Identification and Partial Characterization by Chemical Cross-linking of a Binding Protein for Tissue-type Plasminogen Activator (t-PA) on Rat Hepatoma Cells

A PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1-INDEPENDENT t-PA RECEPTOR*

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Plasma tissue-type plasminogen activator (t-PA) is cleared rapidly in vivo by the liver. Previous studies with the human hepatoma cell line HepG2 have identified a clearance system for t-PA modulated by plasminogen activator inhibitor type 1 (PAI-1). In the present study, a rat hepatoma cell line MH.C1 is shown to contain a PAI-1-independent t-PA clearance system. At 4°C, binding of 125I-t-PA to MH.C1 cells was rapid, specific, and saturable. Scatchard analysis of the binding data yielded a mean estimate of 105,000 high affinity binding sites per cell (Kd = 4.1 nM). When the bound ligand was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the majority (about 90%) of the specific binding was in the form of uncomplexed 125I-t-PA. This is in contrast to HepG2 cells in which specific binding was mainly in the form of a sodium dodecyl sulfate-stable 125I-t-PA-PAI-1 complex. When availability of matrix-associated PAI-1 was blocked by preincubation with anti-PAI-1 antibody or removed by elastase treatment, specific 125I-t-PA binding to MH.C1 cells was unaffected, whereas most of the specific 125I-t-PA binding to HepG2 cells was abolished. Furthermore, when the active site of t-PA was inactivated with diisopropyl fluorophosphate, the diisopropyl fluorophosphate-t-PA specifically competed for binding of 125I-t-PA to MH.C1 cells, but failed to block specific 125I-t-PA binding to HepG2 cells. At 37°C, PAI-1-independent t-PA binding to MH.C1 cells was followed by ligand uptake and degradation with kinetics similar to that seen in HepG2 cells. Chemical cross-linking of t-PA to MH.C1 cells revealed a specific t-PA binding protein with a molecular mass of about 500,000 daltons. Ligand-receptor complexes generated by chemical cross-linking were immunoprecipitable by anti-t-PA antibody, but not by anti-PAI-1 antibody, further supporting the finding that binding of t-PA to MH.C1 cells is PAI-1-independent.

Tissue-type plasminogen activator (t-PA) is an endogenous serine protease which initiates fibrinolysis by catalyzing the conversion of the zymogen plasminogen to the active proteolytic enzyme plasmin (1). Plasmin, in turn, proteolytically degrades fibrin clots and has been implicated as an initiator of a number of physiological processes ranging from thrombolysis to cell migration and tissue remodeling (2, 3). The role of t-PA as an initiator in thrombolysis has been exploited for clinical therapeutics. Exogenously administered t-PA is capable of eliciting prompt thrombolysis in experimental animals with induced coronary artery thrombosis (4) and in patients with evolving myocardial infarction (5, 6). However, the major drawback for the clinical use of t-PA is its rapid in vivo clearance from the blood. The half-life of t-PA in the circulation ranges from 2–4 min in rodents (7–12) to 5–10 min in humans (13, 14).

Although the mechanism of t-PA clearance is poorly understood, the liver appears to be the major organ of clearance (7–12). Exogenously administered t-PA delivered intravenously rapidly accumulates in the liver and is subsequently degraded. Autoradiographic studies in mice (12) demonstrated that the hepatocyte was responsible for t-PA clearance. In rats, a specific, high affinity mechanism for t-PA clearance has been identified in isolated hepatocyte preparations (15). Seeking the human counterpart to this clearance process, we have previously reported that the well differentiated hepatoma cell line HepG2 possesses an oligosaccharide-independent high affinity clearance system (16). This catabolic process is modulated by the rapidly acting inhibitor plasminogen activator inhibitor type 1 (PAI-1). Previously reported studies from our laboratory indicate that endogenous PAI-1 is distributed throughout the extracellular matrix bound to vitronectin but is absent from the HepG2 cell surface. Upon exposure of the HepG2 monoclonal to exogenous t-PA, a sodium dodecyl sulfate (SDS)-stable t-PA-PAI-1 complex is rapidly formed and released from the matrix. Following specific binding to the surface of HepG2 cells, the t-PA-PAI-1 complex is internalized and degraded (16–19). Our studies have also suggested that specific binding of t-PA-PAI-1 complexes to HepG2 cells requires elements of the PAI-1 moiety and/or parts of the protease domain of t-PA (20).

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The abbreviations used are: t-PA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DFP, diisopropyl fluorophosphate; DTSSP, dithiobis(sulfosuccinimidylpropionate); LRP, low density lipoprotein receptor-related protein; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline.
ent t-PA clearance mechanism in the rat hepatoma cell line MHClC. We demonstrate in the current report that this cell line possesses a high affinity, PAI-1-independent t-PA receptor system. Binding of t-PA to this cell line requires neither the active site of t-PA nor the presence of bioactive PAI-1. Subsequent to surface binding, t-PA is internalized and degraded with rapid kinetics.

While kinetic studies of the clearance process suggest the existence of a t-PA receptor, little is known about its structure. Here we report the detection by chemical cross-linking of a specific t-PA binding protein on the surface of hepatoma cells with a molecular mass of about 500,000 daltons.

**Experimental Procedures**

**Materials**

Single-chain recombinant human t-PA expressed in Chinese hamster ovary cells was kindly supplied by Genentech (Lot 9124AX). Carrier-free sodium [125]iodide, [35]S]methionine, [35]S]cysteine, Amplify, and Hyperfilm-MP were purchased from Amersham. Phenylmethanesulfonyl fluoride (PMSF), bovine serum albumin (fraction V), cytochrome c, Tween 80, Triton X-100, sodium deoxycholate, and laminin were from Sigma. PONase was obtained from Calbiochem, and elastase was from Boehringer Mannheim. Human serum albumin was obtained from the American Red Cross. Fetal calf serum and elastase was from Boehringer Mannheim. Human serum albumin and human serum albumin, and 1 mM PMSF ("Immunomix"). SDS was included in the Laemmli sample buffer.

**Methods**

**Cell Culture**—Rat hepatoma MHClC cells were cultured in Earle’s minimum essential medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml), and were incubated at 37°C in humidified air containing 5% CO2 (51). The maintenance of Hep2 cells has been described before (21). All cultures were supplemented with fresh media 12 h prior to use. Cell monolayers were generally used at 80–90% confluence.

**Protein Labeling**—Recombinant t-PA, normal rabbit IgG, and anti–PAI-1 IgG were labeled with [125]Iodide using IODO-GEN. Briefly, protein (50 µg) in a minimum volume was added to a glass tube precoated with IODO-GEN (10 µg) according to manufacturer’s instructions. Phosphate buffer (0.2 M final concentration, pH 7.4) and 1 mCi of [125]Iodoide were added, and the reaction was allowed to proceed for 10 min at room temperature. The reaction was quenched by adding 2% nonfat dry milk and cytochrome c, each to a final concentration of 2 mg/ml. The [125]I protein was then separated from unreacted [125]I-iodide by gel filtration chromatography using a PD-10 Sephadex column which had been pre-equilibrated with phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 80. The specificity of the 4% phosphotungstic acid/20% trichloroacetic acid-insoluble [125]I-protein was determined by γ scintillation spectrometry. The specific activity was 5–10 µCi/µg of protein.

**Ligand Binding Assays**—Cells were seeded into multwell (12 wells/plate) disposable plastic tissue culture plates 2–3 days prior to assay. Ligand binding buffer was composed of PBS containing 1 mM CaCl2, 0.5 mM MgCl2 containing 10 mM α-amino-n-caproic acid. Binding experiments were performed at 4°C to prevent possible concomitant internalization during the binding interval. Cell monolayers were washed three times on ice with prechilled binding buffer. Binding was initiated by adding 0.5 ml of binding buffer containing selected concentrations of [125]I-t-PA in the absence or presence of an excess of various competitors. After incubation at 4°C for 1.5 h (unless otherwise specified), buffer containing unbound ligand was removed. Cells were then washed three times with binding buffer and lysed in 0.0625 M Tris-HCl, pH 6.8, containing 0.2% (w/v) SDS, 0.5 M NaCl, 0.1% (v/v) glycerol ("low-SDS lysis buffer"). Radioactivity of cell lysates was quantified by γ scintillation spectrometry. For some experiments, cells lysates were further analyzed by SDS-PAGE and autoradiography as described below. Total binding was determined in the presence of excess unlabeled t-PA (1 µM). Specific binding was defined as the difference between total and non-specific binding.

**Elastase Treatment of Cell Monolayers**— Cultures were washed three times with PBS and incubated with PBS containing 10 µg/ml elastase at 4°C for 0.5 h. Cell monolayers were then washed extensively with binding buffer prior to ligand binding assays.

**Ligand Uptake andDegradation**—Ligand uptake experiments were performed by allowing cells to bind ligand at 4°C as described above. After washing with binding buffer, cells were incubated in buffer containing 1 µM unlabeled t-PA. At selected intervals, buffer overlying each cell monolayer was removed and saved. Dishes were then quickly chilled on ice and washed with prechilled binding buffer to prevent any further ligand uptake. The cell monolayer was treated with 0.25% (w/v) Pronase in PBS for 30 min at 4°C to remove residual surface-bound ligand. This treatment also detached cells from the culture wells. The detached cells were then separated from buffer by centrifugation. Radioactivity associated with cell pellets represents internalized protease-resistant ligand, whereas radioactivity in the supernatant fraction represents surface protease-sensitive ligand. Degraded ligand was defined as the radioactive fragments in the overlying buffer that were soluble in 4% phosphotungstic acid/20% trichloroacetic acid. Each determination represents the specific signal (difference in the absence and presence of 1 µM unlabeled t-PA) and is a mean value of triplicate determinations.

**Metabolic Labeling**—Cells growing in 10-cm dishes at about 80% confluence were incubated for 30 min at 37°C in two changes of Earle’s minimum essential medium lacking either l-methionine or l-cysteine and containing 2 mM L-glutamine. Metabolic labeling was initiated by the addition of the above medium supplemented with corresponding [35]S]methionine or [35]S]cysteine (200 Ci/mM). Following incubation for 5 h at 37°C, media containing secreted proteins were collected. Cell monolayers were washed with binding buffer and were used for t-PA binding and chemical cross-linking experiments as described below.

**Chemical Cross-linking**—Experiments were performed with either [125]I-labeled t-PA cross-linked to unlabeled cells or unlabeled t-PA cross-linked to [35]S]cysteine metabolically labeled cells. After ligand binding at 4°C, each cell monolayer was washed three times with PBS. Chemical cross-linking was performed by incubating cell monolayer with PBS containing 0.5 mM DTSSP. After 30 min at 4°C, the reaction was quenched by washing cell monolayer two times with PBS containing 10 µg/ml of rabbit serum. Another PBS containing 1% (v/v) Triton X-100 and 1 mM PMSF ("lysis buffer") for 30 min at 4°C with occasional vortexing. The solubilized cell lysate was centrifuged at 14,000 × g for 2 min to remove nuclei, and the supernatant was subsequently used for immunoprecipitation.

**Immunoprecipitation**—A aliquots of conditioned media following 35S-labeling or cell lysates from cross-linking experiments were brought to 0.5 ml with lysis buffer and mixed with 0.5 ml of PBS containing 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 1% (w/v) SDS, 0.5% (v/v) human serum albumin, 0.5% (w/v) bovine serum albumin, and 1 mM PMSF ("Immunomix") and 10 µg of α-lactalbumin in the Immunomix to reduce nonspecific immunoprecipitation. Primary antibody (10 µg of IgG or 10 µl of rabbit serum) was added, and the samples were rocked overnight at 4°C followed by incubation for 1 h at room temperature with 50 µl of protein A-agarose. Nonbound radioactivity was removed by washing protein A-agarose beads three times with Immunomix and three times with PBS. The immunoprecipitated material was then released from the beads by boiling each sample for 5 min in 0.0625 M Tris-HCl, pH 6.8, 2% (w/v) SDS, and 10% (v/v) glycerol ("Laemmli sample buffer") and was analyzed by SDS-PAGE as described below. For some experiments, SDS-PAGE was conducted under reducing conditions by including 5% (v/v) 2-mercaptoethanol in the Laemmli sample buffer.

**SDS-PAGE and Autoradiography**—Samples of cell lysates or from immunoprecipitations were analyzed by SDS-PAGE using 6%, 7.5%, or 10% (w/v) polyacrylamide slab gels (22) under reducing or non-reducing conditions. The following prestained molecular weight standards from Bio-Rad were used: myosin, 205,000; β-galactosidase, 117,000; bovine serum albumin, 80,000; ovalbumin, 50,000. Rainbow molecular weight markers from Amersham were also used: myosin, 200,000; phosphorylase B, 97,000; bovine serum albumin, 69,000; bovine serum albumin, 46,000; and cyanogen bromide cleaved bovine serum albumin, 30,000. Laminin was used for 6% SDS-PAGE (large subunit 400,000). Autoradiography of 125I-labeled proteins was performed with dried polyacrylamide gels using Kodak XAR-5 films. For fluorography of 35S-labeled proteins, gels were impregnated with Amplify, dried, and exposed to Amersham Hyperfilm-MP. Films were placed at −70°C for various periods of time as specified in each figure prior to development.

**General Procedures**—Protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard.
RESULTS

Specific Binding of $^{125}$I-t-PA to MH$\text{H}_1\text{C}_1$ Cells—The rat hepatoma cell line MH$\text{H}_1\text{C}_1$ was characterized with respect to specific $^{125}$I-t-PA binding. Binding isotherms were performed at 4 °C to avoid possible ligand uptake. In a preliminary experiment, 10 mM $\epsilon$-amino-$n$-caproic acid was found to reduce nonspecific $^{125}$I-t-PA binding presumably to lysine residues without affecting specific $^{125}$I-t-PA binding. This reagent was therefore included in the binding buffer. Over the concentration range of 1–16 nM, nonspecific ligand binding increased linearly, while total binding increased in a curvilinear fashion, demonstrating saturable, specific $^{125}$I-t-PA binding (Fig. 1A). Saturation of specific binding was observed at $^{125}$I-t-PA concentrations in excess of 16 nM. Scatchard (23) analysis of the binding data from three such experiments yielded a mean estimate of 105,000 ± 41,000 S.D. homogeneous high affinity surface binding sites per cell with an apparent $K_d = 4.1 \pm 1.2$ nM (3.7 nM for the binding isotherm shown in Fig. 1A). When cell lysates from selected ligand concentrations were analyzed with SDS-PAGE (Fig. 1B), both uncomplexed $^{125}$I-t-PA and SDS-stable $^{125}$I-t-PA-PAI-1 complex (17) were observed. While $^{125}$I-t-PA-PAI-1 complex binding was saturated at lower ligand concentrations, an increase in specific binding at higher ligand concentrations was observed as a result of uncomplexed $^{125}$I-t-PA binding.

To determine whether the specific $^{125}$I-t-PA binding was on the cell surface, cultured MH$\text{H}_1\text{C}_1$ cells were removed from culture dishes with PBS containing 3 mM EDTA as described before (18). The remaining growth substratum was tested for $^{125}$I-t-PA binding. Of several experiments performed, less than 5% of the specific $^{125}$I-t-PA binding on intact cell monolayers was observed. This result suggested that specific binding of $^{125}$I-t-PA to cell monolayers was associated with the cell surface but not with the growth substratum.

Comparison of Specific $^{125}$I-t-PA Binding Species on MH$\text{H}_1\text{C}_1$ and HepG2 Cells—Previous studies have shown that specific $^{125}$I-t-PA binding to human hepatoma HepG2 cells was mainly due to $^{125}$I-t-PA-PAI-1 complex binding which represented 80–90% of the entire complement of specifically bound ligand (17). To examine the nature of the ligand bound to MH$\text{H}_1\text{C}_1$ and HepG2 cells, binding experiments were performed with 8 nM $^{125}$I-t-PA to both hepatoma cell lines. After binding at 4 °C, both overlying buffer and cell lysates were analyzed by SDS-PAGE. As shown in Fig. 2, a similar amount of $^{125}$I-t-PA-PAI-1 complex was released from both hepatoma cell lines into the overlying buffer (lanes 1 and 5). However, when postbinding cell lysates were analyzed by SDS-PAGE, the majority (>90%) of specific $^{125}$I-t-PA binding to MH$\text{H}_1\text{C}_1$ cells was in the form of uncomplexed $^{125}$I-t-PA (lanes 3 and 4), while most (about 90%) of the specific $^{125}$I-t-PA binding associated with HepG2 cells was in the form of $^{125}$I-t-PA-PAI-1 complex (lanes 6 and 7). These differences of binding species on the two hepatoma cell lines suggest that binding of $^{125}$I-t-PA to MH$\text{H}_1\text{C}_1$ cells is independent of PAI-1, whereas binding of $^{125}$I-t-PA to HepG2 cells is PAI-1-dependent.

PAI-1 Production by MH$\text{H}_1\text{C}_1$, Cells and HepG2 Cells—To determine whether the differences in apparent ligand specificity between the two hepatoma cell lines resulted from differential PAI-1 production and secretion, the amount of PAI-1 in the culture media and on the cell monolayers was analyzed for each cell line. Cells were metabolically labeled with $^{35}$S-methionine, and the media overlying cell monolayers were harvested and immunoprecipitated with normal rabbit serum, anti-t-PA antibody, or anti-PAI-1 antibody. The amount of media used for each immunoprecipitation was normalized on the basis of cell number. The immunoprecipitated materials were then analyzed by 10% SDS-PAGE. Since the anti-PAI-1 antibody was generated using purified human PAI-1 with a fragment of vitronectin attached, this antibody also recognized human vitronectin but not rat vitronectin. As shown in Fig. 3A, the amount of PAI-1 secreted into the overlying media was similar for the two hepatoma cell lines. This experiment also showed that the rat hepatoma MH$\text{H}_1\text{C}_1$ cells synthesize and secrete t-PA. The secreted t-PA in this experiment formed t-PA-PAI-1 complex with matrix PAI-1 and was released to the overlying medium as shown in the figure (lanes 3 and 5). This t-PA-PAI-1 complex was specifically immunoprecipitated by both anti-t-PA antibody and anti-PAI-1 antibody, confirming the identity of the complex. The amount of secreted t-PA was very small compared to secreted PAI-1 since no free t-PA was observed, and the amount of t-PA-PAI-1 complex was less than 5% of the total.
secreted PAI-1. The endogenous t-PA-PAI-1 complex was not detected from the overlying medium of HepG2 cells.

Previous experiments have shown that exogenously added t-PA could bind to active PAI-1 on the cell matrix and form an SDS-stable t-PA-PAI-1 complex which was then released from the matrix to the overlying media (18). To compare the available amount of bioactive PAI-1 on each hepatoma cell line, [35S]methionine-labeled cell monolayers were examined for t-PA binding (5 nM unlabeled t-PA). After 1 h at 4°C, overlying buffer from each cell line was harvested and immunoprecipitated with normal rabbit serum, anti-t-PA antibody, or anti-PAI-1 antibody. The immunoprecipitated materials were then analyzed by SDS-PAGE (Fig. 3B) under nonreducing conditions. The 95-kDa t-PA-PAI-1 complex was immunoprecipitable with both anti-t-PA antibody and anti-PAI-1 antibody and was immunoprecipitated from the two hepatoma cell lines at a similar level after normalization for cell number. No detectable vitronectin was released from the HepG2 cell monolayer during the 1-h incubation period. The above experiments demonstrated that the two hepatoma cell lines synthesized and secreted similar amounts of bioactive PAI-1 and that PAI-1-independent binding of t-PA to MH4C1 cells was not due to decreased production and availability of bioactive PAI-1.

PAI-1-independent and -dependent 125I-t-PA Binding—To further examine the role of PAI-1 in mediating 125I-t-PA binding to MH4C1 and HepG2 cells, several experiments were performed. PAI-1 was removed from cultured cell monolayers by elastase treatment. As shown in Table I, prior elastase treatment did not affect nonspecific binding of 125I-labeled normal rabbit IgG, but reduced the binding of 125I-labeled anti-PAI-1 IgG significantly on both hepatoma cell lines indicating an effective removal of PAI-1 from cell monolayers. When 125I-t-PA was incubated with either cell monolayers or cell growth substratum, no detectable 125I-t-PA-PAI-1 was observed in the overlying buffer, suggesting that active PAI-1 was not available (data not shown). When binding of 3 nM

**TABLE I**

Effects of elastase treatments on 125I-t-PA binding

<table>
<thead>
<tr>
<th>Ligand</th>
<th>% total binding</th>
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<tr>
<td>125I-IgG (N.R.)</td>
<td>HepG2</td>
</tr>
<tr>
<td></td>
<td>102 ± 6.0</td>
</tr>
<tr>
<td>125I-IgG (α-PAI-1)</td>
<td>HepG2</td>
</tr>
<tr>
<td></td>
<td>32.2 ± 3.5</td>
</tr>
<tr>
<td>125I-t-PA</td>
<td>HepG2</td>
</tr>
<tr>
<td></td>
<td>33.1 ± 4.2</td>
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</table>

Cultured hepatoma cell monolayers were washed three times with PBS(C) and were incubated with PBS(C) containing 10 μg/ml elastase. After 0.5 h at 4°C, cells were washed with binding buffer prior to ligand binding. 125I-Labeled ligands (3 nM) were allowed to bind cells for 1.5 h at 4°C. Total binding for each ligand was then determined. 100% total binding was 125I-t-PA binding to untreated cells. Nonspecific binding was 21 ± 2.5% for HepG2 cells and was 18 ± 3.1% for MH4C1 cells. Each reported value represents an average of triplicate determinations.
125I-t-PA was performed following elastase treatment, the majority of specific binding of 125I-t-PA to MHIC1 cells was unaffected, whereas most of the specific 125I-t-PA binding to HepG2 cells was abolished. These data suggested that specific binding of 125I-t-PA to HepG2 cells depended on the availability of PAI-1, while binding to MHIC1 cells was independent of PAI-1.

Complex formation between t-PA and PAI-1 requires a functional t-PA active site present in the serine protease domain. Accordingly, destruction of this t-PA active site with DFP should prevent 125I-t-PA-PAI-1 complex formation. If specific binding of 125I-t-PA requires 125I-t-PA-PAI-1 complex, then DFP-treated t-PA should not be able to specifically bind to the cell. Thus, we hypothesized that DFP-treated t-PA should not compete for 125I-t-PA binding on HepG2 cells but should still be able to compete for 125I-t-PA binding on MHIC1 cells. To test this hypothesis, t-PA was inactivated with DFP (24) and subsequently used for competition studies of native 125I-t-PA binding. As shown in Table II, most of the 125I-t-PA binding to MHIC1 cells was competed by DFP-treated t-PA, whereas DFP-treated t-PA did not compete for specific 125I-t-PA binding to HepG2 cells. Furthermore, when cell monolayers were preincubated with Fab fragments of anti-PAI-1 antibody to block the availability of PAI-1 for complex formation, specific 125I-t-PA binding to HepG2 cells was abrogated while binding of 125I-t-PA to MHIC1 cells remained largely unaffected. The above data confirm the earlier observations that specific binding of 125I-t-PA to MHIC1 cells is PAI-1-independent, whereas it is PAI-1-dependent on HepG2 cells. Although not shown, 125I-DFP-treated t-PA does not form an SDS-stable complex with either native PAI-1 from HepG2 extracellular matrix or recombinant PAI-1. It also does not specifically bind to HepG2 cells.

Uptake and Degradation of 125I-t-PA by MHIC1 Cells—Previous studies (16) have shown that t-PA bound to HepG2 cells was subsequently endocytosed and degraded. To test if MHIC1 cells can also mediate t-PA uptake and degradation, binding of 125I-t-PA (3 nM) was performed at 4°C followed by incubation at 37°C with subsequent monitoring of the fate of radioactive ligand. After removal of unbound 125I-t-PA and replacement with prewarmed media containing a 6% SDS-polyacrylamide gel with a molecular mass greater than 40,000 daltons. In these experiments, the cross-linked material from both hepatoma cell lines appeared as a broad smear migrating on the top of a 6% SDS-polyacrylamide gel with a molecular mass greater than 600,000 daltons. Fig. 5 shows one such experiment with 125I-t-PA cross-linked to MHIC1 cells and analyzed under nonreducing (Fig. 5A) or reducing (Fig. 5B) conditions. In this experiment, the pattern of specific 125I-t-PA binding is similar to those observed in the experiments shown in Figs.

![Graph](image)

**FIG. 4.** Distribution of 125I-t-PA during a single cycle of endocytosis in MHIC1 cells. Binding of 3 nM 125I-t-PA to MHIC1 cells was performed at 4°C for 1.5 h in the presence or the absence of 1 μM unlabeled t-PA. After washing, cells were incubated at 37°C for selected intervals in the presence of unlabeled t-PA, 1 μM. Overlaying buffer was then added and cells were chilled rapidly prior to treatment with Pronase. Cell-associated ligand which was Pronase-sensitive (open circles) and Pronase-resistant (triangles) was quantified. Buffer-associated ligand (squares) as well as the fraction degraded (filled circles, percent trichloroacetic acid-soluble) were determined. Symbols represent the specific signals (difference in the absence and presence of unlabeled t-PA) and means of triplicate determinations.

**TABLE II**

<table>
<thead>
<tr>
<th>Competitor</th>
<th>% total binding</th>
<th>HepG2</th>
<th>MHIC1</th>
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<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>t-PA</td>
<td>15.1 ± 1.8</td>
<td>24.4 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>DFP-t-PA</td>
<td>86.5 ± 2.7</td>
<td>33.3 ± 6.1</td>
<td></td>
</tr>
<tr>
<td>α-PAI-1 IgG Fab</td>
<td>15.5 ± 2.4</td>
<td>84.5 ± 3.8</td>
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</table>

Inhibition of 125I-t-PA binding to hepatoma cells

Cultured hepatoma cell monolayers were preincubated with binding buffer alone or binding buffer containing a 1 μM concentration of the indicated competitors. After 1 h at 4°C, 3 nM 125I-t-PA was added to each binding assay. 125I-t-PA binding was continued for 1.5 h at 4°C before total bound ligand was determined. Each value represents the average of triplicate determinations.
was analyzed by SDS-PAGE under reducing conditions, with 0.5 mM DTSSP. Cell lysates without or with cross-linking were exposed to film for absence or the presence of excess unlabeled t-PA.

Binding of '*'I-t-PA (10 nM) to MHICl cells was able to immunoprecipitate lZ5I-t-PA. PAI-1 complex, although less efficiently than anti-t-PA antibody, which failed to immunoprecipitate any detectable high molecular weight cross-linked material. When the cross-linked material in the nonreducing gel is marked with a bracket. The positions of 125I-t-PA and 125I-t-PA:PAI-1 are indicated.

Although less efficiently than anti-t-PA antibody, anti-PAI-1 antibody could also immunoprecipitate bound 125I-t-PA:PAI-1 complex so formed was not very stable to the reducing reagent as it mostly disappeared under reducing conditions. Each cell lysate for immunoprecipitation was prepared from about 106 cells. Gels were exposed to film for 3 days prior to developing. The region of cross-linked material was marked with a bracket. The positions of 125I-t-PA and 125I-t-PA:PAI-1 are indicated.

1B and 2 indicating the majority of the available radioactive ligand prior to the addition of cross-linker was uncomplexed 125I-t-PA (Fig. 5A, lane 1). Although less efficiently than anti-t-PA antibody, anti-PAI-1 antibody could also immunoprecipitate bound 125I-t-PA:PAI-1 complex (Fig. 5A, lane 2). The 125I-t-PA:PAI-1 complex so formed was not very stable to the reducing reagent as it mostly disappeared under reducing conditions (Fig. 5B, lane 1). The cross-linked material can be seen on top of the nonreducing stacking gel with a small portion spread across the border between the stacking and separating gel (Fig. 5A, lane 5). Anti-PAI-1 antibody, which was able to immunoprecipitate 125I-t-PA:PAI-1 complex, failed to immunoprecipitate any detectable high molecular weight cross-linked material. When the cross-linked material was analyzed by SDS-PAGE under reducing conditions, uncomplexed 125I-t-PA but not 125I-t-PA:PAI-1 complex was observed (Fig. 5B, lane 5). The amount of 125I-t-PA cross-linked to its binding protein was approximately 20% of the total specifically bound 125I-t-PA ligand (Fig. 5B, compare lane 5 to lane 1). This percentage represents the cross-linking efficiency under these conditions, whereas the non-cross-linked ligands have dissociated from the cells during the 30-min cross-linking period (Fig. 5A, lane 5). The cross-linked material observed in this experiment was specific for t-PA since it was not present when the binding buffer contained an excess of unlabeled t-PA (Fig. 5, A and B, lanes 3 and 7). Cross-linking of 125I-t-PA to HepG2 cells showed similar results with a severalfold weaker signal (data not shown).

To determine the molecular size of the t-PA binding protein, cellular proteins were metabolically labeled with [35S]cysteine. After 5 h at 37°C, cell monolayers were washed and incubated in binding buffer without or with unlabeled t-PA followed by chemical cross-linking. After cross-linking, the cells were lysed and immunoprecipitated with one of the following antibodies: normal rabbit serum, anti-t-PA antibody, or anti-PAI-1 antibody. Fig. 6 shows an SDS-PAGE analysis of the immunoprecipitated products of MHICl cells under nonreducing (Fig. 6A) or reducing (Fig. 6B) conditions. Under nonreducing conditions, the cross-linked material (Fig. 6A, lane 5) appears as high molecular weight smear identical to that seen on top of the nonreducing stacking gel with a small portion spread across the border between the stacking and separating gel (Fig. 5A, lane 5). The positions of 125I-t-PA and 125I-t-PA:PAI-1 are indicated.

A: Non-reducing

Crosslinking: Excess unlabeled t-PA: No Yes
Antibody: t-PA:PAI-1

B: Reducing

Crosslinking: No Yes
Excess unlabeled t-PA: No Yes
Antibody: t-PA:PAI-1

Fig. 5. Chemical cross-linking of 125I-t-PA to MHICl cells. Binding of 125I-t-PA (10 nM) to MHICl cells was performed in the absence or the presence of excess unlabeled t-PA (1 μM). After 1.5 h at 4°C, cells were either lysed directly without cross-linking or were lysed after the ligand has been cross-linked to its binding protein with 0.5 mM DTSSP. Cell lysates without or with cross-linking were then immunoprecipitated with either anti-t-PA antibody or anti-PAI-1 antibody and were analyzed by SDS-PAGE (6% acrylamide) under nonreducing (A) or reducing (B) conditions. Each cell lysate for immunoprecipitation was prepared from about 106 cells. Gels were exposed to film for 3 days prior to developing. The region of cross-linked material was marked with a bracket. The positions of 125I-t-PA and 125I-t-PA:PAI-1 are indicated.

A: Non-reducing

Crosslinking: Excess unlabeled t-PA: No Yes
Antibody: t-PA:PAI-1

B: Reducing

Crosslinking: No Yes
Excess unlabeled t-PA: No Yes
Antibody: t-PA:PAI-1

Fig. 6. Chemical cross-linking of t-PA to [35S]cysteine-labeled MHICl cells. MHICl cells at about 80% confluence were metabolically labeled with [35S]cysteine (200 μCi/ml). Following incubation for 5 h at 37°C, cell monolayers were washed with prechilled binding buffer and were incubated at 4°C in the same buffer without or with unlabeled t-PA (10 nM). After 1 h of incubation, cells were cross-linked with 0.5 mM DTSSP. Cell lysates without or with unlabeled t-PA binding were immunoprecipitated with normal rabbit serum, anti-t-PA antibody, or anti-PAI-1 antibody. Fig. 6 shows an SDS-PAGE analysis of the immunoprecipitated products of MHICl cells under nonreducing (Fig. 6A) or reducing (Fig. 6B) conditions. Under nonreducing conditions, the cross-linked material (Fig. 6A, lane 5) appears as high molecular weight smear identical to that seen on top of the nonreducing stacking gel with a small portion spread across the border between the stacking and separating gel (Fig. 5A, lane 5). The positions of 125I-t-PA and 125I-t-PA:PAI-1 are indicated.

A: Non-reducing

Crosslinking: Excess unlabeled t-PA: No Yes
Antibody: t-PA:PAI-1

B: Reducing

Crosslinking: No Yes
Excess unlabeled t-PA: No Yes
Antibody: t-PA:PAI-1

Lane: 1 2 3 4 5 6

M_r (kDa)

400 - 205 - 117 - 80 - 50

125I-t-PA:PAI-1

125I-t-PA

Lane: 1 2 3 4 5 6

M_r (kDa)

400 - 205 - 117 - 80 - 50

1-PA:PAI-1

1-PA

Fig. 5. Chemical cross-linking of 125I-t-PA to MHICl cells. Binding of 125I-t-PA (10 nM) to MHICl cells was performed in the absence or the presence of excess unlabeled t-PA (1 μM). After 1.5 h at 4°C, cells were either lysed directly without cross-linking or were lysed after the ligand has been cross-linked to its binding protein with 0.5 mM DTSSP. Cell lysates without or with cross-linking were then immunoprecipitated with either anti-t-PA antibody or anti-PAI-1 antibody and were analyzed by SDS-PAGE (6% acrylamide) under nonreducing (A) or reducing (B) conditions. Each cell lysate for immunoprecipitation was prepared from about 106 cells. Gels were exposed to film for 3 days prior to developing. The region of cross-linked material was marked with a bracket. The positions of 125I-t-PA and 125I-t-PA:PAI-1 are indicated.

A: Non-reducing

Crosslinking: Excess unlabeled t-PA: No Yes
Antibody: t-PA:PAI-1

B: Reducing

Crosslinking: No Yes
Excess unlabeled t-PA: No Yes
Antibody: t-PA:PAI-1

Lane: 1 2 3 4 5 6

M_r (kDa)

400 - 205 - 117 - 80 - 50

125I-t-PA:PAI-1

125I-t-PA

Lane: 1 2 3 4 5 6

M_r (kDa)

400 - 205 - 117 - 80 - 50

1-PA:PAI-1

1-PA

Fig. 6. Chemical cross-linking of t-PA to [35S]cysteine-labeled MHICl cells. MHICl cells at about 80% confluence were metabolically labeled with [35S]cysteine (200 μCi/ml). Following incubation for 5 h at 37°C, cell monolayers were washed with prechilled binding buffer and were incubated at 4°C in the same buffer without or with unlabeled t-PA (10 nM). After 1 h of incubation, cells were cross-linked with 0.5 mM DTSSP. Cell lysates without or with unlabeled t-PA binding were immunoprecipitated with normal rabbit serum, anti-t-PA antibody (lanes 2 and 5), or anti-PAI-1 antibody (lanes 3 and 6) and were analyzed by SDS-PAGE (6% acrylamide) under nonreducing (A) or reducing (B) conditions. Gels were exposed to film for 3 days prior to developing. The positions of t-PA and t-PA:PAI-1 complex are indicated. The region of cross-linked material in the nonreducing gel is marked with a bracket, and the position of the t-PA binding protein in the reducing gel is indicated by an arrowhead.
with that seen in Fig. 5A. When the cross-linked material was reduced with 2-mercaptoethanol prior to analysis by SDS-PAGE, a specific protein band with an apparent molecular mass of 500,000 daltons was observed (Fig. 6B, lane 5). This t-PA binding protein cross-linked to its ligand was specifically immunoprecipitated by anti-t-PA antibody (Fig. 6, A and B, lane 5) but not by anti-PAI-1 antibody (Fig. 6, A and B, lane 6) indicating that most, if not all, of the cross-linked ligand was uncomplexed t-PA but not t-PA-PAI-1 complex. This result further confirmed the conclusion that binding of t-PA to rat hepatoma MHICl cells is PAI-1-independent. De novo synthesized t-PA and t-PA-PAI-1 complex were observed in the absence or the presence of exogenously added unlabeled t-PA. However, the 500-kDa t-PA binding protein was only detected following incubation of the monolayer with exogenously added unlabeled t-PA. This indicates that this binding protein requires binding of the exogenously added t-PA for its immunoprecipitation by anti-t-PA antibody. Since the 500-kDa t-PA binding protein was not observed in the absence of t-PA binding, the endogenous t-PA or t-PA-PAI-1 complex does not appear to specifically bind to this protein. Only t-PA but not PAI-1 was [35S]cysteine-labeled in this experiment due to the lack of cysteine in the mature PAI-1. Cross-linking of t-PA to [35S]-labeled HepG2 cells showed a binding protein band of similar molecular size with a more than 10-fold weaker signal (data not shown).

**DISCUSSION**

Hepatic parenchymal cells have been shown to be responsible for rapid in vivo clearance of circulating t-PA. However, the mechanism(s) responsible for this hepatic clearance are controversial and not well understood. It is generally believed, largely upon kinetic data, that the process involves receptor-mediated endocytosis. Previous studies from our laboratory have shown that the human hepatoma cell line HepG2 exhibits specific catabolism of 125I-t-PA following rapid endocytosis and delivery of ligand to intracellular degradative compartments (16). This system in HepG2 cells is modulated by PAI-1 (17). Similar results have also been reported by Wing et al. (25) utilizing primary human hepatocytes. They have described a specific clearance system for t-PA-PAI-1 complexes and showed, by kinetic analysis, that the system participated in t-PA clearance.

In this report we have identified and partially characterized a high affinity, PAI-1-independent t-PA specific surface receptor system on the rat hepatoma cell line MHICl. At 4 °C, binding of 125I-t-PA to MHICl cells is rapid, specific, and saturable (Fig. 1). Binding parameters at 4 °C are similar to those for other ligand-receptor systems that involve receptor-mediated endocytosis (26). The affinity (Kd = 4.1 nM) of the surface t-PA receptor system observed in this study is similar to that observed on HepG2 cells (16) (Kd = 3.7 nM). In addition, it is similar in magnitude to other reported high affinity receptor systems such as the asialoglycoprotein receptor system in HepG2 cells (21) (Kd = 7.0 nM) and the mannose receptor system described using rat alveolar macrophages (27) (Kd = 10 nM).

The nature of the t-PA receptor on MHICl cells appears distinct from that observed on HepG2 cells in that the presence of biologically active PAI-1. When PAI-1 was removed by elastase treatment or blocked by preincubating cell monolayers with anti-PAI-1 antibody, the majority of specific 125I-t-PA binding to MHICl cells remained unaffected. The same treatments, however, abolished most of the specific 125I-t-PA binding to HepG2 cells (Tables I and II). Specific binding of 125I-t-PA to MHICl cells does not require a functional active site, as DFP-treated t-PA can also compete for specific 125I-t-PA binding. Furthermore, we show herein that the rat hepatoma cell line MHICl also produces and secretes biologically active PAI-1 at a level similar to that seen with HepG2 cells (Fig. 3). In spite of this, when the binding species to the two hepatoma cell lines were compared, specific binding of 125I-t-PA to MHICl cells was primarily in the form of uncomplexed 125I-t-PA. This is in contrast to 125I-t-PA binding to HepG2 cells in which specific binding correlates with the formation of an SDS-stable 125I-t-PA-PAI-1 complex (Fig. 2). The PAI-1-independent t-PA receptor on MHICl cells could be either a different receptor system than that present on HepG2 cells or could reflect species or cell-type specific differences between these experimental systems.

The PAI-1-independent t-PA clearance system reported in this study on MHICl cells also exhibits receptor-mediated endocytosis of the bound ligand (Fig. 4). Several earlier studies have also suggested both t-PA active site and PAI-1-independent hepatocyte clearance mechanisms. Bakhit et al. (15) demonstrated 125I-t-PA uptake and degradation by isolated rat hepatocytes when PAI-1 availability was limited. Studies in mice (12) showed that clearance of FMSF, 125I-t-PA, which has a functional site-inhibited, was identical with the active enzyme. It is possible that both PAI-1-dependent and -independent t-PA receptor systems exist in vivo. Analysis of t-PA clearance by different hepatoma cell lines and by isolated primary hepatocytes from various species may be necessary to further examine this paradox.

Little is known about the molecular nature of the t-PA receptor. Hajjar and Hamel (28), utilizing ligand blotting analysis, reported that t-PA binds to a 40-kDa membrane protein on cultured human endothelial cells. However, to date, no molecular details are available for any hepatocyte t-PA binding protein. We now report the first biochemical data on a putative t-PA binding protein present on rat hepatocytes. In this study, we have shown by chemical cross-linking that the putative t-PA receptor on rat hepatoma cells has a molecular mass of about 500,000 daltons. Cross-linking of 125I-t-PA to unlabeled cells generated cross-linked products which migrated as a broad smear with the majority of the material localized to the top of a 6% SDS-polyacrylamide gel (Fig. 5). This could be due to a series of various numbers of 125I-t-PA molecules cross-linked to different receptors. Alternatively, the proximity of the ligand-receptor complexes on the cell surface may have resulted in cross-linking of the complexes to one another under these conditions. At the present time, the stoichiometry of the number of t-PA molecules bound and cross-linked to a single binding protein is not known. When unlabeled t-PA was cross-linked to [35S]cysteine-labeled MHICl cells and analyzed by SDS-PAGE, similar results were obtained (Fig. 6). The cross-linked ligand was predominantly uncomplexed t-PA since an anti-PAI-1 antibody, which immunoprecipitated the t-PA-PAI-1 complex, failed to immunoprecipitate any detectable ligand-binding protein complex. When the cross-linked material was reduced with 2-mercaptoethanol and analyzed on 6% SDS-PAGE, a distinct protein band with a molecular mass of approximately 500,000 daltons was observed (Fig. 6). The estimate of the molecular mass was only approximate, since molecular weight standards larger than 400,000 daltons were unavailable.

The t-PA receptor identified in this study does not appear to share characteristics associated with the urokinase receptor (29). Not only are the sizes of the two receptors distinctly different (u-PA receptor Mr = 58,000), the functions of the
two receptors are distinct as well. The receptor for urokinase plasminogen activator serves primarily to localize ligand at the cell surface with little subsequent internalization and degradation. This function is further supported by the fact that the urokinase receptor lacks a transmembrane domain and is anchored to the cell membrane by glycosyl-phosphatidylinositol (30). In contrast, binding of t-PA to its putative receptor leads to rapid ligand internalization and degradation, suggesting that the primary function of the t-PA receptor is catabolic in nature.

A low density lipoprotein receptor-related protein (LRP) with a molecular mass of 600 kDa (composed of 515-kDa and 85-kDa subunits) has been isolated and characterized by Herz et al. (31). This protein was shown to mediate binding and endocytosis of β-migrating very low density lipoproteins. Strickland et al. (32) have recently reported the identity of LRP to the receptor for α2-macroglobulin, thus demonstrating multiple functions of LRP. The apparent molecular mass of the putative t-PA receptor identified in our cross-linking experiments on MH,1C1 cells is similar to LRP. Both t-PA receptor and LRP are relatively liver-specific receptors and perform similar functions of receptor-mediated endocytosis. Therefore, the unusual, but similar, large sizes of these two membrane proteins may imply some relationship between them. Further experiments will be required to define the exact relationship, if any.

REFERENCES