Internalization of the Urokinase-Plasminogen Activator Inhibitor Type-1 Complex Is Mediated by the Urokinase Receptor*

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The role of the urokinase receptor (uPAR) in the internalization of the urokinase-plasminogen activator inhibitor type-1 (uPA-PAI-1) complex has been investigated. First, exploiting the specificity of uPA binding, we show that mouse LB6 cells (that express a mouse uPAR) were unable to bind or degrade the human uPA-PAI-1 complex. On the other hand, LB6 clone 19 cells, which express a transfected human uPAR, degraded uPA-PAI-1 complexes with kinetics identical to the human monocytic U937 cells. We also show by immunofluorescence experiments with anti-uPA antibodies that in LB6 clone 19 cells, the uPA-PAI-1 complex is indeed internalized. While at 4°C uPA fluorescence was visible at the cell surface, shift of the temperature to 37°C caused a displacement of the immunoreactivity to the cytoplasmic compartment, with a pattern indicating lysosomal localization. If uPA-PAI-1 internalization/degradation is mediated by uPAR, inhibition of uPA-PAI-1 binding to uPAR should block degradation. Three different treatments, competition with the agonist amino-terminal fragment of uPA, treatment with a monoclonal antibody directed toward the binding domain of uPAR or release of uPAR from the cell surface with phosphatidylinositol-specific phospholipase C completely prevented uPA-PAI-1 degradation. The possibility that a serpin-enzyme complex receptor might be primarily or secondarily involved in the internalization process was excluded since a serpin-enzyme complex peptide failed to inhibit uPA-PAI-1 binding and degradation. Similarly, complexes of PAI-1 with low molecular mass uPA (33 kDa uPA), which lacks the uPAR binding domain, were neither bound nor degraded. Finally we also show that treatment of cells with uPA-PAI-1 complex caused a specific but partial down-regulation of uPAR. A similar result was obtained when PAI-1 was allowed to complex to uPA that had been previously bound to the receptor. The possibility therefore exists that the entire complex uPA-PAI-1-uPAR is internalized.

All these data allow us to conclude that internalization of the uPA-PAI-1 complex is mediated by uPAR.

Plasminogen activators are serine proteases which regulate a highly complex proteolytic cascade, thus playing a fundamental role in a variety of cellular processes which require extracellular proteolysis, including endothelial cell migration, monocytes differentiation, tumor cell invasiveness, and metastasis (1–8). Urokinase plasminogen activator (uPA) is the plasminogen activator that has been most definitely involved in these processes. A very large body of information demonstrates that uPA-mediated plasminogen activation is highly regulated. Regulation occurs not only at the level of synthesis, but also at the levels of enzymatic activity and cell surface location (reviewed in Refs. 2, 9, and 10). The key role of uPA-mediated proteolysis is demonstrated by a series of experiments which have shown that inhibition of uPA activity blocks metastasis or invasion (5, 6, 11, 12).

The cellular location of uPA is dictated by a specific receptor (uPAR) which binds uPA with high affinity at the enzyme’s amino-terminal fragment (ATF), a domain different from that responsible for catalytic activity (13, 14). The importance of the cell surface location of uPA is demonstrated by the block of the invasion of basement membranes and of in vitro extracellular matrix degradation by inhibitors of surface localization of uPA (15–17). The surface localization of plasminogen activation (18) allows a strong, local amplification of the proteolytic signal as surface-bound plasmin is inaccessible to its plasma inhibitors (19), paracrine or autocrine activation of the single chain pro-urokinase (16, 20–24), and regulation of these reactions by uPA-specific inhibitors (25–27).

A very important novel regulatory mechanism has recently been uncovered. While receptor-bound uPA and pro-uPA are not internalized or degraded (13, 14, 18), the enzymatically inactive uPA-PAI complexes are internalized and degraded (28–30). Thus cells are able to regulate not only the amount but also the location of the cell surface uPA activity. This mechanism would thus explain an essential element in directional proteolysis, where the location of uPA activity marks the direction of cellular migration and invasion (31).

Available data suggest but do not demonstrate that uPAR is essential in uPA-PAI internalization/degradation. This is a central question that requires a direct answer. We now report experiments which demonstrate the direct and primary

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1 The abbreviations used are: uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; PAI-1 and PAI-2, plasminogen activator inhibitor type 1 and 2, respectively; ATF, amino-terminal fragment of uPA; SEC, serpin-enzyme complex; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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involvement of uPAR in the removal of inactive uPA-PAI-1 complexes from the cell surface. The data also show that uPAR undergoes down-regulation from the cell surface.

**EXPERIMENTAL PROCEDURES**

**Materials**—Two-chain uPA and low molecular mass uPA (33 kDa uPA), the catalytically active carboxyl-terminal fragment of uPA, were obtained from Leptep SpA, courtesy of Dr. M. L. Noll; ATF, the amino-terminal fragment of uPA (residues 1-144) containing the receptor binding site (14) was obtained from Abbott Laboratories, courtesy of Dr. Jack Henkin. Active PAI-1 was purified from serum-free conditioned medium of HepG2 cells by affinity chromatography on immobilized anhydrourokinase as described (32). Phosphatidylinositol-specific phospholipase C from *Bacillus cereus* was from Boehringer Mannheim GmbH; benzamidine-Sepharose 6B was from Pharmacia (Uppsala, Sweden); dimethylphenyl polyisoxazoline was from Sigma. Synthetic peptides SEC395-374 and PAI 347-362 were synthesized according to standard techniques. The sequence of the SEC peptide (SIPPEFKNPKPFVFLM) was chosen according to published data on the α1-antitrypsin peptide sequences blocking the SEC receptor (33, 34); PAI-1 peptide (MAPPEEMDRPELPEV) contains the PAI-1 sequence corresponding to the SEC peptide. Monoclonal anti-uPAR antibodies R3 and R4 have been described. Antibody R3 blocks ligand binding, while R4 does not (35).

**Cells and Cell Culture**—Human monocyte-like U937 cells were grown at 37 °C in Eagle's medium supplemented with 10% newborn calf serum and 2 mM glutamine; they were maintained and harvested at densities of 3-6 × 10⁶ cells/ml. Mouse LB6 and LB6 clone 19 cells, a clone expressing recombinant human uPAR (36), were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 2 mM glutamine, and 10 IU/ml penicillin and streptomycin.

**Iodinations**—15 or 20 mg of protein (ATF or uPA) were iodinated with 1 mCi of Na¹²⁵I (Amersham Corp.) and 12 mg of Iodogen (Pierce Chemical Co.) for 1 h at room temperature. The reaction was stopped by the addition of 0.1 M Tris, pH 8, 0.1% CHAPS, and excess N-acetic anhydride through a G-25 PM PD-10 column (Pharmacia). Specific activities of the proteins ranged between 2.5 and 7.5 × 10⁶ cpm/mmol. Iodinated uPA retaining enzymatic activity was purified by affinity chromatography on benzamidine-Sepharose 6B (37).

**Formation of uPA-PAI-1 Complexes**—uPA-PAI-1 complex was formed by combining uPA and a 20-fold molar excess of PAI-1 at room temperature for 1 h, and its formation was checked by SDS-PAGE as previously described (25). SDS-PAGE analysis of complex binding, internalization, and degradation was carried out as previously described (28).

**Phosphatidylinositol-specific Phospholipase C Treatment of Cells**—LB6 clone 19 cells were washed (see above) and resuspended in RPMI-1640 binding buffer (see below) and treated with phosphatidylinositol-specific phospholipase C (3 units/ml) at 37 °C for 1 h (38).

**Determination of uPA Number by ¹²⁵I-ATF Binding**—The assay was done on ice unless indicated. Typically 2 × 10⁵ U937 cells (10⁶ cells/ml) were centrifuged and the supernatants removed. Cell pellets were loosened and then subjected to acidic conditions (50 mM glycine-HCl, 100 mM NaCl, pH 3) for 3 min. The mixture was then neutralized by the addition of 0.5 volume of 500 mM HEPES, 100 mM NaCl, pH 7. Cells were centrifuged, resuspended in 200 μl of RPMI binding buffer (RPMI-1640 containing 25 mM HEPES, pH 7.4, 2 mM glutamine, and 1 mg/ml bovine serum albumin). Cell number was determined from aliquots of cell samples using a Coulter counter. A saturation mixture of ¹²⁵I-ATF and unlabeled ATF (final concentration 7 nm, specific activity 8-9 × 10⁶ cpm/mg) was added, and samples were incubated on ice for at least 60 min. Nonspecific binding was determined by the addition of 50 nM unlabeled ATF prior to the addition of the saturation mixture. Following this incubation, cells were pelleted by centrifugation (500 × g), resuspended in 100 μl of binding buffer, layered over 85% dimethyl-diphenyl siloxane and centrifuged at room temperature for 15 min at 10-12,000 rpm in an Eppendorf 5415 centrifuge. The upper, aqueous layer containing unbound ligands was removed, and the cell associated radioactivity was counted in an LKB mini-γ counter. Specific binding was determined by subtracting nonspecific counts, and data were calculated based on cell number determinations. In the case of LB6 clone 19 cells, the assay was carried out under these same conditions of ligand excess following the procedure outlined below (see “Degradation Assay”).

**Degradation Assay**—Prior to the assay, U937 cells were acid-washed (see above) and resuspended in RPMI-1640 binding buffer to a density of 5 × 10⁶ cells/ml. LB6 clone 19 cells were plated at a density of 5 × 10⁶ cells/well in 24-well culture dishes 16-18 h prior to the assay. Tissue culture medium was replaced with Dulbecco's modified Eagle's medium containing 25 mM HEPES, pH 7.4, 1 mg/ml bovine serum albumin, 2 mM glutamine, and 10 IU/ml penicillin/streptomycin and cells were transferred to ice. In a typical experiment approximately 0.2 nm (specific activity 10-20 μCi/μg) ¹²⁵I-uPA or ¹²⁵I-uPA-PAI-1 (see “Materials”) were added and allowed to bind for 60 min on ice. When saturation of uPAR was required, a 10 nm concentration of ligand was employed. Free ligand was removed, and cells were washed and then reincubated in fresh binding buffer with no ligand. Appropriate samples were then incubated at 37 °C for different lengths of time. Supernatants were then collected, and cells were subjected to acid wash (see above) and then lysed in 1 N NaOH. Aliquots of supernatants were precipitated with ice-cold 5% trichloroacetic acid for 15 min on ice; precipitates were recovered on glass filters and washed once with ice-cold 5% trichloroacetic acid. Radioactivity associated with cell lysates, acid washes, supernatant aliquots, and corresponding trichloroacetic acid precipitates were counted. Trichloroacetic acid-soluble cpm were determined by subtracting trichloroacetic acid-precipitable counts from total supernatant radioactivity.

**Immunofluorescence**—LB6 clone 19 cells were preincubated with either uPA (10 nm), ATF (10 nm), or preformed uPA-PAI-1 (1 nm uPA, 20 nm PAI-1, see “Materials”) complex at 4 °C for 1 h. Cells were rinsed three times with serum-free culture medium and transferred to 37 °C. Cells were fixed with 4% paraformaldehyde after 0, 30, or 60 min of incubation at 37 °C, permeabilized with 0.2% saponin (Sigma) in phosphate-buffered saline, and then subjected to indirect immunofluorescence labeling with polyclonal anti-uPA antibodies as previously described (31).

**RESULTS**

**Binding to uPAR Is the First Step in uPA-PAI-1 Degradation**—A fundamental question regarding the internalization/degradation of the uPA-PAI-1 complex is whether it is mediated by uPAR. To answer this question, we first tested whether this property could be provided to a mouse cell line by introduction of a human uPAR receptor cDNA. As previously shown, parental LB6 cells did not bind human uPA or uPA-PAI-1 complex at all, while LB6 clone 19 cells, which express the human uPAR, were able to bind. Degradation of the uPA-PAI-1 complex, and not of free uPA, only occurred in the LB6 clone 19 cells indicating that the expression of a surface uPAR is required for internalization/degradation of the uPA-PAI-1 complex (data not shown). We next compared the time course and extent of uPA-PAI-1 degradation between LB6 clone 19 cells previously unincubated human U937 cells, which express a natural uPAR. Both cells were therefore incubated with ¹²⁵I-uPA-PAI-1 complex for 90 min at 4 °C, then washed with cold buffer and incubated for various periods of time at 37 °C. As shown in Fig. 1, with both cell lines a consistent fraction of the bound radioactivity disappeared from the surface (i.e. could not be solubilized by an acid wash), was concentrated within the cells at about 30-60 min, and was later recovered in the cell supernatant in a chloroacetic acid-soluble form. The shape of the various curves in the two cell lines is similar, indicating that the LB6 clone 19 cells internalize and degrade uPA-PAI-1 complexes in a way similar to U937 cells. Actually the extent of degradation was higher and the time zero background lower in LB6 clone 19 cells.

We have previously demonstrated by SDS-PAGE analysis in U937 cells that the uPA-PAI-1 complex is not degraded prior to binding and internalization (28). We have repeated this test for LB6 clone 19 cells and verified with these cells an identical behavior. An ~100-kDa uPA-PAI-1 complex binds to LB6 clone 19 cells and is internalized as such.
were incubated with \(^{125}\text{I}-\text{uPA.PAI-1}\) on ice for 60 min, washed, and binding was determined by competition with 200 nM uPA and subtracted. Results are the average of two independent experiments in duplicate (less than 10% variation). Open circles, acid-washed ligand; solid circles, cell-associated ligand remaining after acid washing; squares, trichloroacetic acid-soluble ligand in the supernatant. Panel A, LB6 clone 19 cells; panel B, U937 cells.

![Fig. 1. Time course of internalization and degradation of the uPA-PAI-1 complex in LB6 clone 19 and U937 cells.](image)

of the 100-kDa band followed internalization (data not shown).

The internalization of uPA-PAI-1 in LB6 clone 19 cells has been tested by immunofluorescence, using anti-uPA antibodies. Positive staining on the cell surface was observed after incubation of the cells with uPA-PAI-1 at 4 °C (Fig. 2a). 30 and 60 min after raising the temperature to 37 °C (Fig. 2, b and c), the pattern of immunofluorescence shifted from a cell surface-type to the interior of the cell in a punctate form, reminiscent of lysosomal localization. The internalization and the signal were specific as they were not observed with cells incubated with free uPA only or without ligand, respectively (data not shown).

We then tested whether inhibition of binding to uPAR causes inhibition of degradation. Inhibition has been accomplished in three ways: 1) competition with the non-internalized ATF of uPA, 2) incubation with anti-uPAR antibodies, and 3) treatment of the cells with phosphatidylinositol-specific phospholipase C.

ATF is neither internalized nor degraded despite its ability to bind uPAR (28). ATF and uPA competed equally well with the uPA-PAI-1 complex in binding experiments carried out at 4 °C with human U937 and LB6 clone 19 cells (data not shown). In an internalization-degradation experiment, in which after binding at 4 °C the temperature was raised to 37 °C for 2 h, about 60% of the ligand was degraded. The presence of ATF, however, drastically inhibited both binding and degradation of the uPA-PAI-1 complex (Table I). It is important to note that excess unlabeled ATF was present during the 37 °C incubation. These data indicate that degradation requires binding to uPAR and not to another receptor specific for the uPA-PAI-1 complex.

Additional support to this conclusion was obtained with two well characterized anti-uPAR monoclonal antibodies. Antibody R3 recognizes the 1–87 amino-terminal, ligand-binding fragment of uPAR and is able to block ligand binding. Antibody R4 recognizes the carboxy-terminal 88–282 fragment and does not interfere with binding (35). As shown in Table I, in both U937 cells and LB6 clone 19 cells antibody R3 blocked both binding and degradation of uPA-PAI-1; antibody R4, on the other hand, had little or no effect on either parameter. Treatment of cells with phosphatidylinositol-specific phospholipase C removes uPAR from the cell surface (38). As shown in Table I, \(B.\) cereus phosphatidylinositol-specific phospholipase C removed greater than 95% of uPA-PAI-1 binding sites from LB6 clone 19 cells. This treatment also inhibited degradation of the uPA-PAI-1 complex by the same extent. Taken together, the results of Table I show that the uPA-PAI-1 complex binds to the uPA receptor of U937 and LB6 clone 19 cells and that this binding is essential for the subsequent internalization and degradation of the uPA-PAI-1 complex. This is also substantiated by experiments in which iodinated 33-kDa uPA-PAI-1 complexes were used. In fact, PAI-1 also forms an SDS-stable complex with the carboxy-terminal moiety of uPA (33-kDa uPA), a molecule that

**Table I**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Effector*</th>
<th>Binding*</th>
<th>Degradation*</th>
</tr>
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<tbody>
<tr>
<td>U937</td>
<td>ATF (^d)</td>
<td>100%</td>
<td>60%</td>
</tr>
<tr>
<td>U937</td>
<td>+ATF</td>
<td>&lt;10%</td>
<td>15%</td>
</tr>
<tr>
<td>U937</td>
<td>-mAb (^e)</td>
<td>100%</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>U937</td>
<td>+R3</td>
<td>&lt;5%</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>U937</td>
<td>+R4</td>
<td>90%</td>
<td>52%</td>
</tr>
<tr>
<td>LB6 CLONE 19</td>
<td>-mAb (^f)</td>
<td>100%</td>
<td>63%</td>
</tr>
<tr>
<td>LB6 CLONE 19</td>
<td>+R3</td>
<td>22%</td>
<td>9%</td>
</tr>
<tr>
<td>LB6 CLONE 19</td>
<td>+R4</td>
<td>&gt;95%</td>
<td>55%</td>
</tr>
<tr>
<td>LB6 CLONE 19</td>
<td>+PI-PLC (^g)</td>
<td>100%</td>
<td>61%</td>
</tr>
<tr>
<td>LB6 CLONE 19</td>
<td>+PI-PLC (^h)</td>
<td>&lt;5%</td>
<td>&lt;5%</td>
</tr>
</tbody>
</table>

* In all cases, addition of the effector preceded that of the ligand.

* Percentages of \(^{125}\text{I}-\text{uPA-PAI-1}\) binding are relative to that bound in the absence of effector (100%). In all cases nonspecific binding was estimated by competition with 250 nM uPA and subtracted. About 8,000 cpm (±20%) were bound at 4 °C in the different experiments, with a nonspecific binding of about 300 cpm.

* Degradation was estimated by measuring trichloroacetic acid-soluble radioactivity as described under "Experimental Procedures" after 2 h at 37 °C and is expressed in percent of the ligand bound at 4 °C.

* 200 nM ATF. In the degradation assay, the samples are shifted to 37 °C without the removal of excess, unbound ligands. Values are the average of two independent experiments with less than 10% variation.

* Monoclonal antibodies specific for the human uPAR. Culture media of the hybridoma cells were diluted 13-fold and preincubated with the cells for 60 min on ice before the start of the experiment. Values are the average of two independent experiments with less than 10% variation.

* As in footnote e except that culture media of the hybridoma cells were diluted 3.3-fold.

* Phosphatidylinositol-specific phospholipase C treatment was carried out with 1.7 units/ml for 15 min at 37 °C prior to the start of the experiment.

**Fig. 2. Immunofluorescence view of uPA-PAI-1 internalization.** LB6 clone 19 cells were preincubated with uPA-PAI-1 complex at 4 °C, washed, and then shifted to 37 °C for 0 (panel a), 30 (panel b), or 60 min (panel c), fixed, and permeabilized. Cells were then probed with anti-uPA antibodies followed by a fluorescein isothiocyanate-conjugated secondary antibody. Negative controls (not shown), included the omission of the specific antibody and the omission of the binding of cells to uPA-PAI-1 and showed no staining.
cannot bind to uPAR (13, 14). However, 125I-33 kDa uPA-PAI-1 complex neither bound nor was degraded by either U937 or LB6 clone 19 cells (data not shown) further demonstrating that the receptor-binding amino-terminal fragment of uPA is required for the internalization and degradation of the uPA-PAI-1 complex.

We next considered the possibility that uPAR might deliver the uPA-PAI-1 complex to another surface molecule responsible for the translocation of the complex across the membrane. A candidate secondary receptor molecule is the serpin-protease complex (SEC) receptor that internalizes and degrades the α1-antitrypsin-elastase and α1-antitrypsin-trypsin complexes (33, 34). This effect can be competed for by a synthetic peptide (the 105-Y peptide) reproducing a specific sequence from α1-antitrypsin (33, 34). We have tested an essentially identical peptide, here termed SEC peptide, as well as a peptide derived from the homologous sequence in PAI-1, as competitors of binding and degradation of uPA-PAI-1 complexes in U937 and LB6 clone 19 cells (both at a 5,000-fold excess, 10 μM). Neither the SEC nor the PAI-1 peptide had any effect on uPA-PAI-1 binding or degradation in either cell line, strongly suggesting that an SEC family receptor is not involved in the internalization and degradation of the uPA-PAI-1 complex (data not shown). It must be noted that the same preparation of the SEC and PAI-1 peptides used in internalization and degradation in either cell line, strongly suggesting that an SEC family receptor is not involved in the internalization and degradation of the uPA-PAI-1 complex (data not shown).

PAI-1 Induces a Moderate Down-regulation of uPAR from the Surface of U937 Cells—We next tested whether internalization of the uPA-PAI-1 complex would result in a variation of uPAR number. Excess preformed uPA-PAI-1 complexes or free uPA were incubated with U937 or LB6 clone 19 cells at 37°C for 0–120 min. The cells were transferred to ice and acid-treated to dissociate bound ligand, and the number of receptors titrated with a 125I-ATF binding assay (see "Experimental Procedures"). As shown in Fig. 3, when cells were incubated with preformed uPA-PAI-1 complex, a time-dependent reduction in uPAR number (37% after 2 h at 37°C) was observed. Therefore, degradation of the uPA-PAI-1 complex was accompanied by a down-regulation of uPAR. Incubation with free uPA resulted in a slight reduction of uPAR number (92% remaining after 2 h at 37°C). No reduction was observed after 2 h at 4°C. A low level degradation of exogenous free uPA has been previously shown to depend on the presence of endogenous uPA inhibitors produced by the cells (28). In conclusion, with both cell lines the data show a moderate uPA-PAI-1-dependent down-regulation of uPAR, which indicates that uPAR may be internalized subsequent to uPA-PAI-1 complex binding.

In a separate set of experiments, we tested whether uPAR down-regulation could be observed by allowing the uPA-PAI-1 complex to form on the cell surface. U937 cells were incubated with saturating (10 nM) concentrations of uPA at 4°C for 90 min in the presence of excess 33 kDa uPA to scavenge any endogenous inhibitor. A 17-fold molar excess of PAI-1 was then supplemented and the temperature raised to 37°C. Control were either PAI-1-supplemented cells kept at 4°C or cells shifted to 37°C without addition of PAI-1. After 3 h at 37°C, the cells were acid-washed to remove receptor-bound ligand and the number of titratable uPAR/cell determined using 125I-ATF binding under conditions of excess of ligand. The results are shown in Table II. A 35% reduction of the number of uPAR/cell was observed after 3 h at 37°C; the effect was totally dependent on the addition of PAI-1 and was almost undetectable if cells were kept at 4°C. As expected

D. Perlmutter, personal communication.

FIG. 3. Time-, temperature-, and PAI-1-dependent down-regulation of uPAR in U937 and LB6 clone 19 cells. Cells were incubated at 37°C with a saturating concentration of uPA-PAI-1 (10 nM) for the indicated times and transferred to ice, and the number of uPAR was determined by the 125I-ATF assay and expressed in percent of maximum 125I-ATF binding. The data with U937 cells represent the average of six independent experiments conducted in duplicate with less than 10% variation. Solid triangles, U937 cells, uPA-PAI-1, 37°C; open triangles, U937 cells, uPA-PAI-1, 4°C; open circles, U937 cells, free uPA, 37°C; solid rectangles, LB6 clone 19 cells, uPA-PAI-1, 37°C.

**TABLE II**

<table>
<thead>
<tr>
<th>Incubation temperature</th>
<th>uPAR Number (%)</th>
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<tr>
<td>4°C</td>
<td>100%</td>
</tr>
<tr>
<td>37°C</td>
<td>35%</td>
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The presence of excess 33 kDa uPA completely suppressed background down-regulation in the absence of PAI-1 at 37°C. In conclusion, PAI-1-specific and temperature-dependent reduction in the number of available uPARs was observed during internalization and degradation of the uPA-PAI-1 complex.

**DISCUSSION**

It has been previously demonstrated that uPA can be internalized and degraded only when complexed with PAI-1 or PAI-2 (28–30). We have now analyzed directly the involvement of uPAR in the internalization and degradation of the uPA-PAI-1 complex. We have addressed two main questions: the requirement of uPAR in the process of internalization-
degradation of the uPA-PAI-1 complex, and the fate of ligand and receptor subsequent to surface interaction.

The results reported in this paper show that binding of uPA-PAI-1 to uPAR is an absolute prerequisite for internalization and degradation of the complex. In fact: 1) this property was acquired by mouse cells coincidentally with the expression of the human uPAR (Fig. 1). 2) Inhibition of binding by three different approaches inhibited degradation (Fig. 3, Table I). These results, therefore, show beyond any doubt the primary role of uPAR in the internalization of the uPA-PAI-1 complex. In addition, the data of Fig. 1 also show that mouse LB6 cells, which on their own do not bind nor internalize the uPA-PAI-1 complex, acquire this property upon expression of the recombinant human uPAR. Therefore, either uPAR contains all the determinants required for the internalization process, or, if other proteins are also involved, sufficient inter-species conservation exists in the cellular machinery responsible for the internalization of these proteins.

Receptors that would recognize the uPA-PAI-1 complex through regions different from the uPAR-binding aminoterminal fragment also do not appear to be involved. In fact, the 33 kDa uPA-PAI-1 complex could not be bound nor internalized. As previously shown, excess 33 kDa uPA eliminates any background internalization independent of exogenous PAI-1 (Table II, and Ref. 28). Also the role of the SEC receptor (33, 34) has been excluded since the SEC and the PAI peptides, which do compete for binding to the SEC receptor, had no effect on uPA-PAI-1 binding or degradation.

Using LB6 clone 19 cells, we have followed the fate of the uPA-PAI-1 complex by immunofluorescence using anti-uPA antibodies (Fig. 2). In agreement with previous data showing lysosomal involvement in uPA-PAI-1 degradation (28), we noticed transfer of the fluorescent immunoreactivity from the cell surface to the cytoplasm with a distribution reminiscent of lysosomal accumulation. This also agrees with the presence of the uPA-PAI-1 complex within lysosomal particles in other cell types (30).

The fate of uPAR after surface binding of the ligand has also been addressed. Degradation of a preformed uPA-PAI-1 complex was accompanied by a low-level decrease of available uPAR from the surface of the U937 cells (35% in 2 h at 37 °C), which was PAI-1- and temperature-dependent. This effect was also observed if the uPA-PAI-1 complex was allowed to form on the cell surface, i.e. upon addition of PAI-1 to receptor-bound uPA (Table II). The down-regulation of uPAR suggests that the receptor itself is internalized. However, accurate kinetic and molecular analyses need to be performed to prove this point. In conclusion, this paper brings definitive evidence that internalization of the uPA-PAI-1 complex is indeed mediated by the uPAR.

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REFERENCES