Kinetic Analysis of the Interaction between Vitronectin and the Urokinase Receptor*

Received for publication, November 26, 2001, and in revised form, December 20, 2001
Published, JBC Papers in Press, December 31, 2001, DOI 10.1074/jbc.M111225200

Yuushi Okumura‡, Yuichi Kamikubo‡, Scott A. Curriden‡, Jieyi Wang§, Tatsuto Kiwada¶, Shiroh Futaki‡, Kouki Kitagawa†, and David J. Loskutoff**

From the ‡Department of Cell Biology, Division of Vascular Biology, Scripps Research Institute, La Jolla, California 92037, §Abbott Laboratories, Abbott Park, Illinois 60064, the ¶Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan, and the ¶Niigata College of Pharmacy, Kamishin’ei-cho 5-13-2, Niigata 950-2081, Japan

Although the urokinase receptor (uPAR) binds to vitronectin (VN) and promotes the adhesion of cells to this matrix protein, the biochemical details of this interaction remain unclear. VN variants were employed in BIACore experiments to examine the uPAR-VN interaction in detail and to compare it to the interaction of VN with other ligands. Heparin and plasminogen bound to VN fragments containing the heparin-binding domain, indicating that this domain was functionally active in the recombinant peptides. However, no significant binding was detected when uPAR was incubated with this domain, and neither heparin nor plasminogen competed with it for binding to VN. In fact, uPAR only bound to fragments containing the somatomedin B (SMB) domain, and monoclonal antibodies (mAbs) that bind to this domain competed with uPAR for binding to VN. Monoclonal antibody 8E6 also inhibited uPAR binding to VN, and this mAb was shown to recognize sulfated tyrosine residues 56 and 59 in the region adjacent to the SMB domain. Destruction of this site by acid treatment eliminated mAb 8E6 binding but had no effect on uPAR binding. Thus, there appears to be a single binding site for uPAR in VN, and it is located in the SMB domain and is distinct from the epitope recognized by mAb 8E6. Inhibition of uPAR binding to VN by mAb 8E6 probably results from steric hindrance.

Vitronectin (VN) is a 75-kDa adhesive glycoprotein. It circulates in blood in a monomeric (“closed,” “native”) form, but is converted into a multimeric (“extended,” “opened,” “denatured”) form when incorporated into the extracellular matrix or treated with urea (1, 2). The extended form of VN binds to specific receptors on cells (3, 4) and to various other molecules such as the C5b-9 complement complex (5), the thrombin-antithrombin III complex (6, 7), plasminogen activator inhibitor 1 (8-10), uPAR (11), heparin (1, 2, 12, 13), collagen (14-16), plasminogen (13, 17), and b-endorphin (18). These interactions not only promote the attachment, spreading, and growth of cells (19-21) but also influence the coagulation, fibrinolytic, and complement systems (22, 23).

Although a number of investigators have attempted to identify the binding site(s) in VN for these molecules, the literature remains somewhat controversial. For example, three different regions of the VN molecule have been proposed to contain the binding sites for uPAR and PAI-1. The first region, the somatomedin B (SMB) domain (residues 1-44) was identified from direct binding studies (9, 24-27) and from studies showing that soluble SMB competes with uPAR and PAI-1 for binding to denatured VN (9). The second region in VN that has been implicated in uPAR and PAI-1 binding is the heparin binding (HB) domain (residues 348-370). Thus, synthetic peptides from this domain interfere with both uPAR (21) and PAI-1 (28) binding to VN. Moreover, mAB 8E6 (which has been mapped to the HB domain (Ref. 13)) also inhibits the binding of PAI-1 to VN. Although a third region in VN (residues 115-121) was shown to have weak PAI-1 binding activity (29), the relative importance of this region for PAI-1 and uPAR binding remains to be established.

Another potential problem for the interpretation of VN binding studies concerns the molecular forms of VN employed (i.e. native closed form versus the more opened denatured form). These forms of VN have dramatically different binding properties for various ligands including uPAR and PAI-1. For example, although PAI-1 binds to both native and denatured VN, it appears to bind more avidly to the denatured form (30, 31). It was suggested that there is an initial low affinity interaction between PAI-1 and the COOH-terminal domain (including the HB domain) and that this interaction induces a conformational change in native VN, which exposes the partially cryptic NH2-terminal SMB domain (30, 32, 33). However, this hypothesis has not been verified experimentally. Although uPAR binds to denatured VN (11, 19), available evidence suggests that it does not bind to native VN (33).

Because of these inconsistent and sometimes conflicting results, we decided to reexamine the binding sites in VN for uPAR and PAI-1. We constructed and purified several recombinant VN variants, and also developed a series of mAbs against VN. We then employed these molecules in real-time biomolecular interaction analysis experiments (BIACore) to more precisely identify the uPAR and PAI-1 binding sites in denatured VN, and to determine the kinetic constants for their interactions. Our results indicate that the SMB domain is the only high affinity binding site for uPAR and PAI-1 in VN, and that the binding site for mAb 8E6 is distinct from the binding sites for uPAR and PAI-1. The 8E6 epitope is not localized to

* This work was supported by National Institutes of Health Grant HL31950 (to D. J. L.). This is Scripps Research Institute Manuscript 14322-VB. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Dept. of Cell Biology, Division of Vascular Biology, Scripps Research Inst., 10550 N. Torrey Pines Rd., VB-3, La Jolla, CA 92037. Tel.: 858-784-7125; Fax: 858-784-7353; E-mail: loskutoff@scripps.edu.

† The abbreviations used are: VN, vitronectin; uPA, urokinase; uPAR, urokinase receptor; mAb, monoclonal antibody; SMB, somatomedin B; PAI, plasminogen activator inhibitor; HB, heparin binding; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Fmoc, N-(9-fluorenyl)methoxycarbonyl; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; AP, alkaline phosphatase; PVDF, polyvinylidene difluoride; RU, resonance unit(s).
the HB domain, but rather is present in the connecting region immediately adjacent to the SMIB domain. Sulfation of tyrrosines 56 and 59 in this region is essential for recognition by this mAb. These observations suggest that results from previous studies using either mAb 8E6 or synthetic peptides to identify binding sites in VN may actually reflect indirect effects or steric hindrance.

Experimental Procedures

Materials—Bovine serum albumin (BSA), plasminogen, heparin, and heparin-albumin-biotin were purchased from Sigma. Human urokinase type plasminogen activator (uPA) was a kind gift from Winthrop Laboratory (Rensselaer, NY). Monoclonal antibody (mAb) 8E6 was purchased from Roche Molecular Biochemicals, whereas mAbs 153, 1244, and 1330 were developed in our laboratory under standard hybridoma techniques using denatured VN as immunogen. Polyclonal antibodies against human VN, PAI-1, and uPAR were raised in rabbits. Biotin-labeled secondary antibodies as well as streptavidin alkaline phosphatase and its substrate were from Zymed Laboratories Inc. (South San Francisco, CA). The BIAcore running buffer (HBS-EP: 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20), Sensor Chip (CM5), and Amine Coupling kit, were purchased from BIAcore (BIACORE, Uppsala, Sweden). The recombinant stable active form of human PAI-1 was purified from expression clone 14-1b (a kind gift from Kvassman and Shore (34). The recombinant soluble form of human uPAR (amino acid residues 1–281) was expressed and purified as described previously (35). Native VN was purified from human plasma by a modification of the original procedure of Dahlback and Podack (36), using heparin-Sepharose (Amersham Biosciences AB, Uppsala, Sweden) to remove contaminating albumin and to improve the purity as described by Bittorf et al. (37). The extended or denatured form of VN was purified from urea-treated human plasma as described by Yatohgo et al. (38).

Construction of VN Variants—VN variants (1–44, 1–97, and 40–459) were constructed from a human VN cDNA by using standard PCR amplification methods. The sense (5′) and the antisense (3′) oligonucleotide primers containing a restriction enzyme site were as follows: VN 1–44, 5′-TAGAACCTTCCATGACCAAGTGCTATGCA-3′ (5′- and 5′-AATACCTGAGTAAGCTGCTCTGCTCA-3′); VN 1–97, 5′-TAGAACCTTCCATGACCAAGTGCTATGCA-3′ (5′- and 5′-GCTACCTGAGTACGAAGCTGCTCTGCTCA-3′); VN 40–459, 5′-ATATGTTAATGATCGCAGCAGTTAGCTG-3′ (5′- and 5′-TTATATACCGAGCTCAGATGCGCAGCAGGAG-3′). The single point mutant (VN 1–97Y28A) was generated using a two-step PCR method as follows: Using the full-length cDNA as template, a 5′ PCR fragment was constructed using one 5′ specific primer (5′-TTTCATACGTGCCGTGCTGGCAT-3′) and the reverse complementary mutagenic primer (5′-CAGCAACGCCGCCGCCGCTGCTCTGCT-3′); the 5′ fragment was amplified using the 5′ specific primer (5′-CTCCTGGCTTACACGCTCAGAGCTG-3′) and a 3′ specific primer (5′-GCCTTGTTCCCAGGGTCTGCTCAASAAC-3′). For the second round of PCR, the 5′ and 3′ PCR fragments were used as templates and amplified by the same primer designed for VN 1–97. For protein expression, PCR-amplified products were digested with NcoI and XhoI or BamHI and XhoI, and ligated into the pET-32a (+) expression vector (Novagen, Madison, WI). To generate a double deletion mutant (VN 40–342,377–450), the VN 40–459 expression clone was used as template and amplified by the sense primer (5′-CCTCGTTCTTCGATCTCAGAGTGATGCTG-3′) and the antisense primer (5′-GGTGGTCATGGGGATGATGATGATG-3′) starting from residue 377 and the antisense primer (5′-GGTGGTCATGGGGATGATGATGATG-3′) starting from residue 342. E. coli AD494 cells were transformed with various expression plasmids and grown at 37 °C in LB medium containing ampicillin (100 μg/ml) and kanamycin (15 μg/ml) to a density of ~2 × 10^8 cells/ml. The cells were transferred to room temperature, induced by the addition of isopropyl-β-D-thiogalactopyranoside (1 mM) for 3 h, collected by centrifugation, resuspended in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and lysed using a pressure cell press (Spectroscopic Instruments, Rochester, NY). The cold osmotic shock fluid was centrifuged at 31,000 × g for 30 min, and the supernatant was employed for the purification of the target peptides using TALON metal affinity resin (CLONTECH, Palo Alto, CA). The eluted peptides were then dialyzed against a 0.10 concentration of phosphate-buffered saline (PBS) and concentrated by lyophilization. The concentration of the purified proteins was determined using the BCA protein assay reagent (Pierce).

Characterization of VN Variants by ELISA—To determine the heparin binding capacity of the VN variants, wells were coated overnight at 4 °C with VN or VN variants (5 μg/ml). Excess sites on the wells were blocked by incubation with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS: 20 mM Tris-HCl, pH 7.5/0.5% NaCl) for 2 h at 37 °C, and then the wells were washed with TBS. Biotin-conjugated goat anti-rabbit IgG (0.5 μg/ml) was added and the plates were incubated for 1 h at 37 °C, and then the wells were washed with TBS containing 0.1% Tween 20. The bound heparin-albumin was detected using streptavidin alkaline phosphatase (AP) and a chromogenic substrate for AP. To determine the plasminogen binding capacity of the VN variants, wells were coated with plasminogen (1 μg/ml), blocked, and washed as before. Biotin-conjugated goat anti-plasminogen IgG (0.5 μg/ml) was added and the wells were incubated for 1 h at 37 °C. After washing, the bound VN variants were detected by incubating the wells with rabbit anti-vitronectin IgG (1:1000 dilution) in TBS containing 0.1% Tween 20 for 45 min at 37 °C. The wells were washed and then incubated sequentially with biotin-conjugated goat anti-rabbit IgG, with streptavidin AP, and finally with the chromogenic substrate. To determine the extent of uPAR and PAI-1 binding, wells were first coated with VN or VN variants (0.5 μg/ml), blocked, and washed. Then, uPAR plus uPA (5 μg/ml each) was added, and the wells were incubated for 1 h at 37 °C. The wells were washed and bound protein was detected by incubating them with 2 μg/ml of either rabbit anti-uPAR or rabbit anti-PAI-1 IgG in TBS containing 0.1% Tween 20 for 45 min at 37 °C. The wells were blocked and then incubated sequentially with biotin-conjugated goat anti-rabbit IgG, streptavidin AP, and a chromogenic substrate. The wells were added, and the concentration of bound protein was determined kinetically by monitoring the change in color at 405 nm (milli optical density per min; mOD/min).

Characterization of VN Variants by Real Time Biomolecular Interaction Analysis (BIAcore)—All experiments were carried out at 25 °C using a constant flow rate (10 μl/min) of running buffer. Briefly, denatured VN or VN variants were immobilized on the sensor chip using the amine coupling method as described by the manufacturer, and then various concentrations of uPAR plus uPA, or PAI-1 alone, were injected. Thus, in these experiments, the VN concentrations were constant and the concentrations of uPAR and PAI-1 were varied. This experimental set-up minimizes the influence of differences in the percent of properly folded VN, on the calculation of binding constants. The association, dissociation, and regeneration phases were followed in real-time by monitoring the change in signal expressed in resonance units (RU). Sensorgrams were analyzed using BIAevaluation Software, version 3.0. Kinetic constants were obtained from the association and dissociation curves generated from different concentrations of analyte by using the integrated binding model (A + B + CB). The model gave a single-binding site model of one analyte to the immobilized ligand. Comparison fitting with a more complex binding model did not give a better interpretation of the data. Between experiments, the chips were regenerated as recommended by the manufacturer by washing with regeneration buffer (0.1 n HCl). This treatment does not alter the binding properties of the VN-coated sensor chip (31). In some experiments, denatured VN was coated on sensor chip and then a preincubiation mixture containing increasing concentrations of native or denatured VN (0–4 μM) with either uPAR containing uPA (1 μM each) or PAI-1 (50 nM) was injected. The preincubitation time for VN with uPAR plus uPA was 30 min at room temperature, whereas that for VN with PAI-1 was 10 min at room temperature. The amount of bound uPAR or PAI-1 was quantified by monitoring binding rates (RU/s).

Experiments to Determine the Effect of Heparin and Plasminogen on the Binding of uPAR and PAI-1 to VN—Wells were first coated with denatured VN (0.5 μg/ml) by incubation overnight at 4 °C. Excess sites were blocked by incubating the wells with 5% BSA in TBS for 2 h at 37 °C. The wells were then washed and preincubated with increasing concentrations of heparin or plasminogen (0–10 μg/ml) for 1 h at 37 °C. After this incubation, a 0.5 volume of uPAR plus uPA (500 nM each containing 1 μM apotinin, or 0.1 μM each) was added, and the plates were incubated for an additional 1 h at 37 °C. After washing, the wells were incubated with 2 μg/ml rabbit anti-uPAR or anti-PAI-1 IgG in TBS containing 0.1% Tween 20 for 1 h at 37 °C. The wells were then washed, and bound antigens were detected as described above by monitoring the change in color at 405 nm.

Purification and Analysis of VN Fragments Prepared by Acid Cleavage—Acid hydrolysis of VN was performed as described previously (9).
Briefly, purified denatured 65-kDa VN (2 mg) was incubated with 70% formic acid for 24 h at 37 °C. The resultant sample was diluted 1:10 with distilled water and lyophilized. The sample was dissolved in 10 mM Tris-HCl, pH 7.5, containing 5 mM EDTA and 8 M urea, and applied to a heparin-agarose column (heparin-agarose type II-S Sigma); bed volume for about 2 vol of VN was determined by adding 2 μl of 1 M Tris. The samples were diluted to a final concentration of 1 μg/ml and then coated onto the wells by incubation overnight at 4 °C. Excess sites were blocked by incubating the wells with 5% BSA in TBS for 2 h at 37 °C. The wells were then washed and incubated with either uPAR plus uPA (5 μg/ml each), with PAI-1 (1 μg/ml), or with mAbs (153, 8E6, 1244, and 1330: 2 μg/ml) for 1 h at 37 °C. After washing, the wells were incubated sequentially in biotinylated anti-rabbit or anti-mouse IgG, streptavidin alkaline phosphatase (AP), and finally the chromogenic substrate. Bound antibodies were visualized as described above by monitoring the change in color at 405 nm.

**SDS-PAGE and Immunoblotting Analysis**—SDS-PAGE was performed in slab gels (12%) according to Laemmli (39). For immunoblotting analysis, proteins in the gels were electrophoretically transferred to PVDF membranes. Excess sites on the membranes were blocked by incubation with 5% casein in TBS for 2 h at room temperature. After washing once with TBS, the membranes were probed with 2 μg/ml mAb 8E6 in 3.5% casein in TBS by incubation overnight at 4 °C. After washing three times with TBS containing 0.1% Tween 20, the membranes were incubated for 1 h at room temperature in a 1:2500 dilution of peroxidase-labeled anti-mouse IgG in TBS containing 3.5% casein. Immunoreactive proteins were visualized using the ECL detection system (Amersham Biosciences AB).

**Cell Adhesion Assays**—Cell adhesion assays were performed as previously described (19) using the human histiocytic lymphoma-derived cell line U937 (ATCC CRL 1593) obtained from the American Type Culture Collection (Rockville, MD). Briefly, microwell plates (0.32 cm²; 96-well coated overnight at 4 °C with denatured VN (1 μg/well) and 2 μg/ml mAb 8E6) were blocked by incubation for 2 h at room temperature with 7.5% BSA and washed twice with RPMI containing 0.02% BSA (RPMI/BSA). RPMI/BSA medium (100 μl) was added to the wells with or without PAI-1 (40 μg/ml) or various mAbs (40 μg/ml). The wells were incubated for 1 h at room temperature and then washed with the RPMI/BSA solution. After the U937 cells (3 × 10⁵ cells/well) were added, the microwell plates were incubated for 2 h at 37 °C, then agitated twice for 1 min (Molecular Devices Vmax plate reader), and gently washed with RPMI/BSA. The number of remaining adherent cells was determined by crystal violet staining as follows. The medium was first removed from the wells, and then the cells were fixed with 100% methanol, stained with crystal violet, washed extensively with water, and extracted into 10% acetic acid. The amount of stain in the extract was quantified by absorbance at 590 nm.

**RESULTS**

**Initial Characterization of VN Variants**—Intact human VN is 459 amino acids long and is composed of distinct domains (Fig. 1). Experiments were performed to evaluate the role of two of these domains (i.e. the SMB and HB domains), in uPAR binding to VN. In these experiments, we directly compared the ability of uPAR, PAI-1, plasminogen, and heparin to bind to VN fragments lacking either the SMB domain or the HB domain (see Fig. 1). As shown in Fig. 2, heparin (panel A) and plasminogen (panel B) bound to denatured VN and to VN variants containing the HB domain, but did not bind to VN variants lacking this domain. Importantly, the magnitude of binding of these molecules to VN 40–459 was similar to that for their binding to denatured VN. Thus, the majority of the recombinant VN 40–459 molecules in this preparation appear to be properly folded and functionally active. Despite this, neither uPAR (panel C) nor PAI-1 (panel D) bound significantly to VN fragments containing this functional HB site if a functional SMB domain also was lacking (i.e. the Y28A mutant or mutants lacking the SMB domain). They bound only to intact VN and to VN fragments containing the SMB domain. Thus, the HB domain does not appear to be required for the binding of uPAR and PAI-1 to VN, whereas the SMB domain is necessary and sufficient for these interactions. These conclusions are supported by the data summarized in Fig. 3. In these experiments, the effect of increasing concentrations of heparin or plasminogen on the binding of uPAR (Fig. 3A) and PAI-1 (Fig. 3B) to denatured VN was assessed. As shown, high concentrations of heparin and plasminogen had no effect on the binding of uPAR or PAI-1 to denatured VN. These results are again consistent with the conclusion that the binding of uPAR and PAI-1 to denatured VN does not involve the HB domain itself.

**Binding Properties of VN Variants**—Surface plasmon resonance was employed to analyze the interactions between the various VN variants and uPAR and PAI-1 in more detail (Fig. 4). As shown in Fig. 4A, uPAR again binds to immobilized VN variants containing the SMB domain (i.e. VN 1–44, VN 1–97, and VN 1–217), but does not bind to VN mutants lacking a functional SMB domain (i.e. VN 1–97Y28A, VN 40–459, and VN 40–342,377–459). Similarly, PAI-1 only binds with a high rate of association and a low rate of dissociation to immobilized VN variants which contain the functional SMB domain (panel B). Although PAI-1 also binds to the Y28A variant with a high association rate, the bound peptide is rapidly released from it. Thus, the SMB domain of VN is the only region detected in these BIAcore experiments that is involved in the high affinity binding of uPAR and PAI-1 to VN. In agreement with previous studies (19, 24), residue Tyr²⁸⁸ in VN is critical for these interactions. In additional experiments of the type shown in Fig. 4, the kinetic constants for the various interactions were determined from the BIAcore association and dissociation curves obtained by varying the concentration of uPAR or PAI-1 (Table 1). The KD values for the binding of uPAR to denatured VN and to VN variants containing the functional SMB domain were similar and ranged from 0.14 to 0.5 μM. Again, no binding was detected when uPAR was incubated with either the Y28A mutant, or with mutants lacking the SMB domain. The approximate KD values for the interaction of PAI-1 with denatured VN and with...
VN variants containing a functional SMB domain also were similar (0.15–0.36 nM), whereas the K₅₀ for the interaction of PAI-1 with the Y28A mutant was almost 2000 times lower. Again, no binding was detected in these experiments when PAI-1 was incubated with VN mutants lacking the SMB domain. These results are consistent with the data shown in Fig. 2 and support the conclusion that the SMB domain of VN is the only region involved in the binding of uPAR and PAI-1.

![FIG. 1. Vitronectin variants.](Image)

The top bar drawing shows the domain structure of the 75-kDa form of human VN (SMB, somatomedin B domain; RGD, Arg-Gly-Asp sequence; CR, connecting region; H1 and H2, heparin-binding-like domains 1 and 2; HB, heparin-binding domain). All mutants were constructed from human VN cDNA, and the expressed proteins were purified as thioredoxin fusion peptides. Trx, thioredoxin; His, histidine tag. The 1–44 variant contains only the SMB domain. The Y28A mutant is derived from the 1–97 variant by making a single amino acid change in the SMB domain, from tyrosine to alanine at position 28. The 40–459 variant is a deletion mutant lacking the SMB domain, and variant 40–342,377–459 is a deletion mutant lacking both the SMB domain and the heparin-binding domain. The 1–217 variant is the 40-kDa VN fragment purified from acid cleaved VN.

![FIG. 2. Initial characterization of vitronectin variants by ELISA.](Image)

To compare the binding properties of heparin (panel A), uPAR (panel C), or PAI-1 (panel D), wells were first coated with VN or VN variants (5 μg/ml for the heparin and uPAR experiments, 0.5 μg/ml for the PAI-1 experiments) and then incubated with biotin conjugated heparin-albumin (0.5 μg/ml), with uPAR/uPA (5 μg/ml, each), or with PAI-1 (1 μg/ml). To determine plasminogen binding to denatured VN and VN variants (panel B), wells were first coated with plasminogen (1 μg/ml) and then the appropriate form of VN (5 μg/ml each) was added. In all cases, bound molecules were detected by using a chromogenic substrate for AP. Each column reflects the average of triplicate wells ± S.D. The details of these assays are described under “Experimental Procedures.” Statistical significance is indicated by asterisks as follows: *, p < 0.05; **, p < 0.01.
assays are described under experimental procedures. It was noted that the fragment migrated at the same molecular weight in the presence or absence of 2-mercaptoethanol (data not shown), suggesting that it is a monomer. Amino acid sequencing revealed that the cleavage site in VN was located between Arg	extsuperscript{217} and Pro	extsuperscript{218} (data not shown). Moreover, the purified 40-kDa fragment (i.e. VN 1–217) bound uPAR and PAI-1 (Figs. 2 and 4; Table 1) but not plasminogen or heparin (Fig. 2).

As shown in Fig. 6A, harsh acid treatment (1 N HCl, 85 °C) of the purified 40-kDa VN fragment for 4 min had little effect on the amount of storable protein detected in the sample, but it completely destroyed the binding site for mAb 8E6.

Direct binding studies (Fig. 6B) revealed that, although the binding of mAb 8E6 was greatly diminished by this treatment, the binding of uPAR, PAI-1 and mAbs 153, 1244, and 1330 was not affected (Fig. 6B). Taken together, these results indicate that the binding sites for uPAR and PAI-1 in VN are distinct from the epitope recognized by mAb 8E6.

Acid treatment at high temperature is known to hydrolyze ester bonds on sulfated tyrosines, releasing the sulfate groups and making it impossible to detect sulfated tyrosine in proteins (41). These results raise the possibility that sulfated tyrosine residues in VN 1–217 are necessary for the binding of mAb 8E6, but not for the binding of uPAR, PAI-1, or the other mAbs. To more directly investigate this possibility, we prepared sulfated and nonsulfated synthetic peptides containing residues 48–68 of VN, and tested their reactivity toward mAb 8E6. As shown in Fig. 7, mAb 8E6 showed the strongest reactivity toward sulfated VN peptide 1, which contained the two sulfated tyrosines at positions 56 and 59. The mAb was much less reactive toward peptides 2 and 3, which only contained one sulfated tyrosine, and it did not recognize the nonsulfated VN peptide (peptide 4). These results indicate that tyrosine sulfation at residues 56 and 59 is critical for the reactivity of mAb 8E6. Moreover, these results place the epitope for mAb 8E6 immediately downstream from the SMB domain of VN (i.e. in residues 48–68 in the connecting region).

Inhibition of the Binding of uPAR and PAI-1 to Immobilized VN by Various mAbs—Experiments were performed to determine the effects of the various mAbs on the binding of uPAR and PAI-1 to VN. All of the mAbs that were tested appeared to inhibit uPAR binding to immobilized denatured VN to some extent (Fig. 8A). However, the magnitude of inhibition varied over a wide range with mAbs 1244 and 1330 only beginning to inhibit at very high concentrations. In fact, the magnitude of inhibition appears to increase the closer the epitope recognized by each mAb is to the SMB domain. Although the inhibition curves for mAbs 153 and 8E6 were similar, only mAb 153 completely inhibited uPAR binding in these experiments. As shown in Fig. 8B, mAb 153 was the only mAb that inhibited PAI-1 binding to immobilized denatured VN. Taken together, these results again support the conclusion that the SMB domain contains the primary binding sites in VN for uPAR and PAI-1. The fact that some mAbs (e.g. 8E6) inhibit uPAR but not PAI-1 binding (Fig. 8, compare A and B) suggests that the uPAR and PAI-1 binding sites are distinct but overlapping.

Effect of Various mAbs on the Adhesion of U937 Cells to VN—To confirm that the SMB domain is necessary for the binding of cellular uPAR to denatured VN, the anti-adhesive properties of the mAbs were studied. The uPAR-mediated adhesion of U937 cells to immobilized denatured VN was effectively inhibited by PAI-1 and by mAbs 153 and 8E6, but not by mAbs 1244 or 1330 (data not shown). Thus, the anti-adhesive effect of the mAbs is similar to the uPAR binding data shown in Fig. 8A.
A. uPAR binding

B. PAI-1 binding

FIG. 4. Binding properties of vitronectin variants. In these typical BIACore experiments, VN variants were immobilized on a BIACore sensor chip (20 μg/ml) and then 500 nM each of uPAR with uPA (panel A) or 400 nM PAI-1 (panel B) were injected. The results for the thioredoxin control and for the non-immobilizing control were subtracted from each sensorgram (●, VN 1–44; ▲, VN 1–97; ■, VN 1–97Y28A; ◆, VN 40–459; ▼, VN 40–342, 377–459; ⋄, VN 1–217).

DISCUSSION

Although a number of observations indicate that the biological activity of VN can be modulated through interactions with uPAR and PAI-1 (11, 13, 19–21, 42), the nature of these interactions remains somewhat controversial. For example, some groups suggest that uPAR and PAI-1 bind to the amino-termi-
To directly compare the uPAR-binding properties of the SMB and HB domains of VN, we studied the isolated domains themselves, as well as recombinant fragments of VN that specifically lacked one of the two domains. The following observations support the conclusion that the only binding site for uPAR in VN resides in the NH₂-terminal SMB domain. First of all, uPAR only bound to VN fragments containing the SMB domain (Fig. 2). No binding could be detected with VN containing a functional HB-domain but lacking the SMB domain (Fig; Table I). Importantly, the kinetic constant for the interaction of uPAR with the isolated SMB domain was similar to that for its interaction with intact VN (Table I). Thus, the binding of uPAR to the SMB domain mimics its interaction with VN itself. Second, purified recombinant SMB inhibits the binding of uPAR to denatured VN (19), and mAb 153 (which recognizes an epitope in the SMB domain) blocks uPAR and PAI-1 binding to this VN (Fig. 8). In contrast, the presence of relatively high concentrations of heparin and plasminogen, two molecules known to bind to the HB domain (13), have no effect on the binding of uPAR to VN (Fig. 3). Finally, mAb 8E6 blocks the binding of purified uPAR (Fig. 8) and U937 cells (data not shown) to VN. It does so not by binding to the HB domain, but by binding to a site that is immediately adjacent to the SMB domain (i.e. the sulfated tyrosines at residues 56 and 59; Figs. 6 and 7).

The fact that mAb 8E6 recognizes an epitope in the NH₂-terminal region of VN provides a powerful new argument for the conclusion that the COOH-terminal HB-domain is not involved in uPAR binding. The evidence that mAb 8E6 binds to residues 56 and 59, and not to a site in the HB domain, is rather compelling. For example, Seiffert (32) showed that this mAb interacts with a 35-kDa fragment of VN containing residues 52–217 (i.e. lacking the HB domain), and Tomasini-Johnson et al. (44) showed that inhibition of sulfation decreased the reactivity of this mAb toward VN. These studies first raised the possibility that the epitope recognized by mAb 8E6 resides in the NH₂-terminal half of the molecule and includes the sulfated tyrosines in the connecting region. This conclusion is supported by observations in this report. Thus, brief acid treatment to hydrolyze ester bonds on sulfated tyrosines (41) destroyed the binding site in VN recognized by mAb 8E6 (Fig. 6). Importantly, this treatment had no effect on the binding of uPAR, PAI-1, or mAb 153 to VN. Thus, the epitope in VN recognized by mAb 8E6 is distinct from the binding sites recognized by uPAR and PAI-1. Perhaps more importantly, mAb 8E6 bound to synthetic peptides derived from VN if they contained sulfated tyrosines 56 and 59 (Fig. 7). It did not bind to related peptides containing nonsulfated tyrosines. The fact that the magnitude of binding decreased dramatically if only one of the tyrosines was sulfated suggests that both of the sulfated tyrosines contribute to the epitope. Finally, mAb 8E6 did not recognize any of the recombinant peptides shown in Fig. 1, even though uPAR, PAI-1, and other Mabs bound to them as expected (data not shown). This is an important observation because recombinant proteins expressed in E. coli do not contain sulfated tyrosines (41, 45). The ability of mAb 8E6 to recognize sulfated tyrosines may account for the observation that this mAb also recognizes a 30-kDa protein that is synthesized by nonhepatic cells and is not related to VN (44).

Taken together, the above observations indicate that the epitope recognized by mAb 8E6 is in the connecting region of VN and not in the HB domain, and that it is distinct from the site in VN recognized by uPAR. Moreover, these results suggest that inhibition of uPAR binding by mAb 8E6 is an indirect (i.e. steric) effect and not the result of direct competition for the same site.

If the hypothesis that the SMB domain is the only domain...
involved in uPAR binding to denatured VN is correct, then how do we account for the observation that synthetic peptides from the HB-binding domain (i.e., residues 364–375 and 371–380) compete with uPAR for binding to immobilized denatured VN (21)? This observation has been offered as evidence that the HB domain may contain a second binding site for uPAR (21). However, no data were presented in these studies to show that the peptides actually bind to uPAR. Without this information, it is difficult to distinguish between the assumed direct effects of the peptides (e.g., binding to uPAR) versus indirect mechanisms (e.g., binding to critical regions in VN itself). In this regard, VN contains a stretch of acidic amino acids between residues 53 and 64, whereas the synthetic peptides contain a cluster of basic residues. Thus, it is possible that an ionic interaction between these acidic peptides and the basic residues at the NH$_2$ terminus of VN may mask the binding site in SMB and/or alter the three-dimensional structure of this domain.

Based on the above considerations, we suggest that there is a single binding site for uPAR in VN, and that it is located in the SMB domain (Fig. 9). The BIAcore and mAb results (Table I; Figs. 4 and 8) also suggest that PAI-1 binds to a single site in VN, and that this site is again located in the SMB domain. These conclusions are consistent with other studies showing that PAI-1 binds to the SMB domain of VN (9, 24–27) with a stoichiometry of 1:1 (31). However, our results and their interpretation differ somewhat from those of Podor et al. (46). For example, whereas we showed the mAb 8E6 had no effect on PAI-1 binding (Fig. 8), Podor et al. (46) observed that this mAb inhibited PAI-1 binding by 50%. Similarly, whereas mAb 153 completely inhibited PAI-1 binding in our studies (Fig. 8), it only inhibited PAI-1 binding by 50% in their studies. Complete inhibition was only observed by Podor et al. (46) when both mAbs were present. Taken together, these observations led Podor et al. (46) to conclude that there are 2 distinct binding sites for PAI-1 in VN, one recognized by mAb 153 and a second recognized by mAb 8E6. This conclusion is consistent with their subsequent analytical ultracentrifugation data (e.g., a 4:2 stoichiometry was suggested for the binding of PAI-1 to VN). How-
ever, the in vivo significance of this observation is difficult to assess because the experiments were performed at relatively high concentrations of PAI-1 (i.e. at a 1:1 molar ratio of PAI-1 to VN). The ratio of PAI-1 to VN in the circulation is less than 1:1000, and high concentrations of PAI-1 are known to promote the multimerization of VN (47).

The different results with the same antibodies appear to reflect the different assay systems and conditions employed in the two studies. For example, when we used an ELISA system (VN-coated wells) instead of the BIAcore to study PAI-1 binding, we also observed that mAb 8E6 partially (50%) inhibited PAI-1 binding (data not shown). These differences may result from conformational changes in the bound VN because of the flowing conditions or chemical coupling procedures employed in the BIAcore studies. The binding sites in VN for both PAI-1 (30) and mAb 8E6 (6, 7) are known to be conformationally dependent. Thus, in the BIAcore system, there may be a change in the conformation of VN, which moves the binding sites for PAI-1 and mAb 8E6 far enough apart to eliminate steric hindrance. This conformational change may not occur in the ELISA system. Similarly, the failure of Podor et al. (46) to observe complete inhibition of PAI-1 binding with mAb 153 may reflect the relatively low levels of competing mAb they employed. In this regard, we also observed 50% inhibition at the concentration of mAb 153 employed in their studies (Fig. 8). As shown in Fig. 8, complete inhibition was only achieved at the higher concentrations. Whatever the mechanism for these differences, it seems that both mAbs can inhibit PAI-1 binding under some conditions. However, this observation does not necessarily lead to the conclusion that there must be two distinct binding sites for PAI-1 in VN. The fact that the SMB domain contains all of the structural and sequence information required for PAI-1 binding (Table I), together with the observation that mAb 8E6 does not directly block PAI-1 binding (Figs. 6 and 7), argues that the observed inhibition reflects steric interference of PAI-1 binding to one site rather than the existence of two distinct PAI-1 binding sites.

In summary, the studies presented here indicate that the SMB domain of VN is the only region in VN involved in the binding of uPAR and PAI-1 (Fig. 9). They suggest that the PAI-1 and uPAR binding sites in the SMB domain are partially overlapping, that the uPAR site is located closer to the 8E6 epitope than the PAI-1 site, and that the epitope recognized by mAb 153 is very close to both binding sites. Although the PAI-1 binding site was recently localized to 6 residues in the central region of the SMB domain (24, 48), the exact relation of this site to the uPAR binding site remains to be determined. These studies also suggest that the epitope recognized by mAb 8E6 is located outside of the SMB domain but in the connector region immediately adjacent to the SMB domain, and that this epitope is distinct from the binding sites for uPAR and PAI-1. The
Vitronectin and uPAR

ability of mAb SE6 to inhibit uPAR and PAI-1 binding to native and denatured VN would seem to result from indirect steric effects on the site in the SMB domain and not from blocking a second distinct site. Future NMR or x-ray crystallography approaches will help us to further understand these interactions and how they are regulated.

Acknowledgments—We thank Nancy Wagner for technical support and Alicia Palestini and Marcia McRae for preparing the manuscript.

REFERENCES

1. Barnes, D. W., Reing, J. E., and Amos, B. (1985) J. Biol. Chem. 260, 9117–9122
2. Hayashi, M., Akama, T., Kono, I., and Kashiwagi, H. (1985) J. Biochem. (Tokyo) 98, 1135–1138
3. Pytela, R., Pierschbacher, M. D., and Ruoslahti, E. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5766–5770
4. Pytela, R., Pierschbacher, M. D., Ginsberg, M. H., Flow, E. F., and Ruoslahti, E. (1986) Science 231, 1559–1562
5. Hogasen, K., Molnes, T. E., and Harboe, M. (1992) J. Biol. Chem. 267, 23076–23082
6. Tomasini, B. R., and Mosher, D. F. (1988) Blood 72, 903–912
7. Tomasini, B. R., Owen, M. C., Fenton, J. W. I., and Mosher, D. F. (1989) Biochemistry 28, 7617–7623
8. Declerck, P. J., De Mol, M., Alessi, M.-C., Baudner, S., Paques, E.-P., Preissner, K., Muller-Berghaus, G., and Collen, D. (1988) J. Biol. Chem. 263, 15454–15461
9. Seifert, D., and Loskutoff, D. J. (1991) J. Biol. Chem. 266, 2824–2830
10. Wisman, B., Almeida, A., Sigurdardottir, O., and Lindahl, T. (1988) FEBS Lett. 242, 125–128
11. Wei, Y., Waltz, D. A., Rao, N., Drummond, R. J., Rosenberg, S., and Chapman, H. A. (1997) J. Clin. Invest. 100, 58–67
12. Hayman, E. G., Pierschbacher, M. D., Ohgren, Y., and Ruoslahti, E. (1983) J. Biol. Chem. 258, 13401–13408
13. Pytela, R., Pierschbacher, M. D., Ginsberg, M. H., Flow, E. F., and Ruoslahti, E. (1986) Science 231, 1559–1562
14. Gebb, C., Hayman, E. G., Engvall, E., and Ruoslahti, E. (1986) J. Biol. Chem. 261, 1689–16703
15. Ishikawa, M., and Hayashi, H. (1992) Biochim. Biophys. Acta 1121, 173–177
16. Izumi, M., Shimo-Oka, T., Morishita, N., Ii, I., and Hayashi, M. (1988) Cell Struct. Funct. 13, 217–225
17. Preissner, K. T. (1993) Biochem. Biophys. Res. Commun. 168, 966–971
18. Hildebrand, A., Schweigerer, L., and Teschemacher, H. (1988) J. Biol. Chem. 263, 2436–2441
19. Deng, G., Curriden, S. A., Wang, S., Rosenberg, S., and Loskutoff, D. J. (1996) J. Cell Biol. 134, 1563–1571
20. Stefansson, S., and Lawrence, D. A. (1996) Nature 383, 441–443
21. Waltz, D. A., Naitkin, L. R., Fujita, R. M., Wei, Y., and Chapman, H. A. (1997) J. Clin. Invest. 100, 58–67
22. Preissner, K. T., and Seifert, D. (1998) Thromb. Res. 91, 1–21
23. Loskutoff, D. J., Curriden, S. A., Hu, G., and Deng, G. (1999) APMIS 107, 54–61
24. Deng, G., Royle, G., Wang, S., Crain, K., and Loskutoff, D. J. (1996) J. Biol. Chem. 271, 12716–12723
25. Philips, M., Johnsen, H., and Thorsen, S. (2000) Fibrinolysis Proteolysis 14, 22–34
26. Seifert, D., Ciambrone, G., Wagner, N. V., Binder, B. B., and Loskutoff, D. J. (1994) J. Biol. Chem. 269, 2659–2666
27. Sigurdardottir, O., and Wisman, B. (1992) Fibrinolysis 6, 27–32
28. Gechtman, Z., Sharma, R., Kreizman, T., Fridkin, M., and Shaltiel, S. (1993) FEBS Lett. 315, 293–297
29. Mimuro, J., Muramatsu, S., Kurano, Y., Uchida, Y., Iikada, H., Watanabe, S., and Sakata, Y. (1993) Biochemistry 32, 2314–2320
30. Lawrence, D. A., Palaniappan, S., Stefansson, S., Olson, S. T., Francis-Chmura, A. M., Shore, J. D., and Ginsburg, D. (1997) J. Biol. Chem. 272, 7676–7680
31. Ehnehom, J., Bjorquist, P., Sigurdardottir, O., and Deinum, J. (2000) Fibrinolysis Proteolysis 14, 47–57
32. Seifert, D. (1995) FEBS Lett. 368, 155–159
33. Seifert, D., and Smith, J. W. (1997) J. Biol. Chem. 272, 13705–13710
34. Kvassman, J., and Shore, J. D. (1995) Fibrinolysis 9, 215–221
35. Higazi, A. A. R., Mazar, A., Wang, J., Reilly, R., Henkin, J., Kniss, D., and Cines, D. (1996) Blood 87, 3545–3549
36. Dahlback, B., and Podack, E. R. (1985) Biochemistry 24, 2368–2374
37. Bittorf, S. V., Williams, E. C., and Mosher, D. F. (1993) J. Biol. Chem. 268, 24838–24846
38. Yatsuho, T., Izumi, M., Kashiwagi, H., and Hayashi, M. (1988) Cell Struct. Funct. 13, 281–292
39. Laemmli, U. K. (1970) Nature 227, 680–688
40. Kitagawa, K., Aida, X., Fujiwara, H., Yamagami, T., Kogure, M., Ida, J., and Inoue, K. (2001) J. Org. Chem. 66, 1–10
41. Huttner, W. B. (1984) Methods Enzymol. 101, 200–223
42. Preissner, K. T., and Jenne, D. (1991) Thromb. Haemostasis 66, 123–132
43. Gechtman, Z., Belleli, A., Lechpammer, S., and Shaltiel, S. (1997) Biochem. J. 325, 339–349
44. Tomasini, B. R., Mosher, D. F., and Pierschbacher, M. D. (1993) Matrix 13, 203–214
45. Jenne, D., Hille, A., Stanley, K. K., and Huttner, W. B. (1989) Eur. J. Biochem. 185, 391–395
46. Podor, T. J., Shaichnessey, S. G., Blackburn, M. N., and Peterson, C. B. (2000) J. Biol. Chem. 275, 25402–25410
47. Seifert, D., and Loskutoff, D. J. (1996) J. Biol. Chem. 271, 29644–29651
48. Royle, G., Deng, G., Seifert, D., and Loskutoff, D. J. (2001) Anal. Biochem. 296, 245–253
