The intact urokinase receptor is required for efficient vitronectin binding: receptor cleavage prevents ligand interaction

Gunilla Høyer-Hansen[a,*], Niels Behrendt[a], Michael Ploug[a], Keld Danø[a], Klaus T. Preissner[b]

[a] *Finsen Laboratory, Rigshospitalet, Strandboulevarden 49, DK-2100 Copenhagen Ø., Denmark*
[b] *Max-Planck-Institut, Haemostasis Research Unit, Kerckhoff-Klinik, D-61231 Bad Nauheim, Germany*

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Abstract The urokinase receptor (uPAR) is a receptor for both urokinase plasminogen activator (uPA) and the adhesion protein vitronectin. There are two forms of cell surface-bound uPAR; intact uPAR and a cleaved form, uPAR(2+3), which is formed by uPA-catalyzed cleavage of uPAR. In ligand-blotting experiments we found that vitronectin binds uPAR but not uPAR(2+3). In real-time biomolecular interaction analysis using recombinant, soluble uPAR (suPAR) both plasma and multimeric forms of vitronectin bound to intact, antibody-immobilized suPAR. Monoclonal antibodies against domain 1 of uPAR blocked suPAR binding to vitronectin and vitronectin did not interact with suPAR(2+3). Both suPAR(2+3) and the isolated domain 1 failed to compete with the intact suPAR in binding to vitronectin. We therefore conclude that the intact receptor is required for efficient vitronectin binding.

Key words: Receptor cleavage; Urokinase; Urokinase receptor; Vitronectin; Real-time biomolecular interaction analysis

1. Introduction

Plasminogen activation catalyzed by the urokinase-type plasminogen activator (uPA) is crucial for the proteolytic degradation of the extracellular matrix and basement membrane during tissue remodeling including cancer invasion [1,2]. A cell surface receptor for uPA (uPAR) and type-1 plasminogen activator inhibitor (PAI-1) play key roles as regulators of this process, uPAR by enhancing cell surface plasmin generation and PAI-1 by inhibiting both receptor-bound uPA and uPA in solution [3,4]. uPAR is a highly glycosylated protein with a carboxy-terminal glycolipid membrane anchor [5,6]. The protein contains three homologous domains, each consisting of approximately 90 amino acids and characterized by a unique pattern of cysteine residues [7,8]. uPA binding is mediated predominantly by the amino-terminal domain 1 of uPAR [7], but the rest of the protein is also required for the high affinity binding [9,10]. Incubation with uPA or plasmin results in the liberation of domain 1 from the remaining membrane-bound part (designated uPAR(2+3)) of the receptor, as demonstrated in a purified system as well as on the surface of U937 histiocytic lymphoma cells [11,12]. Thus, uPA-catalyzed cleavage of uPAR is a putative negative feedback regulation mechanism for cell surface plasminogen activation [12].

In addition to uPA, binding of the multimeric form of the adhesion protein vitronectin to uPAR has been demonstrated on various cell types and purified systems [13–17] and uPAR-mediated adhesion of vitronectin correlates with the formation of membrane complexes comprising integrins, caveolin and uPAR itself [15]. The affinity of vitronectin binding to uPAR was reported to increase in the presence of pro-uPA, whereas active PAI-1 totally abrogated the interaction [14,16,17]. Based on some initial antibody competition studies Wei and co-workers [13] proposed that the vitronectin binding site is located on domains 2 and 3 of uPAR. However, Kanse et al. [14] found that antibodies against domain 1 of uPAR could block binding of uPA as well as vitronectin to endothelial cells. This prompted us to study the interactions between uPAR and vitronectin in more detail in a purified system using real-time biomolecular interaction analysis. Our results indicate that domain 1 of uPAR is required but not sufficient for efficient binding of vitronectin, in analogy with the mechanism demonstrated for uPA binding to uPAR [10].

2. Materials and methods

2.1. Proteins

Vitronectin was purified from human plasma according to a published procedure [18]. Multimeric vitronectin was generated from the plasma form by incubation in 6 M urea for 1 h at 37°C followed by extensive dialysis against PBS [19]. Glycolipid anchored uPAR and uPAR(2+3) were purified by immunoaffinity chromatography of clarified detergent phase fraction from Triton X-114 lysate of phorbol 12-myristate 13-acetate stimulated U937 cells by first retaining uPAR on immobilized monoclonal antibody (Mab) R3 and uPAR(2+3) on a R2 column as described previously [12]. Recombinant soluble uPAR (suPAR) was purified from the conditioned media of Chinese hamster ovary (CHO) cells transfected with a uPAR expression vector [20], with the only modification that immunoaffinity purification was carried out using immobilized Mab R2 coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.). suPAR(2+3) and domain 1 were obtained by chymotrypsin (Worthington Enzyme Corporation, Freehold, NJ) cleavage of suPAR followed by purification by gel filtration [9]. uPA was purchased from Serono (Aubonne, Switzerland) and inactivated by treatment with diisopropyl fluorophosphate (DFP) [21]. Recombinant, active PAI-1 was a kind gift from Dr. T.M. Reilly (DuPont Merck, DW). Both forms of vitronectin and DFP-uPA were biotinylated according to published procedures [22]. Plasma vitronectin and suPAR were iodinated using the Iodogen procedure [10].

2.2. Antibodies

Monoclonal antibodies to uPAR were obtained from a fusion where the mouse had been injected with uPAR purified from cell lysates of phorbol ester stimulated U937 cells [23]. The antibodies R2, R4 and R8 bind to epitopes on domain 2 or 3 of uPAR, whereas R3, R5 and R9 bind different epitopes on domain 1 [20,23,24].

*Corresponding author. Fax: +45 31385450.

Abbreviations: uPA, urokinase-type plasminogen activator; pro-uPA, pro-enzyme form of uPA; uPAR, uPA receptor; uPAR(2+3), uPAR containing only domains 2 and 3; suPAR, soluble form of uPAR; PAI-1, plasminogen activator inhibitor 1; DFP, diisopropyl fluorophosphate; RU, resonance unit; Mab, monoclonal antibody

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2.3. Ligand-blotting analysis and Western blotting

Protein samples were separated by SDS-PAGE [25] and electroblotted onto polyvinylidene difluoride (PVDF) membranes. Ligand-blotting was performed using 10 nM biotinylated DFP-uPA or 10 nM biotinylated multimeric vitronectin (calculated average number of monomeric vitronectin molecules per multimer = 4) using previously described procedures [26]. Western blotting [23] was performed using 10 μg/ml of R2.

2.4. Real-time biomolecular interaction analysis

All measurements were done employing the BIAcore 2000 instrument (BIACORE, Uppsala, Sweden). Rabbit anti-mouse antibody (RAM Fcγ, BIACORE) was immobilized on the sensor chip CM 5 (BIACORE) at a concentration of 30 μg/ml using the amine coupling method according to the procedure provided by the manufacturer. For immobilization of suPAR, the protein was pre-treated with neuraminidase for removal of sialic acids, since immobilization of non-treated suPAR (pI 4.8 [8]) was found to be very inefficient. The neuraminidase treated suPAR was immobilized on the sensor chip in the same way as RAM Fcγ. In single-layer experiments DFP-uPA or vitronectin, in combinations with or without suPAR or suPAR fragments, were used for injection. In multi-layer experiments, anti-uPAR Mabs were bound to the RAM Fcγ capturing molecules. Then suPAR or suPAR(2+3) was added and interaction was measured after adding varying concentrations of vitronectin. In some experiments uPA or PAI-1 was included. The eluent in single-layer experiments was 10 mM glycine-HCl pH 1.0 and in multi-layer experiments 1 M formic acid. Calculations of kinetic constants were attempted using the BIAevaluation 2.1 software. The validity of the calculated data was assessed using derived logarithmic plots as described [27]. Since the association and dissociation rates measured in a multi-layer experiment are dependent on the dissociation rate of several components, separate curves were recorded with buffer as the last component of the multi-layer and these curves were subtracted from the curves obtained with the varying vitronectin concentrations.

2.5. Immunoprecipitation

suPAR (2 nM or 5 nM) was incubated for 1 h at 4°C with ^{125}I-labeled plasma vitronectin (0.5 nM or 1 nM) in 0.1 M Tris-HCl pH 8.1, 0.3 M NaCl, 0.1% Chaps (binding buffer) in a final volume of 15 μl. Subsequently, 185 μl binding buffer was added prior to further incubation with 2 μg of the Mab R2 for 1 h at 4°C. Finally, 50 μl of a 1:1 slurry of Protein A-Sepharose in binding buffer was added and the samples were end-over mixed for an additional hour at 4°C, after which Sepharose was pelleted by centrifugation and the supernatant withdrawn. The pellet was washed 6 times in binding buffer containing 0.1% (w/v) of bovine serum albumin, Sepharose being transferred to new tubes during the washing procedure. The Sepharose pellets were boiled in sample buffer, the supernatants analyzed by SDS-PAGE [25] with detection of radio-labeled bands by autoradiography.

3. Results

3.1. The intact three-domain structure of uPAR is essential for binding to vitronectin

Since the binding site for vitronectin on uPAR has been claimed to reside in domains 2 and/or 3 [13], we wanted to investigate if the cleaved form of uPAR, uPAR(2+3), could bind vitronectin. Therefore, uPAR and uPAR(2+3), purified from lysates of phorbol ester stimulated U937 cells, were probed with either biotinylated multimeric vitronectin or DFP-uPA in ligand-blotting assays (Fig. 1). Both ligands showed binding to intact uPAR only (Fig. 1, lanes 1, 2 and 5), indicating the requirement for domain 1 of uPAR to obtain vitronectin binding, as has previously been shown for uPA binding to uPAR [7,9,10].

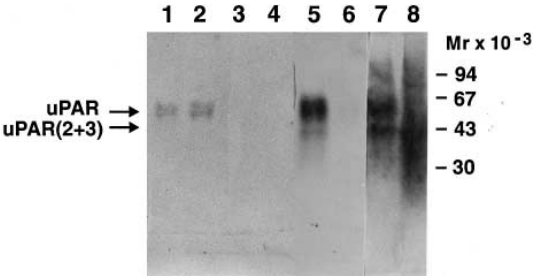

Fig. 1. Ligand-blotting with multimeric vitronectin or DFP-uPA. Immunoaffinity purified uPAR (lanes 1, 5, 7, ~50 ng and lane 2, ~75 ng) and immunoaffinity purified uPAR(2+3) (lanes 3, 6, 8, ~50 ng and lane 4, ~75 ng) were separated by SDS-PAGE and blotted onto PVDF membranes. The samples in lanes 1 to 4 were probed with biotinylated multimeric vitronectin (10 nM) and in lanes 5 and 6 with biotinylated DFP-uPA (10 nM). Lanes 7 and 8 show Western blot with 10 μg/ml Mab R2. The arrows indicate the position of cell surface-bound uPAR and uPAR(2+3). The faint band seen in lane 5 represents intracellular uPAR (M_r 43 000) present in lysates from phorbol ester-stimulated U937 cells [5]. Electrophoretic mobility of standard proteins are indicated to the right.

In order to study structural aspects of the interaction between uPAR and vitronectin we employed real-time biomolecular interaction analysis. In these studies recombinant soluble uPAR (suPAR) [20] and chymotryptic fragments thereof [7] had to be employed in order to obtain sufficient amount of purified protein and fragments (see also Section 4). To ensure a uniform orientation of the molecule, suPAR was captured on the sensor chip by various Mabs. The panel of anti-uPAR Mabs employed included the domain 1 specific R3, R5 and R9, as well as R2, R4 and R8 recognizing epitopes on domain 2 or 3 [20,23,24]. suPAR captured by R2, R4 or R8 all bound significant amounts of vitronectin, whereas no binding was observed with suPAR captured by R3, R5 or R9 (Table 1). These findings showed that immobilization of suPAR via domain 1 prevented the binding of vitronectin, possibly due to competition between vitronectin and the respective antibody.

In the subsequent experiments Mab R2, giving the highest signal with vitronectin (Table 1), was chosen for anchoring of suPAR. The binding of vitronectin to intact suPAR as well as to the chymotryptic cleavage product suPAR(2+3), was investigated (Fig. 2A and B). While intact suPAR bound both plasma and multimeric forms of vitronectin, suPAR(2+3) bound very little if any vitronectin, in accordance with the result found above with glycolipid anchored uPAR.

Domain 1 is likewise known to be an essential element in the binding of uPAR to uPA [7], but it has recently been shown that the integrity of the multi-domain structure of uPAR is required for high affinity binding to uPA [9,10]. In order to investigate whether vitronectin binding to suPAR had similar characteristics, a Biacore-based competition assay was designed, where either intact suPAR, suPAR(2+3) or domain 1 was allowed to interact with vitronectin prior to pas-

Table 1
Interaction between suPAR and vitronectin using anti-uPAR antibodies as capturing molecules

Capturing Mab 333 nM Mab (RU)		First analyte 200 nM suPAR (RU)	Second analyte 200 nM VN (RU)
R2	2418.7	908.5	203
R3	1406.1	238.4	−16
R4	1996.2	773.6	121
R5	1628.5	557.9	−136
R8	1307.5	548.6	92
R9	1198.9	295.8	−84

RU values for analytes were corrected for non-specific binding but not for the variable dissociation of suPAR from the different Mabs.

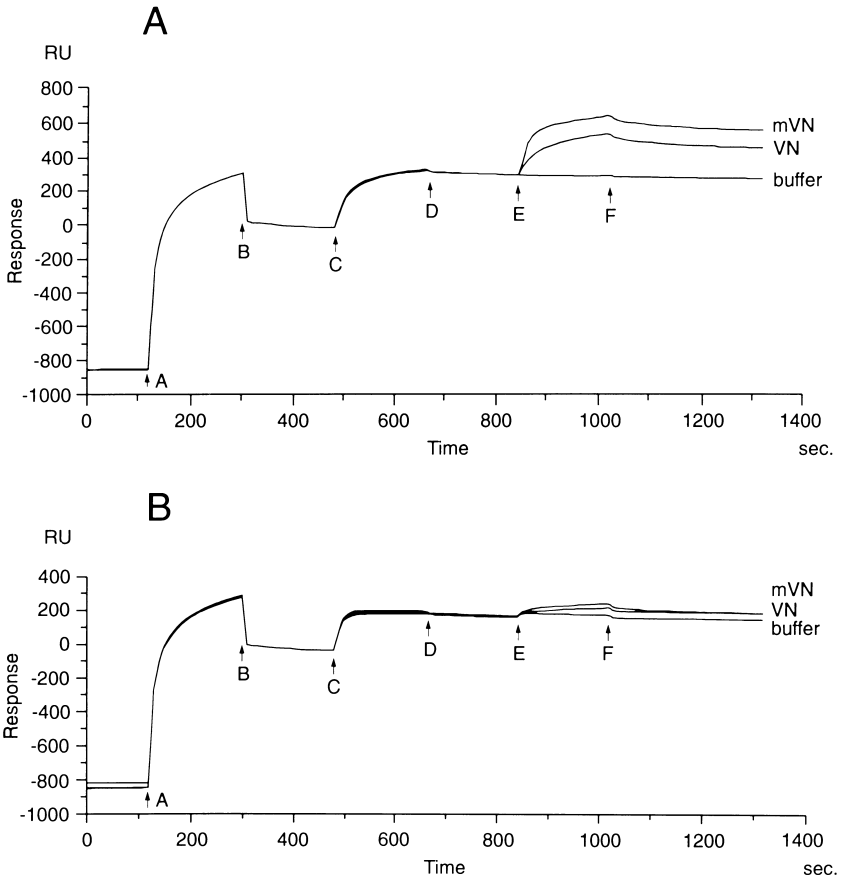

Fig. 2. Real-time measurement of interactions between vitronectin and suPAR or suPAR(2+3). suPAR (A) or suPAR(2+3) (B) were anchored on a sensor chip using Mab R2 as the capturing molecule. The arrow A indicates start of injection of 333 nM R2 and B is R2 injection stop, arrows C and D indicate start and stop, respectively, of injection of 100 nM suPAR (A) or 100 nM suPAR(2+3) (B) and arrows E and F indicate start and stop of injection of 200 nM (15 μg/ml) plasma vitronectin or 15 μg/ml multimeric vitronectin. The flow rate was 10 μl/min. Each injection lasted for 3 min after which the chip was flushed with buffer to allow dissociation for 3 min.

sage through a flow cell with immobilized intact suPAR. For this particular experiment the antibody capture assay could not be employed, since both suPAR and suPAR(2+3), added to the injected vitronectin, would bind any unoccupied binding sites on Mab R2. Therefore, suPAR had to be immobilized directly on the sensor chip. For technical reasons (see Section 2) this required desialylation of suPAR prior to immobilization. However, it was ascertained that this modification of suPAR still allowed binding of both vitronectin and uPA. In both cases the binding was competed efficiently by pre-mixing the injected protein with unmodified suPAR (Fig. 3). Only the intact suPAR prevented the binding of vitronectin to the immobilized suPAR, whereas concentrations as high as 800 nM of either suPAR(2+3) or domain 1 had hardly any effect on vitronectin binding (Fig. 3A). Using this assay we also confirmed that intact suPAR is required for high affinity uPA binding (Fig. 3B) [9,10].

3.2. Effect of occupancy of uPAR with uPA

Binding of uPA to uPAR has been reported to augment the binding between uPAR and vitronectin [13,14]. Since our results show that uPAR domain 1 is essential for both vitronectin and uPA binding, we wanted to study the quantitative details of this simultaneous binding. Therefore, we performed kinetic analysis of the interaction between vitronectin and suPAR in the absence or presence of uPA. This revealed that at any of the vitronectin concentrations used, more vitronectin was bound to suPAR when occupied with uPA than to suPAR alone (Fig. 4). This qualitative observation could however not be interpreted in terms of a simple kinetic model. Thus, no reasonable fit was obtained, when the binding curves were fitted to a second order monovalent association model and a first order dissociation model.

3.3. Both forms of plasma vitronectin bind suPAR

Plasma vitronectin is present as a mixture of the intact protein (M_r 75 000) and a cleaved form (M_r 65 000) due to a cleavage site C-terminal to the heparin binding domain. In order to study the complex formation between suPAR and the individual forms of vitronectin, ^{125}I-labeled vitronectin was mixed with unlabeled suPAR in solution, followed by precipitation with Mab R2, which was also the capturing antibody in the real-time biomolecular interaction analyses. It was ascertained that vitronectin could be precipitated with Mab R2 only when suPAR was present (Fig. 5, lanes 1 and 2). The electrophoretic pattern of precipitated, radio-labeled vitronectin was indistinguishable from that obtained with a polyclonal anti-vitronectin antibody (Fig. 5, lane 5). Thus, suPAR complex formation occurred with both intact and cleaved vitronectin.

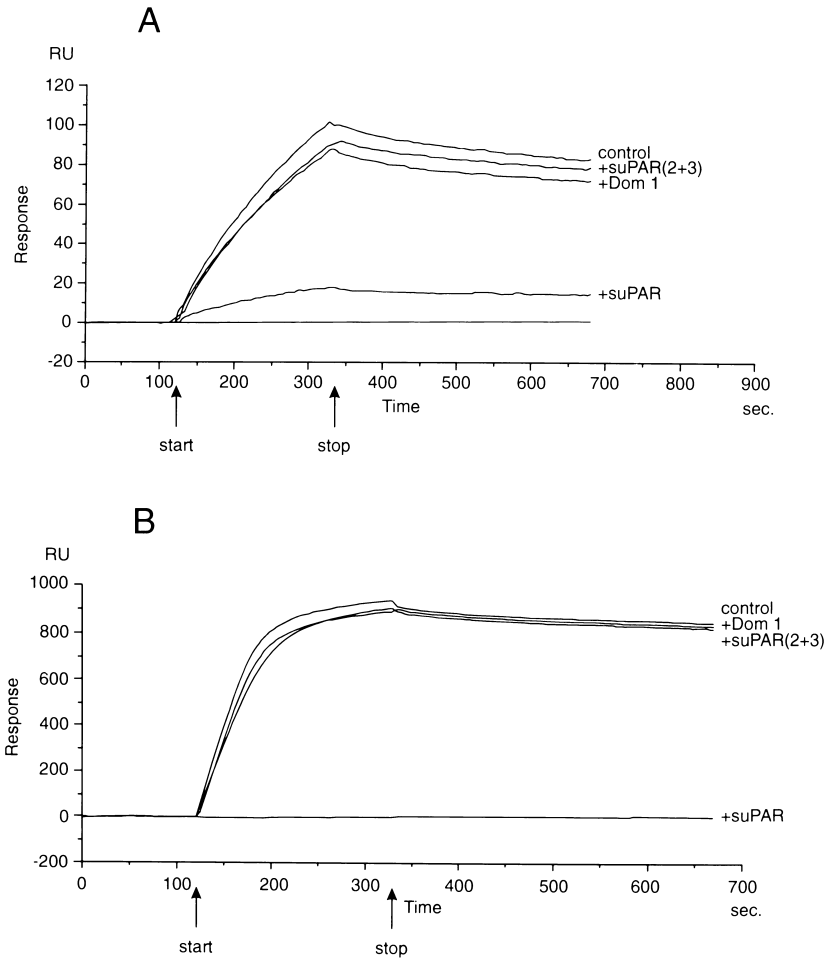

Fig. 3. Competition studies on suPAR vitronectin binding. Neuraminidase treated suPAR (30 μg/ml) was immobilized using 10 mM sodium acetate buffer, pH 5.0. In A the injected protein was 100 nM vitronectin alone (control) or mixed with 800 nM of either domain 1, suPAR(2+3) or intact suPAR. In B the analyte was 25 nM DFP-uPA alone (control) or mixed with 200 nM of either domain 1, suPAR(2+3) or intact suPAR. The flow rate was 10 μl/min and the injection was stopped after 3.5 min after which the chip was flushed with buffer to allow dissociation for 2.5 min.

3.4. PAI-1 inhibits suPAR binding to vitronectin

PAI-1 binds with high affinity to the amino-terminal region of vitronectin [28,29]. To quantitate the effect of PAI-1 on the interaction between suPAR and vitronectin, increasing concentrations of active PAI-1 were pre-incubated with vitronectin prior to passage through a flow cell with Mab anchored suPAR. The amount of bound vitronectin was found to decrease as a function of increasing doses of PAI-1 (Fig. 6). The inhibition was maximal at equimolar concentrations of vitronectin and PAI-1.

4. Discussion

The demonstration of an interaction between uPAR and vitronectin [13,14] is particularly interesting because of the potential function of uPAR as an adhesion protein in addition to its role in governing cell surface proteolysis. The results presented in this report elucidate a number of structural aspects of this interaction. We have demonstrated that domain 1 of uPAR is an essential part of the molecule in binding to vitronectin, but that the intact receptor is indeed required for efficient binding, since domain 1 alone will not compete with suPAR for binding to vitronectin (Fig. 3). The importance of this observation is emphasized by the fact that in vivo uPAR is cleaved on the cell surface [11,12]. Ligand-blotting experiments with glycolipid anchored uPAR(2+3) revealed the complete lack of binding of vitronectin, whereas vitronectin bound to full length uPAR (Fig. 1). The real-time biomolecular interaction analysis of vitronectin binding was done with recombinant suPAR and chymotryptic fragments, i.e. after cleavage of uPAR between Tyr^{87} and Ser^{88} [7]. The uPAR(2+3) purified from U937 cells arise from cleavage at very close positions in the linker region between domains 1 and 2 and actually is a mixture of molecules with N-terminal amino acids Ala^{84} or Ser^{90}. The same cleavage sites are obtained by incubation with uPA [12]. In our Biacore studies, uPA treatment of suPAR would influence the subsequent binding studies (Fig. 4). However, studies with the chymotryptic fragment, suPAR(2+3) were in complete accordance with the ligand-blotting results found with uPAR(2+3) from U937 cells. Thus, the results strongly suggest that uPA-catalyzed uPAR cleavage on the cell surface prevents both vitronectin and uPA binding.

Conflicting results have previously been reported concerning the localization of the vitronectin binding site on uPAR [13,14]. Our observation in real-time biomolecular interaction

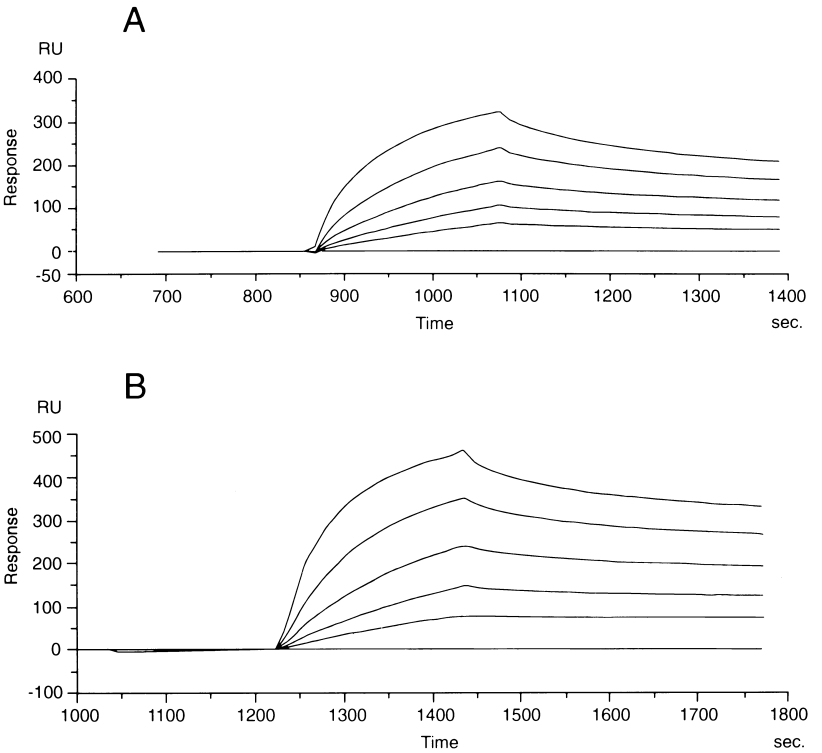

Fig. 4. Effect of uPA on the interaction between suPAR and vitronectin. suPAR (100 nM, 575 RU immobilized) was captured on Mab R2 (200 nM) as in Fig. 1. In B 100 nM uPA (674 RU) was subsequently bound to suPAR. The figure shows the curves obtained in the final injection step where increasing amounts of vitronectin (12.5 nM, 25 nM, 50 nM, 100 nM and 200 nM) was added at a flow rate of 10 μl/min.

analyses, that anchoring suPAR with any of the domain 1 specific antibodies prevented vitronectin interaction, is in agreement with studies of uPAR on endothelial cells in which pre-incubation with domain 1 specific antibodies prevented both uPA and vitronectin binding [14]. This is in contrast to another study, where Mab R4 (directed against domain 2+3) was found to displace suPAR from vitronectin, whereas an antibody to domain 1 showed no effect [13]. Even though different experimental conditions were used in the two studies, the background for this discrepancy is not clear. Our study clearly showed that the antibodies directed against domains 2 and 3, specifically including Mab R4, could be used for anchoring of suPAR to allow subsequent binding of vitronectin.

Along this line it was reported that partially purified suPAR(2+3) inhibited binding of uPAR to vitronectin, whereas purified domain 1 had no effect, leading to the proposal that determinants within domains 2 and 3 were exclusively responsible for binding [13]. However, the preparation of suPAR(2+3) employed in those studies was reported to be 90% pure [13] and could therefore contain intact suPAR in sufficient amounts to explain the inhibition observed. Thus, the observations as such are in complete accordance with our findings.

Our finding that domain 1 is involved in interactions with both uPA and vitronectin prompted us to study the mutual influence of these two ligands on the binding process. We found that simultaneous binding of uPA and vitronectin is indeed possible as reported previously [13–16]. Furthermore, we confirmed in a qualitative manner that binding of uPA to suPAR increases suPAR's capacity to bind vitronectin (Fig. 4). However, in the curve fitting analysis [30,31] simple kinetic models for bimolecular reactions failed to fit the experimental data. Thus, the present binding did not allow the assignment of kinetic constants, even when correcting for the individual

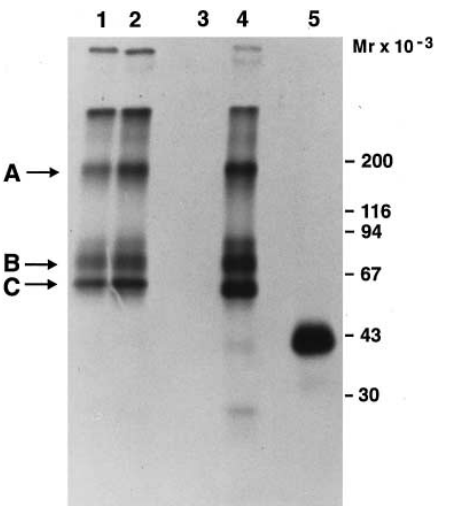

Fig. 5. Immunoprecipitation of suPAR-vitronectin complexes. Purified preparations of suPAR and ^{125}I-vitronectin (final concentrations indicated) were mixed and subjected to immunoprecipitation. The precipitated proteins were analyzed by SDS-PAGE on a 6–16% gradient gel under non-reducing conditions and autoradiography. Lane 1: suPAR (2 nM) mixed with ^{125}I-vitronectin (0.5 nM) was precipitated with 2 μg R2. Lane 2: suPAR (5 nM) mixed with ^{125}I-vitronectin (1 nM) was precipitated with 2 μg R2. Lane 3: ^{125}I-vitronectin (0.5 nM) was precipitated with 2 μg R2. Lane 4: ^{125}I-vitronectin (0.5 nM) was precipitated with 10 μg polyclonal antibody to vitronectin. Lane 5: ^{125}I-suPAR (1 nM, M_r 43 000) was precipitated with 2 μg R2. The arrows indicate A: vitronectin dimers, B: intact vitronectin monomer (M_r 75 000) and C: cleaved vitronectin monomer (M_r 65 000).

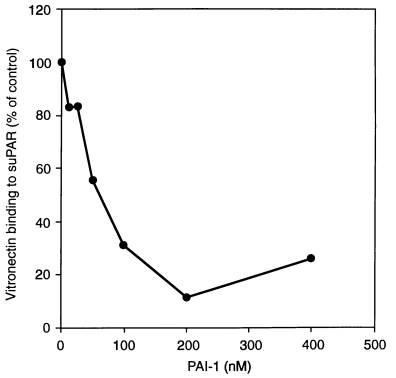

Fig. 6. Effect of PAI-1 on the interaction between suPAR and vitronectin. suPAR (100 nM, 471.5 ± 1.5 RU immobilized) was captured on a BIACORE sensor chip using Mab R2 (667 nM). Vitronectin (200 nM) mixed with increasing amounts of PAI-1 (12.5–400 nM) was added at a flow rate of 10 μl/min. The injection was stopped after 3 min and the chip was flushed with buffer to allow dissociation for 3 min. The increase in RU obtained after injection of vitronectin ± PAI-1 is plotted against PAI-1 concentration.

dissociation of the antigen-antibody complex and the uPA-suPAR complex, respectively. Therefore, the resulting binding situation may not be a simple equilibrium between a single type of complex and two well-defined reaction partners as suggested previously [13], but rather a more complex situation, which is also indicated by a recent report showing that different parts of the vitronectin molecule interacts with uPAR [17].

As PAI-1 binds to vitronectin, it was in addition important to study the influence of PAI-1 on the present molecular ensemble. Since complex formation with PAI-1 was found to prevent uPAR binding, uPAR and PAI-1 binding sites on vitronectin are likely to be localized in close proximity. While this manuscript was under preparation, Deng and co-workers showed that both uPAR and PAI-1 bound to the amino-terminal portion of vitronectin [16], in agreement with our competition data. However, also the carboxy-terminal end of vitronectin was recently shown to be important for uPAR interactions [17], since peptides spanning residues 364–380 of vitronectin, between the plasmin cleavage site after Arg^{361} [32] and the endogenous cleavage site after Arg^{379}, blocked binding of uPAR to vitronectin.

A complex pattern of interactions is emerging between uPA, uPAR, PAI-1 and vitronectin, that apparently serves to modulate cell-matrix interactions. uPA interacts with uPAR through its growth factor domain and PAI-1 inactivates uPA protease activity, thereby balancing pericellular proteolysis. Independent of their enzymatic and inhibitory activities, both uPA and active PAI-1 serve to modulate the physical binding of uPAR to vitronectin [14,16,17]. uPA and vitronectin bind to distinct areas of uPAR, in both cases requiring the intact receptor for efficient binding, whereas PAI-1 and uPAR recognize adjacent/overlapping sites on vitronectin. Upon uPA-catalyzed cleavage of uPAR, liberating domain 1 [11,12], this complex system of protein interactions falls apart. Interestingly, the remaining uPAR(2+3) is present on the cell surface of many neoplastic cell lines [11,33–35]. It remains to be investigated whether the recently reported complexes of uPAR with β_1-integrin [15] are dependent on the described ligand interactions and whether cleaved uPAR is involved in these interactions.

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