Effect of purified, soluble urokinase receptor on the plasminogen–pro-urokinase activation system

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Abstract The extracellular proteolytic pathway mediated by the urokinase plasminogen activator (uPA) is a cascade system, initiated by activation of the zymogen, pro-uPA. Pro-uPA as well as uPA binds to the cellular uPA receptor (uPAR) which has a central function in cell-dependent acceleration of the cascade system. This role of uPAR is generally assumed to be a positioning effect since uPAR-expressing cells exclusively stimulate the activation of cell surface-bound plasminogen (Ellis et al. (1993) *Methods Enzymol.* 223, 223–233). However, it was recently reported that a recombinant, soluble uPAR (suPAR) was capable of accelerating plasminogen activation in solution (Higazi et al. (1995) *J. Biol. Chem.* 270, 17375–17380). In this work we show that suPAR as such has no accelerative role. In contrast, the progress of the activation reactions in a soluble system with pro-uPA and plasminogen was found to be attenuated by suPAR. This delay of the activation system was shown to include a partial inhibition of the plasmin-mediated activation of pro-uPA as well as of the uPA-mediated activation of plasminogen.

Key words: Plasminogen activator; Proteolytic cascade; Extracellular proteolysis; Fibrinolysis

1. Introduction

Urokinase plasminogen activator (uPA) is a serine protease which specifically converts the inactive zymogen, plasminogen, into active plasmin. Through this function, uPA plays an important role in a number of extracellular proteolytic processes which involve plasmin activity, notably in the context of tissue remodeling phenomena occurring in certain normal physiological conditions as well as in cancer invasion [1].

The active uPA molecule emerges by proteolytic activation of the single-chain pro-enzyme, pro-uPA, which has very low intrinsic proteolytic activity [2]. While several proteases have been shown to activate pro-uPA in vitro [2–9], the physiological initiation mechanism for this system is still unknown. However, as soon as the first plasmin molecules are formed, a strong feed-back mechanism, mediated by plasmin-catalyzed activation of pro-uPA, leads to a dramatic amplification of the activation system (see [2,10] for a discussion). For this reason, in in vitro experiments with mixtures of pro-uPA and plasminogen, increasingly rapid activation of both zymogens proceeds once the system is initiated, e.g., by trace amounts of one of the active enzymes.

Both pro-uPA and active uPA bind to the cellular uPA receptor (uPAR) [11,12] which participates in localizing and accelerating the cascade at the cell surface. Thus, in the presence of pro-uPA a number of cell types are able to stimulate plasminogen activation and this acceleration can be abolished by uPAR-blocking reagents [13,14]. However, the molecular mechanism of this stimulatory function has not been clarified. The accelerative effect of uPAR-expressing cells is strictly dependent on the additional cellular binding of plasminogen [13], suggesting that the role of uPAR is mostly an orientation and concentration effect. In contradiction to this view it has been suggested that receptor-ligand binding as such augments the enzymatic properties of pro-uPA [15].

Recently, a stimulatory effect of a recombinant, soluble uPAR on the activation system in the liquid-phase was reported [16]. Due to the role of the uPA system in invasive processes the interference with plasminogen activation at the cell surface may have important therapeutic perspectives [17] and in this context, an accelerative effect caused directly by uPAR would be a central potential target. In order to study the functional role of uPAR at the molecular level, we have now investigated the influence of the purified receptor on the activation reactions which proceed in solution with different combinations of the pro-enzymes and active enzymes of the cascade.

2. Materials and methods

2.1. Purified proteins and protease substrates

Recombinant, human soluble uPAR (suPAR) was isolated from the culture medium of transfected Chinese Hamster Ovary cells by immunoaffinity chromatography as described [18] and subsequently subjected to gel filtration on a Superdex 75 prep grade column (Pharmacia Biotech, Uppsala, Sweden), using PBS as the eluent, for removal of trace amounts of IgG. Purified, recombinant human pro-uPA was a kind gift from Dr. P. Sarmientos, Farmitalia Carlo Erba, Milan, Italy. Human two-chain uPA was purchased from Serono, Aubonne, Switzerland. Purification of human Glu-plasminogen from fresh plasma of healthy donors and preparation of active plasmin were performed as described [19]. Purified bovine serum albumin (BSA) and purified ovalbumin were purchased from Behringwerke AG, Marburg, Germany and Sigma, St. Louis, MO, respectively. The amino-terminal fragment (ATF) of uPA, and the chromogenic substrate for plasmin, H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide (Nle-HHT-Lys-pNA; Spectrozyme PL), were purchased from American Diagnostica Inc., Greenwich, CT. The chromogenic uPA substrate, L-pyroglutamyl-glycyl-L-arginine-*p*-nitroanilide (< Glu-Gly-Arg-pNA; S2444) was purchased from Chromogenics AB, Mölndal, Sweden.

2.2. Protease activity assays

Chromogenic assays were performed in the wells of microtitre plates [9] (Nunc, Roskilde, Denmark) in a total reaction volume of

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Abbreviations: uPA, urokinase plasminogen activator; pro-uPA, single-chain proenzyme for uPA; uPAR, uPA receptor; suPAR, soluble uPAR; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ATF, amino-terminal fragment of uPA; tPA, tissue-type plasminogen activator

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200 μl. All samples were tested in duplicate. Pro-enzymes, active enzymes, other proteins and chromogenic peptide substrates were mixed as indicated and the substrate conversion was followed at 37°C by readings of the absorbance at 405 nm. The plasmin activity was expressed as the rate of conversion of the substrate, Nle-HHT-Lys-pNA (ΔA_{405}/min), measured at each time point indicated [20,21]. The amidolytic activity of uPA, resulting from conversion of pro-uPA, was measured in the same manner except that the substrate, < Glu-Gly-Arg-pNA, was used [2].

3. Results

3.1. Binding of pro-uPA to purified suPAR attenuates plasminogen activation

In order to study the function of receptor-bound pro-uPA in a cell-free system, we employed a recombinant, water-soluble uPAR variant (suPAR) which comprises all of the three extracellular domains of the receptor and which binds uPA with the same affinity as cell-bound uPAR [22,23]. Purified suPAR was added to mixtures of pro-uPA and Glu-plasminogen and the generation of plasmin was followed by the conversion of a chromogenic plasmin substrate (Fig. 1). Two parallel sets of samples were analyzed. In one series of samples we included a carrier protein, BSA (0.5% w/v), in the buffer in order to prevent the problems of denaturation and surface adsorption usually encountered with dilute protein solutions. In another series, the samples were made up without carrier proteins in order to reproduce the conditions under which a stimulatory function of suPAR has been reported [16].

This difference in conditions turned out to be critical for the outcome. In the carrier-containing samples, the addition of

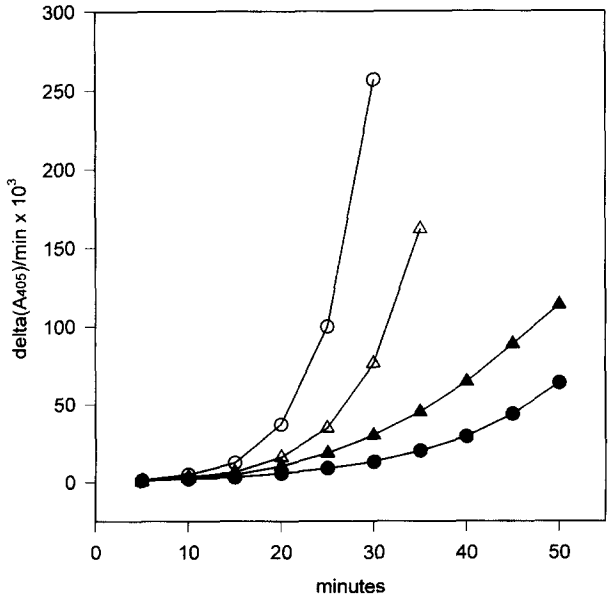

Fig. 1. Effect of purified suPAR on a coupled activation system with pro-uPA and plasminogen. The assay was performed using PBS with 0.5% BSA as the solvent (○, △) or PBS only (●, ▲). Pro-uPA (2 nM) and Glu-plasminogen (1 μM) were incubated with the chromogenic plasmin substrate, Nle-HHT-Lys-pNA (0.5 mM) in the absence (○, ●) or presence (△, ▲) of suPAR (2 nM). The absorbance at 405 nm was followed and the plasmin activity (expressed as the substrate conversion rate; ΔA_{405}/min) was plotted vs. time. Each point is the mean of a double determination. Parallel samples, prepared in the same manner but without pro-uPA, all had activities below 2×10^{-3} AU/min throughout the assay.

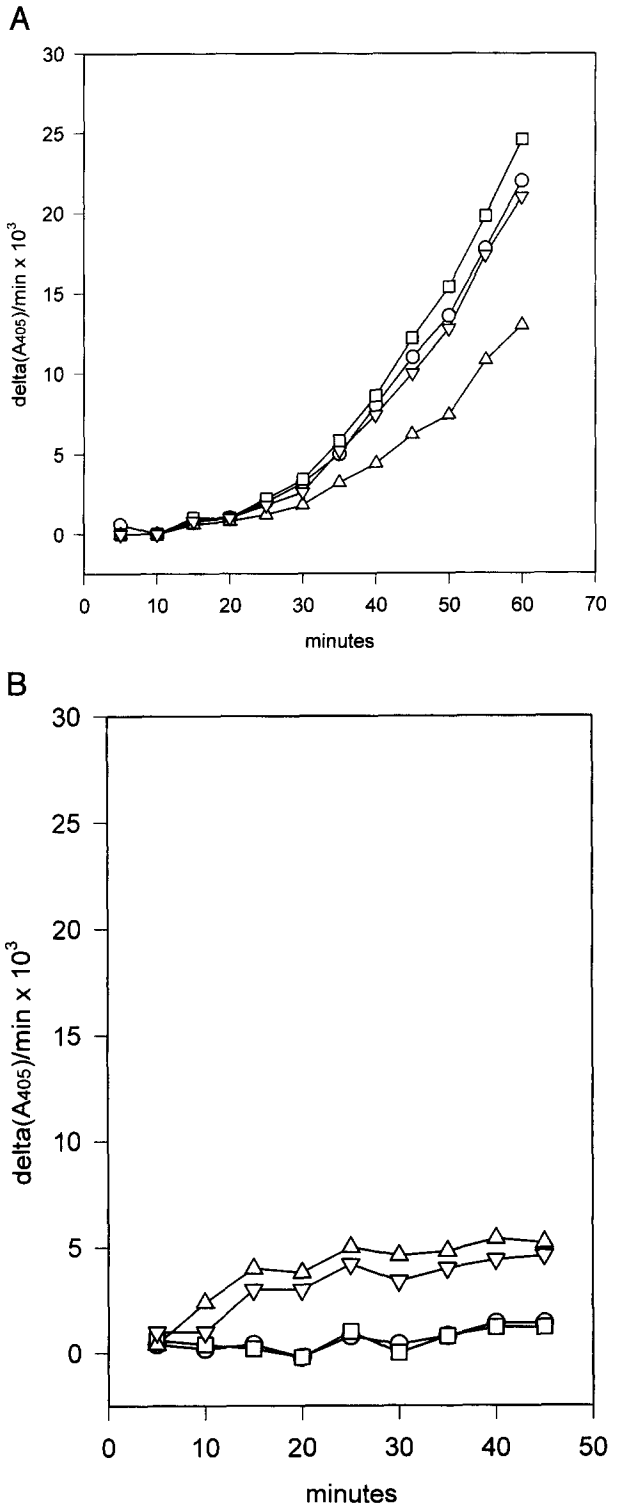

Fig. 2. Effect of blocking suPAR against pro-uPA binding. Pro-uPA (5 nM) and Glu-plasminogen (10 nM) were incubated with 0.5 mM of Nle-HHT-Lys-pNA, either alone (○) or in the presence of the following reagents: 5 nM of suPAR (△), 50 nM of ATF (□) or a pre-formed mixture of suPAR and ATF (final concentrations 5 and 50 nM, respectively) (▽). The plasmin activity was plotted as described in the legend to Fig. 1. Each point is the mean of a double determination. (A) Results obtained using PBS with 0.5% BSA as the solvent. (B) Results obtained using PBS as the solvent without carrier proteins added. Note that under these conditions the generation of active plasmin (slope of the curves) declines after 30–40 min, probably due to surface adsorption phenomena.

Fig. 3. Concentration dependence of suPAR. Pro-uPA (5 nM) and Glu-plasminogen (10 nM) were incubated with 0.5 mM of Nle-HHT-Lys-pNA in the absence of suPAR (○) or in the presence of the following concentrations of suPAR: 5 nM (△), 25 nM (□), 100 nM (◇). The plasmin activity was plotted as described in the legend to Fig. 1. Each point is the mean of a double determination. (A) The assay was performed using PBS with 0.5% BSA as the solvent. (B) The solvent was PBS only. In this case prolongation of the incubation period beyond 30–40 min led to a decrease in the plasmin generation; see the legend to Fig. 2B.

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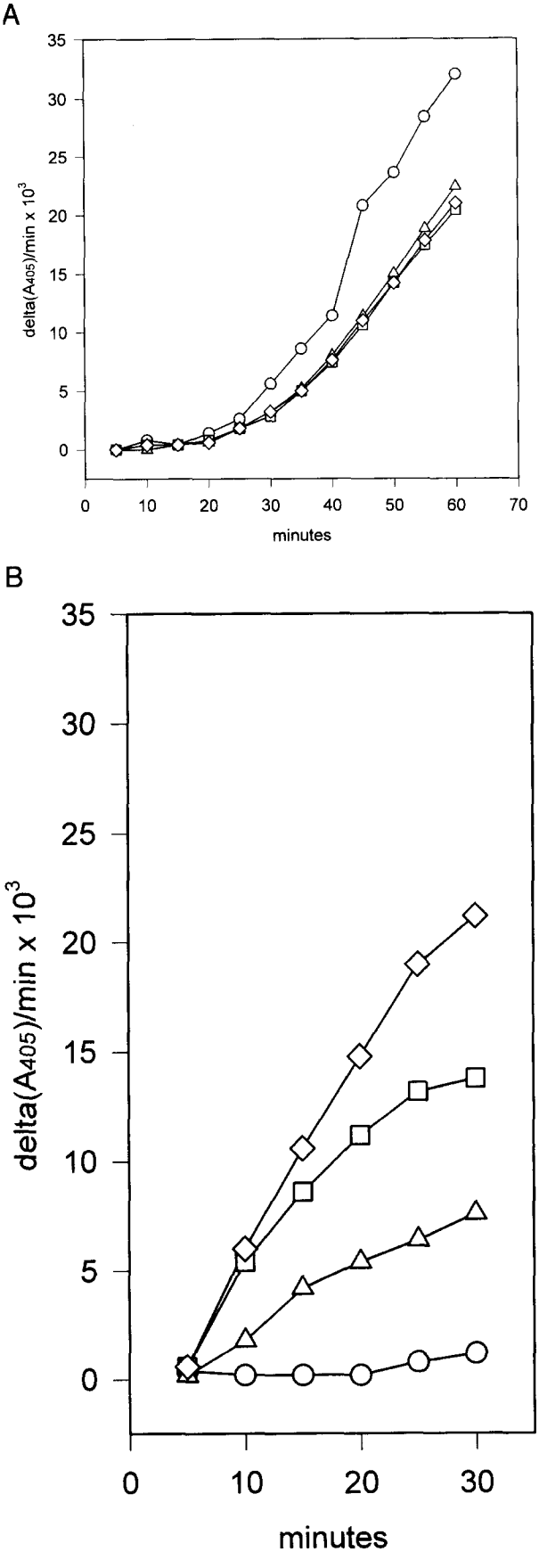

suPAR had no stimulatory effect on the activation system. Actually, a delayed progress of the cascade reaction was noted, relative to the samples without suPAR (Fig. 1, ○, △). The same result was found if another carrier protein, ovalbumin, was used instead of BSA (results not shown). In contrast, in the carrier-free system (Fig. 1, ●, ▲), the samples containing the purified suPAR preparation showed a faster activation profile than the samples without suPAR. The latter result was thus in accordance with the results the results reported [16] but under these conditions, both in the presence and absence of suPAR, the progress of the activation system was slower than that observed in the BSA-containing samples. This observation was likely to reflect surface adsorption of part of the material; see also Figs. 2 and 3, below.

The apparent discrepancy between the effects of suPAR addition found in the presence and absence of carriers could be explained if only one of the effects was caused by the pro-uPA–suPAR binding reaction per se, while other factors were responsible for the opposing effect. Therefore, it was necessary to test the specificity of the interactions further. Since the feed-back conversion of pro-uPA, mediated by the active plasmin generated, soon becomes a dominating factor under the conditions used above, we adjusted the conditions by decreasing the concentration of plasminogen 100-fold and increasing the pro-uPA concentration to 5 nM in order to analyze the early activation phase in more detail. Under these conditions, an inhibitory effect of suPAR was again observed in the presence of the carrier protein while stimulation was found in the samples devoid of carrier (Fig. 2A and B, respectively; compare the curves represented by circles and triangles). Using this system, we studied the effect of blocking suPAR binding by means of the receptor-binding, amino-terminal fragment (ATF) of uPA which lacks the catalytic domain of the enzyme [24]. suPAR was pre-incubated with ATF and subsequently added to the mixtures of pro-uPA and plasminogen (Fig. 2A,B, ▽). In the system with carrier proteins present, ATF blocked the inhibitory capacity of suPAR completely (Fig. 2A), in accordance with the expected pattern for inhibition dependent on the binding of pro-uPA to suPAR. In contrast, ATF did not influence the stimulation of the cascade resulting from addition of suPAR to the carrier-free samples (Fig. 2B). The latter finding is contradictory to the results reported [16] and strongly suggests that the stimulatory effect, observed in these samples, was a result of events other than the binding reaction between suPAR and pro-uPA.

The most obvious interfering factor would be the unavoidable proteolytic initiation of the feed-back system, which would be caused by even minute amounts of contaminating proteases within the purified suPAR preparation. Such an effect would be expected to be most dominant in carrier-free samples; see Section 4. In the next experiment, we varied the

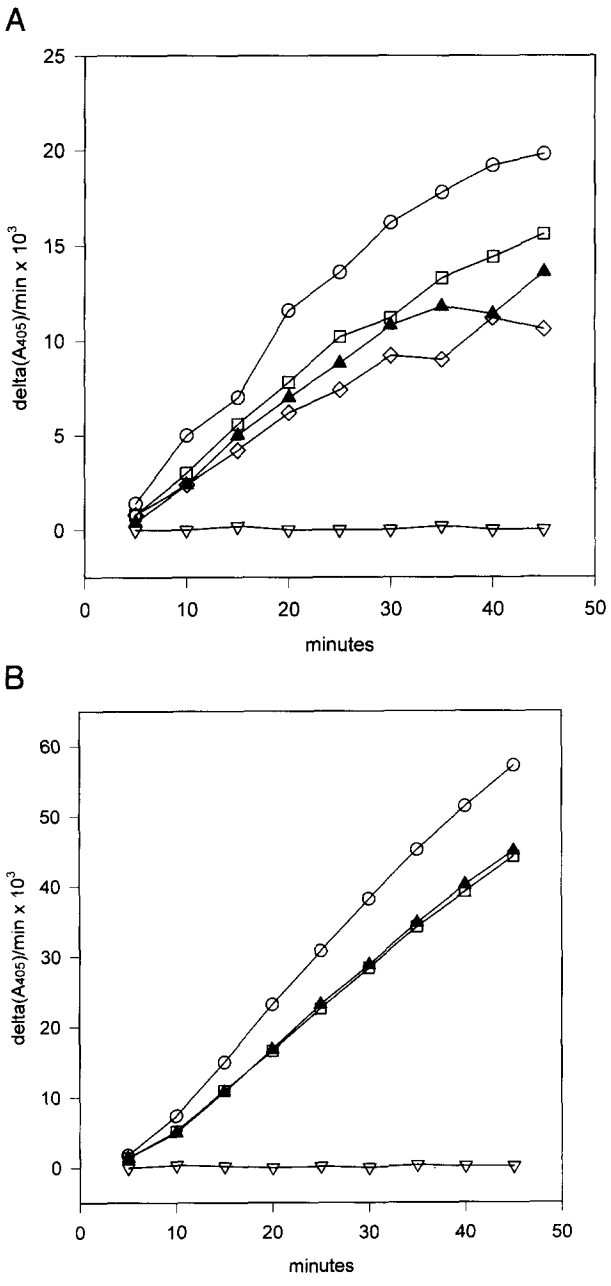

Fig. 4. Effect of suPAR on the reciprocal activation processes of the feed-back system. (A) Activation of pro-uPA, catalyzed by plasmin. Pro-uPA (10 nM) was incubated with the uPA substrate, <Glu-Gly-Arg-pNA (0.4 mM) and 100 ng/ml of plasmin in the absence (○) or presence (▲) of suPAR (10 nM), or with the following concentrations of plasmin in the absence of suPAR: 60 ng/ml (□) or 40 ng/ml (◇). The curve represented by inverted triangles (▽) shows the result obtained with pro-uPA and chromogenic substrate alone. All samples were dissolved in PBS with 0.5% BSA. The uPA activity (ΔA_{405}/min) was plotted vs. time. Each point is the mean of a double determination. (B) Activation of plasminogen, catalyzed by active uPA. Glu-plasminogen (100 nM) was incubated with 0.5 mM of Nle-HHT-Lys-pNA and 0.25 nM of two-chain uPA in the absence (○) or presence (▲) of suPAR (2 nM), or with 0.20 nM of two-chain uPA in the absence of suPAR (□). The curve represented by inverted triangles (▽) shows the result obtained with plasminogen and chromogenic substrate alone. All samples were dissolved in PBS with 0.5% BSA. The plasmin activity was plotted vs. time. Each point is the mean of a double determination.

concentration of suPAR from equivalence to 20-fold excess, relative to pro-uPA (Fig. 3). It was clear that, in the carrier-free samples, the stimulatory effect was concentration dependent even when a large molar excess of suPAR was used (Fig. 3B). Already at equivalence the suPAR concentration was about 10 times higher than the K_d of the interaction [10,23], implying that pro-uPA was largely saturated with suPAR in the whole concentration range employed. In the light of the concentration dependence observed, the stimulatory effect could therefore not be ascribed to binding of the added suPAR to pro-uPA. On the other hand, the pattern observed was in complete accordance with a proteolytic effect, leading to concentration-dependent amplification of the activation system.

In contrast, the moderate inhibitory effect of suPAR which was noted in the BSA-containing samples showed no dose dependence in the present concentration range (Fig. 3A). This observation was consistent with a true effect produced by receptor binding.

3.2. suPAR inhibits the conversion of pro-uPA as well as plasminogen

In addition to studies on the initial phase in the coupled activation system, we performed separate analyses of the two steps responsible for feed-back activation (Fig. 4). In the light of the findings described above, these studies were limited to carrier-containing systems.

Samples of pro-uPA in the absence or presence of equimolar amounts of suPAR were subjected to activation with varying amounts of active plasmin, and the formation of active uPA was followed by conversion of a chromogenic uPA substrate. The addition of suPAR led to a delay in pro-uPA activation (Fig. 4A). The activation profile obtained with 100 ng/ml (1.1 nM) of plasmin in the presence of suPAR was compared to a series of curves obtained with lower concentrations of plasmin in the absence of suPAR. The curves obtained with 40 and 60 ng/ml of plasmin, respectively, are included in Fig. 4A, showing that addition of suPAR led to an approx. 50% inhibition of the activation efficiency under these conditions. This inhibition was directed against pro-uPA activation and not against the activity of the generated uPA towards the chromogenic substrate since we found that suPAR had no effect on the amidolytic activity of two-chain uPA with <Glu-Gly-Arg-pNA (results not shown).

Likewise, we studied the activation of plasminogen with active (two-chain) uPA and the influence of suPAR on this process (Fig. 4B). A weak inhibition of the reaction was noted, leading to an activation curve identical to the curve obtained with a 20% lower amount of uPA in the absence of suPAR. In a similar reaction system with detergent-containing samples the inclusion of uPAR, isolated from membrane fractions of U937 cells, has been shown to yield a 30–40% inhibition of plasminogen activation [25]. Thus, the use of suPAR instead of uPAR and the change in reaction conditions did not change the qualitative effect of addition of receptor.

4. Discussion

In order to elucidate the functional consequences of suPAR-binding to pro-uPA, we studied the proteolytic cascade under several experimental conditions. By using the condi-

tions reported to reveal a stimulatory function of suPAR [16], we could reproduce an apparently accelerative effect on the activation system with pro-uPA and plasminogen. In a parallel experimental setting, we chose to include albumin as a carrier protein in the samples since the conformation and aggregation state of the zymogens and enzymes under these conditions would be more likely to reflect the physiological state, as compared to carrier-free samples with a very low total protein concentration. Under these conditions, we found that the cascade was not stimulated by suPAR but was in contrast moderately attenuated.

This discrepancy was not likely to reflect a gross difference in the receptor-ligand binding. Thus, BSA is conveniently used as a carrier protein that will fully allow the interaction between suPAR and pro-uPA, e.g., in ligand-binding assays [26], as well as in enzymatic studies with uPAR on U937 cells [20]. Furthermore, the result obtained with another carrier protein, ovalbumin, was identical to that obtained with BSA, thus excluding a specific effect of the identity of the carrier. Consequently, we employed two independent criteria to examine the relation between receptor-binding of pro-uPA and the effects observed, i.e., (1) the effect of blocking of suPAR with ATF and (2) the concentration dependence of suPAR. According to both of these criteria the delay of the activation reactions, observed in the carrier-containing samples, was consistent with an effect of the binding of pro-uPA to suPAR. In contrast, neither criterion supported that this binding reaction could be responsible for the stimulatory effect seen in the carrier-free system.

Even though the interfering factor in the carrier-free system was not positively identified, the concentration dependence observed favored the assumption that contaminating proteases gave rise to cascade initiation under these conditions. This interpretation is supported by the reported observation [16] that preincubation of pro-uPA with suPAR enhances the apparent stimulatory effect of the latter. A proteolytic effect of this kind would be expected to be counteracted by carrier proteins since these proteins would act as competing substrates for proteolysis and would also reduce the aggregation of active proteases with the highly hydrophobic pro-uPA. In the coupled system, even an extremely small proteolytic effect at an early point would appear as a strong effect after feedback amplification (see, e.g. [2] for experimental demonstration of this phenomenon). It should be noted that this highly amplifying capacity is a major problem in any attempt to quantify the putative activity of pro-uPA in terms of initial reaction rates since these rates are likely to reflect the content of active enzymes from the start of analysis [2]. Furthermore, the shape of the curves observed in Figs. 2B and 3B suggests that surface adsorption phenomena in the microtitre wells represents an additional problem in the carrier-free system.

We conclude that the functional consequence of binding of pro-uPA to suPAR is a moderate inhibition of the activation of pro-uPA as well as plasminogen, and that some inhibition likewise results when plasminogen activation is studied with active uPA bound to suPAR. The finding that inhibition is only partial, even when complex formation is total, points to an incomplete steric hindrance of the activation reactions, rather than a specific inhibition directed against an active enzymatic site. For this reason, these inhibitory phenomena may not be of profound physiological importance. The important finding, in contrast, is that the present receptor does not by itself stimulate enzymatic activity. This finding is in line with the result that uPAR-expressing cells become unable to stimulate plasminogen activation if the binding of plasminogen to the cell surface is prevented [13,25].

Together, these results support the view that the stimulatory role of uPAR-expressing cells is caused by the steric organization of uPAR and plasminogen-binding molecules on the cell surface [27]. This property of the uPA cascade marks an important difference from the tissue-type plasminogen activator (tPA) system in which the binding of single-chain tPA to fibrin directly enhances the enzymatic activity of the single-chain enzyme, in addition to localizing the activity (see, e.g. [28,29]).

Studies on the mechanism responsible for accelerated plasminogen activation on the cell surface are of crucial importance for the understanding of extracellular proteolysis in the context of invasive processes [17]. As evident from the discussion above, important aspects of these studies will be the identification of additional components on the cell with which uPAR and the enzymes of the cascade interact, as well as specific steric features of the plasma membrane allowing the interplay of all of the components involved.

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