Plasmin-dependent elimination of the growth-factor-like domain in urokinase causes its rapid cellular uptake and degradation

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Urokinase-type plasminogen activator (uPA) and its receptor (uPAR) act in concert to mediate pericellular proteolysis and to stimulate intracellular signalling responsible for cell migration and proliferation. uPA is composed of three domains, a proteolytic domain (PD), a kringle domain (KD) and a growth-factor-like domain (GFD), the last of which mediates the interaction with uPAR. We demonstrate that uPA, associated with the surface of U937 cells, undergoes plasmin-mediated cleavage of the Lys^{46}-Ser^{47} bond with elimination of the GFD. Using recombinant forms of uPA, we show that a uPA variant lacking the GFD (r-uPAΔGFD) and unable to associate with uPAR is rapidly cleared from the cell surface. Binding and internalization of r-uPAΔGFD are markedly decreased in the presence of 39 kDa receptor-associated protein (RAP), the antagonist of several endocytic receptors of the low-density lipoprotein receptor family, suggesting that this protein clearance pathway is used for r-uPAΔGFD. In contrast with rapidly internalized r-uPAΔGFD, the intact recombinant single-chain urokinase with wild-type structure (r-uPAwt) bound to uPAR is retained on the cell surface. Soluble uPAR protects uPA from cleavage by plasmin that results in the elimination of GFD, suggesting that uPAR might protect cell-bound urokinase from plasmin-mediated cleavage between the GFD and KD and subsequent degradation.

Key words: internalization, proteolysis, urokinase receptor.

INTRODUCTION

Urokinase-type plasminogen activator (uPA) is important in cell invasion, tissue remodelling, inflammation and vascular development [1–3]. uPA is a highly specific serine protease that converts plasminogen into plasmin, a protease with broad substrate specificity. Urokinase is secreted as a single-chain zymogen, pro-urokinase (scuPA), possessing little or no intrinsic enzymic activity. scuPA is converted into the active two-chain enzyme (tcuPA) by plasmin-mediated cleavage of the bond between Lys^{158} and Ile^{159} [4,5].

The cell-surface location of uPA is mediated primarily by a specific glycolipid-anchored receptor (uPAR), which binds the enzyme with high affinity via its growth-factor-like domain (GFD), located in the N-terminal non-catalytic portion of the molecule [6]. Binding of the uPA to uPAR might localize plasmin generation to an area of the cell surface to enhance the directional migration of cells [7]. The proteolytic activity of uPA is essentially irreversibly inhibited by the specific plasminogen activator inhibitors PAI-1 and PAI-2 and the protease nexin [8–10]. Urokinase bound to its receptor is retained on the cell surface for a long time, being internalized or degraded to a minor extent, whereas the uPAR-bound tcuPA–PAI-1 complex undergoes rapid endocytosis and degradation [11]. Results from many studies demonstrate that the multiligand low-density lipoprotein receptor (LDLR)-related protein (LRP) mediates the endocytosis of uPAR–tcuPA–PAI-1 complexes [12,13]. Thus, in addition to localizing the proteolytic uPA activity, uPAR participates in the clearance pathway of the irreversibly inhibited enzyme.

Studies on the uPA–uPAR interaction and a detailed kinetics analysis have correlated the binding of uPA and plasminogen to the cell surface with enhanced plasmin generation [14,15]. However, the precise functional significance of uPAR in regulating pericellular plasmin generation is not clear, because results have been provided that uPA-catalysed plasminogen activation is stimulated by cells lacking uPAR, suggesting other cell-surface binding sites for uPA [16]. Plasmin clearly up-regulates further plasmin production by cleaving scuPA to tcuPA. Taking into account the results showing the poor ability of PAIs to inactivate surface-bound uPA [17,18] and their different localizations on the cell surface [19], it is feasible to suggest that plasmin could down-regulate its own generation by limiting the available substrate even under conditions where PAI-inhibitors are limiting.

In the present study we demonstrate that cell-associated uPA undergoes plasmin-mediated cleavage at Lys^{46}-Ser^{47}, which is located within the linker region between the kringle domain (KD) and the GFD, with elimination of the latter. The remaining uPA fragment, containing the KD and the proteolytic domain (PD), is susceptible to rapid uptake and degradation. In addition, we suggest a new role of uPAR in protecting bound uPA from this kind of processing.

EXPERIMENTAL

Proteins and reagents

Recombinant scuPA with wild-type structure (r-uPAwt) and recombinant scuPA with deletion of the N-terminal 43-residue

Abbreviations used: ATF, N-terminal fragment of uPA; GFD, growth-factor-like domain of uPA; KD, kringle domain of uPA; LDLR, low-density lipoprotein receptor; LMM-uPA, low-molecular-mass uPA; LRP, LDLR-related protein; PD, proteolytic domain of uPA; RAP, receptor-associated protein; r-uPAΔGFD, recombinant urokinase lacking the GFD; r-uPAwt, recombinant single-chain urokinase with wild-type structure; scuPA, single-chain uPA; suPAR, soluble uPAR; TCA, trichloroacetic acid; tcuPA, two-chain uPA; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; PAI, plasminogen activator inhibitor.

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fragment (the GFD) (r-uPAΔGFD) were expressed in *Escherichia coli* and purified as described [20]. The N-terminal 166-residue fragment of uPA was a kind gift from Dr R. Beabealashvilly (Cardiology Research Center, Moscow, Russia). Glycosylated high-molecular-mass urokinase expressed in Schneider 2 cells with the *Drosophila* expression system (Invitrogen) and soluble uPAR (suPAR) were a kind gift from Dr D. Cines (University of Pennsylvania, Philadelphia, PA, U.S.A.). Receptor-associated protein (RAP) was kindly provided by Dr T. Willnow (Max-Delbrück Center for Molecular Medicine at Humboldt University, Berlin, Germany). Polyclonal and monoclonal (UIG-1) anti-uPA antibodies, plasminogen and plasmin were provided by Dr S. Domogatsky (Cardiology Research Center, Moscow, Russia). Aprotinin (Trasylol) was from Bayer (Leverkusen, Germany); α_2-antiplasmin was from Sigma (Deisenhofen, Germany). Iodo-Gen, sulphosuccinimidyl 6-(biotinamido)hexanoate, horseradish-peroxidase-conjugated neutravidin, goat anti-mouse horseradish-peroxidase-conjugated IgG, Protein G–agarose and chemiluminescent substrate 'SuperSignal West pico' were from Pierce (Rockford, IL, U.S.A.). A monoclonal mouse antibody clone 3 (R-3-01) that reacts with domain I of human uPAR was from Monozyme (Hoersholm, Denmark). RPMI 1640 culture medium was purchased from Life Technologies (Eggenstein-Leopoldshafen, Germany). PVDF Western blotting membrane were obtained from Boehringer Mannheim (Mannheim, Germany), and CNBr–Sepharose CL-4B was from Sigma.

Cell lines and culture

U937 histiocytic lymphoma cells were grown in RPMI 1640 culture medium containing 10 % (v/v) foetal calf serum, penicillin (100 i.u./ml) and streptomycin (100 μg/ml).

Cleavage of uPA by plasmin

Urokinase (10–20 μM) in PBS was incubated with plasmin (2–5 units/ml) for various durations at 37 °C. The reaction was terminated by the addition of aprotinin (final concentration 100 k-i.u./ml). In some experiments 30 μM suPAR was added to the incubation mixture. Aliquots were subjected to SDS/PAGE in the presence and in the absence of 2-mercaptoethanol.

N-terminal sequencing

Protein samples were subjected to SDS/PAGE [15 % (w/v) gel] followed by electroblotting to Immobilon-P PVDF membrane (Millipore) for 2 h at 15 °C and 400 mA in 0.025 M $NaHCO_3$ buffer, pH 9.0, containing 20 % (v/v) methanol and 0.1 % SDS. The membrane was washed with methanol; the protein bands were detected by staining with a 0.1 % aqueous solution of Amido Black 10B. The amino acid sequences were established with an Applied Biosystems Model 477A Pulsed Liquid Phase Protein/Peptide Sequencer (Foster City, CA, U.S.A.). Phenylthiohydantoin derivatives of the amino acids were identified with an Applied Biosystems Model 120A PTH Analyzer.

Measurement of cell-associated uPA proteolysis

The forms of uPA were biotinylated with sulphosuccinimidyl 6-(biotinamido)hexanoate reagent in accordance with the manufacturer's instructions. All procedures were performed at 0 °C. U937 cells were washed twice with binding buffer (Hanks salt solution containing 20 mM Hepes, pH 7.4, and 1 mg/ml BSA) and resuspended at 2×10^7 cells/ml. Saturation of plasminogen-binding sites on cells was achieved by incubation with 1 μM human plasminogen for 60 min, followed by extensive washing with the binding buffer. The cells were then resuspended in the binding buffer at 5×10^6 cells/ml and incubated in the presence of biotinylated r-uPAwt (5 nM) for 120 min. The cells were then washed with PBS to remove unbound ligand and extracted with lysis buffer {1 % (v/v) Triton X-114/5 mM 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonic acid ('CHAPS')/0.1 M Tris/HCl/5 mM EDTA/100 k-i.u./ml aprotinin/10 μg/ml leupeptin/10 μg/ml pepstatin/0.5 mM PMSF, pH 8.1}. Cell-associated biotinylated r-uPAwt was immunoprecipitated from cell lysates with polyclonal goat anti-human urokinase antibodies and collected with Protein G–agarose. Immunoprecipitates were subjected to SDS/PAGE followed by protein transfer to PVDF blotting membrane, and were then probed with horseradish-peroxidase-conjugated neutravidin. Detection of proteins was performed with the chemiluminescent substrate.

Internalization and degradation assay

Urokinase variants (60 μg) were iodinated with 1 mCi of $Na^{125}I$ and 0.1 mg of Iodo-Gen (Pierce) for 8 min at room temperature, and the reaction was terminated with excess L-tyrosine. Purification of iodinated proteins was performed by chromatography on PD-10 columns (Amersham Pharmacia Biotech) equilibrated with PBS. Iodinated proteins were precipitated (95–98 %) by trichloroacetic acid (TCA) and had specific radioactivities of $(3.0–4.0) \times 10^5$ c.p.m./pmol. Before assay, U937 cells were washed twice and resuspended in ice-cold binding buffer at 5×10^6 cells/ml. In a typical experiment, 0.5–5 nM ^{125}I-labelled r-uPAwt or ^{125}I-labelled r-uPAΔGFD was added to cell suspension for 90 min at 0 °C. In blocking experiments, cells were preincubated with different concentrations of RAP for 30 min before the addition of iodinated uPA forms. An excess of non-bound radioactive ligands was then removed by centrifugation of cell suspension at 800 ***g***, after which the cells were washed twice and resuspended in fresh binding buffer at 5×10^6 cells/ml. Aliquots of the cell suspension were incubated at 37 °C for different durations. Samples were centrifuged at 800 ***g*** to pellet the cells. The supernatants were collected and ice-cold TCA [final concentration 10 % (w/v)] was added for 15 min. The precipitates were collected by high-speed centrifugation; supernatants were considered to be degraded fractions of uPA forms. Glycine/HCl buffer [50 mM glycine/HCl (pH 3.0)/100 mM NaCl] was added to the cells for 3 min to remove surface bound ligands; glycine/HCl supernatants were collected as well. The radioactivities remaining in the cells, glycine/HCl supernatants and TCA precipitates and supernatants were measured with a gamma-counter. Non-specific binding was assessed in the presence of 200-fold molar excess of unlabelled proteins and was typically between 20 % and 40 %.

RESULTS

Cleavage of scuPA by plasmin

The time course of plasmin cleavage of recombinant scuPA was studied. When we analysed samples of urokinase cleavage products by SDS/PAGE under reducing conditions, we found that scuPA was converted into tcuPA within the first 10 min (Figure 1A). The starting material migrated as a single major band with an apparent molecular mass of 48 kDa (lane 0). After a 10 min incubation with plasmin, a 29 kDa band (band 1) was readily apparent, thought to correspond to low-molecular-mass uPA (LMM-uPA), as reported previously [21]. However, two bands with apparent molecular masses of 20.5 kDa (band 2) and

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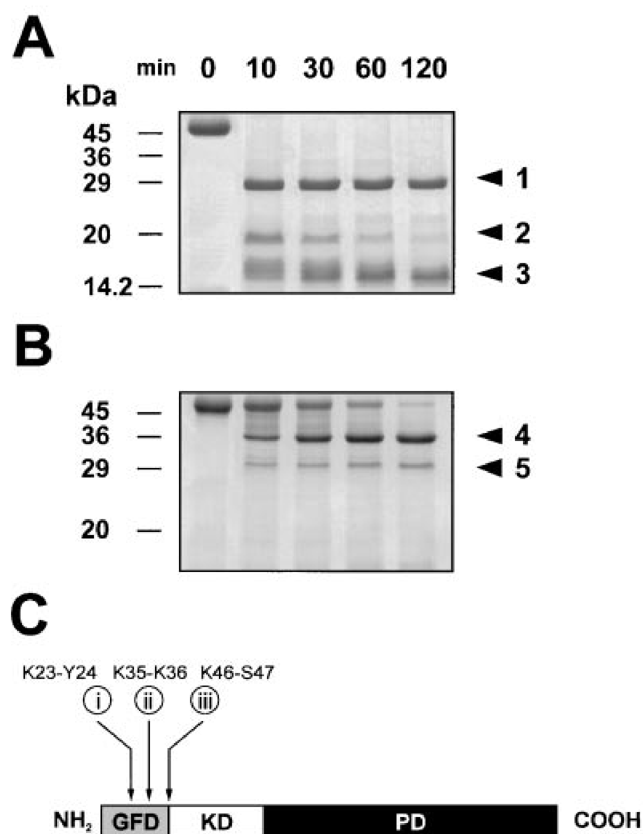

Figure 1 Proteolysis of r-uPAwt by plasmin

r-uPAwt (20 μM) in PBS was incubated with 5 units/ml plasmin at 37 °C as described in the Experimental section. Aliquots were subjected to SDS/PAGE [12.5% (w/v) gel] in the presence (**A**) and in the absence (**B**) of 2-mercaptoethanol. The intact r-uPAwt is shown in lane 0. Arrows indicate the positions of the 31 kDa LMM-uPA (1), the 20 kDa N-terminal fragment of uPA (2), the 17 kDa fragment of uPA (3), the 36 kDa fragment of uPA (4) and the 29 kDa uPA fragment (5). (**C**) Schematic representation of urokinase. The grey box represents the GFD, the white box represents the KD and the black box represents the PD. Arrows indicate the potential sites of cleavage by plasmin within the GFD of uPA.

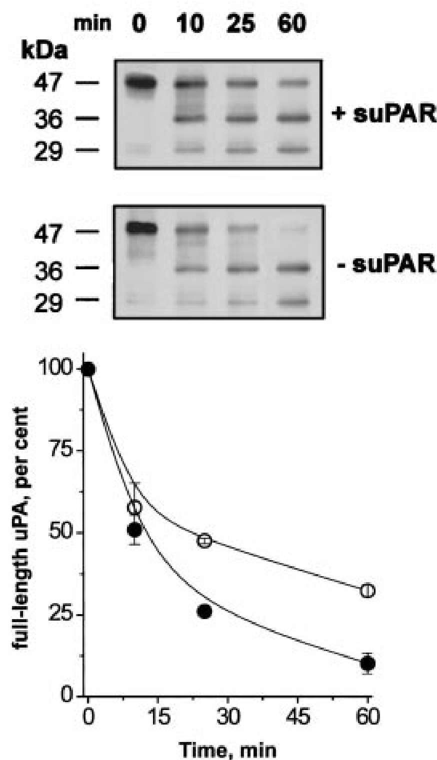

Figure 2 Proteolysis of free and suPAR-bound r-uPAwt by plasmin

Upper panel: r-uPAwt (10 μM) in PBS was incubated with 5 units/ml plasmin at 37 °C for the indicated durations in the absence and the presence of 30 μM suPAR as described in the Experimental section. Aliquots were subjected to SDS/PAGE [12.5% (w/v) gel] in the absence of 2-mercaptoethanol; the separated proteins were analysed by Western blotting with anti-uPA monoclonal antibody. Lower panel: protein bands corresponding to the uPA proteolytic fragments were subjected to densitometry and computation analysis with PCBAS 2.08 software. Values are expressed as percentages of the initial amounts of uncleaved r-uPAwt at zero time before the addition of plasmin in the absence (●) and presence (○) of suPAR. Results are means $\pm$ S.D. for triplicate points from three separate experiments.

17 kDa (band 3) were detected at the 10 min time point as well. The intensity of the 20.5 kDa band decreased over the first 60 min and was faint by 120 min, whereas the intensity of the 17 kDa band increased steadily. When the same products of uPA degradation were analysed under non-reducing conditions, a protein band with an apparent molecular mass of 36 kDa (Figure 1B, band 4) was detected. Incubation with plasmin for a further 120 min led to the complete degradation of full-length uPA and the formation of two fragments with apparent molecular masses of 36 and 29 kDa (band 5) when analysed under non-reducing conditions. Formation of the smaller fragment (band 5) occurred very slowly in comparison with the 36 kDa band. We suggest that the 29 kDa band corresponds to LMM-uPA generated after cleavage of the molecule between Lys^{135} and Lys^{136} and contains the catalytic B-chain of uPA.

If this interpretation is correct, the 36 kDa uPA fragment might comprise a part of uPA from which either the N-terminus or the C-terminus has been removed. However, Figure 1(B) demonstrates that the 29 kDa fragment corresponding to LMM-uPA remained uncleaved during the same time frame in which proteolytic digestion of uPA was evident. Moreover, the intensity of the 20.5 kDa band (band 2), presumably representing the N-terminal fragment of uPA, and containing the GFD and the KD, decreased rapidly and simultaneously with formation of the 36 kDa band visualized under non-reducing conditions (band 4). Taken together, these observations suggest that the 36 kDa band emerging from the proteolysis of uPA by plasmin represents a fragment of uPA formed by deleting a portion of its N-terminus.

Sequence analysis within the N-terminus of uPA revealed the presence of two putative sites favouring plasmin-specific proteolysis located within the linker region between the GFD and the KD: Lys^{46}-Ser^{47} and Lys^{48}-Tre^{49}. To localize the cleavage site within the N-terminus of uPA, plasmin-mediated hydrolysis of the recombinant N-terminal fragment (residues 1–166) of native uPA was performed. The proteolytic fragments obtained were subjected to partial N-terminal amino acid sequencing. Sequence analysis indicated three sequences (single-letter amino acid codes): Y^{24}FSNIHXXN, K^{36}FGGQHXEID and S^{47}KTXYEGNGH. The results demonstrated that there were at least three putative sites for plasmin cleavage within the N-terminal portion of uPA: (i) Lys^{23}-Tyr^{24}, (ii) Lys^{35}-Lys^{36} and (iii) Lys^{46}-Ser^{47} (Figure 1C). Because of the complex pattern of disulphide bonds within the GFD of uPA, cleavage at the first two sites is less likely to occur, resulting in the generation of the 36 kDa uPA fragment observed in Figure 1(B). We therefore suggested that digestion of the Lys^{46}-Ser^{47} bond was responsible

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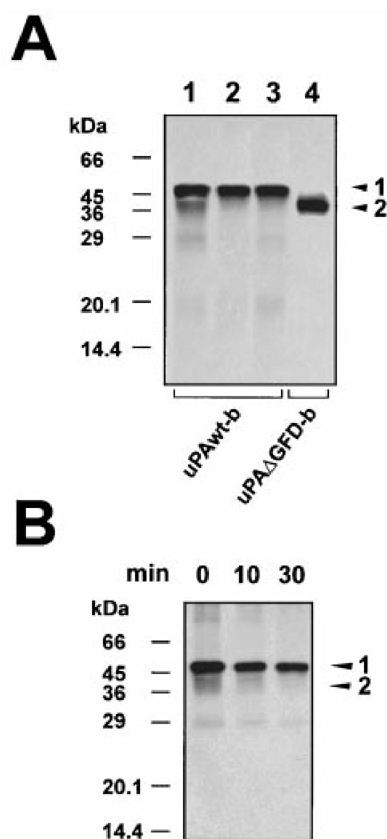

Figure 3 Proteolytic processing of biotinylated r-uPAwt (r-uPAwt-b) on the surface of U937 cells

U937 cells were washed with the binding buffer and resuspended as described in the Experimental section. (**A**) Cells were incubated with 1 μM human plasminogen for 60 min, followed by extensive washing with the binding buffer. They were then incubated with r-uPAwt-b (5 nM) for 120 min (lanes 1–3) in the absence (lane 1) or in the presence of 100 k-i.u./ml aprotinin (lane 2) or 1 μM α_2-antiplasmin (lane 3). Cells were washed with PBS to remove unbound ligand and extracted with lysis buffer as described in the Experimental section. Cell-associated r-uPAwt-b was immunoprecipitated from cell lysates with polyclonal goat anti-(human urokinase) antibodies. The immunoprecipitates were analysed by SDS/PAGE [12.5% (w/v) gel] and Western blotting. The biotinylated proteins were detected with horseradish-peroxidase-conjugated neutravidin. The migration of a biotinylated recombinant uPA-variant lacking the GFD (r-uPAΔGFD-b) is shown to define the position of the proteolytic products of r-uPAwt-b (lane 4). (**B**) Cells were preincubated with r-uPAwt-b in the absence of inhibitors, as described for (**A**), and were warmed to 37 °C for the indicated durations. Cells were washed, lysed, subjected to immunoprecipitation and analysed as described above. The positions of molecular-mass markers are indicated at the left.

for separation of the GFD and releasing the truncated form of uPA. To ensure that the plasmin-mediated elimination of GFD in recombinant bacterially expressed uPA was not an artefact of a certain degree of mis-folding, glycosylated uPA, expressed in Schneider 2 cells, was subjected to cleavage by plasmin in the same conditions. The pattern of partially degraded glycosylated uPA fragments after SDS/PAGE analysis was similar to that illustrated in Figure 1 for bacterially expressed uPA (results not shown).

The cleavage site is located in the vicinity of the uPAR-binding region of the GFD of uPA. It was plausible to suggest that interaction with uPAR might interfere with plasmin digestion at this position. In fact our results indicate that r-uPAwt bound to suPAR was more resistant to plasmin-mediated deletion of the GFD than was free urokinase (Figure 2), suggesting that uPAR might protect bound uPA from this type of processing.

Cell-surface-associated proteolytic cleavage of scuPA

To test whether proteolytic digestion of uPA by plasmin at Lys^{46}-Ser^{47} followed by the release of the GFD might occur on the cell surface, we analysed the degradation of biotinylated r-uPAwt bound to U937 cells. U937 cells were incubated with biotinylated r-uPAwt at 4 °C and lysed as described in the Experimental section. The degradation products of biotinylated r-uPAwt were immunoprecipitated by polyclonal anti-uPA antibodies, separated by SDS/PAGE and detected on PVDF blotting membrane (Figure 3A). We detected two major bands: one at 50 kDa (band 1) corresponding to biotinylated r-uPAwt and one at 38–40 kDa (band 2) representing a truncated uPA fragment. Cleavage of cell-surface-associated biotinylated r-uPAwt was highly dependent on plasmin, because formation of the 38–40 kDa uPA fragment was markedly inhibited in the presence of the plasmin inhibitors α_2-antiplasmin and aprotinin.

When these results were analysed with reference to the pattern of uPA digestion by plasmin in the absence of cells, it was likely that the 38–40 kDa uPA fragment generated from the cell-surface-associated scuPA represented a uPA fragment (residues 47–411) lacking the GFD. Moreover, as shown in Figure 3(A), the position of the 38–40 kDa uPA fragment corresponds to position of the biotinylated recombinant uPA variant lacking the GFD (r-uPAΔGFD-b) described previously [20,22]. It is worth noting that formation of the truncated uPA derivative was observed only when the cells were incubated at 4 °C. When cells with pre-bound biotinylated r-uPAwt were warmed to 37 °C, the band corresponding to the cleaved uPA fragment disappeared within 30 min (Figure 3B). This finding suggests that the separation of GFD from uPA promotes the cellular uptake and degradation of the rest fragment, whereas full-length uPA is retained on the cell surface.

U937 cells internalize full-length uPA and the uPA lacking the GFD with different efficiencies

To examine the fate of the truncated uPA form after cell-surface-associated elimination of the GFD, we compared the internalization and degradation rates of ^{125}I-labelled r-uPAΔGFD and ^{125}I-labelled r-uPAwt by U937 cells. The specific bindings of ^{125}I-r-uPAΔGFD and ^{125}I-r-uPAwt at 0 °C were 20–25 and 35–40 fmol/10^6 cells respectively. Nearly 50 % of the surface-bound fraction of ^{125}I-r-uPAwt at 4 °C was retained on the cell surface when the cells were warmed to 37 °C for 60 min. The fraction of degraded ^{125}I-r-uPAwt reached a low steady-state level of approx. 10 % of specifically bound ligand (Figure 4A). In contrast, ^{125}I-r-uPAΔGFD disappeared rapidly from the cell surface, and more than 80 % of it was degraded by cells within 30 min under the same conditions (Figure 4B). Thus ^{125}I-r-uPAwt and ^{125}I-r-uPAΔGFD differed significantly in the efficiencies of their internalization and degradation by U937 cells. We proposed that these differences might be explained by the inability of r-uPAΔGFD to bind uPAR on the surface of U937 cells. Indeed, with the use of affinity chromatography we demonstrated that r-uPAwt, but not r-uPAΔGFD, specifically precipitated uPAR from U937 cell lysates (Figure 4C).

To examine a cell clearance pathway for uPA lacking GFD, we performed experiments with 39 kDa RAP, an antagonist of the endocytic receptors from the LDLR family that is known to mediate the internalization of uPA in complexes with its inhibitors. We demonstrated that a 200-fold excess of RAP competed for the binding of uPAwt (10 nM) and uPAΔGFD

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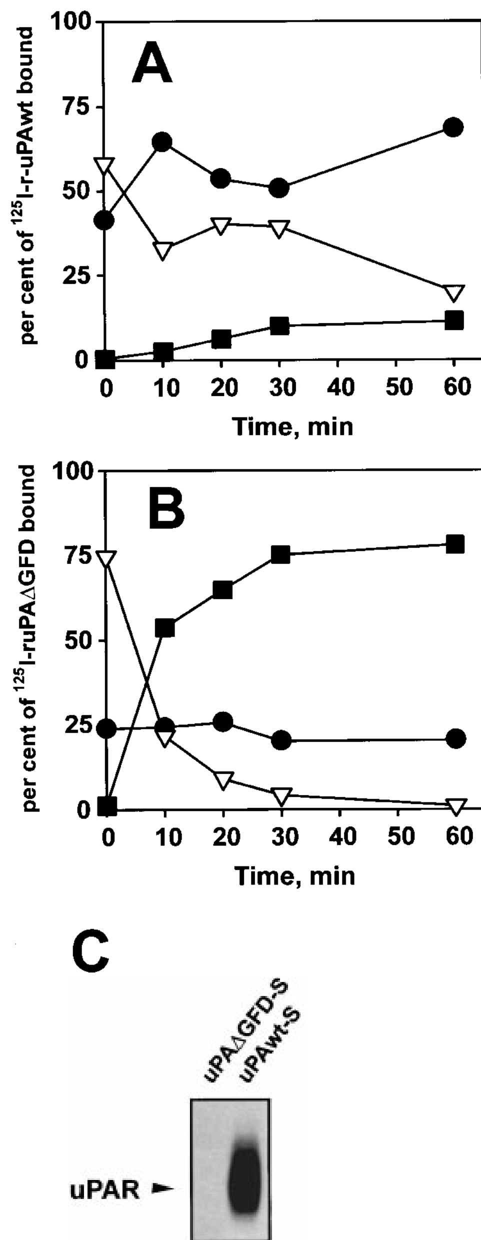
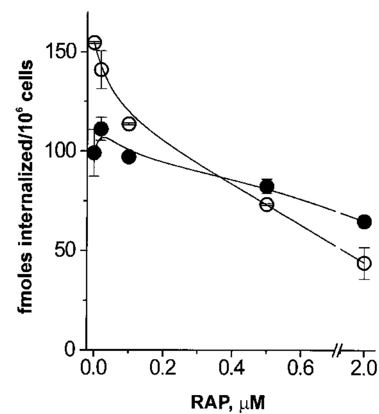


Figure 4 Binding properties of uPAwt and uPAΔGFD

(**A**, **B**) Binding, uptake and degradation of ^{125}I-uPAwt (**A**) and ^{125}I-uPAΔGFD (**B**) by U937 cells. U937 cells were incubated for 90 min at 4 °C with ^{125}I-r-uPAwt (**A**) and ^{125}I-uPAΔGFD (**B**). The cells were then washed with ice-cold binding buffer and incubated at 37 °C in the absence of ligands for the indicated durations as described in the Experimental section. Results are expressed as percentages of the total counts recovered at zero time. Symbols: ▽, surface-bound ligand; ●, internalized non-acid-extractable ligand; ■, degraded ligand (TCA-soluble radioactivity). (**C**) Western blot analysis of the proteins eluted from affinity matrices. r-uPAwt and r-uPAΔGFD were conjugated to CNBr–Sepharose. U937 cell lysates were incubated with affinity matrices. Bound proteins were eluted from matrices, subjected to SDS/PAGE and Western blotting, then probed with anti-uPAR monoclonal antibody.

(10 nM) by 30–40 % and 90–95 % respectively (Figure 5, lower panel). Furthermore, the internalization of ^{125}I-r-uPAΔGFD, but not that of ^{125}I-r-uPAwt, by U937 cells was markedly and dose-dependently inhibited by RAP (Figure 5, upper panel). These results suggest that the uPA variant lacking GFD, being formed after processing with plasmin and being unable to bind uPAR, might associate with an endocytic receptor from the LDLR family, resulting in subsequent rapid degradation.



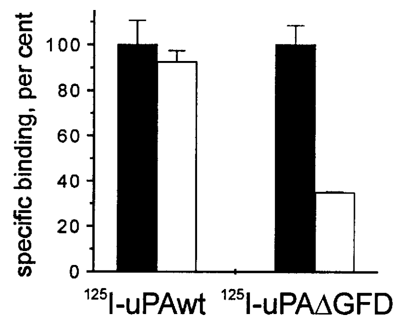


Figure 5 Inhibition of ^{125}I-uPAwt and ^{125}I-uPAΔGFD internalization by RAP

Upper panel: U937 cells were washed and incubated for 30 min at 4 °C in the binding buffer in the presence of the indicated concentrations of RAP. Then ^{125}I-uPAwt and ^{125}I-uPAΔGFD were added to the cell suspension (final concentration 10 nM) and incubation proceeded for a further 60 min. The cells were then transferred to 37 °C and incubated for 30 min. Cells were washed thoroughly, treated with glycine/HCl buffer, pH 3.0, to remove surface-bound ligands, and the amounts of non-acid extractable (internalized) fraction of ^{125}I-r-uPAwt (●) and ^{125}I-uPAΔGFD (○) were determined. Lower panel: cells were incubated in the absence (filled bars) and presence (open bars) of RAP and radioactive ligands, as described above. Before transfer to 37 °C, cells were washed thoroughly with ice-cold PBS and cell-associated radioactivity was counted as described in the Experimental section. Results are means ± S.D. for triplicate points from three separate experiments.

DISCUSSION

Our findings provide evidence that the plasmin-catalysed processing of uPA on cell surfaces results in the appearance of a new uPA form lacking the GFD that is unable to bind uPAR and undergoes rapid cellular uptake and degradation.

There is abundant evidence that plasmin converts the relatively inert scuPA into the active tcuPA by cleavage at the Lys^{158}-Ile^{159} peptide bond [23]. The two resulting chains, the 18 kDa non-catalytic A chain comprising the GFD and the KD, and the 33 kDa catalytic B chain, remain connected via a single disulphide bond, Cys^{194}-Cys^{222}. Prolonged incubation of tcuPA with plasmin results in the release of LMM-uPA commencing at Lys^{136} and corresponding roughly to the catalytic domain [4]. However, our results demonstrate that the conversion of scuPA into tcuPA by plasmin is followed by additional cleavage resulting in the elimination of the GFD. Moreover, accumulation of the uPA form lacking the GFD predominates over the formation of the 33 kDa LMM-uPA. Kobayashi et al. [24] reported that the N-

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terminal portion of uPA (ATF), containing the GFD and the KD, is generated by the action of plasmin on scuPA. However, our observations indicate that ATF is a minor reaction product. Rather, uPA lacking the GFD is the dominant product when scuPA and plasmin are incubated in the presence of U937 cells. Recently we demonstrated that a recombinant uPA variant lacking the GFD (r-uPAΔGFD) binds to the surface of smooth-muscle cells and HEK-293 cells to a membrane target distinct from uPAR [22]. Once formed on the cell surface under the action of plasmin, the uPA derivative lacking the GFD remains associated with the cell surface at 0 °C, but is quickly degraded when the cells are warmed to 37 °C.

Considerable information has been collected on the occurrence, structure and properties of the 55 kDa glycosylphosphatidylinositol-anchored urokinase receptor uPAR/CD87. These studies have shown that cells expressing this receptor are able to bind to scuPA and tcuPA with high affinity and that bound ligands express proteolytic activity [25]. Thus plasmin generation is localized to the leading edge of migrating cells [26–28]. Other interactions involving uPAR have also been identified, such as binding of the uPA–PAI-1 complexes in concert with LRP [12], association with vitronectin and integrins resulting in the modulation of cell adhesion and migration [29–34] and the proteolysis-independent activation of intracellular signalling upon ligand binding [35–39].

In the present study we have demonstrated that uPAR protects bound uPA from plasmin-mediated cleavage within the linker region between the GFD and the KD. Previous studies have demonstrated that the uPA–uPAR complex is stable on the cell surface [40,41]. Several papers have demonstrated that LRP and very-low-density lipoprotein receptor (VLDLR) are able to bind uPA and mediate its internalization and degradation, and that uPAR prevents uPA from binding to the endocytic receptor [42,43]. Our results suggest that uPA associated with the cell surface but bound to a membrane receptor distinct from uPAR might be more susceptible to cleavage by plasmin, with subsequent rapid clearance. We demonstrate that association with suPAR protects uPA from plasmin-mediated cleavage and elimination of the GFD.

Taken together, our results support the concept that uPA undergoes plasmin-dependent proteolytic cleavage on the cell surface, with subsequent formation of a fragment lacking the GFD. The resultant GFD-deficient uPA is unable to bind uPAR and becomes susceptible to the rapid cellular uptake and degradation via a member of an LDLR family. uPAR protects full-length uPA from loss of the GFD by plasmin, which promotes its retention on the cell surface. The results presented here help one to understand the mechanism by which uPAR could protect associated urokinase from degradation, thereby retaining enzymically active urokinase on the cell surface.

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