A High Affinity Interaction of Plasminogen with Fibrin Is Not Essential for Efficient Activation by Tissue-type Plasminogen Activator*

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Fibrin (Fn) enhances plasminogen (Pg) activation by tissue-type plasminogen activator (tPA) by serving as a template onto which Pg and tPA assemble. To explore the contribution of the Pg/Fn interaction to Fn cofactor activity, Pg variants were generated and their affinities for Fn were determined using surface plasmon resonance (SPR). Glu-Pg, Lys-Pg (des(1–77)), and Mini-Pg (lacking kringles 1–4) bound Fn with Kd values of 3.1, 0.21, and 24.5 μM, respectively, whereas Micro-Pg (lacking all kringles) did not bind. The kinetics of activation of the Pg variants by tPA were then examined in the absence or presence of Fn. Whereas Fn had no effect on Micro-Pg activation, the catalytic efficiencies of Glu-Pg, Lys-Pg, and Mini-Pg activation in the presence of Fn were 300- to 600-fold higher than in its absence. The retention of Fn cofactor activity with Mini-Pg, which has low affinity for Fn, suggests that Mini-Pg binds the tPA-Fn complex more tightly than tPA alone. To explore this possibility, SPR was used to examine the interaction of Mini-Pg with Fn in the absence or presence of tPA. There was 50% more Mini-Pg binding to Fn in the presence of tPA than in its absence, suggesting that formation of the tPA-Fn complex exposes a cryptic site that binds Mini-Pg. Thus, our data (a) indicate that high affinity binding of Pg to Fn is not essential for Fn cofactor activity, and (b) suggest that kringle 5 localizes and stabilizes Pg within the tPA-Fn complex and contributes to its efficient activation.

Fibrinolysis, the process that degrades fibrin (Fn)3 clots, is initiated when tissue-type plasminogen activator (tPA) converts the zymogen plasminogen (Pg) to plasmin (Pn), the enzyme that solubilizes Fn (1, 2). A distinguishing feature of tPA is its affinity for Fn. By serving as a template onto which both tPA and Pg bind, Fn promotes enzyme-substrate interaction through formation of a ternary complex. Consequently, the catalytic efficiency of Pg activation by tPA is 3 orders of magnitude greater in the presence of Fn than it is in its absence and 2 orders of magnitude greater in the presence of fibrinogen (Fg), the soluble precursor of Fn (3–5). These features render tPA Fn-specific; enhanced Pn generation on the Fn surface promotes clot dissolution, whereas inefficient Pn activation in the presence of Fg limits systemic Fg degradation. Urokinase-type plasminogen activator (uPA), in contrast to tPA, does not bind to Fn (2). Consequently, the catalytic efficiency of Pg activation by uPA in the presence of Fn is similar to that in its absence, or in the presence of Fg. Because Pn generation is not localized to the Fn surface, uPA lacks Fn specificity. The functional differences between tPA and uPA identify Fn affinity as a critical determinant of Fn specificity. Thus, the capacity of Fn to promote Pg activation on its surface is dependent on the Pg activator/Fn interaction.

In contrast to the role played by the activator/Fn interaction, the contribution of the Pg/Fn interaction to the potentiating effect of Fn is unclear. The predominant form of Pg, termed Glu-Pg, is a single-chain glycoprotein consisting of an NH2-terminal peptide, five kringle domains, and a protease domain (6, 7). Lys-Pg, a truncated Pn-derived form that lacks the NH2-terminal 1–77 peptide is produced during fibrinolysis (Fig. 1A) (8). Whereas Glu-Pg assumes a closed conformation because of intramolecular links between the NH2-terminal peptide and

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The abbreviations used are: Fn, fibrin; Pg, plasminogen; Pn, plasmin; Fg, fibrinogen; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; SPR, surface plasmon resonance; RU, response units; VFKck, Val-Phe-Lys-chloromethyl ketone; FPRck, Phe-Pro-Arg-chloromethyl ketone; ε-ACA, ε-aminocaproic acid.
because uPA does not bind to Fn, we postulated that Fn would have little or no effect on the kinetics of activation of all four forms of Pg by uPA.

**EXPERIMENTAL PROCEDURES**

**Materials**—H-D-Val-Leu-Lys-p-nitroaniline-dihydrochloride (S2251) was from Chromogenix (Milan, Italy), whereas benzamide-Sepharose was from Amersham Biosciences. Streptavidin-agarose, Tween 80, Tween 20, and human serum albumin were obtained from Sigma. Precast 4–15% SDS-polyacrylamide gels were from Bio-Rad. Human neutrophil elastase was purchased from Elastin Products Co. (Owensville, MO). Human thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Val-Phe-Lys-chloromethyl ketone (VKck) and Phe-Pro-Arg-chloromethyl ketone (FPRck) were purchased from EMD Chemicals (Gibbstown, NJ). Biotinylated FPRck (biotin-FPRck) was obtained from Hematologic Technologies Inc. (Essex Junction, VT). Recombinant tPA (Activase) was kindly provided by Dr. B. Keyt (Genentech, South San Francisco, CA), whereas uPA was purchased from Calbiochem. Fg was prepared using the procedure of Straughn and Wagner (11) with the modifications described by Walker and Nesheim (12), except that the first PEG-8000 cut was performed at 1.2%, which increased the final yield of Fg to ~0.4 mg/ml of starting plasma. Isolated Fg was stored at −80 °C. The integrity of all purified proteins was confirmed by SDS-PAGE analysis, and concentrations were determined by photospectrometry.

**Isolation of Glu-, Lys-, Mini-, and Micro-Pg—**Native Glu-Pg was isolated from citrated fresh frozen human plasma by lysine-Sepharose (Lys-Sepharose) affinity chromatography using the method of Castellino and Powell (13) and the modifications described by Stewart et al. (14). Pn was generated by incubating 24 μM Glu-Pg in 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4 (TBS), with 48 nM uPA for 60 min at 25 °C with constant mixing, prior to addition of another 48 nM uPA and a further 60 min incubation. The extent of Pn generation was determined by monitoring the rate of S2251 hydrolysis. Once a maximal rate was achieved, the mixture was loaded onto a 2.5 × 20-cm Lys-Sepharose column and equilibrated with TBS. After washing with TBS to remove uPA, Pn was eluted with TBS containing 20 mM e-ACA (14). Protein-containing fractions were pooled, dialyzed against TBS, concentrated, and stored in aliquots at −80 °C. Concentration was determined by photospectrometry.

Lys-Pg was generated by Pn hydrolysis of Glu-Pg as described previously by Nesheim et al. (15) with the modifications of Stewart et al. (14). Briefly, Glu-Pg (10 μM) was converted to Lys-Pg by incubation for 2 h at 25 °C with 0.16 μM Pn in 0.02 M Hepes, 0.15 M NaCl, pH 7.4 (HBS), and 0.01% Tween 80 (HBST) containing 50 mM e-ACA. Additional Pn (0.16 μM) was added and the mixture was incubated for another 2 h. To remove Pn, a 10-fold molar excess of biotin-FPRck was added and incubated for 2 h at 25 °C while monitoring aliquots for Pn activity by S2251 hydrolysis. When no Pn activity was detected, the mixture was dialyzed against 4 × 1 liter of HBST prior to addition of streptavidin-agarose suspended in HBST. After centrifugation, the solution was filtered to remove any traces of streptavidin-agarose, and after confirming the absence of resid-
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ual biotin-FPRck by showing no reduction in Pn-mediated S2251 hydrolysis, the solution was stored in aliquots at −80 °C. Concentration was determined by photospectrometry.

Mini-Pg was generated as described previously (16) with some modifications. Briefly, after incubating 33 μM Glu-Pg with 0.67 μM elastase in TBS for 60 min at 25 °C, the reaction was terminated by adding 2 mM phenylmethylsulfonyl fluoride. The mixture was dialyzed against 0.1 M sodium phosphate, pH 8.0 (NaPi), for 2 h at 4 °C, and then loaded onto a Lys-Sepharose column pre-equilibrated with NaPi. After washing with the same buffer, the flow-through, which only contained Mini-Pg, was collected and protein-containing fractions were pooled, dialyzed against TBS, and concentrated by centrifugation. Concentration was determined by photospectrometry using an extinction coefficient of 16.0 (ε1% cm⁻¹ (280 nm)) and molecular mass of 38,000 Da, as reported previously (16). Mini-Pg was then stored in aliquots at −80 °C.

Micro-Pg was prepared as described previously (17) with modifications. Briefly, 190 μM Glu-Pg was incubated with 3 μM uPA-free Pn for 48 h at 25 °C with constant mixing. The mixture was then applied to a Lys-Sepharose column pre-equilibrated with NaPi. After washing with the same buffer, protein-containing fractions were pooled and traces of Pn, detected by monitoring S2251 hydrolysis, were removed by loading the mixture onto a benzamidine-Sepharose column that was pre-equilibrated with NaPi. Pn-free, protein-containing fractions were pooled, dialyzed against NaPi, and equilibrated with NaPi. Mini- or Micro-Pg, in concentrations up to 20 μM, was then adsorbed to a SA sensor chip at a flow rate of 5 μl/min to an RU of 1800. Binding studies were carried out with Glu-, Lys-, Mini-, or Micro-Pg, in concentrations up to 20 μM, diluted in HBSC-Tween 20 containing a 2-fold molar excess of VFKck relative to Pg. Samples were injected at a rate of 20 μl/min for 2 min and flow cells were then washed with HBSC-Tween 20 to monitor dissociation. Between injections, the flow cells were regenerated with 0.02 M HEPES, 1 M NaCl, pH 7.4, containing 25 mM FPR-tPA and the various Pg derivatives in a range of concentrations were co-injected into flow cells containing immobilized biotinylated Fn. Pg binding to Fn in the presence of tPA was corrected for tPA binding to Fn alone. The total RU change at saturation was then plotted against the starting concentration of Pg and the K_d was determined by nonlinear regression as described above.

Binding of Pg Variants to tPA—Binding interactions were studied using SPR. tPA was inactivated by incubation with biotin-FPRck. Biotin-FPR-tPA, dissolved in HBSC-Tween 20, was then adsorbed to a SA sensor chip at a flow rate of 5 μl/min to an RU of 1800. Binding studies were carried out with Glu-, Lys-, Mini-, or Micro-Pg, in concentrations up to 20 μM, diluted in HBSC-Tween 20 containing a 2-fold molar excess of VFKck relative to Pg. Samples were injected at a rate of 20 μl/min for 2 min and flow cells were then washed with HBSC-Tween 20 to monitor dissociation. Between injections, the flow cells were regenerated with 0.02 M HEPES, 1 M NaCl, pH 7.4, containing 20 mM ε-ACA. To normalize RU values, the RU change at saturation with each Pg variant was then divided by its molecular mass and the normalized values were then plotted against the starting concentration of Pg.

Affinities of Pg Variants for Fg or Fn—Binding interactions were studied by SPR using a Biacore 1000 (Piscataway, NJ). Fg and albumin were biotinylated by coupling to a biotin-EZ-Link spacer consisting of sulfo succinimidyl-6-(biotinamido)hexanoate (Thermo Fisher Scientific Inc., Rockford, IL). Biotinylated Fg or albumin were biotinylated by coupling to a biotin-EZ-Link albumin in HBS containing 2 mM CaCl2 and 0.005% Tween 20. Absence or Presence of Fg or Fn—Microtiter plates were pre-treated for at least 1 h with HBS containing 1% Tween 80 and washed thoroughly with water. For studies with Fg, Glu-Pg (0–18 μM), Lys-Pg (0–5 μM), Mini-Pg (0–18 μM), or Micro-Pg (0–6.5 μM) was incubated at 37 °C with 5 mM CaCl2 and 400 μM S2251 in the absence or presence of 1 μM Fg in HBST. Reactions were initiated by addition of tPA or uPA, and absorbance was monitored at 405 and 450 nm using a SpectraMax Plus microplate reader (Molecular Devices, Sunnyvale, CA). A tPA concentration of 0.25 nM was used with all Pg derivatives; in contrast, 1 nM uPA was used with Glu-, Lys-, and Micro-Pg, whereas 0.5 nM uPA was used with Mini-Pg.

For studies with Fg, immobilized Fg was converted to Fn by injecting 0.5 μM thrombin in HBSC-Tween 20 at a rate of 10 μl/min for 30 min as described previously (18, 19). This procedure was repeated at least twice and was continued until there was no further reduction in RU, indicating complete conversion of Fg to Fn. Flow cells were then washed with 0.02 M HEPES, 1 M NaCl, 2 mM CaCl2, pH 7.4, containing 0.005% Tween 20 and equilibrated with HBSC-Tween 20.

Binding studies were performed with a range of Glu- (0–20 μM), Lys- (0–0.75 μM), Mini- (0–20 μM), or Micro-Pg (0–20 μM) concentrations diluted in HBSC-Tween 20 containing a 2-fold molar excess of VFKck. Samples were injected at a rate of 20 μl/min for 2 min and the cells were then washed with HBSC-Tween 20 to monitor dissociation. Between runs, the cells were regenerated with 0.02 M HEPES, 1 M NaCl, pH 7.4, containing 20 mM ε-ACA. The binding data were analyzed using the steady-state affinity model (Biacore). In the sensorgrams where RU values approached a plateau, RU bound was determined by subtracting the control RU values obtained in the albumin channel from those obtained with Fg or Fn. Corrected RU values were then plotted against the starting concentrations of Pg, and data were fit to a rectangular hyperbola to determine K_d values using SigmaPlot (version 8, SPSS).

Similar studies were performed in the presence of active-siteblocked tPA (FPR-tPA), which was generated by incubating 1 mg/ml of tPA with a 20-fold molar excess of FPRck for 2 h, followed by dialysis against HBS. Analytes, which contained 25 nM FPR-tPA and the various Pg derivatives in a range of concentrations were co-injected into flow cells containing immobilized biotinylated Fn. Pg binding to Fn in the presence of tPA was corrected for tPA binding to Fn alone. The total RU change at saturation was then plotted against the starting concentration of Pg and the K_d was determined by nonlinear regression as described above.

Kinetics of Activation of Pg Derivatives by tPA or uPA in Absence or Presence of Fg or Fn—Microtiter plates were pre-treated for at least 1 h with HBS containing 1% Tween 80 and washed thoroughly with water. For studies with Fg, Glu-Pg (0–18 μM), Lys-Pg (0–5 μM), Mini-Pg (0–18 μM), or Micro-Pg (0–6.5 μM) was incubated at 37 °C with 5 mM CaCl2 and 400 μM S2251 in the absence or presence of 1 μM Fg in HBST. Reactions were initiated by addition of tPA or uPA, and absorbance was monitored at 405 and 450 nm using a SpectraMax Plus microplate reader (Molecular Devices, Sunnyvale, CA). A tPA concentration of 0.25 nM was used with all Pg derivatives; in contrast, 1 nM uPA was used with Glu-, Lys-, and Micro-Pg, whereas 0.5 nM uPA was used with Mini-Pg.

For studies with Fg, Glu-Pg (0–0.7 μM), Lys-Pg (0–0.05 μM), Mini-Pg (0–0.7 μM), or Micro-Pg (0–6.5 μM) was incubated at 37 °C with 5 mM CaCl2 and 400 μM S2251 in the absence or presence of 1 μM Fg in HBST. Reactions were initiated by the addition of 10 nM thrombin and either tPA or uPA, in the same concentrations used for the studies with Fg, and absorbance was then monitored at 405 and 450 nm. Absorbance at 405 nm was corrected for turbidity by subtracting absorbance determined at 450 nm. The rate of Pn generation (r) was determined

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as described previously (20). Using the relationship $A_{\text{corr}} = (SA \times r/2) \times t^{2}$, corrected absorbance values ($A_{\text{corr}}$) were plotted against $t^{2}$ to determine the slope ($SA \times r/2$), where SA is the specific activity of Pn against S2251, $r$ is the rate of Pn generation, and $t$ is time. The rate was then calculated using the SA, which was experimentally determined to be 1.439 $A_{\text{corr}}$/min/μM. Rates were then plotted against the starting concentrations of the Pg derivatives and the data were fit to the Michaelis-Menten equation by nonlinear regression using SigmaPlot to determine the individual $k_{\text{cat}}$ and $K_m$ values. For reactions displaying linear rate vs. substrate relationships, the slope of the line was taken as the catalytic efficiency. Because there is substantial change in turbidity when Fn polymerizes, more rigorous correction was necessary to accurately quantify the rate of Pn generation, as described by Schneider and Nesheim (21). The correction factor was determined experimentally by forming Fn clots in the absence of added tPA or uPA. Absorbance at 405 nm, which reflects turbidity, was 1.7-fold higher than absorbance readings at 450 nm. Therefore, turbidity readings at 450 nm were multiplied by this correction factor prior to subtraction from the 405 nm readings ($A_{\text{corr}} = A_{405} - (1.7 \times A_{450})$) to obtain those readings that solely reflect S2251 hydrolysis. Corrected absorbance values were again plotted against time squared and activation rates were determined as described above. The specific activity of Pn against S2251 in a 1 μM Fn clot was 0.924 $A_{\text{corr}}$/min/μM.

**RESULTS**

Affinities of Various Pg Variants for Fg or Fn—Affinities of the Pg variants for immobilized Fg or Fn were assessed by SPR. The binding of Lys-Pg to Fn is illustrated in Fig. 2; RU values increased with increasing Lys-Pg concentration. The changes in corrected RU values were plotted against the corresponding concentration of Lys-Pg (Fig. 2, inset) and the $K_d$ value was determined by nonlinear regression analysis. A similar approach was taken to determine the $K_d$ values for Glu-, Lys-, and Mini-Pg binding to Fg or Fn. With Micro-Pg, the signal change did not approach saturation, even with concentrations up to 20 μM, indicating a lower limit to the $K_d$ value. Binding data are summarized in Table 1.

The affinity of Glu-Pg for Fn was 4-fold higher than that for Fg; $K_d$ values were 3.1 and 12.5 μM, respectively. In previous reports, the affinity of Glu-Pg for Fn ranged from 13 to 38 μM and there was low or no measurable affinity of Glu-Pg for Fg (14, 22). Therefore, the higher affinity of Glu-Pg for Fn relative to Fg is consistent with previous work, which validates the use of SPR for determination of the affinity for Fg and Fn, as observed previously with thrombin (18, 19). Compared with Glu-Pg, Lys-Pg bound Fg and Fn with 50- and 15-fold higher affinities, respectively; $K_d$ values were 0.25 and 0.21 μM, respectively. As expected, with the loss of lysine-binding kringle domains, Mini-Pg bound Fg and Fn with low affinity: $K_d$ values were 10.5 and 24.5 μM, respectively. This represents an 8-fold decrease in Fn affinity compared with Glu-Pg and a 117-fold decrease compared with Lys-Pg. Micro-Pg, which does not possess any kringle domains, exhibited minimal binding to both Fg or Fn, consistent with previous reports that kringle 1, 4, and 5 mediate the interaction of Pg with Fg or Fn (9, 23, 24).

Affinities of Various Pg Variants for tPA—The Pg/tPA interaction was studied using SPR to explore the influence of this binary interaction on Pg activation. Because changes in RU are directly proportional to the molecular mass of the ligand, the observed RU values were first corrected for the molecular mass of the Pg variant used, and then plotted against the initial Pg concentration (Fig. 3). Pg variants bound to biotin-FPR-tPA, 4655

**TABLE 1**

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|        | $K_d$ (μM) |
|--------|------------|
| Glu-Pg | 12.5 ± 2.9 |
| Lys-Pg | 0.25 ± 0.03 |
| Mini-Pg | 10.5 ± 2 |
| Micro-Pg | >20 |

Values for the interaction of the Pg variants with Fg and Fn as determined by nonlinear regression analysis using the Michaelis-Menten equation (Table 2). Glu-, Lys-, and Mini-Pg activation by tPA in the presence of Fn are illustrated.
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Figure 3. SPR analysis of the interaction of Pg variants with tPA. Glu- (closed circles), Lys- (open circles), Mini- (closed triangles), or Micro-Pg (open triangles), at varying concentrations (0–20 μM), were injected into a SA chip flow cell containing immobilized biotin-FPR-tPA. R U values at equilibrium present.

Analysis of $k_{cat}$ and $K_m$ values (Table 2) reveals that the 18-fold increase in catalytic efficiency of Lys-Pg activation relative to Glu-Pg in the presence of Fn predominantly reflects a decrease in $K_m$, a finding in agreement with previous reports (4, 5). The decrease in $K_m$ is consistent with the fact that Lys-Pg binds Fn with 15-fold higher affinity than Glu-Pg (Table 1). Based on these observations, the catalytic efficiency of Mini-Pg activation by tPA in the presence of Fn would be expected to be lower than that of Glu- or Lys-Pg because Mini-Pg binds Fn with an affinity 8- and 117-fold lower than those of Glu- and Lys-Pg, respectively (Table 1). However, the catalytic efficiency of Mini-Pg activation by tPA in the presence of Fn is 9-fold higher than that of Glu-Pg, resulting from a 3-fold increase in $k_{cat}$ and 3-fold reduction in $K_m$. These data suggest that Mini-Pg is a good substrate for the tPA-Fn complex even though it lacks measurable affinity for fibrin. Individual kinetic parameters for Micro-Pg could not be determined because saturation was not achieved (Fig. 4).

Interestingly, in the absence of a cofactor, Lys- (Fig. 4B, inset), Mini- (Fig. 4C, inset), and Micro-Pg (Fig. 4D) exhibit similar −18-fold enhancements in catalytic efficiencies compared with Glu-Pg (Fig. 4A, inset) (Table 2). This finding raises the possibility that the protease domain of Pg, the only domain common to all three forms of Pg, interacts directly with tPA.

To examine the contribution of the interaction of the Pg activator with Fg or Fn to cofactor activity, we used uPA instead of tPA because uPA does not bind Fg or Fn (2). With uPA, the catalytic efficiency values for each form of Pg in the absence of a cofactor were an order of magnitude higher than those with tPA. Also, as expected, the Pg ratios demonstrate that Lys-, Mini-, and Micro-Pg are better substrates for uPA than Glu-Pg. However, a striking divergence is noted with the cofactor ratios. The cofactor ratios with all forms of Pg were ~1 (Table 4), indicating that Fg and Fn have little effect on the catalytic efficiency of Pg activation by uPA. The lack of cofactor activity is consistent with the fact that uPA does not bind to Fg or Fn and supports the concept that the interaction of the substrate with the cofactor is a minor contributor to cofactor activity.

Influence of Fn on Pg/tPA Interaction—Despite its low affinity for Fg (Table 1), the catalytic efficiency of Mini-Pg activation by tPA is almost 9-fold higher in the presence of Fn than in its absence, raising the possibility that on the Fn surface, there is a direct Pg/tPA interaction that is distinct from the binary Pg/Fn and Pg/tPA interactions. To explore this possibility, we used SPR to examine whether the interaction of the Pg variants with Fn was altered in the presence of FPR-tPA. Pg variants were injected into flow cells containing immobilized Fn in the absence or presence of FPR-tPA. Co-injection of FPR-tPA had little effect on the binding of Glu-Pg, Lys-Pg, or Micro-Pg to Fn (data not shown). In contrast, with Mini-Pg, co-injection with FPR-tPA altered the binding profile (Fig. 5). The $K_d$ values for the interaction of Mini-Pg with Fn in the absence or presence of FPR-tPA were calculated using individual on- and off-rates by fitting either a single- or a two-site model to the sensorgram data. In the absence of tPA (Fig. 5A), Mini-Pg bound Fn with a profile that best fit a single binding site model with a $\chi^2$ value of 3.2% of the $R_{max}$, which is indicative of a good fit (25). Fitting to a one-site model yielded a $K_d$ value of 31.3 μM, a value similar to...
the observed $K_d$ of Mini-Pg for Fn using the steady-state affinity method (Table 1). In the presence of FPR-tPA (Fig. 5B), however, the sensogram with each Mini-Pg concentration exhibited an additional slow-binding phase, suggesting two binding sites. As expected, the binding data exhibited a better fit to a two-site model than to a single-site model ($\chi^2$ values 2.6 and 14.5% of $R_{\text{max}}$, respectively) with $K_d$ values of 35.8 and 131 $\mu\text{M}$ for the high and low affinity sites, respectively. Additionally, the initial fast binding appears to approach a plateau similar to the

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**TABLE 2**

|        | $k_{\text{cat}}$ ($\text{s}^{-1}$) | $K_m$ ($\mu\text{M}$) |
|--------|----------------------------------|-----------------------|
| Glu-Pg | 0.073 ± 0.017                    | 0.41 ± 0.03           |
| Lys-Pg | 0.064 ± 0.016                    | 0.02 ± 0.001          |
| Mini-Pg| 0.220 ± 0.057                    | 0.14 ± 0.004          |

**TABLE 3**

| Cofactor | Catalytic efficiency ($\times 10^{-4} \text{m}^{-1} \text{s}^{-1}$) | Cofactor ratio | Pg ratio |
|----------|---------------------------------------------------------------|---------------|----------|
| Glu-Pg   | None ($0.03 \pm 0.003$)                                       | 1.0           | 1.0      |
|          | Fg ($0.19 \pm 0.006$)                                         | 6.3           | 1.0      |
|          | Fn ($18.25 \pm 5.03$)                                         | 608.3         | 1.0      |
| Lys-Pg   | None ($0.60 \pm 0.04$)                                         | 1.0           | 20.0     |
|          | Fg ($4.91 \pm 0.38$)                                          | 8.2           | 25.8     |
|          | Fn ($336.24 \pm 84.30$)                                       | 560.4         | 18.4     |
| Mini-Pg  | None ($0.54 \pm 0.18$)                                         | 1.0           | 18.0     |
|          | Fg ($10.21 \pm 1.22$)                                         | 18.9          | 53.7     |
|          | Fn ($159.16 \pm 39.48$)                                        | 294.7         | 8.7      |
| Micro-Pg | None ($0.52 \pm 0.09$)                                         | 1.0           | 17.3     |
|          | Fg ($0.54 \pm 0.04$)                                          | 1.0           | 2.8      |
|          | Fn ($2.81 \pm 0.67$)                                          | 5.4           | 0.2      |
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TABLE 4
Catalytic efficiency values for the activation of the Pg variants by uPA in the absence or presence of Fg or Fn

| Cofactor | Catalytic efficiency (units) | Cofactor ratio | Pg ratio |
|----------|-----------------------------|----------------|---------|
| None     |                            |                |         |
| Glu-Pg   | 1.48 ± 0.72                 | 1.0            | 1.0     |
| Fg       | 0.77 ± 0.30                 | 0.5            | 1.0     |
| Fn       | 0.57 ± 0.20                 | 0.4            | 1.0     |
| Lys-Pg   | 14.85 ± 0.47                | 1.0            | 10.0    |
| Fg       | 10.24 ± 0.14                | 0.7            | 13.3    |
| Fn       | 7.54 ± 0.54                 | 0.5            | 13.2    |
| Mini-Pg  | 10.61 ± 0.58                | 1.0            | 7.2     |
| Fg       | 13.33 ± 1.46                | 1.3            | 17.3    |
| Fn       | 10.96 ± 0.68                | 1.0            | 19.2    |
| Micro-Pg | 6.06 ± 0.49                 | 1.0            | 4.1     |
| Fg       | 3.63 ± 0.36                 | 0.6            | 4.7     |
| Fn       | 4.02 ± 0.77                 | 0.7            | 7.1     |

FIGURE 5. SPR analysis of the interaction of Mini-Pg with Fn in the absence or presence of FPR-tPA.

A) RU values observed in the absence of tPA (Fig. 5A), suggesting that the initial binding reflects the tPA-independent interaction of Mini-Pg with Fn. The secondary slow binding, which is only evident in the presence of FPR-tPA, results in a 1.5 ± 0.1-fold increase in total binding with all concentrations of Mini-Pg. This binding pattern is similar to the slow binding of FPR-tPA to Fn alone (Fig. 5B, inset), thus suggesting that this additional binding is tPA-dependent and that the binding characteristics of the Mini-Pg/Fn interaction are altered in the presence of tPA. Furthermore, the dissociation profiles in the presence of FPR-tPA are different from those observed in its absence and exhibit a slower rate of dissociation, suggesting that the Mini-Pg/Fn interaction is stabilized in the presence of FPR-tPA. In contrast to Mini-Pg, there was no measurable binding of Micro-Pg to Fn in the absence or presence of FPR-tPA (data not shown).

DISCUSSION

Fn and, to a lesser extent, Fg serve as cofactors that promote Pg activation by tPA. To exert this cofactor activity, Fn provides a template onto which tPA and Pg assemble (4, 5). Such assembly not only concentrates the reactants in close proximity, but may also induce conformational changes that promote their interaction. The importance of the interaction between tPA and Fn is highlighted by the fact that Fn cofactor activity is lost with uPA, a Pg activator that does not bind to Fn. However, the contribution of the Pg/cofactor interaction to the cofactor activity of Fn or Fg is less clear. To address this gap in knowledge, we used a series of Pg variants to examine the impact of Pg truncation on (a) Fn and Fg affinity, and (b) kinetics of activation by tPA or uPA in the absence or presence of Fg or Fn.

Removal of selected domains from Pg had predictable effects on affinity for Fn. Lys-Pg has higher affinity for Fn than Glu-Pg because its open conformation renders the lysine-binding kringle domains more accessible to Fn (26). Likewise, removal of most or all of the kringle domains progressively reduces the affinities of Mini- and Micro-Pg for Fn and Fg. Our binding data, which were obtained using SPR, complement previously reported results using Fn clots. With SPR, we were not only able to determine affinities of the Pg variants for Fn, but we also were able to compare affinities for Fn with those for Fg. Having verified the production of a panel of Pg variants with varying affinities for Fn and Fg, we next studied the kinetic of their activation by tPA in the absence or presence of Fg or Fn.

Because the Pg variants had different rates of activation, cofactor ratios were used to compare the influence of Fn or Fg on their catalytic efficiencies of activation. Despite the fact that Lys-Pg binds Fn with an affinity 2 orders of magnitude higher than that of Glu- or Mini-Pg, cofactor ratios with Glu-, Lys-, and Mini-Pg are similar. These results suggest that the substrate/cofactor interaction is not the primary determinant of the stimulatory effect of Fn or Fg. This concept is supported by the results with uPA; neither Fn nor Fg has a stimulatory effect on activation by uPA, which does not bind to Fn. Therefore, the cofactor activity of Fn or Fg is dependent on the tPA/Fn or tPA/Fg interaction; a concept supported by the Pg ratios that are higher for Lys-Pg or Mini-Pg than for Glu-Pg, regardless of whether the cofactor is Fn or Fg. These results point to the
tPA/Fn interaction as the primary determinant of cofactor activity.

By studying Micro-Pg, which lacks kringle domains, we were able to further examine the contribution of the substrate/cofactor interaction to cofactor activity. Because it only possesses the protease domain, Micro-Pg should be at least as readily activable as Lys- and Mini-Pg. However, Fn and Fg have minimal effects on the catalytic efficiency of its activation by tPA. In contrast, Fn enhances the catalytic efficiency of Mini-Pg activation by tPA, even though the affinity of Mini-Pg for Fn is very low. The presence of the kringle 5 domain distinguishes Mini-Pg from Micro-Pg. Therefore, the absence of a kringle 5 domain limits Micro-Pg activation by Fn-bound tPA, which raises the possibility that this domain, and possibly the other kringle domains, play a part in Pg activation that extends beyond Fn binding and involves direct interaction with Fn-bound tPA.

Because the tPA/cofactor interaction is critical for expression of cofactor activity, we used SPR to explore the possibility that tPA influences the interaction of Pg with Fn. Compared with injection of Mini-Pg alone, co-injection of FPR-tPA and Mini-Pg enhances Fn binding and increases the stability of the ternary complex, as evidenced by the slower off-rate (Fig. 5B). These observations support the hypothesis proposed by Horrevoets et al. (4) that the stability of the ternary complex is a more important determinant of the catalytic efficiency of Pg activation than the affinity of the enzyme or the substrate for Fn. What remains uncertain is the exact mechanism by which the tPA/Pg interaction stabilizes the ternary complex.

Our data identify at least two mechanisms by which formation of the ternary tPA-Pg-Fn complex enhances Pg activation. First, the interaction between tPA and Mini-Pg is altered when they are localized on the Fn surface (Fig. 6A). tPA binds to Fn predominantly via its finger (F) domain, whereas the kringle 5 domain of Mini-Pg mediates only a weak interaction with Fn. The conformational change that occurs when tPA binds to Fn (27, 28) may promote an interaction between its kringle 2 domain (K2) and the protease domain of Pg. The closed conformation of Glu-Pg and kringles 1 through 4 on Lys-Pg likely render the tPA binding site on the protease domain of Pg less accessible, thereby explaining why Glu-Pg exhibits little or no binding to tPA and Micro-Pg binds tPA with higher affinity than either Mini-Pg or Lys-Pg (Fig. 3).
Kinetics of Plasminogen Binding and Activation

Alternatively, tPA binding to Fn may expose a cryptic binding site on tPA that is specific for the kringle 5 domain of Pg (Fig. 6B). Binding of the kringle 5 domain to the tPA-Fn complex may not only anchor Pg within the ternary complex, but may also better position the substrate for tPA-mediated activation. Binding of Mini-Pg to Fn is increased in the presence of FPR-tPA (Fig. 5B). Although this may reflect increased binding of Mini-Pg and/or tPA to Fn, the slow association of this interaction is similar to that observed when FPR-tPA binds to Fn (Fig. 5B, inset), where the plateau is only reached after 30 s. By comparison, the association of Mini-Pg with Fn (Fig. 5A) or with tPA (data not shown) rapidly reaches a plateau. Taken together, it is likely that Mini-Pg and tPA bind to distinct sites on the Fn surface, and that the additional binding observed when the two are co-injected reflects an interaction of Mini-Pg with the tPA-Fn complex, which is distinct from its interaction with Fn alone. Accordingly, the formation of the initial tPA-Fn complex is the rate-limiting step, which explains why the binding profile resembles that of the tPA/Fn interaction (Fig. 5B, inset). Thus, we hypothesize that the kringle 5 domain of Pg plays a unique role in Pg activation that extends beyond Fn binding. This explains why Fn and Fg potentiate the activation of Mini-Pg by tPA to a similar extent as they do Glu-Pg and Lys-Pg, even though kringle 5 is less important for Fn binding than kringle 1 and 4 (23, 24, 29).

Based on kinetic analyses, other researchers have proposed sequential or parallel models for assembly of the ternary Pg activation complex (4, 5). In the sequential model, Fn binds tPA prior to Pg, whereas in the parallel assembly model, Pg and tPA bind Fn in random order. The parallel model accommodates the apparent Fn concentration dependence of the \( k_{\text{cat}} \) at Fn concentrations below 1 \( \mu \text{M} \) (4). Because these conditions were not examined here, the significance of this aspect of the parallel assembly model is unclear. Our data are consistent with initial tPA binding to Fn to generate a binary enzymatic complex, as suggested by Hoylaerts et al. (5). Further support for the sequential assembly model comes from the fact that formation of binary Pg-Fn or Pg-tPA complexes is likely to be negligible in vivo because of the high \( K_d \) values.

In summary, our data suggest a novel role for the kringle 5 domain of Pg in efficient Pg activation by the tPA-Fn complex. This may represent a key regulatory step in Pg activation that provides a unique target for controlling Fn generation.

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