Vitronectin Interaction with Glycosaminoglycans

KINETICS, STRUCTURAL DETERMINANTS, AND ROLE IN BINDING TO ENDOTHELIAL CELLS*

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Vitronectin (VN) is a high affinity heparin-binding protein. The physiological role of this binding has hitherto received little attention, and its molecular determinants are subject to controversy. In this study, we characterized vitronectin interaction with heparin, heparin analogues, bacterial extracts, and cell surface glycosaminoglycans. As assessed by (i) fluorescence assays, (ii) precipitation with heparin-Sepharose beads, or (iii) Western blotting with antibodies against VN347–361 (the heparin-binding site), we demonstrate an exposure of the VN heparin-binding site in multimeric but not monomeric vitronectin. Through its heparin-binding site, vitronectin also bound other glycosaminoglycans and Staphylococcus aureus extracts. The kinetics of heparin binding to vitronectin were complex. After a fast association phase (τ = 0.3 s), a slow conversion of an unstable to a stable heparin-vitronectin complex (τ = 180 s) occurred. Heparin binding kinetics and transition to a stable complex were mimicked by VN347–361, demonstrating that this area is the fully functional heparin-binding site of vitronectin. Multimeric vitronectin bound to endothelial cells. This binding was blocked by soluble heparin and was not observed when endothelial cells were pretreated with glycosaminoglycan-removing enzymes. Glycosaminoglycan-dependent interaction of endothelial cells with multimeric vitronectin might be a relevant mechanism for removal of multimeric vitronectin from plasma. Conversion of an unstable to a stable glycosaminoglycan-vitronectin complex is likely to be relevant for association with endothelial cells under flow conditions.

Vitronectin (VN), a abundant, multifunctional glycoprotein of plasma and extracellular matrix (1, 2), exists in a monomeric and a multimeric form. VN monomers are synthesized in the liver and secreted into the plasma. Circulating VN is essentially monomeric, while VN in the extravascular space is essentially multimeric (1). VN avidly binds glycosaminoglycans (GAGs; e.g. heparin (2)); however, the role and mechanisms of GAG binding by VN remains poorly understood. (i) What is the physiological role of GAG binding? A role in VN activation and VN deposition in tissues has been hypothesized (2); however, currently only little experimental evidence concerning these hypotheses is available. There is evidence that the heparin-binding to VN allosterically effects ligand binding to other domains of VN (3). Note, however, that the heparin-binding domain of VN does not only interact with glycosaminoglycans but also directly interacts with proteins, including other serum and extracellular matrix proteins (e.g. complement components 4 and osteonectin (5)) as well as with microbial proteins (e.g. Staphylococcus aureus (6–8)). (ii) Is the heparin-binding site exposed in monomeric VN? Several studies have suggested that only multimeric and not monomeric VN is able to bind heparin (1, 9, 10). This was attributed to a heparin-binding sequence, which is cryptic in monomeric but exposed in multimeric heparin (1, 11). A recent study has challenged this point of view, suggesting that monomeric and multimeric VN exhibit similar affinity for heparin and that the differences in heparin binding properties induced upon denaturation are due to self-association in a multivalent form (12). (iii) Which regions of the VN molecule are involved in heparin binding? Several studies have suggested a role of a highly basic VN region (aa 347–361) to be the heparin-binding site (1, 11, 13). However, recent studies have identified novel heparin-binding domains of VN (14). (iv) what are the kinetics parameters of heparin binding to VN? Since most previous studies on heparin-VN interaction were done with discontinuous binding assays, no analysis of the kinetic parameters of the heparin-VN interaction has been performed so far.

In this study, we analyze the kinetics of the interaction between heparin and multimeric VN and characterize VN interaction with soluble and endothelial cell surface glycosaminoglycans.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were purchased from Merck (Basel, Switzerland) or Sigma (Fluka Chemie AG, Buchs, Switzerland). Unless stated otherwise, experiments were performed in phosphate-buffered saline (Life Technologies, Inc., Basel, Switzerland). RPMI 1640 was from Life Technologies, Inc.). Chondroitinase was from Sigma, and heparitinase was from Seikogaku Corp. (Tokyo, Japan). Heparin (mean M, 10,000) and CMDBS (a synthetic polysulfonated carboxymethylxextran, mean M, 45,000; see also Refs. 15 and 16) were kindly provided by J. Jozefovicz (LEM, University Paris Nord Villeurbanne, France).

Cascade Blue®-Heparin: Synthesis and Characterization—Cascade Blue® (Molecular Probes, Inc., Eugene, OR). Concentrations of fluorescent heparin were assayed using the carbazole method (17). Briefly, the fluorescent hepa...
Heparin binding to multimeric, but not to monomeric, VN. A, multimeric VN (lane 1), untreated VN (lane 2), and heparin-Sepharose-treated monomeric VN (lane 3) were subjected to native gel electrophoresis. Note that the band corresponding to the molecular weight of the dimeric form of VN (indicated by an asterisk) is absent after heparin-Sepharose treatment. Molecular weight standards (MWS) are indicated in thousands on the left. Interaction of these three fractions with CB-heparin was analyzed by FRET (λ_{excitation} = 280 nm, λ_{emission} = 426 nm; B–D) and by EDFI (λ_{excitation} = 380 nm, λ_{emission} = 426 nm; E–G). The indicated numbers represent the corresponding fraction subjected to electrophoresis. Note that the untreated VN yields a small signal but not the heparin-Sepharose-treated monomeric VN.

Vitronectin—Monomeric VN was purified from human plasma as described previously (1, 20, 21) and stored at –70°C. Multimeric VN was generated by incubation of the monomeric form for 1 h at 37°C in an atmosphere containing 5% CO₂, in 75-cm² culture dishes, which were heated for 5 min at 95°C. After cooling, SDS was removed by exhaustive dialysis against PBS. Protein content was assayed using a commercially available kit (Pierce method; Socochim, Lausanne, Switzerland) and subjected to SDS-polyacrylamide gel electrophoresis following a silver staining procedure. Extracts showed the characteristic pattern of staphylococcal proteins with a repartition of molecular weight ranging from 20,000 to 140,000.

Endothelial Cell Culture and Digestion of Cell Surface Glycosaminoglycans—Human endothelial cord cells (ECV304, ATCC:CRL-1998) were cultured in RPMI 1640 supplemented with 10% fetal calf serum at 37°C in an atmosphere containing 5% CO₂, in 75-cm² culture dishes, containing at confluence approximately 10⁷ cells. Adherent cells were detached using EDTA 2.5 mM for 5 min. After rinsing twice in PBS, one half of the suspension was suspended in culture medium, whereas the other half was suspended in PBS plus 0.1% albumin (PBSA) containing 50 mIU/ml and 0.5 IU/ml of heparitinase (Seikagaku Kogyo, Tokyo) and chondroitinase ABC (Sigma). Both suspensions were incubated for 3 h at 37°C with constant and gentle shaking. This treatment has been shown to lead to an almost total degradation of cellular glycosaminoglycans (24–26). After glycosaminoglycan removal, cells were washed with PBSA, resuspended in 100 μl of PBSA, and subjected to VN-binding experiments.

Vitronectin Binding Experiments—Plasma VN (2 μg) or a mixture of plasma VN and heparin H108 (2 or 5 μg) was mixed with 1.5 × 10⁶ of enzymatically treated or untreated cells. After an incubation period of 60 min at 4°C, each sample was centrifuged for 2 min at 1000 rpm. The supernatants were then collected in new tubes. In parallel, 2 μg of plasma VN was incubated in the same condition as above but in PBS or in PBSA enriched with heparin.

gel electrophoresis and Western Blotting Procedure—Protein samples were subjected to gel electrophoresis (8% acrylamide) under non-denaturing, nonreducing conditions as described (13). After separation, proteins were transferred to polyvinylidene difluoride membranes using a liquid transblot system (Bio-Rad) in a 20 mM phosphate buffer, pH 6.5, for 2 h under constant voltage (15 V). Membranes were blocked in PBS containing 2.5% BSA and 0.1% Tween 20. All VN forms were detected using a monoclonal antibody against human VN (1:1000; Life Technologies, Inc., Basel, Switzerland), followed by incubation with
FIG. 2. Displacement of bound CB-heparin by native heparin and heparin-like compounds. FRET was recorded with a mixture of VN (50 nM) and CB-heparin (8 nM). A, for displacement curves, VN-CB-heparin was incubated with the indicated concentrations of standard heparin (●), its inactive analogue de-N-sulfated heparin (▲), dextran T-70 (△), CMDBS (■), or chondroitin sulfate (□). Experiments were performed and data were fitted by a logistic equation. The calculated dissociation constants and Hill coefficients were 43 ± 3 nM and 0.73 ± 0.05; 151 ± 13 nM and 1.12 ± 0.1; and 1650 ± 112 nM and 1.22 ± 0.13 for heparin, CMDBS, and chondroitin sulfate, respectively. Values are means ± S.E. of three experiments. B, the addition of extracted cell wall proteins from S. aureus (10 µg/ml) led to the total displacement of the FRET signal by S. aureus extracts. C, reversal of multimeric VN binding to CB-heparin by various compounds as indicated: standard heparin, CMDBS, chondroitin sulfate, dextran T70, de-N-sulfated heparin (each 25 µM), or extracts from S. aureus or S. epidermidis (both 10 µg/ml). Data are mean ± S.E., n = 3.

RESULTS

Fluorescence Properties of CB-Heparin and of VN—The fluorescence intensity of CB-heparin (10 nM) was compared with the fluorescence intensity of its parental compound CB-acetyladizide (10 nM). The ratio was found to be 1.85 molecules, indicating that the CB-heparin had 1–2 mol of CB bound per mole of heparin. The excitation maximum of CB-heparin was around 380 nm. When elicited with an excitation wavelength of 380 nm, maximal emission was between 420 and 430 nm. The fluorescence intensity of CB-heparin was high in a polar solvent (PBS) and decreased as the hydrophobicity of the solvent increased. These fluorescent properties are similar to those of the parental compound CB-acetyladizide (30).

Changes in fluorescence intensity as a function of the hydrophobicity of the environment have been observed previously (12, 30, 31). Since the binding of a ligand to a protein leads to a change in the hydrophobicity of the environment (in general, an increase in hydrophobicity), this approach can be used to monitor directly the kinetics of ligand-protein interaction. We refer to this approach as EDFI. Note that, depending on the fluorescent probe, binding of the ligand to the protein can either lead to a decrease of fluorescence (e.g. CB-coupled ligand in this study) or to an increase in fluorescence (e.g. coumarin-labeled ligand in a previous study) (12).

Due to aromatic amino acids, most proteins display autofluorescence with absorption maximum around 280 nm and an emission maximum around 380 nm. The superposition of the excitation spectrum of CB-heparin and emission spectrum of VN showed an overlapping, confirming that an interaction between both compounds might yield to FRET.

CB-Heparin Binding to Multimeric or Monomeric VN—The question of whether heparin binding is restricted to multimeric...
Heparin-Sepharose (see “Experimental Procedures”). The same membrane was blotted with monoclonal antibody recognizing all VN forms (lane 2) or with polyclonal raised against VN$_{147-361}$ peptide (lane 3). B and C, FRET was recorded in a mixture of multimeric VN (50 nM) and CB-heparin (50 nM). Polyclonal antibodies against VN$_{147-361}$ peptide (4 μg/ml) were added and followed by the addition of standard heparin (Hep, 25 μM) (B, upper trace). An equivalent decrease in fluorescence was obtained using heparin only (B, lower trace). After three consecutive additions of anti-VN$_{147-361}$ peptide (4 μg/ml), the addition of standard heparin (Hep, 25 μM) induced only a minor decrease in fluorescence (C, upper trace). Neither the antibody nor heparin affected the fluorescence signal of CB-heparin alone (C, lower trace).

Fig. 3. Displacement of CB-heparin by a polyclonal antibody recognizing the heparin-binding site of VN. A, plasma vitronectin transferred onto polyvinylidene difluoride membrane stained with colloidal gold (lane 1) showing monomeric and several multimeric forms of VN. The same membrane was blotted with monoclonal antibody recognizing all VN forms (lane 2) or with polyclonal raised against VN$_{147-361}$ peptide (lane 3). B and C, FRET was recorded in a mixture of multimeric VN (50 nM) and CB-heparin (50 nM). Polyclonal antibodies against VN$_{147-361}$ peptide (4 μg/ml) were added and followed by the addition of standard heparin (Hep, 25 μM) (B, upper trace). An equivalent decrease in fluorescence was obtained using heparin only (B, lower trace). After three consecutive additions of anti-VN$_{147-361}$ peptide (4 μg/ml), the addition of standard heparin (Hep, 25 μM) induced only a minor decrease in fluorescence (C, upper trace). Neither the antibody nor heparin affected the fluorescence signal of CB-heparin alone (C, lower trace).

VN (13) or also occurs with monomeric VN (12, 14) remains controversial. To investigate this question, we compared the heparin binding to both forms of VN. Continuous FRET recordings were performed with an excitation wavelength of 280 nm and an emission wavelength of 426 nm. Under these conditions, the addition of multimeric VN (Fig. 1B) led to a fluorescence increase. The fluorescence increase was due to specific binding, since it could be completely reversed by the addition of an excess of nonfluorescent heparin. When the same amount of nonfluorescent heparin was added to CB-heparin in the absence of VN, no effect on the fluorescence was observed (not shown). As expected from the decreased CB-heparin fluorescence in a hydrophobic environment, EDFI recording of the fluorescence showed a decrease upon the addition of multimeric VN to the probe (Fig. 1E). The fluorescence decrease was due to specific binding, since it was completely reversed by the addition of an excess of nonfluorescent heparin. When the same experimental protocol was performed with monomeric VN, the signal with FRET was very low to undetectable, while EDFI gave a small signal (~10% of the signal seen with multimeric VN), similar to what has been previously observed (12). The latter observation has been used to challenge the concept that the heparin-binding site is cryptic in monomeric VN and is only exposed in the multimeric form of the protein (12). We considered an alternative possibility, namely spontaneous formation of a heparin-binding form within the monomeric VN preparation. Indeed, nondenaturing gel electrophoresis clearly showed that in addition to the band of monomeric VN of about M, 72,000, a second band of approximately M, 150,000, presumably corresponding to a VN dimer, was present (Fig. 1A, lane 2). To investigate whether this dimer could be responsible for the small proportion of heparin binding observed with the monomer preparation, we absorbed the monomer preparation using heparin-Sepharose (see “Experimental Procedures”). Nondenaturing gel electrophoresis (Fig. 1A, lane 3) revealed that this treatment removed the M, 150,000 band. In contrast, the intensity of the M, 72,000 band was not diminished by the heparin-Sepharose absorption. Indeed, densitometric quantification demonstrated that the intensity of the M, 72,000 band was 106 ± 10% (mean ± range, n = 2) of control in the heparin-Sepharose-treated preparation. When the remaining monomeric VN was tested in the fluorescent binding assays, strictly no binding was detected (Fig. 1, D and G). Thus, our results demonstrate that specific high affinity binding to VN is restricted to the multimeric form of the protein. These results, however, do not exclude additional low affinity binding sites on the protein (14).

Interaction of Other Ligands with the Heparin-binding Site—To study further the properties of heparin binding to multimeric VN, we studied the displacement of CB-heparin (as evidenced by a decrease in FRET) by standard heparin and related compounds. Standard heparin (M, 10,000) displaced CB-heparin with an IC$_{50}$ of 43 nM (Fig. 2A). Other heparin-like compounds able to displace CB-heparin were CMDBS (15) (IC$_{50}$ = 150 nM) and chondroitin sulfate (IC$_{50}$ = 1.6 μM) (32) (Fig. 2C). Dermatan sulfate, de-N-sulfated heparin, or dextran T70 (Fig. 2A) did not displace CB-heparin. Thus, the rank order of affinity of various heparin-like compounds for the VN-binding site is as follows: heparin > CMDBS > chondroitin sulfate [tmt] dermatan sulfate = de-N-sulfated heparin = dextran T70.

It has been previously suggested that the binding sites of VN for S. aureus and for heparin are, at least partially, overlapping (8). Binding of VN to S. epidermidis has also been suggested (23, 33); however, its relationship to the heparin-binding site is undefined. Using preparations of extracts from the two staphylococcal strains in the FRET assay, S. aureus but not S. epidermidis proteins displaced CB-heparin from VN (Fig. 2A).
Identity of the Heparin-binding Site—The high affinity heparin-binding site of VN is thought to lie within the C-terminal region of the protein (aa 347–361; VN347–361) (11). However, additional regions of VN might bind heparin as suggested recently (14, 21). To investigate the identity of the FRET-generating heparin-binding site, we studied the effect of a polyclonal antibody raised against VN347–361. Western blot performed in native conditions revealed that this antibody recognizes dimeric or higher molecular forms of VN but does not recognize monomeric VN, which is the most abundant VN form in the sample (Fig. 3A). The addition of 4 µg/ml of the antibody decreased the FRET signal by approximately 50%. The subsequent addition of free heparin completely abolished the FRET signal (Fig. 3B). Repetitive addition of the antibody almost completely abolished the FRET signal, and the final addition of heparin led only to a minor decrease of the fluorescence signal (Fig. 3C).

To further investigate the role of VN347–361 in heparin binding by VN, we directly analyzed heparin binding to the peptide. No significant FRET signal could be obtained with the VN347–361 peptide. This is expected, since there is only one aromatic amino acid contained within this sequence (Phe362), while in the full-length protein there are several flanking aromatic amino acids (Trp320, Trp382, Trp405, and Trp450). This is also illustrated by the absence of detectable autofluorescence of the VN347–361 peptide (not shown). As opposed to FRET, EDFI does not require the presence of aromatic amino acid, and consequently this assay clearly gave a positive signal with the peptide. The addition of the VN347–361 peptide to CB-heparin led to a rapid decrease in fluorescence (Fig. 4A). The fluorescence decrease was completely reversed by the addition of unlabeled heparin, demonstrating the specificity of the binding. A control peptide (VN371–383) did not have any effect on CB-heparin fluorescence (Fig. 4B). The profile of displacement of CB-heparin by heparin and related compounds was similar for the VN347–361 peptide (Fig. 4C) and the entire protein (Fig. 2C). Heparin (M, 10,000), CMDBS, and chondroitin sulfate efficiently displaced CB-heparin from the VN peptide, while dextran and de-N-sulfated heparin did not. Also, extracts from S. aureus but not from S. epidermidis were able to displace CB-heparin from the peptide, indicating that the binding profile was virtually identical to the one observed for full-length VN.

Kinetics of Heparin-VN Interaction—One of the major advantages of fluorescence binding techniques is the analysis of protein-ligand interactions at high time resolution, allowing detailed time-resolved kinetic analysis. The on-rate kinetics of CB-heparin binding to VN could be fitted by a monoeponential decay (Fig. 5A), suggesting a first order reaction, i.e., presumably by a one-site protein-ligand interaction. If a double exponential decay was fitted to the data (Fig. 5B), the y2 value was not improved, and the F value decreased, suggesting that a double exponential fit was inappropriate (for explanation of y2 test and F test, see "Experimental Procedures"). Surprisingly, however, the kinetics (off rate) of CB-heparin dissociation could not be appropriately fitted by a single exponential decay (Fig. 5C), while a double exponential fit improved both y2 value and F value (Fig. 5D). Thus, the dissociation data indicate that the initial, rapidly dissociable binding of heparin to VN is followed by a conversion to a more stable binary complex. To test for this hypothesis, we allowed CB-heparin and VN to interact for different periods of time and investigated the related kinetics of dissociation by FRET. As shown in Fig. 6 (A–C), the slow phase of unbinding increased with the time allowed for VN CB-heparin interaction. Thus, a time-dependent conversion from loose to stable binding occurred. The time constant of this conversion was ~180 s. An identical kinetic behavior was found when VN-heparin interaction was analyzed by EDFI (not shown).

To investigate whether the structural requirements necessary for a conversion from a loose to a stable VN-heparin complex were met within the VN347–361 peptide, kinetic analysis of the peptide-heparin interaction was performed using EDFI. The kinetic characteristics of the interaction between VN347–361 peptide and heparin were virtually identical as compared with full-length VN (Fig. 6, D–F). Unbinding was biphasic, and the magnitude of the slow phase of this unbinding was determined by the time of heparin-VN347–361 interaction. When the kinetic parameters of full-length VN and the peptide were compared, binding was best fitted by a single exponential decay, but unbinding was best fitted by a double-exponential decay (Table I). The time constants for binding, for conversion to a stable complex, and for the two phases of unbinding were very similar (Table I).

Binding of Vitronectin to Cellular Glycosaminoglycans—A putative biological role of the heparin-binding site of vitronec-
tin is the interaction with cell surface glycosaminoglycans. This might be particularly relevant for circulating vitronectin and its interaction with endothelial cells. To investigate this question, we used a preparation of monomeric vitronectin in which a spontaneous formation of dimers (30% of total vitronectin) had occurred. In a mock incubation without cells (60 min at 4 °C, followed by a 5-min centrifugation at 1000 rpm), this ratio remained stable (Fig. 7, lane 1). In contrast, when vitronectin was incubated with endothelial cells, most of the dimeric vitronectin was removed after centrifugation, indicating the binding of dimeric, but not monomeric, vitronectin to endothelial cells (Fig. 7, lane 2). After removal of cell surface glycosaminoglycans (heparitinase and chondroitinase), endothelial cells were unable to remove dimeric vitronectin (Fig. 7, lane 3). Thus, binding of dimeric vitronectin to endothelial cells is mediated through glycosaminoglycans. Finally, the inclusion of heparin in the solution also prevented removal of dimers by endothelial cells, suggesting that native heparin is competing with cell surface glycosaminoglycans for the same binding site.

DISCUSSION

In this study, we establish that multimeric VN interacts with endothelial cells through a binding of GAGs to the VN aa 347–361 region. Kinetics of GAG binding by VN are complex, indicating the transformation of a loose complex into a stable complex. A peptide corresponding to the VN aa 347–361 region mimics all characteristics of GAG binding by the full-length protein, demonstrating that this area is a fully functional heparin-binding domain of VN.

Putative Exposure of Heparin-binding Sites in Monomeric VN—Binding of heparin to VN was restricted to the multimeric form of the protein. A small heparin-binding fraction, initially observed in the preparations of native VN, could be attributed to VN dimers. Note that such dimers are also present in plasma and in plasma-derived preparations (20). Thus, the high affinity heparin-binding site of VN is not accessible in the monomeric protein molecule. This conclusion is in agreement with several previous studies of heparin-VN interaction (13, 20, 21, 34) but contrasts with a recent study by Zhuang et al. (12), who suggested that native and multimeric VN have similar heparin binding properties. These authors attributed the discrepancy between their results obtained with fluorescent techniques and previous data to the lack of sensitivity of classical biochemical binding techniques. However, this explanation is not valid in the light of our study that was performed with two sensitive fluorescence techniques. Thus, the most likely explanation for the results obtained by Zhuang et al. (12) is the spontaneous formation of VN dimers within a solution of monomeric VN, as documented in our study. Furthermore, the polyclonal antibody raised against VN347–361 peptide clearly indicates that this segment of the protein is only accessible for multimeric forms of VN composed at least of two VN molecules.

Identification of VN347–361 as High Affinity Binding Site for Heparin and S. aureus—Our results clearly demonstrate that...
the FRET-generating high affinity heparin-binding site of VN is localized within the aa 347–361 region of the protein. This conclusion is based (i) on the blockage of binding by an antibody raised against this region and (ii) on the virtually identical heparin binding profile of VN347–361 as compared with full-length VN. Thus, the predominant part of high affinity binding can be attributed to the aa 347–361 region. Additional heparin-binding site(s) may exist; however, their biological relevance is less clear.

*S. aureus* has been previously shown to bind VN, and based on inhibition of this binding by heparin, VN 347–361 has been suggested to function as the *S. aureus* binding site (36). The displacement of heparin-bound VN347–361 by *S. aureus* extracts in the present study directly demonstrates that *S. aureus* binds to VN347–361. In contrast, *S. epidermidis* did not displace heparin, as assessed by the FRET assay, nor did it bind VN347–361. Obviously, however, our results do not exclude a binding of *S. epidermidis* to other parts of the VN molecule (23, 33).

Several *S. aureus* proteins have been implicated in the binding to VN, including the multifunctional adhesive protein (37), and a 80,000-Da surface protein (36). However, the relative importance of the different proteins is still unclear. The techniques described in this study will be useful in resolving this question by comparison of the FRET signal obtained with purified *S. aureus* protein extracts.

**Kinetics of VN-Heparin Interaction**—Both the noncooperative displacement curves (Fig. 2A) as well as the simple kinetics (Fig. 5, A and B) strongly indicate the absence of a cooperativity of heparin binding to VN. Thus, it appears that one heparin molecule binds to a single binding site on VN without influencing subsequent binding events. However, our kinetic data clearly suggest that heparin binding to VN is a biphasic event.

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**TABLE I**

| Properties                          | Full-length VN | VN347–361 |
|-------------------------------------|---------------|-----------|
| Relative F value for binding        | 10 ± 2.5      | 7.5 ± 2   |
| (single/double exponential fit)     |               |           |
| Relative F value for unbinding      | 0.2 ± 0.1     | 0.32 ± 0.1|
| (single/double exponential fit)     |               |           |
| On rate                            | 0.4 ± 0.1     | 0.3 ± 0.1 |
| τ of conversion to stable complex   | 180 ± 47      | 205 ± 22  |
| Off rate 1                         | 1.4 ± 0.2     | 2.4 ± 0.1 |
| Off rate 2                         | 14 ± 3        | 22 ± 4    |

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**FIG. 6.** *Time-dependent conversion from a rapidly dissociable to a slowly dissociable heparin-VN complex.* FRET recordings were performed in a mixture of CB-heparin (50 nM) and multimeric VN (50 nM) (A–C) and EDFI recording in a mixture of VN347–361 (250 nM) and CB-heparin (50 nM) (D–F). CB-heparin was displaced, after the indicated period of time, from the protein or the peptide through the addition of a 500-fold excess of standard heparin (25 μM). The kinetics of displacement were fitted with a double exponential equation. The amplitude of the slow unbinding, expressed as a percentage of the total unbinding, was used to obtain an estimate of the amount of slowly dissociable heparin-VN complex. The two examples for CB-heparin unbinding from multimeric VN (A and B) were obtained after a binding time of 86 and 325 s, respectively. The percentage of slow unbinding under these conditions was 15 and 39%, respectively. The two examples for CB-heparin unbinding from VN347–361 (D and E) were obtained after a binding time of 35 and 480 s, respectively. The percentage of slow unbinding under these conditions was 16 and 36%, respectively. The percentage of slow CB-heparin unbinding as a function of the binding time is shown in C (multimeric VN) and F (VN347–361).
in which the initial binding of heparin leads to the formation of a rapidly dissociable (loose) complex, followed by formation of a stable complex. This suggests the following reaction scheme.

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\begin{align*}
\text{Heparin + VN} & \quad \tau = 0.3 \text{s} \\
\text{Heparin - VN} & \quad \tau = 1.3 \text{s} \\
\text{Heparin - VN} & \quad \tau = 180 \text{s} \\
& \quad \tau = 15 \text{s}
\end{align*}
\]

**Scheme 1**

Importantly, all structural requirements for the transformation of the loose heparin-VN complex into a stable complex are contained within VN347–361. Modeling of the heparin-binding domain of VN indicated the formation of a hydrophilic pocket that wrapped around and folded over a heparin octosaccharide, yielding tight binding between complementary structures (38). Collectively, these data suggest a two-step mechanism. The first step includes a recognition step, possibly mediated by complementary charged residues. At this point, the heparin-VN interaction is rapidly reversible. Subsequently, the second step of the interaction includes a conformational change that allows the protein to fold over acidic portions of the heparin moiety. Through this folding step, the stability of the complex is achieved. Transitions of a loose to a stable complex do not appear to be restricted to the VN heparin interaction. Indeed, the interaction between heparin and antithrombin (39) is characterized by an initially metastable complex that becomes stabilized through conformational transition(s).

**Physiological Role of VN Interaction with GAGs**—Our results suggest that endothelial cell surface GAGs efficiently bind multimeric VN via its heparin-binding site. What could be the physiological relevance of such an interaction? Two functions, not mutually exclusive, appear possible: (i) recruitment of multimeric VN to the extracellular matrix and (ii) removal of multimeric VN from the circulation and subsequent degradation. Indeed, previous studies have suggested a role of cell surface GAGs for hepatic removal of ternary VN-thrombin-antithrombin complexes (40) and for degradation of multimeric VN by fibroblasts (41). Note, however, that for degradation binding to cell surface GAGs was not sufficient, and internalization by integrin-type VN receptors was necessary (41). Thus, depending on the number and the internalization of integrin-type VN receptors, GAG-bound dimeric VN might be either internalized or associate with the extracellular matrix (Fig. 8). The need for adhesion of VN to endothelial cells under flow conditions in the blood stream provides a pertinent explanation for the complex kinetics of GAG-VN binding described in our study. Indeed, in order to bind under flow conditions, VN should rapidly associate with cell surface GAGs, but to avoid dissociation, a transition to a stable complex is necessary. This potential antithrombotic role of VN is in accordance with a recent paper by Fray et al. (42), showing that VN knockout mice exhibited a significantly enhanced rate of thrombus formation.

There might, however, be additional physiological functions for the observed transition. They might reflect conformational changes necessary for intramolecular signaling from the heparin-binding site to other parts of the VN molecule. For exam-

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**Fig. 7.** Binding of multimeric, but not monomeric, vitronectin to endothelial cell surface glycosaminoglycans. A, a solution of monomeric (~70%) and dimeric (~30%) vitronectin was incubated with or without cells for 2 h at 4°C, centrifuged at 1000 rpm, and subjected to native polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride, and blotted with an monoclonal anti-vitronectin antibody. Lane 1, no cells; lane 2, untreated endothelial cells; lane 3, endothelial cells pretreated with heparitinase/chondroitinase; lane 4, untreated endothelial cells and native heparin. B, densitometric analysis of data described in A. Data are calculated as percentage of dimeric vitronectin, as described under “Experimental Procedures.” Results are mean ± S.E. of three independent experiments.

**Fig. 8.** Putative biological role of endothelial cell glycosaminoglycans in vitronectin homeostasis. 1, the bulk part of circulating vitronectin is in its monomeric form and does not bind to glycosaminoglycans on the vascular surface; 2, a small fraction of circulating vitronectin multimerizes spontaneously; 3, multimerized vitronectin binds loosely to the vascular surface through its glycosaminoglycan binding sites; 4, a conformation change leads to a tight interaction of vitronectin with glycosaminoglycans; 5, the adherent multimeric vitronectin is either internalized and degraded or used as an element of the extracellular matrix.
ple, initial binding of basic fibroblast growth factor to heparin-like structures at the cell surface leads to a conformational change that allows subsequent high affinity binding to growth factor receptor. It will be of importance to investigate such postoccupancy events with regard to interaction of VN with VN receptors (43).

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