Two Collagen-binding Domains of Vitronectin

Michiko Ishikawa-Sakurai and Masao Hayashi

Department of Biology, Ochanomizu University, Bunkyo-ku, Tokyo 112, Japan

Key words: vitronectin/collagen/binding/protein fragmentation/peptide separation/domain structure

Abstract. Vitronectin is a cell-adhesive glycoprotein present in animal blood and extracellular matrix. To establish the molecular basis of vitronectin interactions with extracellular matrix macromolecules, the binding site of vitronectin to collagen has been investigated. Vitronectin fragments obtained by formic acid cleavage were separated by heparin-affinity chromatography followed by gel filtration chromatography. The collagen-binding activity of the fragments was assayed in terms of inhibitory activity on the binding of 125I-vitronectin to immobilized collagen. There were two groups of collagen-binding fragments. One group consisted of 5 heparin-binding fragments with estimated molecular masses of 12 kDa, 14 kDa, 16 kDa, 18 kDa, and 19 kDa in SDS-polyacrylamide gel electrophoresis. The other group consisted of 2 heparin-nonbinding fragments migrating at 18 kDa and 40 kDa. These results indicate that there are two collagen-binding sites in the vitronectin molecule: one located close to the heparin-binding domain in the COOH-terminal half and the other located in the NH2-terminal half of vitronectin.
Interaction between vitronectin and collagen.

**MATERIALS AND METHODS**

**Vitronectin and vitronectin fragments.** Vitronectin was purified from human outdated plasma according to Yato-go et al. (29). Purified vitronectin was extensively dialyzed against 10 mM NH₄HCO₃ and then lyophilized. Vitronectin (17.2 mg) was cleaved with 17 ml of 70% formic acid at 50°C for 16 h according to Suzuki et al. (30). Formic acid was removed by lyophilization after addition of 9 vol of distilled water. The vitronectin fragments were taken up in 0.13 M NaCl, 8 M urea, 5 mM EDTA, and 10 mM Na-phosphate buffer (pH 7.7), and applied to a heparin-Sepharose 4B column (5 ml bed volume). The flow-through fractions were collected, then heparin-binding fragments were eluted from the column with 0.5 M NaCl, 8 M urea, 5 mM EDTA, 10 mM Na-phosphate buffer (pH 7.7). Both heparin-binding and -nonbinding fragments were concentrated and fractionated by gel filtration on a column of Sephacryl S-200 (16 mm x 94 cm) in 0.05 M NaCl, 8 M urea, 5 mM EDTA, 10 mM Na-phosphate buffer (pH 7.7) at a flow rate of 0.065 ml/min/cm² at room temperature.

**Collagen-binding assay.** Binding of vitronectin to collagen was measured by using ¹²⁵I-labeled vitronectin and microtiter plates bearing immobilized collagen as described previously (31). Collagen-binding ability of vitronectin fragments was assayed in terms of inhibitory activity on the binding of ¹²⁵I-labeled whole vitronectin to collagen. Briefly, native type I collagen from porcine skin (Nitta Gelatin, Osaka, Japan) was incubated at a concentration of 10 μg/ml in 96-well soft microtiter plates made of polyvinyl chloride at 4°C overnight. The plates were rinsed, and blocked with 0.2% skim milk. Fifty μl of ¹²⁵I-labeled vitronectin in 0.05% Tween-20 and 10 mM Na-phosphate buffer (pH 7.4) was then added to each well. To examine the collagen-binding activity of vitronectin fragments, the fragments (0-50 μg/ml) were mixed with 0.5 μg/ml of ¹²⁵I-labeled vitronectin at this step. To examine the activity of Sephacryl S-200 fractions, each fraction was diluted to 1/20 with 0.05% Tween-20 and 10 mM Na-phosphate buffer (pH 7.4) after dialysis against 8 M urea, 5 mM EDTA, and 10 mM Na-phosphate buffer (pH 7.4). After incubation at 37°C for 1 h, the plates were rinsed 4 times with 0.05% Tween-20 and 10 mM Na-phosphate buffer (pH 7.4), and cut into individual wells with scissors. The radioactivity that remained in each well was counted with a gamma counter, Aloka ARC-500.

**RESULTS**

**Vitronectin fragmentation and collagen-binding.** Vitronectin (a mixture of 75 kDa and 65 kDa; Fig. 1, lane 1) was cleaved by 70% formic acid into many fragments ranging in size from 12 kDa to 70 kDa (Fig. 1, lane 2). Formic acid fragments of vitronectin interfered with the binding of ¹²⁵I-labeled vitronectin to collagen, though they were several-fold less active than intact vitronectin (Fig. 2). Thus, these fragments retained their collagen-binding activity. Vitronectin fragments were separated by heparin-affinity chromatography into heparin-nonbinding fragments (Fig. 1, lane 3) and heparin-binding fragments (Fig. 1, lane 4). In SDS-polyacrylamide gel electrophoresis, major heparin-nonbinding fragments migrated to positions corresponding to 40 kDa, 39 kDa, 33 kDa, 18 kDa, and 15 kDa, while major heparin-binding fragments migrated to 32 kDa, 23–26 kDa, 20 kDa, 19 kDa, 18 kDa, 16 kDa, 14 kDa, and 12 kDa. Both heparin-nonbinding and -binding fragments were examined for their ability to interfere with ¹²⁵I-labeled vitronectin binding to collagen. The mixture of heparin-binding fragments had activity as strong as that of intact vitronectin. The mixture of heparin-nonbinding fragments also had similar, but weaker, activity (Fig. 2).

**Collagen-binding of heparin-binding fragments.** Heparin-binding fragments were fractionated by gel filtration chromatography on Sephacryl S-200 in the pre-
sence of 8 M urea. Each fraction was tested for ability to interfere with the binding of $^{125}$I-labeled vitronectin to collagen, as well as protein concentration. There was an interfering activity around fraction number 77, which corresponded to the third peak of the elution profile in terms of absorbance at 280 nm (Fig. 3). SDS-polyacrylamide gel electrophoresis of serial fractions from 70 to 82 revealed several bands (Fig. 4). The profile of the activity in Fig. 3 corresponds well to the appearance of 5 bands of less than 19 kDa in Fig. 4. Their molecular masses were 12 kDa, 14 kDa, 16 kDa, 18 kDa, and 19 kDa. These results suggest that one collagen-binding site of vitronectin is contained in these five heparin-binding fragments.

Collagen-binding of heparin-nonbinding fragments. Heparin-nonbinding fragments were similarly fractionated by gel filtration chromatography. Five major fractions were pooled and examined for collagen-binding activity and for polypeptide composition by SDS-polyacrylamide gel electrophoresis. Fractions 4 and 1 had relatively strong activity and fraction 2 had weak activity, while fractions 3 and 5 had no activity (Fig. 5A). Fraction 4 contained mainly an 18 kDa polypeptide and fraction 1 contained only a 40 kDa band, which was also present in fraction 2 (Fig. 5B). These results suggest that active fragments among the heparin-nonbinding fragments are those with molecular masses of 40 kDa.

---

Fig. 2. Collagen-binding assay of vitronectin and vitronectin fragments. Collagen-binding activity was measured in terms of ability to inhibit the binding of $^{125}$I-labeled vitronectin ($^{125}$I-VN) to microtiter plates bearing immobilized collagen. Increasing concentrations of vitronectin or vitronectin fragments were examined; vitronectin (●), formic acid digest of vitronectin (○), heparin-nonbinding fragments (□), heparin-binding fragments (△), and bovine serum albumin (▲) as a control.

Fig. 3. Gel filtration of heparin-binding fragments of vitronectin. Heparin-binding fragments of vitronectin were chromatographed on a Sephacryl S-200 column (16 mm × 94 cm) in 0.05 M NaCl, 8 M urea, 5 mM EDTA, 10 mM β-mercaptoethanol, and 10 mM Na-phosphate buffer (pH 7.7). Each fraction was assayed for protein concentration in terms of absorption at 280 nm (○) and collagen-binding activity (●).

Fig. 4. SDS-polyacrylamide gel electrophoresis of heparin-binding fragments of vitronectin. Fractions 70 to 82 in Fig. 3, spanning the peak of collagen-binding activity, were examined for protein composition by SDS-polyacrylamide gel electrophoresis. Open triangles indicate collagen-binding fragments of 12 kDa, 14 kDa, 16 kDa, 18 kDa, and 19 kDa bands. Molecular mass (in kDa) is indicated at the left.
Fig. 5. Collagen-binding activity (A) and SDS-polyacrylamide gel electrophoresis (B) of heparin-nonbinding fragments of vitronectin. Heparin-nonbinding fragments of vitronectin were further separated by Sephacryl S-200 column chromatography. (A) Five major peaks of absorption at 280 nm were pooled separately and their collagen-binding activities were examined, i.e., fraction 1 (△), fraction 2 (□), fraction 3 (▲), fraction 4 (●), and fraction 5 (☆), in addition to whole vitronectin (●) and unfractionated heparin-nonbinding fragments (○). (B) The same 5 fractions as in (A) were examined for protein composition by SDS-polyacrylamide gel electrophoresis. Molecular mass (in kDa) is indicated at the left.

and 18 kDa.

DISCUSSION

We have identified several vitronectin fragments which interfere with the binding of ¹²⁵I-whole vitronectin to immobilized collagen. Vitronectin fragments produced by 70% formic acid were previously mapped by Suzuki et al. (30), who concluded that the heparin-nonbinding 40 kDa and 18 kDa fragments are located in the NH₂-terminal half, while the heparin-binding 12-19 kDa fragments are located in the COOH-terminal half of vitronectin. The heparin-nonbinding 40 kDa fragment almost entirely spans the NH₂-terminal half and includes the heparin-nonbinding 18 kDa fragment, but it does not share any region with the heparin-binding 12-19 kDa fragments. Therefore, there should exist two collagen-binding sites (site 1 and site 2) in vitronectin, as shown in a structural model of vitronectin (Fig. 6), in which site 1 may be located near the NH₂-terminus or in the central part of the molecule. Figure 6 also presents a plausible mapping of all the vitronectin fragments referred to in this report. We previously showed that one collagen-binding site is located in the NH₂-terminal half of the molecule on the basis of inhibition analysis of collagen-binding by monoclonal antibodies (31). The NH₂-terminal collagen-binding site identified by use of the monoclonal antibodies seems to correspond to site 1 in this report.

Site 2, reported here for the first time, is located near the heparin-binding region of vitronectin. This site was active in smaller, but not larger, fragments (Fig. 3). Because of the vicinity of site 2 to the heparin-binding site, the larger fragments should contain site 2 according to the mapping by Suzuki et al. (30). It is not clear why the collagen-binding activity of site 2 is detected in smaller,
Two Collagen-binding Domains of Vitronectin

![Diagram of collagen-binding domains of vitronectin](image)

Fig. 6. A plausible mapping of collagen-binding fragments of vitronectin. Vitronectin fragments generated by formic acid in Fig. 1 and collagen-binding fragments identified in Fig. 4 and Fig. 5 are mapped according to the domain model of vitronectin proposed by Suzuki et al. (30). Note that there are two possible arrangements in the NH₂-terminal half. Hep indicates heparin-binding site. Site 1 and site 2 indicate two collagen-binding sites.

but not larger, fragments. One possibility is that site 2 is cryptic in the larger fragments but is exposed at the molecular surface in the smaller fragments, by analogy with the conformational conversion of the heparin-binding site. Högasen et al. (32) recently reported that vitronectin spontaneously forms polymers. The polymerization is accelerated by treatment with 8 M urea or ligand-binding. Our purified vitronectin is polymeric (32). The adsorption of vitronectin and vitronectin fragments on collagen may result in some conformational change during the collagen-binding assay used in this report.

Among many collagen-binding proteins, only a few have been analyzed at the amino acid level to elucidate the mechanism of the binding. Roth et al. (33) identified two collagen-binding domains of von Willebrand factor and proposed an amino acid sequence VL(K,Q)Y-ID(R,V)P-A(L,H)LL as a common collagen-binding motif. In contrast, Pareti et al. (34) isolated two collagen-binding fragments of von Willebrand factor, both of which were different from those identified by Roth et al. (33). Takagi et al. (35) determined the collagen-binding site of propolypeptide of von Willebrand factor as a decapptide, WREPSFCALS. Owens and Baralle (36) proposed the 14 amino acid sequence AAHEEICTTNE GVM as the collagen-binding sequence of fibronectin. de Souza and Brentani (37) reported that two peptides, TKTLRT and SSNTLRS, are responsible for the collagen-binding of fibroblast and polymorphonuclear collagenases, respectively. None of these sequences, however, is present in vitronectin. We have conducted some preliminary experiments to identify the collagen-binding amino acid sequence in vitronectin, using synthetic peptides based on sequences around the heparin-binding site. Surprisingly, a heparin-binding synthetic peptide KKQRFRHRNRKGYRC promoted, but did not inhibit, the binding of 125I-vitronectin to plates bearing immobilized collagen (M. Ishikawa-Sakurai and M. Hayashi, unpublished results).

Deposition of vitronectin on tissues seems to be due to its binding to extracellular matrix macromolecule(s) or cell surface vitronectin receptor. Collagen is a candidate for such an extracellular matrix macromolecule, since purified vitronectin binds to collagen in vitro, although most endogenous blood vitronectin does not (25, 26). The activation of the binding can be at least partially understood as a result of conformational change of vitronectin (26).

The binding of vitronectin to collagen is sensitive to
ionic strength (25, 31). The main reasons for the usage of low ionic strength conditions in this study were to obtain higher binding activity, and to allow comparison of the results with data in our previous paper (31). When 0.15 M NaCl was added to the binding solution used in this paper, the amount of vitronectin bound to collagen decreased to only about 10%. This level of binding was reproducible, and vitronectin clearly binds to collagen \textit{in vitro} in such physiological salt solutions, although the physiological meaning of the binding \textit{in vivo} is still uncertain. Collagen is likely to form complexes with proteoglycans \textit{in vivo}, since collagen readily interacts with proteoglycans (38-40). Such complexes may be more effective for vitronectin binding. Many aspects of the interaction between vitronectin and collagen in the extracellular matrix both \textit{in vitro} and \textit{in vivo} still remain uninterpretable at present. Mechanisms of vitronectin deposition on tissues will need to be elucidated if an understanding of the organization of vitronectin \textit{in vivo} is to be reached.

Acknowledgments. This work was supported in part by research grants from the Ministry of Education, Science, and Culture of Japan. We thank W.R.S. Steele for linguistic advice and K. Hayashi for secretarial assistance.

REFERENCES


Two Collagen-binding Domains of Vitronectin


(Received for publication, July 29, 1993 and in revised form, September 13, 1993)