

Minireview

Receptor-mediated endocytosis of plasminogen activators and activator/inhibitor complexes

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Abstract

Recent findings have elucidated the mechanism for clearance from the extracellular space of the two types of plasminogen activators, urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA), and their type-1 inhibitor (PAI-1). Activator/PAI-1 complexes and uncomplexed t-PA bind to the multiligand receptors α_2 -macroglobulin receptor/low density lipoprotein receptor-related protein (α_2 MR) and epithelial glycoprotein 330 (gp330). These receptors mediate endocytosis and degradation of u-PA/PAI-1 complex bound to the glycosyl phosphatidyl inositol-anchored urokinase receptor (u-PAR) on cell surfaces, and participate, in cooperation with other receptors, in hepatic clearance of activator/PAI-1 complexes and uncomplexed t-PA from blood plasma. The α_2 MR- and gp330-mediated endocytosis of a ligand (u-PA/PAI-1 complex) initially bound to another receptor (u-PAR) is a novel kind of interaction between membrane receptors. Binding to α_2 MR and gp330 is a novel kind of molecular recognition of serine proteinases and serpins.

Key words: α_2 -Macroglobulin, Glycosyl phosphatidyl inositol, Plasminogen activator, Proteolysis, Receptor, Serine proteinase, Serpin

1. Introduction: the plasminogen activation system

The extracellular formation of the serine proteinase plasmin from the zymogen plasminogen is catalysed by either one of two other serine proteinases, the urokinase-type plasminogen activator (u-PA) and the tissue-type plasminogen activator (t-PA). u-PA-catalysed plasmin generation is implicated in events involving pericellular proteolysis, like tissue remodelling, cell migration and invasion, and in activation of growth factors (reviewed in [1–3]). t-PA-catalysed plasmin generation is active primarily in fibrinolysis (reviewed in [4–6]).

u-PA is released from cells as a single-chain zymogen, pro-u-PA, which is converted to the active two-chain enzyme by limited proteolysis (reviewed in [1]). t-PA is also released from cells as a single-chain form; but single-

chain t-PA has a measurable activity, although it is about 50-fold lower than that of its two-chain counterpart (reviewed in [5]). Both activators contain a C-terminal serine proteinase domain (the B-chain) and an N-terminal A-chain, containing in t-PA a fibronectin-type II finger domain, an epidermal growth factor (EGF) domain and two kringles, and in u-PA an EGF domain and a kringle (reviewed in [1]; Fig. 1A).

The glycosyl phosphatidyl inositol (GPI)-anchored u-PA receptor (u-PAR) binds to the N-terminal growth factor domain of u-PA. u-PAR contains 3 homologous domains, the N-terminal one being ligand binding (Fig. 1B). u-PAR serves to accelerate pro-u-PA activation and localize plasminogen activation to cell surfaces (reviewed in [3,7]). Analogously, t-PA activity is strongly stimulated by binding to fibrin (reviewed in [4–6]).

The activity of u-PA and t-PA is controlled by two types of inhibitors, PAI-1 and PAI-2. They belong to the serpin superfamily and act by forming stable equimolar complexes with the activators. PAI-1 is the primary inhibitor, being produced by many cell types and present in normal blood plasma, while PAI-2 has a more restricted occurrence and is a slow inhibitor of t-PA (reviewed in [2,3]).

The activity of plasminogen activators is also terminated by their clearance from the extracellular space by

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Abbreviations: α_2 M, α_2 -macroglobulin, α_2 MR, α_2 -macroglobulin receptor/low density lipoprotein receptor related protein, RAP, α_2 -macroglobulin receptor-associated protein, EGF, epidermal growth factor, gp330, epithelial glycoprotein 330, GPI, glycosyl phosphatidyl inositol, PAI-1, type-1 plasminogen activator inhibitor, PAI-2, type-2 plasminogen activator inhibitor, t-PA, tissue-type plasminogen activator, u-PA, urokinase-type plasminogen activator, u-PAR, urokinase receptor.

endocytosis and degradation. Clearance may occur locally, from cell surfaces or from the pericellular space in the tissues where the activators are produced, or the activators may diffuse to blood plasma and be cleared by the liver. The total concentrations of u-PA and t-PA in normal human plasma is around 20 pM and 100 pM, respectively; both activators occur mostly in the form of complexes with PAI-1 [8–13]. Clearance of the activators from plasma is of pharmacological interest because of their use in thrombolytic therapy (reviewed in [4,6]).

A number of investigations published in 1992 and 1993 have implicated the cell membrane proteins α_2 -macroglobulin receptor/low density lipoprotein receptor-related protein (α_2 MR) and epithelial glycoprotein 330 (gp330) in endocytosis and degradation of plasminogen activators and activator/inhibitor complexes. The purpose of this communication is to review these discoveries, which have been done in experiments with cell cultures, and relate them to previous findings concerning clearance of plasminogen activators and activator/PAI-1 complexes in vivo.

2. α_2 MR and gp330

The amino acid sequence of α_2 MR was determined in two independent ways. Herz et al. [14] cloned a cDNA, which was related to low density lipoprotein receptor by its nucleotide sequence, hence the name LRP, low density lipoprotein receptor-related protein. α_2 MR protein was purified from rat liver and human placenta, utilizing its ability to bind α_2 -macroglobulin (α_2 M)/proteinase complexes [15–17]. From partial peptide and cDNA sequences of human placental α_2 MR, it was found to be identical to LRP [18,19]. α_2 MR consists of an M_r 515,000 ligand binding α -chain and an M_r 85,000 membrane spanning β -chain, encoded by a single mRNA [20]. It contains several clusters of three types of modules, of which two, the EGF domains and the so-called YWTD repeats, were previously found in the EGF precursor, and the third in the terminal complement components C6–C9 (Fig. 2A). An -Asn-Pro-Xaa-Tyr-sequence in the cytoplasmic tail is likely to be essential for endocytosis through clathrin-coated pits. An M_r 40,000 protein (RAP, receptor associated protein) copurifies with α_2 MR [16,17] and inhibits the binding of all other known ligands [21–23]. gp330, first identified by Kerjaschki and Farquhar [24], has been partially sequenced from rat sources and shown to be a homologue of α_2 MR [25]. It has an M_r of more than 500,000 [26]**.

α_2 MR and gp330 bind and mediate endocytosis of a number of ligands besides plasminogen activators and

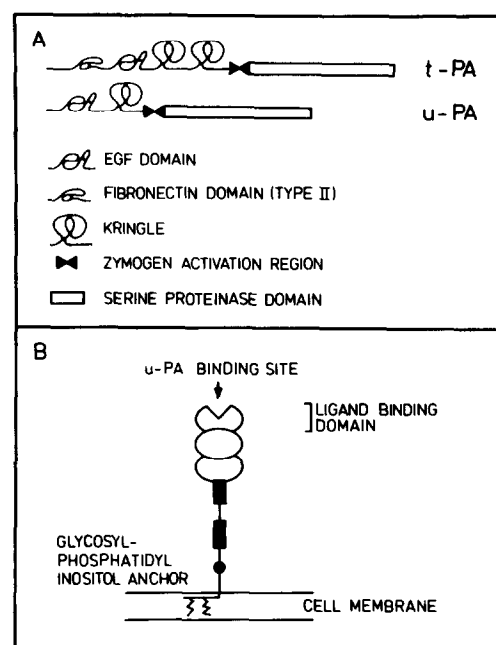


Fig. 1 Structural aspects of u-PA, t-PA and u-PAR. (A) Schematic presentation of the domain structure of u-PA and t-PA. (B) Schematic presentation of u-PAR. The receptor contains 3 homologous domains, the most N-terminal one binding the EGF domain of u-PA.

activator/PAI-1 complexes [21,27–31]. Although the two receptors have many common ligands, including RAP, it is noteworthy that gp330 does not bind α_2 M/proteinase complexes [26,30].

3. α_2 MR and gp330 bind and internalize u-PA/PAI-1 complex in cell cultures

Pro-u-PA and active u-PA bound to u-PAR on cultured human cells are endocytosed slowly if at all (reviewed in [7]). u-PAR-bound active u-PA is, however, susceptible to inhibition by PAI-1 [32], and in a variety of different human cell types, reaction of u-PAR-bound u-PA with PAI-1 leads to rapid degradation of the formed complex. The degradation can be blocked by inhibitors of lysosomal hydrolases. Electron microscopy demonstrated endocytosis into lysosome-like vesicles [33–36]. The degradation of u-PA/PAI-1 complex is blocked by RAP and antibodies to α_2 MR in human monocytes [37] and in African green monkey COS kidney cells [38]. A RAP-inhibitable binding of u-PA/PAI-1 complex to a protein present in membranes from rat and rabbit liver and human placenta and comigrating with α_2 MR in electrophoresis was demonstrated by a ligand blotting assay [28,37–39]. u-PA/PAI-1 complex binds to immobilized purified α_2 MR [37]. These findings strongly suggest that α_2 MR binds and mediates endocytosis of u-PAR-bound u-PA/PAI-1 complex.

u-PA/PAI-1 complex binds to purified rat kidney

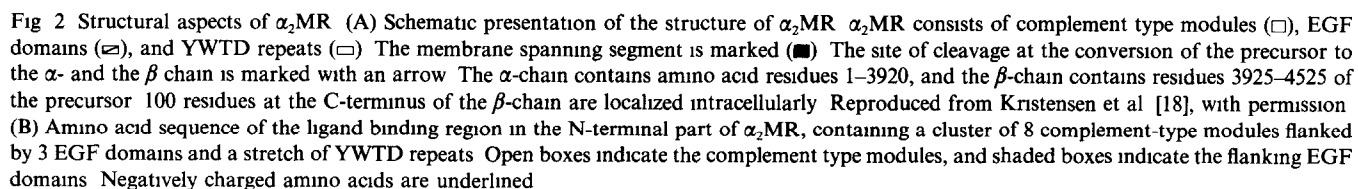
**The abbreviation gp330 is derived from an early determination of the M_r to 330,000.

[46], and in rat H4 hepatoma cells, around 20 percent of the t-PA degradation is independent of the active site [39]. By criteria like those mentioned above, binding and degradation is mediated by α_2 MR in both cases [39,47]. It appears, therefore, that α_2 MR can bind and internalize free t-PA in addition to t-PA/PAI-1 complex, at least in some rodent cells.

t-PA/PAI-1 complex binds in an RAP-inhibitable manner to a rat kidney membrane protein with an electrophoretic mobility identical to that of gp330 [28], and purified gp330 binds t-PA/PAI-1 complex [26], suggesting that also gp330 can mediate endocytosis of this ligand. t-PA/PAI-1 and u-PA/PAI-1 complexes compete for binding [26].

5. Possible models for in vivo clearance of plasminogen activators and activator/inhibitor complexes

From the cell culture studies described above, *in vivo* clearance of u-PAR-bound u-PA/PAI-1 complex from *cell surfaces* appears to be mediated by α_2 MR (Fig 3) gp330, being expressed in other cell types than α_2 MR,



could have a similar function. It is unknown whether u-PAR is endocytosed together with the u-PA/PAI-1 complex and α_2 MR (or gp330). The complex could dissociate from u-PAR concomitantly with the binding to α_2 MR. Alternatively, a quarternary complex between u-PAR, u-PA, PAI-1 and α_2 MR could be internalized. If so, it becomes a question whether u-PAR, like α_2 MR [48], is recycled to the cell surface after intralysosomal dissociation of the ligand. Likewise, in vivo clearance of t-PA and t-PA/PAI-1 complex from the pericellular space around producer cells may be mediated by α_2 MR and gp330.

α_2 MR is expressed in human and rodent liver, in human liver, it has been shown to be present in parenchymal cells and Kupffer cells [15,49]. Is hepatic α_2 MR also responsible for in vivo clearance of activators and activator/PAI-1 complexes from blood plasma?

Human u-PA is cleared from plasma by the liver when injected intravenously into rodents (reviewed in [1]). The plasma half-life is 0.45–4 min. Clearance occurs largely by uptake into lysosomes of liver parenchymal cells [50,51]. Clearance is not dependent on accessibility of the active site [52]. The plasma half-life of intravenously injected human t-PA ranges from 1–4 min in rodents to 5–10 min in humans. In vivo studies with rodents have also shown that t-PA accumulates in the liver, where it is degraded by lysosomes, and have implicated endothelial cells, parenchymal cells as well as Kupffer cells, accounting for approximately 45%, 45% and 10% of the clearance, respectively. The clearance mechanism of endothelial cells is mannose dependent, and seems to be dependent on the glycosylation site at kringle 1. The clearance mechanism of parenchymal cells is mannose-independent. It requires the finger and growth factor domains, but not an available active site. Also t-PA/PAI-1 complex is cleared by the liver in rodents, and is cleared in perfused rat liver (reviewed in [4,6,53–55]). For obvious reasons, similarly detailed evidence is not available for humans.

Attempting to correlate these in vivo data with the findings with cell cultures, assumption of liver parenchymal cell α_2 MR being an indispensable part of the in vivo mechanism for plasma clearance of u-PA/PAI-1 complex is seen to be in agreement with the published in vitro binding studies with rat and rabbit liver membrane proteins and the data on human cell lines (see section 3). These data suggest that α_2 MR-mediated plasma clearance of u-PA is greatly accelerated after complex formation with PAI-1, and plasma clearance mechanisms for free u-PA remain to be characterized. Cultured rat liver parenchymal cells do bind and degrade u-PA [50,51], but it has not been shown that α_2 MR is involved.

With respect to t-PA, the mannose dependence of in vivo plasma clearance by rodent liver endothelial cells is in agreement with studies with cultured rat liver endothelial cells, in which binding, endocytosis and lysosomal

degradation of t-PA has been found to be mediated by the M_r 175,000 mannose receptor [56–60] (Fig. 3). Furthermore, available data are consistent with the assumption that the mannose independent in vivo plasma clearance by liver parenchymal cells involves α_2 MR (Fig. 3). The in vivo observations of mannose and active site independence and growth factor and finger domain dependence have been reproduced with cultured rat liver parenchymal cells and rodent cell lines of hepatic origin [39,46,47,56,58,61–63]. Some of these studies have directly implicated α_2 MR, and point to rodent liver α_2 MR being able to bind free t-PA as well as t-PA/PAI-1 complex (see section 4). The experiments with cultured human cells consistently point to t-PA/PAI-1 complex being endocytosed much faster by α_2 MR than free t-PA (section 4), and the possible role of liver α_2 MR in in vivo clearance of free t-PA in humans remains to be studied. Direct evidence for the involvement of α_2 MR in plasma clearance of t-PA in vivo is beginning to become available. RAP has recently been found to delay clearance of intravenously injected t-PA in rats [64].

Endocytosis of preformed u-PA/PAI-1 complex by monocytes is strongly inhibited by an N-terminal fragment of u-PA, which blocks its binding to u-PAR, but

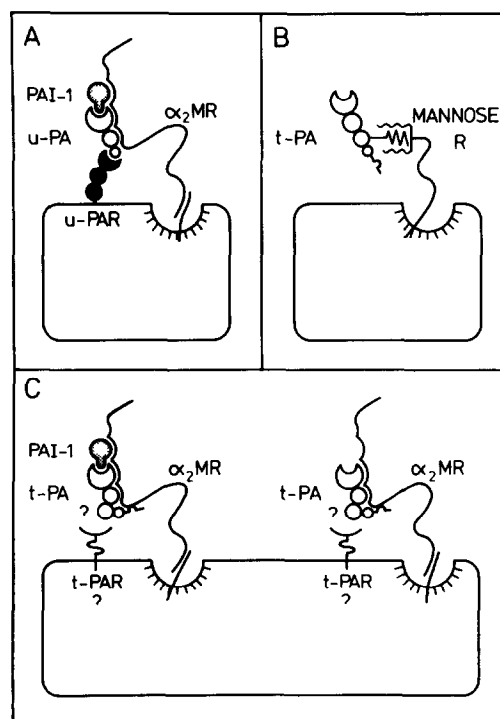


Fig. 3 Cartoon showing models for α_2 MR-mediated clearance of (A) u-PAR-bound u-PA/PAI-1 complexes from the surface of liver parenchymal cells or extrahepatic cells, (B) mannose receptor-mediated clearance of t-PA by liver endothelial cells, (C) α_2 MR-mediated clearance of t-PA/PAI-1 complexes and free t-PA by liver parenchymal cells or by extrahepatic cells, with the hypothetical involvement of a t-PA receptor. α_2 MR and mannose receptor are drawn as situated in coated pits.

not to α_2 MR [37]. This is likely to be caused by a faster association to α_2 MR of u-PAR-bound complex than of complex free in solution, u-PAR binding creating a local high concentration of complex on the cell surface. Similarly, hepatic u-PAR may serve to accelerate clearance of u-PA/PAI-1 complex from plasma by concentrating it on liver cell surfaces and presenting it to α_2 MR. Additional binding proteins could be involved in concentrating activators and activator/PAI-1 complexes on liver cell surfaces, for instance putative distinct rodent hepatic u-PA binding proteins [50,51] and putative hepatic t-PA and t-PA/PAI-1 receptors [62,63,65] (Fig. 3). Other t-PA binding proteins [66–68] may have similar functions in clearance from the pericellular space in extrahepatic tissues.

It should be noted that the various *in vivo* clearance studies referred to above have been done with highly variable amounts of activator being injected intravenously, in many cases leading to plasma concentrations far above physiological ones and far in excess of plasma PAI-1 concentrations. Although the use of such high concentrations are relevant to the *pharmacological* situation during thrombolytic therapy, it may not necessarily give results that are relevant for evaluating the relative importance of different receptor systems in *in vivo* clearance of *physiological* activator concentrations. A further complicating factor is the use of *human* u-PA in *rodents*, rodent u-PAR binds human u-PA with only a low affinity [7].

6. Structural aspects of plasminogen activator and activator/PAI-1 complex binding to α_2 MR and gp330

Like other ligands, activator/PAI-1 complexes and t-PA bind to the α -chain of α_2 MR [37,39,47]. The binding is dependent on Ca^{2+} [37–39]. u-PA/PAI-1 complex, α_2 M/proteinase complexes and RAP have been shown to bind to a region in the N-terminal part of α_2 MR, containing a cluster of eight complement-type modules, flanked N-terminally by one EGF-domain and C-terminally by two EGF-domains and a stretch of YWTD-repeats [69] (see Fig. 2). The binding sites for other ligands have not been localized within the α -chain, and α_2 MR and gp330 have more than one binding site for RAP [23,26].

In most studies, activator/PAI-1 complexes have been found to bind to α_2 MR and gp330 with higher affinity than each moiety alone [26,28,37,39]. Some observations suggests that the activator moiety as well as the inhibitor moiety make contact with α_2 MR: free u-PA and free PAI-1 inhibit the binding of u-PA/PAI-1 complex to α_2 MR with a low affinity, and u-PA/PAI-1 complex binding is inhibited by monoclonal antibodies against u-PA as well as against PAI-1 [37]. Thus, the higher affinity of activator/PAI-1 complexes, as compared to each component alone, may be due to the receptors mak-

ing contacts to both moieties. The A-chain as well as the serine proteinase domain of u-PA and t-PA (Fig. 1A) seem to be involved in binding. Thus, studies of PAI-1 and α_2 MR dependent endocytosis of t-PA by vascular smooth muscle cells led to the finding that a t-PA mutant lacking the finger and growth factor domain was endocytosed less efficiently than wild-type t-PA [45]. Substitution in t-PA of Tyr-67 with Asn created a new N-linked glycosylation site in the growth factor domain; this substitution delayed degradation of t-PA by H4 rat hepatoma cells [70], which is mediated by α_2 MR [39]. None of these mutations affected the reaction with PAI-1. One possible interpretation of these findings is that the mentioned domains are involved in t-PA/ α_2 MR contacts, but the findings need to be confirmed with purified α_2 MR, as other receptors may be involved in endocytosis (see section 5). Recent experiments with purified α_2 MR, a complex between PAI-1 and the M_r 30,000 degradation product of u-PA, which lacks the growth factor domain and the kringle, and with monoclonal anti-u-PA and anti-PAI-1 antibodies with known epitopes further support a binding mechanism with multiple independent contacts between u-PA/PAI-1 complex and α_2 MR [71].

t-PA degradation by the rat hepatoma cell line MH1C1 is PAI-1 independent, in contrast to degradation by human HepG2 hepatoma cells and around 80 percent of the degradation by the rat H4 hepatoma cells (see section 4). This is not due to major differences in PAI-1 production between the cell lines [46]. Taken at face value, this observation suggests that α_2 MR in MH1C1 cells has a relatively higher affinity to uncomplexed t-PA, as compared to t-PA/PAI-1 complex, than α_2 MR in the other cells. It may be speculated that binding specificity of α_2 MR is rendered cell-specific through differential splicing and/or post-translational modifications.

There is no universal cross-competition between the many ligands, for instance, α_2 M/proteinase complexes and u-PA/PAI-1 complex do not compete for binding to α_2 MR [37], in spite of the fact that these two sets of ligands bind to the same region of the receptor (see above). Neither are there any obvious common structural motifs in the many ligands to α_2 MR and gp330. It is noteworthy, however, that binding of many ligands, including u-PA/PAI-1 complex, is inhibited by heparin. Heparin does not bind to α_2 MR [21], and the strongly negatively charged heparin could act by binding to positive charges on the ligands. Binding of α_2 M/proteinase complexes is not inhibited by heparin [21], but derivatization of lysine residues in the human α_2 M receptor binding domain destroys receptor binding [72,73]. The complement-type modules in the region implicated in ligand binding in α_2 MR (see above) is rich in negatively charged residues (Fig. 2B). Ligand/receptor binding may thus depend on interaction, in a hydrophobic environment, between these negative charges and properly posi-

tioned positive charges on the surface of the ligands. The many negative charges may contribute to endowing this region, and putative other ligand binding regions, with a highly versatile ligand binding ability. Heparin binds to the u-PA kringle [74] and to PAI-1 [75]. The increased affinity of activator/PAI-1 complexes, as compared to each component alone, may be due to complex formation positioning groups of positive charges optimally for receptor binding.

7. Perspectives

The findings described here have important implications for our understanding of the plasminogen activation system. α_2 MR and gp330 mediated clearance of u-PA/PAI-1 complex from cell surfaces may be of functional importance for the u-PA-dependent pathway of plasminogen activation. Serine proteinase enzyme systems generally function by the sequential activation of zymogens, a period of activity of the active enzyme, and reaction with inhibitors [76]. Continued activity therefore depends on continued activation of the zymogens. Without clearance, u-PA/PAI-1 complex could accumulate and occupy the u-PAR-sites that are necessary for an efficient conversion of pro-u-PA to u-PA and subsequent u-PA catalysed plasminogen activation. Some cells are unable to degrade u-PAR-bound u-PA/PAI-1 complex, presumably due to lack of clearance receptors [35], future studies should reveal whether this results in a shorter duration of cell-surface-organized plasminogen activation.

The findings also show aspects that are novel to cell biology and biochemistry in general. The great versatility in high affinity ligand binding exhibited by α_2 MR and gp330 is unusual in receptor biochemistry. The binding of plasminogen activators and activator/inhibitor complexes to α_2 MR and gp330 represents a new type of molecular recognition of serine proteinases and serpins. Further studies of its structural basis by the use of mutagenesis, chemical crosslinking and determination of three-dimensional structures are highly desirable. Generally, internalization of GPI-anchored membrane proteins does not seem to follow the classical clathrin-coated pit pathway (reviewed in [77]). Some GPI-anchored proteins have been reported to be endocytosed via non-coated invaginations, so-called caveolae (reviewed in [78]). The α_2 MR and gp330 mediated endocytosis of the ligands initially bound to u-PAR is an alternative mode of endocytosis of ligands to GPI-anchored receptors, and perhaps of GPI-anchored receptors themselves.

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