Urokinase-catalysed cleavage of the urokinase receptor requires an intact glycolipid anchor

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Urokinase (uPA) has the striking ability to cleave its receptor, uPAR, thereby inactivating the binding potential of this molecule. Here we demonstrate that the glycosylphosphatidylinositol (GPI) anchor of uPAR, which is attached to the third domain, is an important determinant in governing this reaction, even though the actual cleavage occurs between the first and second domains. Purified full-length GPI-anchored uPAR (GPI-uPAR) proved much more susceptible to uPA-mediated cleavage than recombinant truncated soluble uPAR (suPAR), which lacks the glycolipid anchor. This was not a general difference in proteolytic susceptibility since GPI-uPAR and suPAR were cleaved with equal efficiency by plasmin. Since the amino acid sequences of GPI-uPAR and suPAR are identical except for the C-terminal truncation, the different cleavage patterns suggest that the two uPAR variants differ in the conformation or the flexibility of the linker region between domains 1 and 2. This was supported by the fact that an antibody to the peptide AVTYSRSRYLE, amino acids 84–94 in the linker region, recognizes GPI-uPAR but not suPAR. This difference in the linker region is thus caused by a difference in a remote hydrophobic region. In accordance with this model, when the hydrophobic lipid moiety was removed from the glycolipid anchor by phospholipase C, low concentrations of uPA could no longer cleave the modified GPI-uPAR and the reactivity to the peptide antibody was greatly decreased. Naturally occurring suPAR, purified from plasma, was found to have a similar resistance to uPA cleavage as phospholipase C-treated GPI-uPAR and recombinant suPAR.

Key words: glycosylphosphatidylinositol, linker region, phospholipase C treatment, plasminogen activator, proteolytic cleavage.

INTRODUCTION

The cellular receptor (uPAR) for urokinase (uPA) is a glycosylated membrane protein engaged in the plasminogen activation system [1]. uPAR is essential for cell-surface-associated plasminogen activation mediated by its ligand uPA [2,3]. This serine protease converts the proenzyme plasminogen into plasmin, which takes part in degradation of extracellular matrix proteins in a variety of biological processes where tissue destruction and cell migration are involved, including cancer invasion [4].

uPAR is anchored in the plasma membrane by a glycosylphosphatidylinositol (GPI) moiety and it contains 283 amino acids in its processed form, which is designated GPI-anchored uPAR (GPI-uPAR) in this work [5,6]. The protein consists of three domains, and each domain contains approx. 90 amino acids connected by linker regions of 15–20 amino acids each [7,8]. The N-terminal domain 1 is needed for the binding of uPA as well as vitronectin, but the entire uPAR is required for high-affinity binding of both ligands [9–11].

In addition to activating plasminogen, uPA is capable of cleaving GPI-uPAR after Arg^{83} and Arg^{89} in the linker region between domains 1 and 2 [12], and this region is also sensitive to cleavage by other proteinases [6,13]. uPA-mediated cleavage of GPI-uPAR is a relatively slow process in solution but is accelerated on the cell surface, where it was found that specific receptor binding of active uPA is required for the high-efficiency cleavage [12]. The cleavage reaction liberates domain 1 and leaves the cleaved form containing only domains 2 and 3, uPAR(2+3), on the cell surface. This form has been identified on several cell lines of neoplastic origin [12–17]. Also extracts of experimental tumours contain considerable amounts of uPAR(2+3) [16].

A soluble form of uPAR, suPAR, has been identified in the plasma of healthy individuals [18] and a recombinant suPAR containing amino acids 1–277 is often used for functional studies [9–11,19]. The level of suPAR is elevated in patients with colorectal cancer [20]. Furthermore, suPAR has been demonstrated in ascites fluid from patients with ovarian cancer [21] and recently also in ovarian cystic fluids, where in addition a soluble, cleaved form was identified [22].

In this work we demonstrate a striking effect of the GPI anchor of uPAR on the cleavability of this membrane protein by its ligand uPA, opening the possibility that it is functionally different from the suPAR of plasma.

EXPERIMENTAL

Enzymes and antibodies

uPA was purchased from Serono (Aubonne, Switzerland) and plasmin from Roche Molecular Biochemicals (Mannheim, Germany). The monoclonal antibodies raised against human GPI-uPAR, R3, recognizing an epitope on domain 1, and R2 and R4, recognizing different epitopes on domain 3, have been described previously [3]. The monoclonal antibody against chemically reduced uPAR and suPAR, S1, was obtained after immunization of a mouse with chemically reduced and alkylated recombinant suPAR [12]. The polyclonal anti-peptide antibody against amino acids 84–94 of uPAR was obtained from a rabbit that was immunized multiple times with the peptide AVTYSRSRYLE. The peptide was assembled on to a branching lysine core with four copies of the peptide by solid-phase synthesis

Abbreviations used: uPA, urokinase; uPAR, uPA receptor; GPI, glycosylphosphatidylinositol; GPI-uPAR, GPI-anchored uPAR; uPAR(2+3), uPAR containing only domains 2 and 3; suPAR, soluble uPAR; Pi-PLC, PtdIns-specific phospholipase C; CHO, Chinese hamster ovary.

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using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry [23] and purified by gel filtration on a Sephadex G-10 column (Pharmacia). The anti-catalytic monoclonal antibody against uPA, clone 5, reacts with an epitope on the B-chain [24,25].

Cell culture

The histiocytic lymphoma cell line U937 (previously designated U937a [1]) was cultured in RPMI 1640 with 10 % heat-inactivated foetal calf serum, 2 mM L-glutamine, 200 units/ml penicillin and 25 mg/ml streptomycin. The cell density was adjusted to 10^6 cells/ml at the onset of the PMA treatment, which was done by addition of 150 nM PMA (Sigma) and continued cultivation for 72 h.

Two transfected Chinese hamster ovary (CHO) cell lines, one expressing recombinant GPI-uPAR on the cell surface and the other secreting a truncated, recombinant suPAR (amino acids 1–277) lacking the glycolipid anchor, were used for production of GPI-uPAR and suPAR. The transfectant expressing GPI-uPAR was constructed by cloning full-length uPAR cDNA [26] into the *Eco*R1 site of pKCR [27]. The resulting expression cassette containing the simian virus 40 promotor and a poly(A)$^+$ site was excised with *Aat*II/*Sal*I, blunted and inserted into the blunted *Eco*RI site of pAdD26SV(A) [28] containing the dihydrofolate reductase (*dhfr*) gene. CHO *dhfr*$^-$ cells were transfected using the calcium phosphate technique. Resulting clones were treated with 1 μM methotrexate for amplification of transfected plasmids [29] and finally characterized using FACS analysis for expression of GPI-uPAR. The construction of the transfectant secreting suPAR was detailed previously [30].

Both transfectants were grown in α-minimal essential medium with Eagle's salt without ribo- and deoxyribonucleosides (Gibco-BRL) supplemented with 3.7 g/l $NaCO_3$, 10 % dialysed foetal calf serum, 4 mM L-glutamine, 10 nM methotrexate, 100 units/ml penicillin and 100 mg/ml streptomycin. Cells, harvested with a rubber policeman, were acid-treated to remove any endogenously bound uPA [31] and lysed in 0.1 M Tris/HCl, pH 8.1, 1 % Triton X-114, 10 mM EDTA, 10 mg/ml trasylol and 1 mM PMSF (lysis buffer). The clarified lysates were subject to temperature-dependent phase separation [1]. The cells were tested and found to be free from mycoplasma infection.

Metabolic labelling

To prepare [^{35}S]GPI-uPAR, 10 Petri dishes (diameter, 14 cm) with confluent transfected CHO cells (approx. 3.3×10^8 cells) were trypsinized to remove cell-surface uPAR. After centrifugation the cells were resuspended in labelling medium {RPMI 1640 without cysteine and methionine (BioWhittaker), including 12.5 mCi/ml NEG072 EXPRE^{35}S^{35}S [^{35}S] Protein Labelling Mix (NEN, Boston, MA, U.S.A.), 10 % dialysed foetal calf serum, 4 mM L-glutamine, 10 nM methotrexate, 100 units/ml penicillin and 100 mg/ml streptomycin} and plated on to 10 new Petri dishes. For preparation of [^{35}S]suPAR, medium was removed from a confluent layer of transfected CHO cells in 10 tissue-culture flasks and replaced with labelling medium. The cells were then grown for 24 h prior to harvest of either the medium, for purification of [^{35}S]suPAR, or cells, for purification of [^{35}S]GPI-uPAR.

Immunoaffinity purification

GPI-uPAR was purified by immunoaffinity chromatography from clarified detergent fractions after temperature-dependent Triton X-114 phase separation of PMA-stimulated U937 cell extracts and CHO cells transfected with uPAR. suPAR was purified from out-dated human plasma or from the media collected from the CHO cells transfected with suPAR. Purification by immunoaffinity chromatography using the monoclonal antibody R3 coupled to CNBr-activated Sepharose 4B (Pharmacia) was done essentially as described previously [12]. Eluted 1 ml fractions were monitored by Western blotting or autoradiography for purification of biosynthetically labelled GPI-uPAR and suPAR. The concentrations of GPI-uPAR and suPAR were measured by ELISA [30]. To ensure that the buffer composition was identical in each cleavage experiment, 25 ng of suPAR and 25 ng of GPI-uPAR were adjusted to the same volume by neutralized elution buffer (9 vol. of 0.1 M acetic acid/0.5 M NaCl/0.1 % Chaps and 1 vol. of 1 M $NaHCO_3$/0.1 M Na_2HPO_4, with a final pH of 7).

Other procedures

In some cases protein samples were subjected to enzymic deglycosylation before electrophoresis to allow complete separation of full-length and cleaved forms of uPAR and suPAR [13]. For this purpose, the samples were pre-treated by denaturation under mildly reducing conditions followed by treatment with peptide N-glycosidase F (PNGase F; Roche Molecular Biochemicals) [1]. After this treatment, the samples were subjected to SDS/PAGE and Western blotting using published methods [13,32,33]. The reactive bands on the Western blots were visualized using either colour reaction [33] or chemiluminesence, using the ECL detection system (Amersham Pharmacia Biotech). Pure GPI-uPAR (25 ng) was treated with 0.25 unit of PtdIns-specific phospholipase C (Pi-PLC; Boehringer Mannheim) for 30 min at 37 °C before and after overnight cleavage with uPA. Pi-PLC was supplied in 50 mM triethanolamine, 10 mM EDTA and 10 mM sodium azide, pH 7.5. The change in buffer composition by addition of a 1/10 vol. of this buffer did not influence the uPA-mediated cleavage of GPI-uPAR. The success of the Pi-PLC treatment was assayed by temperature-dependent phase separation using Triton X-114. Lysis buffer (100 μl) was added to either purified GPI-uPAR directly or to purified GPI-uPAR after Pi-PLC treatment. After initial phase separation the water and detergent phases were separated and Triton X-114 was added to the initial water phase. The water phase from a blank preparation of lysis buffer that had been subjected to phase separation was added to the initial detergent phase. The phase separation was repeated and the resulting water and detergent phases were analysed after addition of 0.1 M Tris/HCl, pH 8.1, to reach equal volumes, and Chaps.

The radioactive samples were separated by SDS/PAGE prior to staining with Coomassie Brilliant Blue, incubation in Amplify (Amersham Pharmacia Biotech) and autoradiography of the dried gels.

RESULTS

GPI-uPAR and suPAR have different cleavage susceptibility to uPA

The striking susceptibility of uPAR to proteolytic cleavage leading to release of the N-terminal domain was previously studied using uPAR-expressing whole cells or detergent-solubilized intact uPAR [12,13]. For most other molecular studies, it is convenient to use a recombinant truncated, water-soluble uPAR variant, suPAR, which can be produced in large amounts [30] and which is considered equivalent to the purified genuine uPAR (GPI-uPAR) in many respects, including the binding characteristics for both uPA and vitronectin [10,11].

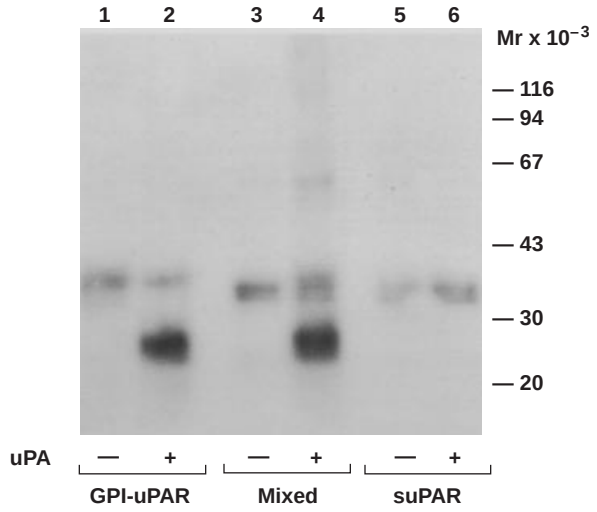

Figure 1 GPI-uPAR and suPAR differ in uPA sensitivity

GPI-uPAR purified from PMA-stimulated U937 cells (lanes 1 and 2) or suPAR purified from the media of transfected CHO cells (lanes 5 and 6) were incubated for 20 h at 37 °C with buffer alone (lanes 1 and 5) or with 100 nM uPA (lanes 2 and 6). Equal amounts of GPI-uPAR and suPAR were mixed and incubated under the same conditions with buffer alone (lane 3) or with 100 nM uPA (lane 4). Each sample was deglycosylated with 0.5 unit of peptide N-glycosidase F (PNGase F) and reduced and alkylated prior to Western-blot analysis using a monoclonal antibody against reduced suPAR, S1 (10 μg/ml). Bands were visualized using chemiluminescence. Note that the apparent molecular masses of deglycosylated uPAR and suPAR are 35 kDa compared with 55–60 kDa for glycosylated GPI-uPAR and 45–50 kDa for glycosylated suPAR. The weak band present in lane 4 with an apparent molecular mass of approx. 60 kDa is most likely the result of incomplete deglycosylation. Electrophoretic mobilities of standard proteins are indicated to the right.

Therefore, we compared the uPA cleavage of suPAR with the previously known pattern for the GPI-containing uPAR (Figure 1). Whereas GPI-uPAR purified from U937 cells (Figure 1, lane 1) is readily cleaved after overnight incubation with 100 nM uPA (Figure 1, lane 2), surprisingly we did not observe any cleavage of suPAR under the same conditions (Figure 1, lane 6). To test whether the purified suPAR preparation contained an inhibitor for the uPA cleavage, GPI-uPAR and suPAR were mixed in equal amounts (Figure 1, lane 3) and incubated with uPA overnight (Figure 1, lane 4). In this mixture approximately half of the material was cleaved as expected, thus excluding inhibition of uPA.

To study this difference in cleavage susceptibility further, we employed GPI-uPAR and suPAR from similar sources. suPAR was purified from the media of CHO cells transfected with this truncated form [30]. To obtain GPI-uPAR, we transfected CHO cells with full-length uPAR cDNA leading to expression of GPI-uPAR (see Experimental and Figure 3, below). Since variation in blotting efficiency of the different receptor variants could not be excluded and the fact that Western blotting does not reveal impurities, we monitored the cleavage reactions by autoradiography. Thus, both transfectants were biosynthetically labelled for 24 h with [^{35}S]methionine and [^{35}S]cysteine. Then [^{35}S]suPAR and [^{35}S]GPI-uPAR were purified from the culture media and the detergent-phase extracts, respectively, of the transfectant cells in question. Since we wanted to isolate the uncleaved receptor we used immunoaffinity purification with immobilized R3 antibody, which does not bind uPAR(2+3). The purified [^{35}S]GPI-uPAR and [^{35}S]suPAR (final concentration, 10 nM) were incubated overnight with increasing concentrations of uPA (Figure 2). The dose–response obtained using the recombinant GPI-uPAR was very similar to the one

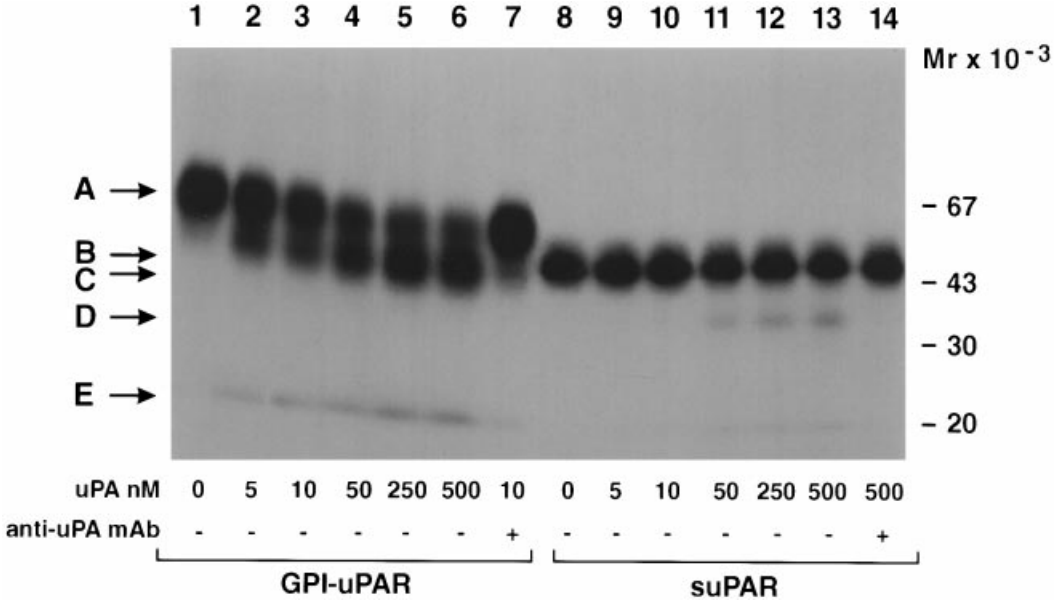

Figure 2 Dose dependence of uPA cleavage of GPI-uPAR and suPAR

^{35}S-Labelled recombinant GPI-uPAR and ^{35}S-labelled suPAR were purified on immunoaffinity columns. Samples of the purified labelled proteins were adjusted to the same buffer composition and incubated at a final concentration of 10 nM with increasing concentrations of uPA in PBS with 0.1% Chaps for 20 h at 37 °C. The samples were separated by non-reducing SDS/PAGE followed by autoradiography. Lane 1 is GPI-uPAR only, lanes 2–6 show GPI-uPAR after incubation with uPA, as indicated, and in lane 7 GPI-uPAR has been incubated with 10 nM uPA after preincubation of the protease for 1 h with 1 μg/ml clone 5, the anti-catalytic anti-uPA antibody. Lane 8 is suPAR only, lanes 9–13 show suPAR incubated with uPA and lane 14 shows treatment of suPAR with 500 nM uPA preincubated for 1 h with 50 μg/ml clone 5. The different uPAR variants are marked with arrows: GPI-uPAR (A), suPAR (B), uPAR(2+3) (C), soluble uPAR(2+3) (D) and uPAR domain 1 (E).

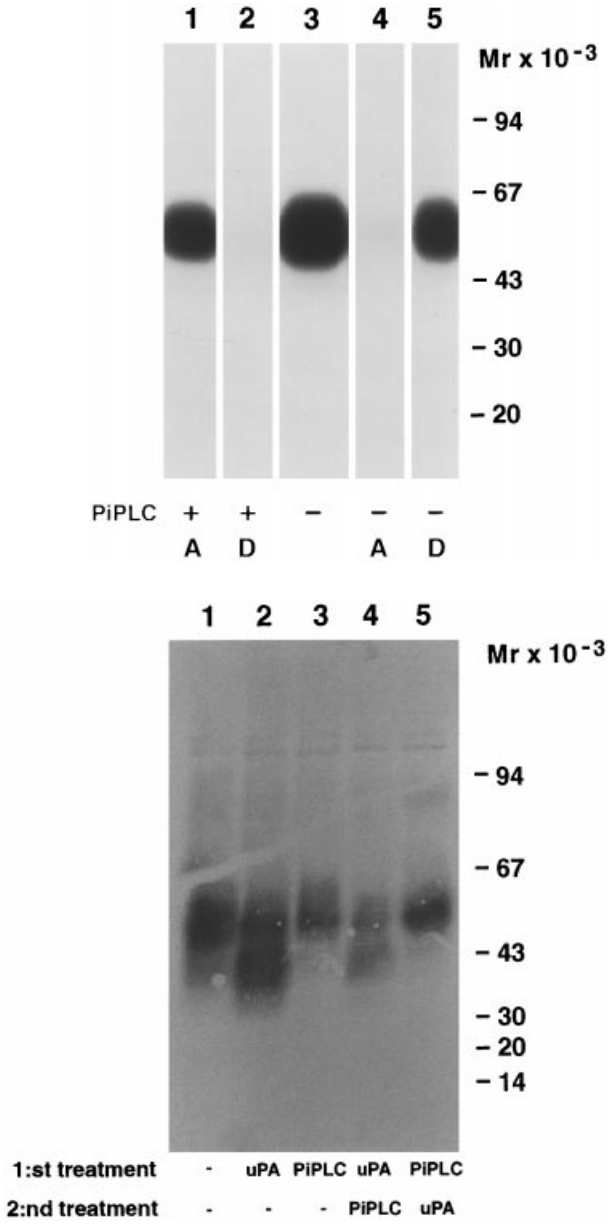

Figure 3 uPA-catalysed cleavage of GPI-uPAR after treatment with Pi-PLC

Top panel: purified recombinant GPI-uPAR (25 ng) after either Pi-PLC or mock treatment was subjected to temperature-dependent phase separation in Triton X-114-containing lysis buffer. Purified [^{35}S]GPI-uPAR was analysed without any treatment (lane 3), after Pi-PLC treatment (lanes 1 and 2) or mock treatment (lanes 4 and 5). Lanes 1 and 4 are aqueous phases (A), lanes 2 and 5 are detergent phases (D). The sample in lane 3 was not subjected to phase separation. Samples were separated by non-reducing SDS/PAGE followed by autoradiography. Bottom panel: purified recombinant GPI-uPAR (25 ng, final concentration 10 nM) was treated with buffer alone or was cleaved with uPA before or after treatment with Pi-PLC. Purified GPI-uPAR was analysed alone (lane 1), incubated for 20 h with 100 nM uPA (lane 2) or for 30 min with 0.25 unit of Pi-PLC (lane 3). Parallel samples of purified GPI-uPAR were cleaved with uPA prior to incubation with Pi-PLC (lane 4) or were incubated with Pi-PLC prior to cleavage with uPA (lane 5). The samples were separated by non-reducing SDS/PAGE prior to Western-blot analysis using the monoclonal antibody R4 (10 μg/ml) recognizing an epitope on uPAR domain 3. Western-blot reactive bands were visualized by colour reaction.

obtained with GPI-uPAR purified from U937 cells [13]. GPI-uPAR was thus readily cleaved by 5 nM uPA (Figure 2, lane 2) but, in accordance with the results using unlabelled molecules (Figure 1), this treatment was completely insufficient in cleaving [^{35}S]suPAR. Actually we found that, under these conditions, 500 nM uPA was needed to cleave appreciable amounts of suPAR (Figure 2, lane 13). At these high uPA concentrations one might suspect that a small contamination of other proteases in the uPA preparation could cause the cleavage. However, pre-incubation of uPA with the anti-catalytic anti-uPA antibody clone 5 prevented the cleavage of both substrates (Figure 2, lanes 7 and 14), showing that uPA was indeed responsible for the cleavage. The released domain 1 is clearly visible and migrates as a single distinct band for both substrate proteins (Figure 2).

We next wanted to investigate whether the above findings reflected a general difference in the proteolytic cleavage susceptibility. GPI-uPAR is also cleaved by plasmin, which has a broader substrate specificity than uPA [13] and consequently we investigated whether suPAR was also resistant to plasmin cleavage. However, overnight incubation of GPI-uPAR and suPAR with different concentrations of plasmin led to cleavage with identical dose-dependence for both uPAR variants (results not shown). The difference between the uPAR variants noted above thus might be a property restricted to the recognition by the more specific protease uPA.

An intact glycolipid anchor is required for efficient uPA cleavage of GPI-uPAR

The GPI anchor is composed of a lipid moiety linked to the protein through a phosphodiester bond and a carbohydrate moiety [5]. We next wanted to study whether the whole of this structure is required to govern uPA susceptibility. Purified GPI-uPAR was treated with Pi-PLC, which removes the lipid moiety alone, leaving the rest of the GPI structure still linked to the polypeptide part [34]. The success of the Pi-PLC treatment of the recombinant GPI-uPAR was monitored using Pi-PLC treatment of [^{35}S]GPI-uPAR followed by temperature-dependent phase separation in Triton X-114-containing buffer. Whereas untreated GPI-uPAR partitioned into the detergent phase (Figure 3, top panel, lane 5), all uPAR was recovered in the water phase after Pi-PLC treatment.

Whereas GPI-uPAR with an intact glycolipid anchor was readily cleaved by 100 nM uPA, the Pi-PLC-treated GPI-uPAR was resistant to cleavage under these conditions (Figure 3, bottom panel, lanes 2 and 5). We ascertained that the Pi-PLC treatment did not prevent us from detecting the cleavage product in Western blotting, since uPAR(2+3) was readily detected if GPI-uPAR was cleaved by uPA prior to incubation with Pi-PLC (Figure 3, bottom panel, lane 4).

suPAR found in blood is thought to be shed from the cell surface by either cleavage of the glycolipid anchor by GPI-specific phospholipase D [35] or polypeptide cleavage mediated by proteases cleaving the protein close to the C-terminus. It would thus seem likely that plasma suPAR would be resistant to uPA cleavage. To investigate this we purified suPAR from human plasma on an R3 immunoaffinity column and performed cleavage experiments as above (Figure 4). Plasma suPAR indeed proved resistant to cleavage by 100 nM uPA (Figure 4, lane 5), just like recombinant suPAR (Figure 4, lane 2). This was not due to a general resistance to proteolysis in the linker region, since plasma suPAR was readily cleaved with plasmin (Figure 4, lane 6).

An antibody specific for the linker region reacts with GPI-uPAR but not with suPAR

The change in susceptibility to uPA cleavage following delipidation by Pi-PLC might be explained by a change in

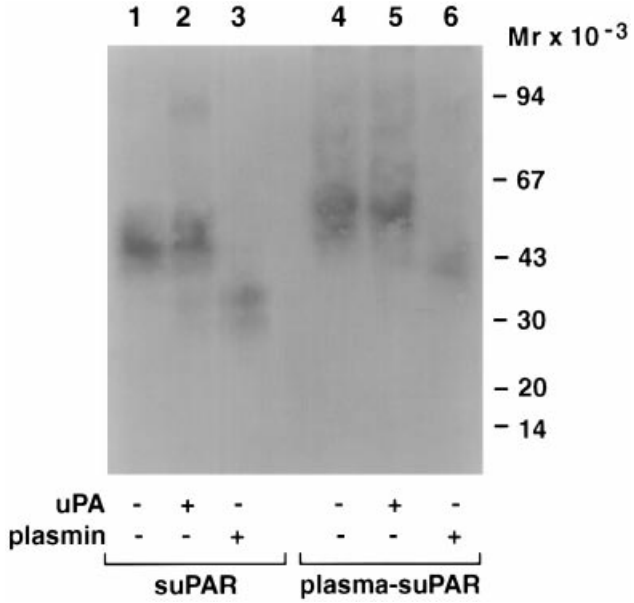

Figure 4 uPA and plasmin cleavage of suPAR purified from plasma

Immunoaffinity-purified recombinant suPAR (lanes 1–3) and suPAR from plasma (lanes 4–6) at a final concentration of 4 nM in PBS with 0.1% Chaps were incubated for 20 h at 37 °C with buffer alone (lanes 1 and 4), with 100 nM uPA (lanes 2 and 5) or with 50 nM plasmin (lanes 3 and 6). The samples were separated by non-reducing SDS/PAGE prior to Western-blot analysis using the domain-3-specific monoclonal antibody R2 (10 μg/ml). Western-blot reactive bands were visualized by colour reaction.

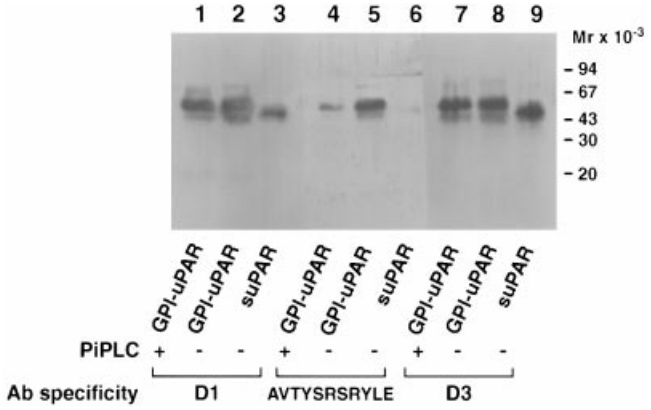

Figure 5 Probing GPI-uPAR and suPAR with an antibody recognizing the linker region between domains 1 and 2

Purified recombinant GPI-uPAR (25 ng) was incubated for 30 min in the presence (lanes 1, 4 and 7) or absence (lanes 2, 5 and 8) of 0.25 unit of Pi-PLC. Purified recombinant suPAR (25 ng; lanes 3, 6 and 9) was electrophoresed directly. After separation by non-reducing SDS/PAGE the samples were subjected to Western-blot analysis using the monoclonal antibody R3 (recognizing an epitope on domain 1, D1; lanes 1–3), the polyclonal anti-AVTYSRSRYLE antibody (lanes 4–6) and the monoclonal antibody R2 (recognizing an epitope on domain 3, D3; lanes 7–9). Equal amounts of protein (25 ng) were applied to each lane and in the Western blotting and the concentration of all antibodies was 10 μg/ml. Western-blot reactive bands were visualized by colour reaction.

conformation or flexibility in the linker region connecting uPAR domains 1 and 2. In some cases conformational changes can be detected with the aid of antibodies against epitopes located in the critical area. To elucidate the putative conformational change in the linker region we raised a polyclonal antibody against a peptide comprising the ten N-terminal amino acids of the uPA-cleaved uPAR, AVTYSRSRYLE, residues 84–94 of the intact protein. When GPI-uPAR, Pi-PLC-treated GPI-uPAR and suPAR were probed with this antibody a strong reaction was found with GPI-uPAR, whereas the reaction with Pi-PLC-treated GPI-uPAR was decreased and the antibody did not react with suPAR (Figure 5, lanes 4–6). Under the blotting conditions used, the reaction of this antibody with GPI-uPAR was comparable in intensity with that found with the monoclonal antibodies R3 and R2 (Figure 5, lanes 2, 5 and 8). The latter antibodies, recognizing epitopes on domains 1 and 3, respectively, detected all three forms of the protein equally well (Figure 5, lanes 1–3 and lanes 7–9). This observation made sure that the difference in reactivity noted with the antibody against the linker region was not caused by a different behaviour of the uPAR variants with respect to immobilization on the blotting membrane.

DISCUSSION

We report here that GPI-uPAR has a much higher susceptibility to uPA-mediated cleavage than soluble forms of uPAR that lack the GPI moiety. Furthermore, these molecular variants of uPAR differ with respect to reactivity with antibodies directed against the cleavage-sensitive linker region. Notably, these changes in molecular properties can be brought about just by the removal of the lipid moiety from the GPI anchor (Figure 3, bottom panel, and Figure 5). These findings suggest that the accessibility of the linker sequence, and thus the conformation or the flexibility of this region of the molecule, is influenced by the hydrophobic group attached to the C-terminus, even though the latter position is remote in terms of primary structure. This effect is probably specifically directed against the linker region, since 16 different monoclonal antibodies we have raised against GPI-uPAR ([3,18,36] and E. Rønne, K. List and G. Høyer-Hansen, unpublished work) react with both GPI-uPAR and suPAR (Figure 5 and results not shown).

Whereas this effect of the GPI moiety was unexpected, a similar situation has indeed been reported for three other GPI-anchored proteins, the Thy-1 glycoprotein [37], Ly-6A.2 [38] and the carcinoembryonic antigen [39]. The antigenic properties of these three proteins thus become altered following delipidation by Pi-PLC, this effect being ascribed to conformational changes.

The sum of these observations suggests that the possibility that conformational effects, imposed by the C-terminal hydrophobic group, may be a more widespread phenomenon among GPI-anchored proteins than previously recognized. If so, the status of the actual cell-bound proteins would deserve separate investigation. For the Thy-1 protein mentioned above the antigenic properties of the cell-bound form follow those of the GPI-containing form in detergent solution [37]. For uPAR, the cell-bound protein has a high susceptibility to uPA [12], making it more similar in this respect to the detergent-solubilized GPI-uPAR than to the water-soluble suPAR. This may seem surprising since the hydrophobic structure is buried in the plasma membrane on the intact cell. However, uPA-mediated cleavage of GPI-uPAR on the cell surface is even accelerated compared with uPA-mediated cleavage of GPI-uPAR in solution. This acceleration on the cell surface requires receptor binding of uPA, which concentrates the enzyme to clusters of uPAR, thereby making the cleavage faster on the cell surface compared with cleavage in solution, where uPA binding is not an important factor for cleavage efficiency [12].

uPAR binds uPA and vitronectin and both of these reactions are abolished upon cleavage of uPAR in the linker region [9–11]. On the intact cell, the cleavage releasing domain 1 is thus likely to affect both pericellular proteolysis and adhesion [11,12,40,41]. Inactivation of pericellular proteolysis by uPA-mediated cleavage of uPAR *in vivo* was suggested in a recent study using transgenic mice overexpressing both uPA and uPAR [42]. It follows from our study that uPA would be unable to accomplish the corresponding inactivation reaction on soluble uPAR.

It is noteworthy that in a completely different line of research the linker region between domains 1 and 2 of uPAR has also been considered functionally important. A chemotactic function of uPAR that has been reported in conjunction with signal-transduction studies was thus found to be exerted only by suPAR cleaved in the linker region [43]. This effect could actually be mimicked by a free peptide, constituting $S^{88}RSRY^{92}$, derived from the linker region [44–46]. It was indeed suggested that as an alternative to proteolytic cleavage a conformational change of uPAR could be sufficient to expose the chemotactic structure [44]. Since intact recombinant suPAR was found not to induce chemotaxis [43], an interesting soluble molecule in this respect might be soluble uPAR(2+3). This component seems to be absent from or very scarce in normal plasma (G. Høyer-Hansen, unpublished work), but it has recently been detected in cystic fluid of ovarian cancer patients [22]. Thus whereas GPI-uPAR functions in pericellular proteolysis and cell adhesion, it is possible that the cleaved GPI-uPAR is shed from the cell surface and the resulting soluble uPAR(2+3) could then be functional in chemotaxis.

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