The pro-urokinase plasminogen-activation system in the presence of serpin-type inhibitors and the urokinase receptor: rescue of activity through reciprocal pro-enzyme activation

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The reciprocal pro-enzyme activation system of plasmin, urokinase-type plasminogen activator (uPA) and their respective zymogens is a potent mechanism in the generation of extracellular proteolytic activity. Plasminogen activator inhibitor type 1 (PAI-1) acts as a negative regulator. This system is complicated by a poorly understood intrinsic reactivity of the uPA pro-enzyme (pro-uPA) before proteolytic activation, directed against both plasminogen and PAI-1. We have studied the integrated activation mechanism under the repression of PAI-1 in a purified system. A covalent reaction between pro-uPA and PAI-1 was positively demonstrated but the reaction of PAI-1 with two-chain uPA was found to be at least 1000-fold faster. However, in spite of this very fast inhibition, two-chain uPA still became the dominant plasminogen activator when plasminogen was incubated with pro-uPA and PAI-1. The activity pattern observed under these conditions revealed an initial lag phase, followed by

a continuous generation of minute amounts of active two-chain uPA, this uPA having a short lifetime before inhibition but still succeeding to generate new plasmin activity, thus preventing a complete inactivation of the feedback system. This property of the activation system was retained even in the simultaneous presence of PAI-1 and α_2 -antiplasmin. Addition of soluble uPA receptor to the system did not change the role of pro-uPA and the same pattern was observed when pro-uPA was bound to the uPA receptor on U937 cells. The present mechanism maintains the system at standby level and may be triggered to increased activity without the need for an external initiating event.

Key words: α_2 -antiplasmin, cascade system, plasminogen activator inhibitor type 1 (PAI-1), proteolysis, urokinase-type plasminogen activator receptor (uPAR).

INTRODUCTION

The urokinase-mediated plasminogen-activation system is an extracellular proteolytic cascade mechanism involved in matrix degradation, fibrinolysis and a number of proteolytic events with specific regulatory roles. The system is active in tissue-remodelling processes that occur under various normal physiological conditions and also in connection with cancer invasion [1].

In this system the serine protease urokinase-type plasminogen activator (uPA), which occurs with low abundance and has a limited tissue distribution, is capable of releasing a large proteolytic potential through the activation of the abundant serine protease zymogen, plasminogen [2]. Therefore, the regulatory events that govern uPA activity are crucial for the whole system. uPA is secreted in the form of a single polypeptide chain pro-enzyme (pro-uPA) with a very low intrinsic activity [3,4]. Proteolytic activation of pro-uPA can be catalysed by plasmin as well as certain other proteases with trypsin-like specificity and leads to the generation of the fully active two-chain uPA [5–10]. The fact that plasmin, the result of plasminogen activation, can efficiently activate pro-uPA leads to a strong positive-feedback mechanism. This reciprocal pro-enzyme activation is a central property of the plasminogen-activation system [4].

The major inhibitor of uPA in serum and most extracellular fluids is considered to be plasminogen activator inhibitor type 1 (PAI-1), which belongs to the serpin family [11]. The fast inactivation of two-chain uPA by PAI-1 is one of the major

mechanisms to keep the accelerative feedback activation under control. Another serpin, α_2 -antiplasmin, is crucially involved in the inhibition of plasmin activity [12].

At least two factors, the necessity for pro-enzyme activation and the subsequent inhibition of the active enzyme, thus serve to limit the level of uPA activity. However, a number of poorly understood, additional events contribute to make the complete picture more complicated. Firstly, it is likely that several routes exist for proteolytic initiation of the cascade system. While a number of proteases have been shown to activate pro-uPA *in vitro*, as mentioned above, there are few examples demonstrating which of those are operative *in vivo*, and under which conditions the activation reaction occurs [10]. Secondly, the importance of the intrinsic activity of pro-uPA has been debated. Even though there is general consensus that this activity is very low, it may have significance as a cascade initiator [13]. Therefore, it is still an open question whether an upstream protease initiator is actually needed in all cases. Thirdly, the selectivity of PAI-1 for the active two-chain form of uPA has been questioned [14,15].

An additional level of complexity arises in terms of the localization of the proteolytic processes. The specific cellular uPA receptor (uPAR) is a central participant in the cascade system and plays important roles in the localization of the activation reactions [16]. Gene inactivation studies in mice have thus demonstrated the physiological relevance of uPA-mediated plasminogen-activation reactions occurring in solution as well as through a uPAR-dependent pathway [17]. Actually, a number of

Abbreviations used: H-D-Val-Leu-Lys-AMC, H-D-Val-Leu-Lys-7-amido-4-methylcoumarin; mAb, monoclonal antibody; PAI-1, plasminogen activator inhibitor type 1; pro-uPA, uPA pro-enzyme; suPAR, recombinant soluble human uPAR; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor.

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complicated mechanisms seem to favour cell-surface-localized proteolysis relative to the activation reactions in solution. These mechanisms include a template function for the assembly of the activation system on the cell surface as well as a protection of surface-bound proteolytic activity against inhibition [18]. Importantly, however, the mere binding of uPA to uPAR is not sufficient for these interactions to occur. The template mechanism for acceleration of the activation system thus is the result of an interplay between uPAR and binding sites for plasminogen [13,19], whereas inhibitor protection is mostly directed to cell-bound plasmin that is efficiently protected against α_2 -antiplasmin [20]. The elucidation of these composite functions requires initial studies of the participating components alone, including investigations on the function of uPAR in a purified system. To this end, as would be expected from the need for plasminogen-binding components as mentioned above, purified, soluble uPAR was found to be devoid of any template function or any other stimulatory role in the activation cascade [21,22]. On the other hand, in terms of inhibitor protection, a function of purified uPAR has actually been suggested [15].

In this paper we have examined the pro-uPA plasminogen feedback system in the presence of PAI-1, focusing on the reactivity of the inhibitor with the pro-enzyme and two-chain uPA respectively, as well as the balance between two-chain uPA and pro-uPA activity under inhibitor repression. Furthermore, we have determined the influence of recombinant, soluble uPAR on the same reactions and studied the behaviour of a cell-bound activation system in the presence of inhibitors.

EXPERIMENTAL

Proteins

The following proteins were purchased from the commercial sources indicated: human pro-uPA, Glu-plasminogen and α_2 -antiplasmin (Technoclone, Vienna, Austria), human two-chain uPA (Serono, Zug, Switzerland), human plasmin (Roche Molecular Biochemicals, Basel, Switzerland) and recombinant human PAI-1 with wild-type sequence (Calbiochem, Bad Soden, Germany). Recombinant human mutant pro-uPA(K158A), expressed in transfected HEK-293 cells and purified from conditioned media [23], was the kind gift of Dr H. H. Petersen (Department of Molecular and Structural Biology, Århus University, Århus, Denmark). Anti-uPA monoclonal antibody (mAb) clone 5 (against human pro-uPA and two-chain uPA) and anti-PAI-1 mAb clone 2 were from the hybridomas described previously [24,25] whereas the zymogen-specific mAb 3901 against human pro-uPA was purchased from American Diagnostica (Greenwich, CT, U.S.A.). Production and purification of recombinant soluble human uPAR (suPAR; residues 1–277) and labelling of pro-uPA and two-chain uPA with ^{125}I were performed as described in [26].

Chromogenic activity assays

Coupled chromogenic assays for plasminogen activation were performed using the experimental set-up and data representation described previously [22], except that the chromogenic plasmin substrate H-D-Val-Leu-Lys-*p*-nitroanilide (S2251; Chromogenix AB, Mölndal, Sweden) was used and that, under standard conditions, the buffer was 0.1 M Tris/HCl, pH 7.4, with 0.1 % Tween 80. In a variant of the assay (see Figure 1B, below), 150 mM NaCl was included in the buffer as specified. Samples with chromogenic substrate, pro-uPA or two-chain uPA and PAI-1 were mixed as indicated in the individual experiments, followed by addition of plasminogen. Parallel samples were

prepared without PAI-1. In some experiments, α_2 -antiplasmin was included in the samples, or pro-uPA was pre-incubated for 10 min with suPAR with concentrations as indicated. All samples were tested in duplicate. The absorbance at 405 nm was followed with time during incubation at 37 °C, time 0 referring to the addition of plasminogen. The absorbance increments were calculated at each time point, based on 5 or 10 min reading intervals. The results are represented in terms of plasmin activity ($\Delta A_{405}/\text{min}$) as a function of time. In experiments with PAI-1 and α_2 -antiplasmin present simultaneously, the direct readings of A_{405} are shown due to the low absorbance increments obtained. Control samples, prepared by excluding uPA from the mixtures above, showed no measurable activity.

Fluorogenic assays

Fluorogenic assays using the plasmin substrate H-D-Val-Leu-Lys-7-amido-4-methylcoumarin (H-D-Val-Leu-Lys-AMC; Bachem, Bubendorf, Switzerland) were performed as described in [27] with the following modifications. Assays were performed in non-transparent 96-well microtitre plates for fluorescence reading (Nunc, Roskilde, Denmark) in a total reaction volume of 200 μl for each sample. Cell-free samples were assayed using the same reaction buffer as specified for the chromogenic assays above whereas cell-containing samples were analysed using 50 mM Tris/HCl, 100 mM NaCl, pH 7.4, with 0.1 % BSA (TBS/BSA). Cell-free samples were mixed as specified above, except that H-D-Val-Leu-Lys-AMC (200 μM) was used instead of the chromogenic substrate. Samples were read at 2.5 min intervals during incubation at 37 °C in a FLUOstar Galaxy fluorescence plate reader (BMG LabTechnologies, Offenbourg, Germany), using filters for excitation at 390 nm and emission at 480 nm, and a gain of 20. The fluorescence increment at each time point was calculated and the plasmin activity ($\Delta F/\text{min}$) was plotted as a function of time. For samples with U937 cells, cells were cultured as described in [28], washed extensively and resuspended in TBS/BSA at a density of 5×10^6 cells/ml. The cells were then preincubated for 1 h at 4 °C with gentle agitation in the presence of pro-uPA (10 nM), two-chain uPA (10 nM) or buffer alone. The cells were washed three times in TBS/BSA for the removal of unbound ligand after which they were added at a final cell density of 1×10^6 cells/ml to mixtures of H-D-Val-Leu-Lys-AMC and inhibitors, as indicated, at 37 °C. Plasminogen was added immediately (at time 0) after which the fluorescence was followed at 37 °C as above, except that 2 min reading intervals were used with a short shaking period after each reading to resuspend the cells. Plasmin activity was calculated as above, subtracting the value for a parallel, cell-free sample without uPA/pro-uPA. All cell-free and cell-containing samples were tested in duplicate.

Analysis of covalent PAI-1 complexes

For formation of complexes, samples of pro-uPA or two-chain uPA were dissolved in 0.1 M Tris/HCl, pH 7.4/0.1 % Tween 80 and incubated with PAI-1 under conditions as indicated. Covalent complexes were analysed by SDS/PAGE as described in [10]. After electrophoresis, the complexes were visualized by direct Coomassie Brilliant Blue staining, or by Western blotting with specific antibodies and an enhanced chemiluminescence detection system [29]. In some experiments, ^{125}I -labelled uPA was used. For these studies, the standard conditions included incubation of 40 nM ^{125}I -pro-uPA or ^{125}I -two-chain uPA with 500 nM PAI-1 at 37 °C. Specific experiments with low concentrations (5 and 10 nM) of PAI-1 were done using 2 nM ^{125}I -two-chain uPA. Differential studies on ^{125}I -pro-uPA wild-type and

Figure 1 Reciprocal pro-enzyme activation in the presence of serpin-type inhibitors

Chromogenic assays were performed by mixing the samples specified below with the chromogenic plasmin substrate H-D-Val-Leu-Lys-p-nitroanilide (final concentration, 400 μM). Plasminogen was then added to all samples at time 0 and the A_{405} was followed during incubation at 37 °C. **(A)** Assay was performed under standard conditions (see the Experimental section). The curves shown represent addition of pro-uPA alone (●), two-chain uPA (tc-uPA) alone (▼), pro-uPA + PAI-1 (○) and two-chain uPA + PAI-1 (▽). The final concentrations of the reactants were 1 μM plasminogen, 0.5 nM pro-uPA and two-chain uPA and 2 nM PAI-1. Plasmin activity ($\Delta A_{405} / \text{min}$) is plotted as a function of time. Each curve shows mean $\pm$ range from a double determination. No indication of range means that the range was smaller than the symbol used. **(B)** The experiment was performed as in **(A)**, except that NaCl (150 mM) was included in the buffer. **(C)** Curves from the same experiment as in **(A)** are shown on an expanded scale. In addition to the pro-uPA-containing samples specified above, curves are shown for samples prepared in the same manner but using pro-uPA(K158A) (0.5 nM) instead of wild-type (wt) pro-uPA; i.e. addition of pro-uPA(K158A) alone (■) and pro-uPA(K158A) + PAI-1 (□). **(D)** A chromogenic assay was performed as in **(A)** except that the concentration of plasminogen was 2 μM and that the following samples were added: Pro-uPA + PAI-1 (○), two-chain uPA + PAI-1 (▽), Pro-uPA + PAI-1 + α_2 -antiplasmin (◇) and two-chain uPA + PAI-1 + α_2 -antiplasmin (□). Pro-uPA and two-chain uPA refer to the wild-type proteins and were both used at a final concentration of 0.5 nM. PAI-1 and α_2 -antiplasmin were used at final concentrations of 2 nM and 1 μM , respectively. Each curve depicts the direct reading of A_{405} as a function of time (mean $\pm$ range from a double determination).

^{125}I -pro-uPA(K158A) were done using standard conditions, except that the concentration of labelled pro-uPA was 80 nM and that samples with ^{125}I -two-chain uPA were replaced by samples with ^{125}I -pro-uPA subjected to plasmin treatment. In these experiments, each of the ^{125}I -labelled pro-uPA variants (80 nM) was incubated with 20 nM human plasmin in 0.1 M Tris/HCl, pH 7.4/0.1% Tween 80 for 2 h at 37 °C, after which the reaction was stopped by addition of 25 $\mu\text{g}/\text{ml}$ basic pancreas trypsin inhibitor (Trasylol). Samples of intact ^{125}I -labelled pro-uPA to be used for comparison in these experiments were

incubated with buffer alone, followed by addition of Trasylol in the same manner. After SDS/PAGE as above, labelled protein complexes were revealed by autoradiography. For quantification of complexes, radiolabelled protein bands were excised from the gels for γ -counting to determine the complexed fraction of the total radioactivity [26].

For studies on complex stability, ^{125}I -labelled complexes were generated after which the reaction was stopped by addition of an excess of unlabelled two-chain uPA and the decay of the complexes was followed with time [14]. In these experiments,

Figure 2 For legend, see facing page

Figure 3 Time course of covalent complex formation

^{125}I -Pro-uPA (left-hand panel) or ^{125}I -two-chain uPA (right-hand panel), both at 40 nM, were incubated in the presence (+) or absence (–) of PAI-1 (500 nM) for the times indicated. Electrophoretic analysis under reducing conditions and autoradiography were performed as in Figure 2(C).

^{125}I -pro-uPA or ^{125}I -two-chain uPA, prepared by plasmin activation as described above, was incubated with PAI-1 under standard conditions at 37 °C. After complex formation for 1 h, 2 μM unlabelled two-chain uPA was added. Separate experiments in which unlabelled two-chain uPA was present from the start of the experiment made sure that this addition would completely prevent the subsequent formation of new labelled complexes. Incubation was now continued for 18 h at 37 °C, after which the labelled PAI-1 complexes remaining were revealed by SDS/PAGE and autoradiography. The labelled complexes were quantified as described above. The amount of labelled complexes remaining after the overnight incubation at 37 °C was compared with that present in a sample stopped immediately after the formation of the labelled complex.

RESULTS

Reciprocal pro-enzyme activation under inhibitor repression

In order to study the integrated activation system in the presence of inhibitors, we examined the effect of PAI-1 on a system with pro-uPA and plasminogen. Pro-uPA and plasminogen were mixed at final concentrations of 0.5 nM and 1 μM , respectively, and the plasmin activity was followed using a chromogenic substrate present in the mixture (Figure 1A). As expected, in the absence of PAI-1 these conditions led to an accelerative accumulation of plasmin activity (Figure 1A, ●), indicative of feedback activation of the two pro-enzymes [4,22]. This activation pattern was distinct from the linear generation of plasmin activity obtained with two-chain uPA (Figure 1A, ▼, and results not shown). Addition of a 4-fold excess of PAI-1 to these samples led to a strong suppression of plasmin generation in both cases. However, the low activity measured under these conditions

clearly developed differently depending on whether two-chain uPA or pro-uPA was studied. When the experiment was done using two-chain uPA (Figure 1A, ▽), only minute plasmin activity was generated and only during the first few min, reflecting the initial uPA activity followed by irreversible inhibition and the lack of formation of new active uPA. On the other hand, when pro-uPA was used (Figure 1A, ○), plasmin activity was generated slowly but kept accumulating during the entire experimental period to levels much higher than those obtained with two-chain uPA. The inclusion of 150 mM NaCl in the buffer did not change this pattern (Figure 1B) although in this case plasminogen activation was slower than that observed in the absence of NaCl, in accordance with previously published studies [30].

The enhanced accumulation of activity in this system, relative to the situation with two-chain uPA, might be assumed to reflect the intrinsic activity of pro-uPA, this activity becoming dominant relative to two-chain uPA due to the inactivation of the latter by PAI-1. Therefore, we examined the progress of activity in detail. Expansion of the scale allowed a clear evaluation of the shape of the curve (Figure 1C; the same experiment as shown in Figure 1A). Interestingly, in the system with pro-uPA and PAI-1 (Figure 1C, ○), the activity did not start to accumulate until after a pronounced lag phase (15–20 min under the conditions used). An equally evident but shorter lag phase (≈ 10 min) was obtained if the concentration of pro-uPA was increased to 1 nM, thus reducing the excess of PAI-1 to 2-fold (result not shown).

The existence of a lag phase was not in ready agreement with the possibility that pro-uPA was the active enzyme activating plasminogen under these conditions because pro-uPA was present at the maximum concentration from the start of the experiment. Accordingly, also in the presence of PAI-1, the activity observed after the lag phase would most likely be ascribed to two-chain uPA, generated by a continuous plasmin-catalysed

Figure 2 Formation of a covalent pro-uPA–PAI-1 complex

(A, B) PAI-1 (2.5 μM) and an equimolar concentration of two-chain uPA (lanes 4) or pro-uPA (lanes 5) were incubated for 1 h at 37 °C before electrophoretic analysis. The same amounts of PAI-1 (lanes 1), two-chain uPA (lanes 2) and pro-uPA (lanes 3) are shown alone for comparison. (A) SDS/PAGE and Coomassie Brilliant Blue staining. Samples were electrophoresed after non-reducing or reducing sample treatment as indicated. (B) Western blotting. Samples were electrophoresed after non-reducing sample treatment. After electroblotting, the following primary antibodies were used for detection: anti PAI-1 mAb clone 2 (left-hand panel), anti-uPA mAb clone 5, reactive with two-chain uPA and pro-uPA (middle panel) and zymogen-specific anti-pro-uPA mAb 3901 (right-hand panel). (C) Comparison of wild-type pro-uPA and pro-uPA(K158A). ^{125}I -Pro-uPA wild-type (left-hand panel) or ^{125}I -Pro-uPA(K158A) (right-hand panel), both at 80 nM, were subjected to treatment with plasmin or buffer alone as indicated, followed by incubation for 1 h in the presence (+) or absence (–) of PAI-1 (500 nM). Electrophoretic analysis was carried out as in (A), using reducing conditions. Labelled proteins, including the stable inhibitor complexes generated, were revealed by autoradiography. The M_r values of marker proteins are indicated to the left.

Figure 4 The effect of suPAR in a coupled activation system with pro-uPA, plasminogen and PAI-1

(A) Chromogenic assay. The same experiment as shown in Figure 1(A) is shown with the inclusion of suPAR-containing samples. All samples contain plasminogen ($1 \mu\text{M}$) and plasmin substrate (H-D-Val-Leu-Lys-*p*-nitroanilide; $400 \mu\text{M}$). The curves shown represent the following additions: pro-uPA alone (●), pro-uPA + PAI-1 (○), pro-uPA + PAI-1 after pre-incubation of pro-uPA with a 100-fold molar excess of suPAR (◇) and pro-uPA pre-incubated with suPAR correspondingly but without PAI-1 (▽). The final concentrations were 0.5 nM pro-uPA and two-chain uPA and 2 nM PAI-1. Results are plotted as in Figure 1(A). (B) Fluorogenic assay with equimolar concentrations of plasminogen and pro-uPA. The following samples were mixed with the plasmin substrate H-D-Val-Leu-Lys-AMC ($200 \mu\text{M}$): buffer alone (▲), pro-uPA alone (●), pro-uPA + PAI-1 (○), pro-uPA + PAI-1 after pre-incubation of pro-uPA with a 20-fold molar excess of suPAR (◇) and pro-uPA pre-incubated with suPAR correspondingly but without PAI-1 (◇). Plasminogen (5 nM) was then added to all samples (at time 0) and the fluorescence was followed during incubation at 37°C . Pro-uPA and PAI-1 were used at final concentrations of 5 and 10 nM , respectively. Plasmin activity ($\Delta F/\text{min}$) is plotted as a function of time (mean $\pm$ range from a double determination).

activation of pro-uPA as a result of the integrated feedback mechanism. This conclusion was confirmed by the use of a pro-uPA mutant protein, pro-uPA(K158A), not activatable by plasmin. In the presence of PAI-1 (Figure 1C, □), pro-uPA(K158A) failed to reproduce the onset of activity found with wild-type pro-uPA, while in the absence of inhibitor, just a negligible and non-accelerative generation of plasmin activity was noted (Figure 1C, ■).

These experiments revealed that the composite system with wild-type pro-uPA has the ability to maintain a low activity by means of continuous, reciprocal pro-enzyme activation, even under conditions where the major part of the uPA activity is constantly being eliminated by irreversible inhibition. This remarkable property of the system might represent a constant source of cascade-initiator activity. Therefore, an intriguing question was whether the pattern observed would still be valid when plasmin activity was also subject to inhibition. To answer this question, we studied samples of pro-uPA and plasminogen in the simultaneous presence of PAI-1 and the major plasmin inhibitor, α_2 -antiplasmin.

In this experiment, as expected, the absorbance increments per unit time were very small and the results, therefore, are shown in terms of direct reading of A_{405} as a function of time (Figure 1D), instead of ΔA values. Strikingly, in the simultaneous presence of both of the two inhibitors, a low but clearly measurable plasmin activity succeeded to survive in the sample with pro-uPA and plasminogen (Figure 1D, ◇), as reflected by a gradual increase in the absorbance with time. Just as noted above, the corresponding sample without α_2 -antiplasmin displayed an accelerative accumulation of absorbance (Figure 1D, ○), reflecting an increasing plasmin activity, whereas the sample with two-chain uPA, plasminogen and PAI-1 only developed plasmin activity for a very short period after which no new activity was formed (Figure 1D, linear increase of A_{405} after the initial phase; ▽). When α_2 -antiplasmin was added to the latter set of components, this inhibitor led to immediate abrogation of all activity (Figure 1D, □). Thus the ability to maintain activity under inhibitor repression was restricted to the reciprocal activation system.

Irreversible reaction of PAI-1 with pro-uPA

The different behaviour of pro-uPA and two-chain uPA observed above made it relevant to study the interaction of PAI-1 with the individual forms of uPA. We therefore compared the products obtained after incubation of PAI-1 with pro-uPA and active (two-chain) uPA (Figure 2). At high equimolar concentrations, both forms of uPA reacted efficiently with PAI-1 to form distinct SDS-stable complexes (Figure 2A, lanes 4 and 5 of both panels). In agreement with results published previously [14], the product formed with pro-uPA migrated with the same apparent M_r (≈ 95000) irrespective of whether reducing or non-reducing conditions were used for SDS/PAGE, unlike the complex generated with two-chain uPA in which the release of the enzyme A-chain was evident after reduction of disulphides. This observation indicated that the former complex was not just the result of proteolytic activation of the pro-enzyme during the experiment. The identity of this product as actually representing a pro-uPA–PAI-1 conjugate was verified by Western blotting using a monoclonal antibody that specifically recognizes the pro-form of uPA (Figure 2B, right-hand panel). This antibody showed a strong reaction with free pro-uPA as well as its $M_r \approx 95000$ complex with PAI-1 (Figure 2B, right-hand panel, lane 5), whereas no reaction occurred with two-chain uPA or its PAI-1 complex (Figure 2B, right-hand panel, lane 4). In contrast, both PAI-1 complexes were recognized by a non-zymogen selective monoclonal antibody reactive with both pro-uPA and two-chain uPA (Figure 2B, middle panel), and by a monoclonal antibody against PAI-1 (Figure 2B, left-hand panel). Note that the PAI-1 complexes of pro-uPA and two-chain uPA migrate with identical electrophoretic mobility in this experiment due to the non-reducing sample treatment necessary for recognition by the antibodies used.

Figure 5 The effect of suPAR on covalent complex formation between pro-uPA and PAI-1

¹²⁵I-Pro-uPA (40 nM) was pre-incubated in the presence or absence of suPAR (100 nM) after which the time course of PAI-1 complex formation was followed, using the same conditions as described for Figure 3. Based on the autoradiography after electrophoresis (insert), labelled protein bands were localized and excised from the gel after which their radioactivity was determined by γ -counting. Each column is the mean from a double determination and shows the radioactivity of the pro-uPA–PAI-1 complex as a percentage of the total pro-uPA radioactivity.

The pro-uPA(K158A) mutant, used above in the activity assays, also formed an $M_r \approx 95\,000$ conjugate with PAI-1 and, as expected, this situation was unchanged after pretreatment of the pro-enzyme with plasmin (Figure 2C, right-hand panel). This was unlike the situation found with wild-type pro-uPA where plasmin-mediated activation was evident (Figure 2C, left-hand panel).

A time-course study with radiolabelled uPA and pro-uPA revealed, however, that the pro-uPA–PAI-1 complex was formed much more slowly than the PAI-1 complex with two-chain uPA. At a PAI-1 concentration of 500 nM, the complex with wild-type pro-uPA kept accumulating during the whole 3 h period studied (Figure 3, left-hand panel) and this time course was indistinguishable from that found with pro-uPA(K158A) (results not shown). In contrast, complex formation with two-chain uPA had reached the end point by the first time point measured (1 min; Figure 3, right-hand panel). Separate experiments indicated that complex formation between two-chain uPA and PAI-1 was virtually complete after 1 min even with a PAI-1 concentration as low as 10 nM, and more than 50% finished after 10 s with 5 nM PAI-1 (results not shown). This experiment also showed that the covalent pro-uPA–PAI-1 complex was not just a consequence of sample denaturation since its occurrence was dependent on the time of incubation under native conditions and the denaturing pre-treatment for electrophoresis was carried out simultaneously and with identical conditions for all samples.

To test the stability of the reaction products, we prepared radiolabelled PAI-1 complexes as described above and incubated the products under conditions preventing *de novo* formation of complexes (see the Experimental section for details). This experiment showed that 60–70% of the radioactivity was still in the form of covalent complexes after 18 h at 37 °C and importantly,

the loss of labelled complexes was the same, irrespective of whether the complexes were formed with pro-uPA or two-chain uPA (results not shown).

Influence of soluble uPAR on the coupled activation system in the presence of PAI-1

As detailed in the Discussion section, the effect of uPAR and its soluble derivative, suPAR, on this system is a matter of dispute. Separate samples with suPAR present were therefore included in the activity assays described above. The curves obtained are shown in Figure 4(A), demonstrating the difference between samples studied in the presence and absence of suPAR. It was evident that the addition of suPAR to the pro-uPA plasminogen-activation system did not provide any protection against PAI-1-mediated inhibition. In the simultaneous presence of PAI-1 and suPAR (Figure 4A, $\diamond$), a suppression of the development of activity was observed that was even stronger than that exerted by PAI-1 alone (Figure 4A, $\circ$). The lag phase thus was increased to approx. 1 h. In accordance with results published previously [22], a more moderate delay in plasminogen activation was noted when suPAR was added to the pro-uPA plasminogen system in the absence of PAI-1 (Figure 4A, $\blacklozenge$).

The lag phase observed before the start of any detectable plasmin generation in the experiment described above showed that the major part of the uPA activity was due to two-chain uPA but a very low fraction would still be likely to represent the intrinsic proteolytic activity of pro-uPA. Thus the high concentration of plasminogen and the low concentration of pro-uPA might lead to a feedback activation so dominant that the intrinsic activity of pro-uPA would not be clearly revealed. In order to favour any contribution from pro-uPA we therefore changed the

Figure 6 Plasminogen activation on U937 cells in the presence of inhibitors

U937 cells were preincubated at 4 °C with 10 nM pro-uPA or two-chain uPA or with buffer alone, after which the cells were washed and a fluorogenic assay with plasminogen (2 μ M) and the substrate H-D-Val-Leu-Lys-AMC (200 μ M) was performed at 37 °C in the presence of inhibitors as indicated. Plasminogen was added to all samples at time 0. The blind-corrected plasmin activity ($\Delta F/\text{min}$; see the Experimental section) is plotted as a function of time (mean $\pm$ range from a double determination). The following samples are shown: (A) cells incubated with pro-uPA, assayed in the absence (●) or presence (○) of PAI-1, cells incubated with two-chain uPA (tc-uPA), assayed in the absence (▼) or presence (▽) of PAI-1, cells incubated with buffer alone, assayed in the presence of PAI-1 (□). PAI-1 was used at a final concentration of 2 nM. (B) Cells incubated with pro-uPA (○), two-chain uPA (▽) or buffer alone (□), all assayed in the presence of PAI-1 (2 nM) and α_2 -antiplasmin (1 μ M).

conditions, increasing the pro-uPA concentration 10-fold and decreasing the concentration of plasminogen 200-fold, thus shifting the balance between the two pro-enzymes to equimolarity. Initial experiments revealed that, under these extreme conditions, the assay format used above did not allow an exact evaluation of the early phase of the activation reactions due to the very low activity levels. Therefore, we switched to a fluor-

escence-based system using the plasmin substrate, H-D-Val-Leu-Lys-AMC, that allows more sensitive detection of plasmin activity [27] (Figure 4B). Again, the sample with pro-uPA and PAI-1 displayed an accelerative formation of plasmin activity, indicating reciprocal pro-enzyme activation (Figure 4B, ○). However, in this case some generation of plasmin activity was evident already at the first points of measurement, suggesting that a measurable contribution indeed originated from the intrinsic activity of pro-uPA. (It should be noted that even though traces of contaminating two-chain uPA might be present in any pro-uPA preparation [4], this contaminant would be rapidly inactivated by PAI-1 during preparation of the samples; see the Experimental section.) Importantly, also under these conditions the preincubation of pro-uPA with suPAR failed to provide any acceleration of the activation reactions or any protection against PAI-1. Thus in the absence of PAI-1 the addition of suPAR led to a moderate delay in plasmin formation (Figure 4B, compare curves with ● and ◆). The simultaneous presence of suPAR and PAI-1 (Figure 4B, ◇) led to a stronger repression of the activation system than that obtained with either PAI-1 alone or suPAR alone (Figure 4B, ○ and ◆, respectively), just like the situation observed in the previous experiment.

In order to study directly whether the reaction of pro-uPA with PAI-1 was influenced by suPAR, we examined the time course of covalent inhibitor complex formation in the absence and presence of suPAR (Figure 5). It was clear that preincubation of pro-uPA with suPAR had no influence on the subsequent reaction of pro-uPA with PAI-1. The concentration of suPAR used was shown to lead to saturation of pro-uPA as verified for the actual batch in other experiments using cross-linking competition [28] (results not shown); note that the binding of pro-uPA to suPAR was also ascertained by its direct effect on the pro-uPA plasminogen-activation system (Figures 4A and 4B, ◆).

Reciprocal pro-enzyme activation on the cell surface in the presence of PAI-1

uPA-mediated plasminogen activation occurs in solution as well as on the cell surface with pro-uPA and uPA bound to uPAR [17,31]. Therefore, finally we asked the question whether the ability of the reciprocal activation system to maintain activity in the presence of PAI-1 was retained in a surface-bound activation system. For these studies, we used human monocyte-like U937 cells because cell-associated plasminogen activation with uPA and pro-uPA bound to uPAR has been characterized extensively on this cell type [19,20,32]. U937 cells were incubated with pro-uPA or two-chain uPA, washed to remove the unbound enzyme and incubated with plasminogen in the absence or presence of PAI-1. The same fluorogenic substrate as used above was included for the detection of plasmin activity since this procedure has been optimized for the study of the low quantities of uPA bound to the surface of washed cells [20,27]. As seen in Figure 6(A), the very efficient plasminogen activation obtained with cells incubated with two-chain uPA (Figure 6A, ▼) was almost completely abolished when PAI-1 was added (Figure 6A, ▽). This finding is in accordance with the notion that the binding of two-chain uPA to uPAR on the cell only marginally affects the reactivity of uPA with PAI-1 [32]. In contrast, when cells incubated with pro-uPA were used, addition of PAI-1 did not prevent the accumulation of plasmin activity (Figure 6A, ○), although a substantial delay was noted relative to the sample without PAI-1 (Figure 6A, ●). In the sample with PAI-1, efficient plasminogen activation did not occur until after the first

5 min of incubation. Thus the pattern observed with cell-bound uPA/pro-uPA was qualitatively similar to that obtained in solution.

When both PAI-1 and α_2 -antiplasmin were added to the cell-containing samples, the activities dropped to very low values, the individual ΔF values being too low to allow a detailed evaluation of the time course of the activation process. Nevertheless, even in this case an increase in plasmin activity with time could be detected with cells pre-incubated with pro-uPA (Figure 6B).

DISCUSSION

The intrinsic proteolytic activity and the inhibitor reactivity of pro-uPA are issues that have been debated extensively as part of the elucidation of the plasminogen-activation system [13,14,21,33]. A complete consensus on these questions is still lacking. In this paper we show that pro-uPA reacts covalently with PAI-1 in an irreversible reaction (see below), but that it does so much more slowly than two-chain uPA. Against this background it could be anticipated that pro-uPA, in spite of its very low proteolytic activity, would be favoured relative to two-chain uPA as a plasminogen activator in a system under inhibitor repression, as suggested by other investigators [14]. However, the activation profiles obtained in a coupled activation system with plasminogen, pro-uPA and PAI-1 did not support this notion. The prolonged lag phase observed before the generation of measurable plasmin activity in the system with wild-type pro-uPA (Figure 1), as well as the extremely low rate of plasmin generation noted with pro-uPA(K158A), thus excluded the possibility that pro-uPA could be a dominant plasminogen activator under these conditions. Our observations are consistent with a dynamic model in which, also in the presence of PAI-1, two-chain uPA is responsible for generating the major part of the plasmin activity measured, this uPA being constantly generated at a low rate by plasmin from the feedback mechanism, and its activity having a certain lifetime before the enzyme gets irreversibly inactivated by the inhibitor.

A distinctive feature of this interplay between a reciprocal pro-enzyme activation system and an irreversible inhibition mechanism is the maintenance of a very low proteolytic potential, comparable with a 'pilot light' that can be up- or down-regulated by moderate changes in the concentration of the reactants. This notion does not contradict a role of pro-uPA intrinsic activity in the very first molecular events of a cascade reaction, but it does emphasize a model for the emergence of activity that is not primarily focused on the initiation mechanism but rather depends on a minute proteolytic stand-by level. Strikingly, we found that this maintenance of activity is retained even in the simultaneous presence of PAI-1 and α_2 -antiplasmin. Under these conditions, both two-chain uPA and active plasmin have a short lifetime but they still succeed in preventing a complete inactivation of the reciprocal system.

A slow reaction between serpins and serine protease zymogens such as chymotrypsinogen and pro-elastase has been reported previously [34–36]. With respect to the uPA system, pro-uPA was found to be largely inert to PAI-1 because the amount of free pro-enzyme was virtually unchanged after co-incubation with the inhibitor under conditions leading to complete inactivation of two-chain uPA [37]. More recently, the formation of SDS-stable pro-uPA–PAI-1 complexes has indeed been reported, even though this reaction was interpreted to be reversible [14]. This interpretation was reasonable at the time of the report because early NMR studies raised the possibility that protease–serpin complexes contain a tetrahedral arrangement around the car-

bonyl group of the scissile bond in the serpin [38]. From this configuration, a dissociation event regaining the intact serpin and protease would be theoretically possible. However, with the more recent consensus that the SDS-stable protease–serpin complexes contain a regular ester bond [39], which also implicates completion of cleavage of the scissile peptide bond of the serpin [40,41], accompanied by a strongly stabilizing conformational change [42,43], a reversible covalent reaction would seem very unlikely. Unlike the previous report [14] we found that the covalent pro-uPA–PAI-1 complex is quite stable with a decay rate equivalent to that of the well-characterized complex of PAI-1 with two-chain uPA. This decay is likely to represent slow release of the cleaved serpin from the relatively stable ester intermediate [43,44], irrespective of whether the complex is formed with pro-uPA or two-chain uPA. It should be stressed, however, that irreversibility of the covalent step does not exclude an important role for reversible, non-covalent complex formation. Thus, the fast covalent reaction of two-chain uPA might lead to a very short lifetime of the initial (non-covalent) Michaelis complex. This adduct may, on the other hand, be a dominant type of complex in the case of pro-uPA. This would explain the observation of reversible pro-uPA–PAI-1 interactions, noted by some investigators [14,21].

In support of the previous report [37] our results show that the covalent reaction of pro-uPA with PAI-1 is at least 1000-fold slower than that of two-chain uPA. The slow reaction is probably explained primarily by the low proteolytic activity of pro-uPA [4]. Early studies on the structural basis for the zymogenicity of serine protease pro-forms have suggested that a very low fraction of the zymogen molecules contain a functional oxyanion hole and represent potentially active enzymes, existing in dynamic equilibrium with the inactive conformation [45,46]. This equilibrium model raises the possibility that the active pro-uPA fraction, present at any time point, might react quite efficiently with PAI-1 without this leading to a fast covalent reaction of the total population of pro-uPA molecules. In accordance with this hypothesis, we have found that pro-uPA(K158A) can be quantitatively inactivated for an extended period by less than equimolar concentrations of PAI-1 (N. Behrendt and P. A. Andreasen, unpublished work).

The covalent reaction of pro-uPA with PAI-1 proceeded with an unchanged, low rate after binding of pro-uPA to suPAR. This observation is in line with the finding that suPAR does not induce any increase in the amidolytic or the proteolytic activity of pro-uPA [21,22,47]. Furthermore, in activity assays on the coupled pro-uPA plasminogen-activation system we found that the simultaneous presence of suPAR and PAI-1 led to an inhibition of the activation system that was even stronger than that noted with PAI-1 alone. We have previously reported that suPAR has an attenuating effect on the coupled feedback-activation system also in the absence of PAI-1, probably caused by moderate steric interference [22]. This effect was also reproduced in the control samples of the present study.

Contrary to our findings, suPAR has been claimed by another group of investigators to exert a protective effect on pro-uPA against PAI-1 [15]. In that report, the PAI-1-mediated inhibition of a pro-uPA–suPAR complex was compared with the inhibition of two-chain uPA. The suggestion of a protective effect of suPAR was based on the observation that, in the presence of PAI-1, the coupled plasminogen-activation system with pro-uPA bound to suPAR actually developed some activity, unlike the system with two-chain uPA. As detailed above, we have now shown that this ability to maintain a finite level of activator potential is an inherent property of the reciprocal activation system under inhibitor repression and not an effect of suPAR.

The necessary comparison of free and suPAR-complexed pro-uPA in terms of reactivity with PAI-1 (Figures 4 and 5 of the present study) was hampered in the previous report [15] due to the postulated suPAR-mediated induction of pro-uPA activity [15,48]. As stated above, experiments done by several groups including ours have rejected the existence of such a mechanism [21,22,47]; for a discussion, see [49,50]. Altogether, the sum of our previous [22] and current observations documents that suPAR has no stimulatory function on the activity of pro-uPA or two-chain uPA, no accelerative effect on the activation of pro-uPA or plasminogen and no protective effect against PAI-1.

The major part of this study was performed in a purified, soluble system to follow the interplay within a limited number of known reactants. Plasminogen activation, mediated by uPA and proceeding in the liquid phase with no requirement for cellular binding, is definitely a physiologically relevant event [17] and our conclusions have immediate relevance to this process. However, it is also clear that in many cases the activation reactions of this system occur on cell surfaces and therefore we also included an experiment with uPA bound to U937 cells. On the cell surface, uPA and uPAR act in the context of an organized ensemble of associated proteins and these additional players provide new mechanisms that in some cases have a strong influence on proteolysis (see [16] for a review). Therefore, rather than attempting to elucidate the complete activation mechanism, we limited ourselves to answering the question of whether the cell-associated, reciprocal activation system would display the same ability as the system in solution to retain activity in the presence of PAI-1. This was clearly the case, since efficient plasminogen activation in the presence of PAI-1 occurred with U937 cells pre-incubated with pro-uPA, while cells pre-incubated with two-chain uPA were unable to support the same reaction. In cell-associated plasminogen activation where molecular orientation effects are involved, it is likely that the intrinsic activity of pro-uPA plays a more important role than that observed in solution [13,16]. Nevertheless, the experiment with U937 cells, pro-uPA and PAI-1 revealed a strongly accelerative activation profile, suggesting that, also in this case, reciprocal pro-enzyme activation was responsible for the major part of the activity.

Cells pre-incubated with pro-uPA generated a measurable plasmin activity even in the presence of both PAI-1 and α_2 -antiplasmin, although this activity was very low. It is important to note that, in this system, the cell-bound pro-uPA is the only pro-uPA present, unlike the experimental set-up with plasminogen activation in solution where pro-uPA is evenly distributed in a homogeneous system. This means that, in the cell-containing system, feedback activation is largely limited to the cell surface, whereas any active plasmin dissociating from the cell will be inactivated after a short time without generating new uPA activity in the surrounding liquid phase. The cell-bound fraction of the active plasmin, however, is protected against α_2 -antiplasmin [20] and this is likely to contribute to the maintenance of a measurable plasmin activity under these conditions.

Our results point to an important property of the pro-uPA plasminogen cascade, common to the cell-bound system and the system in solution, in that a low activity can be maintained continuously in the presence of a large excess of inhibitors. From this situation, activity may be readily boosted to higher levels by temporal or spatial changes in the relative concentrations of the components, such as the changes likely to occur in the local environment of malignant tumours [51]. Since reciprocal pro-enzyme activation and irreversible inhibition of proteases are phenomena quite widespread among proteolytic cascade systems, it is possible that the principles outlined here are relevant also in other proteolytic systems.

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