dissociate on transfer to 37°C. Complexes of u-PA-PAI-1 dissociate slightly faster than u-PA and DFP-u-PA. Figure 3B shows that only ~15% of cell-bound u-PA and DFP-u-PA are degraded to trichloroacetic acid–soluble products. Similar data (not shown) were obtained with labeled pro-u-PA. In contrast, ~50% of the cell-bound u-PA-PAI-1 complex is degraded, i.e., to about the same extent as α_2-macroglobulin-trypsin complex, a ligand previously shown to undergo a rapid internalization and degradation in lysosomes via the α_2-macroglobulin receptor (Davidsen *et al.*, 1985). Similar results were obtained with PAI-2. In another type of experiment, cells were preloaded with ^{125}I-u-PA at 4°C; PAI-2 was added in a 100-fold excess compared with cell-bound ^{125}I-u-PA; and the cells were incubated for additional 15 min at 4°C, then transferred to 37°C. Degradation of receptor-bound ^{125}I-u-PA at 37°C was in that case ~ 40% (47 and 33% in 2 independent ex-

Table 2. Binding of ^{125}I-u-PA-PAI-1 and ^{125}I-u-PA-PAI-2 complexes to JAR cells

	% Bound		
	^{125}I-uPA-PAI-1	^{125}I-uPA-PAI-2	^{125}I-u-PA
Total binding	23.0 ± 5.2	17.6 ± 3.3	17.8 ± 1.8
Binding with 100 nM u-PA	6.0 ± 2.3	0.7 ± 0.2	1.1 ± 0.4
u-PA displaceable binding	17.0 ± 5.7	16.7 ± 3.3	16.7 ± 1.8

The cells were incubated with 5 pM ^{125}I-u-PA, ^{125}I-u-PA-PAI-1, or ^{125}I-u-PA-PAI-2 complexes at 4°C for 16 h with or without 100 nM u-PA. The results are the mean value of four experiments ± 1 SD.

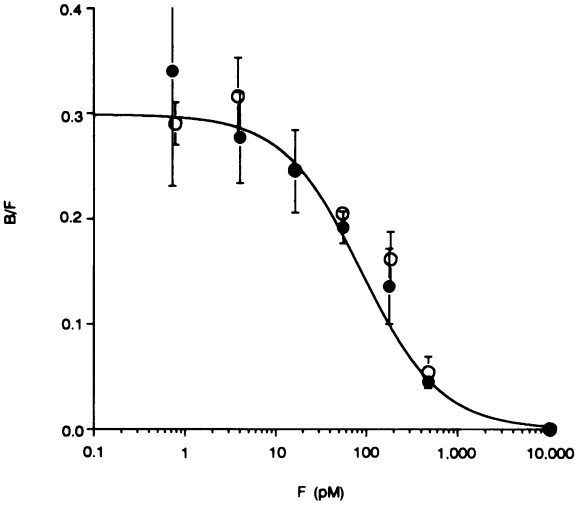


***Figure 2.* Concentration dependence of binding of u-PA and u-PA-PAI-1 complexes.** JAR cells (260 000 cells/well) were incubated for 16 h at 4°C with 1 or 5 pM labeled u-PA (○) or u-PA-PAI-1 complex (●) plus unlabeled u-PA at the free ligand concentration indicated on the abscissa. The bound/free ratio (B/F) value obtained in the presence of 100 nM u-PA has been subtracted from all the points. The curve represents the least-square fit to the equation $B/F = R_o/(K_d + F)$, where K_d is the apparent dissociation constant for u-PA, calculated to 88.7 pM, and R_o is the concentration of u-PA receptors, calculated to 26.5 pM or ~15 000 receptors per cell. Mean ± SD of triplicates are indicated.

periments with triplicate samples in each). Control experiments showed that degradation of the ligands was negligible (~2% in 2 h) in conditioned medium in the absence of cells. Thus, cell-bound u-PA-inhibitor complexes are degraded much more efficiently than cell-bound u-PA, independently of whether the complexes were formed before or after association of u-PA with the receptor.

It is clear from the above experiments that u-PA, pro-u-PA, and DFP-u-PA are degraded by the cells to very similar extents. This excludes the possibility that the degradation of u-PA is caused by complex formation with endogenous inhibitors produced by the cells, because the pro-u-PA and DFP-u-PA are unable to form such complexes. This is in agreement with our observation of the absence of PAI-1 and PAI-2 in conditioned medium, as measured by enzyme-linked immunosorbent assays (Jensen, unpublished). We also excluded the possibility that the difference between u-PA and u-PA-inhibitor complexes was due to the incubation and chromatography used during preparation of the complexes, because the ^{125}I-u-PA used in the experiments was always treated in a similar way, only without the presence of the inhibitors.

We considered the question whether u-PA-PAI-1 complex degradation occurs via the sites responsible for the 20% of the total binding, which could not be inhibited by u-PA. Table 3 shows that ~60% of the complex bound to the u-PA-noninhibitable sites is degraded on transfer to 37°C. However, this degradation, in terms of absolute radioactivity, is far from able to account for the total degradation of complex bound in the absence of unlabeled u-PA. Moreover, the use of larger volumes of medium during the 37°C incubation did not decrease the

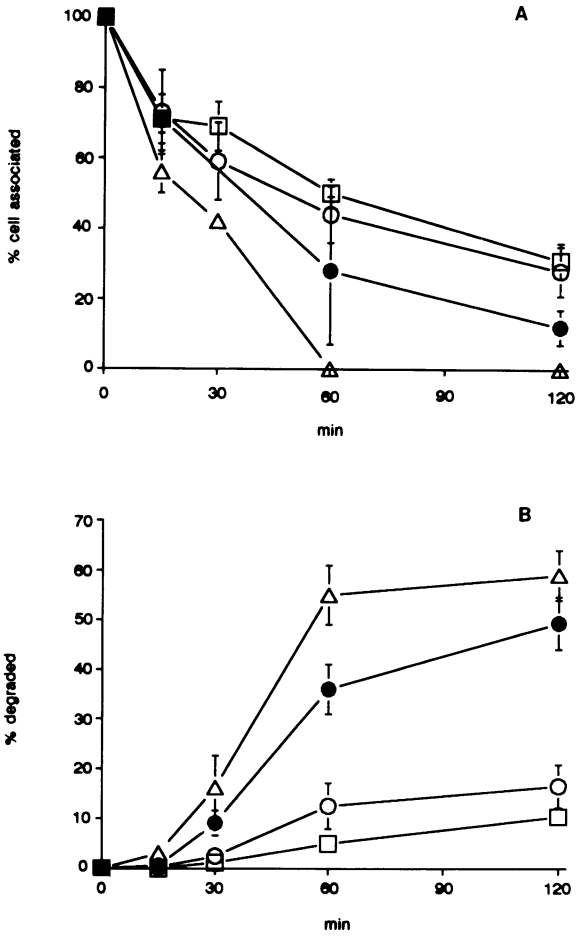


***Figure 3.* Time course of dissociation and degradation of ^{125}I-u-PA and ^{125}I-u-PA-PAI-1 complexes at 37°C.** Cells were incubated with 60 pM labeled u-PA (○), DFP-u-PA (□), u-PA-PAI-1 (●), and α_2-macroglobulin-trypsin complex (△) for 16 h at 4°C, washed, and transferred to 37°C. The ordinate shows cell associated radioactivity (A) or radioactivity soluble in 12% trichloroacetic acid in the medium (B) at the indicated time points after transfer to 37°C, in both cases expressed as percentage of the cell-associated radioactivity present before transfer to 37°C. The points represent mean ± SD of 9, 3, 6, and 3 experiments for u-PA, DFP-u-PA, u-PA-PAI-1 complexes, and α_2-macroglobulin-trypsin, respectively.

Table 3. Degradation of ^{125}I-u-PA-PAI-1 complexes bound to u-PA nondisplaceable sites

Nonradioactive u-PA	^{125}I-u-PA-PAI-1		^{125}I-u-PA	
	0	100 nM	0	100 nM
Bound	6246 ± 216	1531 ± 139	4611 ± 147	320 ± 64
Degraded	3304 ± 135	910 ± 112	800 ± 112	84 ± 36

Cells were incubated at 4°C for 16 h with 95 pM ^{125}I-u-PA or ^{125}I-u-PA-PAI-1 complexes, both equivalent to 47 000 cpm/well, in the absence or presence of 100 nM nonradioactive u-PA. They were then washed and incubated at 37°C for 90 min without or with the 100 nM nonradioactive u-PA as during the 4°C incubation. The medium was collected and trichloroacetic acid added to 12%. Bound tracer is expressed as cpm associated with the cells after the incubation at 4°C. Degraded tracer is expressed as cpm in the medium soluble in 12% trichloroacetic acid. The data show mean ± 1 SD of three experiments.

degraded fraction (data not shown), arguing against the possibility that u-PA-PAI-1 complexes first dissociate from the u-PA receptor and then rebind to different sites, which mediate the degradation. Thus, the u-PA-PAI-1 complex degradation appears to be mediated by the u-PA receptor.

The following experiments were designed to elucidate the nature of the degradation of cell-bound ^{125}I-labeled ligands. Figure 4 shows a gel filtration profile of products released from cells during a 90-min incubation at 37°C after binding of labeled DFP-u-PA or u-PA-PAI-1 complexes at 4°C. With ^{125}I-DFP-u-PA, 84% of the radioactivity eluted as high-M_r species in the void volume (peak a); only a minor fraction coeluted with the monoiodotyrosine standard (peak c). With ^{125}I-u-PA-PAI-1 complexes, ~60% of the radioactivity coeluted with the iodotyrosine standards. Iodine (peak b) represented 4% of the radioactivity both before and after the 37°C incubation, indicating the absence of deiodinase activity in the JAR cells. The inset shows that the majority of the high-M_r radioactivity released to the medium migrated electrophoretically as u-PA or u-PA-PAI-1 complexes, even though a faint band in lane 2 could indicate a minor degradation product of u-PA-PAI-1 complexes with an M_r ~ 70 000. Other experiments showed that the high-M_r radioactivity dissociated from cells loaded with ^{125}I-u-PA-PAI-1 complexes could still be retained on Sepharose-coupled

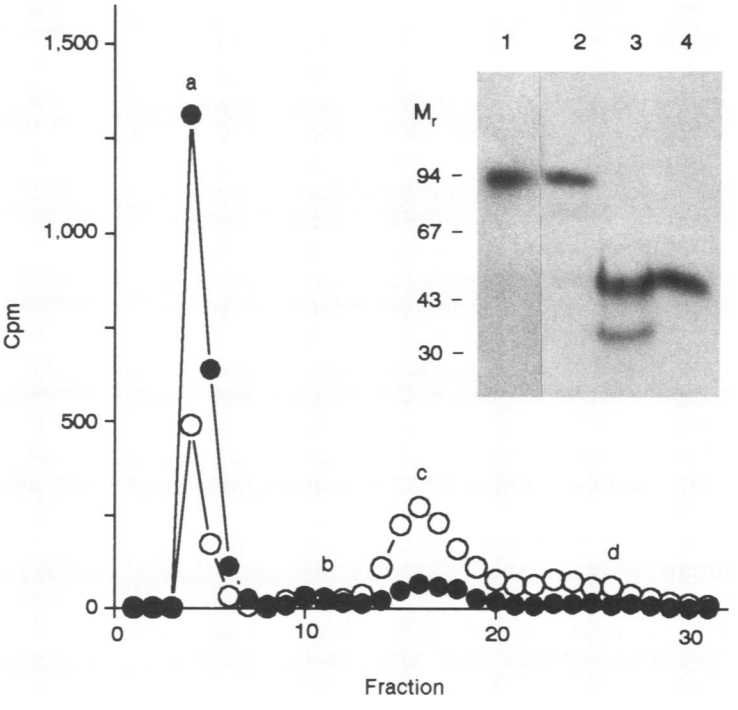


***Figure 4.* Analysis of radioactivity dissociated from cells at 37°C.** Cells were incubated with 60 pM ^{125}I-DFP-u-PA or ^{125}I-u-PA-PAI-1 complexes for 16 h at 4°C, washed, and incubated for 90 min at 37°C. Gel filtration was performed on a Sepharose G25F 1 × 12 cm column equilibrated in 0.5 M acetate, 0.025 M HCl, 0.1% BSA, pH 2.0. ○, ^{125}I-u-PA-PAI-1; ●, ^{125}I-DFP-u-PA. a, b, c, and d correspond to the elution of Blue dextran, $^{125}I^-$, and monoiodotyrosine and diiodotyrosine standards, respectively. Other experiments (not shown) demonstrated that the 4% of the radioactivity eluting as iodine was present in the tracer before incubation with the cells. (Inset) SDS-PAGE and autoradiography of labeled material. (Lane 1) ^{125}I-u-PA-PAI-1 complex tracer. (Lane 2) ^{125}I-u-PA-PAI-1 complex dissociated from the cells after 90 min at 37°C. (Lane 3) ^{125}I-DFP-u-PA tracer. (Lane 4) ^{125}I-DFP-u-PA dissociated from the cells after 90 min at 37°C.

monoclonal anti-PAI-1 antibodies, and comigrated with immunoprecipitated nonincubated ^{125}I-u-PA-PAI-1; in addition, the dissociated u-PA and u-PA-PAI-1 complexes were capable of rebinding to fresh cells (data not shown). Thus, the radioactive material released from the cells during the 37°C incubation represent almost exclusively monoiodotyrosine or have properties indistinguishable from those of added ligands.

Table 4 shows the effects of colchicine, an antitubuline drug capable of disrupting vesicle transport, and the alkalizing agents chloroquine and methylamine, capable of inhibiting lysosomal acid hydrolases. None of the agents caused significant changes in the binding at 4°C (not shown), whereas they inhibited degradation of cell-bound u-PA-PAI-1 complexes by ~80%. Similar results were obtained when using ^{125}I-α_2-macroglobulin-trypsin complex. The low degradation of cell-bound DFP-u-PA was inhibited to proportionally approximately the same extent as u-PA-PAI-1 complexes by the three inhibitors (data not shown).

These results are compatible with the hypothesis that u-PA-PAI-1 complexes, and u-PA to a minor degree, are internalized by vesicular traffic and transferred to lysosomes, where they are degraded to amino acids.

Table 4. The effect of inhibitors on receptor-mediated degradation of u-PA-PAI-1 complex and α_2-macroglobulin-trypsin complex

	% Degraded	
	^{125}I-u-PA-PAI-1	^{125}I-α_2-macroglobulin-trypsin
No inhibitor	52 ± 5	60 ± 7
Colchicine	13 ± 7	10 ± 8
Chloroquine	10 ± 6	19 ± 7
Methylamine	8 ± 5	13 ± 14

Cells were incubated at 4°C for 16 h with 60 pM labeled ligand, washed, and transferred to 37°C. The medium was collected after 90 min and precipitated in 12% trichloroacetic acid. The inhibitors were present in all buffers at the following concentrations: colchicine, 10 mM; chloroquine, 0.1 mM; methylamine, 10 mM. The inhibitory effect is expressed as the percent radioactivity soluble in trichloroacetic acid with inhibitor added as the mean of 5 experiments ± 1 SD.

Localization of bound ligands on the cell surface and in vesicular compartment by transmission electron microscopic autoradiography

Figures 5 and 6 show the ultrastructural localization of plasma membrane–bound ^{125}I-labeled u-PA (Figure 5) and u-PA-PAI-1 complexes (Figure 6) in JAR cells after incubation at 4°C. No difference was observed in the localization of grains resulting from the two ligands. A characteristic distribution of grains was observed, with only a few grains located over the apical cell membrane facing the culture medium (Figure 5A), in contrast to the abundance of grains over the plasma membrane at cell-cell interphases as noted in Figure 5B but also evident in Figure 5A and Figure 6. Grains were also observed over the basal plasma membrane, which makes contact with the substratum (Figure 6).

The cellular compartments responsible for uptake and degradation of cell-bound u-PA and u-PA-PAI-1 complexes were studied by incubating the cells at 37°C for 30 min after they had bound the ligand at 4°C. Figure 7A shows that ^{125}I-u-PA was found over electron-lucent endocytic vesicles and electron-dense lysosome-like bodies. With ^{125}I-u-PA-PAI-1 complexes, more grains were observed over lysosome-like bodies than with ^{125}I-u-PA (Figure 7B). With both ligands, grains were still observed over the plasma membrane.

Table 5 shows a quantitative analysis of the distribution of grains for both ligands after 4 and 37°C incubations. After the 4°C incubation, 80–90% of the grains were located over the plasma membrane, with the remaining grains primarily assigned to the cytoplasm and endocytic-like vesicles. These grains, apparently located intracellularly, may reflect ligand present at deep invaginations of the plasma membrane.

A quantitation of the shift of grains to intracellular compartments after incubation at 37°C showed that about one-third of the grains representing u-PA-PAI-1 complexes were seen over lysosome-like bodies and 21% over endocytic vesicles. The same phenomenon was observed, but to a lesser degree, with u-PA; ~20 and 10% of the grains were localized over lysosome-like bodies and endocytic vesicles, respectively, whereas 57% of the grains were still found over the plasma membrane.

Conclusively, the JAR choriocarcinoma cell line binds u-PA and u-PA-PAI-1 complexes preferentially at the plasma membrane at cell–cell interphases. Plasma membrane–bound u-PA and u-PA-PAI-1 is, to a certain extent, internalized by endocytic vesicles and brought to localizations capable of degrading the ligand. Lysosomes, apparently, are at least partially responsible for this degradative process.

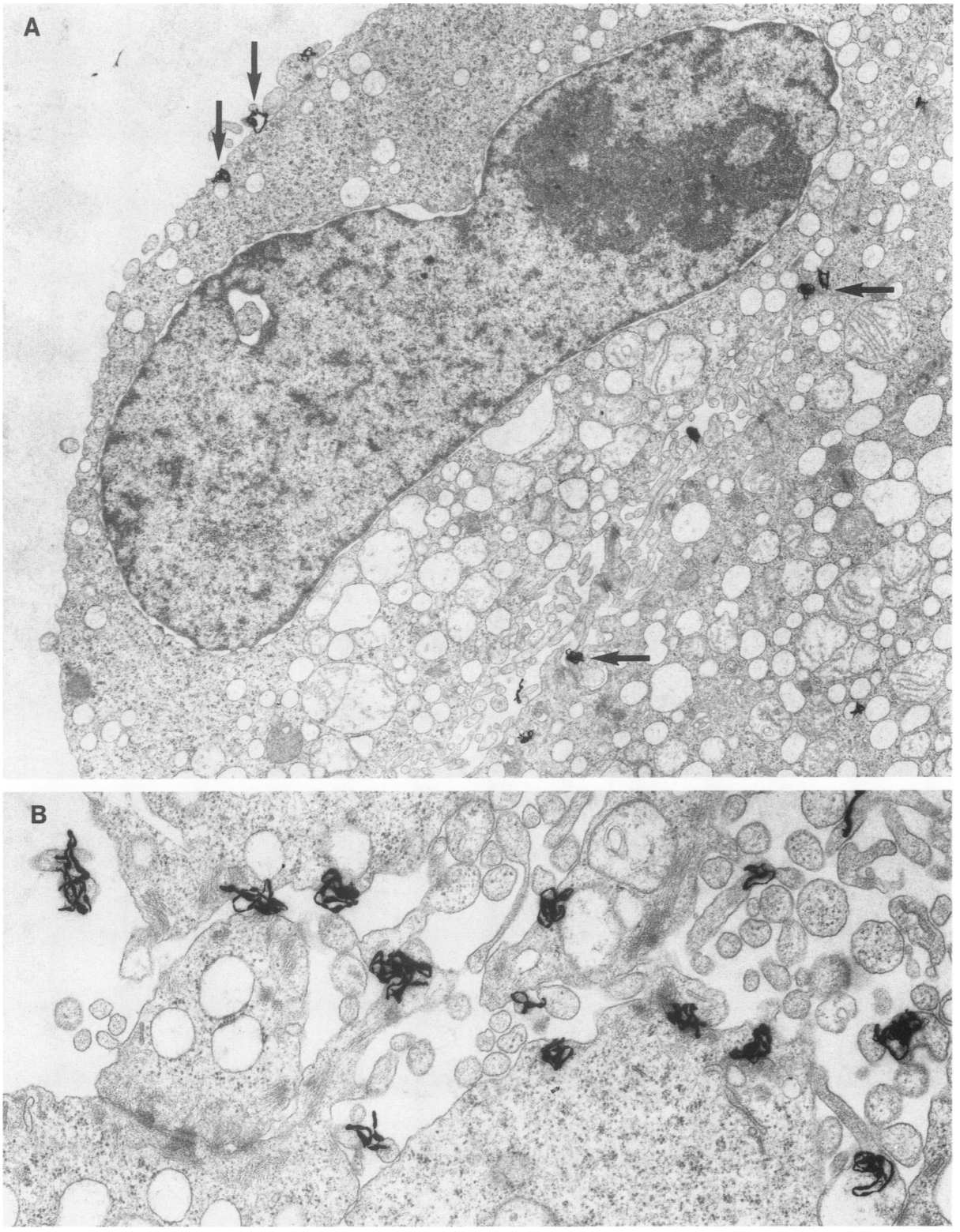


Figure 5. **Electron microscopic localization of ^{125}I-u-PA bound to JAR cells at 4°C.** Cells were incubated with 300 pM labeled u-PA at 4°C for 20 h, washed extensively, and prepared for electron microscopy. (A) Few grains localized over the apical plasma membrane (vertical arrows) compared with the grains over the microvillous membrane in the intercellular space (horizontal arrows). ×9900. (B) Grains located at the cell membrane facing the intercellular space. ×24 900.

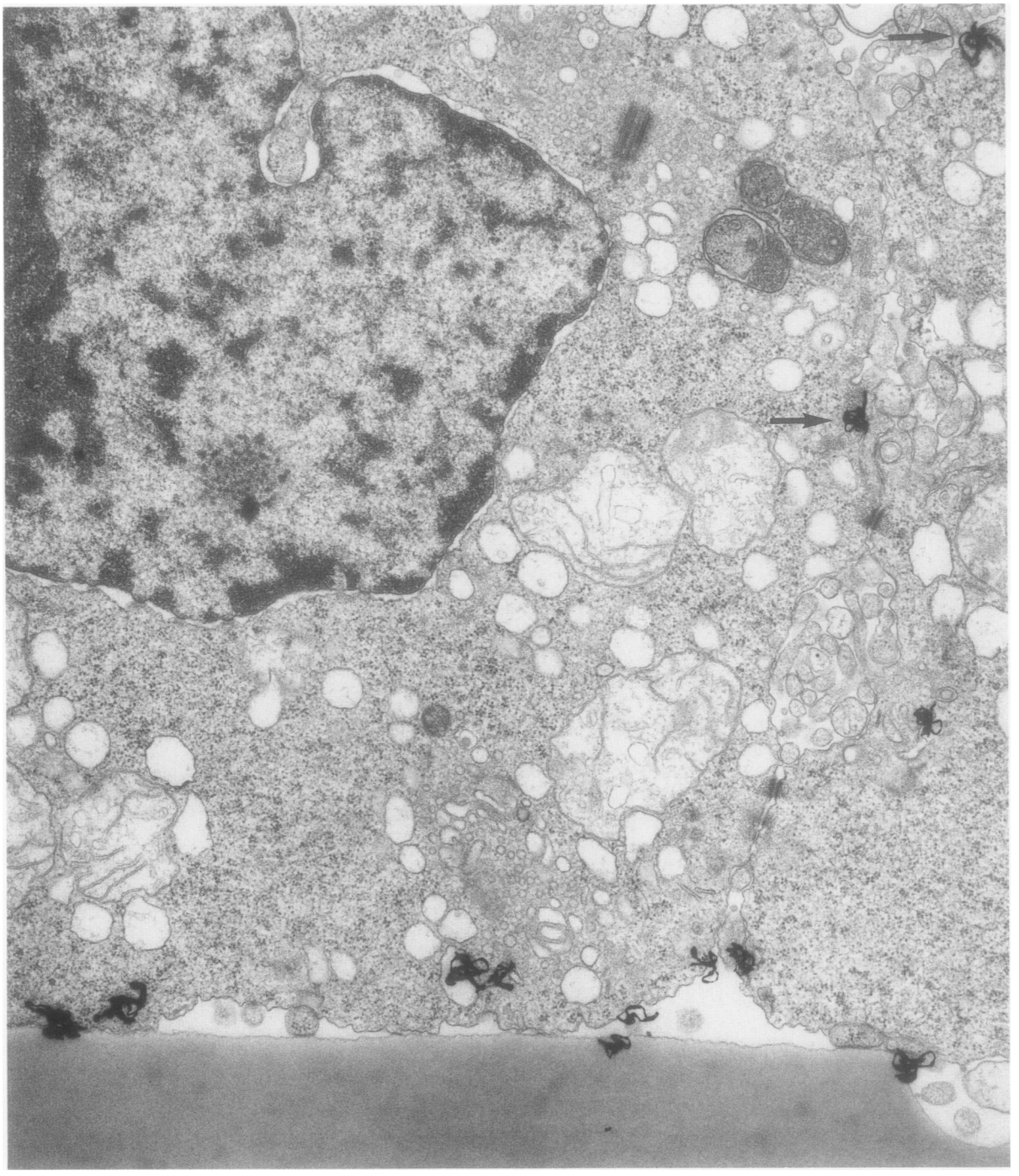


Figure 6. **Electron microscopic localization of ^{125}I-u-PA-PAI-1 complexes bound at 4°C.** Cells were incubated with 300 pM labeled u-PA-PAI-1 complex at 4°C for 20 h, washed extensively, and processed for electron microscopy. Grains are shown over the basal membrane facing the substratum and over the intercellular membrane (horizontal arrows) ×22 100.

Discussion

In this study, we demonstrate that the trophoblastic JAR choriocarcinoma cell line possesses u-PA receptors with properties indistinguishable from those described in other cells (for review, see Blasi, 1988) and that u-PA in complex with its specific inhibitors PAI-1 and PAI-2 binds to the receptors with the same affinity as un-

complexed u-PA at 4°C. On transfer of JAR cells with prebound u-PA or u-PA-inhibitor complexes from 4 to 37°C, a fraction of the bound ligand dissociated from the receptor predominantly in an apparently intact form; but, after a lag period, degradation products in the form of monoiodotyrosine started appearing in the medium. The degradation of u-PA-PAI-1 complexes and u-PA-PAI-2 complexes was about three times higher than the degradation of uncomplexed u-PA. The degradations were suppressible by inhibitors of vesicle transport and lysosomal function. Electron microscopic autoradiography showed that u-PA associated with cells after 4°C incubation was preferentially localized at cell–cell interphases, in agreement with previous investigations (Pöllänen *et al.,* 1987, 1988; Hébert and Baker, 1988; Hansen *et al.,* 1990); but, after 37°C incubation, a significant part of the ligands had been transferred from the cell membrane to lysosome-like bodies.

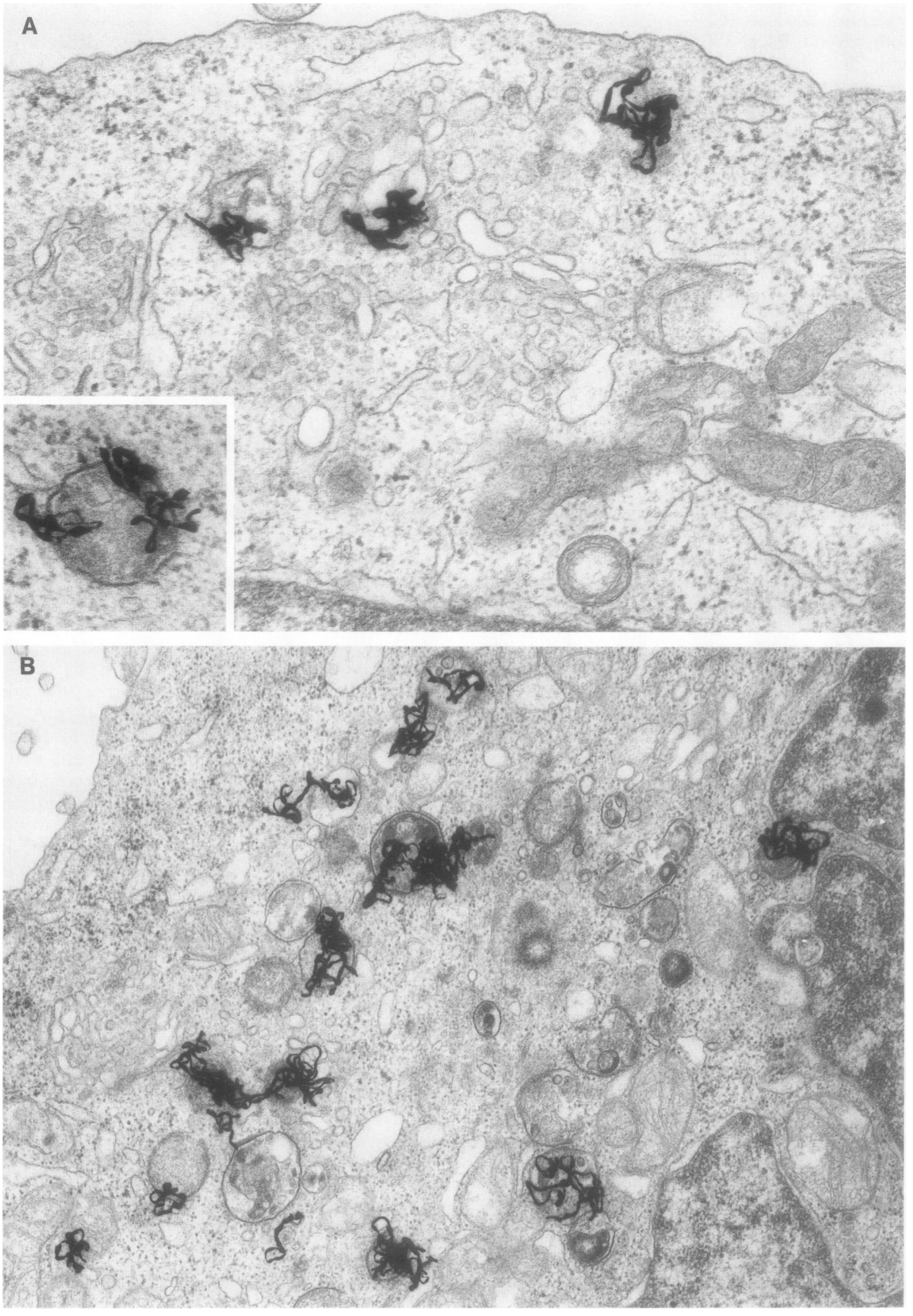
While this work was in progress, biochemical data reported by Cubellis *et al.* (1990) showed a u-PA-receptor–mediated degradation of u-PA-PAI-1 complexes in the monocytoid U-937 cell line growing in suspension culture. These biochemical data are largely in agreement with our results on an adherent cell line. Our electron microscopic autoradiographic results are the first to demonstrate u-PA and u-PA-inhibitor complexes taken up into intracellular organelles reminiscent of lysosomes. The inhibitor-stimulated internalization of u-PA is a novel aspect in the regulation of plasminogen activation. The observations show that the u-PA receptor also participates in the clearance of catalytically inactivated u-PA. This adds a new feature to its function, which previously was thought only to be one of concentrating plasminogen activating activity at the cell surface.

Also, the initiation of internalization by a secondary ligand is a novel phenomenon, which, to our knowledge, has not been described previously for other receptors. It is yet unknown how complex formation of inhibitors with receptor-bound u-PA initiates the increased rate of internalization. Even though the receptor-binding amino-terminal part and the carboxy-terminal inhibitor-binding serine proteinase part of u-PA have been shown to behave as two almost-independent structures by nuclear magnetic resonance analysis (Oswald *et al.,* 1989), the impact of inhibitor binding on this flexible structure remains to be characterized. The recent report by Ellis *et al.* (1990) of a reduced rate of u-PA-inhibitor reaction on binding of u-PA to the receptor does suggest a communication between the two parts of u-PA or between its carboxy-terminal catalytic part and the receptor. Alternatively, the ligand-receptor complexes may make lateral contacts with other components in the membrane, which then initiate the internalization. For example, u-PA has been found capable of binding to ganglioside species (Miles *et al.,* 1989). We observed, in contrast to Cubellis *et al.* (1990), a low but significant degradation of u-PA in the absence of added inhibitors. We excluded the possibility that the degradation was triggered by complex formation with endogenous inhibitors. One can hypothesize that a slow constitutive internalization of the u-PA receptor accounts for this degradation of cell-bound noncomplexed u-PA.

We found that a fraction of the receptor-bound ligands dissociated into the medium at 37°C in a form capable of rebinding to fresh cells. This is in agreement with the data of Nykjær *et al.* (1990) on human monocytes, but in contrast to previous reports suggesting that ligands dissociate from the receptor at best very slowly (Stoppelli *et al.,* 1985; Blasi, 1988). These apparent discrepancies may be due to differences in incubation temperature and choice of ligand; most earlier studies were performed at 4°C with an amino-terminal fragment, and not native u-PA, as the ligand.

Our K_d value for receptor binding of u-PA and u-PA-inhibitor complex was ~100 pM, in agreement with Cubellis *et al.* (1989). Kirchheimer and Remold (1989) reported about a 10-fold lower affinity for the u-PA-PAI-2 complexes than for u-PA binding to cultured human monocytes. However, because they used a tracer concentration of 400 pM, which is higher than the K_d for u-PA-receptor binding, and an incubation temperature of 37°C, at which internalization may take place, their results are not directly comparable with ours.

The invasion of normal human trophoblast cells into the uterine wall is a highly regulated process, being confined to the first trimester of pregnancy. Feinberg *et al.* (1989) found PAI-1 in extravillous invasive trophoblasts in placenta by immunohistochemistry. Our present observations on the trophoblastic cell line suggest that an interplay between u-PA and its receptor and inhibitors may be involved in trophoblast invasion by allowing sequential dissolution and reestablishment of cell-matrix bindings. In third-trimester syncytiotrophoblast microvillous membranes, we recently found u-PA binding sites immunologically related to PAI-2, whereas u-PA receptors were absent (Jensen *et al.,* 1989b). Whether these PAI-2–like molecules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serve a special function at this location or whether their presence represents a step in the cellular release of the protein is unknown at present. By immunohistochemistry, Åstedt *et al.* (1986) and Feinberg *et al.* (1989) found PAI-2 located in the noninvasive villous trophoblastic epithelium. It is tempting to hypothesize that the invasive growth of placenta is arrested by the disappearance of u-PA receptors and the appearance of externally facing, membrane-anchored PAI-2–like molecules quenching the activity of u-PA.

Methods

Proteins

Human two-chain u-PA was purchased from Serono (Aubonne, Switzerland). DFP catalytic site-inactivated two-chain u-PA was prepared as previously described (Jensen *et al.*, 1989b). Pro-u-PA (single-chain u-PA) was purified from serum-free conditioned medium of the human fibrosarcoma cell line HT-1080 cultured in the presence of Trasylol (10 μg/ml). The conditioned medium was applied to a 2-ml column of Sepharose-immobilized monoclonal anti-u-PA IgG from hybridoma clone 6 (Grøndahl-Hansen *et al.*, 1987) equilibrated with 0.1 M tris(hydroxymethyl)aminomethane (Tris), pH 8.1. The column was washed with 1.0 M NaCl, 0.1 M Tris, pH 8.1 and eluted with 1.0 M NaCl, 0.1 M CH_3COOH, pH 2.9. The eluate was neutralized immediately with 0.3 vol of 1 M Tris, pH 9.0. All solutions used for the chromatography contained Trasylol (10 μg/ml). The M_r 33 000 LMW-u-PA (specific activity 6000 U/mg) was purchased from Green Cross (Osaka, Japan). Single-chain and two-chain t-PA were prepared as described (Andreasen *et al.*, 1984).

Human PAI-1 was purified from the serum-free conditioned medium of HT-1080 cells cultured in the presence of dexamethasone. The procedure was as described for pro-u-PA, using Sepharose immobilized monoclonal anti-PAI-1 IgG from hybridoma clone 2 (Nielsen *et al.*, 1986). The buffers used for equilibration, washing, elution, and neutralization were 0.05 M Na_2HPO_4, pH 7.6; 1 M NaCl, 0.05 M Na_2HPO_4, pH 7.6; 1 M NaCl, 0.1 M CH_3COOH, pH 2.9; 1 M Na_2HPO_4, pH 7.6. Human M_r 48 000 PAI-2, a gift from Ingegerd Lecander and Birger Åstedt, Lund University Hopital, Sweden, was prepared from placental tissue as described (Åstedt *et al.*, 1985).

A mouse monoclonal antibody (t-PI-3 F95) against PAI-1 was a gift from Johan Selmer, Novo Nordisk, Denmark. This antibody is capable of inhibiting the binding of t-PA to PAI-1 (Philips *et al.*, 1986). Polyclonal goat antiserum against PAI-2 was that previously described (Åstedt *et al.*, 1986).

α_2-macroglobulin was prepared as described previously (Sottrup-Jensen *et al.*, 1980).

Tracers

Single-chain pro-u-PA and two-chain active u-PA were dialyzed against 0.2 M Na_2HPO_4, pH 8.0, and iodinated using chloramine-T as the oxidizing agent (Jensen *et al.*, 1989b). Briefly, ~200 pmol $^{125}I^-$ (Amersham, Buckinghamshire, UK, 0.4 mCi in ~4 μl) was added to 100 pmol u-PA in a volume of 20 μl 0.2 M Na_2HPO_4, pH 8.0, followed by 2.5 μl chloramine-T (1 mg/ml). Incubation was carried out for 3 min at 20°C and stopped by the addition of 300 μl ice-cold 0.1 M Tris, 0.1% Triton X-100, pH 8.0. Incorporation of iodine into the protein averaged 50%, corresponding to a specific activity of 40 mCi/mg. ^{125}I-labeled pro-u-PA and two-chain u-PA were separated from nonincorporated $^{125}I^-$ by immunoaffinity chromatography as described above.

^{125}I-u-PA-PAI-1 complexes were prepared by incubating ~1 μg/ml ^{125}I-u-PA with SDS-activated PAI-1 (Andreasen *et al.*, 1986) at a concentration of 10 μg/ml in a buffer of 0.1 M Tris, 0.1% Triton X-100, pH 8.1, for 1–2 h at room temperature. The complexes were isolated from the excess of PAI-1 by the use of an anti-u-PA IgG column (Lund *et al.*, 1988). The formed complexes migrated as a single M_r 94 000 band in SDS-PAGE (Figure 4 inset, lane 1) without contaminating uncomplexed ^{125}I-u-PA and could bind to monoclonal

Table 5. Distribution of grains over JAR cells incubated with ^{125}I-u-PA and ^{125}I-u-PA-PAI-1 complexes

	4°C		37°C	
Labeled ligand	u-PA	u-PA-PAI-1	u-PA	u-PA-PAI-1
Plasma membrane region	90.7	78.0	56.7	36.4
Electron lucent vesicles	4.1	9.1	10.0	21.0
Electron dense vesicles	1.0	1.5	17.5	27.2
Cytoplasm	2.6	6.8	8.1	10.0
Nuclei and remaining intracellular components	1.0	0.8	1.0	1.1
Extracellular	0.5	3.8	6.7	4.3

JAR cells were cultured in tissue culture Transwells and incubated with 300 pM ^{125}I-labeled u-PA or u-PA-PAI-1 complex for 16 h at 4°C. Some incubations were terminated then, whereas others were transferred to 37°C and kept there for 30 min before termination. The cells were then processed for electron microscopic autoradiography. The four situations in the table represent 887 grains counted over 3.7×10^4 μm² with >200 grains in each situation with respect to ligand and incubation temperature. About 30% of the cell-associated radioactivity present at 4°C dissociated during the 37°C incubation. The data are presented as percentage of the grains counted in each situation. The background was determined on each grid and averaged 5.4×10^{-4} grains/μm². Incubations of ^{125}I-u-PA plus 100 nM u-PA at 4°C resulted in 3% of the grains found with no unlabeled u-PA present. These grains were not assigned to any organelles, and their amount was not different from the background. For ^{125}I-u-PA-PAI-1 complex plus 100 nM unlabeled u-PA at 4°C, the number of grains above background corresponded to 9% of the grains present with no unlabeled u-PA present; ~75% of these grains were located over the plasma membrane and electron lucent vesicles.

Figure 7. **Electron microscopic localization of ^{125}I-labeled u-PA and u-PA-complexes after 37°C incubation.** Cells were incubated with 300 pM labeled ligand at 4°C for 20 h, washed extensively, reincubated at 37°C for 30 min, and then processed for electron microscopy. (A) u-PA over endocytic vesicles. ×40 000. (Inset) u-PA over lysosome-like body. ×50 000. (B) u-PA-PAI-1 complexes over lysosome-like bodies. ×24 900.

anti-PAI-1 antibodies from hybridoma clone 2 (Nielsen *et al.*, 1986) immobilized on Sepharose (data not shown). ^{125}I-u-PA-PAI-2 complexes were prepared in the same way, using M_r 48 000 PAI-2. For comparative purposes, ^{125}I-u-PA to be used in experiments in parallel with ^{125}I-u-PA-inhibitor complexes was subjected to a parallel incubation (in the absence of inhibitor) and parallel chromatography.

α_2-macroglobulin was iodinated and complexed with trypsin as previously described (Gliemann and Davidsen, 1986).

Cells

Human JAR choriocarcinoma cells (ATCC HTB 144) were from American Type Culture Collection (Rockville, MD). The cells were tested and found negative for mycoplasma. The cells were grown in culture flasks (Nunc, Roskilde, Denmark). For experiments, they were seeded in 24-well (2 cm^2) culture plates (Costar Tissue culture cluster, Cambridge, MA) in RPMI 1640 supplemented with 10% fetal calf serum (non-heat inactivated), 2 mM glutamine, penicillin 100 IE/ml, streptomycin 0.1 mg/ml at 37°C in 5% CO_2 as described (Jensen *et al.*, 1989a). The cells were seeded with 5.5×10^4 cells/well. They were used for experiments after 3 d of culture at a density of $\sim 2.5 \times 10^5$ cells/well. At this density, the cells were multilayered in the center but nonconfluent in the periphery of the well.

Binding studies

The following binding buffer was used: 123.7 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2.5 mM Na_2HPO_4, 0.5% bovine serum albumin (BSA), 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4. For most binding studies, adherent cells cultured as mentioned above were used directly. The culture medium was removed and the wells washed twice in 1000 μl of binding buffer. Then ~5 pM labeled ligand (10^4 cpm/ml) and unlabeled proteins were added in a final volume of 250 μl. The incubations were terminated by aspirating the incubation medium followed by two gentle washes with 1000 μl of incubation buffer. Finally, the cells were hydrolyzed in 500 μl NaOH (1 M), transferred to a plastic tube, and assayed for radioactivity. In studies using ^{125}I-pro-u-PA as tracer, Trasylol (0.1 mg/ml) was present in all buffers.

For binding studies with cells in suspension, the cells were released from culture bottles by incubation in phosphate-buffered saline (PBS), pH 7.4, with 4 mM EDTA for 10 min at 37°C, followed by gentle tapping of the tray against the bench. Single-cell suspensions were made by repeated pipettings through a 0.4-mm-diam canula. After this, the cells could be kept in suspension at 4°C by shaking. The incubation was terminated by transferring the cells to a microfuge tube and pelleting by centrifugation through silicone oil (Andreasen *et al.*, 1974).

Viability was assessed by trypan blue exclusion. The number of cells was measured by counting two wells from each plate after they had been released by EDTA treatment.

Electrophoresis and autoradiography

SDS-PAGE (Laemmli, 1970) was carried out using 8–16% polyacrylamide running gels and 4% stacking gels. The M_r markers were phosphorylase b (M_r 94 000), BSA (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 100), α-lactalbumin (M_r 14 400) (Pharmacia, Uppsala, Sweden). Autoradiography was performed using Hyperfilm MP (Amersham) with exposure at −80°C.

Extraction and cross-linking of cell-bound radioactivity

To analyze cell-associated radioactivity after binding experiments, the cell layers were washed at 4°C and then lysed by the addition of 95°C SDS–sample buffer (2% SDS, 20 mM Tris, 20% glycerol, pH 6.7) and subjected to SDS-PAGE.

For cross-linking, the following procedures were performed at 20°C. After removing unbound ligand by washing, we gently solubilized the cells in 300 μl PBS with 3% Triton X-100. The solution was cleared by centrifugation (100 000 × *g* in an Airfuge, Beckman, Palo Alto, CA) and disuccinimidyl suberate (Pierce, Chester, UK) was added to 5 mM. The cross-linking reaction was allowed to proceed for 15 min, and the disuccinimidyl suberate then quenched by addition of an equal volume of 100 mM ammonium acetate. After 10 min, the solution was mixed with 3.5 ml acetone and placed at −80°C for 30 min followed by centrifugation at 4500 × *g* for 5 min. The pellet was resuspended in SDS–sample buffer and subjected to SDS-PAGE.

Gel filtration

Radioactivity in cell media were analyzed for $^{125}I^-$ and ^{125}I-tyrosine by gel filtration on a G25F Sephadex column equilibrated with 0.5 M acetate, 0.025 M HCl, 0.1% BSA, pH 2.0 (Maceda *et al.*, 1982).

Electron microscopic autoradiography

JAR cells were cultured on 24 mm tissue culture-treated Transwells (Costar) to a density of ~450 000 cells per well. They were rinsed and incubated with 300 pM labeled u-PA or u-PA-PAI-1 complexes for 16 h at 4°C. Nonsaturable binding was measured by including 400 nM unlabeled u-PA. Some cells were reincubated at 37°C for 30 min. The cells were fixed for transmission electron microscopy by placing the culture wells overnight in 1% glutaraldehyde, 0.1 M cacodylate, pH 7.2 at 4°C. The cells were postfixed in 1% OsO_4 in the same buffer, pH 7.2, en bloc stained in uranyl acetate, followed by dehydration in graded alcohols and embedding in epoxy resin (Epon 812). Thin sections (60 nm) were stained with uranyl acetate and lead citrate and then covered with Ilford L-4 emulsion by a modified wire-loop method (Maunsbach, 1966). The sections were exposed for 90 d, developed in Kodak (Rochester, NY) D-19 developer for 90 s and fixed in 20% sodium thiosulphate for 2 min. The sections were studied in a Jeol 100 CX electron microscope. For quantitative determination, two grids from each situation with respect to ligand and incubation temperature were analyzed at a primary magnification of ×5000 and enlarged ×3 to a final magnification of ×15 000. Ten to 15 micrographs and 3 background micrographs from areas with no cells were taken at random from each grid. Grains were assigned to the plasma membrane, electron lucent endocytic-like vesicles and electron dense lysosomal-like vesicles, cytoplasm, nuclei, or other intracellular organelles and extracellular sites. A grain was assigned to the nearest compartment within a distance of less than three half distances for ^{125}I-radiation, corresponding to ~270 nm (Salpeter *et al.*, 1977; Ottosen, 1978). The background was negligible (5.4×10^{-4} grains/μm^2).

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Note added in proof: By the time of submission of this manuscript, another paper appeared, describing the internalization of u-PA-PAI-1 complexes (Estreicher *et al.* (1990) The receptor for urokinase type plasminogen activator polarizes expression of the protease to the leading edge of migrating monocytes and promotes degradation of enzyme inhibitor complexes. J. Cell Biol. *111,* 783–792).

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