Regulation of Single Chain Urokinase Binding, Internalization, and Degradation by a Plasminogen Activator Inhibitor 1-Derived Peptide*

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The internalization and degradation of cell-associated urokinase type plasminogen activator (uPA) through the α2-macroglobulin receptor/low density lipoprotein-related receptor (α2MR/LRP) represent important steps in the control of plasmin formation. Complexes between two chain urokinase (tcuPA) and plasminogen activator type 1 are degraded rapidly whereas single chain urokinase (scuPA) is not, suggesting that α2MR/LRP requires specific epitopes in the receptor for effective function. We report an alternative mechanism that may contribute to this process. The binding of scuPA to LM-TK− cells that lack the uPA receptor was stimulated by the hexapeptide EEIIMD, corresponding to amino acids 350–355 of plasminogen activator type 1, which contacts the sequence RRHGGGS, corresponding to amino acids 179–184 in uPA. EEIIMD increased the Bmax of scuPA binding 4-fold with the half-maximal effect achieved at a peptide concentration of 50 μM. Stimulation was dependent on the charge on the COOH-terminal amino acid but not on the NH2 terminus of the peptide. EEIIMD also stimulated the internalization and degradation of scuPA. Both the binding and internalization of scuPA in the presence of EEIIMD were blocked by recombinant, 39-kDa α2MR/LRP-associated protein as well as by an anti-α2MR/LRP antibody. EEIIMD also stimulated the binding of scuPA to purified α2MR/LRP. EEIIMD had no effect on the binding of tcuPA or of complexes between scuPA and its receptor. These results suggest that EEIIMD regulates the binding of scuPA with α2MR/LRP. These findings also suggest a potential mechanism by which scuPA can be cleared which is independent of activation by plasmin or binding to uPA receptor.

Urokinase type plasminogen activator (uPA)1 has been im-

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5 The abbreviations used are: uPA, urokinase type plasminogen activator; scuPA, single chain urokinase type plasminogen activator; tcuPA, two chain urokinase type plasminogen activator; uPAR, urokinase type plasminogen activator receptor; PAI-1, plasminogen activator inhibitor type 1; α2MR/LRP, α2-macroglobulin receptor/low density lipoprotein-related receptor; suPAR, recombinant soluble urokinase type plasminogen activator receptor; RAP, recombinant soluble 39-kDa α2MR/LRP-associated protein; BSA, bovine serum albumin; TBS, Tris-buffered saline.

The enzymatic activity of tcuPA is regulated in plasma primarily by plasminogen activator inhibitor type 1 (PAI-1) (11–13). The contact region between the two molecules includes the sequence RRHGGGS (amino acids 179–184) in uPA and the sequence EEIIMD (amino acids 350–355) in PAI-1 which has been designated as the “docking site” for uPA (14, 15). The postulated electrostatic interactions between the two regions play a crucial role in forming a stable uPA-PAI-1 complex. However, the scuPA-uPAR complex is less susceptible to inhibition by PAI-1 than is tcuPA or receptor-bound tcuPA (16, 17), although the mechanism of this resistance, including alterations in the interaction involving the docking site, has not been established.

In addition to inhibiting urokinase activity, PAI-1 also promotes the internalization and degradation of uPA through the α2-macroglobulin receptor/low density lipoprotein-related receptor (α2MR/LRP) (18). The complex formed between PAI-1 and tcuPA binds to α2MR/LRP with considerably higher affinity than does either component alone. The increased affinity of the complex results from an independent contribution of epitopes present in each ligand (18), but a possible effect of PAI-1 on the conformation of uPA itself has not been excluded (19). In this context it is of interest that binding of scuPA to its receptor inhibits its binding to α2MR/LRP (18, 19). Again, two mechanisms for this reduced affinity have been postulated. It has been proposed that the contact site between the A chain of scuPA and α2MR/LRP is shielded by uPAR (18). An alternative mechanism is that binding of scuPA to uPAR induces a conformational change that both promotes its binding to integrin ligands and leads to a loss of the epitope recognized by α2MR/LRP (19). The latter possibility is consistent with the observation that soluble scuPA has a higher affinity for α2MR/LRP than does tcuPA (18) and with the loss of affinity for α2MR/LRP which occurs when the active site of tcuPA is occupied by diisopropyl fluorophosphate (18).

To examine in greater detail the mechanism by which uroki-

tion with the docking site of PAI-1, as a result of which the molecule undergoes a conformational change that modulates its biologic activity.

EXPERIMENTAL PROCEDURES

Materials—Recombinant scuPA and recombinant soluble urokinase receptor (suPAR) were purified as reported previously (16, 20) and were the generous gifts of Dr. Jack Henkin (Abbott Laboratories; Abbott Park, IL). The peptides EEIIMD, REIIMD, and EEIIMR were synthesized by the Protein Chemistry Laboratory, Washington University, St. Louis. Glu-plasminogen, tcuPA, PAI-1, and the plasmin chromogenic substrate Spectrozyme PL were obtained from American Diagnostica Inc. (Greenwich, CT). Purified α2MR/LRP and recombinant soluble 39-kDa α2MR/LRP-associated protein (rRAP) were prepared as described previously (21). The LM-TK2 cell line was obtained from the American Type Tissue Collection (Rockville, MD). scuPA, suPAR, and tcuPA were radiolabeled with 125I using IODO-BEADS (Pierce Chemical Co.) as described (22).

Preparation of suPAR:scuPA Complexes—suPAR and scuPA were incubated with each other at a molar ratio of 1.25:1 for 1 h at 37 °C at 10 × the desired final concentration in ligand binding buffer (phosphate-buffered saline supplemented with 1.0% bovine serum albumin (BSA)). To form complexes with PAI-1, the inhibitor was added to scuPA, tcuPA, or complexes containing suPAR:scuPA and suPAR:scuPA at a 1:1 molar ratio in binding buffer for 30 min at 37 °C. The complexes were diluted to the desired working concentration immediately before use.

Plasminogen Activator Activity—tcuPA (5 nM) was incubated in the absence or presence of EEIIMD or REIIMD (200 μM) for 30 min. The mixture was then added to a reaction mixture containing 25 nM PAI-1, 50 mM Glu-plasminogen, and 50 μM chromogenic substrate for the indicated times, and the O.D. at 405 nm was measured continuously (16).

Ligand Binding Assays—Binding of radiolabeled ligands to cells was measured as described (19). Briefly, LM-TK2 cells, resuspended in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal calf serum, were plated in 96-well Falcon multiwell tissue culture dishes (Becton Dickinson) and grown to confluence at 37 °C overnight. The cells were prechilled on ice and washed twice with prechilled binding buffer (phosphate-buffered saline and 1.0% BSA). 125I-labeled ligands, with or without 100-fold molar excess unlabeled ligands, were added to the cells in the presence or absence of the aforementioned peptides for 2 h at 4 °C. The cells were washed four times with binding buffer, solubilized in 0.1 N NaOH, and the extract counted for radioactivity. In other experiments, binding of labeled ligands was performed in the absence or presence of 400 nM rRAP in Tris-buffered saline (TBS) containing 4 mM Ca2+ or in the presence of affinity-purified IgG anti-α2MR/LRP antibody (100 μg/ml).

Solid Phase Binding Assay—To measure the binding of labeled ligands to α2MR/LRP, 96-well microtiter (Dynatech Immunol) was incubated with purified receptor or BSA (3 μg/ml) in TBS containing 4 mM Ca2+ overnight at 4 °C. The buffer was removed, and the unreactive sites were blocked with a solution containing TBS, 4 mM Ca2+, 0.05% Tween 20, and 3% BSA for 1 h and then incubated with the same buffer in the presence or absence of rRAP (200 μM) for another h. Binding of 125I-labeled ligands to immobilized α2MR/LRP was determined as described previously (19, 20).

Internalization and Degradation of scuPA—LM-TK2 cells were grown to confluence in 48-well Falcon multiwell tissue culture dishes (Becton Dickinson) overnight at 37 °C. The cells were prechilled on ice for 1 h, washed twice with binding buffer (TBS, 4 mM Ca2+, 1.0% BSA), and incubated with the same buffer or with buffer supplemented with either 400 nM rRAP or 100 μg/ml IgG anti-α2MR/LRP for 1 h at room temperature. The binding buffer was removed. 125I-scuPA was added alone or in the presence of each of the three peptides and in the presence or absence 400 nM rRAP or 100 μg/ml anti-α2MR/LRP for 2 h at 4 °C. Unbound ligands were removed, and cells were washed five times with binding buffer. Dulbecco’s modified Eagle’s medium containing 4 mM Ca2+ in the presence and absence of 400 nM rRAP or 100 μg/ml anti-α2MR/LRP was added for 18 h at 37 °C. The internalization and degradation of scuPA was measured as described previously (24, 25). Briefly, to measure internalization, the cells were washed twice with the binding buffer and 50 mM glycine, 150 mM NaCl, pH 3.0, was added for 15 min at 4 °C to dissociate cell surface-bound ligands. The cells were dissociated by adding 0.1 N NaOH for 10 min, and the extract was counted. To measure degradation, the media were removed after an 18-h incubation with radiolabeled ligands at 37 °C, trichloroacetic acid was added to a final concentration of 10%, the precipitated protein was separated by centrifugation, and the radioactivity in the supernatant was measured.

RESULTS

The purpose of this study was to determine whether a small peptide, EEIIMD (amino acids 350–355), derived from the putative docking site of PAI-1 (14), would alter the cellular binding and clearance of uPA mediated by α2MR/LRP. To address these questions, we studied the effect of this peptide on the binding, internalization, and degradation of scuPA, scuPAsuPAR complex, and tcuPA by LM-TK2 cells that express α2MR/LRP but lack endogenous uPAR (19).

Effect of EEIIMD on the Cellular Binding of scuPA, the scuPAsuPAR Complex, and tcuPA—The data in Fig. 1A indicate that minimal amounts of scuPA bind to LM-TK2 cells, consistent with previous results (19). However, binding of scuPA to these cells was stimulated by the peptide EEIIMD in a dose-dependent and saturable manner both with respect to scuPA concentration (Fig. 1A) as well as peptide concentration (Fig. 1B). Stimulation was evident at all concentrations of scuPA tested, and the increase in scuPA binding in the presence of EEIIMD occurred in part as a result of a 4-fold increase...
the binding of tcuPA, tcuPA-suPAR, or scuPA-suPAR complexes to the LM-TK− cells could occur as a result of the inability of the peptide to bind to the docking site, or the effect of EEIIMD may be highly dependent on the conformational state of uPA. To address the question of whether EEIIMD can bind to the docking site, we measured the capacity of the peptide to compete with the plasminogen activator inhibitor activity of PAI-1 using tcuPA. The data shown in Fig. 4 indicate that EEIIMD inhibits the activity of PAI-1, whereas the control peptide EEIIMR did not (not shown).

Role of αMR/LRP—It is well described that complexes between urokinase and PAI-1 are internalized and degraded by binding to αMR/LRP (25, 29). Therefore, we asked whether EEIIMD increased the binding, internalization, and degradation of scuPA through this pathway. In support of this hypothesis, EEIIMD stimulated the binding of scuPA to purified αMR/LRP and binding, to the purified protein was inhibited by rRAP (Fig. 5A). In addition, the binding of scuPA to LM-TK− cells in the presence of EEIIMD was inhibited by 400 nM rRAP and by affinity-purified anti-αMR/LRP IgG by approximately 70 and 85%, respectively (Fig. 5B). Furthermore, EEIIMD stimulated the internalization and degradation of scuPA by

Expression of scuPA and its receptor suPAR (scuPA-suPAR) as well as tcuPA are both active enzymes (10) but differ in their susceptibility to PAI-1 (16). This led us to question whether the docking site in the scuPA-suPAR complex is available to PAI-1 as has been reported for tcuPA. To address this question, we compared the effect of PAI-1 and EEIIMD on the binding of scuPA-suPAR and tcuPA to LM-TK− cells. PAI-1 and EEIIMD each caused a minimal increase in the binding of scuPA-suPAR to LM-TK− cells (Fig. 3). On the other hand, PAI-1 clearly stimulated the binding of tcuPA-suPAR as reported by others (18, 29), whereas EEIIMD had no effect (Fig. 3). EEIIMD also failed to stimulate the binding of tcuPA to LM-TK− cells (not shown). The failure of EEIIMD to stimulate

FIG. 3. Effect of PAI-1 on the binding of scuPA-suPAR and tcuPA-suPAR complexes to LM-TK− cells. LM-TK− cells were incubated with suPAR−125I-scuPA (scuPA−125I, 20 nM each) and either PAI-1 (20 nM) or EEIIMD (300 μM) for 2 h at 4 °C. Specific binding was calculated as above. The mean and ± S.D. of three such experiments are shown.
mean degradation and that these consequences are prevented by binding of scuPA is accompanied by its internalization and derived peptide EEIIMD. The observations that the increase in these results suggest a potential mechanism by which scuPA that this augmentation is also inhibited by rRAP. Therefore, This contention is supported further by the finding that EEIIMD and rRAP; anti-2MR/LRP on the binding of EEIIMD and scuPA to 2MR/LRP in this process.

DISCUSSION

Binding of scuPA to its receptor promotes its catalytic activity (10), dampens the inhibitory capacity of PAI-1 compared with tcuPA (16), alters its regulation by peptide substrates of plasmin (30), and promotes its binding to vitronectin (19, 31–35). The results of the current study identify another mechanism by which the function of scuPA can be altered. The binding of scuPA to LM-TK- cells is stimulated by the PAI-1-derived peptide EEIIMD. The observations that the increase in binding of scuPA is accompanied by its internalization and degradation and that these consequences are prevented by rRAP as well as by a specific anti-α2MR/LRP antibody are consistent with the involvement of α2MR/LRP in this process. This contention is supported further by the finding that EEIIMD increases the binding of scuPA to purified α2MR/LRP and that this augmentation is also inhibited by rRAP. Therefore, these results suggest a potential mechanism by which scuPA can be cleared which is independent of receptor binding or proteolytic activation (10).

Although it has been postulated that binding of tcuPA-PAI-1 to α2MR/LRP is mediated by independent epitopes in each molecule (18), such a mechanism is unlikely to explain the stimulatory effect of EEIIMD on scuPA binding in that it is unlikely that the peptide is large enough to accommodate both proteins simultaneously. Further, EEIIMD bound to the docking site in tcuPA, as shown by inhibition of PAI-1 activity, but did not increase its binding to α2MR/LRP. Therefore, the observation that EEIIMD increased the $B_{\text{max}}$ for scuPA suggests that the peptide induces previously unrecognized cellular binding sites that are not available in the native molecule which are recognized by α2MR/LRP, i.e. that the docking sequence to which EEIIMD binds functions as an allosteric site. Consistent with this notion, whereas EEIIMD stimulated the binding of scuPA, the peptide had no effect on the binding of either the scuPA-suPAR complex or tcuPA. These results are consistent with the reported differences in the ability of scuPA, the scuPA-suPAR complex, and tcuPA to interact with α2MR/LRP (18).

The observation that EEIIMD regulates the affinity with which scuPA binds to α2MR/LRP indicates that the docking site for PAI-1 is accessible to the peptide. This finding suggests that the resistance of scuPA to PAI-1 may occur because docking of the serpin does not facilitate its binding to the catalytic site as occurs in tcuPA (14) because of an inappropriate distance between the antigen that is not PAI-1. Differences in the orientation of the docking and catalytic sites in the two molecules, or a failure of the serpin to interact with the catalytic site.

It is likely that EEIIMD fails to stimulate the cellular binding of tcuPA, although it binds to the docking site, because the peptide is unable to induce the site recognized by α2MR/LRP. A similar mechanism may also account for the failure of EEIIMD to induce the binding of the other active form of uPA, scuPA-suPAR. Alternatively, the binding site for α2MR/LRP on scuPA may be induced and shielded by its receptor, consistent with the capacity of uPAR to block the interaction of tcuPA-PAI-1 and scuPA with α2MR/LRP (18, 19). Thus, conversion of scuPA to tcuPA is associated with the loss of the ability
of EEIMD to induce the structure recognized by α₂MR/LRP, presumably as a result of the loss of coordinate interaction between different portions of the molecule. Support for this interpretation comes from the observations that uPAR has little or no effect on the enzymatic activity of tcuPA (10) or its susceptibility to inactivation by PAI-1 (10, 17). These results provide additional support for the concept that the conversion of scuPA to tcuPA is the first step in its inactivation and degradation.

Irrespective of the sequence of events, the capacity of EEIMD to stimulate the binding of scuPA provides additional support for the notion that the biologic activity of scuPA can be regulated through its docking site, as a result of which the internalization and degradation of the protein are accelerated. Thus, it is not necessary that scuPA be converted to tcuPA for the protein to be recognized by α₂MR/LRP (18). It remains to be established whether there exists a physiologic analog for the activity of EEIMD described in this study.

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