Identification of the Plasminogen Activator Inhibitor-1 Binding Heptapeptide in Vitronectin†

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ABSTRACT: We have shown that a heptapeptide which resides in the middle part of vitronectin (VN) is responsible for binding to plasminogen activator inhibitor-1 (PAI-1). A single PAI-1 binding peptide was isolated from human VN after limited proteolysis with protease V8. The amino acid sequence of the fragment corresponded to residues Gly-115–Glu-121 of VN. A murine monoclonal antibody (JYV-1) raised against human VN bond to the same fragment and inhibited binding of PAI-1 to VN. A synthetic peptide (V-115), comprising residues Gly-115–Glu-121 of human VN, competed with VN for both PAI-1 and JYV-1 in a dose-dependent manner. Synthetic peptide V-111 (Ser-111–Glu-121) had a stronger inhibitory effect than V-115 on binding of PAI-1 or JYV-1 to VN. V-111 also inhibited the binding of human PAI-1 to mouse and rabbit VN. The functional half-life of PAI-1 activity was prolonged approximately 2-fold in the presence of V-111 (1 mM). This stabilizing effect of V-111 was equivalent to intact VN, although a 1000-fold higher molar concentration of V-111 over VN was required. These data indicated that VN residues Gly-115–Glu-121 contain a PAI-1 binding site.

Activation of the fibrinolytic system is initiated by two physiological plasminogen activators, tissue-type (tPA)[1] and urokinase-type plasminogen activators (uPA) [Danø et al., 1985; Collen & Lijnen, 1986]. It is thought that fibrin clot lysis in the circulation is initiated mainly by tPA while the fibrinolytic process in tissue depends upon uPA [Danø et al., 1985; Collen & Lijnen, 1986]. The actions of these PAs are regulated primarily by their physiological inhibitor, plasminogen activator inhibitor-1 (PAI-1) [see reviews by Sprengers and Kluft (1987) and Loskutoff et al. (1989)]. PAI-1 is synthesized as an active molecule but is unstable, decaying into an inactive form upon incubation at 37 °C with a functional half-life of 2–3 h [Sprengers & Kluft, 1987; Loskutoff et al., 1989]. Recent studies of PAI-1 binding proteins have shown that PAI-1 binds reversibly to vitronectin [Declerck et al., 1988; Mimuro & Loskutoff, 1989; Salonen et al., 1989; Wun et al., 1989]. Vitronectin (VN) is a cell adhesion molecule present in blood at a relatively high concentration and also in the extracellular matrix of tissue. It has been shown that a wide variety of cells have VN receptors. Thus, VN may play an important role in cell migration and wound healing. VN appears to be a multifunctional protein, binding not only to its cell receptor but also to a number of plasma proteins [Preissner & Jenne, 1991]. VN is identical to S-protein in the complement system [Jenne & Stanley, 1985] which binds to C5–7 complexes, resulting in protection of lipid bilayers from attack by C9 [Dahlbäck & Podack, 1985]. VN also binds to proteins in the blood coagulation and fibrinolysis systems. One of the roles of VN appears to be to stabilize PAI-1, preventing spontaneous inactivation [Declerck et al., 1988]. However, PAI-1 decays even in the presence of VN, although the functional half-life is extended 2-fold [Declerck et al., 1988]. Since PAI-1 is cleared from the circular very quickly with a half-life of 7 min [Sprengers & Kluft, 1987; Loskutoff et al., 1989], the role of VN in the fibrinolytic system is thought to be to localize PAI-1 in tissue. The precise PAI-1 binding domain in the VN molecule is still controversial, since two groups have shown that two distinct CNBr-derived VN fragments contain PAI-1 binding capability using similar experimental procedures [Preissner et al., 1990; Seifert & Loskutoff, 1991b].

In this study, we show that a heptapeptide, isolated from VN digested with protease V8, bound to PAI-1 and to a MoAb that interferes with PAI-1 binding to VN. The peptide was synthesized and also inhibited PAI-1 and MoAb binding to VN. We also show evidence that the synthetic peptide derived from the PAI-1 binding site of VN stabilize PAI-1 activity.

MATERIALS AND METHODS

Reagents. All chemical reagents were the highest analytical grade commercially available and were purchased from as follows: Freund's adjuvant, poly(ethylene glycol) 1540, dimethyl sulfoxide, and 2,6,10,14-tetramethylpentadecane (Pristane) were from Wako Chemicals (Osaka, Japan); Tris-HCl, bovine serum albumin (BSA), and Tween 80 were from Sigma (St. Louis, MO); sodium phosphate was from Seikagaku Kogyo (Tokyo, Japan); Iodo-beads were from Pierce (Rockford, IL); fetal calf serum and reagents for cell culture were from Gibco (Grand Island, NY); horseradish peroxidase-conjugated goat anti-mouse IgG and subclass-specific antibodies against mouse IgG were from Bio-Rad (Richmond,

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[1] Abbreviations: PAI-1, plasminogen activator inhibitor-1; VN, vitronectin; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; CNBr, cyanogen bromide; MoAb, monoclonal antibody; TFA, trifluoroacetic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

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CA) protein A-coupled Sepharose CL-4B was from Pharmacia-LKB Biotechnology (Uppsala, Sweden); and bacterial protease V8 was from Boehringer Mannheim Yamanouchi Biochemical (Tokyo, Japan). Plastic wares were purchased from Corning (New York, NY). The mouse myeloma cell line P3U1 was a generous gift from Dr. T. Watanabe (Kyushu University, Fukuoka, Japan).

Proteins. PAI-1 was purified from the conditioned medium of HT 1080 cells cultured in serum-free medium containing dexamethasone (100 nM) by affinity column chromatography on monoclonal antibody-coupled Sepharose as described [Sakata et al., 1988, 1991]. Purified PAI-1 was radiolabeled with Na [^{125}I] using Iodo-beads as described previously. Radiolabeled and unlabeled PAI-1 was activated as described previously [Hekman & Loskutoff, 1985; Sakata et al., 1988]. After activation, PAI-1 activity was titrated against urokinase using the synthetic substrate S-2444. The specific urokinase-inhibitory activity of radiolabeled PAI-1 was 101% ± 7.8% (n = 5) of the unlabeled PAI-1 activity [(6.2 ± 0.3) × 10^4 units/mg (n = 5)] [Mimuro et al., 1992].

Human vitronectin was isolated by the method of Yatogo et al. (1989) from outdated fresh-frozen human plasma in the presence of proteinase inhibitors (1 mM (*p*-amidinophenyl)-methanesulfonyl fluoride, 100 units/mL aprotinin, and 1 mM benzamidine hydrochloride) as described previously [Sakata et al., 1991]. Mouse and rabbit VN were also isolated from normal mouse and rabbit serum by affinity column chromatography on heparin-coupled Sepharose essentially according to the method of Yatogo et al. (1989). N-Terminal amino acid sequences of mouse and rabbit VN were determined as described below and were consistent with previous reports [Komine et al., 1990; Seifert et al., 1991]. The human, mouse, and rabbit VN preparations consisted of 75- and 65-kDa polypeptides, a 71-kDa single polypeptide, and a single 66-kDa polypeptide on SDS–PAGE under reducing conditions in accordance with previous studies [Dahlbäck & Podack, 1985; Komine et al., 1990; Seifert et al., 1991]. All experiments were done with human proteins, unless otherwise indicated. Single-chain tPA was isolated from the conditioned medium of human melanoma cells cultured in the presence of aprotinin (100 units/mL) as described previously [Sakata et al., 1988, 1991]. Single-chain tPA was converted to the two-chain form by incubation with plasmin-coupled Sepharose as described [Sakata et al., 1991; Kaneko et al., 1991]. Low molecular weight urokinase was purchased from Protogen AG (Läuselfingen, Switzerland). Protein concentrations were determined by the method of Bradford (1976) using BSA or normal mouse IgG as the standard.

Monoclonal Antibodies to Vitronectin. BALB/c mice were immunized with purified human vitronectin, and murine monoclonal antibodies to human VN were selected and cloned according to the method of Köhler and Milstein as described previously [Wakabayashi et al., 1986; Mimuro et al., 1987a,b] Monoclonal antibodies were isolated from mouse ascites using protein A-coupled Sepharose as described previously [Wakabayashi et al., 1986; Mimuro et al., 1987a,b]. MoAbs were radiolabeled with Na[^{125}I] using Iodo-beads according to the manufacturer's directions, and dissociation constants (K'_d) for the binding of MoAbs with vitronectin were determined according to the method of Frankel and Gerhard as described previously [Wakabayashi et al., 1986; Mimuro et al., 1987a,b].

Analysis of the Effect of MoAbs on the Interaction of Vitronectin and PAI-1. Microtiter plastic plates were coated with VN (0.5 μg/mL) in phosphate-buffered saline (PBS) at 4 °C for 16 h. After blocking with 3% BSA in PBS and washing with PBS containing 1% BSA and 0.05% Tween 80 (binding buffer), VN-coated microtiter plates were incubated with [^{125}I]-labeled activated PAI-1 (1 nM) in binding buffer in the presence of increasing concentrations of MoAbs at 4 °C for 16 h. After washing, [^{125}I]-labeled PAI-1 bound to VN was quantified on a γ-counter (Aroka, Tokyo, Japan). Similarly, VN-coated plates were incubated with radiolabeled MoAbs (0.2 nM) in binding buffer in the presence of increasing concentrations of activated PAI-1 at 4 °C for 16 h. After washing, the amount of radiolabeled MoAbs bound to the plates was determined.

Limited Proteolysis of VN with Protease V8 and Isolation of the Digests. Purified VN (1 mg) was subjected to limited proteolysis by bacterial protease V8 (endoproteinase Glu-C) (100 μg/mL) in 0.2 mL of 0.1 M ammonium acetate, pH 4.0, at 37 °C for 24 h after reduction and pyridylethylation as described elsewhere [Maekawa et al., 1991]. The VN fragments were fractionated by reverse-phase HPLC using a $5C_{18}$ column (Toso, Tokyo, Japan). A gradient system of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) was used, and a linear gradient from 0 to 50% solvent B in 120 min was employed. The fractions containing VN fragments were lyophilized and resuspended in 400 μL of H_2O (HPLC grade).

Isolation of a Peptide Fragment of VN That Binds to PAI-1. VN fragments separated by HPLC were diluted 5-fold with H_2O, and 50-μL samples were incubated in plastic microtiter plates at 4 °C for 16 h. After blocking and washing, the plates were incubated in binding buffer containing [^{125}I]-labeled PAI-1 (1 nM) at 4 °C for 16 h. After washing, the binding of [^{125}I]-labeled PAI-1 to VN fragments was quantified using a γ-counter. The amino acid sequence of the VN fragment that bound to PAI-1 was determined using an amino acid sequencer, Model 477A (Applied Biosystems, Foster City, CA).

Digestion of VN by CNBr. Cleavage of VN by CNBr was carried out according to the procedure described by Suzuki et al. (1984). VN (1 mg) was incubated in 70% formic acid containing CNBr (1 mg) at 25 °C for 24 h. After incubation, the digests were lyophilized. CNBr digests were fractionated by SDS–PAGE [Laemmli, 1975] on 7.5–20% gradient slab gels, and binding of MoAb JYV-1 to VN was analyzed by Western blotting as described previously [Mimuro et al., 1987a,b].

Epitope Determination for MoAbs. Samples containing VN fragments generated by V8 treatment were coated on microtiter plates at 4 °C for 16 h. After blocking and washing, the plates were incubated with MoAbs (1 μg/mL) at 37 °C for 1 h. Bound MoAb was detected by an enzyme immunoassay system using enzyme-conjugated goat anti-mouse IgG.

Synthetic Peptides. Peptides encompassing VN residues Gly-115–Glu-121 (V-115) and Ser-111–Glu-121 (V-111) were synthesized using a peptide synthesizer, Model 430A (Applied Biosystems, Foster City, CA). Peptide SV-111 having the scrambled amino acid sequence EPKSEIRSDPG of VN residues Ser-111–Glu-121 was also synthesized and utilized as the control peptide. The amino acid sequences of peptide P-48 and F-7 used in this study were derived from the tPA kringle-2 domain inner loop and the tPA finger domain, respectively [Kaneko et al., 1991; Mimuro et al., 1992]. Each peptide preparation was purified using reverse-phase HPLC on a C_{18} column and appeared to be homogeneous.

Analysis of the Effect of Synthetic Peptides on the Binding of PAI-1 or MoAbs to VN. Radiolabeled PAI-1 (1 nM) was incubated in microtiter plates coated with VN (0.5 μg/mL)

in the presence of increasing concentrations of peptides at 4 °C for 16 h. After washing, the amount of PAI-1 bound to the plates was quantified. Similarly, a fixed concentration of MoAbs (0.2 nM) was incubated with human VN coated plates in the presence of increasing concentrations of peptides at 37 °C for 1 h. After washing, the amount of MoAbs bound to the plates was determined. Another set of experiments was performed to eliminate the effect of immobilization on plastic surfaces on VN PAI-1 binding ability. MoAb JYV-2 was coated on microtiter plates as described above. After blocking, plates were incubated with PBS containing 3% BSA, 0.1% Triton X-100, and VN (1 μg/mL) at 37 °C for 60 min. After extensive washing, plates were incubated with buffer containing radiolabeled PAI-1 in the presence or absence of peptides and the amount of PAI-1 bound to the plate was quantified as described above.

Analysis of PAI-1 Stability. Activated PAI-1 (10 nM) was incubated at 27 °C in 100 μL of 50 mM Tris-HCl and 0.1 M NaCl, pH 7.4 containing 0.01% Tween 80 in the presence or absence of the ligands (peptides, vitronectin). After incubation for various lengths of time, the residual PAI-1 activity was assayed. The plasminogen activator inhibitory activity of PAI-1 was quantified for all the experiments using the synthetic substrate S-2444 (Kabi, Stockholm, Sweden) for urokinase. Aliquots of the reaction mixture (50 μL) also were mixed with the buffer containing 20 nM lower molecular weight urokinase (50 μL) and were incubated further at 37 °C for 30 min. S-2444 (1 mM, final) was added to the reaction mixtures and the rate of S-2444 hydrolysis by lower molecular weight urokinase was determined at 37 °C. Peptides were also incubated in the control experiments during the incubations.

To confirm the data, the same samples were analyzed by another method. Aliquots of the reaction mixture containing PAI-1 were mixed with a 10-fold molar excess of tPA and incubated at 37 °C for 30 min. An enzyme immunoassay for tPA/PAI-1 complexes was used to quantify tPA/PAI-1 complexes as previously described [Kaneko et al., 1991; Sakata et al., 1991]. There was no difference in PAI-1 activity determined in the samples by either assay method.

RESULTS

To study the interaction between PAI-1 and VN, we first sought to isolate a PAI-1 binding fragment of vitronectin by limited proteolysis of VN with protease V8 and separation of the fragments by reverse-phase HPLC (Figure 1). Each eluted fraction was coated on microtiter plates and analyzed for its ability to bind to PAI-1. As shown in Figure 1, PAI-1 bound primarily to a single fragment, E-7 (Figure 1, indicated by arrow). Binding of PAI-1 to this VN fragment was specific since PAI-1 bound to E-7 was reduced by incubation with tPA (1 μg/mL), 0.1 M glycine, pH 2.5, and 1 M arginine at 23 °C for 30 min by 75%, 90%, and 55%, respectively. The amino acid sequence of fragment E-7 was determined and found to be Gly-Ile-Asp-Ser-Arg-Pro-Glu (Table I). The amino acid composition of E-7 was consistent with the amino acid sequence and no other amino acids were detected. Since V8 cleaves the Glu–X peptide bond, the E-7 fragment appeared to be generated by V8 proteolysis of the bonds between Glu-114 and Gly-115 and between Glu-121- and Thr-122.

To study the structure–function relationship of VN, the effect of MoAbs on the interaction between VN and PAI-1 was investigated. Two MoAbs were chosen from six independent clones and their characteristics were further investigated. The immunoglobulin subclass and K'_d values of the two MoAbs are listed in Table II. These two MoAbs had similar affinities for VN. When solid-phase VN was incubated with radiolabeled PAI-1 in the presence of increasing concentrations of MoAb JYV-1, binding of PAI-1 to VN was decreased in a dose-dependent manner (Figure 2A). JYV-2 had no effect on PAI-1 binding to VN. Similarly, VN coated on microtiter plates was incubated with radiolabeled MoAbs in the presence of increasing concentrations of activated PAI-1 and then was analyzed for its accessibility to MoAbs. The

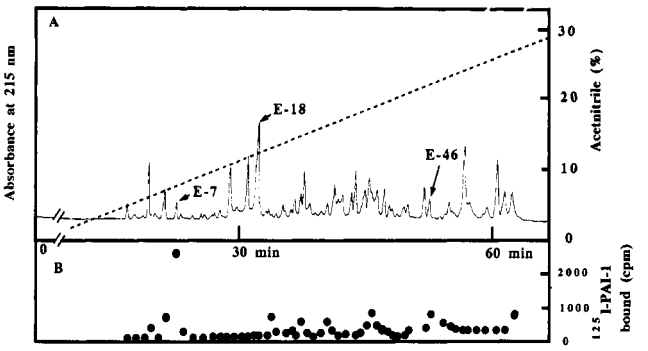

FIGURE 1: Elution profile of VN digests on reverse-phase HPLC and PAI-1 binding to VN digests. (A) VN (1 mg) was subjected to limited proteolysis with bacteria protease V8 as described under Materials and Methods. Half of the sample was applied to a C_{18} column equilibrated in a 0.1% TFA/H_2O solution and VN fragments were eluted with a liner gradient (dashed line) from 0 to 50% acetonitrile in 0.1% TFA/H_2O in 120 min. VN fragments were detected by measuring absorbance at 215 nm. Each absorbance peak was fractionated and numbered. Arrows indicate VN digest peak E-7, E-18, and E-46 (see Tables I and II). Samples containing fragments were lyophilized and resuspended in 400 μL of H_2O (HPLC grade). (B) VN fragments were coated on microtiter plates and were subjected to analysis of PAI-1 binding ability as described under Materials and Methods. The amount of [^{125}I]-PAI-1 binding to VN fragment (●, the mean of duplicate determinations) is shown as a scattergram.

Table I: Amino Acid Sequence and Composition of E-7

amino acid sequence[a]			amino acid composition[b]		
cycle	residue	pmol recovered	amino acid	pmol recovered	ratio (expected)
1	Gly	218.89	Gly	13.97	1.22 (1)
2	Ile	158.26	Ile	12.60	1.10 (1)
3	Asp	20.18	Asp	11.49	1.00 (1)
4	Ser	21.23	Ser	10.00	0.87 (1)
5	Arg	3.97	Arg	10.58	0.92 (1)
6	Pro	29.98	Pro	10.72	0.93 (1)
7	Glu	3.16	Glu	11.27	0.98 (1)
			others	<2	
positions 115–121			total amino acid 143.39 pmol		

[a] The amino acid sequence of E-7 was determined by using a Model 477A sequencer to which a Model 120A phenylthiohydantoin analyzer (Applied Biosystems) was connected. [b] Hydrolysis of peptide E-7 by HCl was carried out in the gas phase at 110 °C for 18 h and analysis of amino acid composition was performed on a HPLC system using Amino Chrome (Ciba-Corning).

Table II: Characteristics of Murine MoAbs to Human VN

MoAb	subclass[a]	K'_d[b] (M)	V8 digest: sequence[c] (position)
JYV-1	IgG_1	2.0×10^{-10}	E-7: GIDSRPE (115–121)
JYV-2	IgG_1	6.7×10^{-10}	E-18: TLHPGRPQPPALE (122–133)

[a] Subclasses of MoAbs were determined by an immunodiffusion method using subclass-specific rabbit polyclonal antibodies to mouse immunoglobulin. [b] Dissociation constants (mean of two determinations) were determined according to the method of Flankel and Gehard as described under Materials and Methods. [c] Amino acid sequences of digests were determined as in Table I and are shown using the standard single letter code.

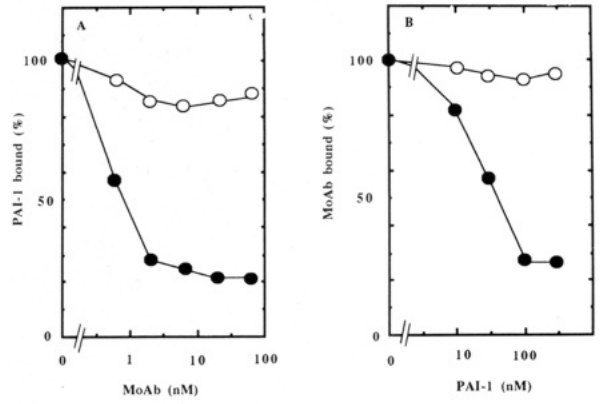

FIGURE 2: Analysis of interaction of MoAb JYV-1 with VN. (A) [^{125}I]-PAI-1 was incubated with VN-coated plates in the presence of increasing concentrations of MoAb (●, JYV-1; ○, JYV-2) at 4 °C for 16 h as decribed under Materials and Methods. The amount of bound PAI-1 in the presence of MoAb was quantified and was expressed as a percentage of that in the absence of MoAb (each value is the mean of triplicate experiments). (B) [^{125}I]-MoAb (0.2 nM) was incubated in VN-coated plates in the presence of increasing concentrations of activated PAI-1 as described under Materials and Methods. The amount of bound MoAb (●, JYV-1; ○, JYV-2) was expressed as above (each value is the mean of triplicate experiments).

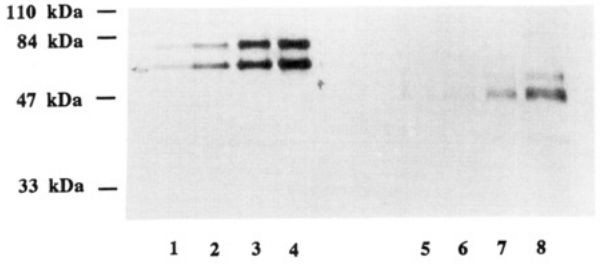

FIGURE 3: Analysis of binding of JYV-1 to VN and VN CNBr digests by Western blotting. Increasing amounts of untreated VN (lane 1, 100 ng; lane 2, 200 ng; lane 3, 500 ng; lane 4, 1 μg) and CNBr digests (lane 5, 1 μg; lane 6, 2 μg; lane 7, 5 μg; lane 8, 10 μg) were separated by SDS–PAGE and were subjected to Western blotting analysis for the binding to MoAb JYV-1 as described under Materials and Methods. The positions of molecular weight marker proteins are indicated by solid bars.

binding of JYV-1 to VN was diminished in the presence of PAI-1 in a dose-dependent manner, while no inhibitory effect of PAI-1 on JYV-2 binding to VN was observed (Figure 2B). These data indicated that the epitope of JYV-1 was located at the PAI-1 binding site or in its vicinity in the VN molecule. Thus, the epitope for JYV-1 was determined. When analyzed by Western blotting, JYV-1 bound to a M_r 43 000 fragment of the CNBr digest (Figure 3), which appeared to correspond to the middle part of VN described by Suzuki et al. (1984). JYV-1 did not react with polypeptides that migrated around 10 kDa. The binding of JYV-1 to the fragment was considerably weaker than to the intact form (Figure 3). The VN V8 fragments, isolated by reverse-phase HPLC as shown in Figure 1, were also analyzed for MoAb binding. Fragments were coated on microtiter plastic plates and incubated with MoAbs at 37 °C for 1 h. After washing, bound MoAbs were detected by enzyme-conjugated goat anti-mouse IgG antibodies. JYV-1 bound to V8 fragment E-7 and E-46 (not shown). E-46 was presumably a partially digested fragment since it was eluted from the column at a relatively high acetonitrile concentration. JYV-2 bound to fragment E-18 (Figure 1), whose amino acid sequence was Thr-Leu-His-Pro-Gly-Arg-Pro-Gln-Pro-Pro-Ala-Glu (residues 122–133) (Table II). The observation that JYV-2 bound to the peptide segment (residues 122–133), immediately carboxyl-terminal to the E-7 fragment (residues 115–121), and did not inhibit

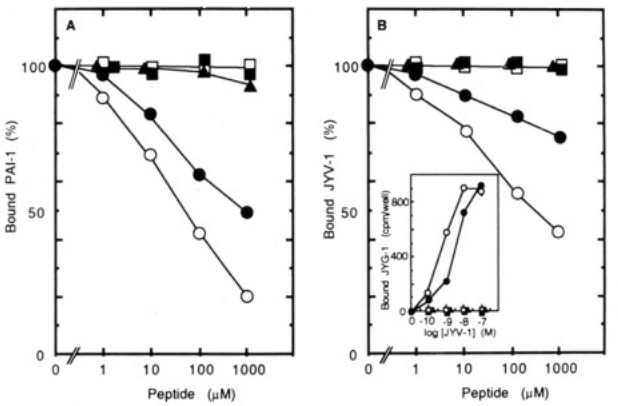

FIGURE 4: Effect of synthetic peptides on the binding of PAI-1 or MoAb JYV-1 to VN. (A) [^{125}I]-PAI-1 (1 nM) was incubated in human VN coated plates in the presence of increasing concentrations of peptides (●, V-115; ○, V-111; ▲, SV-111; ■, F-7; □, P-48) as described under Materials and Methods. Bound PAI-1 in the presence of peptides was quantified and was expressed as a percentage of that in the absence of peptide (each value is the mean of triplicate experiments). (B) [^{125}I]-JYV-1 (30 ng/mL) was incubated in VN-coated plates in the presence of increasing concentrations of peptide, and bound MoAb JYV-1 was expressed as described above. Inset represents the binding of JYV-1 to plates coated with peptides (●, V-115; ○, V-111; ▲, SV-111; ■, F-7; □, P-48).

PAI-1 binding to VN, suggested that the putative PAI-1 binding site (residues 115–121) was conformationally separate from the adjacent polypeptide segment.

To confirm these observations, peptides comprising VN residues Ser-111–Glu-121 (V-111) and Gly-115–Glu-121 (V-115) were synthesized and utilized for further experiments. As the segment residues Gly-115–Glu-121 appeared to separate from its C-terminal segment (E-18) and Glu-114 appeared to be conserved in three species of VN (see Figure 7), we synthesized peptide V-111 in addition to V-115. A scrambled peptide SV-111 was also synthesized and used as the control peptide. Peptides V-115 and V-111 inhibited binding of PAI-1 to VN in a dose-dependent manner (Figure 4A). The inhibitory effect of V-111 was stronger than that of V-115 and 1 mM V-111 inhibited the PAI-1 binding to VN by approximately 90%. PAI-1 binding to VN immobilized on MoAb JYV-2 was also inhibited in the presence of 1 mM V-111 by 79.5% (n = 3). No inhibitory effect of peptide SV-111 was observed on PAI-1 binding to VN. These peptides also inhibited JYV-1 binding to VN in a dose-dependent manner (Figure 4B). Similar to the effect on PAI-1 binding to VN, V-111 was a better inhibitor of MoAb JYV-1 binding to VN relative to V-115, and JYV-1 bound to both peptides. These data indicate that the VN residues Gly-115–Glu-121 are essential for PAI-1 binding and residues Ser-111–Glu-114 have a supporting sequence for PAI-1 binding or rectify the PAI-1 binding conformation of the peptide segment (115–221). PAI-1 binding to VN was not inhibited by tPA-derived peptide P-48 or F-7 (Figure 4A), which binds to PAI-1 [Kaneko et al., 1991; Mimuro et al., 1992], indicating that the VN binding site in the PAI-1 molecule is different from the reversible binding sites for tPA noncatalytic domains.

We also studied the effect of peptide V-111 on PAI-1 binding to mouse and rabbit VN. As reported, human PAI-1 bound to mouse VN [Seifert et al., 1991] and rabbit VN in a dose-dependent manner (not shown). When human PAI-1 (1 nM) was incubated in VN-coated plates in the presence of peptide V-111, PAI-1 binding to both mouse and rabbit VN was inhibited in the presence of peptide V-111 in a dose-dependent manner (Figure 5), indicating that VN molecules isolated from these three species bound to the same peptide segment

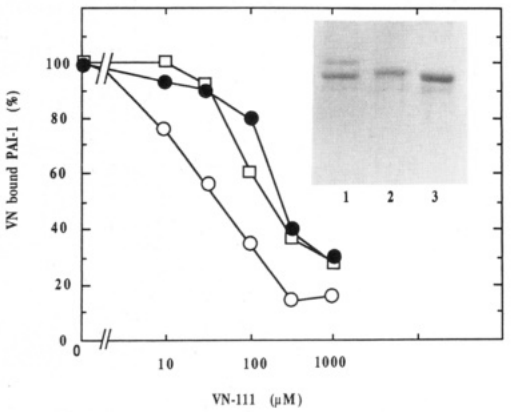

FIGURE 5: Inhibition by peptide V-111 of PAI-1 binding to human, mouse, and rabbit VN. [^{125}I]-PAI-1 (1 nM) was incubated in microtiter plates coated with human (○), mouse (□), and rabbit (●) VN (1 μg/mL) in the presence of increasing concentrations of peptide V-111. After washing, the amount of PAI-1 bound to VN was quantified and was expressed as a percentage of that in the absence of V-111 (each value is the mean of duplicate experiments). Inset represents SDS–PAGE analysis (Laemmli, 1970) of VNs (lane 1, 5 μg of human VN; lane 2, 3 μg of mouse VN; lane 3, 3 μg of rabbit VN).

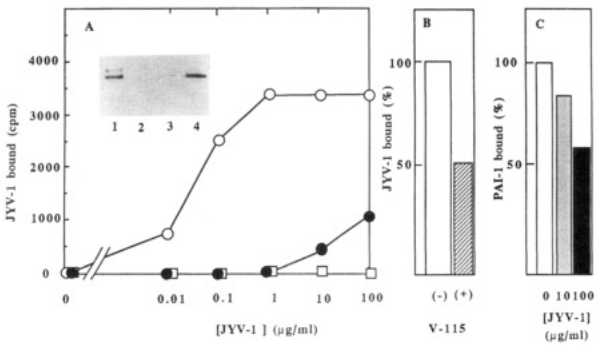

FIGURE 6: Binding of MoAb JYV-1 to human, mouse, and rabbit VN. (A) Binding of MoAb JYV-1 to VNs was investigated by a solid-phase assay. Microtiter plates coated with human (○), rabbit (●), and mouse (□) VN were incubated with increasing concentrations of [^{125}I]-JYV-1. After washing, the amounts of JYV-1 bound to the plates were quantified. Inset shows Western blotting analysis of human VN (lane 1, 200 ng), mouse VN (lane 2, 2 μg), and rabbit VN (lane 3, 200 ng; lane 4, 2 μg) for the binding to MoAb JYV-1 (each value is the mean of triplicate experiments). (B) Binding of JYV-1 to rabbit VN in the presence (hatched bar) or absence (open bar) of 10 μM peptide V-115 was investigated. The amount of JYV-1 bound to rabbit VN in the presence of peptide was expressed as the percentage of that bound in the absence of peptide (each value is the mean of triplicate experiments). (C) Binding of human PAI-1 to rabbit VN in the presence or absence of MoAb JYV-1 was investigated. The amount of PAI-1 bound to rabbit VN in the presence of MoAb JYV-1 was investigated. The amount of PAI-1 bound to rabbit VN in the presence of MoAb JYV-1 (shaded bar, 10 μg/mL; solid bar, 100 μg/mL) was expressed as the percentage of that in the absence of JYV-1 (open bar) (each value is the mean of triplicate experiments).

of the PAI-1 molecule.

The binding of MoAb JYV-1 to human and rabbit VN was compared (Figure 6A) to see if it bound to rabbit VN. Mouse VN was used as a negative control. Although JYV-1 binding to rabbit VN was approximately 1000–2000-fold weaker than that to human VN, JYV-1 bound to rabbit VN significantly as compared with the lack of binding of JYV-1 to mouse VN (Figure 6A and inset). Binding of JYV-1 (60 nM) to rabbit VN was inhibited 50% by peptide V-115 (10 μM) (Figure 6B), indicating that JYV-1 recognizes residues in the V-115 sequence that are common to both human and rabbit VN. Human PAI-1 binding to rabbit VN was inhibited 40% by

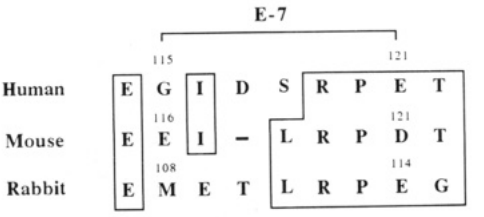

FIGURE 7: Comparison of the human VN PAI-1 binding amino acid sequence with mouse and rabbit VN. Mouse and rabbit VN sequences were searched for sequences homologous with the human VN PAI-1 binding amino acid sequence derived from fragment E-7 using HIBIO-Prosis version 4.0 (Hitachi Software Engineering Co., Ltd., Yokohama, Japan). Each amino acid is represented by the standard single-letter code. Identical or homologous residues in two or three VN species are boxed. The amino acid sequences and their numbering were adopted from previous studies (Jenne & Stanley, 1985; Komine et al., 1990; Seifert et al., 1991).

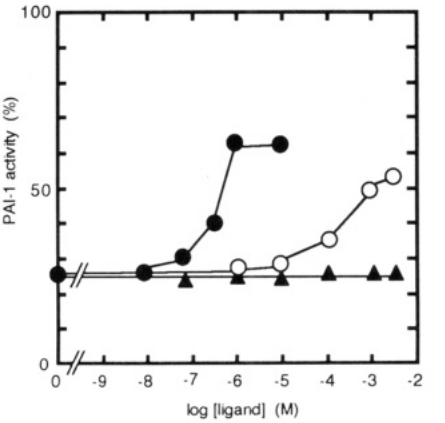

FIGURE 8: Residual activity of PAI-1 after incubation at 37 °C in the presence of ligands. Activated PAI-1 (10 nM) was incubated at 37 °C for 4 h in the absence or presence of increasing concentrations of ligands (●, VN; ○, V-111; ▲, SV-111). The residual plasminogen activator inhibitory activity toward low molecular weight urokinase was determined as described under Materials and Methods and was expressed as a percentage of the control sample kept on ice in the presence of each ligand (each value is the mean of triplicate experiments).

100 μg/mL JYV-1 (Figure 6B), suggesting that the JYV-1 epitope may overlap the PAI-1 binding site in human and rabbit VN.

Figure 7 shows a comparison of the PAI-1 binding amino acid sequence in human VN with the corresponding amino acid sequences of mouse and rabbit VN. After a homology search using the program Hibio-Prosis, the peptide segment of mouse VN Glu-115–Thr-122 corresponded to residues Glu-114–Thr-122 of human VN, and rabbit VN contained the corresponding segment in residues Glu-107–Gly-114. With minimal adjustments to the sequences, all three species share a Glu residue 4–5 residues amino-terminal to an Arg-Pro-Glu/Asp sequence.

On the basis of previous observations that PAI-1 activity was stabilized upon incubation with VN [Declerck et al., 1988], we studied the effect of VN and synthetic peptides derived from VN on PAI-1 stability. PAI-1 activity declined linearly upon incubation at 37 °C without ligands, with a functional half-life ($t_{1/2}$) of 2 h. After a 4-h incubation at 37 °C, residual PAI-1 activity was only 36% of that kept on ice for 4 h. In the presence of VN or V-111, the residual PAI-1 activity after incubation at 37 °C for 4 h increased in a dose-dependent manner (Figure 8). Approximately 1000-fold higher concentration of V-111 over VN was required to obtain the equivalent stabilizing effect of VN. However, control peptide SV-111 showed no stabilizing effect.

DISCUSSION

We have shown that limited proteolysis of VN with protease V8 yields a fragment that is capable of binding to PAI-1 and that its amino acid sequence corresponds to VN residues Gly-115–Glu-121. The observation that MoAb JYV-1, which inhibited PAI-1 binding to VN, bound to the same fragment supports the idea that the peptide segment from Gly-115 to Glu-121 may well be a PAI-1 binding site. These observations were confirmed by experiments demonstrating that the synthetic peptide V-115 consisting of this amino acid sequence inhibited both PAI-1 binding to VN and MoAb JYV-1 binding to VN. Peptide V-111, which has a four amino acid extension at the N-terminus (Ser-111–Glu-121), inhibited PAI-1 binding to VN by 90%. Moreover, V-111 has a stabilizing effect on PAI-1 activity. Although positively charged amino acids have a stabilizing effect on PAI-1 activity [Keijer et al., 1990] or an inhibitory effect on PAI-1 binding to VN [Mimuro et al., 1987c; Seifert et al., 1990], the effect of peptide V-111 on PAI-1 activity and PAI-1 binding to VN may not be the charge effect of positively charged amino acids included in peptide V-111, because the scrambled peptide SV-111 has no effect and ~20–1000-fold higher concentrations of isolated amino acid were required to stabilize PAI-1 or to inhibit PAI-1 binding to VN.

Binding of human PAI-1 to human, mouse, and rabbit VN appears to be mediated by the same VN binding site in the PAI-1 molecule. While the residues from Gly-115 to Glu-121 of human VN are not completely conserved among human, rabbit, or mouse VN, their amino acid sequences are homologous and share partialy sequence identity. Our observation that peptide V-111 inhibited binding of human PAI-1 to all three species of VN supports the notion that the segment is a PAI-1 binding site. In addition, JYV-1 cross-reacted with rabbit VN, JYV-1 inhibited the binding of human PAI-1 to rabbit VN, and peptide V-115 inhibited the JYV-1 binding to rabbit VN. Taken together, these observations are consistent with the conclusion that the conserved Arg-Pro-Glu sequence may be the JYV-1 epitope and may be critical for PAI-1 binding to human and rabbit VN.

There have been contrasting reports concerning the localization of the PAI-1 binding site in VN since two different CNBr fragments of VN were shown to possess PAI-1 binding capability. Using ligand blotting, Preissner et al. (1990) showed that PAI-1 bound to a CNBr fragment containing the heparin-binding domain in the C-terminal part of VN. Wun et al. (1989) reported that the N-terminal fragment of VN copurified with PAI-1 by affinity chromatography on anhydrourokinase-coupled Sepharose. The latter observation was confirmed by Seifert and Loskutoff (1991a,b), who showed, using ligand blotting, that a M_r 6000 CNBr fragment of human VN and M_r 38 000 thrombin-cleaved fragment of bovine VN containing the N-terminus of VN could bind to PAI-1. These varying observations raise the possibility that VN has multiple PAI-1 binding sites. However, it is also possible that denaturation, chemical modification, or cleavage of peptide bonds causes conformational changes resulting in alteration of PAI-1 binding conformation and/or exposure of alternate PAI-1 binding site(s). In fact, binding affinity of MoAb JYV-1 to the M_r 43 000 CNBr fragment of VN containing the VN middle part was considerably lower than to intact VN. This finding indicates that the epitope of JYV-1 (115–121), which contains a PAI-1 binding site, may be conformationally altered by CNBr treatment in 70% formic acid. Our results are consistent with the observation that PAI-1 binds to a thrombin-cleaved VN fragment containing the N-terminus of VN but contrasts with the idea that PAI-1 binds to the C-terminal heparin-binding domain or that PAI-1 binds to the M_r 6,000 somatomedin B domain generated by CNBr treatment. PAI-1 has been shown to contain at least three reversible tPA binding sites and these sites appear to be different from the VN binding site. The Asn-Arg-Arg-Leu PAI-1 binding sequence in the tPA kringle-2 domain has a sequence homology with the Asn-Arg-Lys-Gly sequence (355–358) in the heparin-binding domain of VN. When we examined lysyl endopeptidase digests of VN for their ability to bind to PAI-1, there was a weak association of PAI-1 with a fragment containing residues Gln-348–Lys-357 from the heparin-binding domain (not shown). Since heparin enhanced the PAI-1 binding capacity of VN [Preissner et al., 1990], such a site in the C-terminal heparin-binding domain may be exposed allosterically upon heparin binding to VN. Alternatively, a buried PAI-1 binding site may be exposed during denaturation that inevitably occurs during CNBr digestion in formic acid. Thus, interpretation of ligand blotting data derived from partially denatured protein can be complicated by unavoidable conformational changes. This is also applicable with our current findings because we exposed VN to reduction, pyridylethylation, proteolysis, and HPLC in TFA, all of which would directly or indirectly alter native conformation. However, our results with peptides V-111 and V-115 suggest that this segment is exposed naturally on the surface of VN and is involved in the association with PAI-1.

It is possible that the V-115 peptide (or V-111) bound to PAI-1 altered the PAI-1 conformation in such a manner that resulted in loss of VN binding at a site far from the peptide binding site. However, the observation that MoAb JYV-1 inhibits PAI-1 binding by binding to VN suggests that this possibility is not likely. It is also possible that two discontinuous peptide segments function together to form a single PAI-1 binding domain in the VN molecule. This kind of interaction is observed between von Willebrand factor and platelet glycoprotein Ib in that two domains separated by a linear sequence of 205 residues are involved in the interaction with glycoprotein Ib. Synthetic peptides derived from these two distinct segments independently compete with binding of von Willebrand factor for glycoprotein Ib and for a MoAb which inhibited the interaction of von Willebrane factor and glycoprotein Ib [Mohri et al., 1988]. This possibility is most likely to account for the conflicting observations of VN and PAI-1 binding; however, more precise determinations of the PAI-1 binding site in the somatomedin B domain and in the heparin-binding domain may be necessary to clarify this issue.

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