Urokinase Regulates Vitronectin Binding by Controlling Urokinase Receptor Oligomerization*

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Adhesion of monocytes to the extracellular matrix is mediated by a direct high affinity interaction between cell-surface urokinase-type plasminogen activator (uPA) receptor (uPAR) and the extracellular matrix protein vitronectin. We demonstrate a tight connection between uPA-regulated uPAR oligomerization and high affinity binding to immobilized vitronectin. We find that binding of soluble uPAR (suPAR) to immobilized vitronectin is strictly ligand-dependent with a linear relationship between the observed binding and the concentration of ligand added. Nevertheless, a comparison of experimentally obtained binding curves to those generated using a simple equilibrium model suggests that the high affinity vitronectin-binding pro-uPA/suPAR complex contains two molecules of suPAR. In co-immunoprecipitation experiments, using different epitope-tagged suPAR molecules, suPAR/suPAR co-immunoprecipitation displayed a similar uPA dose dependence as that observed for vitronectin binding, demonstrating that the high affinity vitronectin-binding complex indeed contains oligomeric suPAR. Structurally, the kringle domain of uPA was found to be critical for the formation of the vitronectin-binding competent complex because the amino-terminal fragment, but not the growth factor-like domain, behaved as a full-length uPA.

Our data represent the first demonstration of functional, ligand-induced uPAR oligomerization having extensive implications for glycosylphosphatidylinositol-anchored receptors in general, and for the biology of the uPA/uPAR system in particular.

Cell migration and invasion are important processes in many pathophysiological conditions such as tumor invasion, angiogenesis, and inflammation. Plasminogen activators, their inhibitors, and their cell-surface receptor(s) play central roles in these processes by regulating extracellular proteolysis, cell adhesion, and signal transduction. In tissues, extracellular proteolysis is controlled by the production of plasmin that is generated by plasminogen activators, mainly urokinase (uPA)1 (1), and is involved in cell attachment, migration and proliferation (2–6). Although binding to uPAR is always required, these latter processes are often independent of the proteolytic activity of uPA, strongly suggesting that other protein interactions are involved.

Indeed, several data indicate that a conformational change in uPAR is capable of profoundly modifying its biological properties. First, it has been shown that uPA binding to uPAR causes the appearance of novel binding sites for vitronectin (Vn) (7–10), thrombospondin (8), uPAR-associated protein (11), and the appearance of binding sites for the αv-macroglobulin receptor (8). Furthermore, uPA-induced chemotaxis (12–16) can be mimicked by proteolytic cleavage of uPAR that generates uPAR fragments that act as potent inducers of chemotaxis in cells lacking endogenous uPAR (16, 17). However, although extensive evidence has been presented that uPAR entertains complex interactions with other proteins, very little is known about how these interactions are regulated at the molecular level.

In this paper we have addressed the possibility that uPA binding influences uPAR oligomerization and that uPAR oligomerization is a major determinant for its interaction with other proteins. As a paradigm, we have employed the ability of uPAR to bind Vn, a function that has been shown previously to induce cell adhesion (7, 10) and to change gene expression during the differentiation of human myeloid U937 cells (18).

EXPERIMENTAL PROCEDURES

Materials—A soluble variant of uPAR (residues 1–277) was expressed and purified from culture supernatants of transfected CHO cells as described previously (16). SuPAR/FLAG was purified from transiently transfected COS-7 cells as described previously (17). Proteolytically processed and purified from eukaryotic cell culture supernatants, was a kind gift from Dr. Jack Henkin (Abbott). The amino-terminal fragments (ATF) of uPA, purified from eukaryotic cell culture supernatants, was a kind gift from Dr. Steve Rosenberg. The exact molarity of suPAR and proteolysed uPA were established by amino acid analysis (Research Consortium Inc.)

Urea-purified vitronectin was purchased from Promega. The monoclonal antibody R2 was kindly provided by Dr. Gunilla Höyer-Hansen (Finsen Laboratories, Copenhagen, Denmark). The monoclonal antibody M2 and the NPP substrate were obtained from Sigma. Secondary antibodies were from Amersham Biosciences (peroxidase-conjugated) and Dako (alkaline phosphatase-conjugated).

Binding Assay—Binding assays were performed in 96-well plates (NUNC Maxisorb) coated with Vn (0.1 μg/ml, 1 μg/ml in 0.05 μ phosphate buffer, pH 9.6) overnight at 4 °C and blocked with 0.15 ml of 2% BSA in PBS for 1 h. All subsequent incubations were performed with reagents diluted in dilution buffer (PBS containing 1% BSA) at 27982

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In this assay, suPAR displays specific, high affinity, and ligand-dependent binding to Vn (Fig. 1A), indicating that the high affinity Vn-binding form of uPAR is a complex between uPAR and pro-uPA (7, 10). However, although pro-uPA was clearly required for suPAR binding to Vn, it was also strongly inhibitory when present in excess of suPAR. This suggests that the mechanism of suPAR binding to immobilized Vn was more complex than a simple reaction in which one molecule of suPAR binds one molecule of pro-uPA forming a heterodimeric high affinity Vn-binding complex. To exclude the possibility that this unusual dose dependence of binding was an artifact caused by our detection system, we repeated the experiments using different antibodies to detect bound suPAR (a polyclonal rabbit antibody and two different mouse monoclonal antibodies), as well as using 125I-radiolabeled suPAR in which case no secondary reagent was required. However, independent of the method of detection, qualitatively identical binding curves were observed in all cases (results not shown), demonstrating that the behavior of the binding curves indeed reflects the ligand dependence of suPAR binding to immobilized Vn.

To address the ligand dependence of suPAR binding to Vn, we further analyzed the extent of suPAR binding to immobilized Vn and its dependence on pro-uPA concentration (Fig. 1B). No binding of suPAR was observed in the absence of low concentrations of pro-uPA. With increasing pro-uPA, we found a linear increase in suPAR binding, the extent of which was dependent only on the pro-uPA concentration (in the initial tract the curves superimpose for all concentrations of suPAR). Binding reached a maximum at a pro-uPA concentration close to one-half of the suPAR concentration and then declined at higher pro-uPAs. This suggests the existence of an optimal pro-uPA:suPAR stoichiometry for Vn binding, possibly 1:2. At pro-uPA concentrations above those of suPAR, the curves settle to a plateau close to one-third of the maximum, suggesting the formation of complex with 1:1 stoichiometry, less efficient in binding Vn.

Curves of suPAR binding to Vn for increasing concentrations of suPAR (Fig. 1C) rise linearly at suPAR concentrations up to one-half pro-uPA concentration (and superimpose at all concentrations of pro-uPA). At higher suPAR concentrations the slopes increase (and more so for higher pro-uPA concentrations) until the curves rather abruptly plateau at suPAR concentrations about twice those of pro-uPA. This again points to an optimal 1:2 pro-uPA:suPAR stoichiometry for Vn binding. The region of linear increase in Vn binding (pro-uPA:suPAR >1/2) and the more than linear increase between stoichiometry 2:1 and 1:2 again point to lower binding efficiency for complexes with 1:1 stoichiometry.

Qualitatively, the experimental data thus suggest that suPAR binding to Vn may be explained by the binding of two stoichiometrically different pro-uPA:suPAR complexes. A high affinity 1:2 complex forms when suPAR is in excess of pro-uPA, and a low affinity 1:1 complex preferentially forms when pro-uPA is in excess of suPAR. To address mathematically the validity of this mechanism, a simple equilibrium model was fit to the experimental data shown in Reactions 1 and 2,

where L and R represent pro-uPA and suPAR, respectively, and total suPAR binding to Vn is represented by $B = a[LR_2] + b[LR]$. The effectiveness ratio ($b/a$) represents the relative Vn

RESULTS

Biphasic Effect of Pro-uPA on suPAR Binding to Vn—To study the mechanism of uPAR binding to Vn, we have exploited a simple in vitro binding assay in which Vn-coated plastic surfaces are incubated with suPAR in the presence or absence of the reagents to be tested. After removal of unbound reagents, bound suPAR was quantitated by sequential incubations with reagent-specific primary antibodies, secondary enzyme-conjugated antibodies, and finally a colorimetric substrate (see "Experimental Procedures").
binding activity of the LR and LR₂ complexes.

The curves predicted by this model were slightly sensitive to the \( K_D \) values for Reactions 1 and 2, and best fits were obtained for low values of these parameters, \( K_D(i) < 10^{-10} \) and \( K_D(i) \) even lower (some 50-fold). This suggests that binding under the applied experimental conditions might constitute an almost irreversible reaction, and an equilibrium model might not be the best way to describe the system. However, most aspects of the experimental binding data represented in Fig. 1 were fully and quantitatively accounted for by this model. Experimental estimates are available in the literature for the affinity of the uPA/uPAR interaction (\( K_D(i) \approx 10^{-10} \) \( M \) (20–24)). Therefore, fits were recalculated by constraining \( K_D(i) \) to this value (Fig. 1, B and C, dotted lines). The resulting estimate for \( K_D(i) \) was 0.77 nM, and an effectiveness ratio (\( b/a \)) of 0.061 was estimated for binding of LR and LR₂ complexes to Vn. As can be seen in Fig. 1, crossing of the curves in B and the decrease to plateau in A are well predicted.

Although the above binding model closely predicts the binding curves, it should be noted that it does not take into account the likely presence of different multimeric forms of Vn in the binding assay. In fact, it is possible that the high affinity pro-uPA/\( \text{suPAR} \)/Vn complex may have a 1:2:2 stoichiometry and the low affinity complex an 1:1:1 stoichiometry, etc. However, binding experiments using different preparations of Vn (native and denatured) resulted in identical binding curves (data not shown) suggesting that after adsorption to plastic Vn exposes the same \( \text{suPAR} \) binding epitopes independently of its original state of oligomerization.

Pro-uPA has also been reported previously (25) to interact directly with Vn, and we therefore addressed the alternative possibility that pro-uPA in some way competes for an overlapping binding site on Vn. However, for several reasons this could not explain the inhibitory effect. First, the inhibitory effect of pro-uPA (Fig. 1) occurs at concentrations well below its apparent \( K_m \) value for Vn (97 nM (25)). Second, the minimal concentration of pro-uPA required to observe inhibition was not fixed but correlated with the concentration of \( \text{suPAR} \) (Fig. 1). Third, preincubation of immobilized Vn with pro-uPA failed to prevent the concomitant binding of pro-uPA/\( \text{suPAR} \)/Vn complexes (Fig. 2A, columns F–I). Another possibility is that pro-uPA causes the release of pro-uPA/\( \text{suPAR} \) complexes from Vn. This might, for example, occur by proteolytic cleavage of \( \text{suPAR} \) by trace amounts of active two-chain uPA present in the pro-uPA preparation. However, even this did not explain the inhibitory effect as pro-uPA/\( \text{suPAR} \) complexes bound to Vn were resistant to release by an excess of free pro-uPA (Fig. 2A, columns J–M).

Taken together the data suggest that \( \text{suPAR} \) binding to Vn is inhibited by elevated pro-uPA due to a shift from binding-competent to less effective pro-uPA/\( \text{suPAR} \) complexes for binding to Vn. To test this possibility we performed binding experiments using pre-formed pro-uPA/\( \text{suPAR} \) complexes containing optimal amounts of pro-uPA and \( \text{suPAR} \) (Fig. 2B). After a 1-h incubation, these pre-formed complexes were supplemented with increasing concentrations of pro-uPA, incubated for another hour, and then assayed for Vn binding activity (Fig. 2B, columns F–H). For comparison, we analyzed the reaction having ground absorbance observed in BSA-coated wells (see A) and is presented in arbitrary units (% of the maximal binding observed in the experiment). Individual data points of duplicate determinations are shown. C, is same as B but with a 2-fold dilution curve of \( \text{suPAR} \) in the absence (triangles) or presence of 4.3 nM pro-uPA (circles), 8.7 nM pro-uPA (diamonds), or 17.3 nM pro-uPA (squares). Dotted curves in B and C represent the binding curves predicted from a simple equilibrium model: \( (i) L + R \leftrightarrow RL, \) (ii) \( RL + R \leftrightarrow R_L L \), where R and L represent \( \text{suPAR} \) and pro-uPA, respectively, and \( \text{suPAR} \) binding to Vn is represented by \( B = a[R_L L] + b[RL] \) (see text).
FIG. 2. Resistance of the high affinity Vn binding pro-uPA-suPAR complex to excess pro-uPA. suPAR and pro-uPA were mixed and incubated with immobilized Vn in the concentrations and sequences indicated above the graph columns, and the Vn-bound suPAR was quantified as described in the legend to Fig. 1. A, columns A–E, wells were incubated for 1 h with samples containing 20 nM suPAR and increasing concentrations of pro-uPA as indicated; columns F–I, wells were first incubated for 1 h with concentrations of pro-uPA as indicated, washed, and then incubated for 1 h with 20 nM suPAR and 10 nM pro-uPA; columns J–M, Vn-coated wells were first incubated with dilution buffer containing 20 nM suPAR and 10 nM pro-uPA, washed, and then incubated with increasing concentrations of pro-uPA as indicated. B, equal volumes of dilution buffer containing the indicated concentrations of suPAR and pro-uPA were mixed and incubated for 1 h in the absence of Vn. Another volume of dilution buffer with pro-uPA, as indicated, or without pro-uPA (columns A–E) was then added, and the samples were left for 1 h in the absence of Vn. Finally, the samples were transferred to Vn-coated wells and assayed as above. (Note that this mixing scheme generates three pairs of samples (C/F, D/G, and E/H) having identical final concentrations of suPAR and pro-uPA, but a different mixing sequence.) Specific binding is presented as the percentage of the maximal binding observed in the experiment, and values represent the mean (± S.D.) of triplicate determinations. Similar data were observed in four independent experiments.
ing the same final concentrations of both components, but prepared without the two-step addition of pro-uPA (Fig. 2B, columns B–E). Indeed, the capacity of excess pro-uPA to inhibit suPAR binding to Vn was strongly reduced when the pro-uPAsuPAR complexes were allowed to form before the addition of the excess pro-uPA.

These data demonstrate that what determines the level of suPAR binding to Vn is the ratio between suPAR and pro-uPA and not the absolute concentration of the two proteins. Consequently, the pro-uPAsuPAR complexes that bind with high affinity to Vn cannot be simple 1:1 complexes.

**Urokinase Regulates High Affinity suPAR Binding to Vn by Controlling suPAR Oligomerization**—A fundamental prediction of the model used to explain the binding data is the existence of complexes between pro-uPA and two (or more) molecules of suPAR.

To address this possibility directly, we performed co-immunoprecipitation experiments using two different epitope-tagged suPAR preparations (Fig. 3). We had previously constructed, expressed, and purified a suPAR variant in which a short peptide epitope had been engineered onto Pro-274 of uPAR generating a recognition epitope for the monoclonal anti-FLAG antibody M2 (10, 17). The addition of this epitope involved the removal of the three carboxyl-terminal amino acids (Asp-Leu-Asp, amino acids 275–277) present on “wild-type” suPAR (amino acids 1–277), and resulted in the complete destruction of the recognition epitope for the monoclonal anti-uPAR antibody R2. In contrast to the differential recognition by monoclonal antibodies, these two suPAR variants (from here on termed suPAR and suPAR/FLAG) display indistinguishable pro-uPA and Vn binding (Fig. 3 and results not shown). We mixed the two forms of suPAR and immunoprecipitated the mixture with the M2 anti-FLAG antibody. When the immunoprecipitate was blotted with R2 antibody, R2 did not recognize any protein in the M2 precipitate (Fig. 3A, lane 7). However, in the presence of pro-uPA, suPAR was readily identified in the M2 immunoprecipitate (Fig. 3A, lane 8). The specificity of the co-immunoprecipitation procedure was verified by immunoprecipitation of samples in which one, two, or all three reactants had been omitted (Fig. 3A, lanes 1–6). In these samples no R2 reactive material was observed. We next compared the pro-uPA dose dependence of suPAR co-immunoprecipitation (Fig. 3B) and Vn binding (Fig. 3C). In these experiments an exact correlation between suPAR oligomerization, as evidenced by co-immunoprecipitation, and Vn binding was observed, demonstrating that the generation of the high affinity Vn-binding complex involves pro-uPA-induced suPAR oligomerization.

As the suPAR:uPAR co-immunoprecipitation experiments demonstrated the existence of higher order suPAR:pro-uPA complexes in the absence of Vn, we next sought to identify these complexes by size exclusion chromatography. To this end we subjected purified suPAR, pro-uPA, and mixtures of the two proteins to analytical gel filtration on a Superdex™ 200 column (Fig. 4A). Analyzed individually, both suPAR (dotted curves) and pro-uPA (gray curves) filtered as single peaks with highly reproducible retention times (24.80 ± 0.07 (n = 3) and 27.67 ± 0.04 (n = 3) min, respectively). When mixtures of the suPAR and pro-uPA were analyzed (black curves), a single new peak (retention time, 22.91 ± 0.02 min (n = 5)), corresponding to the 1:1 pro-uPAsuPAR complex was observed. No other complexes could be identified. Furthermore, no apparent difference in the retention time of the pro-uPAsuPAR complex could be observed when the complex was formed in the presence of excess pro-uPA (22.91 ± 0.01 min (n = 2)) or in the presence of excess suPAR (22.92 ± 0.01 min (n = 2)). To exclude that the failure to detect ternary complexes by gel filtration was caused by a lack of resolution, the column performance was validated using mixtures of reference proteins (Fig. 4B). In these experiments an excellent resolution was observed with a linear correlation (correlation coefficient 0.99) between the Kav and the log-transformed molecular weights of the reference proteins (see “Experimental Procedures”). Based on the calibrations curve, the relative molecular masses of pro-uPA, suPAR, and the 1:1 complex were calculated to be 28.1 ± 0.6, 75.2 ± 1.1, and 144.2 ± 2.2 kDa, respectively. It thus appears that in contrast to pro-uPA, which behaves as a very compact molecule, both suPAR and the 1:1 complex may have an extended (non-spherical) shape in solution. Within the presented series of gel filtrations, the maximal difference in retention time between independent runs of the same protein (11 different proteins) was 0.13 min. A reduction in the retention time for the 1:1 pro-uPA:suPAR complex of twice this size (0.26 min) translated into a molecular mass increase of 13.4 kDa (9.2%). We therefore concluded that if the ternary complex had formed stochiometrically, we would have observed it even if its molecular size was only marginally different from that of the 1:1 complex.

The gel filtrations presented in Fig. 4 were all performed in the presence of 0.5 M NaCl to prevent interactions between the solid phase and pro-uPA which was otherwise observed at physiological salt concentrations (data not shown). This is unlikely to explain the failure to detect higher order complexes as suPAR binding to Vn was unaffected under these conditions (data not shown). Although the use of physiological salt concentrations did not allow a quantitative recovery of injected pro-uPA, both suPAR and pro-uPAsuPAR complexes filtered normally under these conditions (not shown). However, even under these conditions no material eluting prior to the 1:1 complex was ever observed (not shown). The gel filtration experiments thus indicated that either the higher order pro-uPAsuPAR complexes are only marginally larger that the 1:1 complex or not abundant enough for detection using this method.

In any case the gel filtration experiments allowed us to compare the relative abundance of free pro-uPA, suPAR, and pro-uPAsuPAR complexes, with the Vn binding activity. To this end, aliquots of the samples applied to the gel filtration column were diluted and assayed for their Vn binding activity (Fig. 4C). Samples containing excess suPAR (filtrations I and II) displayed approximately twice the Vn binding activity of samples containing equimolar concentrations of the two proteins (filtration III) or an excess of pro-uPA (filtrations IV and V). Indeed, the chromatograms of samples displaying a high Vn binding activity (those in filtrations I and II) were associated with the presence of “free” suPAR (the left “shoulder” of the peak corresponding to the 1:1 complex) supporting the importance of unoccupied suPAR for efficient Vn binding.

**The Kringle Domain of uPA Is Required to Induce uPAR Binding to Vn**—It has been demonstrated (9, 10) that the high affinity interactions of uPA and Vn with uPAR require intact uPAR. We therefore focused our attention on the structural requirements to the ligand. Like uPAR, uPA is a modular protein composed of three domains as follows: a small amino-terminal growth factor-like domain (GFD, uPA amino acids 1–48); a central kringle domain (KD, uPA amino acids 49–135); and a carboxyl-terminal catalytic domain (CD, LMW-uPA). Although the catalytic activity of uPA is confined to the CD, the entire uPAR binding activity has been mapped to the GFD that binds to uPAR with an affinity indistinguishable from that of intact pro-uPA, uPA, or ATF (a proteolytic uPA fragment composed of the GFD and the KD) (23, 26, 27). To investigate the structural basis for uPA promotion of suPAR
oligomerization and Vn binding, we tested the capacity of different uPA derivatives to promote suPAR binding to Vn (Fig. 5A). Whereas ATF promoted suPAR binding to Vn with the same efficiency and concentration dependence as pro-uPA, both GFD and CD failed to do so efficiently. The failure of the GFD to promote suPAR binding to Vn was not caused by a failure to bind suPAR as both the GFD and ATF competed equally well for suPAR binding to pro-uPA (Fig. 5B). The weak but consistently observed stimulatory effect of the CD observed in Vn-binding assays and in pro-uPA competition experiments is likely to be explained by low levels of contaminating uPA and/or ATF in the commercial CD preparation used in this study and was not addressed further.

As the GFD is required and sufficient for high affinity interaction with uPAR, we concluded that receptor binding was not sufficient to promote Vn binding. In particular, the data demonstrate that regions within the KD are also required.

**DISCUSSION**

Based on the data presented in this paper we conclude that pro-uPA controls high affinity suPAR binding to Vn by regulating suPAR oligomerization. Direct evidence for this hypothesis comes from the particular pro-uPA concentration dependence of suPAR binding to Vn and from pro-uPA-dependent suPAR:suPAR co-immunoprecipitation.

Based on these data we propose a simple mechanism for the formation of the oligomeric pro-uPA:suPAR complex (Fig. 6). The first step in this reaction is the formation of the heterodimeric pro-uPA-suPAR complex and the second the association of an unoccupied receptor molecule to form the high affinity Vn-binding ternary pro-uPA:suPAR complex. Based on the pro-uPA dependence of suPAR binding to Vn, we were able to demonstrate that this ternary complex is a stable entity when bound to Vn ($K_d = 0.77 \text{ nM}$, assuming a $K_d = 0.1 \text{ nM}$ for
Ternary complex(es) also form in the absence of Vn, as evidenced by suPAR/suPAR co-immunoprecipitation (Fig. 3). Even though the Vn-binding curves do not allow us to calculate the stability of the ternary complex in the absence of Vn, the binding experiment presented in Fig. 2 strongly suggests that in the absence of Vn these complexes are also stable. However, despite its predicted stability, we have not been able to identify this complex by size exclusion chromatography (Fig. 4). How can this apparent discrepancy be explained? Although it cannot be excluded that the ternary complex has dimensions very close to that of the 1:1 complex, preventing its identification by gel filtration, the most likely explanation is that the complex is much less abundant than the 1:1 complex that forms quantitatively (Fig. 4). In support of this is the fact that even though suPAR interaction with Vn is apparently of considerable affinity (specific binding is detectable down to sub-nanomolar levels), only a small fraction (estimated to be less than 5%, not...
Formation of a high affinity heterotrimeric pro-uPA

In the presence of excess suPAR the presence of pro-uPA will lead to the formation of high affinity Vn-binding complexes between suPAR and pro-uPA is shown. In vitro (squares), ATF (diamonds), GFD (circles), or LMW-uPA (triangles). After washing, bound suPAR was quantified as described above. B, pro-uPA-coated wells were incubated with 10 nM suPAR in combination with increasing concentrations of pro-uPA (squares), ATF (diamonds), GFD (circles), and LMW-uPA (triangles). After washing, the amount of bound suPAR was determined as described above.

The predicted high affinity interaction between a heterodimeric pro-uPA-suPAR complex and an unoccupied suPAR molecule when bound to Vn suggests that the pro-uPA-suPAR₂ complex may be stabilized by its interaction with Vn. In this context the oligomeric state of Vn may be particularly important. Thermodynamically, higher order pro-uPA-suPAR-Vn complexes, for example with a 1:2:2 stoichiometry, are expected to be more stable than a corresponding 1:1:1 complex. In fact, a selective binding of uPA to multimeric Vn would make sense as cells in this way could maintain the capacity to interact with the matrix form of Vn even if surrounded by the high concentrations of native Vn present in the circulation. Future investigations on the role of Vn oligomerization in its interaction with uPAR are highly warranted.

Implied in our model is the existence of homophilic uPAR/uPAR interaction(s) and/or divalent pro-uPA uPAR binding. Although both pro-uPA and suPAR are believed to be monomeric proteins with monovalent interactions with each other, increasing evidence is accumulating that this may not always be true. Although the mammalian cell expressed suPAR utilized in this study behaves as a single defined molecular species when analyzed by gel filtration (Fig. 4), it was recently shown that when suPAR is expressed and purified from insect cell cultures it may exist as dimeric and/or multimeric aggregates (28). The aggregation of suPAR observed by Shliom et al. (28) may represent a natural homophilicity of suPAR, which is augmented by the reduced glycosylation and/or the artificial amino-terminal amino acids present in those recombinant suPAR variants. In support of the existence of direct homophilic uPAR/uPAR interaction(s), it was recently shown that uPAR-derived peptides may interact directly with uPAR and affect its interaction with both uPA and Vn (29).

We have documented that, besides being required, pro-uPA causes a strong and consistent inhibitory effect on suPAR binding to Vn and on suPAR/suPAR co-immunoprecipitation when present in excess over suPAR (Figs. 1 and 3). Although the mechanism of this inhibition is unclear, it evidently occurs during the formation of the oligomeric complex and not after it has formed (Fig. 2B). The most likely mechanism is that excess pro-uPA causes a very rapid saturation of suPAR, preventing the subsequent association between heterodimeric pro-uPA-suPAR complexes and unoccupied suPAR. Interestingly, the fact that once formed, this high affinity Vn-binding active form of suPAR is relatively long-lived, as shown by the effect on uPAR binding and the fact that it can be observed in vivo.
complex is resistant to excess pro-uPA, suggests that it may fail to bind another pro-uPA molecule. Why has ligand-induced uPAR oligomerization escaped discovery until now? The key observation leading us to the discovery of ligand-induced dimerization of suPAR was the peculiar uPA dose dependence of suPAR binding to immobilized Vn (Fig. 1). However, although data on the uPAR/Vn interaction has been presented by several groups, similar binding curves have never been described before. The reason for this apparent discrepancy is likely to be explained by the different experimental approaches used in these studies. Waltz et al. (7) measured binding of radiolabeled Vn to cell-surface uPAR. Wei et al. (30) measured binding of Vn to plastic-adsorbed uPAR. Hayer-Hansen et al. (9) studied the binding of Vn to antibody-immobilized suPAR by surface plasmon resonance. The common denominator of these experimental approaches is that the mobility of uPAR is restricted by being bound to the cell surface, adsorbed to plastic, or immobilized on antibodies. Under these conditions uPAR dimerization may occur but will be less dynamic (bound to the cell surface) or even completely static (after adsorption to plastic or antibodies). In our binding assay uPAR is free to self-associate and to bind uPA and Vn.

The three domains of uPAR (D1, D2, and D3) belong to the Ly-6/uPAR domain family that includes glycosylphosphatidylinositol-anchored single-domain membrane proteins (e.g. CD59, E48, and Ly-6) as well as a large number of secreted single domain α-neurotoxins (31). Although none of the mammalian members of this domain family have been shown to oligomerize, the κ-bungarotoxin subfamily of the α-neurotoxins dimerize in solution through a strong homophilic interdomain interaction (32). It is therefore tempting to speculate that one or more of the uPAR domains may engage in intermolecular homophilic contacts. Indeed, it has been proposed that the binding of uPA to uPAR involves a binding pocket formed by a tight intramolecular contact between domains 1 and 3 of uPAR (33). If true, this may leave domain 2 free to form intermolecular homophilic contacts. This may also suggest why Vn binding requires pro-uPA binding.

uPA is composed of an amino-terminal GFD, a kringle domain (KD), and carboxy-terminal catalytic domain. Whereas the ATF and GFD fragments of uPA have identical affinities for uPAR, only ATF efficiently promotes Vn binding (Fig. 4). This suggest that uPA KD, which is present in ATP but absent in GFD, is required to induce uPAR dimerization. In accordance with this notion it was recently found that both the GFD and the KD of uPA are required for efficient induction of chemotaxis in smooth muscle cells (34). However, the molecule binding the KD still has to be determined. In fact, the exact role of the KD in uPAR dimerization remains to be ascertained.

Several lines of evidence suggest that uPA promotes uPAR binding to Vn by changing the conformation of the receptor without itself directly participating in the interaction with Vn. First, overexpression of uPAR in cells expressing no uPA may result in strong binding/adhesion to Vn (10, 30). Second, Vn binds to immobilized uPAR in the absence of uPA (9, 30). The evident correlation between Vn binding activity and uPAR oligomerization presented in this study strongly suggests that the critical event that determines high affinity uPAR binding to Vn is receptor dimerization and not simply ligand binding or a general conformational change.

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