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A Battery of Monoclonal Antibodies That Induce Unique Conformations to Evolve Cryptic but Constitutive Functions of Plasminogen[1]

Seiji Madoiwa,* Koichi Arai,* Yasunobu Ueda,† Masahiro Ishizuka,† Jun Mimuro,* Shinji Asakura,* Michio Matsuda,* and Yoichi Sakata*,[2]

*Division of Hemostasis and Thrombosis, Institute of Hematology, Jichi Medical School, Minamikawachi-machi, Tochigi 329-04; and the †Research Biochemist, Cosmo Research Institute, Saitama 340-01

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Two groups of anti-plasminogen monoclonal antibodies, whose epitope was either in the kringle 1+2+3 domain (F3P2, F11P5, F11P6, and F12P18) or the kringle 5 domain (F1P6 and F12P16), were isolated and their effects on the conformation of plasminogen were explored. All antibodies except F1P6 had 3- to 10-fold higher affinity toward Lys-plasminogen than Glu-plasminogen. F1P6 exhibited a comparable affinity to Glu- and Lys-plasminogen. Among these, only F11P5 binding was inhibited by ε-amino-n-caproic acid (EACA) in a concentration-dependent manner, with half maximal inhibition at 3 mM. From a competition assay, we concluded that the epitopes of F11P5, F11P6, and F12P18 should be very close, and located at or near the low affinity lysine binding site on the kringle 2+3. These three antibodies dramatically enhanced the binding of Glu-plasminogen to the other antibodies, except to F1P6. Interestingly, F3P2, whose non-overlapping epitope was in the kringle 2+3 domain, also augmented the binding of Glu-plasminogen to the other antibodies. In contrast, we did not observe enhanced binding of Lys-plasminogen to one antibody in the presence of the other antibodies, and the binding of Glu-plasminogen to these antibodies did not increase in the presence of 10 mM EACA. In the presence of these antibodies, including F1P6, Glu-plasminogen bound more efficiently to immobilized degraded fibrin, with a binding profile similar to Lys-plasminogen. All antibodies except F1P6 enhanced the conversion rate of plasminogen to plasmin remarkably. Taken together, we propose that these two groups of monoclonal antibodies can dissociate the intramolecular interactions of Glu-plasminogen and induce the conformational transition of Glu-plasminogen to Lys-plasminogen. In addition, the kringle 2+3 and kringle 5 structures of Glu-plasminogen liganded with EACA are distinct from the Lys-plasminogen structure.

Key words: conformation, fibrin, kinetics, monoclonal antibody, plasminogen.

Plasminogen is the inactive precursor of the serine protease, plasmin, that plays a key role in fibrinolysis, cell migration (*1*), and tissue remodeling (*2*). Native plasminogen, Glu-plasminogen (Glu-Plg), is a single-chain polypeptide of 791 amino acids consisting of an NH_2-terminal glutamic acid, followed by an amino terminal peptide, five tandem structures called "kringle" domains, an activation cleavage site (Arg-561 and Val-562), and a trypsin-like serine protease domain (*3*). It has been demonstrated by neutron scattering analysis that Glu-plasminogen exhibits a very tight spiral structure bringing the amino terminal peptide in close contact with the protease domain (*4*). Glu-plasminogen contains two classes of binding sites with respect to affinity for EACA: one high affinity site with a K_d of 9 μM, and possibly four or five low affinity sites with a K_d of 5 mM (*5*). The binding of lysine and its analogues to the low affinity lysine binding sites of Glu-plasminogen results in the transition from a closed to an open conformation (*4, 6*). A similar change is observed when the amino terminal fragment is removed from Glu-plasminogen by plasmin cleavage to yield Lys-plasminogen with lysine, valine, or methionine as the NH_2-terminal amino acid (*7*).

The interaction between Glu- or Lys-plasminogen and fibrin is mediated through lysine binding sites on the plasminogen. It has been suggested that when Glu-plasminogen binds to intact fibrin, the primary interaction is likely to be between the aminohexyl site of kringle 5 (*8*) and a fibrin intra-chain lysine residue (*9*). In the process of fibrinolysis, plasmin-degraded fibrin generates a number of carboxyl terminal lysine residues that serve for lysine

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[2] To whom correspondence should be addressed. Tel: +81-285-44-2111, Fax: +81-285-44-7817

Abbreviations: Glu-Plg, Glu-plasminogen, native plasminogen with NH_2-terminal glutamic acid (Glu_1-Asn_{791}); Lys-Plg, Lys-plasminogen, proteolytically modified form of plasminogen, predominantly residues Lys_{78}-Asn_{791} with NH_2-terminal lysine; K, kringle; K1+2+3, kringle 1+2+3 of plasminogen (Tyr_{80}-Val_{338} and Tyr_{80}-Val_{354}); mini-Plg, kringle 5 and serine protease domain of plasminogen (Val_{443}-Asn_{791}); NTP, NH_2-terminal peptide of Glu-plasminogen, predominantly residues Glu_1-Lys_{78}; u-PA, urokinase type plasminogen activator; EACA, ε-amino-n-caproic acid (6-aminohexanoic acid).

binding sites on kringle 1 and kringle 4, which exhibit high and moderate affinity for EACA, respectively (*8*). The lysine binding site on kringle 4, that is shielded in Glu-plasminogen due to partial blockage of this site by kringle 1+2+3 (*10, 11*), becomes exposed in Lys-plasminogen with great enhancement of fibrin affinity (*7, 12*). It has also been suggested that the compact form of Glu-plasminogen masks its activation cleavage site from access by plasminogen activators (*6*). Both Glu-plasminogen, bound with EACA and its analogues sufficiently enough to saturate low affinity lysine binding sites, and Lys-plasminogen, show accelerated conversion to plasmin, because the conformational change to an open form exposes the activation cleavage sites for plasminogen activators (*13, 14*).

It is difficult to evaluate separately the role of each lysine binding site on plasminogen conformation, since each site is equivalent in the interaction with lysine and its analogues. Castellino and co-worker showed that a monoclonal antibody directed against a single low affinity EACA site on kringle 4 accelerated the activation of Glu-plasminogen (*15*). In the present study we demonstrate that some of our monoclonal antibodies to kringle 2+3 and kringle 5 of plasminogen induce a change in the conformation of Glu-plasminogen which is similar to that in the case of Lys-plasminogen.

MATERIALS AND METHODS

Materials—Materials were purchased from the following sources: ethylenediaminetetraacetic acid (EDTA), dithiothreitol, (*p*-aminophenyl)methane-sulfonyl fluoride hydrochloride (pAPMSF), and porcine pancreatic elastase from Wako Pure Chemical Industries, Osaka; Tween 20, bovine serum albumin (BSA), and human thrombin from Sigma, St. Louis, MO, USA; bisacrylamide, ammonium persulfate, *N,N,N′,N′*-tetramethylenediamine, 6-aminohexanoic acid, and a 25% (v/v) aqueous solution of glutaraldehyde from Nacalai Tesque, Kyoto; chromogenic substrate, H-D-Val-Leu-Lys-pNA (S-2251), from Kabi Vitrum AB, Stockholm, Sweden; and CNBr-activated Sepharose 4B and Sephacryl S-200 HR from Pharmacia Biotech, Uppsala, Sweden. Aprotinin and urokinase type plasminogen activator were kind gifts from Mochida Pharmaceutical, Tokyo. All other chemicals were of the best reagent grade commercially available.

Plasminogen and Its Derivatives—Human Glu-plasminogen was purified from fresh frozen plasma using lysine-Sepharose and Ultrogel ACA 44 (IBF Biotechnics, Villeneuve-la Garenne, France) as described previously (*16*). Lys-plasminogen was generated by limited proteolysis of Glu-plasminogen with plasmin-immobilized Sepharose 4B (*17*). Both plasminogens were considered to be more than 99% pure, as judged on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), autoradiography of the ^{125}I-labeled products, and amino terminal sequence analysis. NH_2-terminal sequence analysis by Edman degradation revealed only glutamic acid for Glu-plasminogen, and lysine, valine, or methionine for Lys-plasminogen. Plasmin was generated with two-chain u-PA-coupled Sepharose as described (*18*). Kringle 1+2+3, kringle 4, and mini-plasminogen were prepared by limited proteolysis of plasminogen with porcine pancreatic elastase, followed by separation of the resulting fragments by gel filtration on a Sephacryl S-200 HR column and affinity chromatography on lysine-Sepharose 4B essentially as described previously (*19*). Protein concentrations were determined spectrophotometrically using the following $E^{1\%}_{1cm}$ at 280 nm values and molecular weights: Glu-Plg, 17.0 and 92,000 (*20, 21*); Lys-Plg, 17.0 and 84,000 (*22*); and mini-Plg, 16.0 and 38,000 (*19*). The $E^{1\%}_{1cm}$ at 280 nm value used for kringle 1+2+3 was 8.08×10^4 $M^{-1}\cdot cm^{-1}$ (*11*). Kringle 1+2+3 was subsequently subjected to limited proteolysis with endoproteinase Glu-C from *Staphylococcus aureus* V8 (Boehringer Mannheim, GmbH, Germany) to yield kringle 1 and kringle 2+3 as described (*23*) with the following modifications: the enzyme/substrate ratio was 1:30 and the digestion was allowed to proceed for 18 h at 37°C.

Fibrinogen—Fibrinogen was purified as described previously (*24*). Briefly, fresh human plasma was passed through lysine-Sepharose and gelatin-Sepharose columns at 25°C. Then fibrinogen was precipitated from the plasma by 25% (w/v) ammonium sulfate saturation. The fibrinogen fractions were dissolved in appropriate volumes of 0.3 M NaCl, which were extensively dialyzed against the same solution and stored frozen in small aliquots at −80°C until used. Protein concentrations were determined using an $E^{1\%}_{1cm}$ at 280 nm of 15.1 (*25*).

Radioiodination of Plasminogen and Its Derivatives—Glu- and Lys-plasminogen, and the kringle 1+2+3 fragment of plasminogen were radiolabeled with $Na^{125}I$ using IODOBEADS (Pierce, Rockford, IL, USA) according to the manufacturer's procedure with the following modifications (*26*). Protein [200 μg in 0.05 M Tris-HCl, pH 7.4, 0.14 M NaCl (TBS)] and 200 μCi of the radioisotope were added to the reaction tube in the presence of a bead coated with Iodogen. After iodination for 15 min at 25°C, the labeled protein was separated from free $Na^{125}I$ by gel filtration on an Ampure SA column (Amersham, Buckinghamshire, England). The specific activities obtained were 1.65×10^6 cpm/μg for Glu-plasminogen, 1.36×10^6 cpm/μg for Lys-plasminogen, and 1.85×10^6 cpm/μg for the kringle 1+2+3 fragment.

Preparation of Fibrin Surfaces—Solid-phase fibrin was prepared as previously described (*27*). In brief, polyvinylchloride-bound stable polyglutaraldehyde derivatives were first produced by treating microtitration polyvinyl chloride plates with 50 μl/well of 2.5% (v/v) glutaraldehyde in 0.1 M sodium bicarbonate buffer, pH 9.5, for 2 h at 22°C. Fibrinogen (50 μl/well, 0.3 μM in 0.1 M sodium phosphate buffer, pH 7.4, containing 1 mM $CaCl_2$) was then covalently fixed for 18 h at 4°C. Unreacted fibrinogen was discarded, and the plate was transformed into a fibrin surface by treatment with 50 μl/well of human thrombin (10 NIH units/ml) in 0.05 M sodium phosphate buffer, pH 7.4, containing 0.08 M NaCl, 0.1% (w/v) BSA, 0.01% (v/v) Tween 20, and 1 mM $CaCl_2$ at 37°C for 1 h. The excess thrombin was eluted by three washes with 0.5 M NaCl and 0.05% (v/v) Tween 20. The wells were washed with 5 mM sodium phosphate buffer, pH 6.8, containing 0.05% (v/v) Tween 20, and stored at 4°C in 100 μl/well of 50 mM sodium phosphate buffer, pH 6.8, 0.08 M NaCl, and 0.1% (w/v) BSA until use.

Preparation of Degraded Fibrin Surfaces—A microtitration plate containing a solid-phase fibrin surface prepared as described above was washed three times with 0.05 M sodium phosphate buffer, pH 7.4, containing 0.08 M NaCl,

0.1% (w/v) BSA, and 0.01% (v/v) Tween 20 (assay buffer). A solution of 25 nM plasmin in assay buffer (50 μl/well) was then incubated with the fibrin surface for 1 h at 37°C. The fluid phase with the plasmin was discarded, and then the surface was washed three times with assay buffer and finally incubated for 24 h at 25°C with assay buffer containing 0.2 M EACA and 50 units/ml aprotinin. The surface was washed three times with assay buffer, and then the absence of plasmin amidolytic activity was verified by adding 100 μl/well of 1 mM chromogenic substrate, S-2251, in assay buffer, followed by measurement of the absorbance at 405 nm after 24 h incubation at 37°C.

Preparation of Antiplasminogen Monoclonal Antibodies—BALB/c mice were immunized three times intraperitoneally with affinity-purified Glu-plasminogen. Fusion of spleen cells with myeloma cells, and hybridoma isolation were performed as described previously (*28*). Antibody-secreting hybridomas were selected using an ELISA technique with the purified antigens adsorbed to the surface of the plate. The selected hybridomas were cloned by limiting dilution and retested for antibody production. The subclones were grown in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (Filtron Pty Ltd, Brooklyn, Australia), and then in the abdominal cavities of pristane-primed BALB/c-mice (*29*). The antibodies were purified from ascites fluid by adsorption to a Protein A agarose column (BioRad Laboratories, Richmond, Canada), elution with 0.1 M citrate buffer, pH 5, and immediate collection in 1 M Tris base, pH 11, to neutralize the solution. The purified monoclonal antibodies were dialyzed against 0.05 M sodium phosphate buffer, pH 7.4, and 0.14 M NaCl (PBS), and then stored at 4°C. For preparation of $F(ab')_2$ fragments from IgG, the monoclonal antibodies, at 5 mg/ml in 0.1 M sodium acetate, pH 5.5, containing 3 mM EDTA, were digested with 5 μg of pepsin from porcine gastric mucosa (Boehringer Mannheim) at 37°C for 12 h. Then the $F(ab')_2$ fragments were separated by ion-exchange and gel filtration as described previously (*30*).

Antibody Binding Assay—The apparent dissociation constants ($K_{d\,app}$) of ^{125}I-labeled Glu-plasminogen, Lys-plasminogen, and the kringle 1+2+3 fragment for antiplasminogen monoclonal antibodies were assessed by means of a solid-phase assay. A 96-well Immuno Plate I (Nunc, Roskilde, Denmark) was incubated with 5 μg/ml monoclonal antibodies in PBS at 4°C for 18 h. The wells were blocked by incubation with TBS containing 1% (w/v) BSA at 37°C for 2 h. The wells were then washed three times with TBS containing 0.02% (v/v) Tween 20 and 0.1% (w/v) BSA (TBS-Tween-BSA). Then various concentrations (0-200 nM) of ^{125}I-labeled Glu-plasminogen, Lys-plasminogen, and the kringle 1+2+3 fragment in TBS-Tween-BSA were added to the coated wells, and followed by incubation at 37°C for 3 h. The solution was removed by aspiration, and the wells were washed three times with TBS-Tween-BSA. After the final wash, each well was removed and subjected to γ-counting in an autowell gamma counter ARC-2000 (Aloka, Tokyo). Control wells were coated with only BSA and the resultant background counts (60 to 80 cpm) were subtracted from all total binding counts. The bound concentrations of plasminogen and its derivatives, and the dissociation constants were calculated as described (*31*).

Competition between Antibodies for Plasminogen—Polyvinyl microtiter plates were coated with antiplasminogen monoclonal antibodies (5 μg/ml) at 4°C for 18 h. After washing, the plates were blocked with 1% (w/v) BSA in TBS at 25°C for 3 h, and then washed three times with TBS-Tween-BSA. ^{125}I-labeled Glu- or Lys-plasminogen was added to the wells and a subsaturating level of bound plasminogen was determined for each monoclonal antibody. An excess of each monoclonal antibody (50 and 500 nM) was added with a constant ^{125}I-labeled Glu- or Lys-plasminogen concentration (5 nM) to the antibody-coated plates, followed by incubation at 37°C for 3 h. After washing five times with TBS-Tween-BSA, the wells were separated and the bound radioactivity determined. The maximal binding of both ligands was similar, with 5,000-8,000 cpm bound for ^{125}I-Glu-plasminogen and 4,000-7,000 cpm bound for ^{125}I-Lys-plasminogen. The results are expressed as percentages of ^{125}I-labeled Glu- or Lys-plasminogen bound in the absence of competing liquid phase antibodies.

Binding of Plasminogen and Its Derivatives to Fibrin Surfaces—^{125}I-labeled Glu- or Lys-plasminogen (0-1,000 nM) was preincubated in the presence or absence of a 10-fold molar excess of monoclonal antibodies at 37°C for 3 h in TBS-Tween-BSA containing 50 units/ml aprotinin (binding buffer). The solutions were then incubated at 4°C for 18 h with intact or degraded fibrin surfaces prepared in microtiter plates as described above. Each supernatant was collected, the surface was washed three times with binding buffer, and then the extent of binding was determined by counting the radioactivity in the wells with a γ-radiation counter. The amount of plasminogen bound to the surface of intact or degraded fibrin was calculated by dividing the radioactivity in each well by the specific activity (cpm/mol) of the plasminogen added. Non-specific binding of ^{125}I-labeled plasminogen to the fibrin surfaces was observed in similar experiments in the presence of a 100-fold molar excess of cold ligand or 25 mM EACA.

Amidolytic Activity Measurement—The effects of antiplasminogen monoclonal antibodies on Glu- and Lys-plasminogen activation by u-PA were monitored as follows: Glu- or Lys-plasminogen was incubated with a 2-fold molar excess of each monoclonal antibody or pre-immune mouse IgG at 37°C for 3 h in TBS-Tween-BSA. The reaction mixture for plasminogen activation consisted of 1 μM plasminogen, 2 μM monoclonal antibody, 1 mM chromogenic substrate S-2251, and 2 nM u-PA in TBS-Tween-BSA. The generation of plasmin was monitored continuously by measuring the absorbance at 405 nm at 25°C (*32*).

Determination of Kinetics Parameters—For kinetic analysis, plasmin or a mixture of plasmin (0.2 μM) and monoclonal antibodies (0.4 μM) was preincubated at 37°C for 30 min, and then incubated with chromogenic substrate S-2251 (0.125-1 mM) at 25°C in TBS-Tween-BSA. The hydrolysis of S-2251 was monitored as described above, and at all concentrations of substrate, <10% of the substrate was utilized. The V_{max} and K_m (apparent Michaelis constant) were determined from a Lineweaver-Burk plot of 1/[substrate (s)] *versus* 1/velocity (v). To determine k_{cat} (catalytic rate constant), the amidolytic activity of a known amount of plasmin toward S-2251 was measured in the absence or presence of monoclonal antibodies (*33*).

Miscellaneous—SDS-PAGE on slab gels was performed with 7.5 or 9% resolving gels and 4% stacking gels according to Laemmli (*34*). Some samples in the gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) by electroblotting, and developed using rabbit antiplasminogen polyclonal antibodies and peroxidase-conjugated anti-rabbit IgG.

NH_2-terminal sequence analyses of plasminogen and its derivatives were performed on samples electroblotted onto PVDF membranes that had been stained with Coomassie Brilliant Blue, and sequenced directly with an Applied Biosystems Inc. Model 476A protein sequencer according to the method previously described (*35*).

RESULTS

Characterization of Monoclonal Antibodies against Plasminogen—Mouse monoclonal antibodies were prepared against intact molecules of human plasminogen and initially characterized by Western blotting. Antibodies F3P2, F11P5, F11P6, and F12P18 bound to both Glu- and Lys-plasminogen, Lys-plasmin, and the kringle 1+2+3 fragment only under non-reducing conditions, whereas antibodies F1P6 and F12P16 bound to Glu- and Lys-plasminogen, the heavy chain of Lys-plasmin, and the kringle 5 fragment of mini-plasmin under both reducing and non-reducing conditions (data not shown). To further identify the epitope recognized by F3P2, F11P5, F11P6, and F12P18, the kringle 1+2+3 fragment was digested with endoproteinase Glu-C to yield kringle 1 and kringle 2+3 fragments (*23*). Endoproteinase Glu-C cleavage generated a distinct set of fragments, with molecular masses ranging from 10 to 55 kDa. When these fragments were examined by immunoblotting with F3P2, F11P5, F11P6, and F12P18 under non-reducing conditions, F3P2 and F11P6 were found to react with 20 and 25 kDa fragments. We sequenced the first 6 residues of these polypeptide band materials, and identified 3 to 5 different amino acid residues in each cycle (data not shown). We determined that the 20 and 25 kDa fragments were both derived from the kringle 2+3 fragment with NH_2-terminal residues of Glu_{164} and Glu_{165}. However, reduction of plasminogen and its derivatives with dithiothreitol resulted in the complete loss of reactivity with these antibodies, suggesting that the conformation plays a role in the formation of the binding epitopes. The binding constants for the ^{125}I-labeled Glu- and Lys-plasminogen as to monoclonal antibodies obtained by Scatchard plot analysis (*31*) are shown in Table I. Monoclonal antibody F1P6 exhibited a similar affinity for Glu- and Lys-plasminogen, whereas the other antibodies had 3- to 10-fold higher affinities for Lys-plasminogen as compared to Glu-plasminogen.

Competitive Binding of Monoclonal Antibodies to Plasminogen—A constant and subsaturating level of ^{125}I-Glu-plasminogen was added simultaneously with various antibodies in a competition assay (Fig. 1). The F3P2 antibody did not compete with the other antibodies for binding to Glu-plasminogen (Fig. 1A). Monoclonal antibodies F11P5 and F12P18 (Fig. 1, B and D) or F11P6 and F12P18 (Fig. 1, C and D) competed with each other for binding to Glu-plasminogen, although their different affinities influenced the binding. Interestingly, the presence of antibodies F3P2, F11P5, and F11P6 enhanced the binding to Glu-plasminogen (Fig. 1, A, B, and C). There was also an augmentation of Glu-plasminogen binding to solid phase antibody when

TABLE I. **Affinity of monoclonal antibodies for Glu- and Lys-plasminogen.**

Monoclonal antibody	Domain[a]	$K_{d\,app}$[b] (nM)	
		Glu-plasminogen	Lys-plasminogen
F3P2	K2+3	14.2	5.2
F11P5	K1+2+3	25.5	5.2
F11P6	K2+3	17.2	3.5
F12P18	K1+2+3	119.7	11.6
F1P6	K5	8.4	8.5
F12P16	K5	48.1	5.8

[a]Domains were determined by immunoblotting technique described under "MATERIALS AND METHODS." [b]For determination of the apparent dissociation constant, a variation of Scatchard analysis of ^{125}I-Glu- and Lys-plasminogen binding was performed as described under "MATERIALS AND METHODS."

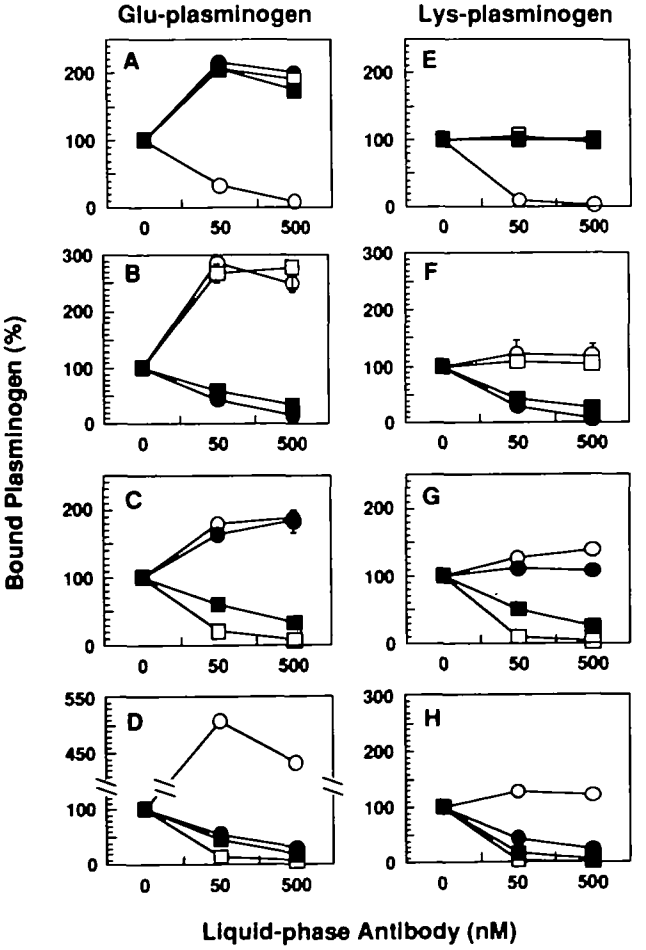

Fig. 1. **Competition between antibodies for binding of ^{125}I-Glu- and Lys-plasminogen.** ^{125}I-Glu- and Lys-plasminogen (5 nM) were incubated with the indicated concentrations of F3P2 (○), F11P5 (●), F11P6 (□), and F12P18 (■). The mixtures were added to wells coated with monoclonal antibodies F3P2 (A and E), F11P5 (B and F), F11P6 (C and G), and F12P18 (D and H) at 4°C for 16 h in TBS-Tween-BSA. After washing, the bound ^{125}I-Glu- or Lys-plasminogen was determined and is shown as a percentage of that bound in the absence of a competing antibody. Error bars indicate standard error of triplicate assays.

F3P2 and F12P18 were assayed (Fig. 1, A and D). In contrast, the binding of Lys-plasminogen did not increase with any combination of monoclonal antibodies (Fig. 1, E-H).

Then we observed the effect of monoclonal antibodies which recognized the kringle 5 domain on the binding of Glu-plasminogen to solid phase antibody F11P5 whose epitope is in the kringle 1+2+3 domain. Antibody F1P6 did not affect the binding of Glu-plasminogen to F11P5 antibody, whereas antibody F12P16 remarkably enhanced the affinity of Glu-plasminogen (Fig. 2A). When monoclonal antibody F1P6, whose affinities for Glu- and Lys-plasminogen are comparable, was used as the solid phase antibody, the binding of Glu-plasminogen was unaltered by the other antibodies. The results obtained with two representative antibodies, F3P2 and F12P16, are shown in Fig. 2B. Additionally, the binding of Lys-plasminogen to the solid phase antibodies did not increase in the presence of F1P6 or F12P16 antibodies (data not shown). With the exception of F1P6, these results suggest that the monoclonal antibodies which recognize two different domains, kringle 1+2+3 or kringle 5, induce a conformational transition in plasminogen from the Glu-form to the Lys-form.

Competition between EACA and Monoclonal Antibodies for Binding to Plasminogen—Since the effect of these monoclonal antibodies on plasminogen is similar to that of EACA on plasminogen, we examined whether the binding regions of these monoclonal antibodies overlap the EACA binding site of plasminogen. As shown in Fig. 3, the binding of Glu-plasminogen to the F11P5 antibody was inhibited by EACA in a concentration-dependent manner, as expected. The binding of Lys-plasminogen and kringle 1+2+3 fragment to this antibody was also inhibited by EACA in a similar manner (data not shown). EACA, however, failed to inhibit the binding of F3P2, F11P6, and F12P18 antibodies. Furthermore, excess EACA, which is supposed to induce a conformational transition of Glu-plasminogen to Lys-plasminogen (*20*), did not enhance the binding of Glu-plasminogen to these monoclonal antibodies (Fig. 3).

Binding to Fibrin Surfaces—The above results suggest that the conformational changes induced in Glu-plasminogen by these antibodies were not the same as those induced by EACA. We thus examined the binding of Glu- and Lys-plasminogen to fibrin in the absence or presence of the antibodies. A solid phase fibrin was used for the binding of plasminogens, and nonspecific binding in the presence of 25 mM of EACA was subtracted to yield values for specific binding. The binding of Glu- and Lys-plasminogen to fibrin surfaces was specific and saturable. For Glu-plasminogen, the B_{max}, maximum binding, and $K_{d\ app}$ values were 0.30 mol/mol fibrin and 0.64 μM, respectively, and for Lys-plasminogen the respective values were 0.26 mol/mol fibrin and 0.66 μM. When immobilized fibrin was digested with plasmin under these experimental conditions (*27*), the binding of Glu-plasminogen to this degraded fibrin surface was dependent on the period of plasmin digestion and reached a plateau within 1 h. Glu-plasminogen binding did not decrease even if fibrin was treated with plasmin for 3 h (data not shown). Figure 4 shows data from the binding of ^{125}I-labeled plasminogens to fibrin surfaces which had been pretreated with plasmin for 2 h. Non-linear, least-squares regression analysis of untransformed raw data fit a simple hyperbolic binding curve for the binding of Glu-plasminogen to degraded fibrin (Fig. 4A, inset). A plot of bound plasminogen/free plasminogen *vs.* bound Glu-plasminogen according to Scatchard analysis was linear, and the B_{max} and $K_{d\ app}$ values were 1.0 mol/mol fibrin and 0.21 μM, respectively (Fig. 4A). In contrast, Lys-plasminogen adsorbed to a plasmin-degraded fibrin surface gave a non-hyperbolic

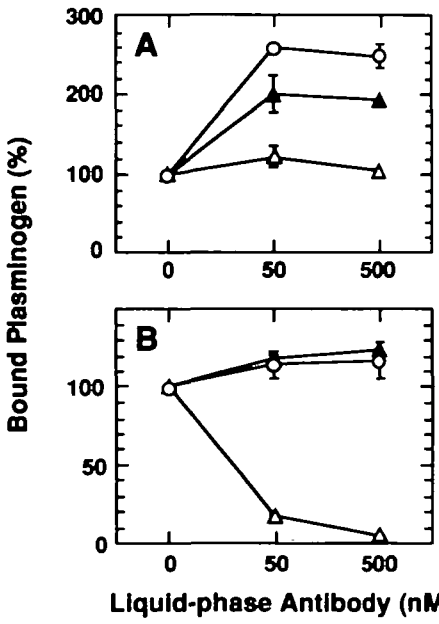

Fig. 2. **Effect of liquid-phase antibodies on the binding of ^{125}I-Glu-plasminogen to solid phase antibodies.** ^{125}I-Glu-plasminogen (5 nM) was incubated with the indicated concentrations of F1P6 (△), F12P16 (▲), and F3P2 (○), and then added to wells coated with monoclonal antibodies F11P5 (A) and F1P6 (B) at 4°C for 16 h in TBS-Tween-BSA. After washing, the bound ^{125}I-Glu-plasminogen was determined and is shown as a percentage of that bound in the absence of a competing antibody. Error bars indicate standard error of triplicate assays.

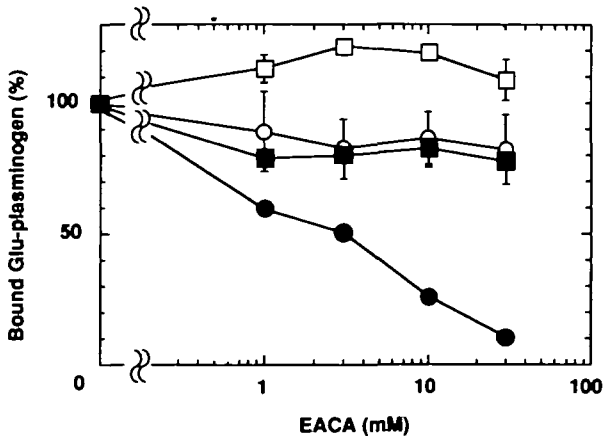

Fig. 3. **Effect of EACA on the binding of ^{125}I-Glu-plasminogen to monoclonal antibodies.** ^{125}I-Glu-plasminogen (5 nM) was added to wells coated with monoclonal antibodies F3P2 (○), F11P5 (●), F11P6 (□), and F12P18 (■) at 4°C for 16 h in the presence of varying concentrations of EACA. The bound ^{125}I-Glu-plasminogen is shown as a percentage of that bound in the absence of EACA. Error bars indicate standard error of triplicate assays.

curve (Fig 4B, inset), and the interaction of Lys-plasminogen with degraded fibrin corresponded to a two-site model of high and low affinity sites (Fig. 4B), with $K_{d\ app} = 0.019$ μM ($B_{max} = 0.49$ mol/mol fibrin) and 0.17 μM ($B_{max} = 0.97$ mol/mol fibrin). Interestingly, when Glu-plasminogen binding was determined in the presence of 10-fold molar excess of F3P2, whose epitope is localized in the kringle 2+3 domain and is independent of the EACA site, the affinity for degraded fibrin remarkably increased with concomitant expression of two sites, similar to Lys-plasminogen binding (Fig. 4C). The $K_{d\ app}$ and B_{max} of the low-affinity site were 0.097 μM and 0.97 mol/mol fibrin, respectively. The $K_{d\ app}$ of the high-affinity site was 0.012 μM ($B_{max} = 0.66$ mol/mol fibrin). Monoclonal antibody F1P6, which recognizes the kringle 5 domain, unexpectedly enhanced Glu-plasminogen binding to a degraded fibrin surface, with $K_{d\ app} = 0.14$ μM ($B_{max} = 0.93$ mol/mol fibrin) and 0.015 μM ($B_{max} = 0.38$ mol/mol fibrin) for the low-affinity and high affinity binding sites, respectively (Fig. 4D). Control mouse IgG or monoclonal antibody 3H1E6, whose epitope is located in the NH_2-terminal peptide of Glu-plasminogen, did not enhance the binding of Glu-plasminogen to degraded fibrin (data not shown).

Competition experiments with kringle 1+2+3 were performed to ascertain whether the increased affinity of the Glu-plasminogen-F1P6 complex for degraded fibrin is an interaction at the plasminogen lysine binding site or due to newly expressed binding sites owing to conformational changes of Glu-plasminogen. As shown in Fig. 5, when a preincubated mixture of 10 nM ^{125}I-labeled Glu-plasminogen and 100 nM F1P6 was added to degraded fibrin surfaces with various concentrations of kringle 1+2+3, the binding of the ^{125}I-labeled Glu-plasminogen-F1P6 complex was inhibited in a concentration-dependent manner. In the presence of 2 μM kringle 1+2+3 fragment, the binding decreased to the levels obtained with ^{125}I-labeled Glu-plasminogen and normal mouse IgG. The half maximal inhibition occurred at 0.2 μM. The binding of the ^{125}I-labeled Glu-plasminogen-F1P6 complex to fibrin was also inhibited by EACA (data not shown). These results suggest that the interaction between lysine binding sites of plasminogen molecule and the lysine residues of fibrin contributes to the increased binding of Glu-plasminogen in the presence of kringle 5-recognizing monoclonal antibody F1P6 to degraded fibrin.

Effects of Monoclonal Antibodies on Plasminogen Activation—To determine whether the binding of monoclonal antibodies to Glu-plasminogen altered its rate of activation

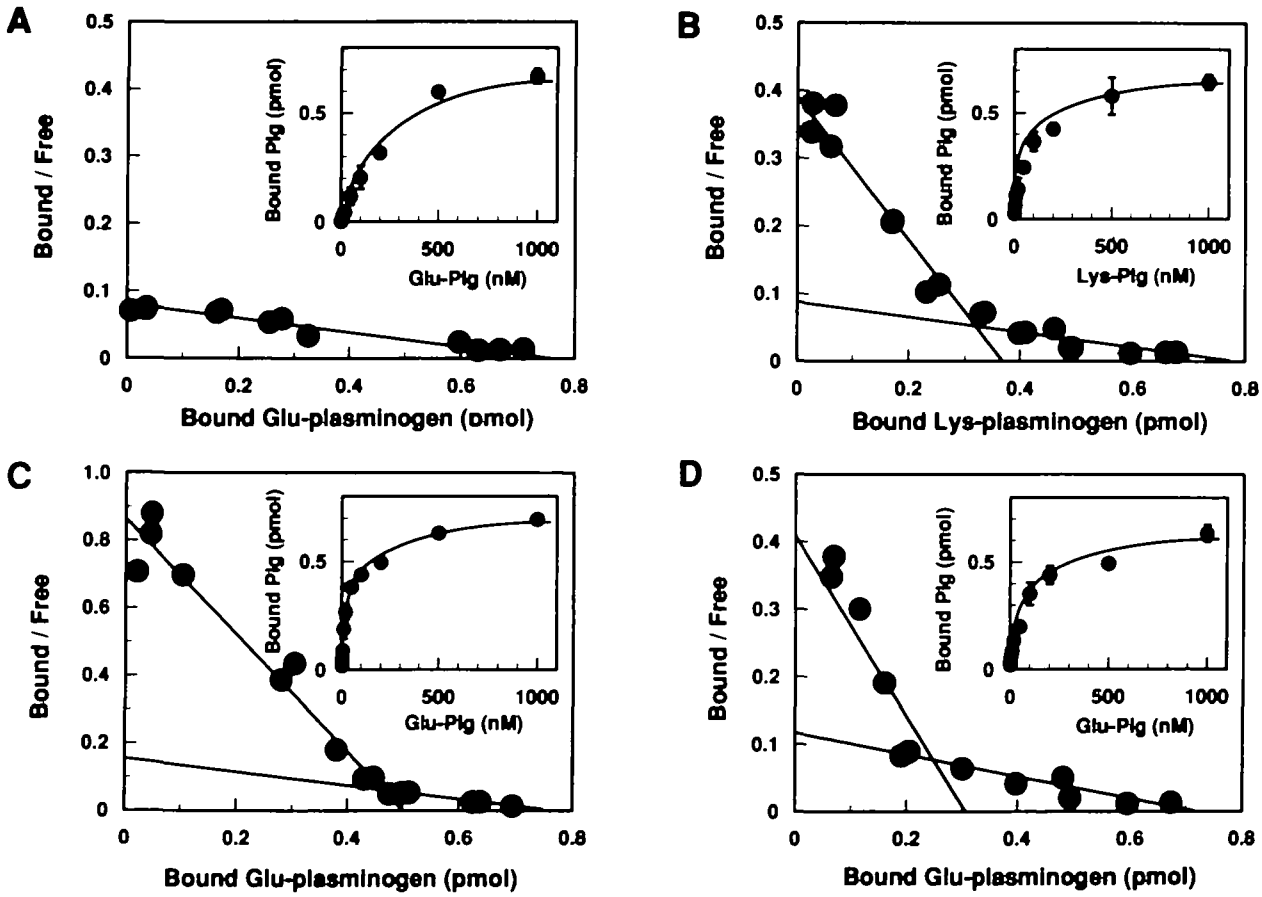

Fig. 4. **Effects of monoclonal antibodies on the binding of ^{125}I-plasminogen to degraded fibrin.** Increasing concentrations of ^{125}I-Glu-plasminogen (A, C, and D) or ^{125}I-Lys-plasminogen (B) were incubated for 16 h at 4°C with a plasmin-degraded fibrin surface in the absence (A and B) or presence of a 10-fold molar excess of monoclonal antibody F3P2 (C) or F1P6 (D), as described under "MATERIALS AND METHODS." Scatchard plots of bound/free *vs.* bound plasminogen are shown with the data fitted by linear regression analysis. Inset: plots of bound plasminogen *vs.* the concentration of added plasminogen. The data shown are the means±SD of three independent experiments.

by u-PA, as occurs in the presence of EACA, we monitored the amidolytic activity of plasmin formed after the addition of a catalytic amount of u-PA to Glu-plasminogen in the presence of a 2-fold molar excess of monoclonal antibodies (Fig. 6). The conversion of single-chain plasminogen to two-chain plasmin in the mixture was also analyzed by SDS-PAGE under reducing conditions (Fig. 6, inset). Lys-plasminogen was activated to plasmin more rapidly than Glu-plasminogen by a catalytic amount of u-PA. Interestingly, when Glu-plasminogen was preincubated with F11P5 antibody, which recognizes the kringle 1+2+3 domain and competes for binding with EACA, plasmin generation was accelerated significantly to a level similar to Lys-plasmino-

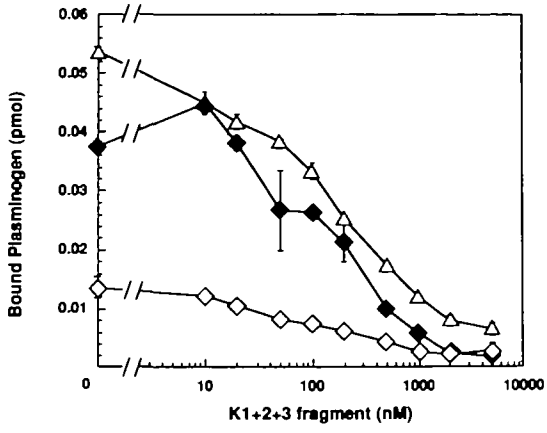

Fig. 5. **Effect of the K1+2+3 fragment on the binding of plasminogens to a degraded fibrin surface.** ^{125}I-Plasminogen (10 nM) was incubated with degraded fibrin-coated plates in the presence of increasing concentrations of K1+2+3 fragment at 4°C for 16 h. ◇, ^{125}I-Glu-plasminogen preincubated with a 10-fold molar excess of normal mouse IgG; ◆, ^{125}I-Lys-plasminogen preincubated with a 10-fold molar excess of normal mouse IgG; △, ^{125}I-Glu-plasminogen preincubated with a 10-fold molar excess of F1P6 antibody. The bound radioactivity was transformed into picomoles of plasminogen bound per well. The results are shown as means±SD of three independent experiments.

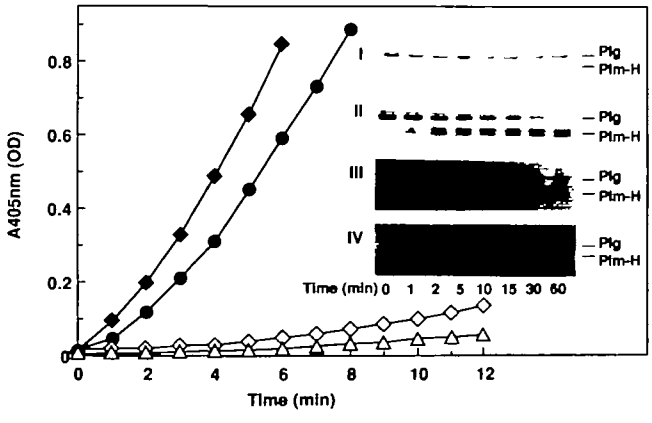

Fig. 6. **Effects of monoclonal antibodies on u-PA catalyzed plasminogen activation.** Glu-plasminogen was incubated with a 2-fold molar excess of normal mouse IgG (◇), or monoclonal antibody F11P5 (●) or F1P6 (△), and Lys-plasminogen was incubated with a 2-fold molar excess of normal mouse IgG (◆) at 37°C for 2 h in TBS-Tween-BSA. The concentrations of u-PA, plasminogen, and H-D-Val-Leu-Lys-pNA (S-2251) in the assay mixture were adjusted to 2 nM, 1 μM, and 1 mM, respectively. Plasmin generation was monitored by measuring the absorbance at 405 nm at 1 min intervals at 25°C. (Inset) Glu-plasminogen (I), Lys-plasminogen (II), or Glu-plasminogen (5 μM) with an equimolar concentration of monoclonal antibody F11P5 (III) or F1P6 (IV) were incubated with u-PA (10 nM) at 37°C in TBS-Tween-BSA. Samples were taken at various times, and then fractionated by SDS-PAGE (12.5% resolving gel) under reducing conditions. The gel was stained with Coomassie Brilliant Blue (I and II), or transferred to PVDF membranes and processed by immunoblotting with anti-plasminogen polyclonal antibodies (III and IV).

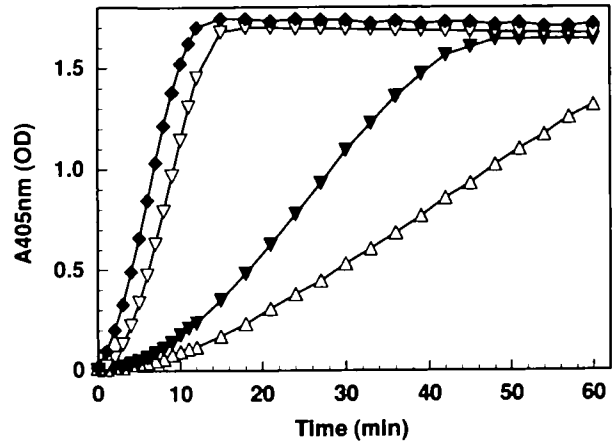

Fig. 7. **Effect of EACA on u-PA catalyzed Glu-plasminogen activation in the presence of F1P6 antibody.** Glu-plasminogen was incubated with a 2-fold molar excess of F1P6 antibody, and Lys-plasminogen was incubated with a 2-fold molar excess of normal mouse IgG at 37°C for 2 h in TBS-Tween-BSA. The concentrations of u-PA, Glu-plasminogen, and S-2251 were adjusted to 2 nM, 1 μM, and 1 mM, respectively, in the presence of varying concentrations of EACA (△, 0 mM; ▼, 1 mM; ▽, 5 mM). Lys-plasminogen (◆) was incubated under similar conditions to as described above in the absence of EACA. Plasmin generation was monitored by measuring the absorbance at 405 nm at 1 min intervals at 25°C.

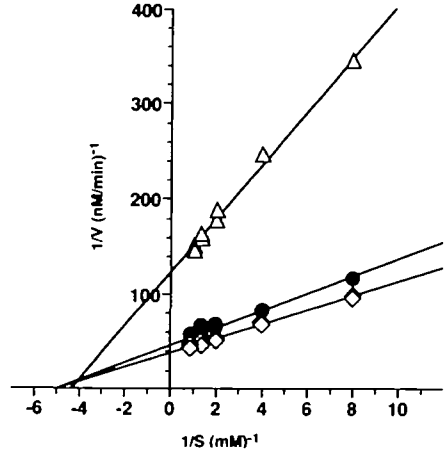

Fig. 8. **Analysis of the amidolytic activity of plasmin toward S-2251.** Lys-plasmin was incubated with a 2-fold molar excess of normal mouse IgG (◇), or monoclonal antibody F11P5 (●) or F1P6 (△) at 37°C for 30 min in TBS-Tween-BSA. The S-2251 substrate (final concentration, 0.125 to 1 mM) was added to this mixture containing a final concentration of 0.1 μM Lys-plasmin, and its hydrolysis was determined at 25°C as described under "MATERIALS AND METHODS."

TABLE II. **Kinetic constants of amidolytic activity of plasmin in the presence of monoclonal antibodies.**[a]

Monoclonal antibody	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1}/s^{-1})
F3P2	0.22	1.97	8.87
F11P5	0.20	3.64	18.60
F11P6	0.23	3.13	13.61
F12P18	0.21	2.31	10.77
F1P6	0.23	1.36	5.91
F12P16	0.18	2.71	15.13
IgG	0.20	4.47	22.11

[a]Kinetic parameters were obtained by linear regression analysis of Lineweaver-Burk plots, and means for three independent experiments are shown.

gen. In contrast, F11P5 antibody did not accelerate the conversion of Lys-plasminogen to plasmin (data not shown). These phenomena were also found in monoclonal antibody F3P2, F11P6, F12P18, or F12P16 (data not shown). Although the F1P6 antibody enhanced the binding of Glu-plasminogen to fibrin, it did not accelerate the conversion of Glu-plasminogen to plasmin (Fig. 6 and inset IV). Interestingly, the addition of EACA to this Glu-plasminogen and F1P6 mixture increased the activation rate of Glu-plasminogen to approximately that in the case of Lys-plasminogen (Fig. 7).

Effects of Monoclonal Antibodies on Kinetic Parameters of Plasmin—The hydrolysis of the chromogenic substrate, S-2251, by Lys-plasmin in the absence or presence of monoclonal antibodies obeyed Michaelis-Menten kinetics, as evidenced by linear plots of reciprocal *p*-nitroaniline generation ($1/v$) *versus* reciprocal substrate concentration ($1/[s]$) (Fig. 8). The antibodies had little effect on the K_m of Lys-plasmin for S-2251, but the k_{cat} value was variably decreased. In particular, in the presence of monoclonal antibody F1P6, the k_{cat} decreased remarkably (Table II). These results were basically similar even if the $F(ab')_2$ fragments of these monoclonal antibodies were used instead of intact IgG (data not shown).

DISCUSSION

In this study we characterized the conformational changes of plasminogen induced by two groups of monoclonal antibodies which recognize either the kringle 1+2+3 domain or the kringle 5 domain of human plasminogen. Since the conformation of plasminogen determines the epitopes of these monoclonal antibodies, we constructed an epitope map by determining each $K_{d\ app}$ (Table I), by means of a plasminogen binding competition assay (Figs. 1 and 2), and by observing the effect of EACA on plasminogen-monoclonal antibody complex formation (Fig. 3). The monoclonal antibody, F3P2, did not compete with any other monoclonal antibodies which recognized the kringle 1+2+3 structure in the binding to plasminogen. The monoclonal antibody F11P5 binding site is probably localized at the low affinity "lysine binding site" of the kringle 1+2+3 domain (*5, 36, 37*), because the antibody binding to Glu-, Lys-plasminogen and the kringle 1+2+3 fragment was inhibited by EACA, with half-maximal inhibition at 3 mM (Fig. 3). The F12P18 antibody competed with F11P5 and F11P6 for plasminogen binding, although F11P5 and F11P6 bind to distinct sites. Accordingly, the epitopes of F11P5, F11P6, and F12P18 may be localized at or near the lysine binding site within the kringle 2+3 structure of plasminogen, and are different from the epitope of monoclonal antibody F3P2.

Interestingly, we observed increased binding of Glu-plasminogen to one monoclonal antibody, for example F11P5, in the presence of another monoclonal antibody (F3P2, F11P6, or F12P16). However, when Lys-plasminogen was used as a ligand or if the plates were coated with F1P6, that exhibits a similar affinity for both Glu-plasminogen and Lys-plasminogen, no enhanced binding was observed. Taken together these results suggest that the binding of a monoclonal antibody to one site of Glu-plasminogen probably induces a conformational transition in the Glu-plasminogen molecule that results in more effective exposure of the epitope recognized by the second monoclonal antibody.

Glu-plasminogen adopts three distinct conformations involving two intramolecular interactions. One is mediated by regions of the NH_2-terminal peptide and kringle 5, and is competed for by EACA or benzamidine. The other interaction may be between kringle 3 and kringle 4, and is competed for by EACA but not by benzamidine (*38*). It is well known that EACA induces conformational changes in Glu-plasminogen, which have been detected by a decrease of the sedimentation constant, by changes in intrinsic fluorescence, and also by changes in circular dichroism and gel filtration parameters (*39-41*). Since IgG is a large molecule with its own intrinsic fluorescence, similar approaches to monitor the conformational changes were not feasible. We thus evaluated the conformational changes by comparing the effects of EACA, by measuring the binding to fibrin, and by estimating the initial activation rate of plasminogen by u-PA in the presence of monoclonal antibodies. Although it is known that EACA induces the conformational transition from the Glu-form to the Lys-form of plasminogen (*4*), EACA neither decreased (no competition) nor enhanced the binding of Glu-plasminogen to F11P6 or F12P18, despite the observation that their affinities for Lys-plasminogen were 5-10-fold higher than those for Glu-plasminogen. The simplest explanation is that the EACA-induced conformation of Glu-plasminogen is not the same as that of Lys-plasminogen.

To determine the effects of these monoclonal antibodies on the binding of plasminogen to fibrin, we evaluated the binding profiles of Glu- and Lys-plasminogen to fibrin. The binding affinities of Glu- and Lys-plasminogen to intact fibrin were similar, with a single binding site. The binding of Glu-plasminogen to the degraded fibrin showed a single class of binding site in the concentration range investigated, although the number of molecules bound per fibrin molecule increased, accompanied by a decreasing dissociation constant. In contrast, Lys-plasminogen bound to two distinct sites, a low affinity binding site with a dissociation constant similar to that of Glu-plasminogen, and a high affinity binding site with a 10-fold higher affinity (Fig. 4). Although previous reports have indicated that plasminogen binds to degraded fibrin with a higher affinity than to intact fibrin, the dissociation constants and number of bound plasminogen molecules per fibrin molecule are variable, probably due to the different experimental approaches (*27, 42-47*). In this study we used fibrin or degraded fibrin linked to a solid support with polyglutaraldehyde for the plasminogen binding (*27*). Our results for Lys-plasmino-

gen binding to the degraded fibrin surface differ from those reported by Fleury and Anglés-Cano, who employed a similar method (*27*). This may be related to the lower concentration range of plasminogen, without dilution of radiolabeled molecules, used in the current study. Both groups of monoclonal antibodies enhanced Glu-plasminogen binding to the degraded fibrin surface and two classes of binding sites were observed, similar to the interaction between Lys-plasminogen and degraded fibrin (Fig. 4). This effect was not observed using monoclonal antibody 3H1E6, which recognizes the NH_2-terminal peptide of Glu-plasminogen.

It has been shown that the binding of plasminogen to intact fibrin, which provides inter-chain lysine residues, is determined mainly by the kringle 5 domain (*9, 48*). It is also known that the carboxyl-terminal lysine residues of fibrin generated by cleavage with plasmin may interact with kringle 1 and kringle 4 of plasminogen, since both domains bind to carboxyl-terminal lysine analogues with strong and moderate affinity, respectively (*8, 36, 49, 50*). The kringle 4 domain, which harbors a potential high affinity lysine binding site, is normally shielded in Glu-plasminogen but becomes exposed in Lys-plasminogen, with a concurrent increase in affinity for carboxyl-terminal lysine analogues (*7, 11, 12, 47*). The enhanced effects of monoclonal antibodies against the kringle 5 domain on the binding of Glu-plasminogen to degraded fibrin were inhibited by the kringle 1+2+3 fragment, and the concentration of kringle 1+2+3 required to inhibit 50% of the plasminogen binding was 0.2 μM (Fig. 5). The kringle 1+2+3 fragment also blocked the binding of Lys-plasminogen at a concentration similar to previous data (*9, 44*). These results suggest that when our monoclonal antibodies bind at or near the lysine binding sites in kringle 2+3 and kringle 5, association of the NH_2-terminal peptide might be disrupted (*51, 52*), and the Glu-plasminogen-antibody complex changes its conformation toward that of EACA-liganded Glu-plasminogen, similar to the open form of Lys-plasminogen (*7, 40*). Thus, exposure of the kringle 4 domain may result in increased affinity to a degraded fibrin surface.

It has been shown that Lys-plasminogen is more susceptible to activation by plasminogen activators than Glu-plasminogen (*53*), and EACA produces a conformational change in Glu-plasminogen so as to make it easily activatable like Lys-plasminogen (*54*). Castellino and co-worker showed that antibody binding to the EACA binding site on the kringle 4 domain accelerated the activation of Glu-plasminogen by u-PA (*15*). Most of the monoclonal antibodies in this study had an accelerating effect on the activation of Glu-plasminogen but Lys-plasminogen. The monoclonal antibody, F1P6, however, neither enhanced the binding of Glu-plasminogen to the other monoclonal antibodies in this study, nor accelerated the conversion of plasminogen to plasmin (Fig. 6). Binding of F1P6 increased Glu-plasminogen binding to fibrin, and the co-presence of EACA could accelerate the conversion rate even in the presence of the antibody (Fig. 7). In addition, in the presence of F1P6, the k_{cat} of plasmin for S-2251 decreased to one-third of the control value (Table II). Taken together, the binding of antibody F1P6 induces a conformational change in Glu-plasminogen resulting in more efficient binding to fibrin, but incomplete exposure of the Arg-561 and Val-562 peptide bond. Since the epitope of F1P6 is in kringle 5, it is possible that the large antibody sterically shields this bond and the active site of plasmin. In any case, the additive effect of EACA in the presence of F1P6 on the conversion of plasminogen to plasmin suggests the existence of several transitional conformations of plasminogen from the compact Glu-form to the open Lys-form. It has been suggested that Glu-plasminogen can change its conformation from "closed" to "open" and from "open" to "more open" by the disruption of the interactions between the NH_2-terminal peptide and kringle 5, and between kringle 1+2+3 and kringle 4 by occupying lysine binding sites (*38*). The current study demonstrated that our both groups of monoclonal antibodies can dissociate these intramolecular interactions, and that the conformational changes in Glu-plasminogen may be induced by antibodies interacting at or near only one of the lysine binding sites.

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