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Low density lipoprotein receptor-related protein/α_2-macroglobulin receptor is an hepatic receptor for tissue-type plasminogen activator

(hepatocyte/clearance/endocytosis/39-kDa protein)

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ABSTRACT **Tissue-type plasminogen activator (t-PA), a serine protease that catalyzes the initial and rate-limiting step in the fibrinolytic cascade, is cleared rapidly *in vivo* by the liver. Using chemical crosslinking, we have recently identified a plasminogen-activator inhibitor type 1 (PAI-1)-independent t-PA clearance receptor on rat hepatoma MH_1C_1 cells with a relative molecular mass of ≈500 kDa. Another recently identified membrane receptor, low density lipoprotein receptor-related protein/α_2-macroglobulin receptor (LRP/α_2MR), was also detected on MH_1C_1 hepatoma cells by using immunoprecipitation with anti-LRP/α_2MR antibody. When analyzed by SDS/PAGE, we found the t-PA receptor identified on MH_1C_1 cells comigrated with the large subunit of LRP/α_2MR. The t-PA receptor was immunoprecipitated by an anti-LRP/α_2MR antibody after chemical crosslinking of specifically bound ^{125}I-labeled t-PA to its receptor. Through chemical crosslinking studies, we found that t-PA and methylamine-activated α_2-macroglobulin could bind to LRP/α_2MR simultaneously without competing with one another for binding, suggesting that the two ligands bound to two independent sites on the LRP/α_2MR molecule. Furthermore, a 39-kDa protein, which modulates ligand binding to LRP/α_2MR, was also found to inhibit t-PA binding to its receptor. These data thus show that the t-PA clearance receptor identified on MH_1C_1 hepatoma cells is LRP/α_2MR.**

Tissue-type plasminogen activator (t-PA) plays an essential role in the fibrinolytic process by catalyzing the conversion of the zymogen plasminogen to plasmin (1, 2). The plasmin thus activated can proteolytically degrade the fibrin network associated with blood clots. The role of t-PA as an initiator in thrombolysis has been exploited as a thrombolytic agent (3–5). However, the half-life of t-PA in plasma is very short, ranging from 1–4 min in rodents (6–11) to 5–10 min in humans (12, 13).

In vivo studies have shown that the liver is the major organ responsible for t-PA clearance (6–11). Kinetic studies have suggested that specific receptors exhibiting characteristics of receptor-mediated endocytosis are involved in the clearance process. At least two classes of clearance mechanisms exist: a noncarbohydrate-mediated pathway via hepatocytes and a carbohydrate-mediated pathway via endothelial cells (14). In hepatocytes, detailed studies of the clearance mechanism have revealed two types of clearance receptor: plasminogen-activator inhibitor type 1 (PAI-1)-dependent (15–20) and -independent (11, 21) receptors. Recent studies from our laboratory have identified a PAI-1-independent t-PA receptor on rat hepatoma MH_1C_1 cells (22). Binding of t-PA to this receptor occurs predominantly via the free form and requires neither the protease active site nor the presence of bioactive PAI-1. This situation contrasts with the PAI-1-dependent t-PA receptor we have previously characterized on human hepatoma HepG2 cells, for which the predominant specific-binding species is the t-PA–PAI-1 complex (16–19, 22). Similar to the PAI-1-dependent receptor, the PAI-1-independent t-PA receptor also exhibits receptor-mediated endocytosis and degradation of t-PA with a rapid kinetics (22). Using chemical crosslinking and immunoprecipitation with anti-t-PA antibody, we have identified this receptor as a 500-kDa polypeptide (22).

A low density lipoprotein receptor-related protein/α_2-macroglobulin receptor (LRP/α_2MR, hereafter abbreviated as LRP) with a molecular mass of 600 kDa (composed of 515-kDa and 85-kDa subunits) has recently been isolated and characterized (23–26). This plasma-membrane receptor was shown to mediate the binding and endocytosis of both apoprotein E-enriched β-migrating very low density lipoprotein (24, 27) and protease- or methylamine-activated α_2-macroglobulin (α_2M*) (28). These observations thus demonstrate the multifunctional nature of LRP. The apparent molecular mass of the t-PA receptor identified on rat hepatoma cells resembles that of the large subunit of LRP. In addition, both t-PA receptor and LRP are relatively liver-specific and perform similar functions of receptor-mediated protein clearance. These similarities between the two receptors prompted us to examine the identity and possible relationship between them. Our data strongly suggest that the t-PA receptor identified on MH_1C_1 cells is identical to LRP.

MATERIALS AND METHODS

Cell Culture. Rat hepatoma MH_1C_1 cells were cultured in Earle's minimum essential medium/10% fetal calf serum/2 mM L-glutamine/penicillin at 100 units/ml/streptomycin at 100 μg/ml and were incubated at 37°C in humidified air/5% CO_2. Cultures were supplemented with fresh media 12 hr before use. Cell monolayers were generally used at 80–90% confluence.

Metabolic Labeling. Cells growing in 10-cm dishes at ≈80% confluence were washed two times with Earle's minimum essential medium lacking L-methionine and containing 2 mM L-glutamine and were incubated for 30 min at 37°C in two changes of the above medium. Metabolic labeling was initi-

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Abbreviations: t-PA, tissue-type plasminogen activator; PAI-1, plasminogen-activator inhibitor type 1; LRP, low density lipoprotein receptor-related protein; α_2MR, α_2-macroglobulin receptor; α_2M*, protease- or methylamine-activated α_2-macroglobulin; GST, glutathione-*S*-transferase; LDL, low density lipoprotein; PBSc, phosphate buffered saline/1 mM $CaCl_2$/0.5 mM $MgCl_2$.

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ated by addition of the above medium/[^{35}S]methionine at 200 μCi/ml (1 Ci = 37 GBq). After incubation for 5 hr at 37°C, cell monolayers were washed with binding buffer and used for t-PA binding and chemical crosslinking experiments.

Ligand-Binding Assays. Cells were seeded into either 10-cm dishes or multiwell (12 wells per plate) culture plates 2–3 days before assay. Ligand-binding buffer for t-PA and glutathione-*S*-transferase (GST)–39-kDa protein was composed of PBSc (phosphate-buffered saline/1 mM $CaCl_2$/0.5 mM $MgCl_2$) supplemented with 10 mM ε-amino-1-caproic acid (22). Dulbecco's modified Eagle's medium/bovine serum albumin at 6 mg/ml/5 mM $CaCl_2$ was used for α_2M* binding. When binding of multiple ligands was examined simultaneously, the binding buffer for α_2M* was used but was supplemented with 10 mM ε-amino-1-caproic acid. Cell monolayers were washed three times with binding buffer and were incubated with either unlabeled or ^{125}I-labeled ligands in binding buffer for 1 hr at 4°C. Cells were then washed three times with PBSc to remove unbound ligands and were either lysed directly to determine specific binding or used for chemical crosslinking. When ^{125}I-labeled ligands were used to quantitate ligand binding, radioactivity was quantified by γ scintillation spectrometry. Protein iodination was done as described before (22). Single-chain recombinant human t-PA expressed in Chinese hamster ovary cells was supplied by Genentech (lot 9124AX). Recombinant GST and GST–39-kDa protein were provided by Joachim Herz (University of Texas Southwestern Medical Center, Dallas).

Chemical Crosslinking. After ligand binding at 4°C, each cell monolayer was washed three times with PBSc and was incubated with PBSc/0.5 mM water-soluble, thio-cleavable crosslinker dithiobis(sulfosuccinimidylpropionate) (Pierce). After 30 min at 4°C, the crosslinking reaction was quenched by washing the cell monolayer two times with Tris-buffered saline. Cells were then solubilized in PBSc/1% (vol/vol) Triton X-100/1 mM phenylmethylsulfonyl fluoride (lysis buffer) for 30 min at 4°C and were used for immunoprecipitation.

Immunoprecipitation. Cell lysates after chemical crosslinking were mixed with equal volume of PBSc/1% (vol/vol) Triton X-100/0.5% (wt/vol) sodium deoxycholate/1% (wt/vol) SDS/0.5% (wt/vol) human serum albumin/0.5% (wt/vol) bovine serum albumin/1 mM phenylmethylsulfonyl fluoride (immunomix). Primary antibody was added, and the samples were rocked overnight at 4°C. The amounts of antibody used in immunoprecipitation (10 μg of total rabbit IgG, 2 μg of affinity-purified IgG, or 10 μl of rabbit antiserum) were added in excess of the corresponding antigens, and the resulting immunoprecipitations were, therefore, quantitative. Protein A–agarose beads (Repligen, Cambridge, MA) were then used to precipitate antigen–antibody complexes by incubation at room temperature for 1 hr. Nonspecifically bound radioactivity was removed by washing protein A–agarose beads three times with immunomix and then washing three times with PBSc. The immunoprecipitated material was released from the beads by boiling each sample for 5 min in 62.5 mM Tris·HCl, pH 6.8/2% (wt/vol) SDS/10% (vol/vol) glycerol (Laemmli sample buffer) (29) and was analyzed by SDS/PAGE. If the immunoprecipitated material was analyzed under reducing conditions, 5% (vol/vol) 2-mercaptoethanol was included in the Laemmli sample buffer. Rabbit anti-t-PA and anti-PAI-1 polyclonal antibodies were described earlier (22), as was rabbit anti-LRP polyclonal antibody (30).

SDS/PAGE and Autoradiography. Samples from immunoprecipitation were analyzed by SDS/PAGE using 5% (wt/vol) polyacrylamide under nonreducing or reducing conditions. The following prestained molecular mass standards from Bio-Rad were used: myosin, 205 kDa; β-galactosidase, 117 kDa; bovine serum albumin, 80 kDa. Laminin was also used as a molecular mass marker (large subunit, 400 kDa). For fluorography of ^{35}S-labeled proteins, gels were impregnated with Amplify (Amersham) before being dried. Autoradiography was done with Hyperfilm-MP (Amersham). Films were generally placed at −70°C for 2–3 days before developing.

RESULTS

Identity between LRP and the t-PA receptor was first investigated by comparing the molecular sizes of the two proteins. With chemical crosslinking and immunoprecipitation, both proteins were identified and characterized on MH_1C_1 cells. Fig. 1*a* shows an experiment in which MH_1C_1 cells were metabolically labeled with [^{35}S]methionine before t-PA binding. Chemical crosslinking was then done by using a thio-cleavable crosslinker followed by immunoprecipitation with selected antibodies and analysis via SDS/PAGE under reducing conditions. The t-PA receptor complexed to its ligand was detected by anti-t-PA antibody as a single protein band with a molecular mass of ≈500 kDa. Although t-PA–PAI-1 complex binding could be detected on MH_1C_1 cells as ≈5% of the total specific binding (22), the complex did not appear to bind to this t-PA receptor because anti-PAI-1 antibody, which could immunoprecipitate t-PA–PAI-1 complex, failed to detect any t-PA receptor molecule. This observation is consistent with our previous findings that the t-PA receptor identified on MH_1C_1 hepatoma cells recognizes t-PA independent of the presence of PAI-1 (22). The large subunit of LRP was also detected on this cell line by an affinity-purified anti-LRP antibody. To compare the exact molecular size of the t-PA receptor to that of the large subunit of LRP, we applied similar amounts of radioactive material immunoprecipitated by anti-t-PA antibody or by anti-LRP antibody to SDS/PAGE independently or together. As shown in Fig. 1*b*, the two proteins migrated to the same position (lanes 1 and 2) and comigrated as a single band after mixing (lane 3). These experiments confirmed that t-PA receptor and the large subunit of LRP have identical mobilities on SDS/PAGE. Although the small subunit of LRP (85 kDa) was also detected by anti-LRP antibody (data not shown), no specific band was observed at this position after immunoprecipitation with anti-t-PA antibody. It is likely that the small subunit of LRP was not crosslinked to the large subunit to which t-PA bound

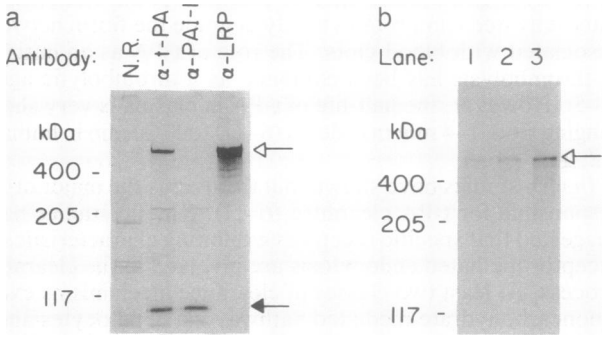

FIG. 1. Comparison of t-PA receptor and LRP from MH_1C_1 cells. (*a*) t-PA binding to [^{35}S]methionine metabolically labeled MH_1C_1 cells followed by chemical crosslinking and immunoprecipitation with normal rabbit serum (N.R.), anti-t-PA antibody (α-t-PA), anti-PAI-1 antibody (α-PAI-1), or anti-LRP antibody (α-LRP). The immunoprecipitated material was analyzed on SDS/5% PAGE under reducing conditions. (*b*) Size comparison of t-PA receptor to LRP. As in *a*, material was immunoprecipitated by using anti-t-PA antibody (lane 1) or anti-LRP antibody (lane 2) and analyzed by SDS/5% PAGE. Lane 3 shows the mixture of materials applied in lanes 1 and 2. Positions of t-PA receptor/LRP are indicated by open arrows; positions of t-PA–PAI-1 complex are indicated by a closed arrow.

and was, therefore, dissociated from the t-PA–large subunit complex during immunoprecipitation with the stringent washing conditions.

To examine whether the anti-LRP antibody could recognize the t-PA receptor complexed to t-PA, we performed ^{125}I-labeled t-PA binding to MH_1C_1 cells with or without excess unlabeled t-PA, followed by chemical crosslinking and immunoprecipitation. Fig. 2*a* shows the autoradiography of the immunoprecipitated products analyzed by SDS/PAGE under nonreducing conditions. The t-PA receptor complexed to ^{125}I-labeled t-PA was immunoprecipitated by both anti-t-PA antibody and anti-LRP antibody but not by anti-PAI-1 antibody. This material migrated at the top of the 5% gel with a molecular mass >600 kDa. Because anti-LRP antibody does not crossreact with ^{125}I-labeled t-PA (data not shown), the immunoprecipitated ^{125}I-labeled t-PA must have resulted from the interaction of anti-LRP antibody with the t-PA receptor. This interaction shows that the t-PA receptor and LRP are immunologically indistinguishable. The immunoprecipitated material was specific for t-PA binding because it was not seen when the binding buffer contained an excess amount of unlabeled t-PA.

To confirm that the crosslinked material at the top of the gel represented complexes of t-PA with its receptor, we crosslinked unlabeled t-PA to [^{35}S]methionine-labeled MH_1C_1 cells before immunoprecipitation with normal rabbit serum or anti-t-PA antibody (Fig. 2*b*). When the immunoprecipitated material was analyzed by SDS/PAGE under nonreducing conditions, it appeared identical to that seen in Fig. 2*a*. However, when the same material was analyzed under reducing conditions, a specific band (≈500 kDa) was observed, indicating that the material at the top of the nonreducing gel consisted of t-PA linked to its receptor.

Although both t-PA and α_2M* bind specifically to MH_1C_1 cells, they do not compete with each other for binding to MH_1C_1 cells (data not shown). To confirm that the t-PA-binding protein can also bind α_2M*, we performed ^{125}I-labeled α_2M* binding to MH_1C_1 cells with or without selected competitors, followed by chemical crosslinking, immunoprecipitation, and analysis by SDS/PAGE under nonreducing (Fig. 3*a*) or reducing conditions (Fig. 3*b*). In the absence of t-PA, LRP complexed to ^{125}I-labeled α_2M* was immunoprecipitated only by anti-LRP antibody (lane 3). However, when the binding solution contained unlabeled t-PA, radioactive

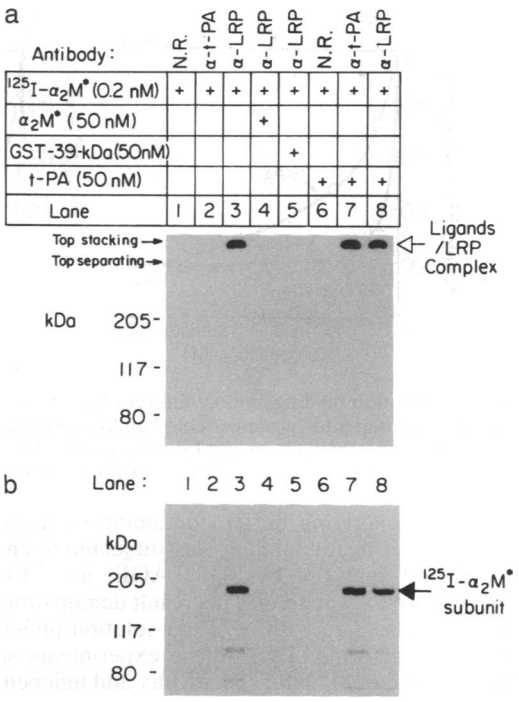

FIG. 3. Both ^{125}I-labeled α_2M* and t-PA bind to LRP. Binding of ^{125}I-labeled α_2M* (0.2 nM) was done with or without various competitors as indicated. Chemical crosslinking was done followed by immunoprecipitation with the indicated antibodies. The immunoprecipitated material was analyzed via SDS/5% PAGE under nonreducing (*a*) or reducing (*b*) conditions. Positions of ligands–LRP complex in the nonreducing gel and ^{125}I-labeled α_2M* subunit in the reducing gel are indicated by an open and a closed arrow, respectively. N.R., normal rabbit serum.

material was seen not only with anti-LRP antibody (lane 7) but also with anti-t-PA antibody (lane 8). Because anti-t-PA antibody does not crossreact with ^{125}I-labeled α_2M*–LRP complex alone (lane 2), t-PA and ^{125}I-labeled α_2M* must have simultaneously bound to LRP and formed an ^{125}I-labeled α_2M*–t-PA–LRP trimeric complex that was immunoprecipitable by both anti-t-PA and anti-LRP antibodies. To confirm that t-PA and α_2M* were not bound to and crosslinked to

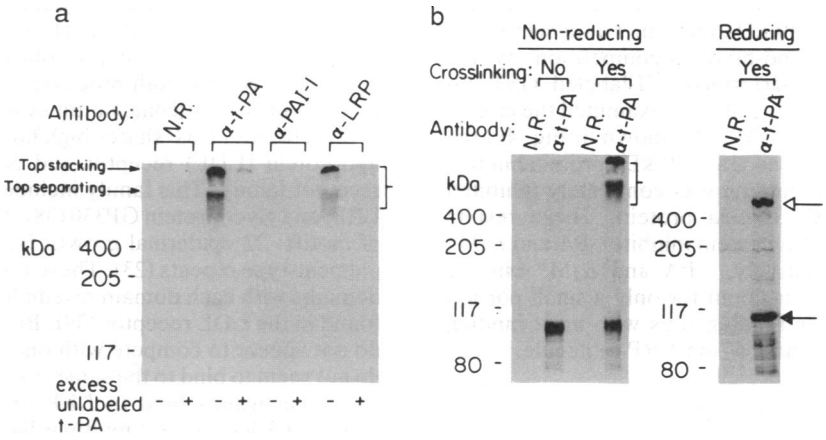

FIG. 2. Anti-LRP antibody crossreacts with t-PA receptor. (*a*) ^{125}I-labeled t-PA (10 nM) was used for ligand binding without (−) or with (+) excess unlabeled t-PA (1 μM). Chemical crosslinking was then done followed by immunoprecipitation with the same set of antibodies used in Fig. 1*a*. The immunoprecipitated material was analyzed by SDS/5% PAGE under nonreducing conditions. The region of immunoprecipitated material is marked with a bracket. (*b*) Control experiments showing the high-molecular-weight material contains t-PA complexed to its receptor. Binding of t-PA was done on [^{35}S]methionine-labeled MH_1C_1 cells. Cells were then lysed either directly without crosslinking or after the ligand had been crosslinked to the receptor. Cell lysates without or with crosslinking were then immunoprecipitated with normal rabbit serum (N.R.) or anti-t-PA antibody (α-t-PA) and were analyzed by SDS/5% PAGE under nonreducing or reducing conditions. Positions of the t-PA receptor and t-PA–PAI-1 complex are indicated by an open arrow and a closed arrow, respectively.

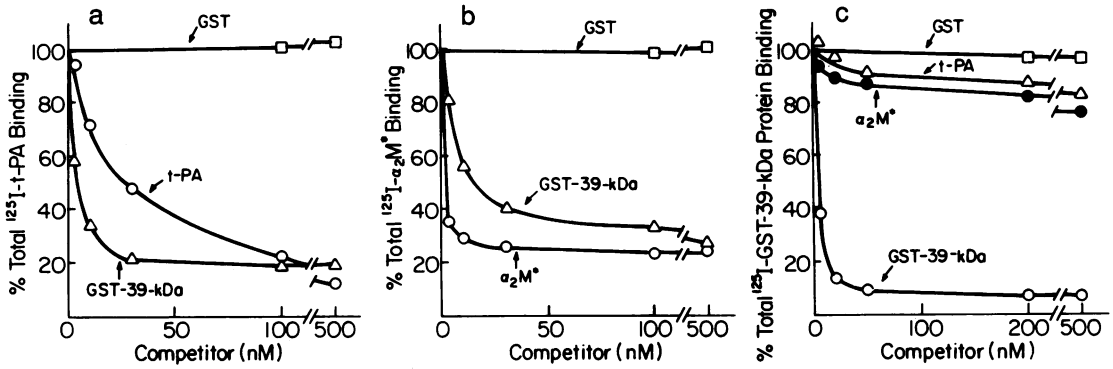

FIG. 4. Competition binding among different ligands on MH_1C_1 cells. Binding of ^{125}I-labeled ligands was done without or with increased concentrations of competitor proteins. One hundred percent binding was determined without competitor protein. Each symbol represents the average of duplicate determinations. (*a*) Inhibition of ^{125}I-labeled t-PA (3 nM) binding by GST–39-kDa protein. (*b*) Inhibition of ^{125}I-labeled α_2M* (0.2 nM) binding by GST–39-kDa protein. (*c*) Competition of ^{125}I-labeled GST–39-kDa (5 nM) protein binding by t-PA or α_2M*.

each other, the overlying buffer containing t-PA and ^{125}I-labeled α_2M* after ligand binding was subjected to chemical crosslinking and analyzed by SDS/PAGE; no ^{125}I-labeled α_2M*–t-PA complex was seen. This result demonstrates that t-PA does not associate with α_2M* in solution under these experimental conditions. Thus, these experiments showed that both t-PA and α_2M* bind specifically and independently to LRP.

To further establish the relationship between the t-PA receptor and LRP, the effect of 39-kDa protein on t-PA binding to MH_1C_1 cells was examined. The 39-kDa protein associates with the LRP on the cell surface (30) and inhibits the uptake of apoprotein E-enriched β-migrating very low density lipoprotein (31) and the binding of α_2M* (31–33) to LRP. We used a recombinant 39-kDa protein expressed as a fusion protein with GST (GST–39-kDa protein). Binding of ^{125}I-labeled GST–39-kDa protein to MH_1C_1 cells was specific and saturable (data not shown). Fig. 4*a* shows that binding of ^{125}I-labeled t-PA to MH_1C_1 cells was inhibited in a dose-dependent manner by both excess unlabeled t-PA (apparent K_i = 20 nM) and excess unlabeled GST–39-kDa protein (apparent K_i = 3.3 nM) but not by GST. Fig. 4*b* shows that the binding of ^{125}I-labeled α_2M* to LRP on MH_1C_1 cells was inhibited by both excess unlabeled α_2M* (apparent K_i = 0.86 nM) and excess unlabeled GST–39-kDa protein (apparent K_i = 14 nM) but not by GST. These data indicate that the ligand-binding properties of both the t-PA receptor and the LRP are modulated by the 39-kDa protein. To further examine the nature of the ligand-binding competition by the GST–39-kDa protein, we performed ^{125}I-labeled GST–39-kDa protein binding to MH_1C_1 cells and examined the effects of excess unlabeled t-PA or α_2M*. As shown in Fig. 4*c*, the two ligands reduced ^{125}I-labeled GST–39-kDa protein binding only slightly, whereas the binding was completely inhibited by excess unlabeled GST–39-kDa protein. These results suggest that the GST–39-kDa protein inhibits t-PA and α_2M* binding indirectly. Alternatively, t-PA and α_2M* binding sites on MH_1C_1 cells might account for only a small portion of the GST–39-kDa protein-binding sites with most binding sites distributed on other parts of the LRP molecule.

DISCUSSION

Hepatic clearance of t-PA is mediated by liver parenchymal cells and, to a lesser degree, endothelial cells. Mannose receptors have been shown to be responsible for t-PA clearance by endothelial cells (14, 34). However, t-PA receptors on parenchymal cells are oligosaccharide independent. In studying t-PA clearance by hepatocytes, our laboratory has previously identified and characterized two types of t-PA receptor systems: a PAI-1-dependent receptor on human hepatoma cell line HepG2 (15–19) and a PAI-1-independent receptor on rat hepatoma cell line MH_1C_1 (22). Our chemical crosslinking and immunoprecipitation experiments on rat hepatoma cells revealed a PAI-1-independent receptor protein with a molecular size of ≈500 kDa. In the present report we demonstrate that this PAI-1-independent t-PA receptor and the previously identified LRP have identical mobilities on SDS/PAGE, are immunologically indistinguishable, are both modulated by the 39-kDa protein, and can simultaneously bind two independent ligands (t-PA and α_2M*). These data thus demonstrate an interaction of t-PA with LRP and provide strong evidence for a role of LRP in t-PA metabolism.

In vivo the three ligands, α_2M, β-migrating very low density lipoprotein, and t-PA, are each cleared from the circulation predominantly via the liver. Data reported here suggest that these ligands are probably cleared by the same receptor, LRP. Using monoclonal antibodies against various domains of LRP, Herz *et al.* (35) have shown that LRP could mediate endocytosis *in vivo*. Although LRP mRNA is present in various tissues (23), the functional expression of LRP—i.e., receptor-mediated endocytosis—was confined to the liver. *In vivo* clearance studies with α_2M* have traced the majority (85–90%) of the cleared ligand to hepatocytes (36). In general, these LRP-mediated hepatic clearance systems are both rapid and specific, typical characteristics of receptor-mediated endocytosis. Physiologically, activation of α_2M by plasmin and subsequent clearance of the plasmin–α_2M complex will affect systemic fibrinolysis similarly as t-PA clearance because both processes diminish circulating components of the anticoagulation system.

Structurally, LRP shares high homology to the low density lipoprotein (LDL) receptor and is a member of the LDL receptor family. This family includes the LDL receptor (37), LRP, and glycoprotein GP330 (38). LRP consists of two types of motifs: 22 epidermal growth factor repeats and 31 complement-type repeats (23). These repeats are arranged in four domains with each domain resembling the motif organization found in the LDL receptor (39). Because the ligands for LRP do not appear to compete with one another for binding, they do not seem to bind to the same site. It is likely, however, that the three ligands bind to LRP on independent sites. This ability of LRP to bind multiple ligands simultaneously was demonstrated in the present report and may be attributed to the large size and repeated structural regions in this receptor molecule. The accommodation of, at least, three different ligands on distinct sites of a single receptor represents a different type of endocytosis receptor. Detailed analysis is required to define the precise binding domain of each ligand.

The 39-kDa protein, which was initially copurified with α_2MR from rat liver (28), has been shown to associate with

the large subunit of LRP and, therefore, is also termed α_2M receptor-associated protein or RAP (30). The fact that this protein inhibits both t-PA and α_2M* binding and, therefore, endocytosis in hepatoma cells clearly suggests a role in regulation of receptor activity. The amount of this protein bound to LRP could modulate receptor activity *in vivo*, as this protein is very abundant in kidney, where the endocytotic activity of LRP is low, but is less abundant in the liver, where the endocytotic activity of LRP is high (data not shown). Uptake of many of the protein ligands for endocytosis (e.g., LDL, transferrin) is highly regulated (40). Thus control of LRP-receptor activity may well play a role in several potential physiological circumstances, in addition to lipoprotein metabolism and coagulation.

Although data reported here strongly support an interaction of t-PA with LRP on hepatoma cells, studies by Orth *et al.* (41) demonstrate binding of t-PA–PAI-1 complexes to LRP via ligand blotting, as well as degradation of t-PA–PAI-1 complexes after binding to LRP on COS cells. The differences in ligand-binding specificity of LRP (i.e., PAI-1-independent or PAI-1-dependent recognition of t-PA) may be the result of different analytic systems used. LRP may exist in different conformations under different conditions and, therefore, exhibit different ligand-binding specificities. Alternatively, low-affinity, unstable t-PA–PAI-1 complexes may mediate the initial recognition with subsequent stable interaction being PAI-1 independent. Nevertheless, these studies together provide strong evidence that LRP plays an important role in t-PA clearance.

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