Regulation of Streptokinase-Human Plasmin Complex by the Plasma Proteinase Inhibitors α₂-Antiplasmin and α₂-Macroglobulin Is Species Specific and Temperature Dependent

By Steven L. Gonias, Nancy L. Figler, and Lela L. Braud

Streptokinase-plasmin complex (SkPI) was prepared with human plasminogen. Regulation of SkPI and plasmin by the plasma proteinase inhibitors, α₂-antiplasmin (α₂AP) and α₂-macroglobulin (α₂M), was studied as a function of temperature in plasminogen-depleted human plasma, mouse plasma, and solutions of purified proteins. The reaction of plasmin with proteinase inhibitors in human plasma was complete. α₂AP was the principal inhibitor. The reaction of α₂M-plasmin recovered was not affected significantly by incubation temperature. In contrast, the reaction of SkPI with human proteinase inhibitors was markedly temperature dependent. The apparent second-order rate constant for the reaction of SkPI with purified α₂AP at 37°C (1.5 x 10⁶ mol/L⁻¹ s⁻¹) was >150-fold higher than the constant derived at 4°C. In human plasma and in solutions containing mixtures of purified human proteins, α₂AP was the principal inhibitor of SkPI. Elevating the temperature enhanced the reaction of SkPI with α₂AP and α₂M comparably. Equivalent results were obtained when incubations were performed in platelet-rich plasma (PRP) or whole blood. In murine plasma, SkPI reacted readily with the proteinase inhibitors. The principal inhibitor of SkPI was α₂M. Maximum reaction between SkPI and murine α₂M was observed at 37°C; however, significant reaction also occurred at 4°C. α₂AP was the predominant inhibitor of plasmin in mouse plasma. Reaction of α₂AP with SkPI in murine plasma was significant only after the α₂M was inactivated with methylamine. These results were not affected by platelets or whole blood cells. We conclude that the thrombolytic efficacy of streptokinase reflects not only the nature of the plasminogen activator complex but also the function of the proteinase inhibitors.

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From the Departments of Pathology and Biochemistry, University of Virginia Medical Center, Charlottesville.

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Address reprint requests to Steven L. Gonias, MD, PhD, University of Virginia Medical Center, Department of Pathology, Box 214, Charlottesville, VA 22908.

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from Amersham (Arlington, IL), and iodobeads were from Pierce (Rockford, IL). All other reagents were of the best grade commercially available.

Proteins. Streptokinase was purified from Kabikinase (Kabi, Stockholm) by the method of Castellino et al. The final preparation demonstrated a single band with an apparent mol wt of 50,000 by sodium dodecyl sulfate SDS-gel electrophoresis. Plasminogen was prepared from human plasma as described by Deutsch and Mertz and activated with catalytic quantities of streptokinase with 4% glycerol added or with <10 CTA U urokinase. α2AP was purified from human plasma by the method of Wiman. Human α2M was purified as previously described and was free of "reacted form" as judged by native gel electrophoresis. Anti-urokinase antibody was a gift of Dr. Salvatore Pizzio (Duke University Medical Center). Fibrinogen, L grade, was purchased from Kabi and further purified by chromatography on lysine-Sepharose. Soybean trypsin inhibitor and trypsin were from Sigma. The trypsin was 60% active as determined by active site titration.

Activity assays. Plasmin activity after activation of purified plasminogen was determined by hydrolysis of S-2251 (0.8 mmol/L) under previously specified conditions. A Hewlett Packard 8450A temperature-controlled diode-array spectrophotometer was used (Hewlett Packard, Sunnydale, CA). Percentage of activity was calculated based on published steady-state kinetic parameters using a molar extinction coefficient of 9,950 for nitroaniline at 405 nm. Plasminogen in plasma preparations was assayed by a similar method after acid precipitation to remove inhibitors. α2M in solutions containing purified proteins and in plasma was assayed at 22°C with S-2251 by the method of Ganrot. α2AP activity was determined based on the depletion of S-2251 amidase activity in plasma or solutions containing plasmin. In these studies, plasminogen was activated with urokinase. The urokinase was then inactivated with a 400-fold molar excess of antiruokinase antibody before the plasmin was added to the solution containing α2AP.

Plasma preparations. Fresh-frozen human plasma was obtained from the American Red Cross (Washington, DC). Whole blood was collected into sodium citrate by antecubital venous puncture. Murine PPP, PRP, and PDP were prepared from the American Red Cross (Washington, DC) by the method of Castellino et al. The temperature was varied. Reactions were terminated by the addition of 5-2251 by the method of Ganrot. α2AP activity was determined based on the depletion of 5-2251 amidase activity in plasma or solutions containing plasmin. In these studies, plasminogen-depleted plasma (PDP) was prepared by chromatography on lysine-Sepharose. The total protein concentration in PDP was decreased by <20% as compared with PPP. The concentrations of α2M and α2AP were decreased proportionately in PPP (10% to 15% decrease), and the concentration of plasminogen was decreased by >95%. Platelets and whole blood cells from a type O Rh-negative donor were washed extensively and reconstituted in PDP.

Fresh whole blood was collected from anesthetized CD-1 female mice (Charles River Labs, Boston) by cardiac puncture or retroorbital venous puncture. Sodium citrate was used as an anticoagulant. Experimental results were not affected by the method of blood collection or by the heparin associated with the capillary tubes used for retroorbital venous puncture. Murine PPP, PRP, and PDP were prepared as described above. The concentrations of α2M, α2AP, and plasminogen in murine PDP were equivalent to the levels in human PDP.

Radioiodination. Human plasminogen was radioiodinated using iodobeads as described by the manufacturer. Desalting was performed on Sephadex G-25. Specific activities ranged between 0.3 and 0.5 μCi/μg.

Kinetic analyses. SkPI was prepared by incubating radiiodinated human plasminogen with a twofold molar excess of streptokinase for 20 minutes at 37°C. The SkPI was analyzed by SDS-gel electrophoresis after denaturation in the presence of reductant (dithiothreitol, 40 mmol/L). The single polypeptide chain of plasminogen, mol wt ~90,000, was entirely converted into the two-chain form characteristic of plasmin. This result indicates that the SkPI studied in this investigation was uniformly streptokinase-plasmin.

SkPI (0.2 μmol/L) was reacted with a sixfold molar excess of α2AP in 20 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.4, at variable temperatures. At various times, aliquots of the reaction mixture were removed and the SkPI was inactivated with 0.1 mmol/L PNPGB. Samples were denatured without reductant and subjected to SDS-polyacrylamide gel electrophoresis on 5% slabs using the imidazole-HEPES (pH 7.3) buffer system described by McLellan as modified by Goni et al. All gels were autoradiographed, and each lane of the dried gel was sliced into 3-mm sections and counted in an LKB model 1275 Minigamma γ counter (counting efficiency for 125I >75%). This procedure quantitates the plasmin in each gel band. Apparent second-order rate constants (kcat) were calculated using the basic equation:

\[ k_{cat} = \frac{1}{[I]_0 - [I]_t} \ln \left( \frac{[E]_t}{[E]_0} \right) \]

with [I]0 and [I]t the initial concentrations of inhibitor and enzyme, respectively, and [E] the concentration of enzyme at time t. All experiments were performed in duplicate, and the results were averaged.

Proteinase inhibitor distribution experiments. SkPI or plasmin was prepared with radiolabeled plasminogen and incubated with various combinations of purified inhibitors, plasma, or modified plasma. Components were diluted in 20 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.4, and incubated for 20 minutes unless otherwise specified. The temperature was varied. Reactions were terminated with 0.1 mmol/L PNPGB and analyzed using the electrophoresis-autoradiography procedure described above. All experiments were performed at least in duplicate.

RESULTS

Effect of Temperature on the Regulation of Plasmin in Human Plasma

Plasmin was incubated with variably diluted PPP at 4, 22, and 37°C. Solutions were then equilibrated at 22°C, and residual plasmin activity was determined using 0.8 mmol/L S-2251. In this system, substrate hydrolysis is catalyzed by the free proteinase and by plasmin bound to α2M at nearly equal rates; the kcat and Km values are 11.8 s⁻¹ and 0.2 mmol/L for plasmin and 8.5 s⁻¹ and 0.21 mmol/L for α2M-plasmin under these exact conditions. Figure 1 shows that when the quantity of α2AP in the PPP was sufficient to inhibit the added plasmin, amidase activity was decreased by 90% to 95%. The residual activity most likely represented plasmin bound to α2M. Temperature did not affect the distribution of the plasmin between α2AP and α2M as determined by this method.

Equivalent experiments were performed with radioiodinated plasmin. Reaction with proteinase inhibitors in PPP was studied at different temperatures using the somewhat more sensitive electrophoresis procedure (Fig 2). The relative mobilities of plasminogen, α2AP-plasmin, and α2M-plasmin are shown in the insets (Fig 2). Approximately 80% to 90% of the plasmin that reacts with α2M forms a covalent bond. This material distributes between two bands representing plasmin bound to two α2M subunits or four α2M subunits. A small correction factor was applied uniformly to the data to account for the fraction of noncovalent α2M-plasmin complex. At each of the temperatures studied, all of the active
variably diluted PPP in mm (active enzyme concentration: 75 nmol/L) was incubated with 225.

was calculated assuming that the plasma concentration of a2AP is 25°C (C) or 37°C (D). Estimated molar ratio of a2AP to plasmin l5 was uniformly predominant, as was detected by sub-

strate assay. A slight decrease in the ability of a2M to compete for proteinase was observed at 4°C; however, the plasmin bound to a2M was much less than 10% at all temperatures.

Regulation of SkPl by Human Plasma Proteinase Inhibitors

The reaction of SkPl with purified a2AP was temperature dependent as expected. Data are summarized in Table 1. The kapp determined at 25°C was midway between the two previously reported values of 0.8 mol/L s" and 1.4 x 102 mol/L s" at 4°C. The reaction rate at 37°C was >150-fold higher than the rate at 4°C.

The reaction of radioiodinated SkPl with proteinase inhibitors was studied in partially diluted PDP (Fig 3). The extent of reaction was markedly temperature dependent. At all temperatures, the major inhibitor of SkPl was a2AP. The distribution of radiolabeled proteinase between a2AP and a2M after incubation at 37°C approximated that observed in the plasmin experiments.

Platelets or whole blood cells were reconstituted in PDP as described. The radioiodinated SkPl distribution experiments were then repeated. The rate of reaction of the SkPl with the proteinase inhibitors and the fractions associated with a2AP and a2M remained unchanged.

Experiments performed with purified proteins confirmed that SkPl reacts preferentially with a2AP at 37°C (Fig 4). a2M–proteinase complex was recovered at significant levels only when a2AP was not included in the incubation. At 4°C, most of the SkPl did not react with either proteinase inhibitor. Table 2 summarizes the data. Equivalent results were obtained when SkPl was formed with unpurified Kabikinase mixture.

Temperature-dependent dissociation of SkPl to regenerate active plasmin was considered as a possible explanation for the reaction of the enzyme with a2AP. Many previous studies indicate that dissociation does not occur.14,21 Studies of the murine system presented below strongly support non-dissociation. As additional evidence, fibrinogenolysis experiments

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>kapp (mol/L s&quot; )</th>
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<tr>
<td>37</td>
<td>1.5 x 102</td>
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<tr>
<td>25</td>
<td>2.3 x 102</td>
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<tr>
<td>15</td>
<td>3.4</td>
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Table 1. Apparent Second-Order Rate Constants for Reaction of SkPl with a2AP

![Fig 1. Plasmin activity after addition to human plasma. Plasmin (active enzyme concentration: 75 nmol/L) was incubated with variably diluted PPP in 20 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.4, for ten minutes. Incubation temperature was 4°C (C), 25°C (D), or 37°C (D). Estimated molar ratio of a2AP to plasmin was calculated assuming that the plasma concentration of a2AP is 1.0 μmol/L. Residual plasmin activity was determined with S-2251.](image1)

plasmin reacted with the proteinase inhibitors. The role of a2AP was uniformly predominant, as was detected by substrate assay. A slight decrease in the ability of a2M to compete for proteinase was observed at 4°C; however, the plasmin bound to a2M was much less than 10% at all temperatures.

Fig 1. Plasmin activity after addition to human plasma. Plasmin (active enzyme concentration: 75 nmol/L) was incubated with variably diluted PPP in 20 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.4, for ten minutes. Incubation temperature was 4°C (C), 25°C (D), or 37°C (D). Estimated molar ratio of a2AP to plasmin was calculated assuming that the plasma concentration of a2AP is 1.0 μmol/L. Residual plasmin activity was determined with S-2251.

![Fig 2. Covalent binding of plasmin to proteinase inhibitors in human plasma. 125I-Plasminogen was activated with 5 U urokinase for ten minutes at room temperature and then treated with antiurokinase antibody. Activation was 97% to 98% complete. The plasminogen (0.17 μmol/L) was incubated with variably diluted PPP for ten minutes at 4°C (C), 25°C (D), or 37°C (D). Samples were analyzed by electrophoresis, autoradiography, and gel slicing. Closed symbols: percentages of a2AP-125I-plasmin. Open symbols: percentages of a2M-125I-plasmin. Insets a and b show autoradiography of unreacted plasmin (PI) and plasmin incubated at 37°C with PPP at an estimated molar ratio of a2AP to plasmin of 0.8, respectively.](image2)

![Fig 3. Reaction of SkPl with proteinase inhibitors in human plasma. SkPl (0.17 μmol/L) was formed with 125I-plasminogen and incubated with PDP diluted into 20 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.4, for 30 minutes at 4, 25, and 37°C. Estimated molar ratios of a2AP to SkPl were calculated for each incubation mixture assuming that the concentration of a2AP in plasma is 1.0 μmol/L. Reactions were terminated with PNPGB and analyzed by electrophoresis. Percentage of recovery of total radioactive plasmin is shown for a2AP-plasmin (C) and a2M-plasmin (D) after incubation at 37°C; a2AP-plasmin (C) and a2M-plasmin (D) after incubation at 25°C; and a2AP-plasmin (D) after incubation at 4°C.](image3)
Fig 4. Reaction of SkPI with purified human α2M and α2AP. SkPI (0.2 μmol/L) was formed with 125I-plasminogen and incubated for 30 minutes in 20 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.4, at 37°C. Included in the incubation mixture was: (a) no other protein; (b) a threefold molar excess of α2M; (c) a threefold molar excess of α2M and a threefold molar excess of α2AP; (d) a threefold molar excess of α2M and a sixfold molar excess of α2AP; and (e) a threefold molar excess of α2M and a ninefold molar excess of α2AP. Samples were subjected to electrophoresis and analyzed by autoradiography.

were performed (Fig 5). In these experiments, SkPI was incubated with plasminogen-depleted fibribogen at 4 or 37°C. Terminal plasmin fibribogen digestion products were not generated by the SkPI. The faint bands with slightly increased mobility as compared with fibribogen indicate that the SkPI did cause limited proteolysis; however, this change was comparable at 4 and 37°C. That this mild proteolysis represented dissociated plasmin is therefore highly unlikely.

Regulation of SkPI by Murine Plasma Proteinase Inhibitors

When human SkPI was incubated with murine plasma, significant reaction with α2M was observed (Fig 6). Radioactive α2M-plasmin was recovered in bands with apparent mol wts of 420,000 and >700,000, which corresponded closely with the human α2M-plasmin bands. The reaction was temperature dependent; however, even at 4°C, nearly 40% of the SkPI formed complex with α2M (Table 3). At 37°C, <20% of the radioactivity was recovered in the free plasmin band. Because these data are not corrected for the fraction of α2M-plasmin that lacks a covalent bond, the reaction at 37°C may have been essentially complete.

α2AP competed poorly with α2M when SkPI was added to the murine plasma. This result was independent of temperature. Extraction of the plasminogen from murine plasma by chromatography on lysine-Sepharose did not affect either the extent of reaction of SkPI with the inhibitors or the distribution between α2AP and α2M. Equivalent results were also obtained when SkPI was incubated with murine PRP or whole blood.

<table>
<thead>
<tr>
<th>Table 2. Reaction of SkPI With Purified Human α2AP and α2M</th>
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<tr>
<td>Concentration (μmol/L)</td>
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<tr>
<td>SkPI</td>
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Incubation time 30 minutes.

Fig 5. Fibrinogenolysis by SkPI in vitro. Fibrinogen was depleted of plasminogen by chromatography on lysine-Sepharose and subjected to electrophoresis in lane a. The same fibrinogen (1.3 μmol/L) was incubated with SkPI (0.1 μmol/L) at 4°C (lane b) or 37°C (lane d) for 30 minutes. Lane C shows fibrinogen after incubation with 0.1 μmol/L plasmin.

Fig 6. Reaction of SkPI with proteinase inhibitors in murine plasma. SkPI was formed with 125I-human plasminogen and incubated with diluted murine plasma for 20 minutes. The concentration of SkPI was 0.1 μmol/L. The incubation temperatures and plasma dilution factors were lane b, 4°C, 1:10; lane c, 4°C, 2:5; lane d, 25°C, 1:10; lane e, 25°C, 2:5; lane f, 37°C, 1:10; lane g, 37°C, 2:5. Lane a shows purified human α2M reacted with SkPI at 37°C as a control. Reactions were analyzed by electrophoresis and autoradiography.
Table 3. Reaction of SkPl With Proteinase Inhibitors in Murine Plasma

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<th>Temperature (°C)</th>
<th>Plasma Dilution Factor</th>
<th>Recovered 125I-plasmin (%)</th>
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<tr>
<td></td>
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<td>Free α2AP-Pl</td>
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<tr>
<td>4</td>
<td>1:10</td>
<td>81</td>
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<td>4</td>
<td>2:5</td>
<td>56</td>
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<td>25</td>
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<td>37</td>
<td>1:10</td>
<td>20</td>
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<td>37</td>
<td>2:5</td>
<td>18</td>
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Concentration of SkPl was 0.1 μmol/L. All incubations were for 20 minutes.

†Values are not corrected for fraction of noncovalent α2M-plasmin.

Experiments were performed to evaluate the role of α2AP as a fibrinolysis inhibitor in murine plasma (Fig 7). Human 125I-plasminogen was activated with catalytic amounts of urokinase or streptokinase and incubated with PPP. Reaction occurred nearly exclusively with α2AP (>95%) confirming previous in vivo studies. Murine plasma was then diluted 1:2 with a solution of 400 mmol/L methylamine, 20 mmol/L Tris-HCl, pH 8.0, and incubated at room temperature for 40 minutes to inactivate the α2M. A second aliquot of plasma was treated similarly except that methylamine was omitted. SkPl reacted readily with α2M and minimally with α2AP in the control plasma. In methylamine-treated plasma, significantly enhanced reaction of SkPl with α2AP was detected. These data suggest that the predominant role of murine α2M in regulation of human SkPl reflects enhanced reactivity with α2M and not decreased reactivity with α2AP.

DISCUSSION

α2AP is a member of the serpin proteinase inhibitor family that includes α2-proteinase inhibitor and antithrombin-III. The reaction of α2AP with plasmin involves at least two steps: a very rapid noncovalent association step followed by covalent bond formation. The active-site serine in plasmin is unable to deacylate after reaction with the α2AP active-site Arg-Met peptide bond.

The reaction of α2AP with proteinase involves a mechanism very different from other inhibitors. Each of the four identical α2M subunits has a centrally located sequence of peptide bonds that is highly susceptible to proteolysis. Reaction of a proteinase with one of these peptide bonds proceeds unhindered through the step of deacetylation; however, the cleavage causes a major conformational change in the α2M that traps the proteinase. Covalent bonds form variably between proteinase lysine groups and specialized thioester bonds in each α2M subunit. The α2M–proteinase complex is irreversible, even when no covalent bonds form.

Based on the difference in reaction mechanism, different factors evidently affect the reactivity of a proteinase with α2AP and α2M. One important consideration in α2M reactivity is proteinase size. Reaction of SkPl with human α2M occurs through facilitated transfer of the plasmin from streptokinase to the inhibitor. The dissociation of the streptokinase in this reaction is probably necessary because the α2M proteinase binding sites cannot accommodate the extensive mass of the entire SkPl complex. A high-energy intermediate involving a temporarily strained SkPl–α2M complex was proposed to explain the temperature dependence of the SkPl–α2M reaction. The importance of temperature helped reconcile reported in vitro studies of the SkPl–human α2M reaction; however, the extremely fast reaction of human SkPl with murine α2M in vivo remained incompletely explained. The data we present demonstrate markedly enhanced reactivity of SkPl with murine α2M as compared with human α2M. This enhanced reactivity was evident in plasma and did not require cell-surface cofactor activity. The SkPl–murine α2M reaction was temperature dependent; however, significant levels of reaction were detected even at 4°C.

With an electrophoresis procedure, second-order rate constants were derived for the reaction of purified human α2AP with SkPl. The value derived at 25°C (23 mol/L 1 s⁻¹) was midway between two previously reported values determined at the same temperature with a substrate assay. The kinetics experiments were performed to demonstrate the marked effect of temperature on the SkPl–α2AP reaction. The constants were calculated based on covalent product which actually forms in the second step of the reaction following noncovalent association. The previously used substrate assays did not require covalent binding to detect complex. Because the rate constants we report are not significantly lower than the previous values, formation of an SDS-stable covalent bond is not the rate-limiting step in the α2AP–SkPl reaction.
The ratio of recovered $\alpha_2$AP-135I-plasmin to $\alpha_2$M-135I-plasmin remained nearly constant when SkPI was incubated in human plasma at different temperatures. This result suggests that increased temperature enhances the rate of reaction of SkPI with both inhibitors comparably.

The reaction of SkPI with $\alpha_2$M in mouse plasma was essentially complete. The reaction of SkPI with human $\alpha_2$AP was not. This result was observed in experiments with purified inhibitors and in human plasma. The initial rates of reaction between human $\alpha_2$AP and SkPI followed the principles of second-order kinetics closely. After slightly more than 50% of the SkPI had reacted with $\alpha_2$AP, the rate of complex formation decreased. These data may reflect heterogeneity in the SkPI preparation. Such heterogeneity could result from the internal digestion of streptokinase in streptokinase-plasmin complex.\textsuperscript{18} The data we present suggest that murine $\alpha_2$M is unique in its ability to bridge any heterogeneity in the SkPI and react with the entire preparation.

Muninglobulin is a proteinase inhibitor found in murine plasma but not in human plasma.\textsuperscript{39} Proteinases that bind to

murinoglobin retain esterase activity, a property attributed to the $\alpha$-macroglobulin class of inhibitors. The mol wt of murinoglobin is 180,000. An SDS-stable complex of murinoglobin with plasmin would have a maximum mol wt of 260,000. The complexes formed during incubation of SkPI with murine plasma were significantly larger than the hypothetical murinoglobin–plasmin complex. We therefore conclude that murinoglobin does not contribute to the regulation of SkPI in the mouse.

Plasminogens from different species differ significantly in ability to bind streptokinase and form activator complex.\textsuperscript{3,12,40} The SkPI complexes that do form demonstrate widely variable activity. In contrast, studies of the proteinase inhibitors, $\alpha_2$M and $\alpha_2$AP, have generally demonstrated conservation of function across species lines.\textsuperscript{32} The reaction of $\alpha_2$AP with SkPI is an exception to the rule, perhaps because SkPI is not a natural mammalian proteinase. We conclude that the thrombolytic activity of streptokinase in a given species can reflect variability in proteinase inhibitors as well as plasminogen.

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