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Binding of Tissue-type Plasminogen Activator by the Mannose Receptor*

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Previous studies have shown that tissue-type plasminogen activator (t-PA) in blood is cleared by the liver partially through a mannose-specific uptake system. The present study was undertaken to investigate, in a purified system, whether t-PA is recognized by the mannose receptor which is expressed on macrophages and liver sinusoidal cells. The mannose receptor was isolated and purified from bovine alveolar macrophages and migrated as a single protein band at M_r 175,000 on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Ligand blotting revealed that this protein specifically bound t-PA. The t-PA-receptor interaction was further characterized in a binding assay, which showed saturable binding with an apparent dissociation constant of 1 nM. t-PA binding required calcium ions and was negligible in the presence of EDTA or at acid pH. Mannose-albumin was an effective inhibitor, whereas galactose-albumin did not have a significant effect. From a series of monosaccharides tested, D-mannose and L-fucose were the most potent inhibitors, *N*-acetyl-D-glucosamine was a moderate inhibitor, whereas D-galactose and *N*-acetyl-D-galactosamine were ineffective. t-PA, deglycosylated by endoglycosidase H, did not interact with the receptor. It is concluded that the mannose receptor specifically binds t-PA, probably through its high mannose-type oligosaccharide.

Receptor-mediated endocytosis refers to a general process which permits cells to internalize macromolecules and particles from the extracellular surroundings (1, 2). A group of receptors involved in this process is formed by membrane-bound lectins, which recognizes specific glycoproteins or glycoconjugates (3, 4). Examples of these lectins are the asialoglycoprotein receptor (3), the Kupffer cell galactose/fucose receptor (5), the chicken hepatic lectin (3), and the mannose receptor of macrophages and hepatic sinusoidal cells. The latter receptor was isolated from rabbit (6, 7), rat (8), and human (9, 10) cells or tissues and appeared to consist of a single subunit of M_r ~175,000. Recently, the primary structure of the human mannose receptor was deduced from the sequence of cDNA clones (11). It appeared that the receptor contains multiple motifs resembling carbohydrate-recognition domains, also found in other surface glycoprotein receptors. The mannose receptor probably has a physiological function in host defense mechanisms by mediating phagocytosis of mannose-containing microorganisms (12, 13). In addition, it has been proposed that the receptor functions as a scavenger for secreted lysosomal enzymes, such as α-hexosaminidase and β-glucuronidase, bearing high mannose-type carbohydrates (14, 15). It has been demonstrated recently (16) that the circulating C-terminal propeptide of type I procollagen is cleared mainly via the mannose receptor in liver endothelial cells.

Tissue-type plasminogen activator (t-PA)[1] is a highly specific proteinase, which is synthesized by vascular endothelial cells and secreted into the bloodstream. The enzyme plays a key role in the fibrinolytic system, which constitutes the natural counterpart of the blood coagulation system and is responsible for a timely degradation of fibrin structures in blood clots and thrombi (17, 18). Recombinant t-PA is presently used as a thrombolytic drug, for instance after a myocardial infarction (19). t-PA is a glycoprotein with a molecular weight of about 70,000 (20). It contains one high mannose-type oligosaccharide and one or two complex-type oligosaccharides (21-24). The high mannose-type oligosaccharide may be involved in the rapid clearance of t-PA from plasma by the liver ($t_{1/2} \approx 5$ min). Kuiper *et al.* (25), as well as other investigators (26-34) have provided evidence that t-PA is partially cleared by a specific uptake system on parenchymal cells and partially by a mannose-specific uptake system on liver endothelial cells and Kupffer cells (reviewed in Ref. 35). The latter route may be mediated by the M_r 175,000 mannose receptor.

The aim of the present study was to establish in a purified system whether or not t-PA fulfils the requirements for a specific interaction with the mannose receptor and thus could be considered as a physiological ligand.

EXPERIMENTAL PROCEDURES

Materials—PMSF, leupeptin, chymostatin, BSA (product A7030), mannose-albumin containing 20-30 mol of monosaccharide per mol albumin (product A4664), galactose-albumin containing 15-25 mol of monosaccharide per mol albumin (product A1159), L-fucose, and D-galactose were obtained from Sigma; iodoacetamide from BDH Chemicals (Poole, UK); D-mannose and *N*-acetyl-D-galactosamine from Aldrich-Chemie (Steinheim, Germany); *N*-acetyl-D-glucosamine from Janssen Pharmaceutica (Beerse, Belgium); and Endo-H from Boehringer Mannheim. t-PA was purified from a human melanoma cell line (36, 37) by Dr. J. H. Verheijen of the IVVO-TNO Gaubius Laboratory and consisted for 70% of the single-chain form and for 30% of the two-chain form; concentrations were based on amino acid analysis. t-PA was radiolabeled with ^{125}I (specific radioactivity 4×10^7 cpm/μg protein) by using the IODO-GEN method (38) and

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[1] The abbreviations used are: t-PA, tissue-type plasminogen activator; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; Endo-H, endoglycosidase H; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

purified as described earlier (39). Mannose-albumin was coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.) at a concentration of 5 mg/ml gel.

Isolation of the Mannose Receptor—Alveolar macrophages were isolated from bovine lung, washed five times with 15 mM phosphate buffer, pH 7.5, 0.15 M NaCl (PBS) at 4 °C, and stored frozen in PBS, containing 1 mM PMSF, 5 mM iodoacetamide, 1 μg/ml leupeptin, and 1 μg/ml chymostatin (and 5% 2-propanol to keep PMSF in solution). Macrophage membranes were prepared and then extracted with 1% Triton X-100 as described by Lennartz *et al.* (7). The PMSF concentration in the buffers was, however, enhanced from 0.1 mM to 1 mM. Fifteen ml of extract (derived from 1.5×10^9 cells) was applied twice on a 4-ml mannose-albumin-Sepharose column, previously equilibrated with 10 mM Tris-HCl, pH 7.4, 1.25 M NaCl, 15 mM $CaCl_2$, 1% Triton X-100, containing the inhibitors specified above. The column was washed with equilibration buffer and eluted with equilibration buffer supplemented with 0.2 M mannose. The column fractions containing the mannose receptor (as shown by SDS-PAGE) were pooled and stored at −70 °C. When indicated, this pool was dialyzed extensively against equilibration buffer to remove mannose and then reapplied on the mannose-albumin-Sepharose column. Elution was accomplished as described above, but now without Triton X-100 in the buffer, which resulted in a yield of about 25%. Detergent-free mannose receptor was used in the binding assay (see below). All procedures were carried out at 4 °C.

SDS-PAGE and Ligand Blotting—SDS-PAGE was performed according to the method of Laemmli (40). High molecular weight standard protein markers were obtained from Bio-Rad and low molecular weight markers from Pharmacia. The gels were stained by silver staining, subjected to autoradiography, or blotted on nitrocellulose in a 50 mM borate buffer, pH 8.0, containing 10% methanol. The nitrocellulose was washed for 1 h at room temperature with 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 5 mM $CaCl_2$, 0.5% Tween 80, 1 mg/ml BSA and then incubated in the same buffer supplemented successively with 1 μg/ml t-PA (for 1.5 h), with 1000-fold diluted goat anti-t-PA antiserum (for 1.5 h), and with 1000-fold diluted rabbit anti-goat IgG antibody conjugated with alkaline phosphatase (Sigma), for 2 h. Staining was performed by the method of Blake *et al.* (41).

Binding Assay—The wells of polyvinylchloride microtitration plates were coated overnight at 4 °C with 100 μl of 0.15 μg/ml detergent-free mannose receptor in 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl. The wells were washed for 0.5 h at room temperature with the same buffer, supplemented with 5 mM $CaCl_2$, 0.5% Tween 80, 1 mg/ml BSA, and then incubated with 100 μl of 10–13 ng/ml ^{125}I-t-PA (approximately 40,000 cpm) for 1 h at room temperature in the latter buffer. After washing, the wells were cut from their support and counted in a gamma spectrometer. The results were corrected for nonspecific binding by subtracting values obtained in wells which were coated with buffer without mannose receptor (about 200 cpm). Potential inhibitors of t-PA binding were mixed with ^{125}I-t-PA before incubation with the immobilized receptor. Inhibition curves were fitted by nonlinear regression analysis with a computer program (GraphPAD, ISI Software, Philadelphia, PA).

Endo-H Treatment—^{125}I-t-PA (3×10^6 cpm/ml) in 40 mM Tris/80 mM acetate, pH 7.0, 0.8 M NaCl, 0.01% Tween 80 was incubated with or without 40 mU/ml Endo-H for 3 h at 37 °C. The effect of the Endo-H treatment on the structure of ^{125}I-t-PA was studied by SDS-PAGE and autoradiography. The interaction of Endo-H-treated ^{125}I-t-PA with the mannose receptor was determined in the binding assay.

RESULTS

Isolation and Purification of the Mannose Receptor—The mannose receptor was extracted from bovine alveolar macrophage membranes and purified by affinity chromatography on mannose-albumin-Sepharose. The final yield was 10–50 μg of receptor protein per 1.5×10^9 cells. SDS-PAGE of the purified protein, both before and after reduction with 2-mercaptoethanol, showed one main band corresponding with a M_r of 175,000 (Fig. 1*A*). Occasionally a trace component at $M_r > 200{,}000$ was observed (Fig. 1*A*), possibly representing an aggregated form of the receptor. The isolation procedure is a modification of that developed for the mannose receptor from rabbit alveolar macrophages (7). The major modification is that the proteinase inhibitors were not only present during washing and extraction of the macrophage membranes but also during washing and elution of the mannose-albumin-Sepharose column. In addition, the PMSF concentration was enhanced from 0.1 to 1.0 mM. Without these modifications the purified receptor lost its activity rapidly and completely (not shown) and could not be used in binding studies.

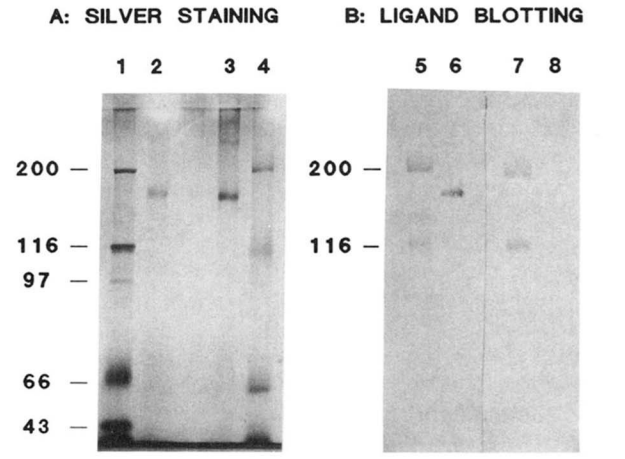

FIG. 1. **SDS-PAGE of the purified mannose receptor (*A*) and ligand blotting with t-PA (*B*).** Aliquots of 0.2 μg of mannose receptor (*lanes 2* and *3*) or standard proteins (*lanes 1* and *4*) were subjected to SDS-PAGE on 6% gels and stained by silver staining (*lanes 1* and *2* reduced, *lanes 3* and *4* nonreduced). Aliquots of 0.8 μg of mannose receptor (*lanes 6* and *8*) or standard proteins (*lanes 5* and *7*) were run under nonreduced conditions, transferred to nitrocellulose, incubated with 1 μg/ml t-PA in the absence (*lanes 5* and *6*) or presence (*lanes 7* and *8*) of 0.1 M mannose, and immunostained as described under "Experimental Procedures." The standard proteins were myosin (200,000), β-galactosidase (116,250), phosphorylase *b* (97,400), BSA (66,200), and ovalbumin (42,699).

Ligand Blotting—The mannose receptor was subjected to SDS-PAGE, blotted onto nitrocellulose, incubated with t-PA, and stained by using antibodies against t-PA. Fig. 1*B* (*lane 6*) shows a clear band at M_r 175,000, suggesting that the mannose receptor bound t-PA. No other bands were visible, indicating that the receptor preparation did not contain other proteins which interacted with t-PA in this technique. Two out of the five molecular weight markers were also stained (*lane 5*), which may possibly be ascribed to the affinity of t-PA for some denatured proteins (42). The binding of t-PA to the mannose receptor was fully blocked by addition of 0.1 M mannose (*lane 8*), pointing to a specific binding phenomenon. By contrast, the apparent binding of t-PA to the molecular weight markers was unaffected by mannose (*lane 7*).

Binding Assay—In order to further investigate the interaction between the mannose receptor and t-PA, a binding assay was developed by immobilizing detergent-free mannose receptor onto microtitration plates. The immobilized receptor was incubated with iodinated t-PA and bound radioactivity was counted. Fig. 2 shows that the amount of bound ^{125}I-t-PA increased with increasing receptor concentrations employed for coating the microtitration plates. A concentration of 0.15 μg/ml or 0.85 nM was used in all further binding studies. Binding of iodinated t-PA was blocked by an excess of unlabeled t-PA (Fig. 3), demonstrating that the binding was specific and saturable. Curve fitting by nonlinear regression analysis showed 50% inhibition at 1.2 nM unlabeled t-PA (corresponding with an apparent dissociation constant of 1.0 nM) and a number of binding sites of 3.3 fmol/well.

Pilot experiments had shown that optimal binding was achieved at neutral pH and in the presence of calcium ions. Binding of ^{125}I-t-PA decreased from 1837 ± 195 cpm/well (mean ± S.D., $n = 4$) under standard conditions to 6 ± 27

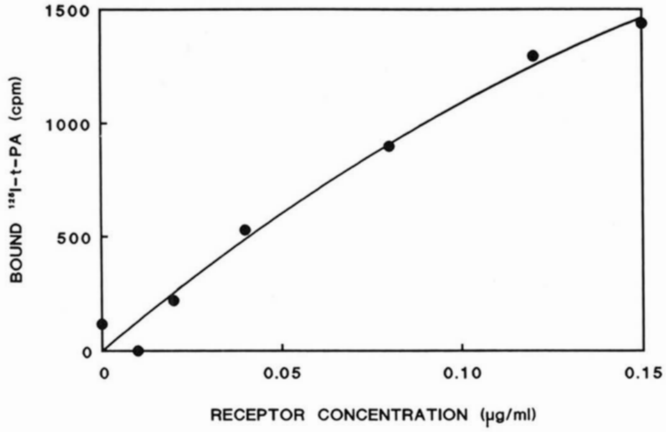

FIG. 2. **Effect of mannose-receptor concentration on the binding of ^{125}I-t-PA in the binding assay.** The wells of microtitration plates were coated with varying concentrations of the mannose receptor (0–0.15 μg/ml) and then incubated with ^{125}I-t-PA, washed and counted as described under "Experimental Procedures."

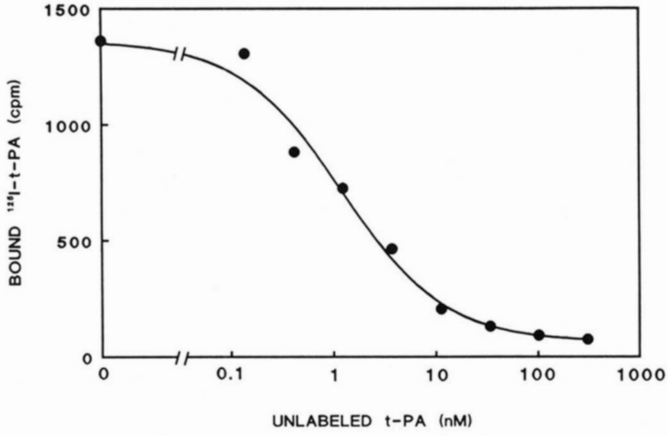

FIG. 3. **Inhibition of ^{125}I-t-PA binding to the mannose receptor by unlabeled t-PA.** ^{125}I-t-PA (13 ng/ml or 0.22 nM) was mixed with varying concentrations of unlabeled t-PA (0–300 nM) and incubated with immobilized receptor in the binding assay (see "Experimental Procedures"). The sigmoid curve was calculated with a fitting program.

cpm/well in the presence of 10 mM EDTA instead of 5 mM $CaCl_2$ and to 28 ± 2 cpm/well in a buffer containing 20 mM acetate, pH 4.0, instead of 20 mM Tris-HCl, pH 7.4.

Carbohydrate Specificity—The involvement of carbohydrates in the interaction between the mannose receptor and t-PA was further studied by measuring the extent of inhibition of ^{125}I-t-PA binding in the binding assay by various glycoproteins and monosaccharides. Fig. 4 shows that mannose-albumin, a standard ligand for the mannose receptor, was an effective inhibitor, whereas galactose-albumin did not have a significant effect. The most potent inhibitors of the monosaccharides tested were D-mannose and L-fucose. *N*-Acetyl-D-glycosamine was a moderate inhibitor, whereas D-galactose and *N*-acetyl-D-galactosamine were ineffective (Fig. 5).

Endo-H-treated t-PA—Incubation of ^{125}I-t-PA with Endo-H resulted in a small increase in mobility on SDS-PAGE, corresponding well with the expected elimination of the high mannose-type oligosaccharide of t-PA (Fig. 6, *inset*). Incubation of ^{125}I-t-PA without Endo-H did not change the mobility. Deglycosylated t-PA appeared to have no affinity for the mannose receptor in the binding assay (Fig. 6), indicating that the high mannose-type chain of t-PA is essential for binding.

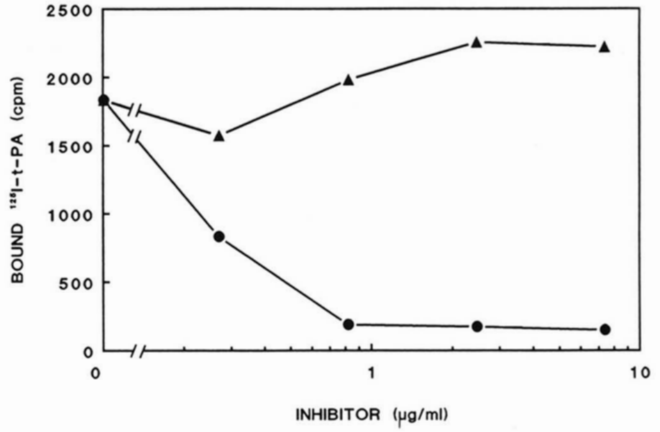

FIG. 4. **Inhibition of ^{125}I-t-PA binding to the mannose receptor by mannose-albumin and galactose-albumin.** ^{125}I-t-PA (10 ng/ml) was mixed with varying concentrations (0–7.4 μg/ml) of mannose-albumin (●) or galactose-albumin (▲) and incubated with the immobilized receptor in the binding assay (see "Experimental Procedures").

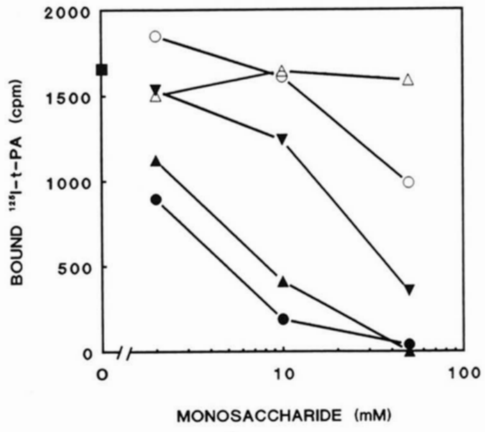

FIG. 5. **Inhibition of ^{125}I-t-PA binding to the mannose receptor by various monosaccharides.** ^{125}I-t-PA (10 ng/ml) was mixed with varying concentrations (0–50 mM) of D-mannose (●), L-fusoce (▲), *N*-acetyl-D-glucosamine (▼), D-galactose (○), or *N*-acetyl-galactosamine (△) and incubated with the immobilized receptor in the binding assay (see "Experimental Procedures").

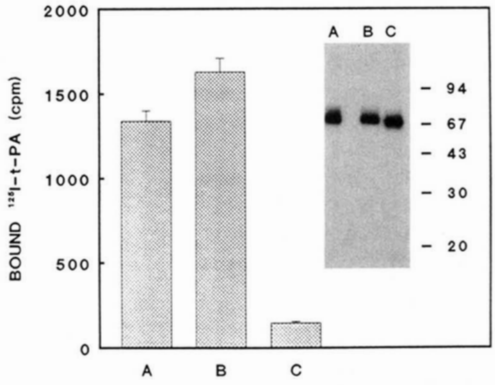

FIG. 6. **Effect of deglycosylation of ^{125}I-t-PA by Endo-H on the binding to the mannose receptor.** ^{125}I-t-PA binding (mean ± S.D.) was determined in the binding assay both before (*A*) and after a 3-h incubation period in the absence (*B*) or presence (*C*) of Endo-H (see "Experimental Procedures" for details). The *inset* shows a radioautogram after SDS-PAGE (10% gel) of the three ^{125}I-t-PA preparations. The standard proteins were phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,100).

DISCUSSION

In this study the mannose receptor was isolated and purified from bovine alveolar macrophages, extending the number of species from which a M_r 175,000 mannose receptor has been isolated. The functional properties of the receptors isolated from rabbit (6, 7), rat (8), human (9, 10), and bovine (this study) cells or tissues are very similar. The isolation of the bovine receptor required, however, extensive use of proteinase inhibitors to prevent proteolytic inactivation of the receptor. It is interesting to note that the inactivation did not lead to a visible change in electrophoretic mobility on SDS-PAGE under reducing conditions (data not shown), pointing to a proteolytic clip near the extracellular (*i.e.* N-terminal) end of the receptor. This finding is difficult to reconcile with the position of the eight potential carbohydrate-recognition domains in the middle of the molecule (11). However, our finding may be in line with a recent suggestion that the ligand-binding domain is located on a N-terminal fragment of M_r 35,000 (43).

The main finding of this study is that the mannose receptor specifically bound t-PA. This was found both in ligand blotting experiments and in a binding assay developed for small quantities of receptor protein. The apparent dissociation constant in the binding assay was 1.0 nM and the number of binding sites 3.3 fmol/well (33 pM). The latter value is low with respect to 0.85 nM, the receptor concentration used for coating of the microtitration plates. This may suggest that only 4% of the receptor molecules were capable of binding t-PA. However, other factors might also explain this low value, such as incomplete immobilization of the receptor solution employed, loss of functional activity of the receptor due to immobilization to the polyvinylchloride, and dissociation of immobilized receptor and/or bound ^{125}I-t-PA during the wash steps of the binding assay.

Binding of t-PA did not occur in the presence of EDTA or at acid pH, which is in line with known properties of the receptor (7–10). In addition, the inhibition profile of monosaccharides, D-mannose and L-fucose being the most potent inhibitors, *N*-acetyl-D-glucosamine a moderate one, and D-galactose and *N*-acetyl-D-galactosamine the least potent ones (Fig. 5), is very characteristic of the mannose receptor (44, 45) and underlines the specificity of the observed t-PA binding.

Both melanoma and recombinant t-PA contain a high mannose-type oligosaccharide in kringle 1, a complex-type in kringle 2 (only present in t-PA variant I), and another complex-type oligosaccharide in the protease domain (22–24). It is probably only the high mannose-type structure which fulfils the minimum requirements for binding and endocytosis by the mannose receptor (46). This was supported by a complete loss of affinity for the mannose receptor of t-PA treated by Endo-H (Fig. 6), which only removes high mannose-type and some hybrid-type chains in glycoproteins (47).

Binding of t-PA to the macrophage mannose receptor, which is very similar to, or identical with, the liver mannose receptor (45), has at least four potentially important implications. First, t-PA is one of the first plasma proteins, shown to be a suitable ligand for the mannose receptor, which is relevant for understanding the function(s) of the mannose receptor especially on liver sinusoidal cells. Although most glycoproteins in plasma have complex-type carbohydrate chains, t-PA is not the only glycoprotein in plasma with a high mannose-type oligosaccharide. Another known example is IgM, which has, however, a relatively long half-life. It has been suggested that the high mannose-type chains in this and other stable proteins are sterically protected from the mannose receptor (48). The high mannose-type oligosaccharide in t-PA should be readily accessible. This assumption is supported by the finding that t-PA, in contrast to many other glycoproteins (47), does not require treatment with agents known to perturb protein configuration in order to become deglycosylated by Endo-H (31, 32, this study).

Second, the finding strongly supports the hypothesis that the mannose receptor is involved in the rapid clearance of t-PA (35). This is helpful in studies developing slower-clearing mutants of t-PA to improve thrombolytic therapy. Not only could glycosylation be prevented as previously described (31), but minor modifications in the carbohydrate structures could also be elaborated by taking into account the minimum required structure for an interaction with the mannose receptor (46). In addition, identification of receptor systems involved in t-PA clearance is essential in strategies to prolong the half-life of t-PA by affecting the receptors directly.

Third, the mannose receptor does not only occur on sinusoidal liver cells but also on macrophages. This may point to an active role of macrophages in regulating t-PA concentrations in tissues and body fluids outside the blood circulation.

Fourth, the binding of t-PA by the mannose receptor could be considered in the light of a recent analysis of the molecular evolution of components of the coagulation, fibrinolysis and complement systems. Patthy (49) has suggested that the coagulation and complement cascades are descendants of a general defense system, which protected the organism both from infection and tissue injury by making use of lectin domains. The present mannose receptor, still being involved in phagocytosis of microorganisms (12, 13), as well as in the haemostatic system (this study), may represent a relic of the ancestral defence system.

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