Soluble Human Urokinase Receptor Is Composed of Two Active Units*

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The mechanism by which single-chain urokinase (scuPA) binds to its receptor (uPAR) is incompletely understood. We report that a fragment comprising the first domain of recombinant soluble uPAR (sDI) as well as a fragment comprising the remaining domains (sDI-DIII) competes with the binding of recombinant full-length soluble uPAR (suPAR) to scuPA with an IC₅₀ = 253 nM and an IC₅₀ = 1569, respectively. sDII-III binds directly to scuPA with K_d = 238 nM. Binding of scuPA to each fragment also induces the expression of plasminogen activator activity. sDI and sDII-DIII (200 nM each) induced activity equal to 66 and 36% of the maximum activity induced by full-length suPAR (5 nM), respectively. Each fragment also stimulates the binding of scuPA to cells lacking endogenous uPAR. Although scuPA binds to sDI and to sDII-DIII through its amino-terminal fragment, the fragments act synergistically to inhibit the binding of suPAR and to stimulate plasminogen activator activity. Furthermore, sDII-DIII retards the velocity and alters the pattern of cleavage of sDI by chymotrypsin. These results suggest that binding of scuPA to more than one epitope in suPAR is required for its optimal activation and association with cell membranes.

Binding of urokinase (uPA)¹ to its receptor may play an important role in inflammation (1, 2) and the development of tumor metastases (3), among other processes. The urokinase receptor (uPAR) is a single-chain glycoprotein that is attached to cell membranes by a glycosylphosphatidylinositol-linked receptor (3, 4). Binding of single chain urokinase (scuPA) to uPAR stimulates its enzymatic activity (5), provides relative protection from plasmin and plasminogen activator type 1 (6), and retards its internalization by the α₂-macroglobulin receptor/lipoprotein-related receptor (8, 10). uPAR is comprised of three domains which share notable sequence similarity (3, 11). The exact mechanism by which scuPA binds to these domains and the mechanism by which scuPA is then activated remains unclear. The growth factor domain of uPA plays an important role in the binding to uPAR (12–14). However, whether the growth factor domain binds to uPAR with the same affinity as uPA remains unsettled (15). It has also been reported that the amino-terminal fragment of scuPA (ATF) can bind to the first domain of uPAR, but with a 1500-fold lower affinity than to the full-length receptor (16). These data were obtained using a preparation of sDI and sDII-DIII that contained sufficient residual suPAR to account for the results leading the investigators to conclude that the capacity of isolated domain 1 to bind to scuPA is negligible (16, 17). This interpretation leaves unresolved the mechanism by which the three domains contribute to the binding energy of scuPA.

The fact that scuPA binds to full-length suPAR with a higher affinity than to sDI can be the result of at least two mechanisms. The second and third domains may stabilize the optimal structure of DI. In other words, DII-DIII may induce conformational changes in DI making it a better ligand for scuPA. An alternative explanation is that DII-DIII participates in the binding of scuPA directly by offering a distinct, low affinity site. Either hypothesis is compatible with the observation that the second and the third domains of uPAR promotes its interaction with scuPA (18).

To study these possibilities in greater detail, we analyzed the binding of scuPA to highly purified fragments of suPAR added alone and together as well as the effect of these fragments on several of its biologic activities. The results indicate that sDII-DIII contributes to the activity of suPAR by providing a second binding site for scuPA.

EXPERIMENTAL PROCEDURES

Materials—scuPA and its amino-terminal fragment (ATF; amino acids 1–135) were prepared and isolated as described (14, 19). scuPA, ATF, and soluble urokinase receptor (see below) were radiolabeled with ¹²⁵I using IODO-BEADS (Pierce Chemical Co.). The LMTK- cell line was obtained from the American Type Tissue Collection (Rockville, MD). The plasmin substrate Spectrazyme PL (H-D-norleucyl-hexa-H-drotyrosyl-lysine-p-nitroanilide diacetate salt) and low molecular weight urokinase were provided by American Diagnostica (Greenwich, CT). EGR-chloromethyl ketone was purchased from Calbiochem (La Jolla, CA) and fish skin gelatin from Sigma.

suPAR Fragments—Recombinant soluble urokinase receptor (suPAR; amino acids 1–281) was expressed and purified as described previously (6). Domain 1 (amino acids 1–87) was separated from a fragment containing domains II and III (amino acids 88–281) by proteolytic digestion and gel filtration. Briefly, suPAR (1 mg/ml) in phos-
phate-buffered saline (PBS) containing 10 mM EDTA was digested with chymotrypsin (5000:1 mol/mol ratio) for 24 h at 4 °C. The reaction was quenched by adding phenylmethylsulfonyl fluoride (final concentration 100 μM). Soluble domain 1 (sDI) was separated from soluble domains II–III (sDII-DIII) by reverse phase high performance liquid chromatography using a Vydac C8 analytical column (Phenomenex, Torrance, CA). Typically, 1.5 ml of the reaction mixture was injected per run and the column was developed using a linear gradient (0–70% acetonitrile containing 0.1% trifluoroacetic acid) over 1 h at a flow rate of 1 ml/min. sDI and sDII-DIII eluted with retention times of approximately 36 and 34 min, respectively. Peaks from repetitive runs were collected, lyophilized, and analyzed by SDS-PAGE. Neither sDI nor sDII-DIII contained full-length suPAR detected by SDS-PAGE. Trace amounts of sDII-DIII were detected in sDI (Fig. 1). sDII-DIII was free of sDI by SDS-PAGE and N-terminal sequence analysis of the peptide showed a single, expected sequence starting at amino acid 88. Laser desorption mass spectral analysis of sDI showed at least 12 peaks ranging from 11,000 to 18,000 mass units. This heterogeneity was completely abolished when the protein was deglycosylated using N-glycanase. Mass spectral analysis of the resultant deglycosylated sDI yielded a single peak of 9759 mass units, consistent with its predicted molecular weight calculated from the primary amino acid sequence (9761 mass units).

Binding of suPAR Fragments to scuPA—Several methods were employed to measure the binding of suPAR and suPAR fragments to uPA. In the first set of experiments, the binding of 125I-suPAR to a stably transfected CHO cell line expressing GPI-anchored scuPA was measured in the presence or absence of increasing concentrations of unlabeled suPAR, sDI or sDII-III, as described (20). In a second set of experiments, binding was measured using a solid phase enzyme-linked immunosorbent assay. Briefly, two-chain urokinase (tcuPA) was catalytically inactivated using Biotin-TEGR-chloromethyl ketone. 96-Well microtiter plates were coated overnight at 4 °C with full-length suPAR (500 ng in 100 μl of PBS). The plates were then washed, unreactive sites were blocked with 3% fish skin gelatin at 37 °C, and the plates were washed again. Various concentrations of competitor (sDI, sDII-DIII, or scuPA) were added in a final volume of 100 μl. Biotin-TEGR-tcuPA (0.5 nm) and avidin-horseradish peroxidase were then added to all wells simultaneously and the incubation was continued for 3 h at room temperature. The plate was washed and the color development at 490 nm was measured using the appropriate substrate.

Measurement of Plasminogen Activation Activity—In one set of experiments, scuPA (5 nm) was added either alone or together with various concentrations of suPAR, sDI, or sDII-DIII to a reaction mixture containing plasminogen (5 nm) and the plasmin chromatographic substrate (900 μM) in PBS at 37 °C, and the light absorbance at 405 nm was measured continuously over time. In another set of experiments, the plasmin-insensitive scuPA variant (scuPA Glu158) (5, 21, 22) was used instead of the wild-type protein.

Effect of sDII-DIII on the Proteolysis of suPAR—The susceptibility of suPAR, sDI, and an unrelated protein (heat shock protein-70) to proteolytic digestion in the presence of sDII-DIII was tested. Each protein was dialyzed against PBS and adjusted to a final concentration of 70 μM. Chymotrypsin (1.500 mol/mol ratio of enzyme:substrate) was added at 22 °C for various periods of time. The samples were diluted 1:1 in SDS sample buffer containing 2% 2-mercaptoethanol and were either flash frozen overnight at −80 °C or processed immediately with identical results. Before analysis, the samples were boiled at 100 °C for 5 min, cooled briefly, and analyzed by SDS-PAGE using either a 10–15% or a 10–27% gradient.

Effect of suPAR Fragments on scuPA Binding to LMTK Cells—Cells were grown to confluency in 48-well Falcon Multiwell tissue culture dishes (Becton Dickinson, Lincoln Park, NJ) at a final density of approximately 5 × 10⁴ cells/well. The cells were prechilled to 4 °C for 30 min and then washed 2 × with prechilled binding buffer. A 150-μl aliquot was taken from a mixture containing either 10 nm 125I-labeled scuPA in PBS containing 1% bovine serum albumin or 10 nm labeled scuPA in the presence of various concentrations of sDI or sDII-DIII or 10 nm suPAR. These aliquots were then added to the cells in the presence or absence of 100-fold molar excess unlabeled low molecular weight uPA. The incubation was continued for 2 h at 4 °C, buffer containing the unbound ligand was removed, the cells were washed four times with binding buffer, 0.1 × NaOH was added for 10 min, and the radioactivity in each well was measured. Nonspecific binding was defined as cell-associated radioactivity not inhibited by excess unlabeled ligand or by low molecular weight urokinase (8). Specific binding was defined as the difference between total and nonspecific binding.

RESULTS

Effect of sDI and sDII-DIII on the Binding of scuPA—Our initial goal was to study the contribution of the first domain of soluble urokinase receptor, sDI, and the other two domains, sDII-DIII, to the binding of scuPA. To do this, we measured the capacity of sDI and sDII-DIII individually to compete with the binding of radiolabeled suPAR to a stably transfected CHO cell line expressing GPI-anchored scuPA. The data in Fig. 2 show that suPAR binds to these cells in a dose-dependent manner by unlabeled suPAR. Half-maximal inhibition occurred at a concentration of unlabeled suPAR of 3.7 nM and binding was totally inhibited at 17 nM suPAR, indicating that all of the binding of labeled suPAR to the cell-bound ligand was specific. sDI inhibited the binding of 125I-suPAR with an IC₅₀ of 5.34 ± 0.19 nM.
The fact that sDII-DIII and sDI contain distinct binding sites for scuPA, with lower avidity than does the full-length receptor. DIII. Thus, sDII-DIII as well as sDI can bind to scuPA, albeit with half-maximal inhibition measured at concentrations of approximately 100 nM for sDI and 1000 nM for sDII-DIII (not shown).

These results indicate that sDII-III as well as sDI can compete with suPAR for binding to scuPA. This inhibition could occur because sDII-DIII binds to scuPA directly, as reported previously for sDII (23), thereby competing with the binding of suPAR, or the fragment could interact with suPAR inhibiting the capacity of the full-length receptor to bind to scuPA. To distinguish between these possibilities, we next examined the capacity of 125I-sDII-DIII to bind directly to the uPA-expressing CHO cells in the presence and absence of unlabeled sDII-DIII, suPAR, or scuPA. sDII-DIII bound specifically to cell-associated scuPA. Half-maximal binding occurred at a ligand concentration of 238 nM. Specific binding of 125I-sDII-DIII was totally inhibited by 100-fold excess soluble scuPA, by full-length suPAR, and by sDI (not shown), confirming that binding of sDII/DIII to the CHO cell line was mediated by cell-associated scuPA. To be certain that the bound radioactivity did not represent contaminating full-length suPAR or sDI, an eluate from these cells was prepared and analyzed using SDS-PAGE (Fig. 3). These results show the labeled protein eluted from CHO-scuPA migrated exclusively with the mobility expected of sDII-DIII. Thus, sDII-DIII as well as sDI can bind to scuPA, albeit with lower avidity than does the full-length receptor.

**sDI and sDII-DIII Interact to Promote the Binding of scuPA**—The fact that sDII-DIII and sDI contain distinct binding sites for scuPA does not establish whether or how these sites function within the intact receptor. Therefore, experiments were performed to determine whether the interaction between the binding epitopes in sDI and sDII-DIII can generate the higher binding affinity of the full-length receptor. To examine this possibility, the capacity of subinhibitory concentrations of sDI and sDII-DIII to compete with the binding of 125I-suPAR was examined. The results shown in Fig. 4 indicate that the simultaneous addition of both fragments exerts a synergistic inhibitory effect on the binding of suPAR.

This result also suggests that sDI interacts with sDII-DIII. This interaction may approximate the two binding epitopes for scuPA, thereby increasing its net affinity for the ligand without either domain undergoing a conformational change. However, it is also possible that sDI undergoes a conformational change in the presence of sDII-III that facilitates its interaction with scuPA or that sDI induces a conformational change in scuPA that facilitates its binding to sDII-DIII.

To test the latter possibility, we examined the effect of sDII-DIII on the susceptibility of sDI to proteolytic cleavage by chymotrypsin. suPAR was incubated with chymotrypsin in the presence and absence of sDII-DIII. As shown in Fig. 5, the addition of sDII-DIII not only inhibited the extent to which sDI was cleaved, but also changed the pattern of its cleavage. Identical results were obtained when the effect of sDII-DIII on chymotrypsin-mediated cleavage of deglycosylated sDI was studied (not shown). These results suggest that the cleavage site in sDI becomes less accessible to the active site of the enzyme in the presence of sDII-DIII, but do not exclude a direct effect of sDII-DIII on chymotrypsin itself.

To explore the mechanism of this effect in more detail, we next asked whether sDII-DIII altered the susceptibility of suPAR to proteolysis in a similar manner. The results shown in Fig. 6 indicate several points. First, sDI is released from suPAR prior to further proteolytic cleavage. Second, the velocity of cleavage of nascent sDI released from suPAR is slower than the cleavage of isolated sDI (compare Figs. 5A and 6A). Third, the pattern of cleavage of sDI newly released from suPAR is similar to that of isolated sDI. Fourth, exogenous sDII-DIII further slowed the velocity and altered the pattern of cleavage of sDI released from suPAR (Figs. 6A and 5A). Finally, sDII-DIII had no effect on the chymotryptic cleavage of an unrelated protein substrate, HSP-70, in the presence and absence of sDII-DIII, studied in parallel (not shown). These latter two results exclude a direct inhibitory effect of sDII-DIII on the enzyme itself. We next examined the last possibility, i.e., that sDI induced a conformational change in scuPA that facilitates the binding of sDII-DIII. To do this, we measured the binding of 125I-sDII-DIII to scuPA in the presence of varying concentrations of sDI. Although sDI at high concentrations inhibited the binding of labeled sDII-DIII to CHO-scuPA, binding of sDII-DIII to scuPA was not augmented at any concentration of sDI tested, including those below which its inhibitory effect was evident (not shown).
shown). This outcome argues against the possibility that sDI induces a conformational change in scuPA that facilitates its binding to sDII-III.

Expression of Plasminogen Activator Activity—We previously reported that suPAR stimulates the plasminogen activator activity of scuPA (5). Therefore, we asked whether the isolated domains of suPAR have a similar effect. As shown in Fig. 7, both sDI and sDII-DIII stimulated the plasminogen activator activity of scuPA. sDI was more potent. At optimal concentrations, sDI and sDII-DIII (200 nM each) stimulated scuPA activity by 66 and 36% of that attained with 5 nM suPAR, respectively. A similar induction of activity was seen using the plasmin-insensitive scuPA variant (scuPA-Glu158). The induction of scuPA activity by sDI and sDII-DIII were both completely abolished by 100-fold excess ATF, suggesting that each fragment exerts its effect through a similar site in scuPA. We then asked whether the two fragments of scuPA when combined would exert a synergistic effect on the activation of scuPA. Plasminogen and scuPA were incubated in the presence of both fragments, each added at substimulatory concentrations. The data shown in Fig. 8 indicate that the combination of both fragments exert a synergistic effect on the plasminogen activator activity of scuPA, an effect similar to their ability to interact and compete with the binding of suPAR shown earlier (Fig. 2).

Binding of scuPA to Cells—We have also previously reported that the suPAR promotes the binding of scuPA to certain cell-associated integrin ligands expressed by LMTK− cells which...
lack uPAR (8). Increased binding occurred as a consequence of induced conformational changes in both the scuPA and uPAR components of the complex. Therefore, we examined the capacity of each fragment of suPAR to induce the binding of scuPA to LMTK<sup>2</sup> cells. The data shown in Fig. 9 indicate that sDI as well as sDII-DIII stimulated the binding of scuPA to these cells. The maximal effects of sDI and sDII-DIII were almost comparable to that of full-length suPAR.

**DISCUSSION**

The results of this study indicate that soluble human urokinase receptor is composed of at least two active units. Both soluble domain I and a fragment containing domains II and III have the capacity to bind scuPA, to stimulate its enzymatic activity, and to promote its association with cell surfaces. These conclusions are based on the capacity of sDI and sDII-DIII to compete with the binding of full-length suPAR to scuPA, the capacity of each fragment to stimulate the plasminogen activator activity of scuPA and to promote the binding of scuPA to LMTK<sup>2</sup> cells which lack endogenous uPAR, and by direct measurement of the binding of each fragment to scuPA. The net affinity of scuPA for full-length receptor can be explained by the presence of two or more lower affinity binding sites in the native receptor.

The observation that sDI and sDII-DIII both bind to scuPA is consistent either with the possibility that each recognizes a different epitope within a single binding site or that each recognizes a distinct binding site. The discrepancy between the affinity with which radiolabeled sDII-DIII binds and activates scuPA and its capacity to compete with full-length suPAR suggests the existence of two binding epitopes in scuPA. One of these sites may be occupied preferentially by DI within the native receptor, the other by DII-DIII, but DII-DIII can occupy both sites when present alone at higher concentrations. If scuPA binds to suPAR through two epitopes, it is expected that each would contribute independently to the final interaction between full-length receptor and scuPA and that the affinity of the final interaction would best be described by the multiple of the individual binding affinities (24). Another plausible explanation is that two sites in suPAR bind to the same epitope in scuPA and cooperate to maintain a high local concentration of ligand. Thus, the results of the present study suggest that the net affinity of scuPA for full-length receptor can be explained by the presence of two or more lower affinity binding sites in the native receptor.

The possibility that scuPA bound to sDII-DIII has been noted by others but was attributed to contaminating full-length suPAR (16). The reagents used in the present study were essentially free of suPAR by several independent criteria. No full-length receptor was evident on gels overloaded with sDI or sDII-DIII nor by amino acid sequencing of the DII-DIII fragment. No suPAR was detected in the <sup>125</sup>I-sDII-DIII eluted from cell-bound scuPA. Furthermore, the fact that neither sDI nor sDII-DIII alone or together activated scuPA to the same extent as suPAR, even when present at saturating concentrations, excludes a role for contaminating full-length receptor as a suitable explanation for the observed results.

The observation that sDI and sDII-DIII both bind to scuPA is consistent either with the possibility that each recognizes a different epitope within a single binding site or that each recognizes a distinct binding site. The discrepancy between the affinity with which radiolabeled sDII-DIII binds and activates scuPA and its capacity to compete with full-length suPAR suggests the existence of two binding epitopes in scuPA. One of these sites may be occupied preferentially by DI within the native receptor, the other by DII-DIII, but DII-DIII can occupy both sites when present alone at higher concentrations. If scuPA binds to suPAR through two epitopes, it is expected that each would contribute independently to the final interaction between full-length receptor and scuPA and that the affinity of the final interaction would best be described by the multiple of the individual binding affinities (24). Another plausible explanation is that two sites in suPAR bind to the same epitope in scuPA and cooperate to maintain a high local concentration of ligand. Thus, the results of the present study suggest that the net affinity of scuPA for full-length receptor can be explained by the presence of two or more lower affinity binding sites in the native receptor.

The results of these experiments do not exclude the possibility that sDII-DIII facilitates the binding of scuPA by inducing a conformational change in sDI or by stabilizing a conformation in which its binding site in sDI is more accessible to the ligand. This notion is consistent with the synergistic effect of sDI and sDII-DIII on suPAR activity, with the synergistic inhibition of suPAR binding to cell-associated scuPA when the two fragments are added together, and with the observation that sDII-DIII modulates the velocity and the pattern of the enzymatic cleavage of sDI and suPAR by chymotrypsin. The observation that sDII-DIII interferes with the cleavage of sDI more completely than it does in the context of full-length suPAR suggests that isolated sDI has a somewhat higher affinity for the re-

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**FIG. 8.** Synergistic effect of sDI and sDII-DIII on the plasminogen activator activity of scuPA. scuPA (5 nM) was incubated with plasminogen (5 nM) and the plasmin chromogenic substrate (900 μM) alone or with sDI (2.5 nM), sDII-DIII (7.5 nM), or with both domains for 40 min and the OD<sub>405 nm</sub> was measured. The results are expressed relative to the activity of scuPA (5 nM) plus suPAR (5 nM) and represent the mean ± S.E. of three experiments, each performed in triplicate.

**FIG. 9.** sDI and sDII-DIII induce the binding of scuPA to cells. LMTK<sup>2</sup> cells were incubated with 10 nM <sup>125</sup>I-scuPA in the absence (gray column) or presence of 10 nM suPAR (■), 50 nM sDI (□), or 50 nM sDII-DIII (●) for 2 h and the cell-associated radioactivity was measured. The results shown are the mean ± S.E. of three experiments, each performed in triplicate.
remaining fragment than does nascent sDI released from suPAR, a difference that is overcome at a somewhat higher concentration of sDI-DIII. However, additional experiments will be required to determine whether such conformational changes actually occur with the first domain of uPAR or whether such changes modulate the binding of scuPA or other proteins that bind to the uPA receptor (7–9, 25).

The finding that sDI stimulates the binding of scuPA to LMTK− cells supports our previous suggestion that the capacity of suPAR to increase the binding of scuPA to cells is due in part to changes in suPAR, specifically within the low molecular weight portion of the molecule (8). This contention is consistent with the observations of others that tcuPA has a biphasic effect on the binding of suPAR, while ATF has only a stimulatory effect and by the observation that the addition of scuPA to suPAR increases the total amount of ligand bound without an increase in its affinity (9). A contribution of the second and third domains of the receptor to the cell binding induced by full-length receptor cannot be excluded by our data (7), since the maximal effect of sDI required 5-fold more protein than did full-length suPAR. Nor do our results exclude the possible contribution of conformational changes in DI induced by scuPA (9). However, the reported ability of a monoclonal anti-uPAR antibody presumed to recognize DI exclusively to block scuPA binding to cells (9) could be due to cross-reactivity with sDII-DIII.2 Nevertheless, the observation that complexes composed of scuPA and sDI, sDII-DIII, or full-length suPAR bind to LMTK− cells to the same extent provides additional support for the involvement of urokinase in this interaction. However, the exact mechanism by which the binding of scuPA to suPAR changes the capacity of the resultant complex to bind to other proteins requires further study.

Note Added in Proof—The presence of multiple binding sites in suPAR has recently been reported (Behrendt, N., Ronne, E., and Dano, K. (1996) J. Biol. Chem. 271, 22888–22894).

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