Monoclonal Antibodies to Discrete Regions of Human Glu_1-Plasminogen†

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ABSTRACT: Monoclonal antibodies to various domains of human Glu_1-plasminogen have been prepared from stable hybrid cell lines consisting of hyperimmune mouse spleen lymphocytes fused to mouse NS-1 myeloma cells. One cell line, 10-F-1, produced a monoclonal antibody which reacted equally well (K_D values in parentheses) with Glu_1-plasminogen [$(1.9 \pm 0.6) \times 10^{-8}$ M], Lys_{77}-plasminogen [$(1.8 \pm 0.4) \times 10^{-8}$ M], and Val_{354}-plasminogen [$(1.0 \pm 0.4) \times 10^{-8}$ M]. In addition, this antibody reacted with isolated plasminogen kringle 4 [$(2.1 \pm 0.6) \times 10^{-8}$ M] and all other isolated plasminogen fragments containing kringle 4, viz., kringles 1–4 [$(2.0 \pm 0.6) \times 10^{-8}$ M] and the Lys_{77}-plasmin heavy chain [$(2.7 \pm 0.6) \times 10^{-8}$ M]. The antibody did not react with Val_{442}-plasminogen or with kringles 1–3. These data show that the antibody 10-F-1 was specific for an epitope on the kringle 4 domain and that this epitope is conserved in all of the above fragments containing kringle 4. This antibody was of the IgG_1/κ class and exhibited one isoelectric form of $pI = 6.6 \pm 0.1$. Another cell line, 10-V-1, produced a monoclonal antibody which reacted equally well with Glu_1-plasminogen [$(0.93 \pm 0.08) \times 10^{-8}$ M], Lys_{77}-plasminogen [$(1.9 \pm 0.4) \times 10^{-8}$ M], the Lys_{77}-plasmin heavy chain [$(2.7 \pm 0.6) \times 10^{-8}$ M], kringles 1–4 [$(1.2 \pm 0.5) \times 10^{-8}$ M], and kringles 1–3 [$(1.4 \pm 0.5) \times 10^{-8}$ M]. It did not interact with Val_{354}-plasminogen, Val_{442}-plasminogen, or kringle 4. These data show that the antibody 10-V-1 recognized an epitope in the kringle 1–3 domain and that this epitope is conserved in the above fragments containing kringles 1–3. This antibody was also of the IgG_1/κ classification and exhibited a single isoelectric form of $pI = 6.8 \pm 0.1$. Antibodies 10-F-1 and 10-V-1 did not compete with one another when both were added to the kringle 1–4 domain, and both were fully bound by this antigen, suggesting that the epitopes on kringles 1–3 and kringle 4 are not in close proximity on the antigen surface. Further, antibody 10-F-1 was completely displaced by ϵ-aminocaproic acid (ϵ-ACA), showing that this antibody was produced against the ϵ-ACA site on kringle 4. On the other hand, ϵ-ACA did not displace antibody 10-V-1, demonstrating that this antibody does not recognize the ϵ-ACA sites on the kringle 1–3 domain.

Human Glu_1-Pg[1] is synthesized as a single polypeptide chain containing 791 amino acids (Wiman, 1973, 1977; Sottrup-Jensen et al., 1978) and is excreted into plasma in two variant forms, separable by affinity chromatography (Brockway & Castellino, 1972), representing two different states of glycosylation of plasminogen (Hayes & Castellino, 1979a–c). The major form of human plasmin, Lys_{77}-Pm, the activated product of plasminogen, contains two polypeptide chains (Robbins et al., 1967), a heavy chain consisting of amino acid residues Lys_{77}–Arg_{560} which is linked by two disulfide bonds (Sottrup-Jensen et al., 1978) to an active site containing light chain, consisting of amino acid residues Val_{561}–Asn_{791}. The latent or actual plasmin heavy chain contains all of the carbohydrate of plasminogen or plasmin (Hayes et al., 1975), as well as the lysine binding sites (Rickli & Otavsky, 1975; Gonzalez-Gronow et al., 1977). These latter sites are believed to be responsible for interaction of plasmin(ogen) with fibrin(ogen) (Thorsen, 1975), for induction of the large conformational alteration in Glu_1-Pg (Violand et al., 1975) and concomitant acceleration of the activation rate of Glu_1-Pg (Claeys & Vermylen, 1974), and for initial rapid interaction of plasmin(ogen) with its major plasma inhibitor, α_2-antiplasmin (Wiman et al., 1978).

After limited elastolytic digestion of Glu_1-Pg or Lys_{77}-Pg, it has been shown that the following fragments of the molecule can be isolated: K1–3, K4, and Val_{442}-Pg (Sottrup-Jensen et al., 1978), as well as K1–4 and Val_{354}-Pg (Powell & Castellino, 1981). Other peptide fragments which have been usefully employed in past studies, such as Lys_{77}-H (Rickli & Otavsky, 1975), Glu_1-H (Gonzalez-Gronow et al., 1977), and Lys_{77}-Pg (Rickli & Otavsky, 1973), have also been generated by chemical or enzymatic methodology. Our laboratory has recently shown, by thermodynamic analysis, that most of these fragments appear to exist as independent domains in Glu_1-Pg (Castellino et al., 1981).

In order to assess the importance of these regions in many of the chemical and functional properties of human plasminogen and plasmin, utilizing the native molecule, we have undertaken a program aimed at the generation of a series of monoclonal antibodies to these domains. Data are presented in this paper which show that antibodies of this type have been generated and can indeed bind to specific domain regions in plasminogen.

Materials and Methods

Proteins and Peptides. Glu_1-Pg was purified from fresh human plasma, as variants 1 and 2, by the gradient elution modification (Brockway & Castellino, 1972) of the Deutsch & Mertz (1970) affinity chromatography method.

Lys_{77}-Pg, variants 1 or 2, was prepared by incubating the appropriate Glu_1-Pg variant with activator-free plasmin, as described by Violand & Castellino (1976).

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[1] Abbreviations: Glu_1-Pg, Lys_{77}-Pg, Val_{354}-Pg, and Val_{442}-Pg, plasminogens containing amino acid residues Glu_1–Asn_{791}, Lys_{77}–Asn_{791}, Val_{354}–Asn_{791}, and Val_{442}–Asn_{791}, respectively; K1–3 and K1–4, plasminogen fragments consisting of amino acid sequences Tyr_{79}–Val_{337}, Val_{353}, and Tyr_{79}–Ala_{439}, respectively; K4, plasminogen fragment containing the sequence Val_{354}–Ala_{439}; K5, plasminogen fragment consisting of amino acid residues Val_{442}–Arg_{560}; Glu_1-H and Lys_{77}-H, purified plasmin heavy chains containing the amino acid sequences of Glu_1–Arg_{560} and Lys_{77}–Arg_{560}, respectively; $NaDodSO_4$, sodium dodecyl sulfate; ϵ-ACA, ϵ-aminocaproic acid; *i*-Pr_2PF, diisopropyl fluorophosphate.

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Val_{354}-Pg and K1–4 were prepared according to the method of Powell & Castellino (1981). Val_{442}-Pg, K1–3, and K4 were generated and purified by slight modifications (Powell & Castellino, 1980) of the procedure of Sottrup-Jensen et al. (1978).

Glu_1-H and Lys_{77}-H were prepared by the procedures of Gonzalez-Gronow et al. (1977) and Rickli & Otavsky (1975), respectively.

Characterization of all of these fragments has been published in the appropriate references cited above.

K5 was generated by elastase digestion of Lys_{77}-H. A quantity of 100 mg of Lys_{77}-H was dissolved in 20 mL of a buffer consisting of 0.1 M phosphate, pH 8.0. To this solution was added 2 mg of porcine pancreatic elastase (Sigma Chemical Co.), in 1 mL of the same buffer, and the digestion was allowed to proceed at room temperature for 30 min. After this point, the reaction was terminated by adjusting the solution to 1 mM in *i*-Pr_2PF. The resulting solution was then dialyzed against a buffer consisting of 0.15 M phosphate, pH 8.0, and passed over a column (2.5 cm × 15 cm) of Sepharose 4B–L-lysine, equilibrated with the same buffer. Upon elution with this buffer, the fragment K5 passed through the column only slightly retarded, whereas the only other fragment of comparable molecular weight, K4, was retained by the column. $NaDodSO_4$ gel electrophoretograms demonstrate that K5 is homogeneous, and amino-terminal amino acid sequence analysis (Powell & Castellino, 1981) shows that the material isolated was indeed K5, in that the first three residues were Val-Asp-Pro and were identical with those in the first three positions of Val_{442}-Pg, which contains K5 as its amino-terminal domain.

Iodination. The procedure of David (1972) was used, with some modification. The protein or peptide of interest was dissolved to an extent of 1.5–2.0 mg/mL, in a buffer consisting of 0.1 M phosphate, pH 8.0. A total of 2 mL of protein or peptide solution was combined with 0.4 mL of Sepharose 4B–lactoperoxidase (Sigma Chemical Co.), in the same buffer, 0.01 mL of 0.01 M KI, in H_2O, and 0.75 mCi of $Na^{125}I$. The iodination reaction was initiated by addition of 0.01 mL of 0.3% H_2O_2. The solution was allowed to incubate at room temperature for 30 min with frequent mixing. After this time, the supernatant was removed from the resin by aspiration, subsequent to low-speed centrifugation. The resin was washed 3 times with 1 mL of protein buffer, and all supernates were combined.

When a protein or peptide was used which possessed affinity for Sepharose 4B–L-lysine, the solution was adsorbed to a small column (3 mL of resin) and washed until excess ^{125}I was removed. The protein or peptide of interest was then eluted from the column with a solution of 0.1 M phosphate/0.012 M ϵ-aminocaproic acid, pH 8.0, and dialyzed against the buffer of interest. In cases where the protein or peptide did not possess affinity for Sepharose 4B–L-lysine, the solution, after iodination, was exhaustively dialyzed against a buffer consisting of 100 mM phosphate/0.15 M NaCl, pH 8.0, until the cpm in the dialysate reached background levels.

The resulting ^{125}I-labeled protein or peptide solutions were subjected to specific radioactivity measurements, utilizing a Beckman Model 4000 γ counter. In all cases, specific radioactivities of 3–5 cpm/ng were obtained.

Immunization Schedule. Six-week-old female BALB/c mice were immunized, subcutaneously, with 0.1 mg of human Glu_1-Pg in Freund's complete adjuvant. After 2 weeks, a booster injection of 0.1 mg (1.0 mg/mL) of the same antigen, in saline, was administered intravenously. Spleens were removed 3 days after the final booster injection.

Preparation and Isolation of Monoclonal Antibody Secreting Cell Lines. Single cell spleen suspensions from immunized mice (10^8 cells) were fused with log-phase cells of an 8-azaguanine-resistant immunoglobulin NS-1 myeloma cell line (a gift of Dr. Ellis Reinherz, Harvard Medical School), grown and maintained in RPMI 1640 media (Gibco), supplemented with γ-globulin-free 10% (v/v) horse serum (Gibco)/2 mM glutamine/1 mM sodium pyruvate/0.1 mg mL^{-1} gentamicin/10^{-4} M 8-azaguanine. Prior to fusion, spleen cells and NS-1 cells were washed with Dulbecco's modified Eagle's medium (high glucose type) containing 50 units mL^{-1} penicillin/50 μg mL^{-1} streptomycin (Gibco).

Fusion was accomplished by addition of 30% (v/v) poly(ethylene glycol) 1000 (Baker) to a ratio of 8:1 (spleen cells:NS-1) cell mixtures, as described by Oi & Herzenberg (1980). The fused cells were added to a 96-well microtiter plate at approximately 10^5 cells/well and cultured in a selective media, containing Dulbecco's modified Eagle's medium (high glucose type), supplemented with 10% (v/v) fetal calf serum (γ-globulin free)/10% horse serum (γ-globulin free)/10% (v/v) NCTC 109 (MA Bioproducts)/2 mM glutamine/1 mM sodium pyruvate/10^{-4} M hypoxanthine/1.6×10^{-5} M thymidine/1 mM sodium oxaloacetate/0.5 mM pyruvic acid/0.2 unit mL^{-1} bovine insulin (HY media)/8×10^{-7} M aminopterin (HAT media).

After 2–3 weeks, those wells with visible cell growth were assayed for antibody production, using the assay described below. Active cell lines were recloned 3 times by limiting dilution. Monoclonal cell lines were obtained from wells diluted to an average of 0.5–1 cell/well during the third recloning.

All cells were grown at 37 °C in an incubator atmosphere of 6.5% CO_2 at 96% humidity.

Enzyme-Linked Immunoadsorbant Assay (ELISA). To each well of a 96-well poly(vinyl chloride) microtiter plate (Costar) was added 1 μg of the antigen to be tested in 0.1 mL of a borate/saline buffer, pH 8.3. After 4 h at room temperature, the wells were washed several times with a 1% bovine serum albumin (BSA) solution in phosphate-buffered saline, pH 8.0. Complete coating of the well surface was accomplished by incubation of 0.25 mL of a 1% BSA solution, in the same buffer, for 1 h at room temperature. Aliquots of 0.1–0.2 mL of the antibody source (ca. 1 μg of antibody when purified antibodies are employed) were added to the coated wells and incubated for 90 min at 37 °C. After this time, the wells were washed several times with a 1% solution of BSA, as above. A total of 0.2 mL of a stock solution of alkaline phosphatase, conjugated to rabbit anti-mouse IgG (Zymed), in phosphate-buffered saline/1% BSA, pH 8.0, was added to each well and incubated therein for 90 min at 37 °C. A total of 0.2 mL of *p*-nitrophenyl phosphate (0.4 mg/mL), dissolved in 10% diethanolamine/0.83 mM $MgCl_2$, pH 9.8, was placed in each well. After 30 min, the reaction was quenched by addition of 0.05 mL of 3 M NaOH. The absorbancy of each well was determined at 405 nm with the aid of an Artek Model 210 automatic microtiter plate reader.

Purification of Monoclonal Antibodies to Regions of Glu_1-Pg. The monoclonal antibody producing cell lines were gradually expanded to several 200-mL tissue culture flasks and allowed to grow to cell density in HY media. The supernate was then passed over a Sepharose 4B–human Lys_{77}-H affinity chromatography column, equilibrated with 100 mM Tris-HCl and 500 mM NaCl, pH 7.4. The column was prepared by coupling 50 mg of human Lys_{77}-H to 10 mL of Sepharose 4B,

activated with cyanogen bromide, as previously described (Brockway & Castellino, 1972). The antibodies were eluted from this column with a solution of 3 M guanidine hydrochloride, dialyzed against H_2O, and lyophilized. Approximately 20 mg of antibody was obtained per L of culture fluid.

In addition, monoclonal antibodies were obtained from the ascites fluid of tumor-bearing mice. Before the hybrid cells were injected into BALB/c retired breeders, the mice were first primed with an intraperitoneal injection of 0.5 mL of pristane (2,6,10,14-tetramethylpentadecane, Aldrich Chemical Co.). Three to six days later, 0.5 mL, containing 10^7 cells of the hybrid cell line of interest, in phosphate-buffered saline (0.10 M phosphate/0.15 M NaCl, pH 8.0), was injected intraperitoneally into the primed mice. Tumors usually appeared within 14 days, and the ascites fluid was drained, repeatedly, when possible. Antibodies were obtained from this fluid, by affinity chromatography on Sepharose 4B–Lys_{77}-H, in the same fashion as described above for the tissue culture fluid. Each cell line produced approximately 2 mg of monoclonal antibody per mL of ascites fluid.

Binding Studies. The avidity of ^{125}I-labeled antibodies for human Glu_1-Pg and its elastolytic fragments was assessed by a solid-phase assay, previously described by Frankel & Gerhard (1979). The plasminogen or fragment of interest was dissolved to a concentration of 15 μg/mL in phosphate-buffered saline, pH 8.0, and the plates were coated, as described above.

Various concentrations of the ^{125}I-labeled antibody, dissolved in 0.2 mL of phosphate-buffered saline, were added to the coated wells and allowed to incubate at 37 °C for 1.5 h. At least five replicate experiments were performed for each antibody concentration, at random wells of the plate. The solution was removed by aspiration, and the wells were washed 3 times with a 1% (w/v) solution of bovine serum albumin, as described above. After the final wash solution was removed by aspiration, the wells were individually placed in 12 mm × 75 mm test tubes and subjected to γ counting.

For each antibody concentration, the results of the replicate wells were averaged. Subtracted from these counts were those from controls in which the same concentration of ^{125}I-labeled antibody was incubated in the wells of a plate fully coated with bovine serum albumin. No more than 1% of the counts found in any sample well were obtained in the wells precoated with serum albumin.

The level of radioactivity found in each well was converted to the concentration of antibody bound (using the original solution volume). Avidity constants were calculated from the following equation (Frankel & Gerhard, 1979):

$$\frac{[A]_B}{[A]_F} = sK_a[P]_T - nK_a[A]_B \quad (1)$$

where $[A]_B$ is the concentration of antibody bound to the plate and $[A]_F$ is the free concentration of antibody (obtained by subtracting the bound concentration from the total). The parameters s, K_a, $[P]_T$, and n represent the valence of the antigen, the association constant of the ligand to the antibody, the total antigen concentration, and the valence of the antibody (2 in the case of a monoclonal antibody), respectively. A plot of $[A]_B/[A]_F$ vs. $[A]_B$ yields a slope of $-2K_a$.

The Sips heterogeneity index, α, was determined from the binding data according to the following equation (Sips, 1948; Karush, 1962):

$$\log [[A]_B(s[P]_T - n[A]_B)] = \alpha \log K + \alpha \log [A]_F \quad (2)$$

where all terms are defined as above. The heterogeneity index, α, has been determined from the slope of the line of a plot of $\log [[A]_B/(s[P]_T - n[A]_B)]$ vs. $\log [A]_F$, with $n = 2$. The parameter of $s[P]_T$ was determined from the y intercept of data plotted according to eq 1.

Table I: ELISA Assay of the Reactivity of Mouse Anti-Human Glu_1-Pg Monoclonal Antibodies toward Various Domain Regions of Plasminogen

plasminogen fragment[a]	absorbance at 405 nm[b]	
	10-F-1	10-V-1
Glu_1-Pg I	0.819	1.300
Glu_1-Pg II	0.787	1.254
Lys_{77}-Pg I	0.851	1.280
Val_{442}-Pg I	0	0
Lys_{77}-H I	0.750	1.100
K1-4 I	0.879	1.111
K1-3 I	0	1.037
K4 I	0.202	0
K5 I	0	0

[a] I and II refer to affinity chromatography variants 1 and 2 of the parent plasminogen. [b] Refers to the amount of *p*-nitrophenyl phosphate hydrolyzed after 30 min, at room temperature, with the ELISA assay described under Materials and Methods. Microtiter plates were coated with the antigen of interest, followed by the sequential addition of bovine serum albumin, the antibody to be tested (alkaline phosphatase coupled to anti-mouse IgG), and the substrate (*p*-nitrophenyl phosphate).

Analytical Techniques. Reduced and nonreduced sodium dodecyl sulfate analytical gel electrophoresis, in 5% polyacrylamide, was conducted essentially as described by Weber & Osborn (1969), with the exception that 6 M deionized urea was used in place of H_2O in the gel buffers and in the protein samples.

Isoelectric focusing of the monoclonal antibodies was performed by using an LKB 110-mL column at 4 °C, over a pH range of 3–10, by previously described methodology (Castellino & Sodetz, 1976).

Immunodiffusion gels consisting of 1% agarose were prepared on microscope slides and used to subtype the monoclonal antibodies. Rabbit anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 as well as rabbit anti-mouse κ and λ light chains were purchased from Miles Laboratories for this purpose. Precipitin bands were visually identified without staining.

Results

After immunization of mice with human Glu_1-Pg, affinity chromatography variant 1, several successful fusions of resulting mouse spleen lymphocytes with NS-1 myeloma cells have been achieved. At least 15 antibody-producing cell lines have been isolated and are in various stages of recloning. In screening these cell lines, with a solid-phase ELISA assay and various domain regions of Glu_1-Pg bound to the plate, we found several that appeared specific for the K1–3 and K4 regions. One cell line for each of these regions has been stabilized by recloning 3 times, each recloning performed on antibody-producing wells which were productive at average dilutions of less than 1 cell/well at each recloning stage, and which visibly demonstrated growth as a single clone of cells.

The monoclonal antibodies were purified by affinity chromatography on Sepharose 4B–Lys_{77}-H either from large-scale tissue culture supernates of each cell line or from ascites fluid of mice previously injected, interperitoneally, with each cell line. ELISA assays of the reactivity of the purified antibodies produced from the cell line 10-V-1, which, in culture, showed specificity for the K1–3 region of Glu_1-Pg, toward various isolated domains of Glu_1-Pg are presented in Table I. The assay was employed under conditions where the antibody concentration was at subsaturation. Here, it can be observed

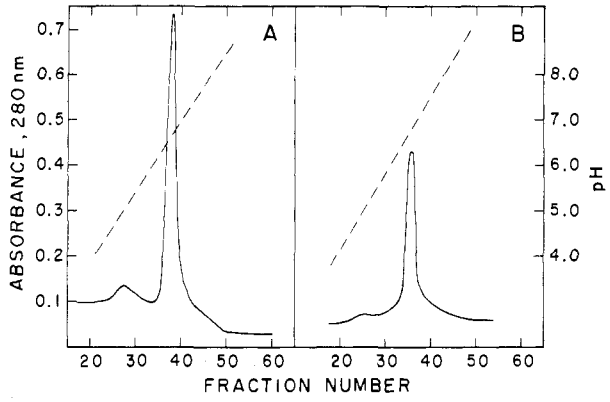

FIGURE 1: Isoelectric focusing profiles of the purified monoclonal antibodies 10-F-1 (A) and 10-V-1 (B). Both proteins were subjected to focusing at 4 °C on a pH 3–10 gradient. The absorbance at 280 nm (—) and the experimental pH gradient (---), determined at room temperature, are indicated on the respective graphs. Each fraction contained approximately 1.5 mL.

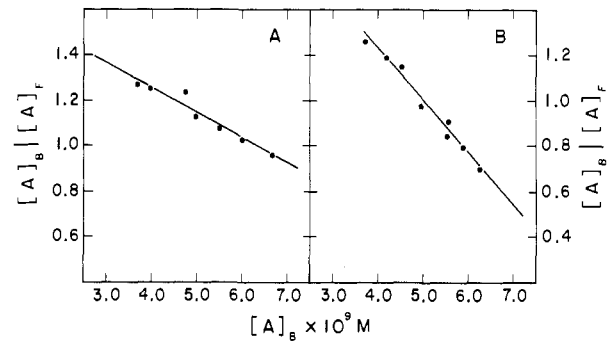

FIGURE 2: Modified Scatchard analysis of the binding of ^{125}I-labeled 10-F-1 antibody to Lys_{77}-Pg (A) and Val_{354}-Pg (B). In each case, the ratio of the concentration of bound ($[A]_B$) to free ($[A]_F$) antibody is plotted against $[A]_B$.

that this antibody reacts with isolated K1–3 and all Glu_1-Pg fragments containing K1–3. The antibody shows no activity toward fragments which do not include this region, viz., Val_{442}-Pg, K_4, and K5. In addition, Table I shows similar results for the purified monoclonal antibody from cell line 10-F-1, which showed specificity, in culture, to the K4 region of Glu_1-Pg. Here, this antibody reacted with isolated K4 and all Glu_1-Pg domains containing K4. It did not react with fragments lacking K4, viz., Val_{442}-Pg, K1–3, and K5. The lower absorbance seen for isolated K4 is likely due to its slightly weaker binding capacity to the plate, and the resulting lower level of enzyme ultimately bound. Also to be noted from this table is the fact that each antibody does not distinguish plasminogen variant 1 from plasminogen variant 2. Therefore, these variants will be referred to interchangeably in this paper.

Reduced and nonreduced $NaDodSO_4$ gel electrophoretograms of antibodies 10-F-1 and 10-V-1 were obtained. The purified antibodies possess intact molecular weights of approximately 150 000 on calibrated gels. When reduced, two molecular weight bands are noted of molecular weights approximately 50 000 and 25 000 on calibrated gels. These data show that the antibodies were of the IgG class of molecules. The purified antibodies were subtyped by microimmunodiffusion analysis against rabbit anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3. In the case of both 10-F-1 and 10-V-1, a clear single precipitin band was observed with IgG_1. No precipitin bands were observed with antisera against any other subclass of IgG, at any combination of concentrations tested. Likewise, antibodies 10-V-1 and 10-F-1 showed a precipitin band, in immunodiffusion analysis, against rabbit anti-mouse κ light chains and produced no precipitin band against rabbit anti-mouse λ light chains, at any combination of concentrations tested. Therefore, both monoclonal antibodies are of the class IgG_1/κ. In addition, each monoclonal antibody was classified according to its isoelectric point. Preparative isoelectric focusing profiles for each purified antibody are shown in Figure 1. Single bands are seen for both 10-F-1 and 10-V-1. From these graphs, the p*I* for antibody 10-F-1 is calculated to be 6.6 ± 0.1, and the p*I* value for antibody 10-V-1 is calculated to be 6.8 ± 0.1.

Binding (avidity) constants for the ^{125}I-labeled 10-F-1 antibody to Glu_1-plasminogen fragments containing the K4 domain have been performed by the assay described under Materials and Methods. Here, the procedure employed is very typical of many studies of this type in the literature. Due to the simplicity of the assay, many replicate experiments could

Table II: Avidity Parameters for Antibodies 10-F-1 and 10-V-1 to Human Glu_1-Pg and Its Isolated Fragments at 37 °C[a]

	antibody 10-F-1		antibody 10-V-1	
antigen	$K_D \times 10^8$	α	$K_D \times 10^8$	α
Glu_1-Pg	1.9 ± 0.6	0.98	0.93 ± 0.08	0.97
Lys_{77}-Pg	1.8 ± 0.4	1.07	1.9 ± 0.4	1.02
Val_{354}-Pg	1.0 ± 0.4	0.97		
Lys_{77}-H	2.7 ± 0.6	0.97	2.7 ± 0.6	1.07
K1–4	2.0 ± 0.6	0.96	1.2 ± 0.5	1.01
K1–3			1.4 ± 0.5	0.96
K4	2.1 ± 0.6	0.90		

[a] K_D (in molar units) refers to the avidity (dissociation) constant. α refers to the Sips heterogeneity index.

be performed for each data point. The data were treated by a modified Scatchard plot, as described by Frankel & Gerhard (1979) and summarized under Materials and Methods. The advantage of this method of data analysis is that the antigen concentration bound to the plate need not be known, as long as its value is constant. Prior to our binding experiments, we have radiolabeled all plasminogen fragments used in this study with $Na^{125}I$ and examined the constancy of their binding to different wells in the same plate. The binding of all fragments to the plate, at saturation, was very reproducible, and no more than a 10–15% variation was noted. Thus, at least five wells, and usually many more, were coated with constant and saturating levels of unlabeled antigen prior to addition of ^{125}I-labeled 10-F-1. Representative plots of the binding analysis for antibody 10-F-1 to Lys_{77}-Pg and Val_{354}-Pg are shown in Figure 2. Avidity (dissociation) constants for each were determined from the slopes of the lines, for three similar experiments, and calculated to be $(1.8 \pm 0.4) \times 10^{-8}$ and $(1.0 \pm 0.4) \times 10^{-8}$ M, respectively. These same experiments were performed on other isolatable fragments of human Glu_1-Pg, which contained K4, and the avidity (dissociation) constants are summarized in Table II. Clearly, the K_D values obtained for the plasminogens and fragments are approximately equal. In addition to the above, the data were replotted according to the Sips equation (Sips, 1948; Karush, 1962) in order that the heterogeneity index, α, could be determined. An example of the results obtained is shown in Figure 3 for Lys_{77}-Pg and antibody 10-F-1. The value for α as determined from the slope of the line is approximately 1.0, showing that 10-F-1 has binding characteristics of a homogeneous antibody. The values of α determined for binding of the antibody to all fragments employed are listed in Table II and closely approximate 1.0 in all cases.

Similar binding studies have been performed with the antibody 10-V-1. Since the data of Table I clearly documented that 10-V-1 was specific for the K1–3 region of human

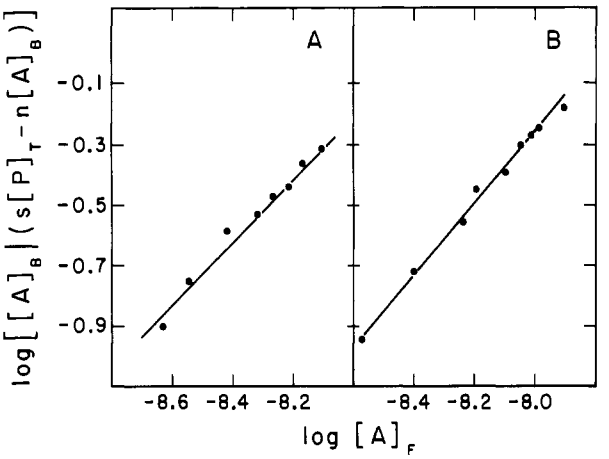

FIGURE 3: Determination of the Sips heterogeneity constant, α, for the binding of antibodies 10-F-1 (A) and 10-V-1 (B) to Lys_{77}-Pg. The equation upon which the graphs are based is given under Materials and Methods.

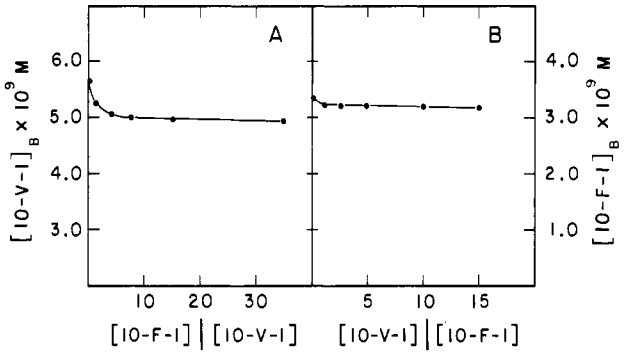

FIGURE 4: Competitive binding of antibodies 10-F-1 and 10-V-1 to K1–4. (A) A constant and subsaturating level of ^{125}I-labeled 10-V-1 was mixed with various concentrations of unlabeled 10-F-1 and added to individual wells of a plate coated with constant amounts of K1–4. The amount of ^{125}I-labeled 10-V-1 bound ($[10\text{-V-1}]_B$) is plotted against the ratio of the molar concentration of [10-F-1]/[10-V-1] added. (B) A constant and subsaturating level of ^{125}I-labeled 10-F-1 was mixed with various concentrations of unlabeled 10-V-1 and added to individual wells of a plate coated with constant amounts of K1–4. The data were plotted as in Figure 4A.

Glu_1-Pg, the avidity constants for 10-V-1 toward all presently identifiable molecules possessing this domain were determined. Scatchard plots of the binding data were obtained as in Figure 2. Avidity (dissociation) constants have been determined by the average of three experiments of this type with each antigen, and the data obtained are presented in Table II. Again, the K_D values for all antigens are very similar to each other. The Sips heterogeneity index, α, for antibody 10-V-1 toward Lys_{77}-Pg, calculated from the data of Figure 3, approximated 1.0. As can be seen from Table II, this is also the case for all fragments to which antibody 10-V-1 binds, suggesting that a homogeneous antibody has been produced.

The steric proximity of the epitopes for each monoclonal antibody has been assessed by competitive binding assays, and the data are presented in Figure 4. The binding of ^{125}I-labeled 10-F-1 antibody to K1–4 was not at all diminished by the presence of antibody 10-V-1 at molar ratios of 10-V-1 to ^{125}I-labeled 10-F-1 up to 20 to 1. Likewise, the binding of ^{125}I-labeled 10-V-1 antibody to K1–4 was not diminished by the presence of antibody 10-F-1, up to the same molar ratios as above. Further, an experiment was designed to test whether both antibodies could simultaneously bind to the smallest antigen on which both antigens were present, K1–4. Under conditions at which a total of 1.38×10^{-3} nmol of ^{125}I-labeled

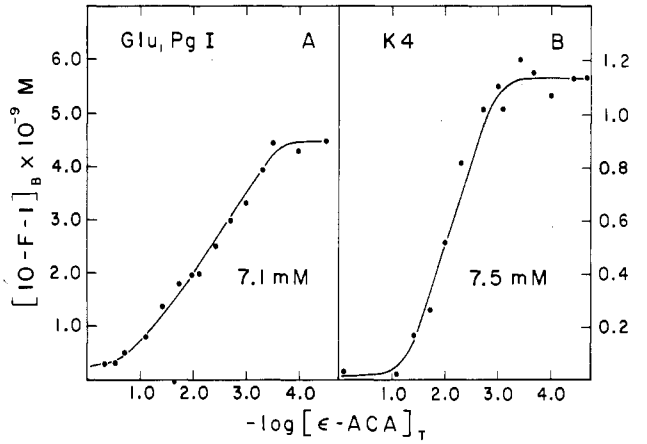

FIGURE 5: (A) Displacement of ^{125}I-labeled 10-F-1 antibody from Glu_1-Pg by various levels of added ϵ-ACA, $[\epsilon\text{-ACA}]_T$. In these experiments, the levels of ϵ-ACA indicated on the abscissa were mixed with a constant and subsaturating level of ^{125}I-labeled 10-F-1 and added to plates, the wells of which were coated with constant levels of the desired antigen. After being washed as indicated under Materials and Methods, the wells were excised and subjected to γ counting for evaluation of the level of antibody bound, which is plotted on the ordinate. The values indicated on the graphs represent the value of $[\epsilon\text{-ACA}]_T$ at the midpoint of each titration curve. (B) Same as in (A) except that the antigen employed was K4.

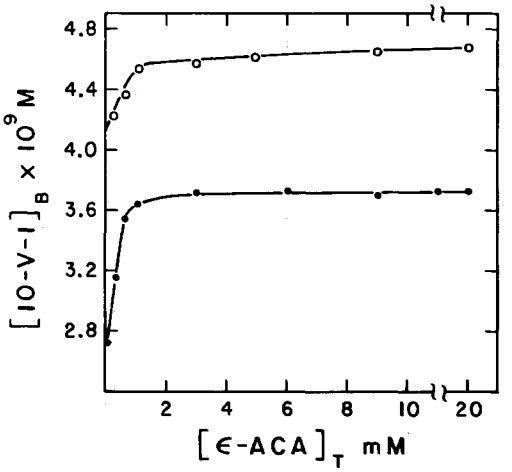

FIGURE 6: Same as Figure 5, except the antibody was ^{125}I-labeled 10-V-1 and the antigens were Glu_1-Pg (○) and K1–3 (●).

10-F-1 antibody and 1.21×10^{-3} nmol of ^{125}I-labeled 10-V-1 antibody were separately bound to K1–4, a total of 2.46×10^{-3} nmol of both antibodies was bound when mixed together in a separate well under the same conditions as used for assessing their binding separately. This clearly shows that both antibodies could be simultaneously accommodated by the antigen surface.

The ability of ϵ-ACA to displace ^{125}I-labeled 10-F-1 antibody, when bound at a subsaturating level to Glu_1-Pg and K4, is shown in Figure 5. In all cases, ϵ-ACA effectively competes with the antibody for its binding site on the K4 domain and displaces 50% of 10-F-1 from isolated K4 and Glu_1-Pg at concentrations of 6–8 mM.

A similar experiment was designed in order to examine whether ^{125}I-labeled 10-V-1 antibody could be displaced from Glu_1-Pg and K1–3, when bound at a subsaturating level, by ϵ-ACA. The data obtained are illustrated in Figure 6. Clearly, no displacement is obtained, up to ϵ-ACA levels of 400 mM. In fact, an enhancement of the binding of antibody is noted in the presence of ϵ-ACA. However, this enhancement is minimal.

Discussion

The latent plasmin heavy chain of human plasminogen, consisting of the amino acid residues 1–560, has been proposed to consist of five kringle structures with interconnecting strands of amino acids (Sottrup-Jensen et al., 1978), each of approximate molecular weight on the order of 10000–12000. By judicious utilization of the protease elastase, many of these kringle regions and other highly interesting plasminogen fragments have been isolated. Based upon studies with several of these fragments, it appears as though these isolated regions reflect many of the properties of native plasminogen and plasmin. For example, in their purification by affinity chromatography, each of the fragments containing kringles 1–4 appears to bind ϵ-ACA (Rickli & Otavsky, 1975; Sottrup-Jensen et al., 1978; Powell & Castellino, 1981; Lerch et al., 1980; Markus et al., 1978a,b). Further, it was found, as a result of employing isolated Lys_{77}-H and the isolated plasmin light chain, that the plasminogen activator streptokinase exerted its function by interactions with the light chain (Summaria & Robbins, 1976; Gonzalez-Gronow et al., 1977). As other examples, the role of the latent plasmin heavy chain in plasminogen activation (Wohl et al., 1980), the role of the plasmin heavy chain in the kinetic properties of human plasmin toward synthetic substrates (Christensen et al., 1979; Wohl et al., 1977; Morris et al., 1981) and toward fibrinogen (Morris et al., 1981), and the role of the plasmin heavy chain in the interaction of human plasmin with α_2-antiplasmin (Christensen et al., 1979; Wiman et al., 1979) have been assessed by using appropriate plasminogens, plasmins, and fragments thereof.

The value of the use of the above fragments as predictors of functional regions of plasminogen, while highly useful, is dependent upon the assumption that the properties of the fragments are not altered when they are liberated from the intact molecule. We have presented evidence, from a thermodynamic perspective, that the thermal denaturation properties of human plasminogen are preserved in the isolated intact fragments, suggesting that the kringle regions exist as independent and noninteracting domains in human Glu_1-Pg, somewhat validating their use as functional probes of plasminogen (Castellino et al., 1981). However, a much more sensitive test of this point would be to determine whether the antigenic determinants of human plasminogen are preserved in the isolated fragments. An initial approach to this problem has been provided by the studies of Hochschwender & Laursen (1981), who showed, using polyclonal antibodies to K4, that many of the K4 determinants were preserved in Glu_1-Pg and Lys_{77}-Pg. We feel that a much more fruitful manner of studying the matter of whether specific antigenic determinants in plasminogen are preserved in the kringle-containing fragments would be to generate monoclonal antibodies to various epitopes in plasminogen and to quantitatively assess their presence in the fragments of interest. This approach, which allows for much more specific information on this matter to be forwarded, is the subject of the present paper.

Subsequent to immunization of mice with human Glu_1-Pg, and fusion of the resulting spleen lymphocytes with a nonimmunoglobulin-secreting line of mouse myeloma cells, we have successfully stabilized two cell lines producing monoclonal antibodies against different epitopes present on the plasminogen molecule. As is clearly seen from the data of Table I, antibody 10-F-1 recognizes an epitope in the K4 region of human plasminogen, and antibody 10-V-1 similarly interacts with an epitope in the K1–3 region of the molecule. The monoclonal nature of these antibodies is evidenced by the fact that the antibody-producing cell lines were recloned at least 3 times at average dilutions of less than one cell per well and that single clones were recognized at each stage, by the fact that only plasminogen fragments containing a given region of human plasminogen reacted with each antibody (Table I), by the observation that the heterogeneity parameter, α, for the binding of each antibody to human Glu_1-Pg approached 1.0 (Figure 3), and by the data of Figure 1 which clearly showed that a single isoelectric antibody molecule is present. With this accumulation of evidence, there can be no reasonable doubt of the monoclonal nature of antibodies 10-F-1 and 10-V-1.

From the binding data of Figure 2 and Table II, it appears as though the avidity (dissociation) constant for antibody 10-F-1 is essentially unchanged in Glu_1-Pg, Lys_{77}-Pg, Val_{354}-Pg, Lys_{77}-H, and K1–4. This shows that the integrity of the K4 epitope which recognizes antibody 10-F-1 is preserved in these fragments. Since antibody 10-F-1 does not appear to react with K1–3 or Val_{442}-Pg, it appears as though the K4 epitope is not present, to a major degree, on K1, K2, K3, or K5. Regarding antibody 10-V-1, it can be seen from the data of Table II that all fragments containing K1–3, i.e., Glu_1-Pg, Lys_{77}-Pg, Lys_{77}-H, K1–4, and K1–3, have preserved the 10-V-1 site on the K1–3 domain, intact. The avidity (dissociation) constants for all of these fragments with antibody 10-V-1 show no significant differences. Since 10-V-1 does not react with isolated K4 or Val_{442}-Pg, it appears as though there is no strong similarity of this particular K1–3 epitope with epitopes on K4 or K5.

It should be emphasized that a solid-state binding assay is a measure of the avidity of antigen to antibody and is very useful on a comparative basis among the given fragments. This approach has been employed successfully on many occasions in the literature. However, the true thermodynamic dissociation constant for antibody to antigen may be different from the avidity constants reported herein.

Information concerning the spacial proximity of the epitopes recognizing antibodies 10-F-1 and 10-V-1 can be forwarded from the competitive binding experiments of Figure 4. Neither antibody 10-F-1 nor antibody 10-V-1 is capable of sterically hindering access of one another to their binding regions, and both are capable of simultaneous occupancy of their binding sites on antigens which can accommodate both. This clearly suggests that these particular epitopes are not in close spacial proximity to one another.

Therefore, we have shown that the epitopes for Glu_1-Pg antibodies present on K4 and K1–3, which are recognized by monoclonal antibodies 10-F-1 and 10-V-1, respectively, are, in the main, conserved in fragments containing the K4 and K1–3 domains. These discoveries significantly contribute positive evidence to the view that the plasminogen kringle structures exist as independent domains in the native molecule.

We report here that antibody 10-F-1 is displaced from Glu_1-Pg and K4 by ϵ-ACA. Titration of the displacement of ^{125}I-labeled 10-F-1 from each antigen, by ϵ-ACA, as illustrated in Figure 5, shows that 50% of the antibody is displaced at ϵ-ACA levels ($C_{0.5}$) of 6–8 mM, in each case, again suggesting that the 10-F-1 epitope on the K4 region of Glu_1-Pg is essentially unaltered upon liberation of K4 from Glu_1-Pg. At this stage, we are not certain whether antibody 10-F-1 partially or fully overlaps the ϵ-ACA site in K4 or whether displacement of the antibody occurs due to long-range effects of ϵ-ACA on the conformation, and the concomitant antibody binding capacity of K4. Interestingly, however, it has been shown that the binding of ϵ-ACA to its weak sites on plasminogen occurs with a K_D of approximately 5 mM (Markus et al., 1978a). If

antibody 10-F-1 is indeed directed to the ε-ACA binding site of K4, our data would support the concept (Plow & Collen, 1981; Hochschwender & Laursen, 1981) that the K4 domain contains a weak ε-ACA binding site(s) of plasminogen. The $C_{0.5}$ for ε-ACA displacement of antibody 10-F-1 from Glu_1-Pg (Figure 5) is in good agreement with similar studies of displacement of polyclonal antibodies to K4 from Glu_1-Pg (Plow & Collen, 1981; Hochschwender & Laursen, 1981) but differs somewhat from the previous studies on isolated K4. Plow & Collen (1981) reported that the displacement of polyclonal antibodies, generated to K4 from isolated K4, occurred at a $C_{0.5}$ of 0.28 mM, whereas the same value, as obtained by Hochschwender & Laursen (1981), was approximately 0.9 mM. While we cannot fully explain these discrepancies, we feel that our study has a distinct advantage in utilizing a monoclonal antibody. In this case, we are certain that ε-ACA is displacing a single antibody population and that a single physical phenomena is responsible for this displacement. In studies employing heterogeneous polyclonal antibodies, it is likely that ε-ACA is displacing different subpopulations of antibodies by different physical behavior (full or partial overlap with ε-ACA sites, as well as long-range conformational effects of ε-ACA). These different effects may occur with different ε-ACA concentration dependencies. It should further be noted that all values estimated for the K_D of ε-ACA on K4, as obtained in all of the above studies employing antibody displacement, are considerably higher, from 10- to 100-fold, than the K_D obtained by direct binding analysis of ε-ACA to K4 (Lerch et al., 1980). However, the K_D obtained in this latter study of 36.4 μM has no counterpart in Glu_1-Pg or Lys_{77}-Pg (Markus et al., 1978a,b) and requires reconfirmation.

Clearly, the availability of monoclonal antibodies 10-F-1 and 10-V-1 has allowed us to gainfully study several important aspects of the plasminogen molecule, as reported in this paper. With the continued utilization of these antibodies, and the many others which we expect to shortly possess, we feel that we will be able to assess the importance of various regions of plasminogen and plasmin in the many functions of these important proteins.

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