Characterization of the Ligand Binding Activities of Vitronectin: Interaction of Vitronectin with Lipids and Identification of the Binding Domains for Various Ligands Using Recombinant Domains†

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ABSTRACT: Vitronectin is a multifunctional plasma glycoprotein which may regulate the systems related to protease cascades such as the coagulation, fibrinolysis, and complement systems as well as cell adhesion. Solid-phase assays and affinity chromatography on immobilized glycolipids indicated that vitronectin purified under denaturing conditions bound to sulfatide (Gal(3-SO_4)β1-1ceramide), cholesterol 3-sulfate, and various phospholipids, but not gangliosides. Only the unfolded or multimeric form of vitronectin bound to sulfatide, suggesting a conformational dependency of the binding activity, while vitronectin bound to cholesterol 3-sulfate regardless of its conformational state. The recombinant domains of human vitronectin and mutants with certain domains deleted were separately expressed in *E. coli* as fusion proteins. Using the recombinants, sulfatide-, phosphatidylserine-, cholesterol 3-sulfate-, Type I collagen-, heparin-, and β-endorphin-binding activities were found to be attributable to hemopexin domain 2 and hemopexin domain 1. The possibility was suggested that the presence of a somatomedin domain and/or connecting region flanking hemopexin domain 1 inactivated its heparin binding. De-*N*-glycosylation of plasma vitronectin significantly affected the cholesterol sulfate- and collagen-binding activities, although its effects were opposite. These findings suggest that diverse ligand-binding activities could be attributed to pexin family motifs but that the interdomain interactions and glycosylations modulate the ligand binding activities of vitronectin.

Vitronectin (VN)[1] is a multifunctional glycoprotein present in mammalian plasma and the extracellular matrix of many tissues (reviewed in refs *1*−*3*). VN may regulate the coagulation, fibrinolysis, and complement systems, and mediate cell−substrate adhesion as a cross-linker. VN may also participate in the matrix remodeling by regulating pericellular proteolysis.

VN interacts with various substances to exhibit diverse biological activities. Most VN in normal plasma does not bind to heparin, complement C5b-7, β-endorphin (*4*), type-1 plasminogen activator inhibitor (PAI-1), Type I collagen (*5*), or urokinase receptors (*6*). VN acquires these binding activities on treatment with urea or heating *in vitro* through a conformational transition of the native inactive form of VN to an active form. Corresponding activation is considered to occur *in vivo* in the presence of heparin or through the formation of VN complexes such as VN−thrombin−antithrombin III (*7*), VN−terminal complement proteins (*8*), and VN−PAI-1 (*9*), while VN is considered to exist in active multimeric tissue forms when deposited in the extracellular matrix or within platelets. The relationships between conformations of VN and its ligand binding activities are complicated and subject to debate (*10*−*13*). Recent progress in research on heparin/glycosaminoglycan binding suggests that the binding site is exposed in the native conformation and that multimerization induced by denaturation enhances heparin binding activities (*12*, *13*). Cell adhesion in plasma vitronectin is reported to be cryptic in native VN (*14*). On the other hand, it was reported that VN released from platelets was cleaved by platelet calpains I and II (*15*), resulting in attenuation of the binding activities toward heparin and cells which maintain the PAI-1 binding activity. These reports suggested that the changes in the intra- and intermolecular interactions of VN accompanying unfolding, multimerization, or proteolysis are essential for VN to express or regulate its function. Therefore, it is necessary to determine the binding characteristics of each structural motif as a basis for a working molecular model of VN as well as

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[1] Abbreviations: The abbreviations: VN, vitronectin; hVN, human vitronectin; pVN, porcine vitronectin; WHHL, Watanabe-heritable hyperlipidemic; sulfatide, Gal(3-SO_4)β1-1ceramide; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; GST, glutathione S-transferase; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PAI-1, type-1 plasminogen activator inhibitor; TBS, 10 mM Tris-Cl buffer (pH 7.5) containing 150 mM NaCl; PBS, 10 mM sodium phosphate buffer containing 0.13 M NaCl.

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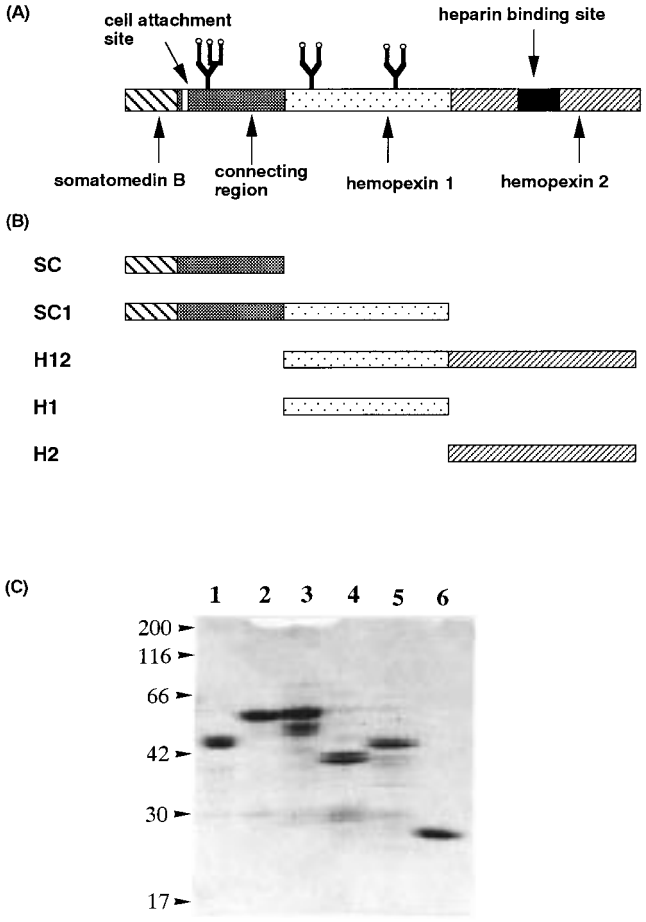


FIGURE 1: The domain structure of hVN (A), recombinant domain mutants and deletion mutants (B), and SDS-PAGE pattern of recombinant proteins (C). (B) The domains of hVN and mutants with certain domains deleted were separately expressed in *E. coli* (DH5) as fusion proteins linked to the C-terminal end of GST. (C) Recombinant proteins purified by glutathione affinity chromatography were subjected to SDS-PAGE on a 11% polyacrylamide gel as a separation gel. Proteins in the gel were stained with Coomassie blue. Markers (in kDa) are shown on the left. Lane 1; SC; lane 2, SC1; lane 3, H12; lane 4, H1; lane 5 H2; and lane 6, GST. Two-pronged and three-pronged forks represent biantennary and triantennary oligosaccharides, respectively.

to identify specific biological ligands.

VN consists of four structurally different domains: somatomedin, hemopexin 1, hemopexin 2, and a connecting region that links somatomedin and hemopexin 1 (*2*), (see Figure 1). The binding sites for heparin and the complement complex have been located in hemopexin domain 2, those for PAI-1 in the somatomedin B domain (*16*, *17*), and/or hemopexin domain 2 (*1*−*3*) for human VN (hVN). The binding sites for other ligands, for example, β-endorphin and Type I collagen, remain ambiguous.

β-endorphin, a 31-residue opioid peptide present in the pituitary (*18*), may be released into the blood during mental and physical stress (*19*, *20*). The interaction with β-endorphin may be significant to the immune and fibrinolysis systems in stress because it has been reported that β-endorphin binds to the soluble complement complex, SC5b-9, via a portion of VN (*21*) and that plasma VN activated with heparin binds to β-endorphin (*4*). It was only indicated that a 55 kDa fragment of VN, whose N-terminal amino acid was Gly45, bound to β-endorphin (*21*). VN showed binding activities toward various types of collagen (II, III, IV, V, and VI) (*5*), in addition to Type I collagen. Based on the inhibition analyses by fragmented peptides, Type I collagen-binding sites have been thought to be located in the N-terminal or internal region of the VN molecule near the heparin binding domain (*22*), but the relative importance of each site has not been established. On the other hand, immunohistochemical studies have demonstrated the deposition of VN in atherosclerotic walls (*23*, *24*) and especially the codeposition of extracellular lipid in the atherosclerosis of Watanabe-heritable hyperlipidemic (WHHL) rabbits (*25*, *26*). However, there have been no reports so far describing the interaction of VN and lipid molecules.

In this paper, we first report that VN can bind to sulfated lipids and phospholipids. The ligand-binding specificities of each isolated domain, including the new lipid ligands, were elucidated using recombinant domains, and the contribution of glycosylation was evaluated.

MATERIALS AND METHODS

Materials. Porcine VN (pVN) and hVN were prepared from porcine and human sera under denaturing conditions using 8 M urea, respectively, as described previously (*27*, *28*). Sulfatide [Gal(3-SO_4)β1-1ceramide] (bovine brain), ganglioside GM1 (bovine brain), GM3, GD3 (bovine brain), GD1b (bovine brain), cholesterol 3-sulfate, phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), sphingomyelin, fucoidan (*Fucus vesiculosus*; sulfur content, 8.5%), and β-endorphin (human) were purchased from Sigma Chemical Co., St. Louis, MO. Ceramide (bovine brain), galactosylceramide (bovine spinal cord), ganglioside GD1a (bovine brain), heparin (porcine intestinal mucosa), and Type I collagen (porcine skin) were from Wako Pure Chemicals, Osaka, Japan. Heparan sulfate (porcine kidney, sulfate content 9.0%) and keratan sulfate (whale nasal cartilage, sulfate content 15%) were prepared in our laboratory as described previously (*29*, *30*). Dermatan sulfate (chondroitin sulfate B, umbilical cord), chondroitin sulfate A (whale cartilage), chondroitin sulfate C (shark cartilage), chondroitin (from chondroitin sulfate A), and hyaluronic acid (umbilical cord) were purchased from Seikagaku Kogyo, Tokyo, Japan. Dextran sulfate (molecular mass ~500000, sulfur content 17%) was from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Anti-pVN antiserum produced in rabbits was purchased from Yagai Inc., Japan. The horseradish peroxidase-labeled (HRP-) antibodies to rabbit IgG (H+L) and to sheep IgG were from Kirkegaard and Perry Lab. Inc., Gaitherburg, MD. The HRP-anti-hVN antibodies and anti-hVN antiserum, both produced in sheep, were from The Binding Site Ltd., Birmingham, England. Bovine serum albumin, fraction V (BSA), and *N*-glycanase were from Boehringer Mannheim, Mannheim, Germany. The affinity gel was prepared by coupling glutathione (reduced form; Boehringer Mannheim, Mannheim, Germany) to epoxy-activated Sepharose 4B (*31*). Antiserum against recombinant glutathione S-transferase (GST)-rat annexin IV was generated by immunization of rabbits in our laboratory (*32*). HRP-heparin and BSA-coupled heparin (BSA-heparin) were prepared by the coupling method using *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline in our laboratory (*27*).

Assaying of Binding of VN to Various Lipids Immobilized on Plastic Plates. The wells of a microtiter plate (Immulon

1, from Dynatech Laboratories, Inc., Chantilly, VA) were coated with 50 μL aliquots of methanol solutions of various concentrations of lipids, and the lipid solutions were dried at 37 °C. All other procedures were performed at room temperature. After the wells were blocked with 5% BSA in 10 mM Tris-Cl buffer (pH 7.5) containing 150 mM NaCl (TBS), 50 μL of hVN or pVN (10 μg/mL) purified under denaturing conditions and corresponding to the multimeric form (*11*) was added to each well, followed by incubation for 1 h. After being washed with the buffer three times, the amount of hVN or pVN bound to the immobilized glycolipids was measured as the absorbance at 490 nm with an anti-hVN or pVN antibody, respectively, HRP-antibodies to goat or rabbit IgG (H+L), and an enzyme-linked immunosorbent assay (ELISA) using the color reaction with *o*-phenylenediamine. To study the inhibition by acidic polysaccharides of the interaction with sulfatide, VN (1 μg/mL) was preincubated with various concentrations of acidic polysaccharides for 30 min and then added to wells precoated with lipids. To study the effects of salt or sulfate ions, VN solutions were prepared in the same buffer with NaCl or sodium sulfate at various concentrations.

Glycolipid Affinity Chromatography. An immobilized glycolipid gel was prepared as described previously (*33*) except for the use of TSKgel Toyopearl HW-50F (Tosoh Corp., Tokyo, Japan) instead of octyl-Sepharose. Briefly, 1 mg of sulfatide, GM3, or GD1a in 4 mL of 0.1 M KCl in 50% ethanol was shaken with 2 mL of HW-50F that had been equilibrated with the same solution at room-temperature overnight. The gel was equilibrated with 10 mM Tris-Cl buffer (pH 7.5) containing 150 mM NaCl and then packed into a column (0.75 × 2 cm). PVN (3 μg) was applied to each column and eluted with 1 M NaCl. Fractions containing VN were detected by ELISA using anti-pVN antibody and HRP-antibodies to rabbit IgG (H+L).

Effect of the Conformational State of VN on the Binding Activities toward Lipids. VN fractions containing human or porcine native VN were prepared by passing each plasma sample through a heparin-Sepharose 4B column with a Sepharose 4B precolumn equilibrated with 10 mM sodium phosphate buffer containing 0.13 M NaCl (PBS), pH 7.7 and 5 mM EDTA. Urea-activated VN was prepared by urea treatment of the native VN fraction at a final urea concentration of 8 M for 2 h at room temperature without a subsequent refolding procedure from the urea-unfolded form. The volumes of the native and urea-activated VN fractions were adjusted with sodium phosphate buffer (pH 7.7) containing 0.13 M NaCl and 5 mM EDTA, and then the fractions were subjected to the binding assay involving ELISA and HRP-anti-hVN antibodies.

Expression of Deletion Mutants and Domain Fragments of hVN as Fusion Proteins in E. coli. HVN is composed of four domains (*2*), see Figure 1A. The deletion mutants and domain fragments of hVN [as shown in Figure 1B] were expressed in *E. coli* DH5 (Toyobo, Tokyo, Japan) as fusion proteins linked to the C-terminal end of GST. The hVN cDNA was amplified with PCR in combination with the following synthetic oligonucleotides as primers. That is, the cDNA encoding the deletion mutant, SC, composed of the somatomedin B and connecting domains, was amplified using primers SOMF and CONR; SC1, composed of the somatomedin B domain, the connecting domain and hemopexin domain 1, SOMF and HP1R; and H12, composed of hemopexin domains 1 and 2, HP1F and HP2R. The cDNA encoding H1, composed of hemopexin domain 1, was amplified using primers HP1F and HP1R; and H2, composed of hemopexin domain 2, HP2F and HP2R.

5′-oligonucleotides		
SOMF	CGGGATCCCCGACCAAGAGTCATG	*Bam*HI
CONF	ATGGATCCAAGTGACTCGCGGGGA	*Bam*HI
HP1F	ATGGATCCCAGCAGAGGAGGAGCT	*Bam*HI
HP2F	ATGGATCCCCAGTCAGGAGGAGTG	*Bam*HI
3′-oligonucleotides		
SOMR	TTGGATCCTATTGGGGCTTGCACTC	*Bam*HI
CONR	ATGGATCCTAGGGCTGAGGTCTC	*Bam*HI
HP1R	ATGGATCCTACTGGTGCTGGAAC	*Bam*HI
HP2R	ATGAATTCCTACAGATGGCCAGGA	*Eco*RI

The PCR products were digested with appropriate restriction enzymes, as indicated above, and then gel-purified, respectively. Each DNA was cloned into the expression vector, pGEX-3X (Pharmacia), digested with *Bam*HI only or *Bam*HI/*Eco*RI. Sequence analyses were performed by the dideoxynucleotide chain termination method using a 373S DNA sequencer (Perkin-Elmer/Cetus, USA). *E. coli* DH5 transformed with the above plasmids was grown at 37 °C for 2 h. After induction with IPTG (at a final concentration of 0.1 mM), the cells were cultured at appropriate temperature and duration to efficiently extract each recombinant protein by sonication only. The cells were collected by centrifugation, suspended in PBS (pH 7.5)-2 mM EDTA, and then sonicated. Each lysate was centrifuged, the supernatant was applied to a glutathione-Sepharose column, and the recombinant fusion protein was eluted with 10 mM glutathione (oxidized form; Boehringer Mannheim). The concentration of the recombinant protein was measured by means of the DC Protein Assay (Bio-Rad, Richmond, VA).

Ligand Binding Activities Using Recombinant Domains. The binding activities of recombinants toward ligands immobilized on plastic plates were analyzed by the following methods including ELISA.

Lipid. Lipid-binding activities were measured as described for the plastic plate assay except for the use of anti-GST rat annexin IV antiserum as the primary antibody and HRP-affinity purified antibody to rabbit IgG (H+L) for detection on ELISA.

β-Endorphin. All procedures were performed at room temperature. The wells of a plastic plate (Immulon 1) were coated with 50 μL of 100 μg/mL or 10 μg/mL β-endorphin in PBS. After blocking with 5% BSA, 50 μL of the recombinant protein solution (10 μg/mL) in TBS was added, followed by incubation for 1 h. After washing with TBS three times, the amounts of recombinants bound to the wells were measured as described above.

Collagen. All procedures were performed at room temperature. The wells were coated with 100 μL of a collagen solution (10 μg/mL) in sodium carbonate buffer (pH 9.0). After blocking with 5% BSA in TBS, 50 μL aliquots of solutions of various concentrations of recombinant proteins in TBS were added, followed by incubation for 1 h. Washing and measurement of recombinants bound were performed as described above.

Heparin. Heparin-binding activity was analyzed by two methods. Using HRP-heparin as a probe, solutions of various concentrations of recombinant proteins were coated on the

wells, followed by blocking with BSA. Fifty microliters of a HRP-heparin solution (1.2 μg/mL) in TBS was added, followed by incubation, and the binding of HRP-heparin was monitored using *o*-phenylenediamine. As another method, 50 μL aliquots of solutions of various concentrations of BSA-heparin were immobilized on the wells. After blocking with 0.5% skim milk, 50 μL aliquots of recombinant protein solutions (10 μg/mL) in TBS were added to the wells, followed by incubation for 1 h. The bound recombinant proteins were determined as described above.

N-Glycanase Digestion of hVN. To study the effects of *N*-glycosylation of hVN on the interactions with ligands, the ligand-binding activity was analyzed using intact and de-*N*-glycosylated hVN. Three units of *N*-glycanase (*Flavobacterium meningosepticum*) was added to 50 μL of plasma hVN (100 μg), which had been purified under denaturing conditions, in 10 mM Tris-HCl (pH 7.5) containing 2 mM EDTA and 0.6% (v/v) 2-mercaptoethanol, and then the mixture was incubated at 37 °C for overnight. The reaction was performed in the presence or absence of enzyme or in the absence of hVN as controls. De-*N*-glycosylation was ascertained by the behavior on SDS-PAGE and the loss of reactivity with HRP-concanavalin A on Western blotting. Ligand-binding assays were carried out using de-*N*-glycosylated hVN or control hVN (10 μg/mL) prepared as described above.

Electrophoresis. SDS-PAGE was performed in the presence of 0.1% SDS on various polyacrylamide running gels with a 3% stacking gel (*34*).

RESULTS

Binding of VN to Acidic Glycolipids and Lipids. Binding of hVN and pVN to various glycolipids and lipids was studied, as shown in Figure 2(A and B). The VNs used were the multimeric form (*11*), which is the biologically relevant tissue form. Both VNs bound in a concentration-dependent manner to sulfatide, cholesterol 3-sulfate, PS, and PA among the tested lipids. Binding of the VNs to PI, PE, PC, and sphingomyelin was observed only with high concentrations of lipids. The VNs did not bind to galactosylceramide or ceramide at the concentrations examined (data not shown), suggesting that a sulfate residue is important for interaction with sulfatide. A difference in the reactivity to cholesterol between hVN and pVN that may be related to the structural difference between them in the connecting region or with C-terminal fragment trimming in pVN (*35*) was observed.

Binding of the VNs with GD1a, an acidic glycolipid containing sialic acid, was not observed even when 5 μg of GD1a was added to the well (Figure 2). Binding with other gangliosides, GM1, GM3, GD3, and GD1b, was not observed either (data not shown). As it has been reported that at least 20% of ^{14}C-labeled GD1a added to plastic wells of Immulon 1 plates remained after repeated washes with buffer (*36*), our findings could be interpreted as an absence of binding of VN for up to 1 μg of GD1a immobilized per well. Therefore, the results suggest that VNs did not bind substantially to the gangliosides under the conditions examined. The presence of EDTA and Ca^{2+} ions had no effect on the interactions with sulfatide and gangliosides (data not shown).

Glycolipid Affinity Chromatography of pVN. The interaction of pVN with sulfatide and gangliosides was also examined by affinity chromatography. Since pVN was nonspecifically adsorbed to octyl-Sepharose and phenyl-Sepharose, but not to Toyopearl HW-50F, glycolipid-immobilized HW-50 gels were prepared. The immobilization of glycolipids was confirmed by extraction of the gels before and after chromatography with chloroform:methanol (1:2), followed by TLC of the extracts. The results of affinity chromatography are shown in Figure 3. Most pVN was adsorbed to the sulfatide-HW-50 column and eluted with 1.0 M NaCl, while it completely passed through the GM3- and GD1a-adsorbed columns, as shown on comparison of the

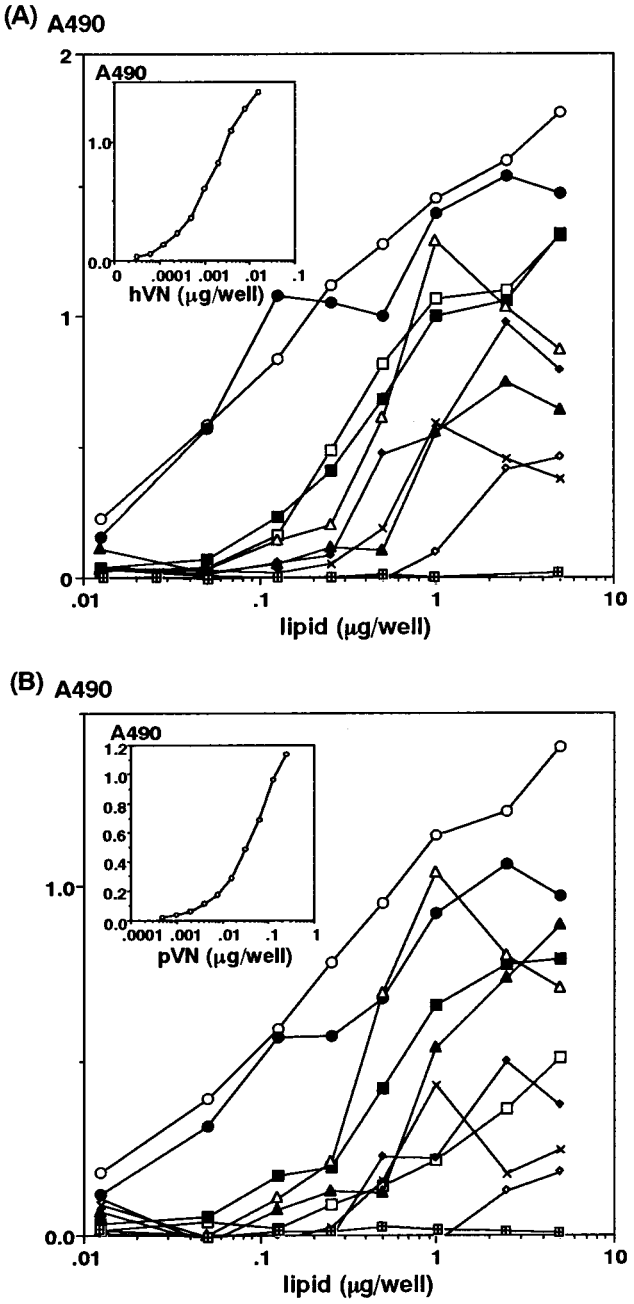


FIGURE 2: Binding of hVN (A) and pVN (B) to various lipids immobilized on plastic plates. Sulfatide (●), cholesterol 3-sulfate (■), PS (△), cholesterol (□), PA (○), PI (▲), PC (◆), PE (×), sphingomyelin (◇), or ganglioside GD1a (⊞) was coated onto a well and then subjected to the assay described under Materials and Methods. The inset shows the dose dependency of the optical response on the amount of immobilized VN. VNs were directly coated onto the wells without lipids at the concentrations indicated on the abscissa and then subjected to the ELISA.

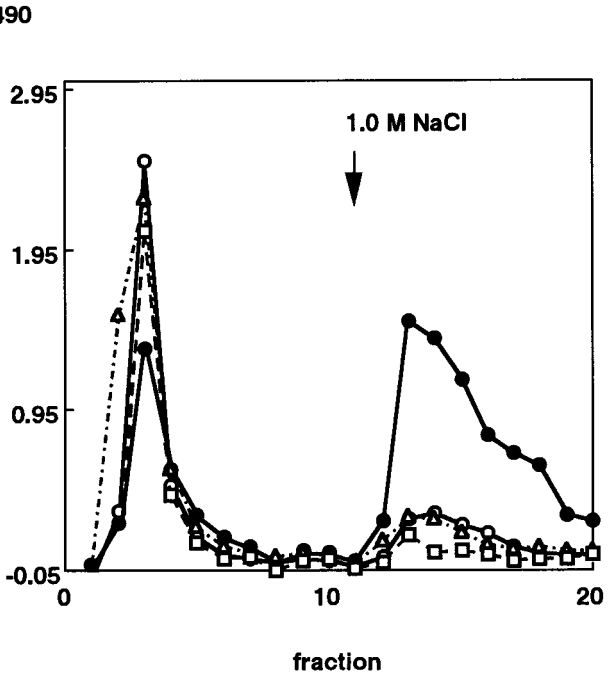


FIGURE 3: Glycolipid affinity chromatography of pVN. Sulfatide (●), GM3 (△), or GD1a (□) was adsorbed to a Toyopearl HW-50F gel, and then glycolipid affinity chromatography was performed as described under Materials and Methods. The elution pattern of an untreated HW-50F column (○) is shown as a control.

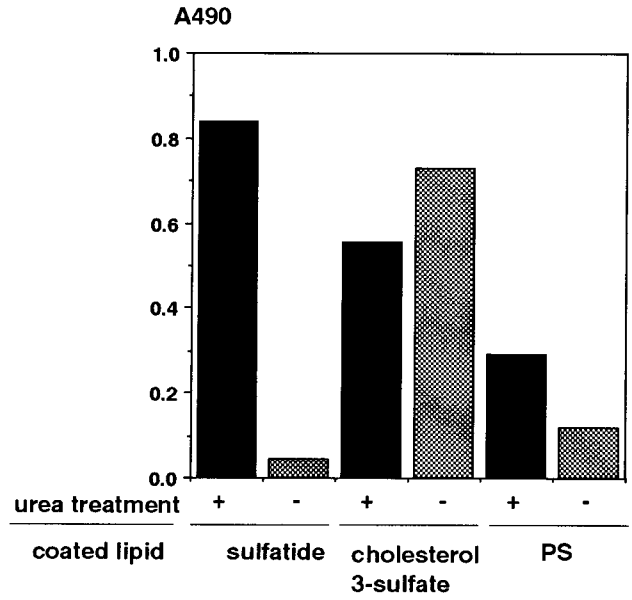


FIGURE 4: The effect of the conformational state on the binding of hVN to sulfatide, cholesterol 3-sulfate, or PS. VN fractions containing the native hVN were prepared from plasma and subjected to a binding assay on a plate precoated with sulfatide, cholesterol 3-sulfate, or PS. The assay was performed before and after 8 M urea treatment of the VN fractions as described under Materials and Methods.

results with those obtained for the control-HW-50 column. The pVN fraction passing through the sulfatide column was not due to overloading, because it always appeared in a certain ratio to the amount of VN applied, suggesting that a part of purified VN is inactive as to sulfatide binding. Together with the results of the solid-phase assay, the results indicate that pVN does not bind to the gangliosides examined.

Dependency on the Conformational State of VN. Various biological activities of VN depend on its conformational state. The native inactive form present in plasma can be converted to the active form by urea treatment *in vitro* (*37*). To determine whether the binding activity toward each lipid depends on the conformational state of VN or not, the native and urea-activated hVN fractions were assayed on plastic plates. As shown in Figure 4, interaction with sulfatide was detected only when the human plasma had been pretreated with urea, which converts VN to its unfolded form. The same result was obtained with pVN fractions (data not shown). Sulfatide binding was observed for multimeric VNs as shown in Figure 2. In an additional experiment, the reactivities of the multimer-form vitronectin used in Figure 2 toward sulfatide were compared in the presence and absence of 2 M urea, the same urea concentration in the incubation mixture for diluted urea-activated VN used in Figure 4, and were found to be unchanged. Thus, the binding activity toward sulfatide was induced by unfolding with denaturant but not by the subsequent multimerization process. On the other hand, binding to cholesterol 3-sulfate was observed both before and after treatment with urea, indicating that the cholesterol 3-sulfate-binding site on VN is exposed in the native form as well as in the denatured form. In the case of PS, the binding of urea-activated hVN increased to 150% of the inactive form, suggesting that the denatured conformation is more capable of PS binding, but that the conformational dependency is not as strict as that to sulfatide.

The Effects of Ionic Strength and Sulfate Ions. The effects of the salt concentration on the interaction of VN with sulfatide, cholesterol 3-sulfate, and PS were examined, as shown in Figure 5A. Maximum sulfatide binding was observed near the physiological salt concentration (from 0.2 to 0.4 M), suggesting that the interaction may occur *in vivo*. In the case of cholesterol 3-sulfate, strong binding was observed at higher salt concentrations, but little binding occurred in the absence of NaCl. Binding of VN to PS decreased with the increasing concentration of NaCl. The binding to sulfatide decreased at higher sulfate ion concentrations, while that to cholesterol 3-sulfate did not change, as shown in Figure 5B. These findings suggest that the binding of VN to sulfatide and PS is due mainly to electrostatic interactions via sulfate or phosphate residues, while that to cholesterol 3-sulfate is due to a hydrophobic interaction.

Inhibition of Sulfatide Binding by Acidic Polysaccharides. The binding of VN to sulfatide was inhibited by dextran sulfate, fucoidan, heparin, and heparan sulfate in a concentration-dependent manner, as shown in Figure 6, whereas chondroitin sulfates A, B, and C, keratan sulfate, and hyaluronic acid had little or no effect on the interaction (data not shown). hVN has been reported to bind to sulfated acidic polysaccharides, i.e., dextran sulfate, fucoidan, heparin, and heparan sulfate (*2*). The inhibition by acidic polysaccharides of the binding to sulfatide showed the same pattern as the specificities of the binding of VN to acidic polysaccharides.

Ligand-Binding Assay Involving Recombinant Proteins. The recombinants were purified by affinity chromatography on a glutathione-immobilized column. Some of the recombinant proteins gave double bands on SDS-PAGE, as shown in Figure 1C, even when they were expressed in other *E. coli* host strains (HB101, JM109, XL-1 blue, and TB1).

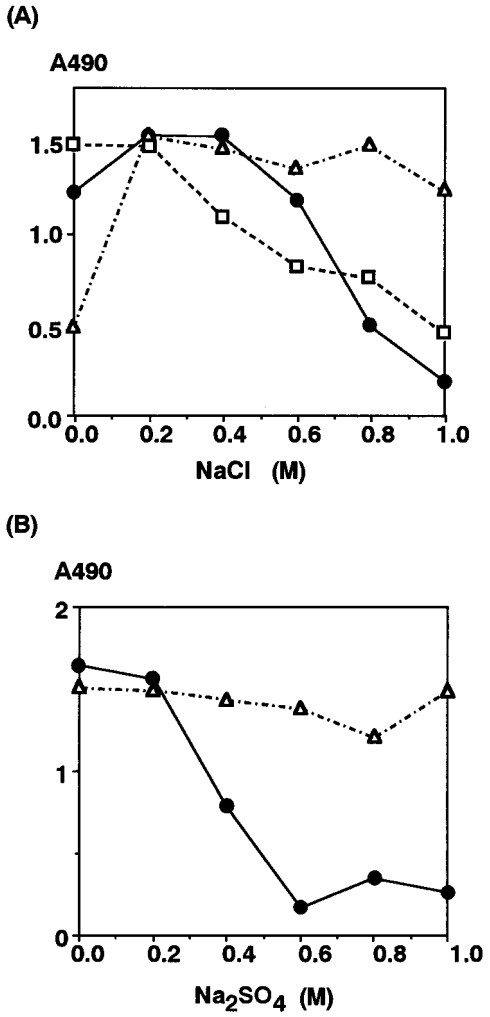


FIGURE 5: The effects of the ionic strength of NaCl (A) and sulfate ions (B) on the binding of pVN to sulfatide, cholesterol 3-sulfate, and PS. The effects of NaCl (A) and sulfate ions (B) on the binding of pVN to the lipids were studied. Sulfatide (●), cholesterol 3-sulfate (△), or PS (□) was coated onto a well and then subjected to the assay described under Materials and Methods.

Lipids. As shown in Figure 7A, recombinant proteins H12, H2, H1, and SC1 bound to sulfatide; therefore, recombinant proteins containing hemopexin domain 2 or 1 were found to exhibit sulfatide-binding activity. The cholesterol 3-sulfate- and PS-binding were also attributable to both H2 and H1, as shown in Figure 7B and 7C. The major difference is that isolated domains H1 and H2 bind relatively less to cholesterol sulfate than construct H12 does, suggesting that interaction with both hemopexin domain 1 and 2 was required for high affinity binding to cholesterol sulfate.

Type I Collagen. The isolated H2 and H1 domains exhibited collagen-binding activity, as shown in Figure 7D, where immobilized Type I collagen interacted with recombinants H12 and H2, and weakly with H1 and SC1.

Heparin. BSA-heparin produced essentially the same result as obtained in the system involving immobilized recombinant proteins and HRP-heparin, as shown in Figures 7E and 7F, respectively. Heparin binding was observed not only for H2 and H12 but also for H1, although neither SC1 nor SC exhibited an affinity for heparin. Both results indicate that hemopexin domain 1 binds to heparin, but the activity may be masked when hemopexin domain 1 is linked to the somatomedin domain and connecting region.

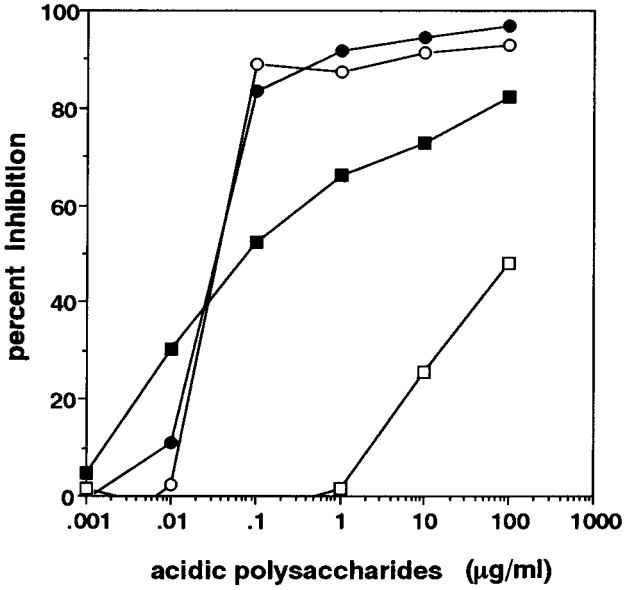


FIGURE 6: The inhibition by various acidic polysaccharides of the binding of hVN to sulfatide. HVN was preincubated with various concentrations of acidic polysaccharides, dextran sulfate (○), fucoidan (●), heparin (■), and heparan sulfate (□), for 30 min at room temperature, and then added to wells precoated with lipids. ELISA was performed as described under Materials and Methods.

β-Endorphin. As shown in Figure 7G, the β-endorphin-binding activity could be attributed mainly to H2, and the contribution of H1 was rather low compared to other ligand-binding activities.

Because both hVN and pVN, which were processed completely at the C-terminal of 9 kDa (*35*), bound to the lipids (Figure 2), collagen, and β-endorphin (data not shown), the trimmed C-terminal fragment in hemopexin domain 2 was not considered responsible for the interaction of these ligands.

The Effects of N-Glycosylation of VN on the Interactions with Ligands. The contribution of covalent glycosylation to VN's binding activities was assayed. The bindings of intact VN and enzymatically deglycosylated VN in the presence of released *N*-glycans toward immobilized ligands were compared in these experiments (Figure 8A−F). The treatment with *N*-glycanase (PNGase F) and release of the *N*-linked oligosaccharides leaves a negative charge in the peptide, as the *N*-linked asparagine residue is converted to an aspartate residue after hydrolysis. Cholesterol 3-sulfate binding decreased in deglycosylated hVN, suggesting that covalent *N*-glycosylation may support the active conformation of VN for cholesterol 3-sulfate, while collagen-binding activity increased compared to that of intact hVN. De-*N*-glycosylation had no effect on the interaction with other ligands. These findings suggest that binding sites for the examined ligands can be attributed to the peptide portion of VN. The results obtained using unglycosylated recombinants expressed in *E. coli* are therefore considered to be applicable to the peptide domain of VN. Ligand-binding activities of recombinant domains and effects of de-*N*-glycosylation on this activity are summarized in Figure 9 together with the conformational dependencies in plasma vitronectin.

DISCUSSION

Hemopexin domain 1 has been regarded as inert in biological activities of VN. When isolated, however, it was

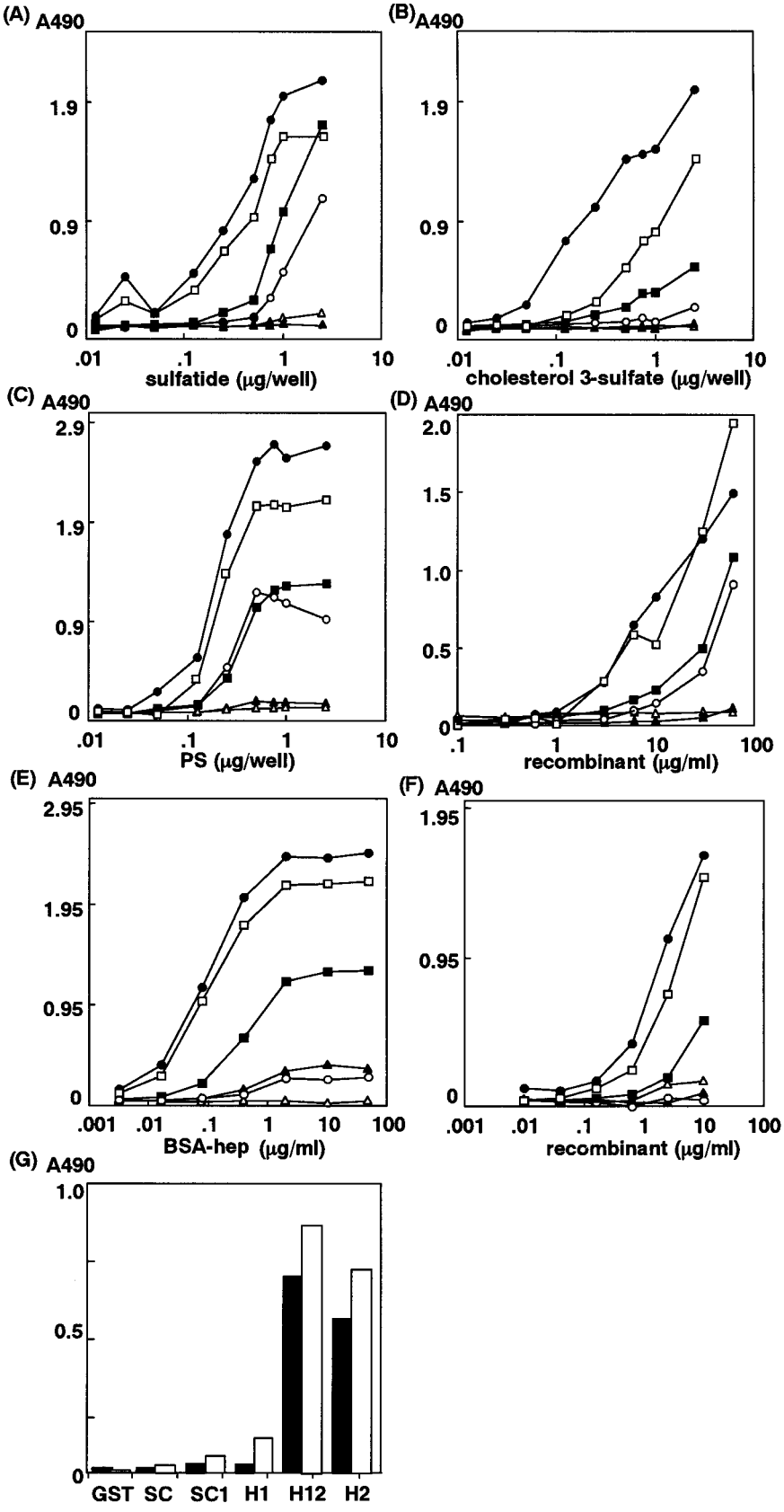


FIGURE 7: Binding assay of recombinant GST-domains toward various ligands. Each recombinant GST-domain (50 μL, 10 μg/mL) was added to wells coated with various concentrations of ligands, sulfatide (A), cholesterol 3-sulfate (B), PS (C), and BSA-heparin (E). (D) Fifty microliter aliquots of solutions of various concentrations of each recombinant were added to wells coated with 100 μL of Type I collagen (10 μg/mL). (F) HRP-heparin (50 μL, 1.12 μg/mL) was added to wells coated with 50 μL aliquots of solutions of various concentrations of recombinant GST-domains. (G) Each recombinant GST-domain (50 μL, 10 μg/mL) was added to wells coated with β-endorphin (50 μL, 10 μg/mL, solid bar; 100 μg/mL, hollow bar). Binding of GST-domains was measured using anti-GST antibodies and HRP-secondary antibodies. The symbols used are: SC (▲), SC1 (○), H1 (■), H2 (□), H12 (●), and GST (△).

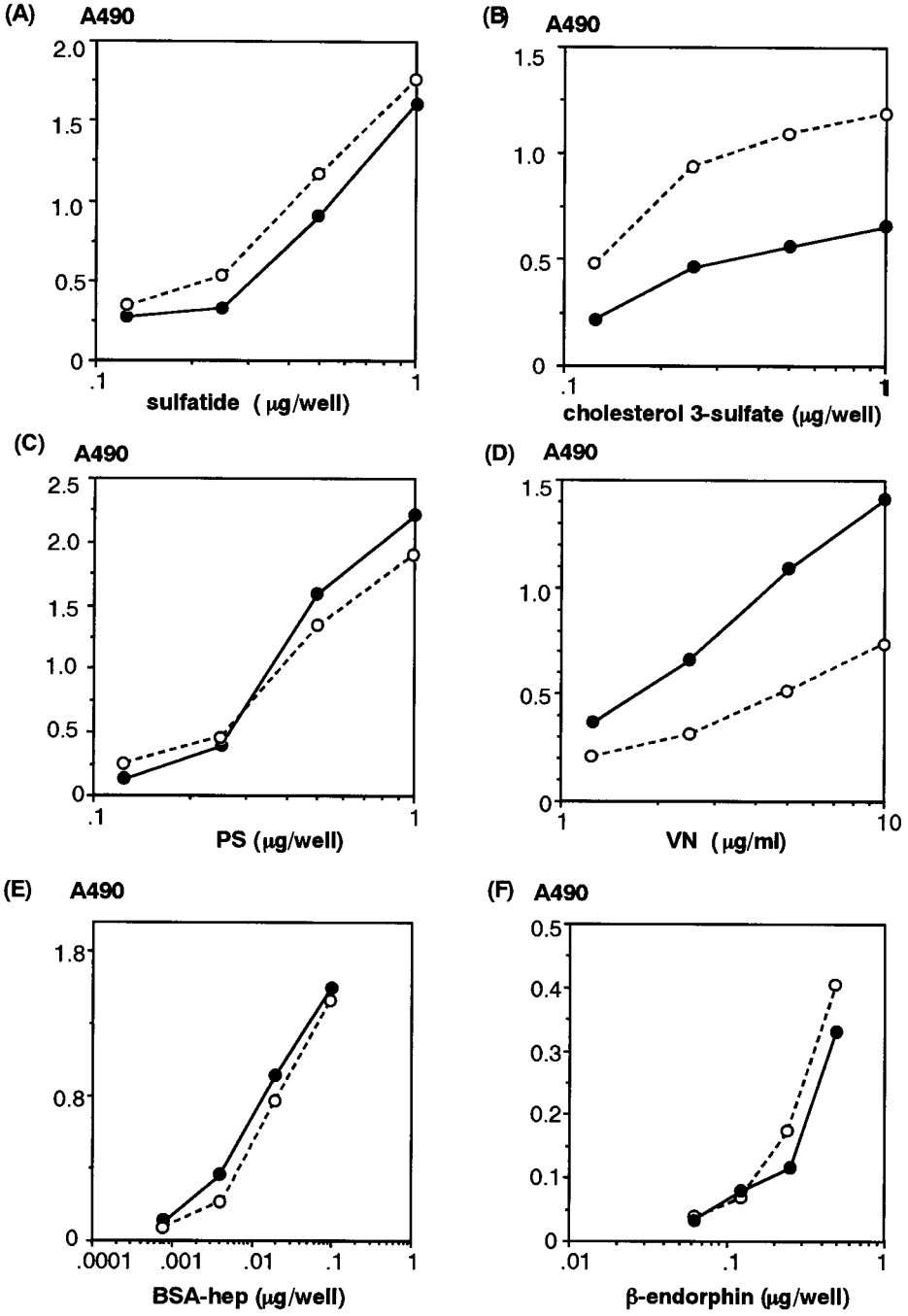
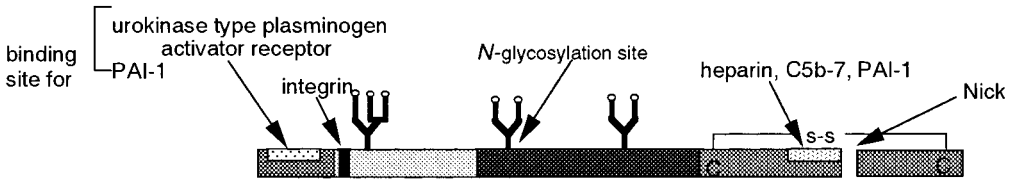


FIGURE 8: The effects of de-*N*-glycosylation of hVN on the interactions with various ligands. The interactions of intact (○) and de-*N*-glycosylated (●) plasma hVN with sulfatide (A), cholesterol 3-sulfate (B), PS (C), Type I collagen (D), BSA-heparin (E), and *β*-endorphin (F) were studied by ELISA.

shown to have ligand binding activities similar to, but attenuated differently from, those of hemopexin domain 2 under physiological conditions. This is notable because the heparin binding activities of VN had been primarily attributed to the heparin binding region in hemopexin domain 2. This oversight might have occurred because the presence of a somatomedin domain and/or connecting region flanking hemopexin domain 1 inactivates its heparin-binding capacity (Figure 7E and 7F). Similarly, a weak masking effect on hemopexin domain 1 by the somatomedin domain and/or its connecting region was observed for sulfatide- and cholesterol 3-sulfate-binding (Figure 7A and 7B). Although the conclusions reached with domain mutants cannot be necessarily related to the interactions of the entire molecule, novel heparin-binding sequences were reported that correspond to Lys^{175}-Asp^{219} in hemopexin domain 1 using phage display techniques in support of our result (*38*). The occurrence of interdomain interaction other than N- and C-terminal ionic interaction, which may stabilize a monomer form, is supported by structural studies of PAI-1-VN interaction in which the heparin binding site undergoes a conformation change by VN's interaction with PAI-1 (*39*) and may induce multimerization (*13*, *9*, *40*).

Recently, native VN was found to exhibit an affinity for heparin similar to multimeric VN and to bind with other glycosaminoglycans as well (*12*). Therefore, the activation can be explained by increased avidity caused by urea-induced multimerization (*12*). Another model presented that the



ligand	domain				effect of de-*N*-glycosylation	conformational dependency
	somatomedin B	connecting	hemopexin 1	hemopexin 2		
sulfatide	−	−	+	+ +	no effect	dependent
cholesterol 3-sulfate	−	−	+	+ +	attenuation	independent
PS	−	−	+	+ +	no effect	dependent
β-endorphin	−	−	−	+ +	no effect	dependent
Type I collagen	−	−	+	+ +	enhancement	dependent
heparin	−	−	+	+ +	no effect	dependent

FIGURE 9: Ligand binding activities of recombinant domains. The upper figure illustrates the ligand binding domains hitherto known for hVN. All the binding activities except that of integrin have been reported to be dependent on the conformation of VN. The characteristics of the binding activity of each recombinant domain found in this study are summarized in the table with the new information obtained here. The conformational dependencies of β-endorphin, Type I collagen, and heparin were previously reported (*2*).

activated glycosaminoglycan binding properties of denatured VN as due, not to unfolding of the VN molecule, but to its release from competition with other heparin-binding proteins to bind with heparin in plasma after urea treatment deactivated the other proteins (*13*). In those reports, however, the existence of a heparin binding site was studied only in hemopexin domain 2, and the existence of one in hemopexin domain 1 was not even considered. As revealed by the binding assay using recombinant protein SC1, the heparin binding site in hemopexin domain 1 may be cryptic in the native form of VN; but when exposed by denaturing treatment, it may contribute to an increased avidity for ligand binding in concert with the one in hemopexin domain 2, in addition to multimerization. The conformational dependencies of the sulfatide and collagen-binding activities together suggest that the ligand-binding sites in hemopexin domain 1 are latent in plasma VN, like those in somatomedin domain. Therefore, hemopexin domain 1 is involved in the conformational transition of VN. In support of this, it has been reported that the conformational transition of VN is not limited to the heparin binding domain (hemopexin domain 2), but that the connecting region and hemopexin domain 1 may be related to it (*41*).

As summarized in Figure 9, lipid binding activities are attributed to hemopexin domains 2 and 1. Different binding sites of VN for cholesterol 3-sulfate and other lipids were, however, suggested by their conformational dependency (Figure 4) and the effect of de-*N*-glycosylation (Figure 8). The consensus sequence for the interaction with cholesterol 3-sulfate is not yet clear, but the sequence C-S-V-T-C-G-X-G-X-X-X-R-X-R has been reported as homologous among sulfatide-binding proteins (*42*). VN, however, does not have this homologous sequence. In hemopexin domain 2, the heparin-binding consensus sequence (XBBXBX, in which B is a basic amino acid) (*43*) corresponding to A347-K-K-Q-R-F352 may be responsible for the sulfatide as well as heparin binding, as has been suggested for thrombospondin. Hemopexin domain 1 should contain a novel sulfatide- and a heparin-binding sequence or a distinctive cluster of positive charges located spatially. Judging from the gene structure of VN (*44*), however, the binding sites in hemopexin domains 1 and 2 could have evolved from a common ancestral repeating unit of the pexin family by divergent evolution.

The binding of hVN with cholesterol 3-sulfate drastically decreased after de-*N*-glycosylation (Figure 8). hVN is *N*-glycosylated on hemopexin domain 1 and the connecting region, but not on hemopexin domain 2. This suggests that *N*-glycosylation may enhance the cholesterol 3-sulfate binding by making the binding site on hemopexin domain 1 more accessible or by promoting the interaction between hemopexin domain 1 and 2, because a cooperative effect was observed for cholesterol 3-sulfate binding (Figure 7B). On the contrary, the release of oligosaccharides from VN increased its binding with collagen. *N*-glycosylation of VN may interfere with the interaction due to the anionic charges of sialic acids just as osteonectins from bone and platelets have differential collagen-binding activities due to tissue-specific glycosylation (*45*).

Various sulfatide-binding proteins have been reported so far: laminin, thrombospondin, von Willebrand factor (*46*), antistasin (*47*), and properdin (*42*). They bind specifically to sulfatide, but not to cholesterol 3-sulfate or other acidic phospholipids, except laminin which binds weakly to cholesterol 3-sulfate and phospholipid. Fibronectin exhibits strong binding activity toward PS and weak activity toward sulfatide and gangliosides (*48*). VN is unique in exhibiting affinity for sulfate and phosphate groups but not carboxyl groups, and in binding to sulfatide only when converted to its unfolded form, while it binds to cholesterol 3-sulfate regardless of its conformation (Figure 4).

The lipid ligands found here may present new cell surface and extracellular binding sites for VN. In view of the fact

that VN binds to cholesterol in addition to sulfatide and cholesterol 3-sulfate, the possibility that the affinity of VN for these lipids may be related to the deposition in atherosclerotic lesions can be strongly proposed. Coincidently, a remarkable increase in the sulfatide content was observed in the aortae of WHHL rabbits (*49*) and localization of VN in the normal and atherosclerotic human vessel wall (*50*). Sulfatide-binding of VN may occur also in the normal brain, where murine VN mRNA was second highest in concentration after liver (*51*, *52*), and may have a specific physiological function. The phospholipid binding of VN may be involved in cells undergoing apoptosis and in platelets stimulated by thrombin or collagen, where PS has become exposed on the outer surface of the cells (*53*). It will be necessary to identify the endogenous lipid ligand for VN in each case to obtain insight into its biological meanings.

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