Receptor-mediated Endocytosis of Tissue-type Plasminogen Activator by the Human Hepatoma Cell Line Hep G2*

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Receptor-mediated endocytosis of tissue-type plasminogen activator (t-PA) was characterized with the human hepatoma cell line Hep G2. At 4°C binding of 125I-t-PA to Hep G2 cells is rapid, specific, saturable, and reflective of a homogeneous population of 76,000 high-affinity surface sites per cell ($K_d = 3.7 \text{ nM}$). The kinetics of 125I-t-PA binding to its receptor are characterized by rate constants for association ($k_1 = 1.2 \times 10^6 \text{ min}^{-1} \text{ M}^{-1}$) and dissociation ($k_{-1} = 0.001 \text{ min}^{-1}$). A specific glycosylation pattern does not appear to be required for binding. Binding does not appear to be mediated by other recognized hepatic receptor systems. At 37°C a single cohort of bound 125I-t-PA molecules disappears rapidly from the cell surface. Ligand then accumulates intracellularly. Thereafter, the intracellular concentration of ligand declines simultaneously with the release of ligand degradation products into the media. In the continued presence of 125I-t-PA at 37°C the concentration of cell-associated ligand plateaus after 30 min with the concomitant appearance of low molecular weight 125I-labeled fragments in the media. Cumulative degradation then increases linearly with time. Under steady state conditions half-maximal ligand uptake and degradation is 26.6 nM and maximal rate of catabolism is 1.2 pmol/10^6 cells/h. At saturating ligand concentrations uptake and degradation by Hep G2 cells continue linearly for at least 6 h even in the absence of protein synthesis. During this period the cumulative ligand uptake exceeds the total cellular capacity of binding sites, consistent with receptor recycling. We conclude that t-PA clearance in human Hep G2 cells involves ligand binding, uptake, and degradation mediated by a novel high-capacity, high-affinity specific receptor system.

Under physiologic conditions tissue-type plasminogen activator (t-PA) initiates fibrinolysis by activating the zymogen plasminogen to the serine protease plasmin via proteolytic cleavage. Fibrin-bound plasminogen is preferentially activated by t-PA compared with free plasminogen. This characteristic of t-PA, itself a serine protease, has been exploited for clinical therapeutics. Exogenously administered t-PA is capable of eliciting prompt thrombolysis in therapeutic doses that do not produce marked fibrinolysis in experimental animals with induced coronary artery thrombosis (1) and in patients with evolving myocardial infarction (2-6). However, because the clearance of t-PA from the circulation is so rapid, continuous infusions have been required. In the several animal species studied (7-15) the half-life of t-PA in the circulation varies approximately inversely with the logarithm of body weight and ranges from 2-3 min in mouse to 5-10 min in man (16).

The liver appears to be the major site of removal and catabolism of t-PA (7-15). Exogenous t-PA delivered intravenously rapidly accumulates in liver and is subsequently degraded. Furthermore, the half-life of circulating t-PA is markedly prolonged in animals subjected to hepatectomy (10, 12). Neither the protease active site nor a specific glycosylation pattern appears to be a major determinant of hepatic recognition and degradation of t-PA in vivo (15), in perfused liver systems (13), or in isolated hepatocytes (17).

Information is limited regarding the particular cell type responsible for clearance of t-PA. In mice results of autoradiography suggest hepatic parenchymal cell rather than sinusoidal cell involvement (15). In rats a specific, high-affinity mechanism for t-PA catabolism has been identified in isolated hepatocyte preparations (17). In humans, however, the cell type responsible for hepatic recognition and catabolism of t-PA has not been identified. Moreover, the cellular events involved in t-PA clearance have not been characterized previously for any species.

The present study was designed to elucidate determinants of clearance of t-PA. With a particular objective of identifying clearance mechanisms, we characterized the continuous well-differentiated human hepatoma cell line Hep G2 (18) and other cell lines with respect to their capacity for specific catabolism of t-PA. We demonstrated that Hep G2 cells possess a homogeneous population of high-affinity specific receptors for t-PA and that, subsequent to surface binding, t-PA is internalized and degraded. Detailed characterization of these phenomena is provided in this report.

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1 The abbreviations used are: t-PA, tissue-type plasminogen activator; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
EXPERIMENTAL PROCEDURES

Materials

Recombinant t-PA expressed in Chinese hamster ovary cells was supplied by Genentech, Inc. (lot 5254-65). Human t-PA from colon fibroblasts was provided by Invitron (lot 38-009-15709). Preparation of asialoorosomucoid has been described previously (19). Mannosylated bovine serum albumin, epidermal growth factor, cytochrome c, cycloheximide, and bovine serum albumin were from Sigma; ovalbumin, transferrin, and Pronase (Streptomyces griseus) were from Calbiochem; insulin was from Novo; human serum albumin was from the American Red Cross. Iron-labeled transferrin (20) and hemoglobin-haptoglobin complexes (21) were prepared with the use of previously described methods. Sodium $^{[125]}$iodide was obtained from Amersham Corp. All other chemicals were analytical reagent grade.

Methods

Labeling of t-PA—Recombinant t-PA was labeled with $^{125}$I with chloramine T (22) as follows: 100 mg of t-PA (2 mg/ml in 0.1 M sodium phosphate containing 0.01% Tween 80, pH 7.5), 1 mM Na$_2$S$_2$O$_4$, and 10 mg of chloramine T were reacted for 30 s at room temperature. The reaction was quenched with basic sodium metabisulfite followed by exclusion chromatography on Sephadex G-10. The resulting product comigrated with unlabeled t-PA on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (Fig. 1). The separation pattern indicates that the parent t-PA is predominantly in the one-chain form with only a small amount of the two-chain form present. Under reducing conditions the single chain form migrates at approximately 68 kDa while the separated chains of the two-chain form migrate at 30–35 kDa. The silver stain also detected the cytochrome c (migrating near the dye front) which, together with 0.01% Tween 80, was added to the ligand buffer to limit nonspecific binding of t-PA to plasticware. In addition, the $^{125}$I-t-PA preserved antigenic determinants recognized in an enzyme-linked immunosorbent assay (23) from American Diagnostica, Inc., eluted from Sepharose 4B as a single peak of molecular mass of approximately 70 kDa, was 96–99% precipitable in 4% phosphotungstic acid/20% trichloroacetic acid, and retained 98 ± 20% S.D. of the fibrinolytic activity of unlabeled t-PA as assessed by fibrin plate assay (1). Each preparation (specific radioactivity 6 × 10$^6$ cpm/mg) was used within 1 week.

Cell Lines—The maintenance of Hep G2 cells has been described (24). The human hepatoma lines Hep 3B and FOCUS as well as the rat hepatoma line H4 were maintained similarly. Human histiocytic lymphoma-derived U937 cells were cultured in RPMI 1640 media containing 10% fetal bovine serum. Murine monocye-macrophage-derived J774-JK cells (gift of S. Diment, Washington University School of Medicine, St. Louis, MO) were maintained in Eagle’s minimum essential media supplemented with 10% fetal bovine serum.

Surface Binding Assays—Adherent cell lines were seeded into multiwell (diameter, 22 mm/well) tissue culture plates 3–5 days prior to assay. Supplemental media were added 12 h before each experiment. Plates were used when cells were 90–95% confluent. Prior to binding, media were removed and cells were washed four times by sequential immersion in 1-liter volumes of phosphate-buffered saline (PBS) at 4°C. To initiate binding, 1 ml of prechilled minimum essential media-Earle’s buffered with 20 mM Hepes at pH 7.3 and containing both 0.1 mg of cytochrome c as well as 0.01% Tween 80 to reduce nonspecific binding (24, 25) (collectively termed “binding media”) was added to each well along with a selected quantity of $^{125}$I-t-PA. After incubation at 4°C on a rocker table for the appropriate time media were removed, plates were rinsed four times by sequential immersion in 1-liter volumes of PBS at 4°C, and 1 ml of 1 M NaOH was added to each well. Radioactivity of the cell lysate was quantitated by gamma scintillation spectrometry.

Uptake, Internalization, and Degradation Assays—Tissue culture dishes were prepared and washed with PBS as above. To initiate uptake 1 ml of prewarmed binding media containing $^{125}$I-t-PA was added, and the dishes were placed in a 37°C incubator. At appropriate intervals media were removed from the wells. Adherent cells were then treated as described above (“Surface Binding Assay”) for determining cell associated radioactivity. Specific uptake was defined as the difference between total and nonspecific surface binding. Total surface binding of ligand was determined in the presence of $^{125}$I-t-PA alone. Nonspecific surface binding was determined by performing the binding assay in the presence of a 100-fold molar excess of unlabeled t-PA (generally 1 μM).

Disassociation Assay—$^{125}$I-t-PA was allowed to bind to Hep G2 cells in the standard manner at 4°C. After binding, cells were washed quickly in PBS as described above. Subsequently, cells were incubated in chilled binding media (1 ml per well) in the absence or presence of unlabeled t-PA. At the appropriate intervals the media were removed and cell-associated radioactivity was quantified.

Uptake, Internalization, and Degradation Assays—Tissue culture dishes were prepared and washed with PBS as above. To initiate uptake 1 ml of prewarmed binding media containing $^{125}$I-t-PA was added, and the dishes were placed in a 37°C incubator. At appropriate intervals media were removed from the wells. Adherent cells were then treated as described above (“Surface Binding Assay”) for determining cell associated radioactivity. Specific uptake was defined as the difference between cell-associated $^{125}$I-t-PA determined in the absence or presence of a 100-fold molar excess of unlabeled t-PA (generally 1 μM).

Degradation of ligand was defined as the appearance of radioactive ligand fragments that were soluble in 4% phosphotungstic acid/20% trichloroacetic acid. Specific degradation was defined as the difference between the specific activity at selected intervals. Degradation that was generated when the incubation with $^{125}$I-t-PA was performed in the presence or absence of 100-fold molar excess of unlabeled t-PA.

FIG. 1. SDS-PAGE analysis silver stain of t-PA. Lane a, recombinant human t-PA expressed in Chinese hamster ovary cells; lane b, silver stain of human t-PA from colon fibroblasts; lane c, autoradiogram of recombinant human t-PA labeled with $^{125}$I as described in the text. Gels were run under reducing conditions. Molecular mass markers are in kilodaltons.
General—For cell lines exhibiting $^{125}$I-t-PA binding cell protein content was determined. Cell number, determined by counting in a hemocytometer, was correlated with protein content. The Bradford (29) assay with bovine serum albumin as standard was employed to determine protein content of cell lysates. In a minimum of 10 determinations per cell line the protein content of $10^6$ cells was 490 $\mu$g (Hep G2), 250 $\mu$g (J774-JK), or 140 $\mu$g (H,S). Concentration of t-PA was determined both by enzyme-linked immunosorbent assay (American Diagnostics) with Bowes melanoma t-PA standards and by Bradford assay. SDS-PAGE was performed by the method of Laemmli (30).

RESULTS

Specificity of $^{125}$I-t-PA Binding by Hep G2 Cells—The human hepatoma cell line Hep G2 was characterized with respect to binding of $^{125}$I-t-PA. Binding isotherms were performed at 4°C to dissociate the binding phenomenon from possible concomitant uptake. As shown by the data in Table 1, Hep G2 cells bind $^{125}$I-t-PA at 4°C. Total binding is reduced by 66% in the presence of a 100-fold molar excess of unlabeled recombinant t-PA of Chinese hamster ovary cell origin and by 81% in the presence of excess t-PA produced by human colon fibroblasts. This reduction in $^{125}$I-t-PA binding is the result of competition for specific binding sites rather than being a dilutional phenomenon, as discussed below (see Fig. 2). Examination of the t-PA preparations by SDS-PAGE (Fig. 1) revealed no unsuspected contaminating proteins that potentially could be responsible for the observed inhibition of $^{125}$I-t-PA binding. Hep G2 cells harbor populations of several well-characterized specific receptors for polypeptide ligands, including asialoglycoproteins (24), transferrin (31, 32), and insulin (31, 32). Addition of a large excess of asialoorosomucoid, ferrotransferrin, or insulin to the incubation media failed to inhibit binding of $^{125}$I-t-PA in these cells (Table I). The heavy chain of t-PA contains an amino acid sequence with a high degree of homology to epidermal growth factor (33, 34). An excess of murine epidermal growth factor was ineffective in competing for $^{125}$I-t-PA binding sites. Several proteins with known hepatic clearance mechanisms including hemoglobin-haptoglobin and mannosylated bovine serum were also screened and found not to inhibit $^{125}$I-t-PA binding by Hep G2 cells. In contrast, both the well-characterized hepatic receptor systems for other ligands screened seem to participate in $^{125}$I-t-PA binding data of unlabeled t-PA, 1 $\mu$M, as described in the text. Table I summarizes specific binding by selected cell lines at 4°C. Tissue culture wells containing $1 \times 10^5$ cells were incubated for 2 h at 4°C with 1 ml of selected amounts of $^{125}$I-t-PA as described in the text. Total (C), nonspecific (O), and specific (A) binding were determined as described. Each symbol represents mean of nine determinations, triplicates of each concentration in three separate experiments. Standard errors are indicated for specific binding. Inset, Scatchard plot of specific binding. B/F, bound/free; B, fmol of bound/10^6 cells. Kd is the dissociation constant determined from the negative reciprocal slope, is 4.7 $nM$ for this selected experiment.

![Fig. 2. Saturation binding of $^{125}$I-t-PA to Hep G2 cells at 4°C. Tissue culture wells containing 1 x 10^5 cells were incubated for 2 h at 4°C with 1 ml of selected amounts of $^{125}$I-t-PA as described in the text. Total (C), nonspecific (O), and specific (A) binding were determined as described. Each symbol represents mean of nine determinations, triplicates of each concentration in three separate experiments. Standard errors are indicated for specific binding. Inset, Scatchard plot of specific binding. B/F, bound/free; B, fmol of bound/10^6 cells. Kd is the dissociation constant determined from the negative reciprocal slope, is 4.7 $nM$ for this selected experiment.](image-url)

### Table I

<table>
<thead>
<tr>
<th>Competition</th>
<th>125I-t-PA bound (fmol/10^6 cells)</th>
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<tbody>
<tr>
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<td>106 ± 5</td>
</tr>
<tr>
<td>t-PAcho</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>t-PAcho-b</td>
<td>20 ± 1</td>
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<tr>
<td>Asialoorosomucoid</td>
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<tr>
<td>Ferrotransferrin</td>
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<td>Insulin</td>
<td>112 ± 5</td>
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<td>Hemepidermal growth factor</td>
<td>105 ± 3</td>
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<tr>
<td>Mannosylated bovine serum albumin</td>
<td>132 ± 4</td>
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<td>Hemoglobin-haptoglobin</td>
<td>96 ± 2</td>
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<tr>
<td>Bovine serum albumin</td>
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<tr>
<td>Cytochrome c'</td>
<td>117 ± 1</td>
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<td>Cytochrome c'</td>
<td>127 ± 4</td>
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</table>

* $^{125}$I-t-PA binding was assessed in minimum essential media-Earle's containing 20 mM Hepes, 0.01% Tween 80, and 0.1 mg/ml bovine serum albumin.

**a** Cytochrome c is of Chinese hamster ovary cell origin.

**b** Cytochrome c is of human colon fibroblast origin.

**c** Binding was assessed in minimum essential media-Earle's containing 20 mM Hepes, 0.01% Tween 80, and 0.1 mg/ml bovine serum albumin.

**d** Cytochrome c' was present at 30 $\mu$M.

G2 cells. In addition, several other proteins, such as bovine serum albumin, that have been shown to reduce hepatocyte binding of certain ligands (19) failed to inhibit $^{125}$I-t-PA binding. These data indicate that $^{125}$I-t-PA binds specifically to Hep G2 cells. None of the well-characterized hepatic receptor systems for other ligands screened seem to participate in $^{125}$I-t-PA binding by Hep G2 cells.

Specific Binding of $^{125}$I-t-PA by Other Cells—In light of previously reported rapid hepatic accumulation and degradation of exogenously administered t-PA in several species (7-15), selected representative cell types possessing differentiated hepatocyte functions or specific monocyte-macrophage characteristics were screened for their capability to bind $^{125}$I-t-PA (Table II). Neither of the other two human hepatoma cell lines tested, Hep 3B and FOCUS, was capable of binding $^{125}$I-t-PA in a specific manner. No specific binding was detected by the human histiocytic lymphoma-derived U937 line, a cell line known to exhibit specific binding of the plasminogen activator urokinase (35-37). In contrast, both the well-differentiated rat hepatoma H4S and the murine monocyte-
macrophage J774-JK cell lines exhibited specific binding of this ligand. Analysis of binding data yielded estimates of the number of binding sites per cell and the dissociation constant $K_d$ (see below). Estimates for these parameters were similar to those determined for Hep G2 cells.

**Saturation Binding of $^{125}$I-t-PA by Hep G2 Cells at 4 °C—** Binding of $^{125}$I-t-PA by Hep G2 cells was examined over a ligand concentration range from $10^{-11}$ to $10^{-7}$ M in isotherms performed at 4 °C. Saturation of specific binding was observed at $^{125}$I-t-PA concentrations in excess of approximately 10 nM (Fig. 2). Scatchard (38) analysis of the binding data from each of 15 such isotherms performed yielded an estimate of 76,000 ± 23,000 S.D. homogeneous high-affinity surface binding sites per cell with $K_d$ of 3.7 ± 0.9 nM S.D. (cf. $K_d$ of 4.7 nM for the particular isotherm depicted in Fig. 2). $K_d$ was determined from the negative reciprocal slope of the bound to free ligand ratio versus bound ligand graph. For each isotherm the slope was determined by least squares analysis.

To determine total number of cellular binding sites (i.e. cell surface plus internal sites) for $^{125}$I-t-PA similar isotherms were performed in Hep G2 cells following their prior exposure to 0.1% saponin at 4 °C. Treatment with this nonionic detergent renders cell membranes permeable to macromolecules (up to 200 kDa) without solubilization of integral membrane proteins (27, 39) such as the asialoglycoprotein receptor (26). Scatchard analysis of $^{125}$I-t-PA binding data from five separate isotherms measured at 4 °C in saponin-treated Hep G2 cells indicates a single population of 488,000 ± 131,000 S.D. binding sites per cell with $K_d$ of 13.4 ± 2.1 nM S.D. (data not shown).

**Effect of Time on $^{125}$I-t-PA Binding by Hep G2 Cells—** The time course for $^{125}$I-t-PA binding by Hep G2 cells at 4 °C was characterized. In the presence of a saturating concentration of ligand, the extent of specific binding reaches a plateau in approximately 1.5 h and remains constant over the ensuing 4 h (Fig. 3). Accordingly, saturation binding experiments were performed with 2-h incubation periods.

**Rate of $^{125}$I-t-PA Binding by Hep G2 Cells—** To characterize the kinetics of approach to equilibrium for $^{125}$I-t-PA binding by Hep G2 cells at 4 °C, the extent of binding at various intervals was examined at six saturating ligand concentrations (Fig. 4A). As a first approximation the association of $^{125}$I-t-PA with its receptor was assumed to be a reversible bimolecular process. The concentration of free ligand ([L]) was assumed to be sufficiently large so that it remained effectively constant during the incubation. With these assumptions the rate of ligand-receptor association could be considered a pseudo-first-order process with respect to unoccupied receptors. Then, an observed rate constant $k_{on}$ can be defined as $k_{on}[L] + k_{-1}$, with $k_{on}$ as the forward rate constant for ligand association with its receptor and $k_{-1}$ as the reverse rate constant for dissociation of the ligand-receptor complex. For each ligand concentration characterized, the $k_{on}$ value could be determined as the negative of the slope of ln(1 - $B_t$ / $B_{max}$) plotted versus $t$, with $B_t$ being ligand bound at time $t$ and $B_{max}$ being maximum binding capacity, i.e. 123 fmol/10⁶ cells in this study. This transformation was applied to the data in Fig. 4A to obtain the straight lines depicted in Fig. 4B. The linearity observed is consistent with a pseudo-first-order kinetics for the association of $^{125}$I-t-PA with its binding site. The time required to achieve half-maximal occupancy of surface binding sites, $t_{1/2}$ ($= 0.69/k_{on}$), decreases from 754 to 65 min as the concentration of $^{125}$I-t-PA increases from 0.5 to 8 pmol/ml. When $k_{on}$ is plotted versus [L] a straight line is obtained whose slope is $k_t$ and whose y intercept is $k_{-1}$. These data are presented in Fig. 4C. Here $k_t$ = $1.2 \times 10^6$ min⁻¹ M⁻¹ and $k_{-1}$ = 0.001 min⁻¹. Symbols are as in A.

**FIG. 4. Rate of specific $^{125}$I-t-PA binding to Hep G2 cells at 4 °C—** A. tissue culture wells containing $1 \times 10^6$ cells were incubated at 4 °C for selected intervals in the presence of 0.5 ( ), 1.0 ( ), 2.0 (A), 4.0 ( ), 6.0 (■) or 8.0 (□) picomoles of $^{125}$I-t-PA in 1 ml. At the times indicated cells were washed and analyzed as described in the text to determine specific binding. Each symbol represents mean of duplicate determinations. $B_t$, the data in $A$, were transformed as described in the text and replotted as a function of fraction of maximal binding. $B_t$, ligand bound at time $t$; $B_{max}$, maximal binding (123 pmol/10⁶ cells). The time for half-maximal binding, $t_{1/2}$, was determined as described in the text and is also depicted for each $^{125}$I-t-PA concentration. Symbols are as in B. C, the pseudo-first-order rate constants, $k_{on}$ for approach to equilibrium were obtained from the slopes of the lines in $B$ as described in the text. Rate constants for $^{125}$I-t-PA receptor association and dissociation were obtained from the slope and y intercept, respectively, of the $k_{on}$ versus ligand concentration plot as discussed in the text. Here $k_t$ = $1.2 \times 10^6$ min⁻¹ M⁻¹ and $k_{-1}$ = 0.001 min⁻¹. Symbols are as in A.
pendent determination was made of the rate of dissociation of prebound 125I-t-PA from Hep G2 cells. Cells were allowed to bind 125I-t-PA at 4 °C in the standard manner, rinsed to remove unbound ligand, and then incubated further in pre-chilled binding media in the absence or presence of unlabeled t-PA. Radioactivity remaining cell-associated was quantitated as a function of time (Fig. 5). Under the experimental conditions employed, no difference was observed in amount of radioactivity remaining cell-associated regardless of whether or not excess unlabeled t-PA was present in the media. Thus, it is unlikely that reassociation of dissociated ligand with unoccupied receptors occurred to any appreciable extent. A plot of $\ln(B_t/B_0)$ versus $t$, where $B_t$ is ligand remaining bound at time $t$ and $B_0$ is that bound at time 0, is shown in the lower panel of Fig. 5. The observed linearity is consistent with a first-order dissociation process. The negative slope of the $\ln(B_t/B_0)$ curve is $k_1$, the rate constant with approximately 50% of bound 125I-t-PA appearing in the media within 30 min (data not shown).

**Uptake and Degradation of 125I-t-PA by Hep G2 Cells**—In addition to binding, Hep G2 cells internalize and degrade 125I-t-PA at 37 °C. The rate of uptake and degradation was examined initially at subsaturating concentrations of ligand.

Uptake of 125I-t-PA by Hep G2 cells proceeds linearly for approximately 30 min at 37 °C (Fig. 6). Thereafter, cell-associated 125I-t-PA plateaus at a steady state level that is maintained for at least 3 h. During the initial 30 min of ligand uptake the medium is devoid of released low molecular weight 125I-t-PA-degraded fragments that are soluble in cold 4% phosphotungstic acid/20% trichloroacetic acid. Only after this lag phase does degraded ligand appear in the media. Degradation subsequently continues in a linear fashion for at least 3 h. The change of total cell-associated and released degraded t-PA appearing in the media within 30 min (data not shown).

FIG. 6. Rate of specific uptake and degradation of 125I-t-PA by Hep G2 cells at 37 °C. Tissue culture wells containing 1 x 10^6 cells were incubated at 37 °C with 1 ml of 125I-t-PA, 3 nM, in the presence or absence of a 100-fold molar excess of unlabeled t-PA for selected intervals as described in the text. At the indicated times media were removed and analyzed for released specific degradation products (O) as described. Specific cell-associated ligand (●) was determined in the standard manner. The sum (∆) of these two was used to define total specific uptake and degradation. Symbols represent the means of triplicate determinations.

FIG. 7. Gel chromatography of 125I-t-PA following degradation by Hep G2 cells. Hep G2 cells were incubated for 2 h at 37 °C in the presence of 125I-t-PA to allow steady state uptake of ligand and release of degraded fragments. An aliquot of media was then explored by gel filtration on a Bio-Gel P-6 column. Cytocrome c and phenol red were used to mark the void and included volumes respectively.

ligand with time can be used to define an overall rate of 125I-t-PA catabolism by Hep G2 cells at this ligand concentration. The value obtained, 0.10 pmol/10^6 cells/h, remains constant throughout several hours of observation. No degraded ligand is detected when 125I-t-PA is incubated at 37 °C for 4 h in identical cell-free wells.

To determine the extent to which internalized 125I-t-PA is degraded by Hep G2 cells, media aliquots were analyzed by gel filtration on a Bio-Gel P-6 column (Fig. 7). When 125I-t-PA was incubated at 37 °C for 2 h with Hep G2 cells, only a moderate amount of the radiolabel in the media (38% by peak area) eluted in the void volume, whereas a substantial amount (62%) appeared as low molecular weight fragments eluting in approximately the same fractions as phenol red (molecular weight 354). Generation of such small 125I-labeled fragments is consistent with degradation of 125I-t-PA to the level of small peptides or individual amino acids. When an aliquot of media from the same incubation was subjected to precipitation by 4% phosphotungstic acid/20% trichloroacetic acid, 40.2% of
the radiolabel was precipitated. The 59.8% of the label that was acid-soluble correlated closely with the proportion of low molecular weight fragments detected by gel filtration.

To determine whether uptake and degradation are saturable, these processes were characterized at 4 h in isotherms at 37 °C over a ligand concentration range from 10⁻¹⁰ to 10⁻⁶ M. Cell-associated ligand, degraded ligand, and their sum, all plateau beyond a ¹²⁵I-t-PA concentration of about 40 pmol/ml (Fig. 8). Inspection of graphs of data (not shown) from four separate steady state isotherms performed yielded estimates of 26.6 ± 18.5 nM S.D. for half-maximal ligand uptake and degradation and 1.2 ± 1.0 pmol/10⁶ cells/h for maximal rate of ¹²⁵I-t-PA uptake and degradation.

To determine whether capacity for ¹²⁵I-t-PA uptake and degradation in Hep G2 cells exceeds the total cellular binding capacity, these phenomena were characterized in cycloheximide-treated cells (Fig. 9). At cycloheximide concentrations employed [³H]leucine incorporation into both cell-associated and secreted proteins is inhibited by more than 99.9% (40). Neither saturation binding of ¹²⁵I-t-PA at 4 °C nor uptake and degradation at 37 °C for 6 h is affected under these conditions. At the rate of uptake and degradation observed (0.25 pmol/10⁶ cells/h) the capacity of the entire complement of surface and internal receptors would be exhausted after only 3 h. However, ligand uptake and degradation continue unabated in the absence of protein synthesis for at least 6 h. These data support the possibility of receptor sites recycling.

Effect of Time on ¹²⁵I-t-PA Distribution in Hep G2 Cells—To define the location of ¹²⁵I-t-PA during a single cycle of endocytosis in Hep G2 cells, the subsequent fate of a pre-bound cohort of ligand molecules was examined (Fig. 10). Ligand was incubated with Hep G2 cells for 2 h at 4 °C to allow surface binding. After removal of unbound ¹²⁵I-t-PA and replacement with prewarmed media containing unlabeled ligand, cells were maintained at 37 °C for selected intervals to allow ligand uptake. Media were removed and cells were quickly cooled to arrest further ligand internalization. Cells then were treated with Pronase at 4 °C to remove residual surface ligand. Internalized ligand was recovered in the cell pellet. Pre-bound ligand disappeared rapidly from the cell surface (protease-sensitive ligand) concurrent with its partitioning into undegraded dissociated (media-associated ligand) and internalized (protease-resistant ligand) fractions. Loss of 50% of surface-bound ligand occurred within 5 min. Concurrently, ligand was internalized rapidly and reached a peak level at 12-15 min before subsequently declining. Ligand appeared in the media simultaneously with the disappearance of cell-surface ligand. Initially, all media-associated ligand was precipitable in cold 4% phosphotungstic acid/20% trichloroacetic acid. It probably represented dissociation of undegraded surface-bound ¹²⁵I-t-PA. Released small molecular weight ligand degradation fragments (soluble in cold 4% phosphotungstic acid/20% trichloroacetic acid) were detected only after a delay of 15-20 min. Their appearance coincided with disappearance of internalized ligand.

**DISCUSSION**

Human t-PA is cleared from the circulation primarily by hepatic mechanisms in man (7). However, the cell type(s)
The parameters characterizing binding of t-PA by Hep G2 cells at 4°C can be compared with those for other representative ligand-receptor systems that involve receptor-mediated endocytosis (for review see Goldstein et al. (46)). The affinity of the cell surface receptor observed here, 3.7 nM, is similar in magnitude to that reported for other high-affinity receptor systems such as transferrin and its receptor, 4.4 nM (31), and asialoorosomucoid and the asialoglycoprotein receptor, 7.0 nM (24), in Hep G2 cells. It is similar also to the binding affinity at 4°C of the mannose receptor for 125I-mannose-bovine serum albumin, 10 nM, in rat alveolar macrophages (47).

Distribution of receptors can be compared as well. In this study 16% of unoccupied t-PA receptors were found to be on the cell surface, similarly, in rat alveolar macrophages 20–30% of mannose receptors are estimated to be on the cell surface (47) while in Hep G2 cells 30% of transferrin receptors are on the cell surface (31). In contrast, a somewhat higher proportion (35–40%) of asialoglycoprotein receptors are on the cell surface in Hep G2 cells (48). Rate constants for ligand-receptor association and dissociation reported here (k₁ = 1.2 × 10⁶ min⁻¹ M⁻¹, k₋₁ = 0.001 min⁻¹) are similar to those reported for asialoorosomucoid and its receptor (k₁ = 0.95 × 10⁶ min⁻¹ M⁻¹, k₋₁ = 0.001 min⁻¹) in Hep G2 cells (24).

Comparison of specific t-PA uptake by Hep G2 cells at 37°C with specific ligand uptake in other systems is informative. In this study half-maximal ligand uptake and degradation under steady state conditions, 26.6 nM, was found to be of similar magnitude to the Kᵦₚₑₘₐₜ of 40 nM reported by Stahl et al. (47) for rat alveolar macrophage uptake of 125I-mannose-bovine serum albumin and to the half-maximal uptake of 10 nM reported by Bakhit et al. (17) for rat hepatocyte uptake of 125I-t-PA. The rate of steady state uptake and degradation of 125I-t-PA by Hep G2 cells (Fig. 6) parallels that observed in several other specific ligand degradation systems. Of particular note, cell release of degraded t-PA fragments was evident only after a lag period of 30 min. A similar delay has been reported for release of degradation products of other ligands known to be degraded in lysosomes (40, 49). Whether t-PA shares the same endocytic pathway as that of other ligands and is delivered to lysosomes for degradation has not yet been determined. However, the observation by Bakhit et al. (17) that lysosomotropic amines inhibit degradation of t-PA by rat hepatocytes (17) is consistent with this possibility.

Internalization by Hep G2 cells of a single cohort of prebound t-PA molecules (Fig. 10) paralleled the fate of prebound ligand in several other specific ligand-receptor systems (28, 47). Maximal rate of t-PA uptake in our study (1.2 ± 1.0 pmol/10⁶ cells/h) was remarkably similar to the previously reported maximal rate of asialoorosomucoid uptake (1.2–1.8 pmol/10⁶ cells/h) by Hep G2 cells (24). Furthermore, when protein synthesis was inhibited with cycloheximide (40), t-PA degradation by Hep G2 cells continued at an undiminished rate for at least 6 h (Fig. 9), by which time the binding capacity of the total cell complement of receptors would be far exceeded. This observation is consistent with receptor recycling.

The receptor identified in this study does not seem to share characteristics of the recently described urokinase receptor (for review see Blasi et al. (50)). The receptor for urokinase plasminogen activator serves primarily to localize the ligand at cell surfaces. Binding to receptor leads to little, if any, subsequent internalization and degradation of ligand. In contradistinction, binding of t-PA to its receptor leads rapidly to ligand internalization and degradation. The amino-terminal fragment of urokinase appears to mediate its receptor binding. Low molecular weight urokinase, a proteolytically active de-
Receptor-mediated Endocytosis of t-PA by Hep G2 Cells

References