THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 8, Issue of February 25, pp. 5770-5775, 1994
Printed in U.S.A.

The Role of Plasminogen Activator Inhibitor Type 1 in the Clearance of Tissue-type Plasminogen Activator by Rat Hepatoma Cells*

(Received for publication, July 6, 1993, and in revised form, October 19, 1993)

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The role of plasminogen activator inhibitor type 1 (PAI-1) in the clearance of tissue-type plasminogen activator (t-PA) by hepatocyte-like cells was studied. Rat (Novikoff) hepatoma cells were able to bind and degrade t-PA in a PAI-1 independent fashion, but PAI-1 markedly increased the rate of degradation and t-PA/PAI-1 was a more efficient inhibitor of ^{125}I-t-PA or of ^{125}I-t-PA/PAI-1 degradation than free t-PA. Competition studies revealed that the effect of PAI-1 is unlikely to involve determinants located on the PAI-1 part of the complex: 1) an excess of free PAI had no effect on the rate of degradation of ^{125}I-t-PA/PAI-1. 2) Complexes of PAI-1 with urokinase-type PA or with a t-PA mutant lacking the finger and growth factor domains were unable to compete for the binding and degradation of free or PAI-1-complexed ^{125}I-t-PA. 3) t-PA KHRR296-299AAAA, a mutant which reacts 2 orders of magnitude slower with PAI-1 than wild type t-PA, behaved similar to wild type t-PA.

The clearance via both the PAI-1-dependent and the PAI-1-independent mechanisms was inhibited by the receptor-associated protein, a general inhibitor of clearance mediated by the LDL receptor-related protein.

We conclude that t-PA can be cleared by rat hepatoma cells in a PAI-1 independent fashion, but after complex formation with PAI-1, binding of t-PA to the cells is increased and clearance accelerated. Both mechanisms seem to involve the same receptor.

Tissue-type plasminogen activator (t-PA),[1] an endogenous serine protease plays a key role in the removal of incipient thrombi and is widely used for the thrombolytic treatment of acute myocardial infarction. Rapid hepatic clearance of t-PA is one of the principal mechanisms by which blood concentrations of t-PA are regulated and involves several distinct mechanisms. Clearance by liver endothelial cells and Kupffer cells involves the mannose receptor (1–4), whereas hepatocytes contain a distinct receptor. Clearance via this receptor was originally reported to depend on previous complex formation of t-PA with plasminogen activator inhibitor type 1 (PAI-1) (5, 6). Furthermore, in a rat liver perfusion system clearance of t-PA/PAI-1 was reported to be faster than that of free t-PA (7). However, a PAI-1-independent clearance of t-PA was observed in rat hepatoma cells (8, 9).

Recently an integral membrane protein homologous to the low-density lipoprotein receptor was identified. The *L*DL *r*eceptor-related *p*rotein (LRP) is a multidomain protein of two subunits of 515 and 85 kDa (10), which mediates the binding, internalization, and degradation of a variety of ligands, including complexes of α_2-macroglobulin with proteases (11, 12) and apolipoprotein E-enriched β-migrating very low density lipoprotein (13). Recent evidence suggests that the LRP also mediates the catabolism of t-PA, but the role of PAI-1 in this process is uncertain: whereas Bu *et al.* (14) observed that t-PA bound in a PAI-1 independent fashion to a LRP-like protein on rat MH_1C_1 hepatoma cells, Orth *et al.* (6) observed that clearance via the LRP on COS cells was dependent on complex formation of t-PA with PAI-1. The LRP may, furthermore, influence the PA system by mediating the clearance of u-PA/PAI-1 (15, 16). Another member of the LDL receptor family, gp330 is normally expressed by epithelial cells but not in the liver (17) and may bind several ligands of the LRP, including t-PA/PAI-1 complexes (18). A 39-kDa protein, the receptor-associated protein (RAP), copurifies with LRP (19, 20) and interferes with the binding and uptake of all known LRP ligands (18, 21–23). It also binds gp330 (24), but not the LDL receptor (21).

The role of PAI-1 in the clearance of t-PA by hepatocyte-like cells is still uncertain. To determine the effect of PAI-1 on t-PA clearance and assess the contribution of determinants on t-PA or PAI-1, we have made a detailed and comparative analysis of the binding, internalization, and degradation of free and PAI-1 complexed t-PA and of t-PA mutants deficient in hepatic clearance or in their reaction with PAI-1. Results obtained with rat hepatoma cells suggest: 1) that these cells are able to clear t-PA in a PAI-1 independent fashion; 2) that binding and clearance are accelerated by PAI-1, but that the PAI-1 part of the complex does not mediate this enhanced binding; and 3) that both PAI-1 dependent and independent clearance are inhibited by RAP, suggesting that in these hepatic cells the LRP mediates both processes.

EXPERIMENTAL PROCEDURES

Materials—Recombinant t-PA (Actilyse®) was provided by Dr. J. Krause (Dr. K. Thomae GmbH, Biberach an der Riss, Federal Republic of Germany); active urokinase of 55 kDa (u-PA) by Serono (Coinsins, Switzerland); the t-PA variant KHRR296-299AAAA by Dr. W. Bennett (Genentech, Inc., San Francisco, CA); recombinant RAP by Dr. D. Strickland (The J. H. Holland Laboratory, Rockville, MD), and PAI-1 by Dr. T. Reilly (DuPont Merck, Wilmington, DE). PAI-1 was over 90% active as judged from its specific activity. For some experiments it was converted into a mainly latent form by 6 h incubation at 37 °C. Bovine serum albumin (BSA) was obtained from Fluka (Buchs, Switzerland); Na^{125}I from Amersham (Amersham, United Kingdom); cycloheximide

* This work was supported by a grant from Dr. Karl Thomae Laboratories, Biberach, FRG, a grant from the Swiss National Fund for Scientific Research (32–29034.90), and by the Foundation for Research on Atherosclerosis and Thrombosis. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: t-PA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; LRP, low density lipoprotein receptor-related protein; u-PA, urokinase-type PA; RAP, receptor-associated protein; BSA, bovine serum albumin.

from Serva (Heidelberg, FRG); aprotinin (Trasylol®) from Bayer (Leverkusen, FRG); all reagents for SDS-polyacrylamide gel electrophoresis and prestained M_r standard proteins from Bio-Rad. All other biochemicals were of analytical grade. Phosphate-buffered saline was composed of: NaCl (8 g/liter), KCl (0.2 g/liter), $NaH_2PO_4 \cdot 2\ H_2O$ (1.44 g/liter), KH_2PO_4 (0.2 g/liter), pH 7.4.

Cell Culture—Rat Novikoff hepatoma cells (American Type Culture Collection, CRL 1604) were grown in suspension culture in 150-cm^2 tissue culture bottles (Corning Glass Works) in RPMI 1640 (Seromed, Biochrom KG, Berlin, FRG), supplemented with 10% fetal calf serum (Life Technolgoies Inc., Paisley, UK). Chinese hamster ovary cells were grown in adherent culture in α-Dulbecco's modified Eagle's medium (Life Technologies Inc.) supplemented with 10% fetal calf serum and 200 μg/ml Geneticin® (G-418 Sulfate, Life Technologies Inc.).

Production and Purification of a t-PA Mutant Lacking the Finger and Growth Factor Domains—Chinese hamster ovary cells, stably expressing a t-PA mutant lacking the finger and growth factor domains (t-PAΔFG) (25) were grown to confluency and, after medium change, maintained for 2 days on serum-free Optimem medium (Life Technologies Inc.) containing 20 KIU/ml aprotinin. This conditioned medium was concentrated 10 times in a Millipore membrane concentrator. Concentrate (150 ml) was incubated for 90 min at 25 °C with 10 mg of immobilized monoclonal anti-t-PA antibody (PAM-2, Biopool, Umeå, Sweden) and the gel washed with phosphate-buffered saline, 0.01% Tween 80. The t-PA mutant was eluted with 5 ml of 50 mM Tris, pH 11, and immediately brought to pH 8.5 with 0.2 M L-arginin, 0.110 M phosphoric acid, 0.01% Tween 80. Electrophoretic analysis on a 7.5% gel (26) revealed a single closely spaced doublet at 56 kDa.

Preparation and Purification of Complexes of PAI-1 with t-PA and t-PAΔFG—The PAs (final concentration 1 μM) were incubated with PAI-1 (1.1 μM) for 1 h at 37 °C and then immunopurified as described above. Electrophoretic analysis revealed a single band of 110 kDa for t-PA/PAI-1 or 100 kDa for t-PAΔFG/PAI-1. Concentrations of the free and PAI-1 bound forms of t-PA were determined by an enzyme-linked immunosorbent assay technique (Tintelize t-PA, Biopool) that measures these forms with equal efficacy. Complexes of labeled PAs with PAI-1 were prepared by incubation of ^{125}I-PAs (at 10 nM) with unlabeled PAI-1 (15 nM).

Production of the RAP-Glutathione Transferase Fusion Protein—The pGEX39-kDa plasmid for the expression of a RAP-glutathione transferase fusion protein (21), kindly provided by Dr. J. Herz (University of Texas, Dallas, TX), was transformed into DH5α bacteria. An overnight culture of plasmid containing bacteria was diluted 1:10 in fresh LB medium and the bacteria grown to mid-log phase (A_{600} = 0.6–1.0). Expression of fusion proteins was induced by addition of isopropylthio-β-D-galactoside to 1 mM. Then, the bacteria were grown for another 4–5 h at 37 °C. Following lysis of the bacteria, the RAP-glutathione transferase fusion protein was purified on a glutathione-Sepharose column (Pharmacia, Uppsala, Sweden) as described by Herz *et al.* (21).

Labeling of Proteins—t-PA and t-PA KHRR296-299AAAA were iodinated using the IODO-GEN method (27) to specific activities of 3–4 μCi/μg.

Binding Assays—Novikoff cells were placed in fresh medium 24 h prior to performing the binding assays. The cells were collected, washed twice with cold Krebs-Henseleit buffer (Krebs buffer: 118 mM NaCl, 5 mM KCl, 1.1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NHCO_3$, pH 7.4) and resuspended to a density of 4×10^6 cells/ml in Krebs buffer containing 1% bovine serum albumin and 0.1% D-glucose (Krebs/BSA). Cell viability was greater than 98% as assessed by trypan blue exclusion. Cells were incubated at 37 °C in Eppendorf tubes with 10,000 cpm of radiolabeled protein (0.06 nM) per million cells, in the presence or absence of competitors. At timed intervals aliquots were taken, the cells centrifuged (2 min, 10,000 × *g* in a microcentrifuge), and washed once with 1 ml of cold buffer.

Internalization and Degradation of Ligands by Rat Hepatoma Cells—The cells (4×10^6 cells/ml) were incubated with 0.06 nM radiolabeled ligand in the presence or absence of competitors. At timed intervals aliquots were withdrawn and the cells centrifuged. To quantify labeled ligand degradation products, trichloroacetic acid was added to the supernatant to a final concentration of 10%. Then, the mixture was incubated for 30 min at 4 °C, centrifuged for 10 min at 10,000 × *g*, and trichloroacetic acid-soluble radioactivity determined.

Determination of Residual Binding Capacity of the Receptor after Having Bound and Degraded a PA-Inhibitor Complex—Cells (4×10^6 cells/ml) were incubated at 4 °C for 2 h with 50 nM t-PA/PAI-1 complex and further incubated at 37 °C to allow internalization of ligand. At timed intervals, 500-μl aliquots were drawn, centrifuged for 3 min at 150 × *g*, and the cells incubated for 3 min in 200 μl of 50 mM glycine-HCl, 100 mM NaCl, pH 3.0, for 3 min at room temperature to remove reversibly bound t-PA/PAI-1 and neutralized with 1:5 volume of 0.5 M HEPES, pH 7.4. The cells were washed twice with 125 mM HEPES, pH 7.4, and resuspended in Krebs buffer to a density of 4×10^6 cells/ml. Labeled t-PA (0.06 nM) was added and after 30 min cell-associated radioactivity counted. Cycloheximide (250 μg/ml) was present throughout the experiment to block protein synthesis.

RESULTS

Effect of PAI-1 on the Binding, Internalization, and Degradation of ^{125}I-t-PA by Rat Hepatoma Cells—The binding of recombinant t-PA and of t-PA complexed with PAI-1 to Novikoff rat hepatoma cells was studied at 37 °C. Binding of ^{125}I-t-PA to the cells increased with time, reached a plateau by 30 min, and remained constant for at least 4 h. Cell-associated radioactivity was reduced by ~60% in the presence of 100 nM unlabeled t-PA (Fig. 1*A*). Binding of ^{125}I-t-PA/PAI-1 was approximately 2-fold higher than binding of ^{125}I-t-PA and reached a maximum after 30 min. Thereafter, cell-associated radioactivity began to decrease. Binding was reduced by ~60% in the presence of 100 nM unlabeled t-PA/PAI-1 (Fig. 1*B*). Binding of labeled ligands was followed by the appearance of degradation products in the cell supernatant. The initial rate of degradation of ^{125}I-t-PA/PAI-1 was significantly faster than that of free ^{125}I-t-PA. However, after 90 min, the rate of degradation of t-PA/PAI-1 began to decrease. Addition of 100 nM unlabeled t-PA and t-PA/PAI-1, respectively, strongly reduced the appearance of trichloroacetic acid-soluble radioactivity in the supernatant (Fig. 1, *C* and *D*).

To ascertain whether the higher degree of binding and the faster rate of degradation of t-PA/PAI-1 was due to the PAI-1 complexed state of t-PA, we first incubated ^{125}I-t-PA with Novikoff cells for 30 min and then added an excess of PAI-1 (100 nM). A higher degree of binding (not shown) and an increased rate of degradation of ^{125}I-t-PA was observed (Fig. 1*C*). Electrophoretic analysis of samples collected at 1 h confirmed that ^{125}I-t-PA was fully complexed to PAI-1 (data not shown).

To investigate the extent to which t-PA and t-PA/PAI-1 share common binding sites we performed cross-competition experiments. t-PA/PAI-1 inhibited ^{125}I-t-PA degradation with the same efficacy as uncomplexed t-PA (Fig. 2*A*), whereas inhibition of ^{125}I-t-PA/PAI-1 degradation was greater when 100 nM t-PA/PAI-1 was used as a competitor (Fig. 2*B*). When binding to the cells was studied, analogous results were obtained (not shown). In dose-response experiments, we observed that t-PA/PAI-1 was a better inhibitor of ^{125}I-t-PA degradation than free t-PA (50% inhibition of degradation reached at 3.6 and 8.4 nM, respectively). At high concentrations both competitors were equally efficient and inhibited degradation of ^{125}I-t-PA by more than 80% (Fig. 2*C*). Also, with ^{125}I-t-PA/PAI-1 as ligand, t-PA/PAI-1 was a more efficient inhibitor; 50% inhibition was obtained at 6 nM, whereas ~50 nM t-PA was needed. Furthermore, even at very high concentrations, t-PA was unable to completely block ^{125}I-t-PA/PAI-1 degradation, a finding which suggests that some of the sites are unique for t-PA/PAI-1 (Fig. 2*D*).

Effect of Free PAI-1 and u-PA/PAI-1 on the Degradation of ^{125}I-t-PA/PAI-1—To determine whether the increased rate of binding and degradation of ^{125}I-t-PA/PAI-1 was due to binding via the PAI-1 part of the complex, we studied the effect of 100 nM "latent" or "active" PAI-1 on the binding and degradation of ^{125}I-t-PA/PAI-1 and observed no effect (Fig. 3).

Previously, Morton *et al.* (5) had shown that u-PA interferred with the binding of t-PA to human HepG2 hepatoma cells, whereas in Novikoff cells u-PA had no effect (9). The inhibitory effect in HepG2 cells could have been due either to competition of u-PA with t-PA for reaction with PAI-1 or to an inhibitory effect of PA bound PAI-1. Therefore, we studied the effect of u-PA/PAI-1 on the binding and degradation of ^{125}I-t-PA and ^{125}I-t-PA/PAI-1. Neither u-PA/PAI-1 nor free, active u-PA had

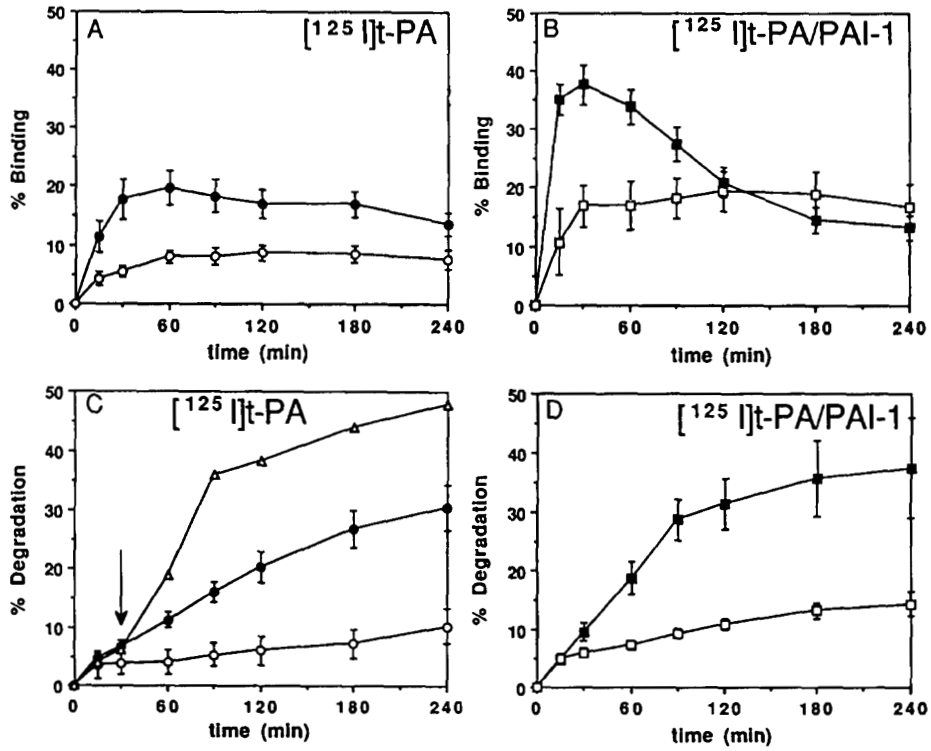

FIG. 1. **Time course of ^{125}I-t-PA and ^{125}I-t-PA/PAI-1 binding by Novikoff cells.** 8 × 10^5 cells in 200 µl of Krebs/BSA were incubated at 37 °C with 0.06 nM ^{125}I-t-PA (*A* and *C*) or ^{125}I-t-PA/PAI-1 (*B* and *D*) in the presence or absence of 100 nM unlabeled ligand. At timed intervals, cell-associated radioactivity was determined and the cell supernatant analyzed for 10% trichloroacetic acid-soluble material as described in the text. *A* and *C*, percentage of ^{125}I-t-PA binding (*A*) and trichloroacetic acid-soluble radioactivity in the supernatant (*C*) in the absence (*closed circles*) or presence of 100 nM unlabeled t-PA (*open circles*). *B* and *D*, percentage of ^{125}I-t-PA/PAI-1 binding (*B*) or trichloroacetic acid-soluble radioactivity in the supernatant (*D*) in the absence (*closed squares*) or presence of 100 nM unlabeled t-PA/PAI-1 (*open squares*). The *open triangles* in *C* represent the degradation of ^{125}I-t-PA after addition at 30 min (*arrow*) of 100 nM PAI-1. The results represent the mean ± S.E. of at least three independent triplicate experiments.

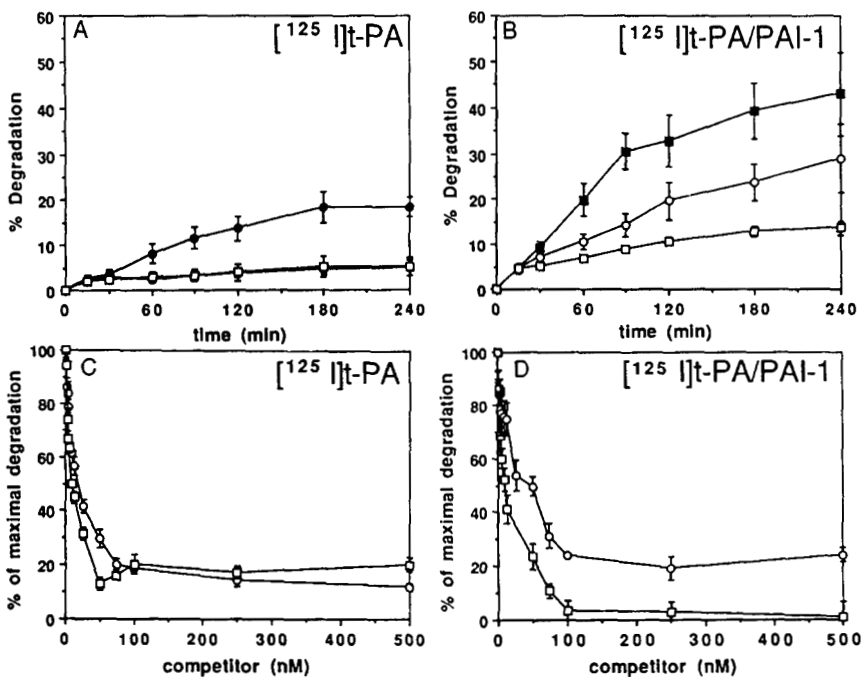

FIG. 2. **Effect of t-PA and t-PA/PAI-1 on the degradation of ^{125}I-t-PA and ^{125}I-t-PA/PAI-1 by Novikoff cells.** *A* and *B*, 8 × 10^5 cells in 200 µl of Krebs/BSA were incubated at 37 °C with 0.06 nM ^{125}I-t-PA (*A*) or ^{125}I-t-PA/PAI-1 (*B*) in the absence (*A, closed circles; B, closed squares*) or presence of 100 nM unlabeled t-PA (*open circles*) or 100 nM unlabeled t-PA/PAI-1 (*open squares*). At timed intervals the cell supernatant was isolated by centrifugation and analyzed for 10% trichloroacetic acid-soluble material. The results represent the mean ± S.E. of at least three independent triplicate experiments. *C* and *D*, 8 × 10^5 cells in 200 µl of Krebs/BSA were incubated at 37 °C for 2 h with 0.06 nM ^{125}I-t-PA (*C*) or ^{125}I-t-PA/PAI-1 (*D*) in the presence of different concentrations of unlabeled t-PA (*open circles*) or unlabeled t-PA/PAI-1 (*open squares*). After 2 h the cell supernatant was isolated by centrifugation and analyzed for 10% trichloroacetic acid-soluble material.

an effect on binding (not shown) and degradation of ^{125}I-t-PA/PAI-1 (Fig. 3) nor of ^{125}I-t-PA (not shown).

Effect of a t-PA Mutant Lacking the Finger and Growth Factor Domains—The results described in the previous section indicate that neither free PAI-1 (active or latent) nor u-PA-bound PAI-1 have an effect on the clearance of t-PA/PAI-1. However, they do not completely rule out a role for the PAI-1 part of the t-PA·PAI-1 complex. Subtle conformational differences may exist between PAI-1 bound to the protease domain of t-PA and free or u-PA bound PAI-1. To investigate whether PAI-1 bound to the protease domain of t-PA had an effect on the binding of t-PA/PAI-1 to the cells we utilized a t-PA mutant lacking the finger and the growth factor domains. This mutant does not bind to the rat hepatoma cells nor is degraded (not shown), which is in accordance with the suggested role for the growth factor domain in the clearance of t-PA (28, 29). First, we established that t-PAΔFG is able to form complexes with PAI-1 (Fig. 4). Competition experiments were then performed, which indicated that neither free nor PAI-1-complexed t-PAΔFG were able to compete for binding (not shown) and degradation of free and PAI-1 complexed ^{125}I-t-PA (Fig. 5, *A* and *B*).

Fate of the Receptor after Having Bound ^{125}I-t-PA/PAI-1—In the present study we observed that the binding of ^{125}I-t-PA reached a plateau after 30 min and remained stable for at least 4 h, whereas the binding of ^{125}I-t-PA/PAI-1 attained a maximum after 30 min and then began to decrease. To establish

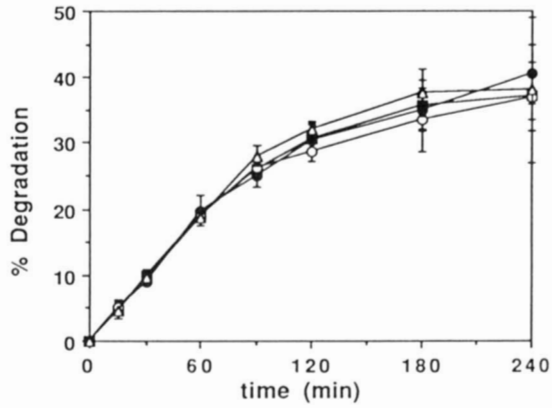

FIG. 3. **Effect of free PAI-1, u-PA, or u-PA/PAI-1 on the degradation of ^{125}I-t-PA/PAI-1 by Novikoff cells.** Cells were incubated with 0.06 nM ^{125}I-t-PAPAI-1 alone (*closed squares*) or in the presence of 100 nM unlabeled u-PA (*open circles*), 100 nM unlabeled u-PA/PAI-1 (*closed circles*), or 100 nM unlabeled PAI-1 (*open triangles*). At timed intervals the cell supernatant was isolated by centrifugation and analyzed for 10% trichloroacetic acid-soluble material. The results represent the mean ± S.E. of at least three independent triplicate experiments.

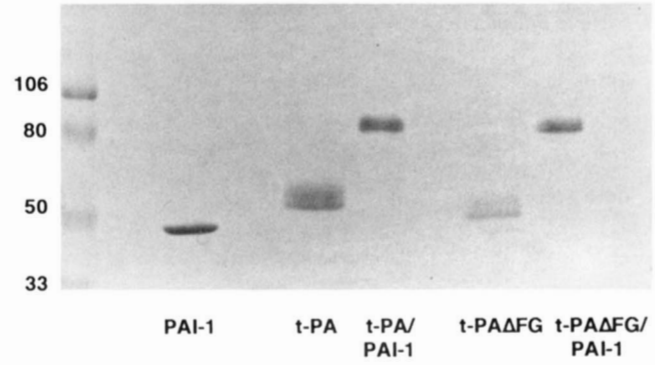

FIG. 4. **Complex formation of PAI-1 with human t-PA and with a t-PA deletion mutant lacking the finger and growth factor domains (t-PAΔFG).** t-PA and t-PAΔFG were incubated with an excess of PAI-1 for 1 h at 37 °C and immunopurified as described in the text. Aliquots were analyzed by SDS-polyacrylamide gel electrophoresis (7.5%) under nonreducing conditions. *Lane 1*, prestained molecular mass standard proteins: phosphorylase *b* (106 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), and carbonic anhydrase (32.5 kDa). *Lane 2*, PAI-1. *Lane 3*, t-PA. *Lane 4*, t-PA after incubation with PAI-1. *Lane 5*, t-PAΔFG. *Lane 6*, t-PAΔFG after incubation with PAI-1.

whether the decrease of binding after 30 min was due to specific receptor down-modulation by t-PA/PAI-1, cycloheximide-treated cells were used. Cycloheximide was maintained at concentrations sufficient to block protein synthesis throughout the experiment. The cells were incubated for 2 h with 100 nM t-PA/PAI-1 at 4 °C, a temperature at which internalization and degradation of ligands are completely blocked and then further incubated at 37 °C to start internalization of ligands. At timed intervals surface bound ligands were removed by acid treatment and the cells incubated with radiolabeled t-PA. Residual binding capacities after 30 and 120 min (24.8 and 28.2%, respectively) were similar to that of control cells (24.6%).

Binding, Internalization, and Degradation of ^{125}I-t-PA KHRR296-299AAAA—Previous reports had shown that the internalization and degradation of t-PA was preceded by complex formation of t-PA with PAI-1 in human HepG2 hepatoma cells (5, 30, 31) and in COS cells (6), whereas such complexes were not observed with Novikoff cells (9). The standard mechanism of complex formation of serine proteases with serine protease inhibitors is that first a reversible complex is formed between the protease and its inhibitor, which then converts into an

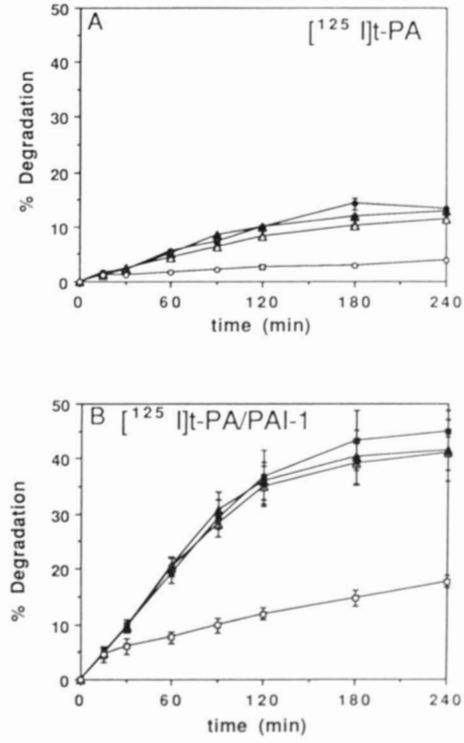

FIG. 5. **Effect of t-PAΔFG, alone or complexed with PAI-1, on the degradation of ^{125}I-t-PA and ^{125}I-t-PA/PAI-1 by Novikoff cells.** 8×10^5 cells in 200 μl of Krebs/BSA were incubated at 37 °C with 0.06 nM ^{125}I-t-PA (*A*) or ^{125}I-t-PA/PAI-1 (*B*) in the absence (*A, closed circles; B, closed squares*) or presence of 100 nM unlabeled t-PAΔFG (*open triangles*), t-PAΔFG/PAI-1 (*closed triangles*), t-PA (*open circles*), or t-PA/PAI-1 (*open squares*). At timed intervals the cell supernatant was isolated by centrifugation and analyzed for 10% trichloroacetic acid-soluble material. The results represent the mean ± S.E. of at least three independent triplicate experiments.

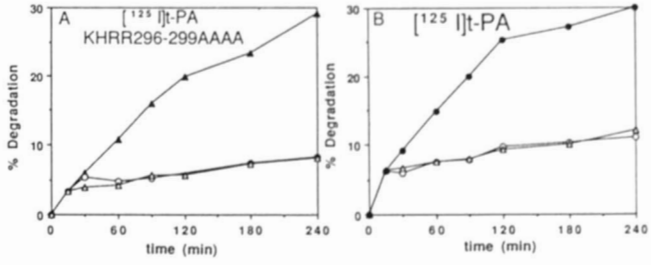

FIG. 6. **Effect of t-PA KHRR296-299AAAA and t-PA on degradation of ^{125}I-t-PA KHRR296-299AAAA and ^{125}I-t-PA by Novikoff cells.** Cells were incubated with 0.06 nM ^{125}I-t-PA KHRR296-299AAAA or ^{125}I-t-PA in the absence (*A, closed triangles; B, closed circles*) or presence of 100 nM unlabeled t-PA KHRR296-299AAAA (*open triangles*) or 100 nM t-PA (*open circles*). At timed intervals the cell supernatant was isolated by centrifugation and analyzed for 10% trichloroacetic acid-soluble material.

irreversible SDS-stable complex. Conceivably, complexes of t-PA with rat PAI-1 formed on the surface of the Novikoff cells could be internalized and degraded before their conversion into irreversible complexes. To examine this possibility, we performed binding and degradation experiments with a t-PA variant KHRR296-299AAAA. This variant lacks an interaction site with PAI-1 and reacts 90 times more slowly with PAI-1 than wild type t-PA (32). When ^{125}I-t-PA KHRR296-299AAAA was incubated with the cells, binding and degradation occurred at a rate similar to that of wild type t-PA. Cell binding (not shown) and degradation were inhibited to a similar extent by 100 nM unlabeled t-PA KHRR296-299AAAA or wild type t-PA (Fig. 6, *A* and *B*) Also, the wild type t-PA and the mutant form

TABLE I

Effect of RAP on the binding and degradation of t-PA, t-PA/PAI-1, and t-PA KHRR296-299AAAA by rat hepatoma cells

Cells (4×10^6/ml) were incubated at 37 °C with 0.06 nM radiolabeled ligand in the presence of 100 nM homologous ligand or 50 nM RAP-glutathione transferase. The effect on binding was determined after 30 min and on degradation after 3 h. The results are expressed as percentage inhibition of binding or degradation induced by the competitor.

Ligand	Competitor			
	Homologous ligand		RAP-glutathione transferase	
	Binding[a]	Degradation[a]	Binding[a]	Degradation[a]
	% inhibition		*% inhibition*	
t-PA	59 ± 9.24	68 ± 5.19	67 ± 4.04	74 ± 2.30
t-PA/PAI-1	58 ± 10.39	65 ± 4.79	68 ± 3.46	71 ± 2.88
t-PA-KHRR296-299AAAA	60 ± 4.04	75 ± 3.46	71 ± 1.73	76 ± 2.30

[a] The results are expressed as mean ± S.E. of three independent experiments.

of t-PA were equally efficient inhibitors of the binding and degradation of ^{125}I-t-PA/PAI-1 (not shown).

Effect of RAP on the Binding, Internalization, and Degradation of ^{125}I-t-PA, ^{125}I-t-PA/PAI-1, and ^{125}I-t-PA KHRR296-299AAAA—We investigated the effect of a RAP-glutathione transferase fusion protein on the binding, internalization, and degradation of ^{125}I-t-PA, ^{125}I-t-PA/PAI-1, and ^{125}I-t-PA KHRR296-299AAAA. RAP inhibited both binding and degradation of the three mentioned ligands by values ranging from 60 to 80% (Table I). The inhibitory effect of RAP was slightly stronger than that of the homologous ligands (Table I).

DISCUSSION

In vivo t-PA is rapidly cleared from the circulation via the liver, partly via specific receptors on hepatocytes. Conflicting results have been obtained on the mechanisms involved in its clearance, in particular on the role of PAI-1. We undertook a comparative study of the binding and degradation of free and PAI-1-bound t-PA by hepatocyte-like cells in order to establish the role of PAI-1 and to identify the determinants that mediate the binding to the clearance receptor. Novikoff rat hepatoma cells were chosen because they grow in suspension culture, thus eliminating possible interferences by extracellular matrix-bound PAI-1 (30). Also, we focused on cell-mediated degradation of t-PA which represents functional binding to the clearance receptor rather than total binding.

Our results confirm and reinforce previous observations of a PAI-1 independent clearance mechanism for t-PA in rat hepatoma cells (8, 9). In the present study we observed that t-PA (KHRR296-299AAAA), a mutant reacting 2 orders of magnitude slower with PAI-1 than wild type t-PA behaved similarly to wild type t-PA, whereas a t-PA deletion mutant lacking the finger and growth factor domains, even in complex with PAI-1, did not compete with wild type t-PA. The latter result agrees with findings suggesting that the growth factor domain is involved in the binding of t-PA to its clearance receptor (28, 29) and that *in vivo* clearance of t-PAΔFG is diminished (25). Taken together these and previous results indicate that t-PA can be cleared in a PAI-1 independent fashion. However, the finding that PAI-1 more than 2-fold accelerated the degradation of t-PA implies an additional PAI-1 dependent clearance mechanism, which is in agreement with many *in vitro* (6, 30, 31, 33) and *in vivo* (7) studies.

For t-PA/PAI-1 we observed a decrease of cell-binding after 30 min, which was not due to a ligand-mediated decrease of receptor levels but rather appears to be due to exhaustion of functional radioligand.

Our results do not allow to draw firm conclusions as to the mechanism of the PAI-1 mediated increased rate of clearance of t-PA by rat hepatoma cells. Two possible, although not mutually exclusive mechanisms are supported by our data: additional binding sites for t-PA/PAI-1 or a higher affinity of t-PA/PAI-1 for its receptor. The observation that an excess of free t-PA could not completely inhibit the clearance of t-PA/PAI-1, suggests distinct binding sites for free and PAI-1 complexed t-PA. However, t-PA/PAI-1 inhibits clearance of labeled t-PA at lower concentrations than wild type t-PA suggesting a PAI-1-mediated increase of the affinity of t-PA for its clearance receptor.

The localization of the determinants on free t-PA and on t-PA/PAI-1 mediating clearance by rat hepatoma cells was investigated. The following observations were made: 1) an excess of active or latent PAI-1 had no effect on the clearance of t-PA/PAI-1 but accelerated that of free t-PA; 2) free nor PAI-1-bound u-PA were able to compete for t-PA/PAI-1 uptake. This is in contrast to the findings in HepG2 cells (5); and 3) free t-PA but not free t-PAΔFG nor t-PAΔFG/PAI-1 competed for clearance of free or PAI-1-bound t-PA. Taken together these results suggest that the determinant mediating the increased rate of clearance of t-PA/PAI-1 in rat hepatoma cells is on the t-PA moiety, most likely on the growth factor domain (28, 29).

Two recent studies suggested that the LRP mediates PAI-1 independent clearance of t-PA in rat hepatoma cells (14) and PAI-1 dependent clearance in COS cells (6). In the present study we observed that RAP, a general clearance inhibitor of LRP ligands, strongly reduced the rate of clearance of both t-PA and t-PA/PAI-1, which is in accordance with a role for LRP.

In conclusion, the results obtained to date on the clearance of t-PA and u-PA by hepatocyte-like cells and other cells suggest three distinct mechanisms for t-PA clearance: 1) a t-PA-specific clearance; 2) accelerated clearance of t-PA/PAI-1, mediated by determinants on the t-PA molecule; and 3) clearance dependent on determinants on the PAI-1 part of the complex, which may also mediate the clearance of u-PA/PAI-1. Apparently these mechanisms are expressed to various extents on human and rat hepatocyte-like cells. The finding that all clearance mechanisms are inhibited by RAP (this paper and Refs. 6, 14, and 15) suggests that the LRP is involved in all three pathways. It remains to be established whether the apparent differences in the degree of expression of these clearance mechanisms are due to accessory factors, such as the u-PA receptor (34) or the 55-kDa protein identified by cross-linking of t-PA to Novikoff rat hepatoma cells (9), or to species-specific differences in the expression or ligand-affinity of the different clearance determinants on the multidomain LRP molecule.

Acknowledgments—We thank Dr. L. Nelles (Leuven, Belgium) for providing Chinese hamster ovary cells stably expressing t-PAΔFG; Dr. J. Herz for the pGEX39-kDa plasmid for expression of a RAP-glutathione transferase protein; Dr. D. K. Strickland for recombinant RAP; Dr. T. Reilly for recombinant PAI-1; Dr. J. Krause for recombinant t-PA; Dr. W. F. Bennett for providing t-PA KHRR296-299AAAA. We thank Dr. Sue Wells for critical reading of the manuscript.

REFERENCES

1. Einarsson, M., Smedsrød, B., and Pertoft, H. (1988) *Thromb. Haemostasis* **59,** 474–479
2. Kuiper, J., Otter, M., Rijken, D. C., and Van Berkel, T. J. C. (1988) *J. Biol. Chem.* **263,** 18220–18224

3. Smedsrød, B., and Einarsson, M. (1990) *Thromb. Haemostasis* **63,** 60–66
4. Seydel, W., Stang, E., Roos, N., and Krause, J. (1991) *Drug Res.* **41,** 182–186
5. Morton, P. A., Owensby, D. A., Wun, T. C., Billadello, J. J., and Schwartz, A. L. (1990) *J. Biol. Chem.* **265,** 14093–14099
6. Orth, K., Madison, E. L., Gething, M. J., Sambrook, J. F., and Herz, J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 7422–7426
7. Wing, L. R., Hawksworth, G. M., Bennett, B., and Booth, N. A. (1991) *J. Lab. Clin. Med.* **117,** 109–114
8. Bu, G., Morton, P. A., and Schwartz, A. L. (1992) *J. Biol. Chem.* **267,** 15595–15602
9. Nguyen, G., Self, S. J., Camani, C., and Kruithof, E. K. O. (1992) *J. Biol. Chem.* **267,** 6249–6256
10. Herz, J., Kowal, R. C., Goldstein, J. L., and Brown, M. S. (1990) *EMBO J.* **9,** 1769–1776
11. Strickland, D. K., Ashcom, J. D., Williams, S., Burgess, W. H., Migliorini, M., and Argraves, W. S. (1990) *J. Biol. Chem.* **265,** 17401–17404
12. Kristensen, T., Moestrup, S. K., Gliemann, J., Bendtsen, L., Sand, O., and Sottrup-Jensen, L. (1990) *FEBS Lett.* **276,** 151–155
13. Kowal, R. C., Herz, J., Goldstein, J. L., Esser, V., and Brown, M. S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 5810–5814
14. Bu, G., Williams, S., Strickland, D. K., and Schwartz A. L. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 7427–7431
15. Nykjaer, A., Petersen, C. M., Møller, B., Jensen, P. H., Moestrup, S. K., Holtet, T. L., Etzerodt, M., Thøgersen, H. C., Munch, M., Andreasen, P. A., and Gliemann J. (1992) *J. Biol. Chem.* **267,** 14543–14546
16. Herz, J., Clouthier, D. E., and Hammer, R. E. (1992) *Cell* **71,** 411–421
17. Kerjaschki, D., and Farquhar M. G. (1983) *J. Exp. Med.* **157,** 667–686
18. Willnow, T. E., Goldstein, J. L., Orth, K., Brown, M. S., and Herz, J. (1992) *J. Biol. Chem.* **267,** 26172–26180
19. Jensen, P. H., Moestrup, S. K., and Gliemann J. (1989) *FEBS Lett.* **255,** 275–280
20. Ashcom, J. D., Tiller, S. E., Dickerson, K., Cravens, J. L., Argraves, W. S., and Strickland, D. K. (1990) *J. Cell Biol.* **110,** 1041–1048
21. Herz, J., Goldstein, J. L., Strickland, D. K., Ho, Y. K., and Brown, M. S. (1991) *J. Biol. Chem.* **266,** 21232–21238
22. Kounnas, M. Z., Morris, R. E., Thompson, M. R., FitzGerald, D. J., Strickland, D. K., and Saelinger, C. B. (1992) *J. Biol. Chem.* **267,** 12420–12423
23. Chappell, D. A., Fry, G. L., Waknitz, M. A., Iverius, P. H., Williams, S. E., and Strickland, D. K. (1992) *J. Biol. Chem.* **267,** 25764–26767
24. Kounnas, M. Z., Argraves, W. S., and Strickland, D. K. (1992) *J. Biol. Chem.* **267,** 21162–21166
25. Lijnen, H. R., Nelles, L., Van Hoef, B., De Cock, F., and Collen D. (1990) *J. Biol. Chem.* **265,** 5677–5683
26. Laemmli, U. K. (1970) *Nature* **227,** 680–685
27. Fraker, P. J., and Speck, J. C. (1978) *Biochem. Biophys. Res. Commun.* **80,** 849–857
28. Nguyen, G., Self, S. J., Camani, C., and Kruithof, E. K. O. (1992) *Biochem. J.* **287,** 911–915
29. Bassel-Duby, R., Jiang, N. Y., Bittick, T., Madison, E., McGookey, D., Orth, K., Shohet, R., Sambrook, J., and Gething, M. J. (1992) *J. Biol. Chem.* **267,** 9668–9677
30. Owensby, D. A., Morton, P. A., and Schwartz, A. L. (1989) *J. Biol. Chem.* **264,** 18180–18187
31. Morton, P. A., Owensby, D. A., Sobel, B. E., and Schwartz, A. L. (1989) *J. Biol. Chem.* **264,** 7228–7235
32. Paoni, N. F., Keyt, B. A., Refino, C. J., Chow, A. M., Nguyen, H. V., Berleau, L. T., Badillo, J., Peña, L. C., Brady, K., Wurm, F. M., Ogez, J., and Bennett, W. F. (1993) *Thromb. Haemostasis* **70,** 307–312
33. Wing, L. R., Bennett, B., and Booth, N. A. (1991) *FEBS Lett.* **278,** 95–97
34. Olson, D., Pöllänen, J., Høyer-Hansen, G., Rønne, E., Sakaguchi, K., Wun, T. C., Appella, E., Danø, K., and Blasi, F. (1992) *J. Biol. Chem.* **267,** 9129–9133