Epsilon Amino Caproic Acid Inhibits Streptokinase−Plasminogen Activator Complex Formation and Substrate Binding through Kringle-Dependent Mechanisms†

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ABSTRACT: Lysine side chains induce conformational changes in plasminogen (Pg) that regulate the process of fibrinolysis or blood clot dissolution. A lysine side-chain mimic, epsilon amino caproic acid (EACA), enhances the activation of Pg by urinary-type and tissue-type Pg activators but *inhibits* Pg activation induced by streptokinase (SK). Our studies of the mechanism of this inhibition revealed that EACA (IC_{50} 10 μM) also potently blocked amidolytic activity by SK and Pg at doses nearly 10000-fold lower than that required to inhibit the amidolytic activity of plasmin. Different Pg fragments were used to assess the role of the kringles in mediating the inhibitory effects of EACA: mini-Pg which lacks kringles 1−4 of Glu-Pg and micro-Pg which lacks all kringles and contains only the catalytic domain. SK bound with similar affinities to Glu-Pg ($K_A = 2.3 \times 10^9$ M^{-1}) and to mini-Pg ($K_A = 3.8 \times 10^9$ M^{-1}) but with significantly lower affinity to micro-Pg ($K_A = 6 \times 10^7$ M^{-1}). EACA potently inhibited the binding of Glu-Pg to SK ($K_i = 5.7$ μM), but was less potent ($K_i = 81.1$ μM) for inhibiting the binding of mini-Pg to SK and had no significant inhibitory effects on the binding of micro-Pg and SK. In assays simulating substrate binding, EACA also potently inhibited the binding of Glu-Pg to the SK-Glu-Pg activator complex, but had negligible effects on micro-Pg binding. Taken together, these studies indicate that EACA inhibits Pg activation by blocking activator complex formation and substrate binding, through a kringle-dependent mechanism. Thus, in addition to interactions between SK and the protease domain, interactions between SK and the kringle domain(s) play a key role in Pg activation.

When plasminogen (Pg)[1] is cleaved (or "activated") by Pg activators, it forms plasmin, the enzyme chiefly responsible for the proteolysis of fibrin (fibrinolysis) and for the dissolution of thrombi in vivo. The kringle domains of both Pg and plasmin [plasmin(ogen)] contain "lysine binding sites" that interact with lysine residues in other molecules (*1*, *2*). These lysine-binding interactions modulate the activity and targeting of plasmin(ogen). Epsilon amino caproic acid (EACA), otherwise known as amicar, mimics the side chain of lysine and interacts with these "lysine-binding sites" (*3*−*6*). Through these interactions, EACA disrupts the binding of plasmin(ogen) to fibrin and potently inhibits fibrinolytic hemorrhage in humans (*4*, *7*, *8*). EACA also profoundly modulates the activity of Pg activators. It markedly stimulates the cleavage of Pg to plasmin by both tissue-type Pg activator and urinary-type Pg activator, (*9*, *10*), but it potently inhibits Pg activation by streptokinase (SK) (*5*, *11*). This physiologically important inhibitory effect of EACA on streptokinase function remains a poorly understood anomaly because EACA and streptokinase bind to distinctly different sites on the kringle and protease domains of plasmin(ogen), respectively (*12*, *13*).

Plasmin(ogen) contains five highly homologous kringle domains (*2*). Kringles 1, 4, and 5 each contain an ω-amino acid or "lysine-binding site" that has a unique binding affinity for ω-amino acids (such as EACA) and appears to interact with different functional ligands (*2*, *14*−*16*). Plasmin(ogen) also contains a catalytic or serine protease domain. Glu-Pg is the naturally occurring form of Pg in vivo (*17*). Lower-molecular-weight forms such as mini-Pg (which lacks kringles 1−4) or micro-Pg (which lacks all kringles and contains the protease domain) can be generated by specific proteolytic cleavage or recombinant methods (*2*). When EACA interacts with Glu-Pg, it induces a conformational change that converts Glu-Pg from a prolate ellipsoid (closed form) to a Debye coil (open form) (*5*, *18*−*23*). The conformational change induced in Glu-Pg by EACA is opposed by negative anions such as Cl^- (*24*, *25*). Because it lacks kringles 1−4, EACA has almost no effect on the conformation of mini-Pg and is believed not to affect the kringle-less micro-Pg (*22*).

SK is not an enzyme and indirectly activates Pg through a unique mechanism. It binds directly to the protease domain of Pg to form a tight SK−Pg complex (*13*). After complex formation, a molecular rearrangement occurs in the protease

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[1] Abbreviations: Pg, plasminogen; SK, streptokinase; EACA, epsilon amino caproic acid.

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domain of the SK−Pg complex that generates a productive active site and creates a catalytic SK−Pg activator complex (*27*, *28*). Like plasmin, this activator complex contains the same active-site residues and catalytically processes small peptide substrates with similar kinetics (*26*). Unlike plasmin, however, the activator complex efficiently cleaves substrate Pg molecules to form the active fibrinolytic enzyme plasmin (*14*, *27*, *28*).

The goal of the present study was to uncover the molecular mechanism for EACA's anomalous inhibition of Pg activation induced by SK. We find that EACA inhibits the activator complex at every key step in the process of Pg activation, including generation of an amidolytically competent active site, binding and complex formation, and the binding of Glu-Pg to the activator complex. These potent inhibitory effects of EACA require the kringle 5 domain of Pg, arguing that this structure plays a critical role in functional interactions with SK.

MATERIALS AND METHODS

Recombinant Proteins. Recombinant SK and micro-Pg were cloned, expressed in bacteria, purified, and characterized as described (*31*, *32*, *45*).

Binding Studies. (i) Saturation Binding Assays. The binding affinities of Glu-Pg, mini-Pg (American Diagnostica Inc, Greenwich, CT) and micro-Pg for rSK were measured by saturation binding assays (Scatchard analyses) that allow efficient separation of the reactants without disrupting binding interactions as we have described (*31*, *32*). In these assays, the saturable, specific binding of Pg to SK was analyzed with a nonlinear least squares curve fitting program [Ligand (*33*)] to derive best statistical estimates of binding site affinity and other parameters.

(ii) Competitive Binding Assays. EACA, L-lysine, and L-arginine (Sigma, St. Louis, MO) were used as inhibitors of the binding interaction between rSK and Glu-Pg. Various concentrations (0−300 mM) of competitor in 1 M stock solution, pH 7.35, were mixed with 5×10^{-10} M of [^{125}I]Glu-Pg and 5×10^{-10} M of recombinant SK at room temperature for 15 min, and the amount of [^{125}I]Glu-Pg bound to rSK was determined by γ counting after immunoprecipitation as described (*31*, *32*). The percentage of specifically bound protein was calculated (after adjustment for background binding) and plotted as a function of the concentrations of competitor. For each inhibitor of the binding between rSK and Glu-Pg or mini-Pg, the K_i was determined with the Ligand program (*33*).

(iii) Solid-Phase Binding Assays. The comparative inhibitory effects of EACA on SK binding to Glu- or micro-Pg were examined. Wells of a microtiter plate were coated for 1 h with 50 μL of Glu-Pg or micro-Pg (5 and 10 μg/mL respectively). After rinsing, nonspecific protein-binding sites were blocked with a 1% BSA solution. Subsequently, EACA (0−1000 mM) was added to the wells with rSK (17 μg/mL, a concentration that yielded ~50% of maximal binding). After 1 h, the wells were rinsed, and affinity-purified anti-MBP antibodies were added to the wells for 1 h to detect bound rSK. The wells were washed and [^{125}I]protein A was added to detect bound antibody. After washing, the amount of radioactivity was determined by γ counting. The amount of inhibition of binding of rSK was determined after accounting for nonspecific rSK binding to wells not coated with Pg (<1% of input cpm). In some experiments, the rSK-Glu-Pg complex was preformed by the same methods. Then after 1 h of incubation with EACA (0−1000 mM), the residual binding of rSK to Glu-Pg was determined as described. To simulate binding of Pg substrate, [^{125}I]Glu-Pg or [^{125}I]micro-Pg was added to the preformed SK-activator complex with or without EACA added as an inhibitor. After 1 h of incubation, the amount of [^{125}I]Pg was determined by γ scintillation counting.

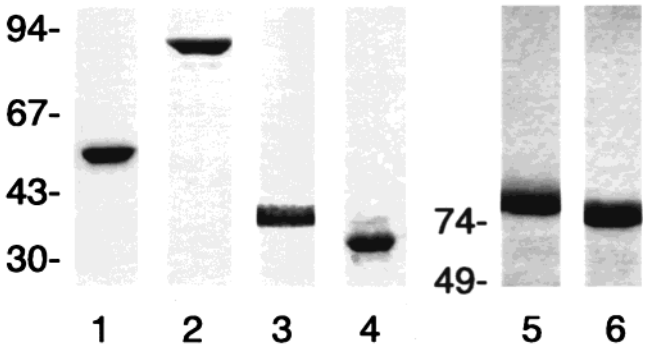

FIGURE 1: SDS−polyacrylamide gel analysis of purified proteins. Proteins (~5 μg) were electrophoresed on 10% gels (lanes 1−4) or 7.5% gels (lanes 5−6) and stained with Coomassie blue dye. Lane 1, SK; lane 2, recombinant SK; lane 3, mini-Pg; lane 4, micro-Pg; lane 5, Glu-Pg; lane 6, Lys-Pg.

Amidolytic Assays. The amidolytic activity of native SK was measured in the presence of various EACA concentrations. EACA, in assay buffer (50 mM Tris, 100 mM NaCl, pH 7.4), was added to microcentrifuge tubes in concentrations ranging from 10^{-1} to 10^{-8} M. After addition of 20 nM native SK (Sigma Chemical Co, St. Louis, MO), 20 nM Glu-Pg (Pharmacia Hepar, Franklin, OH), and assay buffer (50 mM Tris, 100 mM NaCl, pH 7.4), the reaction mixture was incubated at 37 °C for 10 min. Then the tripeptide substrate S2251 (*H*-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride; 500 μM; KabiVitrum, Stockholm, Sweden) was added to the reaction mixture in a total volume of 500 μL and the resulting amidolytic activity of the SK−Pg complex was determined at various times after incubation at 37 °C by measuring the A_{405} in a Hewlett-Packard 8451A spectrophotometer.

RESULTS

Effect of EACA on Amidolysis by Plasmin. The native and recombinant proteins used in these experiments are shown in Figure 1. Because the process of Pg activation is indirectly measured by monitoring the plasmin-mediated cleavage or amidolysis of a small peptide substrate, we first examined how EACA affected plasmin's amidolytic activity. Figure 2 shows that EACA at concentrations ≥1 mM caused a dose-related inhibition of plasmin's amidolytic activity with an IC_{50} of about 200 mM. This value is comparable to EACA's reported effects on plasmin cleavage of other small molecular substrates (*5*, *34*, *35*).

Effect of EACA on Amidolysis by the Activator Complex. The binding of SK to Pg forms an activator complex that cleaves peptide substrates with kinetics similar to that of plasmin (*29*). We examined whether the effects of EACA on the amidolytic activity of the activator complex were similar to its effects on plasmin. EACA inhibited the

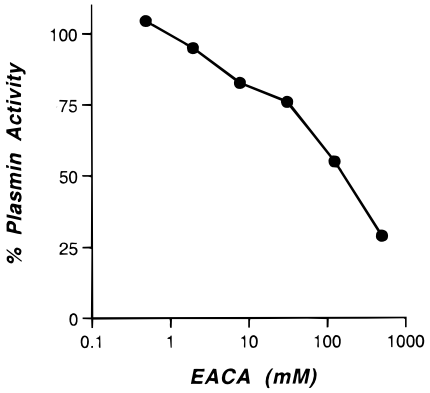

FIGURE 2: EACA inhibits the amidolytic activity of plasmin. Human plasmin (20 nM) was added to a cuvette containing the S2251 substrate in assay buffer (100 mM NaCl and 50 mM Tris-HCl, pH 7.4) and various concentrations of EACA (0−500 mM). The rate of cleavage of S2251 was monitored at 37 °C in a spectrophotometer at 405 nm. The percent residual activity of plasmin was determined by comparison to the rate of S2251 cleavage in the absence of EACA.

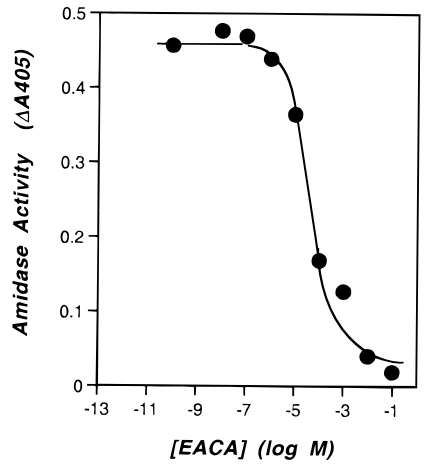

FIGURE 3: EACA inhibits the generation of amidolytic activity by SK and Glu-Pg. Glu-Pg and SK were added to solutions containing various amounts of EACA and 500 μM of a substrate (S2251, *H*-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride) that is cleaved by the SK−PAC. The amount of paranitroanilide substrate cleaved was determined by measuring the absorbance at 405 nm in a spectrophotometer and is plotted as a function of the EACA concentration.

amidolytic activity of the activator complex with an IC_{50} of $\sim 5 \times 10^{-5}$ M (Figure 3). The fact that the amidolytic activity of the activator complex was nearly 10000-fold more sensitive to inhibition by EACA than plasmin (Figure 2) suggested that EACA may specifically affect the primary interactions between SK and Pg that are critical for the generation of the activator complex.

EACA and the Binding of SK to Pg. The binding between SK and Pg is the first critical interaction necessary for the formation of the activator complex. EACA potently inhibited the binding of SK to Glu-Pg with an overall K_i [calculated with the Ligand program (*33*)] of 5.7×10^{-6} M (Figure 4). In contrast, almost 10000-fold higher concentrations of L-lysine and L-arginine ($K_i = 1.8 \times 10^{-2}$ and 6.7×10^{-2} M, respectively) were required to inhibit the binding between SK and Glu-Pg. These experiments indicated that EACA blocks the generation of a functional activator complex by preventing the binding of SK to Glu-Pg.

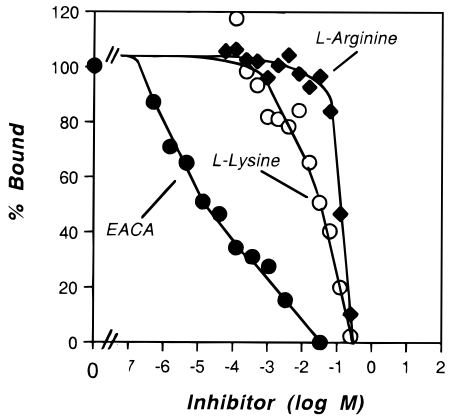

FIGURE 4: Effects of various amino acids on the binding between Glu-Pg and rSK. The binding of rSK to [^{125}I]Glu-Pg in solution was measured in the presence and absence of EACA, L-lysine, and L-arginine at various concentrations. The graph shows the percentage of specifically bound protein (corrected for nonspecific binding) as a function of the log of the inhibitory concentration of each amino acid. The data were analyzed by the Ligand program (*33*) to determine the K_is for each inhibitor.

Table 1: Effects of Kringle Deletion and EACA on the Binding of SK to Plasminogens

	binding affinity	EACA inhibition[b]	
Pg type	$K_A \pm SD \times 10^7$ (M^{-1})[a]	$K_i \times 10^{-6}$ (M)	$IC_{50} \times 10^{-5}$ (M)
Glu-Pg	230 ± 20	5.7 ± 0.6	2.8 ± 1.8
mini-Pg	380 ± 110	81.1 ± 0.3	22 ± 3.3
micro-Pg	6 ± 1	>1 000 000	>100 000

[a] Saturation binding affinities of rSK for [^{125}I]Glu-,[^{125}I]mini-Pg, or [^{125}I]micro-Pg. [b] The dose-related inhibitory effect of EACA on the binding of rSK to [^{125}I]Glu-Pg or [^{125}I]mini-Pg. The association constant (K_A) and inhibitory constant (K_i) and IC_{50} for these binding interactions were calculated with the Ligand program.(*33*)

Role of Kringles in SK-Pg Binding and EACA Inhibition. Although SK is known to interact with the protease domain of Pg, its binding interactions with the kringles of Pg have not been reported. We measured the binding affinity of SK to three types of Pgs: Glu-Pg (which contains kringle 1−5 and the protease domain), mini-Pg (which contains only kringle 5 and the protease domain), and micro-Pg (which contains only the protease domain). In liquid-phase saturation binding assays (in the absence of EACA), Glu-Pg and mini-Pg bound to SK with comparable affinities [$K_A = (2.3 \pm 0.2) \times 10^9$ M^{-1} vs $(3.8 \pm 1.1) \times 10^9$ M^{-1}], suggesting that kringles 1−4 were not important determinants of binding affinity (Table 1). However, micro-Pg bound to SK with a markedly lower (1.5 logs) affinity [$K_A = (6.2 \pm 1.3) \times 10^7$ M^{-1}] than did mini-Pg, indicating that kringle 5 was necessary for tight complex formation (Table 1).

If the inhibitory effect of EACA on SK binding was mediated primarily through its interactions with the kringles domains, its inhibitory effects should be attenuated or lost in Pg fragments lacking these domains. In liquid-phase binding experiments, EACA inhibited the binding of SK to Glu-Pg (Figure 3, Table 1) and mini-Pg (Figure 5, and Table 1). However, EACA had no inhibitory effects on the binding of micro-Pg to SK (Figure 5, Table 1). Taken together, these data indicate that kringle domain 5 is necessary and sufficient to mediate EACA's inhibitory effects on SK binding during activator complex formation.

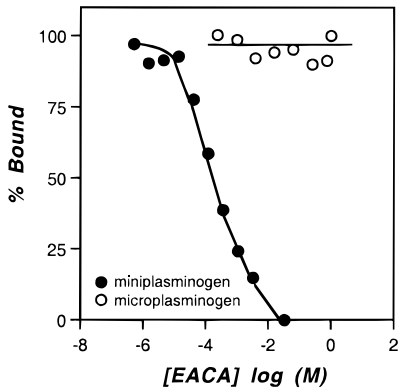

FIGURE 5: Effects of EACA on the binding of rSK to mini-Pg and micro-Pg. The binding of rSK to [^{125}I]mini- or [^{125}I]micro-Pg in solution was measured in the presence of various concentrations of EACA as described in Figure 4.

EACA and the Binding of Glu-Pg and Micro-Pg to the Preformed Activator Complex. A solid-phase assay was developed to examine the effect of EACA on the binding of Pg to the (preformed) activator complex in a manner that might simulate substrate binding. When SK was preincubated with immobilized Glu-Pg, EACA was >100-fold less potent at dissociating the activator complex than it was at preventing activator complex formation when SK and EACA was added synchronously to Glu-Pg (Figure 6). This comparative resistance of the preformed activator complex to dissociation by EACA permitted us to examine the effect of this amino acid on the binding of [^{125}I]Glu-Pg or [^{125}I]micro-Pg substrate. Experiments were performed on ice to minimize proteolysis and SDS−PAGE analysis of the [^{125}I]Pg samples showed that no Pg activation was detectable under these conditions. When compared to wells coated with Glu-Pg alone, wells containing the preformed activator complex formed by SK and Glu-Pg specifically bound [^{125}I]Glu-Pg or, separately, [^{125}I]micro-Pg. Increasing amounts of EACA induced a negligible dissociation of the preformed activator complex and had minimal effects on the binding of [^{125}I]micro-Pg. However, EACA potently inhibited the binding of [^{125}I]Glu-Pg ($IC_{50} \approx 30$ μM) to the activator complex.

DISCUSSION

SK indirectly activates Glu-Pg through a unique three step mechanism that includes (1) high-affinity binding of SK with Glu-Pg to form a complex, (2) productive restructuring of the latent protease domain to generate an active site in the complex, and (3) the binding and proteolysis of substrate Pg molecules by the activator complex (*13*, *24*, *27*, *28*, *31*). Our studies indicate that EACA inhibits indirect Pg activation by interfering with these steps. EACA potently blocks the first stage of this process by preventing the initial binding of SK with Glu-Pg ($K_i = 5.7 \times 10^{-6}$ M) to form an activator complex. By blocking SK and Pg interactions, EACA prevents the development of an amidolytically competent active site in Pg ($IC_{50} \approx 10^{-5}$ M), the second step in this process. EACA potently interferes with the third step of Pg activation by inhibiting the binding of (substrate) Pg molecules by the activator complex. Finally, at very high concentrations EACA (IC_{50} 200 mM) also inhibited plasmin's amidolytic activity with a standard substrate (S-2251), which is consistent with previous reports that at similar concentrations, EACA was a noncompetitive inhibitor of plasmin action (*5*, *34*, *35*).

SK contains a single moderate affinity [$K_d = (3-40) \times 10^{-7}$ M] binding site for the plasmin A chain in addition to its well-known binding to the protease domain (*12*, *44*).

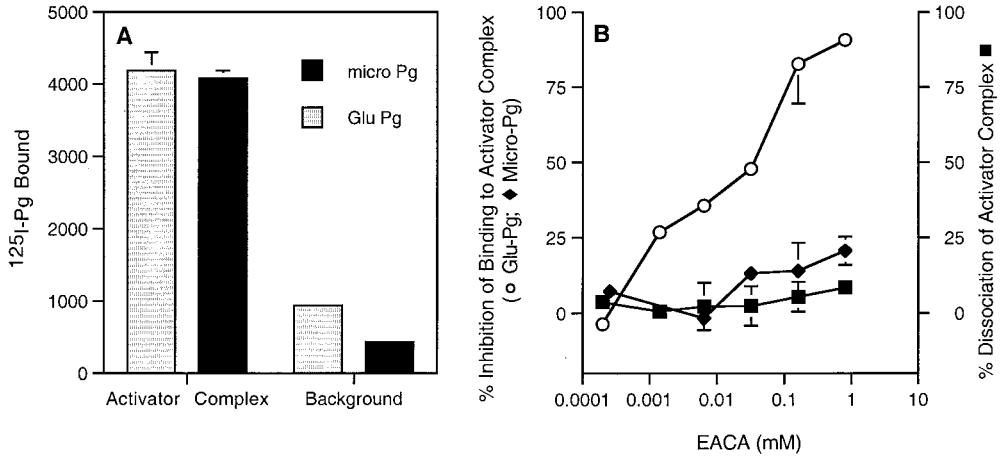

FIGURE 6: Effects of EACA on the binding of [^{125}I]Glu-Pg or [^{125}I]micro-Pg to the activator complex or, on dissociation of the activator complex. (A) The specific binding of [^{125}I]Glu-Pg and [^{125}I]micro-Pg to the SK-Glu-Pg activator complex (in the absence of an inhibitor). Wells of a microtiter plate were coated with Glu-Pg (5 μg/mL, 50 μL) for 1 h and then nonspecific binding sites were blocked with 1% BSA. After washing native SK (20 μg/mL, 50 μL) was added to form an activator complex for 60 min. Subsequently, the wells were washed and blocked with 1% BSA (250 μL for 1 h). After washing, [^{125}I]Glu-Pg (~200 000 cpm/25 μL) or [^{125}I]micro-Pg (~400 000 cpm, 25 μL) was added to the wells for 1 h in the presence of various amounts of EACA (0−500 mm final, 25 μL). After incubation the amount of bound Pg was determined by γ scintillation counting. (B) The effects of EACA on Pg binding to the activator complex and on the stability of the activator complex. Experiments were performed as described above in the presence of various concentrations of EACA. The percentage of inhibition of binding was determined by computing the fractional binding of [^{125}I]Pg to the activator complex in the presence of inhibitor, to that occurring in the absence of an inhibitor, after correcting for nonspecific binding. To determine the dissociation of the preformed SK-Glu-Pg complex, the assay was performed under identical conditions except that EACA (0−500 mM final, 50 μL) was added to the wells for 1 h on ice. After incubation for 1 h on ice and washing, the wells were incubated with pooled monoclonal antibodies against SK for 1 h The wells were rinsed and [^{125}I]antimouse antibodies were added. No dissociation was defined as the amount of anti-SK antibody binding occurring in wells not exposed to inhibitor, and 100% dissociation or background binding was the amount of binding that occurred to wells coated with Glu-Pg but not containing SK, i.e., without the activator complex. The mean and SD are shown.

Because SK binds with comparable affinities to Glu-Pg and mini-Pg, kringles 1−4 are not important for high-affinity interactions in the activator complex. Removal of kringle 5, to create micro-Pg, strongly decreases the binding affinity of SK by ∼1.5 logs, suggesting that interactions between SK and kringle 5 contribute to high-affinity binding. Even so, the protease domain, lacking all kringles, forms an activator complex with SK that has greater catalytic efficiency than the SK−Glu−Pg complex (*29*, *36*). This indicates that the kringle domains of the plasmin or the (activated) Pg moiety in the activator complex are not important in this process per se.

However, the kringle domains of the substrate Pg do appear to be important for efficient Pg activation. In an assay designed to simulate substrate binding by the activator complex, EACA was a potent inhibitor (IC_{50} = 30 μM) of Glu-Pg binding to the activator complex. This inhibitory effect was consistent with two possibilities: the activator complex cannot bind the unfolded form of Glu-Pg induced by EACA or that Pg substrate binding to the activator complex requires a Lys-dependent interaction between the activator complex and the substrate Pg. (In this scenario, SK is the likely source of this Lys-dependent interaction because the binding of [^{125}I]Glu-Pg to Glu-Pg in the absence of SK was minimal). The first possibility can be excluded by kinetic studies: like t-PA and urokinase, the SK activator complex is a much more efficient activator of the Lys-Pg, the unfolded form, than it is of Glu-Pg, the compact form of Pg (*29*). The fact that the SK activator complex can efficiently activate mini-Pg but, unlike urokinase, cannot activate micro-Pg, suggests that efficient catalytic processing of Pg substrate requires Lys-dependent interaction(s) that requires at least kringle 5 (*42*). The SK site that provides this critical interaction has not been formally demonstrated, but in addition to the binding site present in SK between 220 and 414 (*44*), the NH_2-terminus of SK may also be involved because of the marked role it plays in modulating fibrin-independent Pg activation (*45*). Peptide-binding studies have shown that this region can interact with Pg (*46*).

To derive accurate estimates of the inhibitory effects of EACA on activator complex formation, the experiments were performed with SK and Pg concentrations equal to the K_d for their binding (*31*). At these concentrations (∼10^{-10} M), only stoichiometric activator complex binding interactions can be detected between SK and Pg because the binding interactions between the SK−Pg activator complex and Pg substrate are of significantly lower affinity ($K_d \approx 10^{-7}$ M) (*29*). In these solution assays, EACA inhibited the binding of SK to Glu-Pg at concentrations 2−3 logs lower than has been measured in solid-phase assays (*47*). When compared to mini-Pg, the binding inhibition curve for Glu-Pg was complex (Figure 4), reflecting the fact EACA interacts with different kringles with different affinities (*37*, *38*). These complex binding interactions have been deconvoluted by studies with isolated, recombinant kringle domains that bind EACA strongly: kringle 1 (K_d = 11 μM), kringle 4 (K_d = 29 μM), and kringle 5 (K_d = 140 μM). No EACA-binding sites have been identified in kringle 3 or the protease domain of Pg and the binding of EACA to kringle 2 is of low affinity (*16*, *22*, *39*). The overall K_i for inhibition of ∼5 μM is comparable to the reported affinity of EACA for kringle 1 (11 μM) and kringle 4 (K_I = 29 μM). In contrast, when EACA inhibits the binding of SK to mini-Pg (Figure 5), the inhibition curve suggests ligand interaction at one site (K_i =81 μM, IC_{50}= 220 μM), which is consistent with the EACA's binding to only one site in kringle 5 (K_d 140 μM). These inhibitory effects are kringle-dependent because EACA has no inhibitory effects on the binding between SK and micro-Pg. Still, the inhibition of activator complex formation by EACA cannot simply be ascribed to a conformational unfolding of Glu-Pg. That is because SK binds with comparable affinity to mini-Pg, which is naturally unfolded because the primary sites of intramolecular kringle interaction responsible for the tight conformation of Glu-Pg have been removed (the NH_2 terminal peptide-kringle 5, and kringle3-kringle4). Taken together, these data are most consistent with the conclusion that EACA blocks activator complex formation by competing with a Lys-dependent binding interaction between SK and kringle 5 that is important for high-affinity binding. The more complex pattern by which EACA inhibits Glu-Pg and SK binding (Figure 4) may reflect its effects on intramolecular interactions in Glu-Pg (e.g., the NH_2 terminal peptide-kringle 5, etc.) that affect the conformational accessibility of kringle 5 for binding with SK.

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REFERENCES

1. Castellino, F. (1981) *Chem. Rev. 81*, 431−446.
2. Sottrup-Jensen, L., Claeys, H., Eajdel, M., Petersen, T. E., and Magnusson, S. (1978) *Prog. Chem. Fibrinol. Thrombol. 3*, 191−209.
3. Lerch, P. G., Rickli, E. E., Lergier, W., and Gillessen, D. (1980) *Eur. J. Biochem. 107*, 7−13.
4. Okamoto, S. (1957) *British Patent Specification No. 770, 693*, Her Majesty's Stationery Office, London, U.K.
5. Alkjaersig, N., Fletcher, A. P., and S. S. (1959) *J. Biol. Chem. 234*, 832−37.
6. Abiko, Y., Iwamoto, M., and Tomikawa, M. (1969) *Biochim. Biophys. Acta 185*, 424−31.
7. Sweeney, W. M. (1965) *Am. J. Med. Sci. 249*, 576−89.
8. Bok, R. A., Mangel, W. F. (1985) *Biochemistry 24*, 3279−86.
9. Urano, T., de Serrano, V. S., Gaffney, P. J., and Castellino, F. J. (1988) *Arch. Biochem. Biophys. 264*, 222−30.
10. Urano, T., Sator de Serrano, V., Gaffney, P. J., and Castellino, F. J. (1988) *Biochemistry 27*, 6522−8.
11. Roberts, P. S., and Burkat, R. K. (1966) *Biochim. Biophys. Acta 118*, 592−603.
12. Wang, X., Lin, X., Loy, J. A., Tang, J., and Zhang, X. C. (1998) *Science 281*, 1662−5.
13. Summaria, L., and Robbins, K. C. (1976) *J. Biol. Chem. 251*, 5810−13.
14. Castellino, F. J., Ploplis, V. A., Powell, J. R., and Strickland, D. K. (1981) *J. Biol. Chem. 256*, 4778−82.
15. Sehl, L. C., and Castellino, F. J. (1990) *J. Biol. Chem. 265*, 5482−6.
16. Thewes, T., Constantine, K., Byeon, I. J., and Llinas, M. (1990) *J. Biol. Chem. 265*, 3906−15.
17. Holvoet, P., Lijnen, H. R., and Collen, D. (1985) *J. Biol. Chem. 260*, 12106−11.
18. Castellino, F. J., Brockway, W. J., Thomas, J. K., Liano, H. T., and A. B. Rawitch. (1973) *Biochemistry 12*, 2787−91.
19. Sjoholm, I. (1973) *Eur. J. Biochem. 39*, 471−9.
20. Mangel, W. F., Lin, B. H., and Ramakrishnan, V. (1990) *Science 248*, 69−73.
21. Christensen, U., and Molgaard. L. (1992) *Biochem. J. 285*, 419−25.

22. Marshall, J. M., Brown, A. J., and Ponting, C. P. (1994) *Biochemistry 33*, 3599−606.
23. McCance, S. G., and Castellino, F. J. (1995) *Biochemistry 34*, 9581−6.
24. Summaria, L., Ling, C. M., Groskopf, W. R., and Robbins. K. C. (1968) *J. Biol. Chem. 243*, 144−50.
25. Urano, T., Chibber, B. A., and Castellino, F. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4031−4.
26. Wohl, R. C., Summaria, L., Arzadon, L., and Robbins, K. C. (1978) *J. Biol. Chem. 253*, 1402−7.
27. Reddy, K. N., and Markus, G. (1972) *J. Biol. Chem. 247*, 1683−91.
28. Schick, L. A., and Castellino, F. J. (1974) *Biochem. Biophys. Res. Commun. 57*, 47−54.
29. Wohl, R. C., Summaria, L., and Robbins. K. C. (1980) *J. Biol. Chem. 255*, 2005−13.
30. Powell, J. R., and Castellino, F. J. (1980) *J. Biol. Chem. 255*, 5329−35.
31. Reed, G. L., Lin, L. F., Parhami-Seren, B., and Kussie, P. (1995) *Biochemistry 34*, 10266−71.
32. Lin, L. F., Oeun, S., Houng, A., and Reed, G. L. (1996) *Biochemistry 35*, 16879−85.
33. Munson, P. J., and Rodbard, D. (1980) *Anal. Biochem. 107*, 220−39.
34. Iwamoto, M., Abiko, Y., and Shimizu, M. (1968) *J. Biochem. (Tokyo) 64*, 759−67.
35. Menhart, N., Hoover, G. J., McCance, S. G., and Castellino, F. J. (1995) *Biochemistry 34*, 1482−8.
36. Lee, P. P., Wohl, R. C., Boreisha, I. G., and Robbins, K. C. (1988) *Biochemistry 27*, 7506−13.
37. Brockway, W. J., and Castellino, F. J. (1972) *Arch. Biochem. Biophys. 151*, 194−9.
38. Markus, G., DePasquale, J. L., and Wissler, F. C. (1978) *J. Biol. Chem. 253*, 727−32.
39. Marti, D. N., Hu, C. K., An, S. S. A., von Haller, P., Schaller, J., and Llinas, M. (1997) *Biochemistry 36*, 11591−604.
40. Wiman, B., Boman, L., and Collen. D. (1978) *Eur. J. Biochem. 87*, 143−6.
41. Wu, H. L., Chang, B. I., Wu, D. H., Chang, L. C., Gong, C. C., Lou, K. L., and Shi, G. Y. (1990) *J. Biol. Chem. 265*, 19658−64.
42. Peltz, S. W., Hardt, T. A., and Mangel, W. F. (1982) *Biochemistry 21*, 2798−804.
43. Shi, G. Y., and Wu, H. L. (1988) *J. Biol. Chem. 263*, 17071−5.
44. Young, K. C., Shi, G. Y., Wu, D. H., Chang, L. C., Chang, B. I., Ou, C. P., and Wu, H. L. (1998) *J. Biol. Chem. 273*, 3110−6.
45. Reed, G. L., Houng, A., Lin, L., Parhami-Seren, B., Matsueda, L. H., Wang, S., and Hedstrom, L. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 8879−83.
46. Nihalani, D., Kumar, R., Rajagopal, K., and Sahni, G. (1998) *Protein Sci. 7*, 637−48.
47. Conejero-Lara, F., Parrado, J., Azuaga, A. I., Dobson, C. M., and Ponting, C. P. (1998) *Protein Sci. 7*, 2190−9.

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