Assembly of Urokinase Receptor-Mediated Plasminogen Activation Complexes Involves Direct, Non-Active-Site Interactions between Urokinase and Plasminogen†

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ABSTRACT: The binding of the zymogenic form of urokinase-type plasminogen activator (pro-uPA) to its specific cellular receptor, uPAR, leads to a large potentiation of plasmin generation. This is dependent on the concurrent cellular binding of plasminogen, and is completely abrogated by the plasminogen lysine-binding site ligand, 6-aminohexanoic acid. Previous data have provided circumstantial evidence for the formation of specific complexes to mediate the kinetically favorable reciprocal interactions between the protease and zymogen components [Ellis, V., and Danø, K. (1993) *J. Biol. Chem. 268*, 4806−4813]. To further investigate the formation of these putative complexes, we have studied the effect of various lysine-binding site ligands on the binding and activation of plasminogen on U937 cells. Lysine-binding site ligands resembling internal lysine residues, such as N^{α}-acetyl-L-lysine methyl ester, were found to specifically inhibit uPAR-mediated cell-surface plasminogen activation at concentrations up to 40-fold lower than those inhibiting the cellular binding of ^{125}I-labeled plasminogen (IC_{50}s 300 μM vs 8.5 mM). By contrast, 6-aminohexanoic acid, resembling a C-terminal lysine residue, did not display this disparity (IC_{50}s 25 vs 30 μM). These lysine analogues were also found to compete a non-active-site interaction between uPA and plasminogen, detected by surface plasmon resonance (K_d 50 nM), at concentrations correlating with their effect on cell-surface plasminogen activation, suggesting that this interaction is part of the kinetic mechanism. Consistent with this, synthetic peptides corresponding to the sequence uPA$^{149-158}$ (GQKTLRPRFK) and uPA$^{149-157}$ (GQKTLRPRF) specifically abolished the amplification of cell-surface plasminogen activation. These data demonstrate that a novel non-active-site interaction between uPA and plasminogen is necessary for the assembly and efficiency of cell-surface plasminogen activation complexes.

Urokinase-type plasminogen activator (uPA)[1] is one of two specific serine proteases responsible for the conversion of the abundant extracellular serine protease zymogen to the broad-specificity protease plasmin. uPA is expressed in a variety of cell types from which it is secreted as pro-uPA, a single-chain proenzyme form, which can be converted to the fully active two-chain protease by limited proteolysis catalyzed by a number of proteases, including plasmin (*1*, *2*). The action of plasmin leads to the formation of a reciprocal zymogen activation system and a consequent amplification of plasmin generation (*3*).

Both pro-uPA and uPA bind to a specific cellular receptor uPAR, present on the surface of many cell types (*4*). uPAR is a multidomain, glycolipid-anchored protein, and binds these ligands with high affinity (K_d 0.5 nM) via the epidermal growth factor-like module of uPA (*5*). Binding of pro-uPA to uPAR leads to a large increase in the generation of plasmin, due to increased efficiencies of the reciprocal activation of both zymogens (*6*, *7*). A soluble recombinant form of uPAR has no potentiating effect on plasmin generation (*8*) as the effects with cell-associated uPAR are absolutely dependent on the concurrent cellular binding of plasminogen (*6*, *7*). The lack of a direct effect of uPAR on the activity of the system is consistent with the high degree of dynamic independence displayed by the individual domains of uPA (*9*, *10*), suggesting that the serine protease domain would be unaffected by the interaction of the N-terminal epidermal growth factor-like module with uPAR.

The necessity for the cellular binding of plasminogen has been demonstrated using the aminocarboxylic acid analogues of lysine, 4-(aminomethyl)cyclohexanecarboxylic acid and 6-aminohexanoic acid, that act as inhibitors of plasminogen binding (*6*, *7*). These, and other, lysine analogues act by occupying the lysine-binding sites present in the various kringle modules of plasminogen, interfering with the binding of plasminogen to a wide variety of molecules, including its heterogeneous cellular binding sites (*11*−*13*). The lysine-binding sites contain both anionic and cationic centers that bind the ϵ-amino and carboxyl functions of lysine, and an intervening region that interacts with the aliphatic chain (*14*, *15*). However, the structures of the lysine-binding sites of the individual kringles are sufficiently different that they vary in both affinity and selectivity for lysine, other aminocarboxylic acids, and their derivatives (*16*).

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[1] Abbreviations: uPA, urokinase-type plasminogen activator, both the activated two-chain protease and as a generic term; pro-uPA, single-chain form of uPA; uPAR, uPA receptor; AMC, 7-amido-4-methylcoumarin; *p*NA, *p*-nitroaniline; GPI, glycosylphosphatidylinositol; BIA, real time biomolecular interaction analysis; SPR, surface plasmon resonance.

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Although many cells have a very high capacity for plasminogen binding (*11*) and this will lead to a high surface density of plasminogen, indirect evidence suggests that this effect is not the principal mechanism responsible for the efficiency of uPAR-mediated plasminogen activation and its dependence on plasminogen binding. The coincident binding of pro-uPA and plasminogen to an anti-uPA monoclonal antibody, forming a ternary complex, has been shown to both qualitatively and quantitatively mimic the enzyme kinetic effects that occur on the cell surface (*17*). Thus, a simple stoichiometric relationship is sufficient in this situation, in which the antibody acts as a specific template for the catalytically favorable approximation of the protease/zymogen components. The virtually identical kinetic effects observed in both situations strongly imply the involvement of similar mechanisms, and that uPAR-mediated plasminogen activation requires the assembly of specific complexes to enable the interaction of the cell-associated reactants. The mechanism by which the approximation of the reactants on the cell surface may occur is not known, although it appears that uPAR itself does not act as a template for complex formation. This is demonstrated by the observations that a recombinant chimeric mutant of pro-uPA directly anchored to the cell surface via a glycolipid moiety generates plasmin with the same characteristics as the uPAR system (*18*), and that soluble forms of uPAR do not enhance plasmin generation (*8*). Therefore, uPAR does not appear to be directly involved in the interactions leading to complex formation, other than to localize uPA to the cell surface.

In the present study, we have obtained further evidence for the formation of specific activation complexes, and show that their assembly is dependent on a non-active-site interaction between plasminogen and uPA. This interaction can be competed by various lysine analogues and peptides corresponding to $uPA^{149-158}$, implicating the involvement of the kringle-modules of plasminogen and the region N-terminal of the activation cleavage site of uPA in the formation of these functionally relevant complexes.

EXPERIMENTAL PROCEDURES

Proteins and Reagents. Human uPA, pro-uPA, Glu_1-plasminogen, plasmin, and the anti-uPA monoclonal antibody clone 1 were all as described previously (*7, 17*). Soluble, truncated uPAR (residues 1−277) was purified by immunoaffinity chromatography of the conditioned media from transfected Chinese hamster ovary cells as described (*19*), and was a gift from Dr. Michael Ploug (Finsen Laboratory, Copenhagen, Denmark). The plasmin- and uPA-specific fluorogenic substrates H-D-Val-Leu-Lys-AMC and Z-Gly-Gly-Arg-AMC were from Bachem AG, Bubendorf, Switzerland, and the plasmin-specific chromogenic substrate H-D-Val-Leu-Lys-pNA (S-2251) was from Chromogenix AB, Mölndal, Sweden. Carboxypeptidase B from porcine pancreas, carboxypeptidase P (sequencing grade) from *Penicillium janthinellum*, and endoproteinase Glu-C from *Staphylococcus aureus* V8 were obtained from Boehringer Mannheim (Mannheim, Germany). Synthetic peptides were prepared in-house using Fmoc chemistry. Plasminogen was labeled with $Na^{125}I$ as described previously (*7*). The pro-uPA activation site mutant K158E was a gift from Dr. Roger Lijnen (Center for Molecular and Vascular Biology, Leuven, Belgium), and was activated using endoproteinase Glu-C at an enzyme:substrate ratio of 1:100 for 4 h at 37 °C. The various lysine analogues and all other chemicals were of the highest grade commercially available.

Measurements of Cell-Associated Plasminogen Activation. Plasmin generation by uPA or pro-uPA bound to cellular uPAR was determined essentially as described previously (*6, 7*). Briefly, U937 cells (*20*) were washed 3 times in RPMI 1640 buffered in 25 mM HEPES, pH 7.4, and incubated for 3 min with 0.05 M glycine hydrochloride, pH 3.0, 0.1 M NaCl to dissociate endogenously bound uPA, followed by neutralization and further washing. The cells were resuspended at a density of 1×10^7/mL and incubated with uPA or pro-uPA (2 nM) for 20 min at 37 °C, followed by three further washes. In some experiments, a recombinant form of uPA directly anchored to the plasma membrane by a GPI moiety, previously characterized after expression in bovine aortic endothelial cells (*18*), was used after expression by retroviral transduction in U937 cells (Ellis, Lee, and Dichek, unpublished). Cells were incubated (37 °C) at a final density of 1×10^6/mL in 0.05 M Tris-HCl, pH 7.4, 0.1 M NaCl, 0.01% Tween 80 in the presence of 0.2 mM H-D-Val-Leu-Lys-AMC and plasminogen at a fixed concentration of 50 nM or, in the experiments in which kinetic constants were determined, with varying concentrations up to 5 μM. Plasmin generation was determined by recording released AMC fluorescence intensity at 60 s intervals, using a Perkin-Elmer LS-5B spectrofluorimeter at excitation and emission wavelengths of 370 and 470 nm, respectively, and 5 nm slit widths. In some experiments 6-aminohexanoic acid or its derivatives (here collectively termed lysine analogues) were included in the incubation buffer at varying concentrations, as ligands for the lysine-binding sites of plasminogen.

In addition to acting as ligands for the lysine-binding sites of plasminogen, at high concentrations these lysine analogues also behave as competitive inhibitors of the catalytic activity of both plasmin and uPA. The IC_{50} values for the inhibition of plasmin activity, at the concentration of plasmin substrate used above, ranged from approximately 1.5 mM for the various methyl esters to >100 mM for the carboxylic acids. The plasmin concentrations shown in the various figures have been corrected for this inhibitory effect by reference to plasmin activity calibrations for the relevant lysine analogue. The inhibitory effect on uPA activity was corrected for in the calculations of plasminogen activation kinetic constants, which are described below. The use of this approach was validated in control experiments in which the effect of the lysine analogues on solution-phase plasminogen activation by uPA and pro-uPA was determined.

Determination of Inhibition Constants. The effect of uPA-derived synthetic peptides on receptor-mediated plasminogen activation was determined by their inclusion at varying concentrations up to 1 mM together with varying concentrations of plasminogen (0.175−2.62 μM), with experimental conditions otherwise as described above. Although these peptides inhibited plasmin generation, the mechanism of their action did not involve direct inhibition of the active site of uPA. However, the data could be treated in the same manner as direct enzyme inhibition data in order to obtain inhibition constants for the effect of the peptides on plasminogen activation. For this purpose, the data were plotted according to Dixon (*21*). As with the methyl esters, these peptides are substrates for plasmin and act as competitive inhibitors of

plasmin activity with IC_{50}s in the range of 2−5 mM. Therefore, these data were corrected for this effect as described above.

Measurement of Solution-Phase Plasminogen Activation. The solution-phase activation of plasminogen was determined by incubating pro-uPA or uPA (0.5 nM) with plasminogen (85 nM) in 0.05 M Tris-HCl, pH 7.4, 0.1 M NaCl, 0.01% Tween 80, 0.2 mM H-D-Val-Leu-Lys-pNA at 37 °C. Substrate hydrolysis was followed at 405 nm in a Molecular Devices Thermomax plate reader at 60 s intervals. An excess of soluble uPAR (5 nM) was included in some experiments to determine the specificity of the observed effects for cellular uPAR. In some experiments, the anti-uPA monoclonal antibody (clone 1) was included in the incubation at a concentration of 1.5 μg/mL as a specific stimulator of plasminogen activation (*17*).

Kinetic Analysis of Plasmin Generation Curves. In experiments where pro-uPA was the activator initially present, plasmin generation and pro-uPA activation kinetics were calculated using a previously described model (*8*, *17*). In the absence of lysine analogues, the final steady-state rates of plasmin generation were fitted according to the previously reported kinetic constants; the effect of the lysine analogues on the plasmin generation rate was then calculated as a change in the k_{cat}/K_m value. These values were corrected for the inhibitory effect of the lysine analogues on uPA catalytic activity (calculated K_is ranging from 1 mM for N^{α}-acetyl-L-lysine methyl ester and arginine methyl ester to 75 mM for 6-aminohexanoic acid), using the relationship:

$$v = v_{exp}\frac{1 + (K_m/s)(1 + i/K_i)}{1 + K_m/s}$$

in which K_m is the Michaelis constant for plasminogen activation and K_i is the competitive inhibition constant for the lysine analogue, s and i are the concentrations of plasminogen and lysine analogue, respectively, and v_{exp} and v are the experimentally determined and corrected rates of plasmin generation, respectively. The effect of the lysine analogues on the kinetics of plasmin-catalyzed pro-uPA activation was subsequently determined by obtaining the best fit of the associated k_{cat}/K_m values to the experimental plasmin generation curves.

^{125}I-Labeled Plasminogen Binding. The binding of ^{125}I-labeled plasminogen to U937 cells was performed essentially as described previously (*22*). Briefly, cells were prepared as above, and resuspended at a final density of 1×10^7/mL in 0.05 M Tris-HCl, pH 7.4, 0.1 M NaCl, 0.1% bovine serum albumin, containing 10 mg/mL aprotinin. The cells were then incubated for 2 h at 4 °C with ^{125}I-labeled plasminogen, 0.1 mBq/mL, approximately equivalent to 0.6 nM, and various concentrations of 6-aminohexanoic acid or other lysine analogues in the same buffer. One hundred microliter aliquots of cell suspension were then layered onto 200 μL of oil mixture (85:15 v/v of dimethyldiphenyl-polysiloxane and d = 0.88 g/mL mineral oil) in polypropylene microcentrifuge tubes, prior to centrifugation at 14000*g* for 3 min, amputation of the tube tips, and γ-counting.

Carboxypeptidase Treatment of uPA. uPA obtained by plasmin activation of pro-uPA, to ensure an intact A-chain C-terminus (*23*), was subjected to C-terminal digestion with either CpB or CpP. CpB was treated with phenylmethylsulfonyl fluoride prior to use and digestion performed as previously described (*23*). uPA (1 mg/mL) was digested with CpP at an enzyme:substrate ratio of 1:100 in 50 mM sodium citrate, pH 4.5, for up to 24 h at 25 °C. Although at this pH uPA catalytic activity is minimal, parallel incubations in the absence of CpP were made to control for possible autolytic cleavage of uPA. These preparations were assayed for both catalytic and receptor binding activity (*8*).

Real-Time Biomolecular Interaction Analysis (BIA). uPA was inactivated with 4-amidinophenylmethylsulfonyl fluoride as previously described (*17*), and coupled at a concentration of 20 μg/mL to a CM5 sensor chip in a BIACore 2000 instrument (Pharmacia Biosensor AB, Uppsala, Sweden) using a two-stage reaction with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and *N*-hydroxysuccinimide, as recommended by the manufacturers. Plasminogen-binding experiments were performed in 10 mM HEPES, pH 7.2, 0.15 M NaCl, 3.4 mM EDTA, 0.005% surfactant P-20 at 20 °C and a flow rate of 10 μL/min. Controls for the contribution of the bulk refractive index to the surface plasmon resonance signal were performed in a parallel noncoupled flow cell and subtracted from all binding sensorgrams. After each cycle, the sensor chip was regenerated by injection of 0.1 M acetic acid, 0.5 M NaCl, 0.05% surfactant P-20. Plasminogen binding kinetics were performed at varying concentrations of plasminogen (0.5−500 nM). Dissociation rate constants (k_d) were calculated from the slope of plots of ln (R_0/R_t) versus t, where R_0 and R_t are the surface plasmon resonance signals measured at the start of the dissociation phase and at varying times t thereafter. Association rate constants (k_a) were calculated from plots of ln (dR/dt) versus t, which has a slope of $-(k_a[plg] + k_d)$. These calculations were made using the manufacturer's BIAevaluation (version 2.1) software.

The effect of various lysine analogues on plasminogen binding was determined by their inclusion at varying concentrations in the buffer during the association phase at a fixed concentration of plasminogen (50 or 500 nM). As the lysine analogues would be expected to reduce the effective concentration of free ligand during the association phase, their competitive effect was calculated from the maximum SPR signal observed at the end of the association phase when binding is approaching equilibrium.

RESULTS

Inhibition of Cell-Surface Plasminogen Activation by Lysine Analogues. We have previously demonstrated that the kinetically favored substrate for uPAR-bound uPA is cell-associated, rather than solution-phase, plasminogen (*7*). Lysine and its aminocarboxylic acid homologues such as 6-aminohexanoic acid have been demonstrated to inhibit the activity of this uPAR-mediated plasminogen activation system (*6*, *7*) due to competition of the cellular binding of plasminogen (*6*, *11*). This is illustrated in Figure 1A where plasmin generation on U937 cells, initiated by uPAR-bound pro-uPA, is progressively reduced at increasing concentrations of 6-aminohexanoic acid. Figure 1B shows a comparison between this effect on plasmin generation and the effect of 6-aminohexanoic acid on the binding of ^{125}I-labeled plasminogen to the cells. As would be predicted from the model previously proposed (*7*), these competition curves

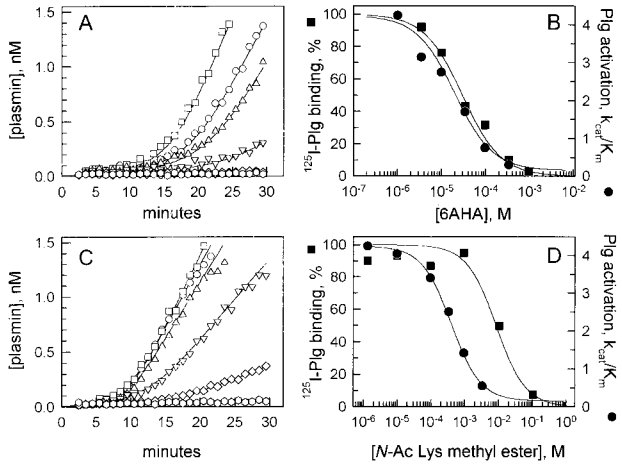

FIGURE 1: Effect of lysine analogues on uPAR-mediated activation and cellular binding of plasminogen. Pro-uPA was pre-bound to U937 cells prior to the addition of plasminogen (50 nM) and plasmin generation determined by hydrolysis of H-D-Val-Leu-Lys-AMC. Plasmin generation is shown for experiments performed in the presence of varying concentrations of either 6-aminohexanoic acid (panel A: 0, 10, 30, 100, 1000 μM) or N^{α}-acetyl-L-lysine methyl ester (panel C: 0, 10, 100, 300, 1000, 3000 μM). The lines shown are the best fit of the experimental data to a model describing this reciprocal zymogen activation system (*17*). In panels B and D, competition curves for the effect of these lysine analogues on the kinetic constants for plasminogen activation (derived from data such as those shown in panels A and C and expressed as k_{cat}/K_m) (●) are compared to their effect on the cellular binding of ^{125}I-labeled plasminogen (■). Similar competition curves were obtained when uPA rather than pro-uPA was bound to the cells (data not shown). The data shown in panels A and C are corrected for the inhibitory effect of 6-aminohexanoic acid and N^{α}-acetyl-L-lysine methyl ester on plasmin catalytic activity. The data in panels B and D have been further corrected for the inhibitory effect on uPA catalytic activity.

coincide, with IC_{50}s of 25 and 30 μM, respectively. However, when derivatives of lysine with blocked α-amino or carboxyl groups were used, discrepancies were found between the concentrations needed to inhibit cell-surface plasminogen activation and those needed to inhibit plasminogen binding.

Figure 1C shows that N^{α}-acetyl-L-lysine methyl ester inhibits plasmin generation in a manner comparable to that of 6AHA, with an IC_{50} of 300 μM. Figure 1D shows that N^{α}-acetyl-L-lysine methyl ester also competes for the cellular binding of ^{125}I-labeled plasminogen, but that this effect has an IC_{50} of 8.5 mM. Kinetic analysis of the plasmin generation data shows that the inhibitory effect is due to an effect on plasminogen activation, with no detectable difference in the rate of pro-uPA activation. A similar IC_{50} was also observed using uPA rather than pro-uPA (data not shown). Therefore, N^{α}-acetyl-L-lysine methyl ester appears to specifically interfere with the activation of cell-surface-associated plasminogen, i.e., inhibit cell-surface plasminogen activation at concentrations which do not affect either the cellular binding of plasminogen or the activation of solution-phase plasminogen (data not shown). Similar effects were observed with N^{α}-acetyl-L-lysine, L-lysine methyl ester, and the equivalent arginine derivatives, as shown in Table 1.

These data suggest that N^{α}-acetyl-L-lysine methyl ester and the other ligands for the kringle lysine-binding sites of plasminogen are interfering with interactions not only between plasminogen and its cellular binding sites but also between plasminogen and another component(s) of the system. Our previous observation that cell-surface plasminogen activation can be mediated in a uPAR-independent manner by a recombinant directly GPI-anchored uPA (*18*) implies that uPAR itself is not involved in an interaction with plasminogen. Consistent with this, the effect of the various lysine analogues on GPI-anchored uPA paralleled their effect on uPAR-bound uPA (data not shown). Therefore, we speculated that uPA may be the additional component interacting with plasminogen through a lysine binding site dependent mechanism.

Table 1: Inhibitory Effects of Lysine Analogues on uPAR-Mediated Plasminogen Activation and Plasminogen Binding on U937 Cells[a]

	Pln generation	^{125}I-labeled plg binding	binding/activation ratio
6-aminohexanoic acid	25 μM	30 μM	1
N^{α}-acetyl-L-lysine	50 μM	150 μM	3
lysine methyl ester	450 μM	3.5 mM	8
N^{α}-acetyl-L-lysine methyl ester	300 μM	8.5 mM	28
N^{α}-acetyl-L-arginine	>1 mM	4.5 mM	1
arginine methyl ester	100 μM	4.0 mM	40

[a] IC_{50} values for the inhibition of plasmin generation initiated by uPAR-bound pro-uPA and ^{125}I-labeled plasminogen binding on U937 cells are shown. These data are taken from the experiments shown in Figure 1 and other similar experiments. The relationship between the two IC_{50} values is also shown expressed as a ratio.

Binding of Plasminogen to uPA Determined by BIA. The potential binding of plasminogen to uPA was investigated using surface plasmon resonance technology. Active-site-blocked uPA was immobilized using amine coupling chemistry to a CM5 sensor chip in a BIACore 2000 instrument. The chip was probed with a dilution series of plasminogen, and, as can be seen in Figure 2A, the SPR signal detected binding at all concentrations. Analysis of the association and dissociation rate constants ($k_a = 3.2 \times 10^4$ M^{-1} s^{-1}, $k_d = 1.6 \times 10^{-3}$ s^{-1}) revealed a dissociation constant of 50 nM for the interaction, in agreement with earlier observations (*24*). To determine whether plasminogen could also interact with uPAR-bound uPA, the sensor chip was pre-injected with soluble recombinant uPAR, uPA−uPAR complex formation was assessed by the SPR signal, and the chip was further probed with plasminogen. The interaction of plasminogen with uPA was found to be unaffected (data not shown), indicating that plasminogen can participate in a ternary complex with uPA and uPAR.

The interaction of plasminogen with uPA was found to be lysine binding site dependent as, at a fixed concentration of plasminogen, inclusion of increasing concentrations of 6AHA in the analysis buffer completely abolished the increase in SPR signal (Figure 2B). Plotting maximum SPR signal against 6AHA concentration revealed an IC_{50} of 30 μM as shown in Figure 2C. This figure also shows competition of the plasminogen−uPA interaction by N^{α}-acetyl-L-lysine methyl ester, with an IC_{50} of 120 μM. Comparison of these data with those in Table 1 shows that these IC_{50}s for the competition of the direct uPA−plasminogen interaction correlate with those for the inhibition of cell-surface plasminogen activation, and that in the case of N^{α}-acetyl-L-lysine methyl ester this is approximately 70-fold lower than the concentration required to compete the cellular binding of plasminogen.

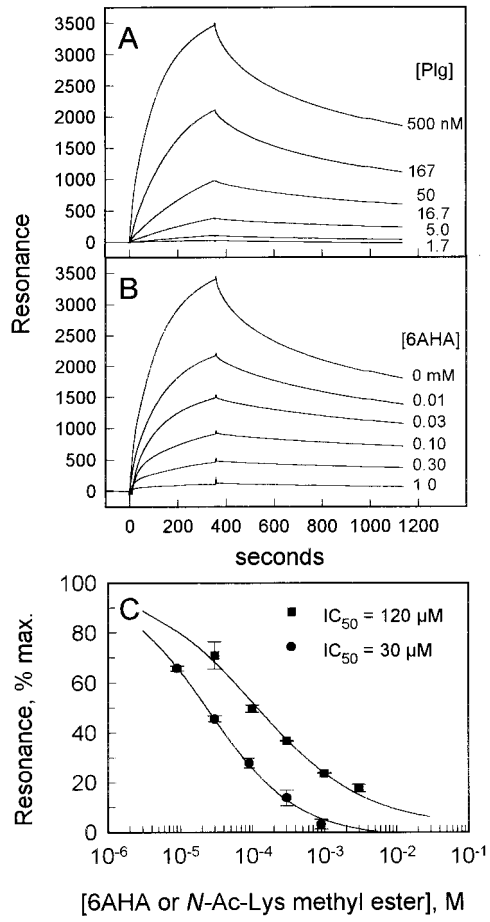

FIGURE 2: Demonstration of an interaction between plasminogen and uPA by real-time biomolecular interaction analysis. uPA inactivated with 4-amidinophenylmethylsulfonyl fluoride was coupled to a CM5 sensor chip in a BIACore instrument. This was then probed with a dilution series of plasminogen as shown in panel A. Analysis of these sensorgrams revealed dissociation and association rate constants of 1.6 ($\pm$0.4) $\times$ 10^{-3} s^{-1} and 3.2 ($\pm$0.6) $\times$ 10^4 M^{-1} s^{-1}, respectively, giving a dissociation constant of 50 nM. Similar binding was observed with noninactivated uPA coupled to the sensor chip (data not shown). Panel B shows the effect of increasing concentrations of 6-aminohexanoic acid on the binding of plasminogen (500 nM). Panel C shows these data plotted as a competition curve of maximum resonance values against 6-aminohexanoic acid (●) and compares this to a similar experiment performed with N^{α}-acetyl-L-lysine methyl ester (■), together with IC_{50} values from the fitted curves.

These data suggest that lysine analogues other than 6-aminohexanoic acid, and in particular N^{α}-acetyl-L-lysine methyl ester, exert their inhibitory effect on plasminogen activation primarily by the antagonism of a direct interaction between uPAR-bound uPA and plasminogen associated with its cellular binding sites. These analogues lacking a carboxyl function resemble internal lysine or arginine residues in a protein sequence, and linear sequences containing a high density of these residues may be candidates for the plasminogen binding site on uPA. uPA contains four such sequences: $uPA^{103-110}$ (RNPDNRRR), $uPA^{151-158}$ (KTLRPRFK), $uPA^{178-181}$ (RRHR), and $uPA^{260-267}$ (KIRSKEGR), and they represent the "heparin binding site" of the kringle module (*25*), the sequence N-terminal of the activation cleavage site (Lys^{158}—Ile^{159}), the "PAI-1 binding site" (*26, 27*), and the VR4 region of the serine protease domain (*28*), respectively. It has previously been shown that in a recombinant chimeric plasminogen activator the sequence N-terminal of the activation cleavage site of uPA can act as a ligand for the lysine-binding kringle 2 module of tPA (*29*). Therefore, we sought to determine whether this sequence could also act as a ligand for the lysine-binding kringle modules of plasminogen.

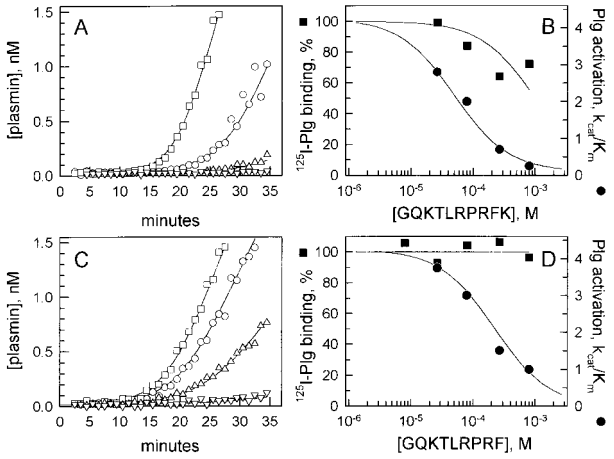

FIGURE 3: Effect of uPA peptide sequences on uPAR-mediated activation and cellular binding of plasminogen. Pro-uPA was prebound to U937 cells prior to the addition of plasminogen (50 nM) and H-D-Val-Leu-Lys-AMC. Plasmin generation is shown for experiments performed in the presence of varying concentrations (0 mg/mL, □; 0.10 mg/mL, ○; 0.33 mg/mL, △; 1.0 mg/mL, ▽) of either GQKTLRPRFK (panel A) or GQKTLRPRF (panel C). In panels B and D, competition curves for the effect of these peptides on the kinetic constants for plasminogen activation (●) are compared to their effect on the cellular binding of ^{125}I-labeled plasminogen (■), as in the legend to Figure 1.

Inhibition of Cell-Surface Plasminogen Activation by $uPA^{149-158}$ Peptides. The peptide GQKTLRPRFK was synthesized, this being the C-terminal sequence of the uPA A-chain immediately adjacent to the interchain disulfide bridge (Cys^{148}—Cys^{289}). This peptide inhibited cell-surface plasminogen activation in a manner qualitatively similar to that observed for the lysine analogues, and with an IC_{50} of 50 μM (Figure 3A,B). Peptides with a C-terminal lysine residue have been shown to compete for the cellular binding of plasminogen (*30*), which may therefore be the inhibitory mechanism of this peptide. Figure 3B shows that the peptide does indeed inhibit the binding of ^{125}I-labeled plasminogen but the IC_{50} is 20-fold higher (1 mM) than that for inhibition of cell-surface plasminogen activation. To further discriminate between these mechanisms, the effect of the analogous peptide lacking this residue (GQKTLRPRF) was tested. As shown in Figure 3C,D this peptide also efficiently inhibited cell-surface plasminogen activation, although with a somewhat higher IC_{50} of 220 μM, but had no effect on ^{125}I-labeled plasminogen binding. Despite the high density of lysine and arginine residues in these peptides, the inhibitory effect appeared to be relatively sequence-specific as a scrambled $uPA^{149-158}$ peptide inhibited plasmin generation with an IC_{50} of 800 μM, 16-fold higher than the authentic sequence. A random octapeptide with a C-terminal lysine residue inhibited with an IC_{50} of 110 μM (Table 2).

The inhibitory mechanism of the $uPA^{149-158}$ and $uPA^{149-157}$ peptides on cell-surface plasminogen activation was further analyzed kinetically. Figure 4A,B shows that at saturating concentrations the effect of both peptides was to increase the K_m for plasminogen activation, from 0.4 μM to >15 μM, with little or no effect on k_{cat}. Repeating these experiments

Table 2: Inhibitory Effects of uPA Sequence-Derived Peptides on uPAR-Mediated Plasminogen Activation and Plasminogen Binding on U937 Cells[a]

		IC_{50}	
sequence	peptide	plasmin generation	^{125}I-plg binding
GQKTLRPRFK	uPA$^{149-158}$	50 μM	1 mM
GQKTLRPRF	uPA$^{149-157}$	220 μM	>10 mM
KRQGPKFTRL	uPA$^{149-158}$ scrambled	800 μM	>10 mM
PKKATELK	C-terminal Lys	110 μM	nd

[a] IC_{50} values for the inhibition of plasmin generation initiated by uPAR-bound pro-uPA and ^{125}I-labeled plasminogen binding on U937 cells are shown. These data are taken from the experiments shown in Figure 3 and other similar experiments.

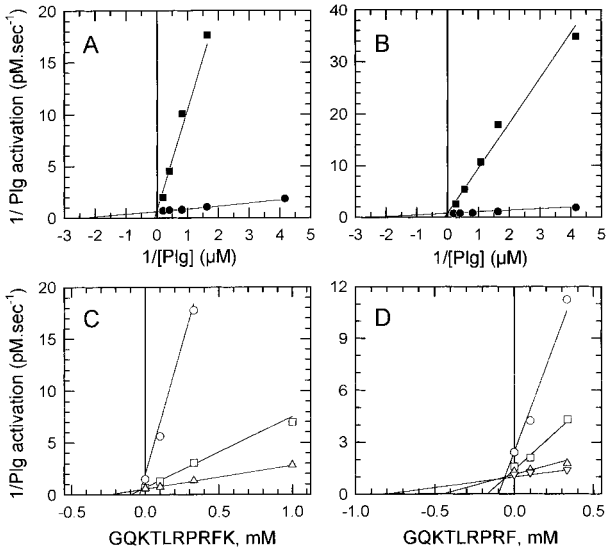

FIGURE 4: Kinetic analysis of the inhibitory effect of uPA peptide sequences on uPAR-mediated plasminogen activation. uPA was pre-bound to U937 cells prior to the addition of plasminogen, peptide, and H-D-Val-Leu-Lys-AMC. In panels A and B, double-reciprocal plots of the kinetics of plasminogen activation are shown in the absence (●) and presence (■) of 1 mM GQKTLRPRFK (panel A) or GQKTLRPRF (panel B). Both peptides had the effect of increasing the K_m for plasminogen activation from 0.4 μM to greater than 15 μM. In panels C and D, the effects of varying the concentrations of the two peptides have been assayed at various fixed plasminogen concentrations (0.175 μM, ○; 0.665 μM, □; 1.31 μM, △; 2.62 μM, ▽). The data are plotted according to Dixon (*21*); the intersection of the extrapolated lines above the *x*-axis demonstrates competitive inhibition with K_i values of 10 μM and 45 μM, respectively.

at a range of peptide concentrations allowed the determination of K_is for the inhibitory effect, these being 10 and 45 μM for uPA$^{149-158}$ and uPA$^{149-157}$, respectively (Figure 4B,D). These plots also demonstrate a competitive inhibition mechanism for both peptides, consistent with the observed increase in K_m for plasminogen activation.

To confirm that the inhibitory mechanism of the peptides is due to an antagonism of the putative uPA−plasminogen interaction, rather than of the cellular binding of plasminogen or a nonspecific effect, we made use of a specific anti-uPA monoclonal antibody which potentiates plasmin generation in a manner kinetically indistinguishable from that of cell-surface uPAR (*17*). The mechanism of this effect involves the coincident binding of uPA and plasminogen to the antibody, i.e., ternary complex formation, and requires both a specific steric arrangement of uPA on the antibody and

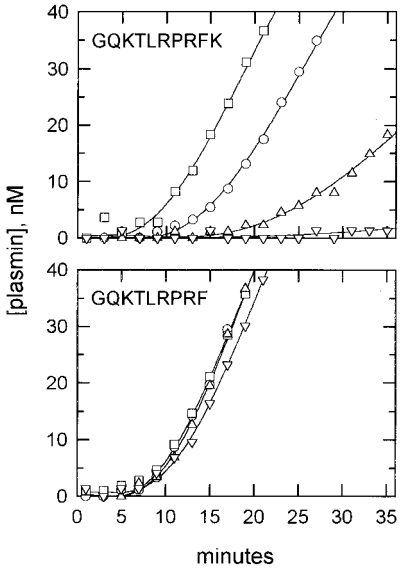

FIGURE 5: Effect of uPA peptide sequences on anti-uPA monoclonal antibody-mediated plasminogen activation. Pro-uPA (0.1 nM) and plasminogen (100 nM) were incubated with anti-uPA antibody (clone 1), as a specific stimulator of plasminogen activation (*17*). Plasmin generation was determined by hydrolysis of H-D-Val-Leu-Lys-*p*NA, and under these conditions, plasmin generation was undetectable in the absence of the antibody (not shown). The two peptides were included in the incubations at varying concentrations (0 mg/mL, □; 0.10 mg/mL, ○; 0.33 mg/mL, △; 1.0 mg/mL, ▽). The IC_{50} for the inhibition of plasminogen activation by GQKTLRPRFK was calculated to be 200 μM.

the presence of a lysine residue at the C-terminus of the immunoglobulin heavy chains to act as a plasminogen-binding site, but does not appear to depend on any other interactions (*17*, *22*). The data in Figure 5 show that the uPA$^{149-158}$ peptide efficiently inhibited plasmin generation (IC_{50} 200 μM), whereas uPA$^{149-157}$ was essentially without effect. This is in contrast to the effects of these two peptides on cell-surface plasminogen activation where both inhibited efficiently. Thus, only the peptide with a C-terminal lysine residue was inhibitory in this model as it could compete for the binding of plasminogen to the immunoglobulin C-terminus.

Effect of uPA A-Chain C-Terminal Sequence on Plasminogen Activation. The data obtained above show that the uPA$^{149-157}$ peptide can act as a specific inhibitor of uPAR-mediated cell-surface plasminogen activation by an antagonism of the uPA−plasminogen interaction. To test whether this sequence within uPA itself also acts as the ligand for plasminogen, uPA was treated with various carboxypeptidases. Both cell-surface and solution-phase plasminogen activation were unaffected after treatment of uPA with the basic carboxypeptidase CpB to remove Lys^{158}. The noninvolvement of Lys^{158} was also demonstrated using recombinant K158E pro-uPA after activation with endoprotease Glu-C. However, when uPA was treated with the general carboxypeptidase CpP, a decrease in cell-surface plasminogen activation relative to the solution-phase reaction was observed. The maximal effect was a reduction in the plasminogen activation rate to 55% that of control values. Although this is a smaller effect than would be predicted from the data above, the extent of C-terminal degradation is likely to be rather limited, due to both the relatively low efficiency of the carboxypeptidase and constraints on the

accessibility of the C-terminus under nondenaturing conditions (*31*).

DISCUSSION

We have shown previously that the presence of uPAR on the surface of cells leads to the formation of an efficient system for the activation of plasminogen. Kinetically this system is characterized by a greatly reduced K_m for plasminogen activation (from >20 μM to 0.1−0.4 μM) and an increased efficiency of plasmin-catalyzed pro-uPA activation (*6*, *7*). These effects are dependent on the cellular binding of plasminogen as they are abolished by aminocarboxylic acid lysine analogues, and consistent with this, soluble forms of uPAR have no potentiating effect on plasmin generation (*7*, *8*). Cell-surface-associated plasminogen is thus the kinetically preferred substrate for uPAR-bound uPA. The simplest model to explain these effects is that the high density of cellular binding sites for plasminogen has a partitioning effect on plasminogen between the solution and immobilized phases leading to high local substrate concentrations (*32*). Alternatively, a more complex mechanism may be involved, dependent on a more ordered interaction between the protease/zymogen components. The latter model is supported by the observation that ternary complex formation, with an anti-uPA monoclonal antibody acting as a specific template for the stoichiometric assembly of uPA and plasminogen, quantitatively mimics the kinetic parameters of the uPAR system (*17*). This suggests that cell-surface plasminogen activation may involve a similar mechanism and therefore also be dependent on specific complex formation. We have previously speculated that uPAR has the characteristics necessary to mediate complex formation (*17*) as it has been shown to have potential associations with other cell-surface components, including integrins (*33*). However, we have demonstrated that efficient cell-surface plasminogen activation can be catalyzed by a recombinant directly GPI-anchored form of uPA (*18*), eliminating the possibility that uPAR has a direct role in the assembly of the putative complexes. The data presented here provide evidence that complex formation is necessary for cell-surface plasminogen activation and demonstrate that these complexes are formed by non-active-site interactions between the protease/zymogen components bound to their cognate cellular binding sites.

This evidence is based on the observation that certain lysine analogues, such as N^{α}-acetyl-L-lysine methyl ester, inhibit cell-surface plasminogen activation at concentrations that do not inhibit either the binding of plasminogen to the cells or the catalytic reactions in the solution phase. Therefore, under these conditions, plasminogen is bound to its cellular binding sites, but no longer behaves as the preferred substrate for receptor-bound uPA. This observation excludes mechanisms involving simple cell-surface concentration and approximation effects, and indicates that the enzymatic interaction between uPA and plasminogen is dependent upon additional interactions to bring about the catalytically favorable juxtaposition of the protease/zymogen components. The demonstration of a direct noncatalytic interaction between uPA and plasminogen by BIA, the antagonism of this interaction by the lysine analogues at concentrations correlating with the inhibitory effects on cell-surface plasminogen activation, and the inhibition of cell-surface plasminogen activation by peptides derived from the sequence of uPA all point to this interaction being necessary for the assembly of the putative plasminogen activation complexes.

The demonstration of the importance of a non-active-site interaction between uPA and plasminogen furthers our understanding of the mechanisms of cell-surface plasminogen activation, but a plausible model for this process needs to account for the lack of effect of complex formation on solution-phase plasminogen activation. The interaction of plasminogen with receptor-bound uPA could be occurring by either of two pathways, both involving the interaction of different kringle modules of plasminogen with uPA and the cell-surface plasminogen binding sites.[2] Cell-associated plasminogen may bind to receptor-bound uPA, thus facilitating the catalytic interaction, or plasminogen may first bind to receptor-bound uPA and then become associated with its cell-surface binding sites. The higher affinity of plasminogen for uPA (K_d 50 nM) compared to its cellular binding sites [K_d 0.8−2.8 μM (*12*)] makes the latter sequence of events more likely. In either case, it can be expected that the two independent interactions put a constraint on plasminogen, which sterically facilitates the catalytic interaction with receptor-bound uPA. In the solution phase this constraint will not be present, and the catalytic interaction will be unaffected. The high K_m for solution-phase plasminogen activation (>20 μM) suggests that the complexed plasminogen is not a substrate for the uPA molecule to which it is bound. In contrast, the apparent K_m for plasminogen activation by receptor-bound uPA under ideal conditions (*7*) is close to the K_d of the uPA−plasminogen interaction (110 nM vs 50 nM) and substantially lower than the K_d of the cellular binding of plasminogen. The antibody model which we have previously used to mimic complex-dependent plasminogen activation (*17*), although reproducing the kinetic effects of uPAR-mediated plasminogen activation, would appear to be independent of the uPA−plasminogen interaction described here. In this case, the juxtaposition of the two reactants in the ternary complex is presumably sufficient to mediate a catalytically favorable interaction in the absence of the interaction described here. Therefore, a peptide specifically interfering with the uPA−plasminogen interaction (i.e., $uPA^{149-157}$) has no effect in this model, although the $uPA^{149-158}$ peptide is inhibitory due its C-terminal lysine residue competing for the binding of plasminogen to the C-terminal lysine of the antibody heavy chain.

The necessity for the cellular binding of plasminogen to realize the effect of the uPA−plasminogen interaction described here is consistent with our previous observation that a soluble recombinant form of uPAR neither potentiates plasminogen activation by uPA nor affects the intrinsic catalytic activity of pro-uPA (*8*), although others have reported that the intrinsic activity of pro-uPA can apparently be increased by soluble uPAR (*34*, *35*). However, the latter effect occurred only under certain very restricted conditions and required the presence of a specific tripeptide plasmin substrate containing nonnatural amino acids, and the effect

[2] The involvement of different kringles in the two processes is suggested by the differential effects of the various lysine analogues on the binding of plasminogen to the cell surface and to uPA, each kringle module of plasminogen having both different affinities and selectivities for these ligands (*40*).

was reversed by low concentrations of carrier molecules such as serum albumin and detergents that would usually be present to prevent losses by adsorption and denaturation. The relevance of these observations to uPAR function is therefore uncertain, and the various lysine analogues and peptides used in this study were found to have no effect on pro-uPA-initiated plasmin generation in the presence of soluble uPAR (data not shown).

The region of uPA identified as being responsible for the interaction with plasminogen is the sequence uPA$^{149-158}$, the C-terminus of the noncatalytic A-chain. The recently published crystal structure of the protease domain of uPA shows this to be a relatively well-ordered region, with Gly^{149}−Leu^{153} unambiguously fitted to the electron density and Arg^{154}−Pro^{155} modeled to an area of weak density (*28*), although there is no conservation of either structure or sequence in this region of the trypsin-like serine proteases. The competition by N^{α}-acetyl-L-lysine methyl ester determined both in cell surface plasminogen activation and by BIA suggests that lysine, or possibly arginine, side chains are the ligands for the lysine-binding site of the kringle modules of plasminogen. The C-terminal residue Lys^{158} does not appear to be critical, as its removal from uPA with CpB had no effect on cell-surface plasminogen activation. However, the 4-fold lower IC_{50} for the synthetic peptide GQKTLRPRFK compared to GQKTLRPRF (and the effect of an irrelevant C-terminal lysine peptide) (Table 1) demonstrates that, although not necessary, a C-terminal lysine residue may be the preferred ligand when present.

Previously we have observed that low concentrations of the polysulfonated naphthylurea compound suramin interfere with cell-surface plasminogen activation without affecting either the cellular binding of plasminogen or the catalytic activity of uPA (*22*). These effects are similar to those observed here with the lysine analogues and the uPA peptides, and may also occur by antagonism of the non-active-site interaction between uPA and plasminogen. However, in contrast to the lysine analogues and uPA peptides, suramin is not a ligand for the kringles of plasminogen. It may therefore have an alternative mode of action, and its polyanionic nature makes it likely that it can bind to the cationic uPA$^{149-158}$ region and thus inhibit the interaction in a manner compatible with the mechanism proposed here.

The interaction of lysine-binding kringle modules with the serine protease domain of uPA has previously been observed in two chimeric plasminogen activators which have the non-lysine-binding kringle module of uPA replaced by the lysine-binding kringle 2 module of tPA (*29*, *36*). Using a chimera in which the two kringles are exchanged, Bakker et al. observed a loss of lysine-binding function and also a lysine analogue-sensitive binding of isolated tPA kringle 2 to both intact uPA and low molecular weight uPA (uPA$^{136-411}$) (*36*). Using a chimera in which the N-terminal region of uPA (uPA$^{1-137}$) was replaced by the corresponding region of tPA (tPA$^{1-274}$), Novokhatny et al. observed by differential scanning calorimetry that the lysine-binding site of kringle 2 was intramolecularly occupied and that tPA and its A-chain fragment bound to both uPA and uPA$^{136-411}$ in a lysine analogue-sensitive manner with K_ds of 50−300 nM in a solid-phase assay and 1−7 μM in a fluid-phase assay (*29*). Furthermore, these investigators also observed that synthetic peptides corresponding to uPA$^{144-158}$ and uPA$^{144-157}$ competed with these interactions with IC_{50}s of 200 and 400 μM, respectively, consistent with our own observation that the C-terminal Lys^{158} is not necessary for the interaction of plasminogen kringles with the uPA protease domain. Preliminary experiments using the chimera described by Bakker et al. point to it having an impaired capability in cell surface plasminogen activation, consistent with the mechanism proposed here (unpublished experiments).

It appears, therefore, that the uPA$^{149-158}$ region is an important functional motif that can act as a ligand for a range of lysine-binding kringles, both in recombinant chimeric plasminogen activators and, more significantly, in the physiologically relevant uPAR-mediated activation of plasminogen. The interaction between uPA and plasminogen may also be of functional significance in other situations in which one or both of these components become immobilized to a surface, and both uPA and plasminogen have been reported to bind to various extracellular matrix components in vitro (*37*, *38*). Such a mechanism may allow for some level of stimulation of plasminogen activation in the absence of uPAR. The observation that in transgenic mice deficiency of uPA has a more profound effect than deficiency of uPAR (*39*) demonstrates that, at least in some circumstances, uPA can function independently of uPAR. Therefore, the uPA/plasminogen interaction may also be involved in the compensatory mechanism for the stimulation of plasminogen activation that is suggested by these observations.

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