Thrombospondin Interaction With Plasminogen. Evidence for Binding to a Specific Region of the Kringle Structure of Plasminogen

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Platelet thrombospondin interacts with plasminogen in a specific and saturable manner. Thrombospondin was found to specifically bind to plasminogen and the nonenzymic chain of plasmin. Preincubation of 125I-labeled thrombospondin with 30 mmol/L lysine was without effect in the binding of thrombospondin to immobilized plasminogen; preincubation of 125I-labeled plasminogen with 30 mmol/L lysine, on the other hand, significantly reduced the binding of plasminogen to immobilized thrombospondin, suggesting that the interaction of thrombospondin with plasminogen is not the direct result of the lysine binding sites of plasminogen. Arginine and benzamidine, ligands known to specifically bind to the kringle 5 domain of plasminogen, blocked the binding of thrombospondin to plasminogen. Limited elastase proteolysis of plasminogen and plasmin resulted in the generation of two distinct thrombospondin binding domains, one of which was retained on lysine-agarose. The isolation and amino-terminal analysis of these domains following elastase proteolysis of plasminogen identified them, respectively, as a domain containing kringle structures 4 and 5 and plasmin and the other domain consisting of kringle 5-plasmin. A 16-residue synthetic peptide, which represents the amino acids linking kringle 4 to kringle 5 (residues 435-450 of native plasminogen), was without effect in either binding to thrombospondin or blocking the binding of thrombospondin to plasminogen. Plasminogen, therefore, possesses a single thrombospondin interactive site that is independent of, but influenced by, the lysine binding site containing kringle structures and most likely is located within the kringle 5 domain.

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MATERIALS AND METHODS

Bovine blood was collected into a final 0.38% sodium citrate solution and processed as previously described.13 High molecular weight standards, type V albumin, L-lysine substituted agarose, L-lysine, l-arginine, benzamidine hydrochloride, and reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE were purchased from Sigma Chemical Co (St. Louis). 6-Aminohexanoic acid was purchased from Aldrich Chemical Co (Milwaukee). Plasminogen was prepared from fresh-frozen human plasma, generously provided by Drs C. Jackson and P. Bock from the Southeastern Michigan chapter of the American Red Cross, by affinity chromatography on L-lysine agaro-lys (dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE was purchased from Sigma Chemical Co (St. Louis). 6-Aminohexanoic acid was purchased from Aldrich Chemical Co (Milwaukee). Plasminogen was prepared from fresh-frozen human plasma, generously provided by Drs C. Jackson and P. Bock from the Southeastern Michigan chapter of the American Red Cross, by affinity chromatography on L-lysine agarose. Using a blot-binding assay, thrombospondin appears to bind specifically to an elastase-generated stable form of miniplasmin; the binding does not appear to directly involve the lysine binding sites of plasminogen.
THROMBOSPONDIN BINDING TO PLASMINOGEN

Purification and iodination of proteins. Thrombospondin was purified from A23187-released bovine platelets using heparin agarose chromatography as previously described.13,14 Thrombospondin was eluted in the 0.55 mol/L NaCl fraction; those fractions that were >0.7 mg/mL, determined using the extinction coefficient of 1.09/mg,15 and were homogeneous by SDS-PAGE16 were used. Approximately 0.6 mmol of thrombospondin were iodinated with 37 MBq of Na125I; IODO-BEADS were added to the thrombospondin, which was in 0.5 mL of 0.15 mol/L Tris containing 0.55 mol/L NaCl, 2 mmol/l CaCl2, and allowed to react for seven minutes. Labeled thrombospondin was separated from unconjugated 125I by chromatography on G-15 in 0.1 mol/L phosphate, pH 7.4. Fractions which was in 0.5 mL of 0.15 mol/L Tris containing 0.55 mol/L NaCl, 2 mmol/l CaCl2, and allowed to react for seven minutes. Labeled thrombospondin was separated from unconjugated 125I by chromatography on G-15 in 0.1 mol/L phosphate, pH 7.4. Fractions of 1 mL were collected into plastic tubes containing 0.1 mL of a 2% bovine serum albumin (BSA) solution. The specific activity of an average iodinated thrombospondin product was calculated to be 9.1 kBq/pmol. Under both reducing and nonreducing conditions, iodinated thrombospondin electrophoresed as a single-banded protein as evaluated by SDS-PAGE, and autoradiography.17 Approximately 2 nmol of plasminogen were iodinated in a manner identical to that described for thrombospondin.

Proteolysis of plasminogen and separation of the resulting kringles. Purified plasminogen was dissolved in 0.1 mol/L NH4HCO3, pH 8.3 and incubated at room temperature with porcine pancreatic elastase for between four and six hours at an enzyme to substrate ratio of 1:100 (wt/wt). For analytical studies, the reaction was terminated by the addition of 10% SDS and heating to 80°C; for preparative studies, the reaction solution was added directly to L-lysine agarose. The lysine agarose column (25 mL bed volume) was washed with at least five column volumes of 0.1 mol/L NH4HCO3 to elute the nonbinding protein before the development of a 300 mL linear gradient consisting of equal volumes of 0.1 mol/L NH4HCO3, pH 8.3, and the same buffer containing 10 mmol/L 6-aminohexanoic acid. Fractions of 6 mL were collected. Individual fractions, both those that did not interact with lysine agarose as well as those that were eluted with 6-aminohexanoic acid, were gel filtered on a Superose 12 column using an FPLC system. This resulted in the purification of the individual kringle structures from plasminogen. The identity of each kringle domain was confirmed by amino-terminal sequence analysis using an Applied Biosystems gas-phase sequencer.28 A peptide that represents the 16 amino acids linking kringle 4 (K4) to kringle 5 (K5), residues 435 to 450, was synthesized by the solid-phase method10 using a Beckman System 990 peptide synthesizer. The peptide was purified by reversed-phase chromatography using a C8 column (PepRPC 5/5 HR, Pharmacia) and characterized by amino acid analysis and amino-terminal sequence.11

Blot-binding assay. Plasminogen, plasmin, and plasminogen kringle structures, as well as standards, were electrophoresed19 on minislab gels containing 10% or 15% acrylamide cast to a thickness of 1.5 mm. All samples were run in triplicate. Following electrophoresis, one of each triplicate was stained with 1% Coomassie Brilliant blue for protein identification. The remaining sample gels were transferred onto nitrocellulose as previously described.17 Plasminogen and plasminogen kringle structures transferred optimally in 30 to 45 minutes. Thrombospondin was transferred as described.17

For binding studies, a nitrocellulose strip was placed in a 150-mm Petri dish and incubated with 4% BSA in PBS-Tween for two hours at 37°C on a rotary shaker. After blocking with BSA, individual nitrocellulose blots of plasminogen, plasmin, and plasminogen kringle structures were incubated for 12 hours with 125I-thrombospondin (10 000 cpm/mL) that had been diluted in 10 mL PBS-Tween. In studies examining the separate effects of lysine, arginine, or benzamidine, after the nitrocellulose sheets containing either plasminogen or thrombospondin were blocked with BSA they were subsequently incubated in 10 mmol/L 6-aminohexanoic acid, 30 mmol/L Lysine, 50 mmol/L arginine, or 50 mmol/L benzamidine in 0.01 mol/L HEPES, pH 7.4, for four hours at 37°C; iodinated thrombospondin or plasminogen was added directly to these solutions and the incubation continued overnight. After incubation, the nitrocellulose strips were washed, dried, and subjected to autoradiography as described.17 The synthetic peptide was assessed for its ability to directly bind to thrombospondin using a dot-blot assay; peptide and control plasminogen kringle domains were immobilized on nitrocellulose using a 96-well manifold obtained from Schleicher & Schuell. This was followed by a BSA blocking step and subsequent incubation of the nitrocellulose with 125I-thrombospondin. The peptide was also incubated with nitrocellulose immobilized thrombospondin for four and 24 hours, at room temperature and 4°C, before the addition of 125I-plasminogen. Lysine binding to plasminogen and the plasminogen kringle domains was assessed by the transfer of these proteins

Fig 1. Binding of 125I-labeled thrombospondin to plasminogen and plasmin transferred to nitrocellulose from SDS-polyacrylamide gels. Proteins were electrophoresed on a 10% SDS-polyacrylamide slab gel and stained with Coomassie Blue (A); lane 1 contains the high molecular mass standard proteins (from the top: myosin, 206 Kd; p-galactosidase, 116 Kd; phosphorylase b, 97 Kd; albumin, 66 Kd; egg albumin, 45 Kd; and carbonic anhydrase, 29 Kd); lane 2 contains native human plasminogen; lane 3 contains reduced human plasmin. (B) A nitrocellulose sheet stained with Amido Black following transfer of the proteins from panel A. (C) An autoradiography of the nitrocellulose following incubation with 125I-labeled thrombospondin. (D) Autoradiography of the nitrocellulose following preincubation of the nitrocellulose strips with 300 ng of thrombospondin overnight before washing and subsequent incubation with 125I-labeled thrombospondin.
onto nitrocellulose followed by BSA blocking before a four hour incubation of the nitrocellulose with 30 mmol/L lysine containing $5 \times 10^5 \text{cpm/mL} \text{H-lysine}$ in PBS-Tween. After incubation, the nitrocellulose was washed, dried, and the respective lanes cut into 5-mm segments and counted in a Packard Minaxi 4000 series liquid scintillation counter.

RESULTS

The interaction of bovine platelet thrombospondin with human plasminogen and plasmin was studied by using blot binding between $^{125}$I-thrombospondin and the nonreduced and reduced proteins transferred to nitrocellulose paper (Fig 1). As can be seen, plasminogen and plasmin bound $^{125}$I-labeled platelet thrombospondin and the interaction with reduced plasmin was specific to the heavy (nonenzyme) chain of plasmin (Fig 1C, lane 3). This binding was specific in that there was no evidence of thrombospondin binding to the enzyme chain of plasmin (Fig 1C, lane 3) and the binding was competitively blocked by preincubation of nitrocellulose strips containing plasminogen and plasmin with thrombospondin before the addition of $^{125}$I-labeled thrombospondin (Fig 1D).

In order to determine whether the interaction of thrombospondin with plasminogen was occurring through the lysine binding sites of plasminogen, the blot-binding assay was used in three different procedures. Initially, plasminogen (Fig 2A) was electrophoresed (Fig 2A, lane 1), transferred onto nitrocellulose (Fig 2A, lane 2), and incubated with $^{125}$I-labeled thrombospondin either in the absence (Fig 2A, lane 3) or presence of 30 mmol/L lysine (Fig 2A, lane 4). Lysine was without effect in the interaction of soluble thrombospondin with immobilized plasminogen. Immobilized plasminogen, and the plasminogen kringle domains K1-3 and K4, but not the domain identified as K5-plasmin, retained the ability to bind $^3$H-lysine as determined in a separate series of experiments (data not shown). This experimental design was then reversed, where thrombospondin was electrophoresed, in reduced form (Fig 2B) or nonreduced form (Fig 2C), transferred onto nitrocellulose (Fig 2B and C, lane 2), and incubated with $^{125}$I-labeled plasminogen either in the absence (Fig 2B and C, lane 3) or presence (Fig 2B and C, lane 4) of 30 mmol/L lysine. As is evident from these figures, when plasminogen was the soluble ligand and thrombospondin was immobilized, lysine was capable of attenuating, but not completely blocking, the interaction of plasminogen and thrombospondin. This pattern of results was similar when 10 mmol/L 6-aminohexanoic acid was substituted for the lysine in the preincubation steps (data not shown). There was evidence of plasminogen binding to a minor component of the reduced form of thrombospondin (Fig 2, lanes 3 and 4); this component represents the non-heparin binding region of thrombospondin and presumably it retains the plasminogen binding site of thrombospondin.

In order to determine whether the interaction of thrombospondin with plasminogen was specific to one of the kringle domains, plasminogen and plasmin were separately subjected to limited elastase proteolysis. As is evident (Fig 3, section I), plasminogen is converted to a series of unique fragments during the four-hour digest and incubation of these fragments with $^{125}$I-labeled thrombospondin resulted in significant specific binding of thrombospondin to one of these fragments, with relatively weak binding to an additional fragment (Fig 3C, section I). Elastase proteolysis of plasmin, on the other hand, resulted in extensive degradation of the kringle domains (Fig 3, section II); incubation of these fragments with $^{125}$I-labeled thrombospondin resulted in specific binding to one kringle domain (Fig 3C, section II, lanes 7 through 10). The low molecular weight, elastase-generated plasmin kringle domain that retains the thrombospondin binding activity does not adsorb onto lysine-agarose (data

Fig 2. Effects of lysine on the interaction between plasminogen and thrombospondin. (A) Plasminogen was electrophoresed on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue (lane 1), transferred to nitrocellulose and stained with Amido Black (lane 2), and separate nitrocellulose strips incubated in the absence (lane 3) or presence (lane 4) of 30 mmol/L lysine for two hours before incubation with $^{125}$I-labeled thrombospondin and autoradiography. (B) Reduced thrombospondin (TSP-r) was electrophoresed on a 10% SDS-polyacrylamide gel and treated as above. Lane 1, Coomassie Blue stained gel; lane 2, Amido Black stained nitrocellulose strip; lane 3, a nitrocellulose strip incubated in the absence of 30 mmol/L lysine, or, lane 4, in the presence of 30 mmol/L lysine for two hours before incubation with $^{125}$I-labeled plasminogen and autoradiography. (C) Identical experimental conditions as described for panel B, except nonreduced thrombospondin (TSP-nr) was electrophoresed on 5% SDS-polyacrylamide gels. Similar results were obtained when 30 mmol/L lysine was replaced with 10 mmol/L 6-aminohexanoic acid.
not shown), suggesting that it is not kringle domain 4, but rather is kringle domain 5.

Plasminogen was next converted into its fragments and passed over a lysine-agarose affinity column, and the resulting fragments isolated and tested for thrombospondin binding (Fig 4). 125I-labeled thrombospondin bound specifically to two separate plasminogen fragments (Fig 4C); one of these fragments was not retained on the lysine-agarose matrix (Fig 4, lane 4), whereas the other fragment that bound thrombospondin was adsorbed by the lysine-agarose matrix (Fig 4, lane 7). Each of the plasminogen fragment regions were subsequently resolved by gel filtration (data not shown) and characterized with respect to their NH2-terminal sequences (Table 1). 125I-labeled thrombospondin bound specifically to "mini-plasminogen" (Fig 4, lane 4) as well as a unique form of plasminogen consisting of kringle 4 and 5 and plasmin (Fig 4, lane 7). In order to determine whether thrombospondin was recognizing a specific peptide...
sequence that linked K4 to K5, the 16 residue peptide having
the sequence Ser-Gly-Thr-Glu-Ala-Ser-Val-Val-Ala-Pro-Pro-
Pro-Pro-Val-Leu-Leu, corresponding to residues 435 to
450 in native plasminogen, was synthesized and tested in an
assay that consisted of dot-blotting the peptide, as well as
bound to those plasminogen fragments that had in common
sequence data is that generated in this study; numbering corre-
sponds to the amino acid position within the mature protein according to
reference 27. Lys-binding indicates whether a given domain interacts
with lysine-agarose. TSP-binding indicates whether $^{125}$I-labeled throm-
bind to K1-3 (Fig 5, blot 6) or to the peptide equivalent to
plasminogen residues 435 to 450 (Fig 5, blot 1). There was no
evidence of thrombospondin binding to this peptide even at a
ten-fold increase in peptide concentration. The peptide, at 5
mmol/L, also failed to prevent plasminogen binding to
thrombospondin. Preincubation of $^{125}$I-labeled thrombospon-
din with plasminogen or “miniplasminogen” (product from
Fig 4, lane 4) blocked the binding of thrombospondin to
plasminogen, whereas the lysine binding kringles of
plasminogen (products from Fig 4, lanes 5 and 6) were
without effect (data not shown), further confirming the
specificity of thrombospondin for the non-lysine binding
kringle region of plasminogen. When nitrocellulose sheets
similar to those shown in Fig 4 were preincubated either with
50 mmol/L arginine or 50 mmol/L benzamidine before
incubation with $^{125}$I-labeled thrombospondin, thrombospon-
din failed to bind to any of the plasminogen components
(data not shown), suggesting that thrombospondin interacts
with plasminogen via these sites that reside within the K5
domain of plasminogen.

**DISCUSSION**

Purified human thrombospondin, which was passively
adsorbed to polystyrene microtiter wells, bound plasminogen
specifically, saturably, and with high affinity (35 nmol/L).22
This binding was confirmed using an affinity assay, where
$^{125}$I-labeled plasminogen bound to thrombospondin-Sepha-
rose. Similar complex formation was demonstrated between
thrombospondin and plasmin, and in such a complex, the
plasmin proteolytic activity was retained.22 These investiga-
tors examined the effects of lysine in such complex formation
and observed that the addition of 10 mmol/L 6-aminohexa-
oxoic acid to the polystyrene assay inhibited the formation of
thrombospondin-plasminogen complexes. This led these
investigators to conclude that such complex formation was
occurring directly through the high affinity lysine binding
sites of plasminogen.23 We have confirmed and extended
these observations using the blot-binding techniques reported
here. In this assay, there is evidence for the direct formation
of a thrombospondin-plasminogen complex, regardless of
which protein is immobilized onto nitrocellulose. However,
the ability of lysine to have an inhibitory effect is directly
dependent on which ligand is immobilized. If plasminogen is
denatured, electrophoresed and transferred onto nitrocellu-
lose, lysine is without effect; when thrombospondin is on the
nitrocellulose sheet and plasminogen is the soluble ligand,
there is an inhibitory effect with lysine. Nitrocellulose
immobilized plasminogen retains the ability to bind lysine;
however, we are not capable of determining whether such
lysine binding is of high or low affinity. We interpret these
observations to indicate that the formation of a thrombo-
spondin-plasminogen complex is not occurring directly
through the lysine binding sites of plasminogen, but rather is
indeed mediated by the lysine binding sites, perhaps through
conformational alterations in plasminogen, which are
induced as a direct result of lysine binding.22 Presumably,
immobilized plasminogen, but not soluble plasminogen, is
restricted with respect to lysine-induced conformational
changes in surface structures that would then be inaccessible.

**Table 1. Characterization of Elastase-Generated
Plasminogen Domains**

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<th>Domain</th>
<th>Kd</th>
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<th>TSP-Binding</th>
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<td>++</td>
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<td>27</td>
<td>Val-Val-Gly-Gly-Cys-Val-Ala-His-</td>
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<td>- -</td>
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<tr>
<td>K5-Pl</td>
<td>38</td>
<td>Val-Ala-Pro-Pro-Pro-Val-Leu</td>
<td>- -</td>
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Abbreviations: Pgn, plasminogen; K, kringles; Pl, plasmin.

Fig 5. Binding of $^{125}$I-labeled throm-
binds to purified plasminogen do-
ments. Plasminogen and purified plasmin-
gen domains were dot-blotted onto
nitrocellulose before incubation with $^{125}$I-
labeled thrombospondin. 1. 25 µg of the
synthetic peptide representing residues
435 to 450 of native plasminogen; 2. 2 µg
plasminogen; 3. 2 µg K5-plasmin; 4. 2 µg
plasmin; 5. 0.5 µg K4-K5-plasmin; 6. 2 µg
K1-3.
for thrombospondin interaction. Our data further suggest that the observed lysine effects are the result of the lysine binding site located in K4, and not the high affinity site located in K1.20,21 This is based in part on the thrombospondin binding seen with plasminogen fragments and the ability of lysine to directly inhibit the interaction of thrombospondin with the K4-K5-plasmin molecule (data not shown).

Since the thrombospondin-plasminogen complex appeared to be the function of the kringle domains of plasminogen, we attempted to generate the specific plasminogen domains using limited elastase proteolysis.27 This resulted in the formation of two fragments that specifically interacted with 125I-labeled thrombospondin. The relative affinity for plasminogen fragments would appear to favor thrombospondin’s interaction with the K4-K5-plasmin complex over its interaction with mini-plasminogen (K5-plasmin) as seen in Figs 3 and 4. We interpret this to reflect conformational differences between these plasminogen structures, based on the following observations. Thrombospondin does not interact with the isolated lysine binding domains of plasminogen, such as K1-3 or K4 (Fig 4 and Table 1). The relative yield of these kringle domains following elastase proteolysis of plasminogen is >90%, whereas the formation of the K4-K5-plasmin structure is <10%, based on gel profiles (Fig 3) and actual protein recovery (Table 1).27 While the formation of the K4-K5-plasmin complex appears to be resistant to further elastase proteolysis, and the amount of this complex that is generated also is relatively constant from preparation to preparation, we have not identified any structural features of this structure that would explain its stability.27 The thrombospondin binding kringle structure generated following elastase proteolysis of plasmin did not adsorb onto immobilized lysine; such a domain would either represent K5 or some degraded structures from the other kringle regions, which have lost their ability to interact with lysine. There is no evidence to suggest that limited elastase proteolysis results in the formation of such non-lysine binding fragments from K1-4.27 Synthesis of the peptide region that corresponds to residues 435 to 450, and represents the peptide structure linking K4 to K5, was without effect either in preventing plasminogen binding to immobilized thrombospondin or in directly binding to thrombospondin. While these synthetic peptide results may reflect weak binding constants or an insufficient peptide length for thrombospondin binding, our results with mini-plasminogen support the interpretation that thrombospondin is interacting with a component of K5 rather than either K4 or the K4-K5 linking peptide. The common feature of the two domains that interact with thrombospondin, as seen in Figs 3 and 4, is the presence of K5, which represents residues 442 to 560 of the mature plasminogen molecule.24,25 Kringle 5 has a highly homologous amino acid sequence with the other four kringle domains, with 28 identical residues; in addition, these kringle structures have extensive homology with prothrombin27 and other vitamin K-dependent proteins.28 Kringle 5 does not contain a lysine binding site, but K5 does bind benzamidine and arginine,43 and probably plays a unique role in fibrinolysis.44 In our assay, lysine was without effect in inhibiting the binding of thrombospondin to “mini-plasminogen” (K5-plasmin) and the presumptive K5 from a plasmin digest (data not shown). Benzamidine and arginine on the other hand each was capable of blocking the binding of thrombospondin to plasminogen and the K5-plasmin form of “mini-plasminogen.” It would thus be probable that thrombospondin is recognizing some aspect of the plasminogen structure that is unique to K5, possibly a peptide sequence that is not present in the other kringle domains. We are currently attempting to address this possibility.

Leung et al have also reported that thrombospondin bound specifically, saturably, and with high affinity to histidine-rich glycoprotein.18 It has been previously reported27 that HRGP interacts with the high affinity lysine binding site of plasminogen. Thrombospondin can form a trimeric complex containing plasminogen and HRGP.24 Such complex formation appears to involve independent binding sites on thrombospondin for plasminogen and HRGP.23 When such a trimeric complex is formed, the initial rate of plasminogen activation is retained, and perhaps even enhanced relative to the activation of plasminogen complexed to thrombospondin.46 We have also been able to demonstrate thrombospondin binding to HRGP using the blot-binding assay4 confirming that independent sites within thrombospondin are mediating such complex formation. Since thrombospondin interacts with urokinase in a manner that retains full urokinase activity, it is possible that platelet secreted thrombospondin and/or extracellular matrix thrombospondin may serve as the template whereupon the cofactors for the initiation or enhancement of fibrinolysis occurs. Such a template might even augment the role that thrombospondin has in complexing with fibrinogen,42 in solution or assembled on the platelet glycoprotein thrombospondin receptor.48

ACKNOWLEDGMENT

We thank Debra McKinley for her assistance in the preparation of thrombospondin, June Snow for the amino acid sequence analyses, and Dr Shinji Satoh for helpful discussions.

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Thrombospondin interaction with plasminogen. Evidence for binding to a specific region of the kringle structure of plasminogen

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