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Receptor-mediated Regulation of Plasminogen Activator Function: Plasminogen Activation by Two Directly Membrane-anchored Forms of Urokinase

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Abstract: The generation of the broad specificity serine protease plasmin in the pericellular environment is regulated by binding of the urokinase-type plasminogen activator (uPA) to its specific glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor, uPAR. This interaction potentiates the reciprocal activation of the cell-associated zymogens pro-uPA and plasminogen. To further study the role of uPAR in this mechanism, we have expressed two directly membrane-anchored chimeric forms of uPA, one anchored by a *C*-terminal GPI-moiety (GPI-uPA), the other with a *C*-terminal transmembrane peptide (TM-uPA). These were expressed in the monocyte-like cell lines U937 and THP-1, which are excellent models for kinetic and mechanistic studies of cell-surface plasminogen activation. In both cell-lines, GPI-uPA activated cell-associated plasminogen with characteristics both qualitatively and quantitatively indistinguishable from those of uPAR-bound uPA. By contrast, TM-uPA activated cell-associated plasminogen less efficiently. This was due to effects on the K_m for plasminogen activation (which was increased up to five-fold) and the efficiency of pro-uPA activation (which was decreased approximately four-fold). These observations suggest that uPAR serves two essential roles in mediating efficient cell-surface plasminogen activation. In addition to confining uPA to the cell-surface, the GPI-anchor plays an important role by increasing accessibility to substrate plasminogen and, thus, enhancing catalysis. However, the data also demonstrate that, in the presence of an alternative mechanism for uPA localization, uPAR is dispensable and, therefore, unlikely to participate in any additional interactions that may be necessary for the efficiency of this proteolytic system. In these experiments zymogen pro-uPA was unexpectedly found to be constitutively activated when expressed in THP-1 cells, suggesting the presence of an alternative plasmin-independent proteolytic activation mechanism in these cells. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: plasmin; plasminogen activator; receptor; serine protease; urokinase

INTRODUCTION

Urokinase-type plasminogen activator (uPA) is one of two specific serine proteases responsible for the conversion of plasminogen, an abundant extracellular serine protease zymogen, to the broad specificity protease plasmin. uPA is expressed in a variety of cell types from which it is secreted as pro-uPA, a single-chain proenzyme form, which can be converted to the fully active two-chain protease by limited proteolysis catalysed by a number of proteases, including plasmin [1,2]. This action of plasmin leads to the formation of a reciprocal zymogen activation system and a consequent amplification of plasmin generation [3]. The mechanisms that exist for the functional regulation of this system include

Abbreviations: 6AHA, 6-aminohexanoic acid; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor.

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various cofactors, inhibitors and cellular receptors or binding sites for the protease components [4].

One of these regulatory components is uPAR, the specific cellular receptor for uPA. uPAR is a multidomain, glycolipid-anchored protein, which binds both pro-uPA and activated uPA with high affinity (K_d 0.5 nM) via the *N*-terminal epidermal growth factor-like module of uPA [5]. Binding of pro-uPA to uPAR leads to a large increase in the generation of plasmin, due to increased efficiencies of the reciprocal activation of both zymogens [6,7]. Although the kinetic mechanisms leading to this potentiation have been well described, the molecular mechanisms involved have yet to be fully elucidated.

The observed kinetic effects are known to have an absolute dependence on the cellular binding of plasminogen [6,7] as determined by the effects of 6-aminohexanoic acid (6AHA) which inhibits the binding by occupying the lysine-binding-sites of the kringle modules of plasminogen [8]. Consistent with this dependency a recombinant soluble form of uPAR does not enhance plasmin generation [9]. The lack of a direct effect of uPAR on the activity of uPA is concordant with the high degree of dynamic independence displayed by the individual domains of uPA [10,11] suggesting that the serine protease domain would be unaffected by the interaction of the *N*-terminal epidermal growth factor-like module with uPAR.

Although uPAR in solution is unable to potentiate plasmin generation, we have identified an anti-uPA monoclonal antibody that is able to do this, and does so with kinetics very similar to those of the cell-associated uPAR system [12]. The mechanism of this effect is ternary complex formation involving the interaction of the antibody with the kringle module of uPA and also with plasminogen, via a *C*-terminal lysine residue on the IgG heavy chains. Thus, the formation of stoichiometric complexes between uPA and plasminogen in the appropriate orientation kinetically mimic the cell-associated uPAR system. We have taken as evidence that these two systems also mechanistically mimic each other and that the cell-associated uPAR system also involves specific complex formation. We have obtained indirect evidence for the existence of such putative complexes [13], and also observed a non-catalytic interaction between uPA and plasminogen in solution [14] that we believe to be involved in their assembly. This uPA·plasminogen interaction is independent of uPAR and this model therefore predicts that alternative modes of confining uPA to the cell surface may also be effective. Consistent with this prediction we have previously shown that uPA directly anchored to the plasma membrane by a *C*-terminal modification leading to addition of a glycosylphosphatidylinositol (GPI)-moiety is a catalytically efficient plasminogen activator [15,16]. We now test this prediction further by determining the activity of both GPI-anchored uPA and a transmembrane-anchored form expressed in two monocyte-like cell lines that are excellent models for kinetic and mechanistic studies of cell-surface plasminogen activation.

MATERIALS AND METHODS

Reagents

Human uPA, pro-uPA, Glu_1-plasminogen, plasmin and the anti-uPA and anti-uPAR monoclonal antibodies were all as described previously [7,12,17]. The plasmin-specific fluorogenic substrate H-D-Val-Leu-Lys-7-amido-4-methylcoumarin was obtained from Bachem, Bubendorf, Switzerland. All other reagents were of the highest grade commercially obtainable.

Cells and Recombinant uPA Constructs

Construction of the LUK + ASN vector encoding uPA cDNA that is modified to include a *C*-terminal sequence from decay accelerating factor (DAF) that directs plasma membrane attachment via a GPI anchor has previously been described [15]. The vector LUKTM encoding uPA cDNA modified at the *C*-terminal to include the transmembrane and first 17 cytoplasmic residues of the rabbit polymeric immunoglobulin receptor [18] was constructed using similar techniques. U937 and THP-1 cells were transduced by incubation with supernatant containing virions with the relevant retroviral vectors as previously described [15]. After transduction, cells were selected in 0.4 mg/ml G418 for at least 14 days prior to being used in experiments. Prior to use in most experiments, cells were briefly incubated in glycine-HCl pH 3.0 to remove traces of uPAR-associated uPA as described previously [19].

Kinetic Assays

Determination of plasmin generation and plasminogen activation kinetics were performed essentially as described previously [7,14] using human Glu^1-plasminogen and fluorimetric detection of plasmin generation at excitation and emission wavelengths

of 370/470 nm in a Perkin–Elmer LS-5B luminescence spectrometer with the substrate H-D-Val-Leu-Lys-7-amido-4-methylcoumarin with reactions performed at 37 °C. Change in fluorescence ($\delta F/\delta t$) was converted to plasmin concentration by reference to data for active-site titrated plasmin. In some experiments, 1 mM 6AHA, a ligand for the lysine-binding kringle modules of plasminogen, was included as a non-competitive inhibitor of the cellular binding of plasminogen.

Kinetic Analysis

Kinetic constants were calculated by non-linear regression analysis of the primary data. The non-linear plasmin generation curves obtained when zymogen pro-uPA was the activator initially present on the cells were analysed as previously described [9,12]. Briefly, the final linear phase of plasmin generation was calculated from the experimentally determined kinetic constants for plasminogen activation by activated uPA. The rate of plasmin-catalysed pro-uPA activation was determined by curve fitting. This procedure was iterated to give the best fit of the data over a range of plasminogen concentrations.

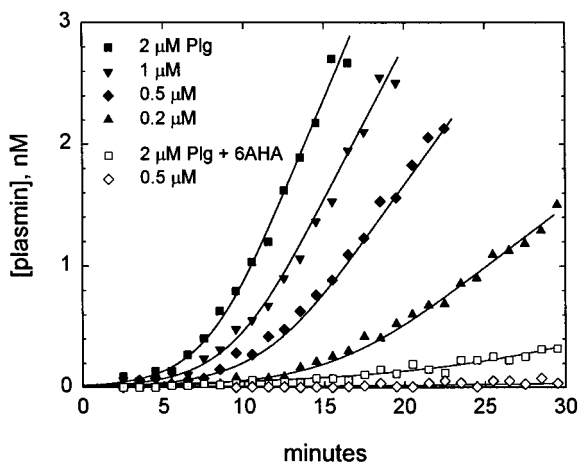

Figure 1 Plasmin generation by GPI-uPA on U937 cells is shown in the presence (open symbols) and absence (closed symbols) of 1 mM 6AHA at varying concentrations of plasminogen. The lines shown are the best fits of the data to a model describing reciprocal zymogen activation using the experimentally calculated kinetic constants for plasminogen activation (shown in Table 1). From these fits the rate of plasmin-catalysed pro-uPA activation were determined and found to be 9.9 $\mu M^{-1}s^{-1}$ and 1.8 $\mu M^{-1}s^{-1}$ in the absence and presence of 6AHA, respectively.

Determination of Cell-Associated uPA

The amount of uPA present on the surface of GPI-uPA and TM-uPA expressing cells was determined by flourescence-activated cell sorter (FACS) analysis using the monoclonal antibody clone 6, recognizing the *N*-terminal fragment of uPA. These data were normalized to wild-type cells saturated with uPA whose uPAR density had been determined by radioligand binding analysis using ^{125}I-labelled *N*-terminal fragment of uPA [7]. Monoclonal antibodies recognizing epitopes in the serine protease domain of uPA gave similar results, demonstrating that the accessibility of the antibodies to the various forms of cell-associated uPA was unaltered, and the quantification therefore valid. Similar experiments were performed using the anti-uPAR monoclonal antibody R3 [17] to exclude the possibility that the directly anchored forms of uPA interacted with uPAR on the cell surface.

In some experiments, cells expressing TM-uPA were treated for 20 min at 37 °C with 1 μg/ml phosphatidylinositol-specific phospholipase C (PI-PLC, Boehringer–Mannheim, Germany) to remove GPI-anchored uPAR, which is constitutively expressed on both U937 and THP-1 cells.

RESULTS

Activity of GPI-uPA

Our previous studies on the mechanism of uPAR-mediated, cell-associated plasminogen activation have utilized the monocyte-like cell U937 as a model, this cell-line expressing both uPAR and plasminogen binding-sites but very little plasminogen activator inhibitors. Its ability to grow and be used experimentally in suspension also facilitates its use in detailed kinetic studies. Therefore, this cell-line was chosen for these with directly-anchored forms of uPA, which were introduced into the cells by retroviral transduction.

Figure 1 shows that GPI-uPA efficiently activates plasminogen with characteristics qualitatively similar to those observed with the uPAR-mediated system on non-transduced cells. These characteristics are; a lag-phase due to reciprocal zymogen activation initiated by the low intrinsic catalytic activity of uPA zymogen (pro-uPA), and a dependency on the cellular binding of plasminogen as shown by effect of the non-competitive inhibitor of this binding, the lysine analogue 6AHA. The similarity between the two systems could be a

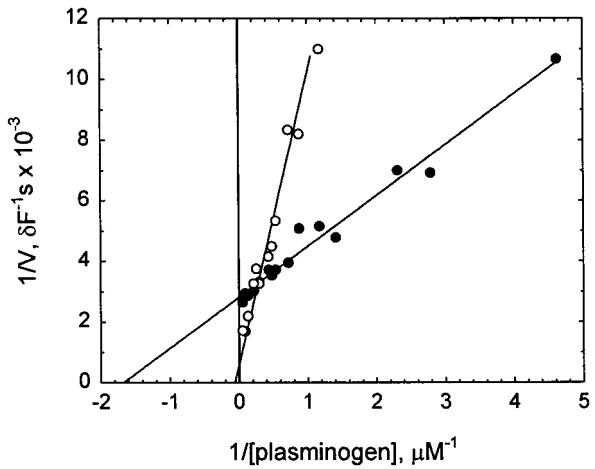

Figure 2 Kinetics of plasminogen activation by GPI-uPA on U937 cells. Data are shown over a range of plasminogen concentrations in the presence (open symbols) and absence of 6AHA (closed symbols).

consequence of the *N*-terminal receptor-binding epidermal growth factor (EGF)-like module of uPA interacting with cellular uPAR. However this was shown not to be the case as; the inclusion of blocking anti-uPAR monoclonal antibodies had no effect, the EGF-like module of uPA was fully accessible to monoclonal antibodies as determined by FACS analysis and the cells had unoccupied uPAR as determined by ^{125}I-labelled uPA binding.

Kinetics of Plasminogen Activation by GPI-uPA

Therefore, to further study the uPAR-independent activity of GPI-uPA, the kinetics of plasminogen activation by uPA were determined, subsequent to plasmin activation of the pro-uPA zymogen. Plasmin generation was found to be linear with time under all conditions and over a wide range of plasminogen concentrations. Lineweaver–Burk plots of these data (Figure 2) clearly show a large decrease in the K_m for plasminogen activation when plasminogen is associated with the cells, i.e. in the absence of 6AHA. Similar experiments were also performed using THP-1 cells expressing GPI-uPA. When the kinetic constants derived from these data are compared to those for uPAR-bound uPA on wild-type U937 and THP-1 cells (Table 1) it can be seen that, although there may be a slight tendency towards a higher K_m for plasminogen activation, within experimental limits GPI-uPA is virtually indistinguishable from uPA·uPAR.

Kinetics of Plasminogen Activation by TM-uPA

To determine whether the similarity in the kinetic constants between directly-anchored uPA and uPA·uPAR was related to both being anchored by a GPI moiety, we performed similar experiments using TM-uPA, with a *C*-terminal transmembrane peptide anchorage sequence. Despite showing qualitative similarities to both the GPI-uPA and uPA·uPAR systems, quantitative analysis shows that plasminogen activation by TM-uPA is of lower efficiency (Table 1). This is primarily due to an increase in the K_m for cell-associated plasminogen, although this is still much lower than that observed in the absence of plasminogen binding (greater than 20 μM, data not shown) or for the reaction in solution [7]. Cells expressing TM-uPA were also treated with PI-PLC to remove GPI-anchored uPAR and, as with the GPI-uPA expressing cells, uPAR was found to play no part in these effects.

Activation of Directly Membrane-Anchored Zymogens

The overall potentiation of plasmin generation by the uPA·uPAR system is due to effects both on plasminogen activation and the reciprocal activation of uPAR-bound pro-uPA by cell-associated plasmin. To determine the activation kinetics of

Table 1 Kinetic Constants for the Activation of Plasminogen by Various Forms of uPA on U937 and THP-1 Cells

	U937 cells			THP-1 cells		
	K_m, μM	k_{cat}, s^{-1}	K_{cat}/K_m, $\mu M^{-1}s^{-1}$	K_m, μM	k_{cat}, s^{-1}	K_{cat}/K_m, $\mu M^{-1}s^{-1}$
uPA·uPAR	0.30 ± 0.18	0.15 ± 0.05	0.50 ± 0.20	0.36 ± 0.22	0.21 ± 0.04	0.57 ± 0.13
GPI-uPA	0.49 ± 0.15	0.25 ± 0.07	0.52 ± 0.11	0.63 ± 0.20	0.18 ± 0.04	0.28 ± 0.09
TM-uPA	1.2 ± 0.3	0.22 ± 0.03	0.18 ± 0.05	1.9 ± 0.9	0.10 ± 0.06	0.053 ± 0.038

In all cases the enzyme is plasmin activated two-chain uPA. Data show the means ± S.D. of at least five independent experiments.

Table 2 Catalytic Efficiencies (k_{cat}/K_m) for the Activation of Various Forms of Pro-uPA on U937 Cells

	−6AHA, $\mu M^{-1}s^{-1}$	+6AHA, $\mu M^{-1}s^{-1}$
uPA·uPAR	11.3	1.8
GPI-uPA	9.9	1.8
TM-uPA	2.5	0.7
In solution	1.1	–

The data shown are obtained by curve fitting of overall reciprocal zymogen activation data, such as those shown in Figure 1.

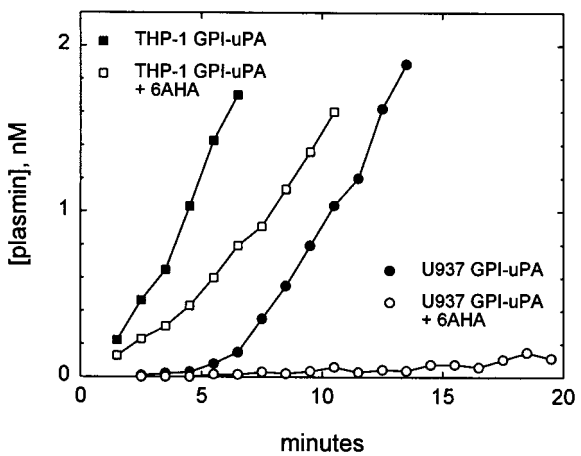

Figure 3 Comparison of plasmin generation by GPI-uPA on U937 and THP-1 cells in the presence and absence of 6AHA. The high initial rates of plasmin generation observed on THP-1 cells demonstrate constitutive activation of pro-uPA on these cells, whereas U937 cells display the lag-phase indicative of zymogen activation.

zymogen GPI-uPA and TM-uPA, overall plasmin generation curves obtained over a range of plasminogen concentrations were analysed. Using the experimentally determined kinetic constants for plasminogen activation and a mathematical model describing reciprocal zymogen activation, rates of pro-uPA activation can be derived. The fitted curves for experiments with GPI-uPA in the presence and absence of 6AHA are shown in Figure 1. As with the plasminogen activation kinetics, pro-uPA activation is again very similar to that of uPAR-bound pro-uPA with GPI-uPA but less efficient with TM-uPA (Table 2). In the absence of plasmin/plasminogen binding, pro-uPA activation is similar in all cases and close to that observed in solution.

Constitutive Activation of uPA Zymogen on THP-1 Cells

In attempting to analyse pro-uPA activation kinetics on THP-1 cells, it was observed that rather than displaying the extended lag-phase observed on U937 cells, plasmin generation was immediate and there was only a small increase in rate with time (Figure 3). These observations are consistent with a high degree of constitutive zymogen activation on these cells, and this could also be reproduced on wild-type THP-1 cells with exogenously added pro-uPA. The effect of 6AHA was also much less marked on THP-1 cells which is again consistent with the presence of active uPA, as under these conditions the additional inhibitory effect of 6AHA on pro-uPA activation by cell-associated plasmin is lost, [7].

DISCUSSION

The observations presented here further our understanding of the mechanisms by which uPAR, through the assembly of specific plasminogen activation complexes, enhances pericellular proteolysis. Using two directly membrane-associated, chimeric forms of uPA, we demonstrate that efficient plasmin generation, equivalent to that mediated by the interaction of uPA with cellular uPAR, can be achieved despite the absence of uPAR. This is consistent with our previous observations demonstrating that the uPAR protein *per se* has no enhancing effect on the enzymatic reactions involved [9], and that an anti-uPA monoclonal antibody can quantitatively mimic the effects of cellular uPAR by binding uPA and plasminogen in a ternary complex [12]. It is also consistent with our hypothesis that a direct, non-active-site interaction between cell-associated plasminogen and cell-associated uPA is a major contributor to the assembly and efficiency of the plasminogen activation complexes [14].

These data do not, however, exclude the possibility that uPAR has a specific function in this system, other than in confining uPA to the cell surface. Both of the directly anchored uPAs studied here are attached to the plasma membrane by *C*-terminal modifications. This must mean that the serine protease domain is proximal to the plasma membrane. In the uPA·uPAR complex, five protein domains separate the serine protease domain from the GPI-anchor, yet GPI-uPA and uPA·uPAR have virtually identical kinetics constants for the activation of cell-associated plasminogen. This suggests that the

serine protease domain of uPA is also in close proximity to the plasma membrane and, therefore, that uPAR has a role in the spatial orientation of uPA relative to the plasma membrane. In this context it is interesting to note that, despite its three domain structure, uPAR is thought to be a compact protein, with strong evidence for functionally significant interactions between the *N*- and *C*-terminal domains [5,20,21]. uPA itself has a high degree of interdomain flexibility, as determined by nuclear magnetic resonance (NMR) studies [10,11], which may allow membrane proximity of the serine protease domain. This could occur either randomly by dynamic motion or potentially additional low affinity interactions may favour this orientation. The kringle module of uPA has the capacity to bind sulphated glycosaminoglycans [22] and, therefore, to be involved in the latter. It may be possible to test the hypothesis that the serine protease domain is proximal to the plasma membrane using fluorescence resonance energy transfer techniques, as has been shown for certain of the protease·receptor complexes of the blood coagulation system when reconstituted into lipid vesicles [23,24].

Although GPI-uPA and uPA·uPAR behave very similarly, the activity of TM-uPA was significantly reduced. This was apparent both as an increased K_m for plasminogen activation and as a reduced efficiency of the activation of the zymogen pro-uPA. There are two possible explanations for these observations, both related to the characteristics that the two types of membrane anchor confer on integral membrane proteins. Firstly, GPI-anchored proteins generally have a higher rate of lateral diffusion within the plasma membrane than transmembrane proteins [25]. Therefore, a decreased mobility of TM-uPA may decrease its accessibility to cell-associated plasmin/plasminogen with a consequent reduction in reaction rates. However if this were the case it would be predicted, at least under ideal conditions, to lead to a reduction in k_{cat}, rather than the increase in K_m that was observed. Secondly, GPI-anchored proteins have a propensity to be sequestered into cholesterol-rich membrane microdomains or 'rafts' [26]. It could be envisaged that these represent preferential sites for plasminogen activation and facilitate catalysis either by co-localizing uPA with plasminogen binding molecules or by clustering leading to increased local reactant concentrations. Either of these would be predicted to give the observed effects on apparent K_m. Disruption of lipid microdomains with cholesterol-binding compounds has been reported to reduce the activity of uPAR-associated uPA in melanoma cells [27], although we have not observed such effects on other cell types [28].

A number of integral membrane proteins occur naturally in both GPI- and TM-anchored forms, due to alternative mRNA splicing, while others have been made as recombinant chimeras, as we have done here. In some cases these proteins display dissimilar behaviour, while in other cases they do not. For example, DAF inhibits complement lysis equally well in either form [29], while the lymphocyte antigen Ly-6 needs to be GPI-anchored to mediate T-cell activation [30]. GPI anchors have also been shown to increase the residency time of proteins on the cell surface [31], and consistent with this a TM-anchored form of uPAR has been reported to alter the internalization of the uPA·PAI-1 complex [32]. However, no general rules for the effect of anchorage method on membrane-associated enzyme systems have previously been reported.

The constitutive activation of membrane-anchored uPA observed on THP-1 cells was unexpected, as with U937 cells uPA was present as the intact zymogen. This activation was not inhibited by aprotinin (data not shown), suggesting a plasmin-independent mechanism which could be involved in the initiation of the uPA-catalysed plasminogen activation pathway on the cell surface. The nature of the protease responsible for this activation is currently under investigation in this laboratory.

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