

# 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

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**Abstract.** Receptor-bound urokinase-type plasminogen activator (uPA) remains associated to the surface of human monocytes for many hours. Monocytes induced to migrate in a chemotactic gradient of f-Met-Leu-Phe rapidly polarize their uPA receptors to the leading front of the cells. Receptor-bound enzyme can be inhibited by plasminogen activator inhibitor 2 (PAI-2), with a kinetics comparable to that determined for the free enzyme, and uPA/PAI-2 complexes can bind to the uPA receptor. In contrast to the active enzyme, the

uPA/PAI-2 complex is rapidly cleared from the monocyte cell surface; this involves an initial cleavage of the complex at the cell surface, followed by endocytosis and degradation. These results indicate that the uPA receptor can function both to focus plasmin-mediated extracellular matrix degradation in front of migrating cells, and to target uPA/PAI-2 enzyme/inhibitor complexes for degradation; they suggest that this receptor is a key determinant in the control of uPA-catalyzed extracellular proteolysis.

THE migration of cells within tissues and between different body compartments requires the proteolytic degradation of components of extracellular matrices. Through the generation of plasmin and in conjunction with other enzymes such as collagenases and elastases, plasminogen activators (PAs)<sup>1</sup> can catalyze the degradation of most proteins of the extracellular space (Bogenmann and Jones, 1983; Heisel et al., 1983; Bergman et al., 1986). The urokinase-type PA (uPA) is produced by a variety of migratory cells, including invasive malignant cells (Danø et al., 1985), implanting trophoblastic cells (Strickland et al., 1976; Sappino et al., 1989), monocytes-macrophages (Vassalli et al., 1984), capillary endothelial cells (Pepper et al., 1987), and inhibition of uPA activity prevents cell migration in different experimental systems (Ossowski and Reich, 1983; Mignatti et al., 1986; Ossowski, 1988a).

A number of cell types express on their surface a binding site for uPA and its single-chain zymogen prouPA (Bajpai and Baker, 1985; Stoppelli et al., 1985, 1986; Vassalli et al., 1985; Cubellis et al., 1986; Blasi et al., 1987). This receptor has recently been characterized; it behaves as an integral membrane protein (Estreicher et al., 1989), and comprises at least one carbohydrate-containing polypeptide chain of 55,000 *M<sub>r</sub>* (Nielsen et al., 1988). Binding occurs through the noncatalytic A chain of the two-chain enzyme (Stoppelli

et al., 1985; Vassalli et al., 1985), and involves the "growth factor-like" domain of the molecule (Appella et al., 1987). The bound zymogen can be cleaved by plasmin into the two-chain enzyme (Cubellis et al., 1986), and the bound enzyme is catalytically active (Vassalli et al., 1985). The source of the enzyme that binds to this receptor may differ for different cell types. In certain cases, the enzyme appears to be synthesized by the receptor-bearing cells themselves, and to bind in an autocrine way after secretion (Stoppelli et al., 1986). Alternatively, cells that do not produce uPA will bind enzyme synthesized by a different cell type (Huarte et al., 1987). Also, prouPA is present in the blood plasma (Tissot et al., 1982; Wun et al., 1982), at a concentration ( $\sim 10^{-10}$  M) in the range of the *K<sub>d</sub>* of its interaction with the receptor, and could bind to receptor-bearing circulating cells. Thus, while uPA is a secreted protein, its subsequent binding to a high affinity cell surface receptor can localize the enzyme to the plasma membrane. The expression of the uPA receptor appears to be a versatile and widespread mechanism to limit the activity of the uPA-plasmin system to the close environment of the cell.

In many cases, however, ligand-plasma membrane receptor interactions lead to rapid internalization of the ligand; if uPA functions as an ectoenzyme, it should not be removed rapidly after binding to the plasma membrane. Also, directed cell migration requires localized proteolysis, and polarized expression of the uPA receptor could be a way to focus plasmin-mediated extracellular matrix degradation to the leading edge of migrating cells. Finally, three serpin class

1. *Abbreviations used in this paper:* fMLP, *N*-formyl-L-methionyl-L-leucyl-phenylalanine; DFP, diisopropylfluorophosphate; PA, plasminogen activator; PAI, PA inhibitor; uPA, urokinase type PA.

antiproteases with high affinity towards secreted uPA have been identified. While it has been suggested that membrane-associated uPA might be protected from inhibition by these serpins (Chapman et al., 1982; Blasi et al., 1987), recent investigations have shown that plasminogen activator inhibitors (PAIs) can react with receptor-bound uPA (Cubellis et al., 1989; Kirchheimer and Remold, 1989); thus, it is also possible that the uPA receptor could function to favor the clearance and degradation of uPA/inhibitor complexes. The experiments reported here were designed to address these issues. To this effect, we have used human monocytes, as well as cells from two human monocytic lines, U937 and THP-1. The uPA receptor of U937 cells has been extensively characterized (Stoppelli et al., 1985; Vassalli et al., 1985; Nielsen et al., 1988), and THP-1 cells (Tsuchiya et al., 1980), while bearing uPA receptors that are indistinguishable from those of monocytes and U937 cells, produce essentially no detectable PAIs. The results obtained help better define the probable physiological roles of the uPA receptor: to focus uPA-catalyzed generation of plasmin to the leading front of migrating cells, and to target uPA/inhibitor complexes for degradation.

## Materials and Methods

### Cell Culture

Human peripheral blood mononuclear cells were prepared by Ficoll-Hypaque sedimentation, and cultured for 20 h in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS (Gibco Laboratories) that had been heated to 56°C for 30 min (HI-FBS) in 35-mm tissue culture Petri dishes (Vassalli et al., 1985).

U937 cells were grown as described (Vassalli et al., 1985). THP-1 cells (Tsuchiya et al., 1980) were a kind gift from Dr. Jean-Michel Dayer; they were grown in RPMI 1640 medium (Gibco Laboratories) supplemented with 25-mM Hepes; 110 U/ml penicillin (Pfizer, Zurich, Switzerland); 110 µg/ml streptomycin (Gruenthal, Bern, Switzerland); and 10% FBS. U937 and THP-1 cells were collected by centrifugation (10 min, 300 g), washed, and resuspended in RPMI 1640 1 mg/ml acid-treated BSA (RPMI-BSA; No. 81-001; Miles Laboratories, Inc., Naperville, IL) (Loskutoff, 1978).

### Iodination of uPA

55,000 and 33,000  $M_r$  two-chain human uPA were obtained from Serono (Denens, Switzerland). Concentrations of the enzymes were determined by active site titration (Chase and Shaw, 1967). uPA was radiolabeled using iodogen (Pierce Chemical Co., Rockford, IL) and Na- $^{125}I$  (Amersham International, Amersham, England), as described (Vassalli et al., 1984) and was used within a month.  $^{125}I$ -uPA had a specific activity of  $1 - 5 \times 10^7$  cpm/µg and was at least 90% enzymatically active.

### Electron Microscopy

Adherent monocytes were washed three times with RPMI 1640, and incubated at 37°C for different time periods in RPMI 1640 containing 25 mM Hepes, 1 mg/ml BSA, and  $1.6 \times 10^{-9}$  M diisopropylfluorophosphate (DFP)-inactivated 55,000  $M_r$   $^{125}I$ -uPA (Vassalli et al., 1985). After extensive washing with ice cold PBS containing 1 mg/ml BSA, the cells were fixed with 2.5% glutaraldehyde in 0.1-M cacodylate buffer (pH 7.4), collected by scraping with a rubber policeman, dehydrated, and processed for autoradiography and electron microscopy as described (Carpentier et al., 1978).

U937 cells were cultured at 37°C for different time periods in presence of  $1.6 \times 10^{-9}$  M DFP-inactivated 55,000  $M_r$   $^{125}I$ -uPA, washed three times with RPMI-BSA, and cell-associated radioactivity was determined. Non-specific binding was determined in presence of a 30-fold excess of nonradioactive ligand, and never exceeded 5% of total binding. The cells were then fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), dehydrated and processed for quantitative EM autoradiography as described

(Carpentier et al., 1978). The analysis of the autoradiographic data as well as the morphometric quantitation were performed as previously described (Carpentier et al., 1979, 1981), and considering that a grain was associated with the plasma membrane when its center was within 250 nm of this structure. A background value of 10% was subtracted from the "internalized" fraction (Carpentier et al., 1978). Binding of  $^{125}I$ -insulin to U937 cells, preparation of the samples and analysis of the data were performed as described (Robert et al., 1984).

### Chemotaxis

Human monocytes were prepared as described above; U937 cells were allowed to attach to the bottom of 35-mm tissue culture Petri dishes by culturing for 24 h in presence of 50 ng/ml PMA. After washing with RPMI-BSA, some of the culture dishes were divided into two concentric chambers: medium was removed and a high vacuum grease-coated 10-mm Teflon ring was fixed to the center of the dish; a glass capillary tube (4-mm long with a 0.1-mm opening) was placed in the grease, below the Teflon ring, so as to connect the two chambers. Culture medium was added to the two chambers (inner, 100 µl; outer, 2.5 ml); when Niagara blue (0.04%) was added to the outer chamber of such culture vessels, dye could be seen to have diffused to ~25% of the inner chamber after 15 min. *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP; Sigma Chemical Co., St. Louis, MO) ( $25 \mu\text{l}$  of a  $10^{-3}$  M solution) was added to the outer chamber, close to the opening of the capillary tube (Fig. 3, B and E). For control cultures (Fig. 3, A and D) and cultures exposed to a uniform concentration of fMLP (C and F), the Petri dishes were not divided in two chambers; where appropriate (C and F), fMLP was added to a final concentration of  $10^{-5}$  M. After 15 min at 37°C, the cells were washed with ice cold PBS-BSA. DFP-inactivated 55,000  $M_r$   $^{125}I$ -uPA ( $1.6 \times 10^{-9}$  M in PBS-BSA) was added, and allowed to bind for 30 min at 4°C. The cells were then washed four times with cold PBS-BSA, and fixed for 30 min at room temperature with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.6); before dismounting the partition between the two chambers, the position of the capillary tube was recorded. Petri dishes were processed for autoradiography, and examined in a Zeiss Axiophot. The proportion of polarized cells was determined by counting 200–600 cells per experimental condition. Experiments were performed four times for both cell types, with similar results.

### SDS-PAGE and Autoradiography

Samples were electrophoresed in the presence of SDS under nonreducing conditions in 10% polyacrylamide gels using the buffer system of Laemmli (1970). Molecular weights were calculated from the position of marker proteins (low molecular weight calibration kit; Pharmacia Fine Chemicals, Piscataway, NJ) electrophoresed in parallel lanes and stained with Coomassie brilliant blue R250.

Autoradiography of the gels was performed as described (Vassalli et al., 1984).

### PAI-2 (PAI Type 2)

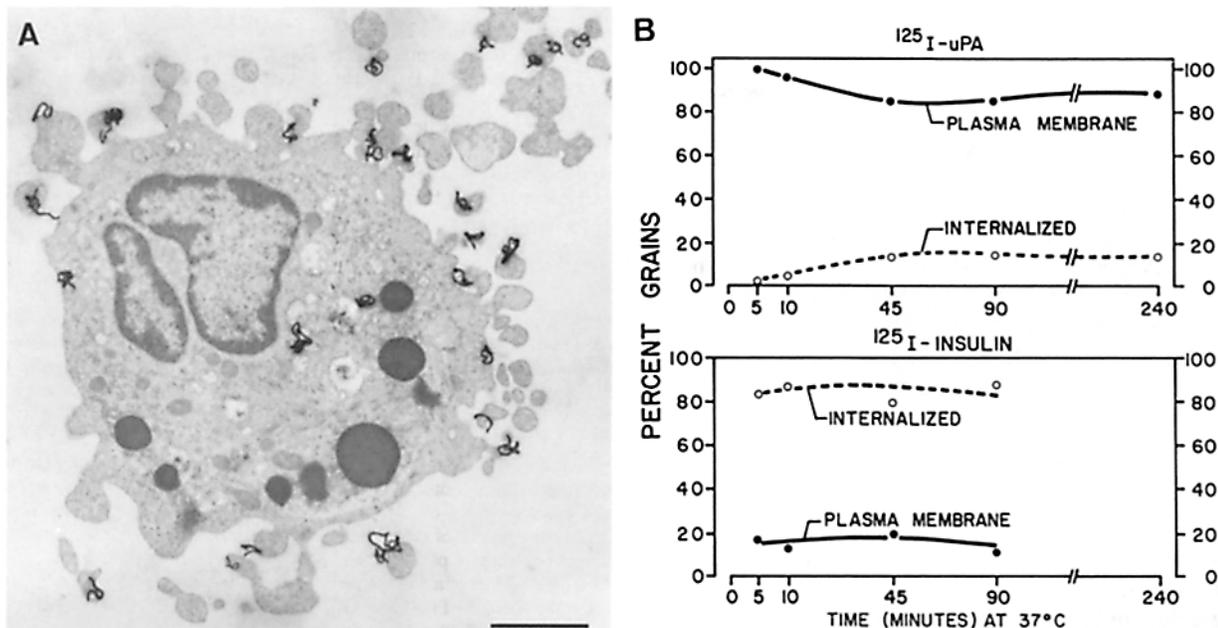
PAI-2, purified as described (Kruithof et al., 1986), was a kind gift of Dr. E. K. O. Kruithof.

### Preparation of Preformed Complexes

33,000- and 55,000- $M_r$   $^{125}I$ -uPA ( $10^{-8}$  M), purified by gel filtration on an Utrogel AcA 54 column in 0.5 M NaCl, 8 mM  $\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , were incubated in the presence of purified PAI-2 ( $5 \times 10^{-7}$  M) for 2 h at 4°C. Quantitative formation of complexes was assessed by SDS-PAGE followed by autoradiography of the gel.

### Single Cell Plaque Assay

THP-1 cells were preincubated in the absence or in the presence of either uPA or purified prouPA ( $2 \times 10^{-9}$  M). Purified prouPA was a kind gift of Dr. W.-D. Schleuning. After 1 h at 4°C, the cells were washed and further incubated at 4°C in the presence or the absence of PAI-2 ( $2 \times 10^{-8}$  M). After washing, aliquots of  $5 \times 10^3$  cells of each condition were resuspended in a mixture of 1.3% casein, 0.8% agar and 40 µg/ml plasminogen in RPMI 1640 medium, and, where indicated,  $2 \times 10^{-8}$  M PAI-2 and plated in 35-mm tissue culture dishes (Falcon Labware, Oxnard, CA). Photographs were taken under dark ground illumination.



**Figure 1.** Localization of receptor-bound <sup>125</sup>I-uPA. (A) EM autoradiograph of a human monocyte cultured 4 h at 37°C in the presence of DFP-inactivated <sup>125</sup>I-uPA; seen at 11,000×. Bar, 1 μm. (B) Upper: quantitative analysis by high resolution EM autoradiography of the distribution of DFP-inactivated <sup>125</sup>I-uPA in U937 cells after different times of culture at 37°C in presence of the ligand. (B) Lower: same as upper, but for <sup>125</sup>I-insulin.

### Interaction of Bound uPA with PAI-2

THP-1 cells (10<sup>7</sup>/ml) were preincubated with 5 × 10<sup>-9</sup> M <sup>125</sup>I-uPA for 2 h at 4°C. After extensive washings, the cells were incubated in the absence or presence of 2 × 10<sup>-8</sup> M PAI-2 at 4°C or 37°C as indicated. At the end of the incubations, the cells were applied onto a 250 μl oil cushion consisting of a 6:4 mixture (vol/vol) of dioctylphthalate (No. 821874; Merck A. G., Darmstadt, FRG) and dibutylphthalate (No. 800919; Merck) in polyethylene microfuge tubes (Milian Instruments S. A., Geneva, Switzerland); and centrifuged at 4°C for 30 s at 12,000 g; the closed tubes were cut and the radioactivity in cell pellets and supernatants counted (Celada et al., 1984). The cells were solubilized in 0.2% Triton X-100. Nuclei were removed by centrifugation (10 min, 800 g). Cells and supernatants were mixed with double strength electrophoresis sample buffer and analyzed by SDS-PAGE and autoradiography of the gels.

For experiments involving tryptic digestion of cell surface-bound ligand, the samples were handled as above, except that before applying on the oil cushion the cells were centrifuged (10 min at 300 g), resuspended in PBS (10<sup>7</sup> cells/ml), and incubated in the absence or in the presence of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated bovine pancreatic trypsin (5 × 10<sup>-7</sup> M) for 10 min at 20°C; trypsin was inactivated by the addition of FBS (15%).

## Results

### Receptor-bound uPA Remains on the Cell Surface

We have localized <sup>125</sup>I-uPA by EM autoradiography at different times after its addition to cultured human monocytes and monocyte-like U937 cells. For both cell types, most autoradiographic grains were found in the vicinity of the plasma membrane at all times studied. Fig. 1 A is one of a series of pictures of monocytes that were used for quantitative analysis: after 4 h of incubation at 37°C, 79% of the radiolabel was associated with the plasma membrane (± 250 nm). A similar analysis performed with U937 cells confirmed that receptor-bound <sup>125</sup>I-uPA remains mostly associated with the plasma membrane of these cells also (Fig. 1 B). By contrast, another cell surface receptor ligand, insulin, was rap-

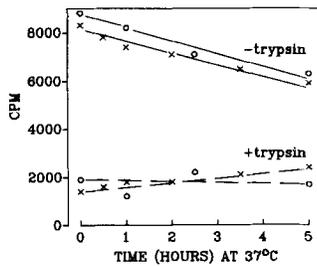
idly internalized in monocytes (Grunberger et al., 1985) and in U937 cells (Fig. 1 B).

The rate of disappearance of receptor-bound <sup>125</sup>I-uPA from the surface of U937 cells (by internalization and/or dissociation) was also determined (Fig. 2). After 5 h of incubation at 37°C, two-thirds of the radiolabel that remained associated with the cells could be removed by trypsin, a result consistent with previous reports by Stoppelli et al. (1985) and Bajpai and Baker (1985). Overall, the amount of cell surface-associated <sup>125</sup>I-uPA decreased with a half-time of ~5 h. Our results are compatible with the idea that a role for the uPA receptor is to localize the (pro)enzyme to the cell surface, thus endowing the cell with a potent mechanism to generate plasmin-mediated proteolysis in its immediate environment.

### The uPA Receptor Is Polarized to the Leading Front of Migrating Cells

Nonuniform distribution of the uPA receptor could be a way to achieve polarization of protease expression to discrete domains of the cell surface. To explore this, we determined the distribution of receptor-bound <sup>125</sup>I-uPA, paying particular attention to its possible association with surface projections. No preferential association was observed in the case of U937 cells; for monocytes, however, <sup>125</sup>I-uPA was found to be more frequently associated with villous surface projections (73%, 500 grains counted) than expected on the basis of the percent of the total cell surface contributed by these structures (57%) (see Fig. 1, A). Thus, at least in the case of freshly prepared human monocytes, surface distribution of the uPA receptor on "resting" cells is not entirely random.

Migration of monocytes can be triggered in response to chemotactic agents (Gallin and Quie, 1978). U937 cells and



**Figure 2.** Fate of receptor-bound  $^{125}\text{I}$ -uPA on U937 cells. Binding of DFP-inactivated 55,000  $M_r$   $^{125}\text{I}$ -uPA (1.6-nM) to U937 cells was carried out for 1 h at 37°C, as described (Vassalli et al., 1985). After washing by centrifugation in RPMI-BSA to remove free ligand, the cells were incubated at 37°C. At various times, cultures were treated for 20 min at 37°C in presence or absence of trypsin (10  $\mu\text{g}/\text{ml}$ ), before washing and counting the cell-associated radioactivity. The results of two different experiments (O, x) are illustrated; each point represents the mean of duplicate cultures.

monocytes attached to tissue culture plates were exposed for 15 min to a concentration gradient of the chemotactic peptide fMLP. The uPA receptor was then localized by binding of  $^{125}\text{I}$ -uPA and autoradiography (Fig. 3). For both cell types, some 30% of the cells expressed a highly asymmetric distribution of the receptor in response to the chemotactic signal; the radioactively labeled pole was always the leading edge of the cell; i.e., that facing the source of the chemotactic gradient. In the case of monocytes, the labeling was most intense on the cytoplasmic projections that were present at the leading edge, while U937 cells showed a uniform intense labeling of the leading edge. Polarized expression of the uPA receptor was a response to the chemotactic gradient, since a uniform concentration of fMLP did not increase the proportion of polarized cells above that observed in absence of the peptide.

**Receptor-bound uPA Can Be Inactivated by PAI-2**

The experiments reported above were performed using DFP-inactivated  $^{125}\text{I}$ -uPA to avoid reaction of the ligand with PAI-2, which is synthesized and released by monocytes and U937 cells (Wohlwend et al., 1987). To explore the fate of receptor-bound active uPA as well as that of its single-chain zymogen prouPA, we used a different cell line of the mononuclear phagocyte lineage, THP-1; THP-1 cells express uPA receptors that are indistinguishable from those of unstimulated monocytes and U937 cells, but they do not produce high levels of a PAI (data not shown, and see below Figs. 8 and 9).

### Receptor-bound uPA Can Be Inactivated by PAI-2

To investigate the susceptibility of receptor-bound uPA and prouPA to inhibition by PAI-2, we first used a catalytic plaque assay, which reveals the activity of the cell-associated enzyme (Vassalli et al., 1985). After incubation with or without uPA or prouPA, THP-1 cells were cultured for 1 h at 4°C in the presence or absence of PAI-2, and washed. They were then embedded in substrate, and the formation of proteolytic plaques was observed (Fig. 4, A). Only few plaques developed when the cells had not been incubated with uPA or prouPA, indicating that THP-1 cells produce only low levels of the enzyme. After incubation with uPA, most cells were surrounded by a lytic zone; incubation of these cells in the presence of PAI-2 prevented the subsequent uPA-dependent proteolytic activity. Incubation of THP-1 cells with prouPA also resulted in the expression of proteolytic activity around the cells; in this case, however, PAI-2

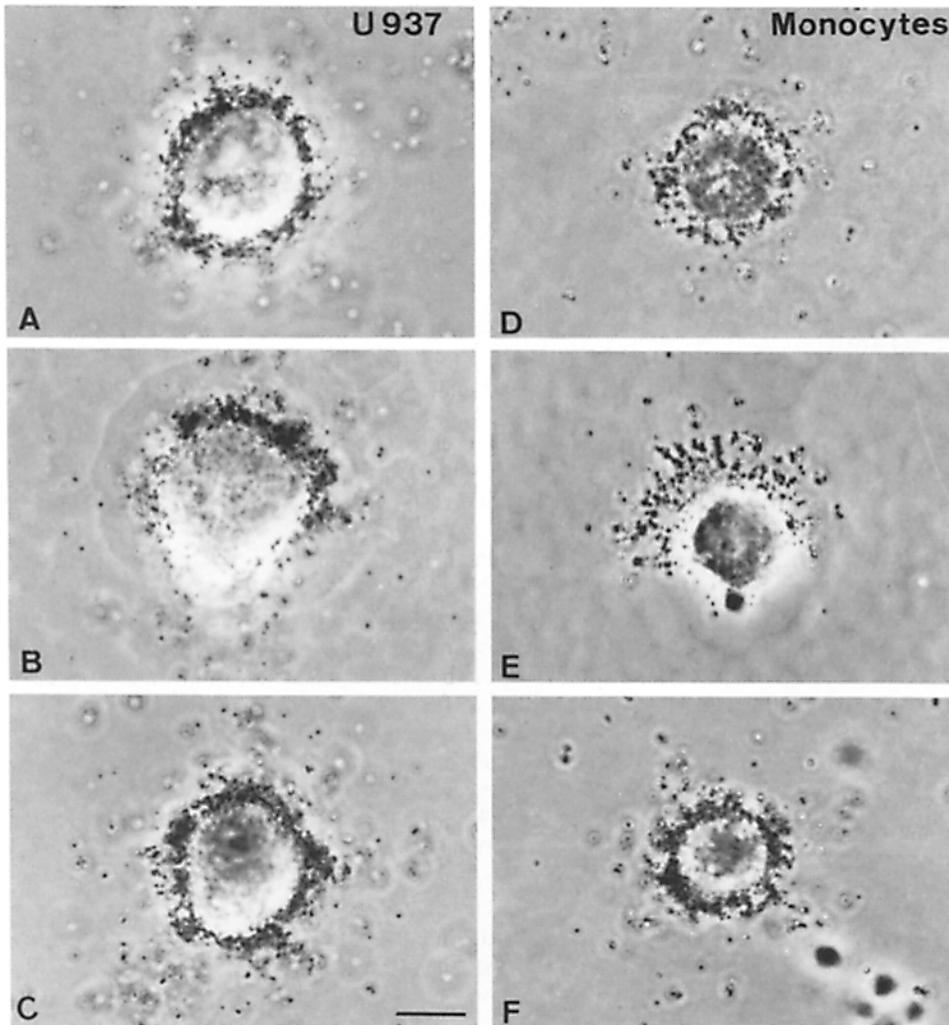
caused only a slight decrease in prouPA-dependent proteolysis. Finally, addition of PAI-2 to the substrate in all cases prevented the formation of proteolytic plaques (Fig. 4, B). We interpret these observations as follows. (a) Receptor-bound uPA is inhibited in the presence of PAI-2, indicating that binding to the receptor does not protect the enzyme from this inhibitor. (b) Binding to the receptor does not render prouPA susceptible to reaction with PAI-2. The slight inhibitory effect of PAI-2 may be because of activation of a fraction of the proenzyme during its binding or during incubation in the presence of PAI-2; alternatively, some PAI-2 may remain bound to the cells, and thus could react with uPA formed during the proteolytic assay. (c) The formation of plaques around prouPA-bearing cells requires the conversion of the proenzyme to active uPA during the catalytic assay (performed in the presence of plasminogen containing trace amounts of plasmin), and this accounts for the effect of PAI-2 when the inhibitor is present in the substrate used for the assay.

The inhibition of soluble and receptor-bound uPA by PAI-2 was compared by taking advantage of the formation of covalent complexes between the enzyme and the inhibitor; these complexes can be resolved from unreacted enzyme by SDS-PAGE, and their abundance provides a quantitative estimate of the proportion of enzyme that has reacted with PAI-2 (Fig. 5). At the enzyme and inhibitor concentrations used in this experiment, half of the soluble (unbound) 55,000  $M_r$   $^{125}\text{I}$ -uPA had reacted with PAI-2 to form a 95,000- $M_r$  complex after a 10–15 min incubation at 4°C; a similar rate of reaction was found for 33,000  $M_r$   $^{125}\text{I}$ -uPA, which contaminates our uPA preparation, and which forms a 73,000- $M_r$  complex with PAI-2. The rate of inhibition of receptor-bound  $^{125}\text{I}$ -uPA was slightly slower: half of the bound 55,000  $M_r$   $^{125}\text{I}$ -uPA had reacted with PAI-2 to form the 95,000- $M_r$  complex after ~40 min. However, this different kinetics may not necessarily reflect a partial protection of the enzyme but could be due to the immobilization of uPA at the cell surface, whereas in solution both enzyme and inhibitor can diffuse freely. Interestingly, a 70,000- $M_r$  radioactive species was observed at late times of incubation of receptor-bound  $^{125}\text{I}$ -uPA with PAI-2; the significance of this species will be discussed below. We conclude from this experiment that the inhibition of receptor-bound uPA by PAI-2 is slightly slower than that of the soluble enzyme; however, the receptor does not shield the enzyme from the inhibitor.

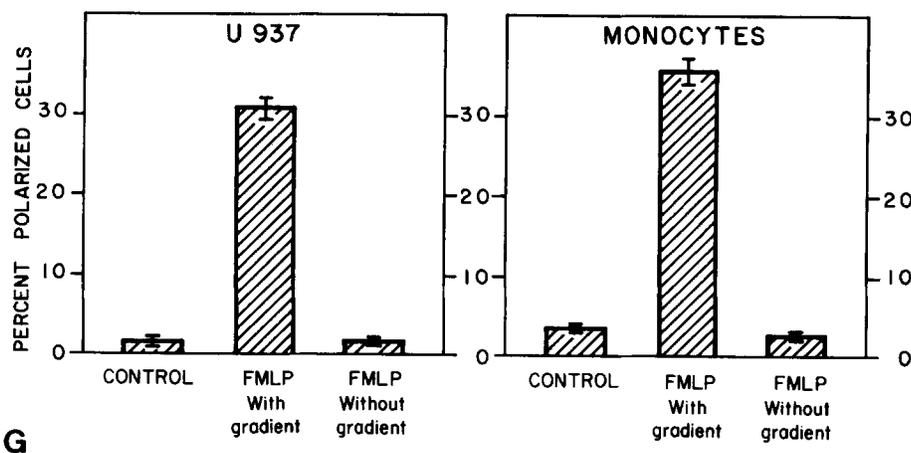
Since PAI-2 reacts only with catalytically active uPA, inhibition of receptor-bound uPA by PAI-2 provided a quantitative means to demonstrate that the enzyme remains functionally active at the cell surface even after prolonged incubation at 37°C. THP-1 cells with receptor-bound  $^{125}\text{I}$ -uPA were exposed to PAI-2 at various times up to 4 h after binding of the enzyme. At all times the majority of intact  $^{125}\text{I}$ -uPA was able to react with PAI-2, to form the covalent 95,000- $M_r$  complex (Fig. 6).

**uPA/PAI-2 Complexes Are Cleared from the Cell Surface**

The lasting presence of receptor-bound uPA at the cell surface together with the ability of the enzyme to interact with PAI-2 suggests that the cell surface could remain encumbered with uPA/PAI-2 complexes for several hours. An alter-

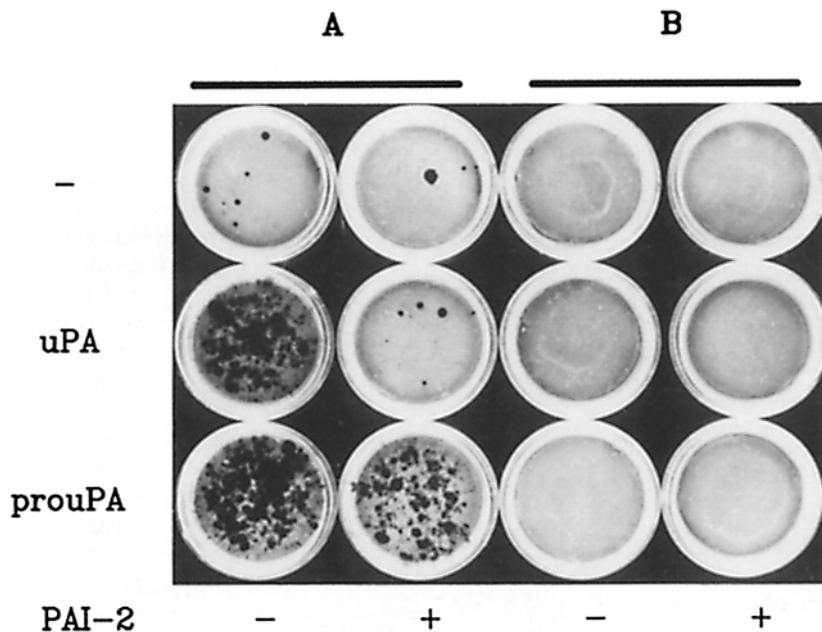


**Figure 3.** Polarized distribution of the uPA receptor on U937 cells (left) and human monocytes (right) in a gradient of the chemotactic peptide fMLP. To localize the uPA receptor, cells were incubated for 30 min at 4°C in the presence of DFP-inactivated 55,000- $M_r$   $^{125}I$ -uPA, washed, fixed and processed for autoradiography. The autoradiographic grains (dark spots) are present throughout the cell periphery in A, C, D, and F, and are restricted to one side of the cells in B and E. (A and D) no fMLP (control); (B and E) two polarized cells in a gradient of fMLP; the source of the gradient is at the top; (C and F) fMLP, no gradient. Bar, 10  $\mu$ m. (G) Proportion of cells showing a polarized binding of  $^{125}I$ -uPA in cultures A, B, C, D, E, and F (mean  $\pm$  SEM).

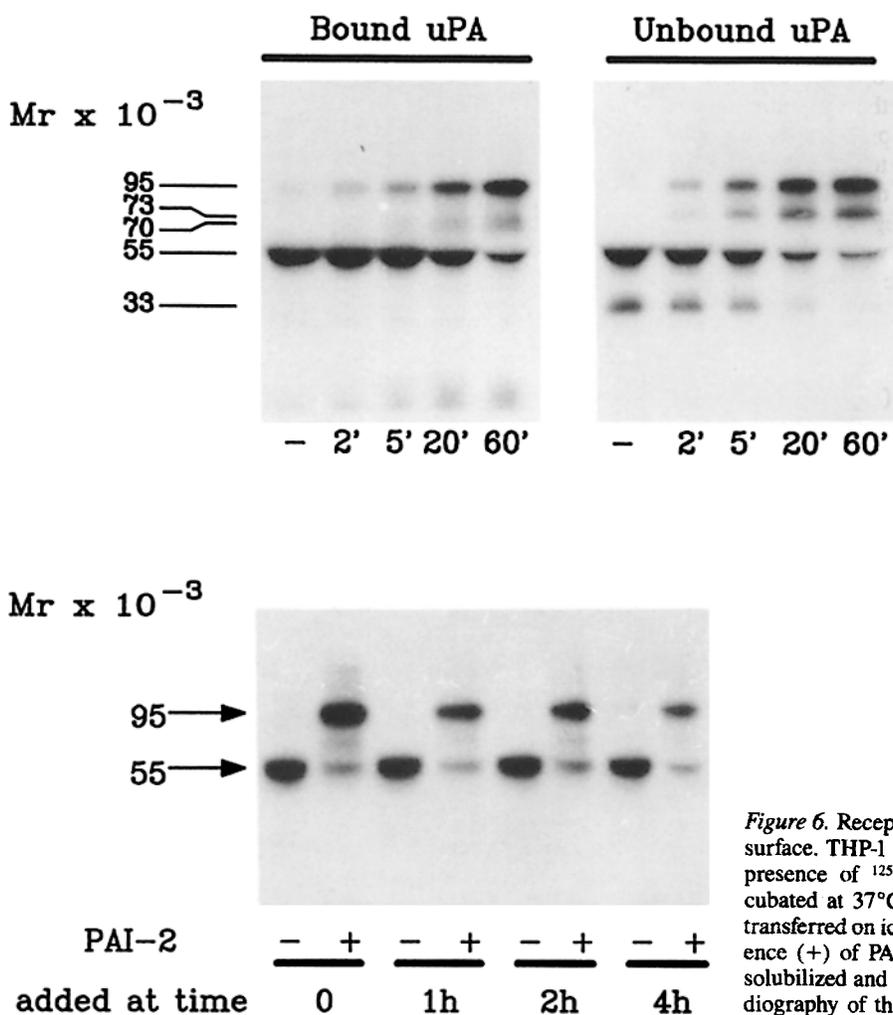


nate possibility is that the fate of receptor-bound active uPA differs from that of the PAI-2-inhibited enzyme. To discriminate between these hypotheses, THP-1 cells were incubated with  $^{125}I$ -labeled prouPA, uPA, or DFP-inactivated uPA, washed, and further incubated at 37°C in the presence or in the absence of PAI-2; the rate of decrease in cell-

associated radioactivity was comparable for prouPA, DFP-inactivated uPA and active uPA (Fig. 7). Addition of PAI-2 to cells that had been incubated with active  $^{125}I$ -uPA resulted in a significantly faster loss in cell-associated radioactivity (Fig. 7); this appeared to require an interaction of PAI-2 with the active site of the enzyme, since PAI-2 did not affect the

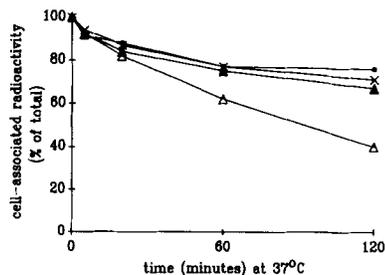


**Figure 4.** Differential sensitivity of receptor-bound uPA and prouPA towards PAI-2. THP-1 cells were preincubated for 1 h at 4°C in the absence (-) or in the presence of either uPA or prouPA, as indicated. The cells were washed and further incubated with (+) or without (-) PAI-2 ( $2 \times 10^{-8}$  M) for 1 h at 4°C. Finally, the cells were washed again and embedded in a mixture of agar, casein, and plasminogen (A) or in the same mixture supplemented with PAI-2 ( $2 \times 10^{-8}$  M) (B). PAI-2 is a highly specific inhibitor of PAs and does not react with plasmin (Kruithof et al., 1986). Photographs were taken after 40 h at 37°C; proteolytic plaques appear as dark circles in a clear background. No plaques were seen in parallel incubations performed in the absence of plasminogen, confirming that lysis of the substrate was mediated by plasmin.



**Figure 5.** Kinetics of interaction of receptor-bound and free uPA with PAI-2. (*Bound uPA*) THP-1 cells were preincubated with  $^{125}\text{I}$ -uPA for 2 h at 4°C. After washing, they were incubated in the presence of PAI-2 ( $2 \times 10^{-8}$  M) for 0, 2, 5, 20, and 60 min at 4°C. The reaction was stopped by centrifugation of the cells through an oil cushion. The cells were solubilized and the samples analyzed by SDS-PAGE and autoradiography of the gel. (*Unbound uPA*) A mixture of 55,000 and 33,000  $M_r$   $^{125}\text{I}$ -uPA containing the same amount of radioactivity as the washed cell suspension (see above) was prepared. It was handled as described above, except that the reaction with PAI-2 was stopped by adding electrophoresis sample buffer directly to the incubation medium.

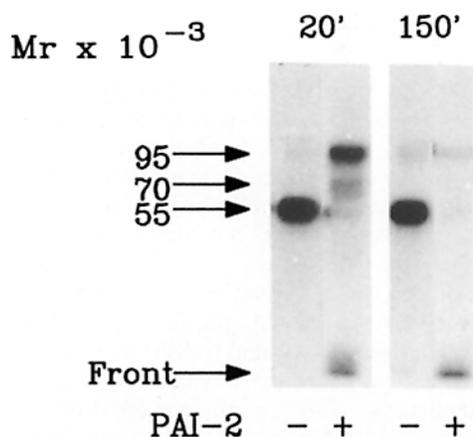
**Figure 6.** Receptor-bound uPA remains functional at the cell surface. THP-1 cells were preincubated for 1 h at 4°C in the presence of  $^{125}\text{I}$ -uPA; they were washed and further incubated at 37°C for 0, 1, 2, and 4 h. The cells were then transferred on ice and kept for 1 h in the absence (-) or presence (+) of PAI-2 ( $2 \times 10^{-8}$  M). The washed cells were solubilized and analyzed by SDS-PAGE followed by autoradiography of the gel.



**Figure 7.** Rate of decrease in cell-associated uPA. THP-1 cells were incubated with either single-chain  $^{125}\text{I}$ -prouPA (●), two-chain active ( $\Delta$ ,  $\blacktriangle$ ), or DFP-treated ( $\times$ )  $^{125}\text{I}$ -uPA for 2 h at  $4^\circ\text{C}$ . After washing, the cells were further incubated in the absence (●,  $\times$ ,  $\blacktriangle$ ) or in the presence ( $\Delta$ ) of PAI-2 for 0, 5, 20, 60, and 120 min at  $37^\circ\text{C}$ . The cells were then centrifuged through an oil cushion, and both cell-associated and free radioactivity were counted. Each point is the mean of duplicate determinations; values differ by  $<2\%$  from the average value.

fate of receptor-bound DFP-inactivated  $^{125}\text{I}$ -uPA (data not shown). These results suggest that when PAI-2 reacts with receptor-bound uPA, the fate of the enzyme/inhibitor complex is different from that of the free enzyme.

To determine the fate of the receptor-bound complexes, THP-1 cells with bound  $^{125}\text{I}$ -uPA were incubated in the presence of PAI-2 for 20 and 150 min at  $37^\circ\text{C}$ , and analyzed by SDS-PAGE and autoradiography of the gel (Fig. 8). After 20 min, the major cell-associated radioactive band was that of the 95,000- $M_r$  uPA/PAI-2 complex. However, two additional bands, one at 70,000- $M_r$  and the other migrating with the electrophoretic front, were also conspicuous; these most likely represent degradation intermediates of the  $^{125}\text{I}$ -uPA/PAI-2 complex. After 150 min, only a small amount of low relative molecular mass radioactive degradation products could be detected; no free uPA and only very little uPA/PAI-2 complex remained associated with the cells. As expected



**Figure 8.** Fate of receptor-bound uPA after interaction with PAI-2. THP-1 cells were preincubated with 55,000- $M_r$   $^{125}\text{I}$ -uPA for 1 h at  $4^\circ\text{C}$ . The cells were then washed, incubated at  $37^\circ\text{C}$  in the absence (-) or presence (+) of PAI-2 ( $2 \times 10^{-8}$  M) for 20 and 150 min and finally centrifuged through an oil cushion. The cells were solubilized and the samples analyzed by SDS-PAGE and autoradiography of the gel.

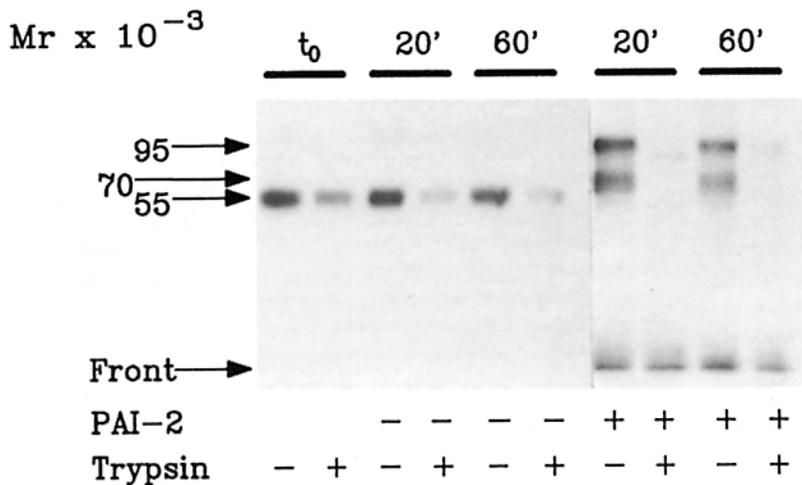
from the experiments reported above, in the absence of PAI-2 the amount of cell-associated  $^{125}\text{I}$ -uPA decreased only slightly between 20 and 150 min of incubation; furthermore, no evidence was obtained for degradation of the bound active enzyme.

These results demonstrate that, as compared to active  $^{125}\text{I}$ -uPA, the  $^{125}\text{I}$ -uPA/PAI-2 complex is rapidly cleared from the cell surface. Experiments in which the medium from cultures of THP-1 cells incubated in the presence or absence of PAI-2 was also analyzed indicate that dissociation of the intact  $^{125}\text{I}$ -uPA/PAI-2 complex from the cell surface was not significantly greater than that of active  $^{125}\text{I}$ -uPA. The earliest detectable evidence of degradation is the formation of a 70,000- $M_r$  radioactive species (Fig. 8). To determine whether this degradation intermediate is present on the cell surface, or whether it is formed upon endocytosis of the uPA/PAI-2 complex, THP-1 cells with receptor-bound  $^{125}\text{I}$ -uPA were incubated at  $37^\circ\text{C}$  in the presence of PAI-2, exposed to trypsin, washed, and analyzed by SDS-PAGE and autoradiography of the gel (Fig. 9). Both the uPA/PAI-2 95,000- $M_r$  complex and the 70,000- $M_r$  degradation intermediate were removed by trypsin, indicating that they are exposed at the cell surface. By contrast, the low relative molecular mass radiolabeled products were not detectably affected; since they were not present in trypsin-treated samples from cells incubated in the absence of PAI-2, they do not result from tryptic digestion of cell surface  $^{125}\text{I}$ -uPA, but must be intracellular, resulting from degradation of the uPA/PAI-2 complex after it has been endocytosed. Further evidence for the initial cleavage of the  $^{125}\text{I}$ -uPA/PAI-2 complex occurring at the cell surface was obtained in experiments performed at  $4^\circ\text{C}$ . Formation of the 70,000- $M_r$  degradation intermediate was also observed under these conditions, which prevent endocytosis (Fig. 6).

Additional experiments were carried out using preformed  $^{125}\text{I}$ -uPA/PAI-2 complexes. At  $4^\circ\text{C}$ , the complexes were found to bind to THP-1 cells only slightly less efficiently than active uPA; their binding was prevented by excess DFP-inactivated 55,000- $M_r$  uPA. By contrast, a complex formed between PAI-2 and 33,000- $M_r$   $^{125}\text{I}$ -uPA did not bind to the cells (Fig. 10). After binding of the preformed complexes, the cells were further incubated at  $37^\circ\text{C}$ ; at various times ranging from 5 to 120 min, they were collected and analyzed by SDS-PAGE and autoradiography of the gel. The cell-associated radioactive 95,000- $M_r$  complex disappeared with a half-time of  $\sim 45$  min, and degradation intermediates similar to those illustrated in Fig. 8 were also observed; by contrast, the radiolabeled 95,000- $M_r$  complex incubated in the presence of THP-1 cells saturated with unlabeled DFP-inactivated uPA remained stable in the medium, indicating that binding is required for degradation of the complex (data not shown). Taken together, these results suggest that the uPA/PAI-2 complex binds to THP-1 cells exclusively through the A chain of the enzyme, and that at least the initial steps in the clearance of uPA/PAI-2 complexes do not depend upon interaction of the PAI-2 moiety of the complex with a putative PAI-2 receptor.

## Discussion

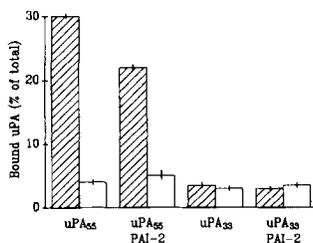
The identification of a plasma membrane binding site specific for uPA has led to the speculation that the enzyme acts



**Figure 9** Cell-associated uPA/PAI-2 complexes are present at the cell surface. THP-1 cells were preincubated with  $^{125}\text{I}$ -uPA for 2 h at  $4^\circ\text{C}$ , washed, and further incubated at  $37^\circ\text{C}$  for 0, 20, and 60 min in the absence (-) or presence (+) of PAI-2 ( $2 \times 10^{-8}$  M). The cells were collected and treated with (+) or without (-) trypsin for 10 min at  $20^\circ\text{C}$ . The reaction was stopped by the addition of FBS (15%) and centrifugation of the cell suspension through an oil cushion. Both cell-associated and free radioactivity were counted; in each condition  $\sim 15\%$  of the radioactivity remained cell-associated after tryptic digestion. The cells were solubilized and analyzed by SDS-PAGE followed by autoradiography of the gel.

primarily while bound at the cell surface, to catalyze spatially restricted generation of plasmin in the context of cell migration and invasion. The uPA receptor may, therefore, play a critical role in controlling localized extracellular proteolysis, and a more detailed understanding of its structure and function is relevant to a variety of physiological and pathological circumstances. The present work has revealed novel aspects of the cell biology of receptor-bound uPA, and thereby provides a sharper view of the probable functions of the receptor.

Using different biochemical and morphological approaches, we have shown that receptor-bound uPA remains exposed at the cell surface with a half-life of 4–5 h. These results extend the observations of Stoppelli et al. (1985) and Bajpai and Baker (1985), who had reported that a large fraction of uPA is accessible to trypsin or acid elution 2–4 h after binding to the cells. The slow endocytosis of uPA is in contrast to the rapid internalization of other ligands of cell surface receptors present on the same cells, for instance insulin. Most importantly, using the property of the active enzyme to react with PAI-2, we have demonstrated that essentially all the  $^{125}\text{I}$ -uPA that remains cell-associated even after long periods of incubation at  $37^\circ\text{C}$  is present in a functional state on the plasma membrane. These observations provide strong support to the notion that the binding of uPA to its receptor can serve to localize enzyme activity to the immediate environment of the cell.



**Figure 10.** Binding of 33,000 and 55,000-M,  $^{125}\text{I}$ -uPA/PAI-2 complexes to THP-1 cells. Cells were incubated in the presence of 33,000 or 55,000-M,  $^{125}\text{I}$ -uPA or of the corresponding uPA/PAI-2 complexes for 2 h at  $4^\circ\text{C}$  without (hatched bars, total binding) or with

(open bars, nonspecific binding) a  $100\times$  molar excess of DFP-treated 55,000-M, unlabeled uPA. The cells were washed by centrifugation through an oil cushion. Both cell-associated and free radioactivity were counted. The mean value and range are indicated.

The rapid redistribution of the uPA receptor in response to a chemotactic signal provides a mechanism to achieve polarized expression of a powerful proteolytic cascade to the leading edge of migrating cells. This allows the highly focused degradation of extracellular matrix components that is likely to be required for the migration of cells within tissues. A number of studies have shown that matrix degradation can occur in a very localized configuration, suggesting that membrane-associated proteases are involved (Chen et al., 1984; Fairbairn et al., 1985; Kramer et al., 1986). Immunohistochemical studies on human fibroblasts and fibrosarcoma cell cultures have demonstrated the presence of uPA at sites of attachment of the cells to the substratum, and its colocalization with the cytoskeletal component vinculin (Pöllänen et al., 1988; Hébert and Baker, 1988). The present work shows that, by binding to its plasma membrane receptor, uPA can be expressed on the cell surface in a controlled configuration, which appears adequate for the directional migration of monocytes towards a chemotactic signal. uPA activity has been shown to be required for the invasion of extracellular matrices by certain malignant cells (Mignatti et al., 1986; Ossowski, 1988a,b); this anarchic behavior could be related to the inappropriate expression of receptor-bound uPA.

It has previously been suggested that receptor-bound uPA may be protected from rapid inhibition by antiproteases (Blasi et al., 1987). Our results clearly show that this is not the case: we observed only a slight decrease in the rate of uPA/PAI-2 complex formation when the enzyme was receptor-bound. Thus, the receptor does not prevent uPA from reacting with either PAI-1 (Cubellis et al., 1989) or PAI-2, and pericellular proteolysis catalyzed by bound uPA is inhibited in the presence of the two PA-specific serpins. A recent paper by Kirchheimer and Remold (1989) has presented results that cannot easily be reconciled with our findings or with those of Cubellis et al. (1989): addition of PAI-1 or PAI-2 did not cause a marked inhibition of exogenous uPA bound to human monocytes; endogenous PAI-2, which is abundant in human monocytes (Wohlwend et al., 1987), may have influenced the results of these experiments, while in our case we have used a monocyte-like cell line selected because it contains and secretes very little endogenous PAI. In any event, these observations should be considered in the broader con-

text of the control of cell surface proteolysis, taking into account the following findings: plasminogen binds to cells that also have uPA receptors (Plow et al., 1986), the binding of plasminogen accelerates the activation of bound pro-uPA and thereby plasmin formation (Ellis et al., 1989), and cell-bound plasmin is protected from inhibition by  $\alpha$ 2-antiplasmin (Plow et al., 1986). Thus, plasmin-catalyzed cell-surface proteolysis may be resistant to inhibition by the major plasma anti-proteases. Regulation of plasminogen-dependent extracellular matrix degradation must therefore rely on the control of uPA synthesis and activity; it is reasonable to envision that PAIs, which are produced, under the influence of hormones and cytokines, by cells that also synthesize uPA and have uPA and plasminogen receptors, could play an important part in modulating the activity of the cell surface uPA/plasmin proteolytic system.

In contrast to the active enzyme and to DFP-inactivated uPA, the uPA/PAI-2 complex, whether formed on the uPA receptor or in solution, is rapidly cleared from the cell surface. This is preceded by an initial cleavage of the cell-associated complex to yield a surface-bound 70,000- $M_r$  degradation intermediate, and is followed by intracellular degradation. Since a complex formed between PAI-2 and 33,000- $M_r$  uPA does not bind directly to the cell surface, and since the 70,000- $M_r$  intermediate is surface-bound, the receptor binding determinants in the amino-terminal region of the A-chain of uPA are probably not removed in this first cleavage. Also, because removal of the carboxy-terminal region of the B chain of uPA (i.e., beyond the active site serine residue) would not suffice to cause a 25,000 D decrease in the apparent relative molecular mass of the complex, it follows that the cleavage(s) must involve the PAI-2 molecule. Alternatively, a change in conformation of the uPA/PAI-2 complex could allow it to remain membrane-bound even following removal of part of the A-chain of uPA. Further studies will be required to decide between these possibilities. Interestingly, clearance of uPA/PAI-2 complexes does not result in a rapid freeing of cell surface uPA receptors: no significant difference in number of receptors available for binding was observed whether the cells had been incubated with or without PAI-2 for 2 h (data not shown). Thus, the receptor could be internalized and degraded concomitantly with the complexes, or the proteolytic cleavage occurring at the cell surface may also affect the receptor and thereby inactivate it. Whatever the precise mechanism for removal of uPA/PAI-2 complexes from the cell surface, the functional consequences of this reaction are the following: (a) the cell surface does not remain encumbered by PAI-2-inhibited uPA; (b) the uPA receptor can serve as a clearance receptor for uPA/PAI-2 complexes, whether these are formed on the cell surface or in solution. Similar results have recently been obtained concerning the fate of uPA/PAI-1 complexes on U937 cells (Cubellis et al., 1990), thus strengthening the proposed role of the uPA receptor in the clearance of serpin-inactivated uPA.

The present studies were performed on cells from the monocyte-macrophage lineage. The colocalization of receptor-bound uPA with the cytoskeleton-associated protein vinculin at cell attachment sites of fibroblasts (Hébert and Baker, 1988) suggests that these cells also can polarize uPA receptors to discrete domains of their plasma membrane. It will be of interest to determine whether the uPA receptors on

cells other than monocytes-macrophages can function in the clearance of uPA/PAI-2 complexes. In any event, together with the previously reported modulations of the number and affinity of uPA receptors (Stoppelli et al., 1985; Estreicher et al., 1989; Picone et al., 1989), our results further illustrate the dynamic nature of uPA/receptor interaction.

A number of possible physiological functions have been proposed for the uPA receptor. Specific binding of the enzyme has been shown to be required for uPA-dependent invasion of the extracellular matrix by malignant cells (Ossowski, 1988b), for uPA-induced chemotaxis of endothelial cells (Fibbi et al., 1988) and for uPA stimulation of cell proliferation (Kirchheimer et al., 1987, 1989). The experiments reported here provide strong support in favor of a role of the uPA receptor in focusing uPA activity to the leading front of migrating cells, and demonstrate that it also acts as a clearance receptor for uPA/PAI-2 complexes. The uPA receptor appears to be a key determinant in the control of uPA-catalyzed proteolysis.

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