Association of Thrombin-Antithrombin III Complex with Vitronectin in Serum*

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Purification of vitronectin by identical procedures from serum instead of plasma results in the coisolation of an additional protein component with mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of 82 kDa. We show that this component is the thrombin-antithrombin III complex based on the following evidence. Similar to a complex constructed using purified thrombin and antithrombin III, the 82-kDa component has a reduced molecular size of 69 kDa if it is not boiled prior to SDS-PAGE. Upon prolonged boiling in SDS it dissociates into 56- and 32-kDa components which co-migrate in SDS-PAGE with purified antithrombin III and thrombin, respectively. The 82- and 56-kDa components react with an antiserum against antithrombin III, and an antiserum prepared against the 82-kDa complex reacts with purified antithrombin III. Thrombin-antithrombin III complex, from either serum or recalcified clotted plasma, bound to vitronectin immobilized on Sepharose or plastic. However, purified antithrombin III which had not reacted with thrombin lacked affinity for vitronectin as did antithrombin III from citrated plasma. Purified antithrombin III acquired affinity for immobilized vitronectin if it was complexed with thrombin or was modified by radioiodination. Binding of vitronectin to antithrombin III coated on plastic was demonstrated using enzyme-linked immunosorbent assay. These results demonstrate that vitronectin binds thrombin-antithrombin III complexes through a cryptic site in antithrombin III which can be exposed when antithrombin III is radioiodinated, bound to plastic, or complexed with thrombin. Since vitronectin can interact with cells, the binding of vitronectin to the thrombin-antithrombin III complex may serve to facilitate the interaction of this complex with cell surfaces.

The cell attachment-promoting activity and structural characteristics of vitronectin have recently been described (1–5). Vitronectin has two polypeptides which have mobilities on SDS-PAGE corresponding to Mr = 75,000 and 65,000 and which are related to one another through proteolysis (4). Like fibronectin, vitronectin promotes attachment and spreading of a variety of normal and neoplastic cells. Vitronectin also interacts with glycosaminoglycans, and this interaction is mediated by a binding site distinct from the cell attachment site. Vitronectin is present in tissues, at cell surfaces, and in plasma (2). Its concentration in plasma and serum is about 200–300 μg/ml (2, 3). The function of the soluble form of vitronectin in plasma is unknown; its cell-binding activity has only been demonstrated when it is immobilized on a solid surface.

Antithrombin III is a serine protease inhibitor considered to be the major regulator of intravascular coagulation (6, 7). Deficiency of this protein results in thrombotic disease (8, 9). Antithrombin III inhibits thrombin activity by forming a tight complex with this protease. The formation of the complex is associated with cleavage of a peptide bond between an arginine and serine residue within the inhibitor (10). The thrombin-antithrombin III complex is very stable in the presence of denaturants such as SDS or guanidine HCl (11) but can be dissociated by high pH (12, 13) or prolonged incubation at 37°C yielding active thrombin and inactive antithrombin III (14). Antithrombin III has been shown to be produced by the liver (15) and endothelial cells (16), and an antithrombin III-like activity is expressed at the cell surfaces in various normal and malignant cells (17). In the studies presented here we show that the thrombin-antithrombin III complex binds to vitronectin through an activatable site in antithrombin III.

EXPERIMENTAL PROCEDURES

Proteins and Affinity Chromatography—Vitronectin was purified from citrated human plasma or from serum. The purification procedure was essentially the same as reported previously consisting of a combination of monoclonal antibody-Sepharose and heparin-Sepharose affinity chromatography (2). Two fractions of serum vitronectin were collected from the heparin-Sepharose affinity by first eluting with 0.12 M and then with 0.5 NaCl in 0.05 M Tris, pH 7.4. Elution was carried out with NaCl/P, containing 8 μl NaCl. Elution was carried out with NaCl/P, containing 8 M urea. The resultant proteins were dialyzed against distilled water and lyophilized. Antithrombin III was purchased from Sigma and Behring Diagnostics.

The homogeneity of the preparations was confirmed by SDS-PAGE. The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate polycrylamide gel electrophoresis; NaCl/P, phosphate-buffered saline; kDa, kilodaltons; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.

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1 The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate polycrylamide gel electrophoresis; NaCl/P, phosphate-buffered saline; kDa, kilodaltons; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.
Copurification of an 82-kDa Serum Component with Vitronectin—Comparison of vitronectin preparations obtained from either serum or plasma showed that an 82-kDa protein copurifies with vitronectin from serum through the monoclonal antibody-Sepharose and heparin-Sepharose affinity chromatography steps used (Fig. 1, lanes 2–4). Densitometric scanning of the gels showed that the 82-kDa protein accounted for an average of 15% (three independent preparations) of the protein in serum vitronectin preparations obtained in this manner. A higher proportion (about 25%) of the 82-kDa component relative to vitronectin was found in preparations taken through the antibody chromatography step only, suggesting that some 82-kDa component is lost in the heparin-Sepharose step (results not shown). The 82-kDa component was also obtained from serum decalcified with citrate, showing that the failure to obtain this component from plasma was not likely to be due to the absence of calcium in the citrated plasma.

To begin to characterize the 82-kDa protein the band was cut out from gels and the protein was eluted from the gel for analyses. When rerun on SDS-PAGE the material isolated from the 82-kDa band appeared as 56- and 32-kDa bands with a small amount of the original 82-kDa component present (Fig. 2, lane 3). The presence of the 82-kDa component in serum but not in plasma, the molecular size of its breakdown products, as well as the fact that the major amino-terminal sequence (seven residues) obtained from the gel-isolated 82-kDa component was identical to that of thrombin heavy chain (data not shown), suggested that the 82-kDa protein was the thrombin-antithrombin III complex. We, therefore, pursued a series of immunological studies to establish this fact.

Reaction of the 82-kDa Serum Vitronectin Component with Antibodies against Antithrombin III—Immunoblotting of serum vitronectin preparations containing the 82-kDa component separated by SDS-PAGE showed that the 82-kDa component and its 56-kDa breakdown product reacted with antibodies to antithrombin III (Fig. 3, lanes 1–3). The 56-kDa band co-migrated with a purified preparation of antithrombin III (Fig. 3, lane 4). Similar to what was found with protein staining, prolonged (90 min) boiling of the serum vitronectin complex in SDS prior to gel electrophoresis gave decreased antibody reactivity in the position of the 82-kDa band while the reactivity in the position of the 56-kDa band was increased (compare lanes 2 and 3). The antithrombin III antibodies did not react with the 32-kDa component (presumably thrombin heavy chain) or with the 65- and 75-kDa vitronectin polypeptides. When the serum vitronectin preparation was analyzed without any boiling of the sample prior to gel electrophoresis, the immune reactivity ran with a reduced molecular size of 69 kDa (Fig. 3, lane 1), suggesting that the complex has a compact structure which requires boiling in SDS in addition to reduction to unfold.

![Fig. 1. Copurification of an 82-kDa serum component with vitronectin.](image1)

![Fig. 2. Elution of the 82-kDa component of serum vitronectin from SDS-PAGE.](image2)
prepared by fractionating serum on monoclonal antibody-Sepharose without boiling. The 82-kDa component was dissolved in SDS and 2-mercaptoethanol and eluted from the heparin-Sepharose with 0.12 M NaCl (M). The 82-kDa component was dissolved in SDS and 2-mercaptoethanol without boiling (lane 1) or boiled for 5 min (lane 2) or 90 min (lane 3). Lane 4, purified antithrombin III (10 µg) boiled for 5 min.

Because it seemed that the copurification of the 82-kDa complex with vitronectin could depend on the binding of the 82-kDa complex to vitronectin that was retained on the antibody-Sepharose, we asked whether a column of vitronectin coupled directly to Sepharose would bind the complex. Whole serum or plasma was passed through a vitronectin-Sepharose column, and the eluates were separated by SDS-PAGE, transferred to nitrocellulose filters, and immunoblotted with antisera prepared against antithrombin III or the anti-82-kDa protein complex. The 82- and 56-kDa components obtained from serum by vitronectin-Sepharose chromatography were the same as those obtained from the antibody column. No immunoreactive protein was found in eluates obtained by fractionation of plasma on vitronectin-Sepharose (Fig. 4, A and B). A 50-kDa protein weakly reactive with the anti-82-kDa serum was occasionally present in various preparations (Fig. 4A). This component has not yet been characterized. We have also observed this 50-kDa protein using antibodies to antithrombin III (result not shown), and it may, therefore, be a degradation product of antithrombin III or a protein cross-reactive with it. Fibrinogen was also tested because its β chain has a molecular size of 56 kDa (24) and because fibrinogen can bind thrombin (8, 25). It gave no reactivity with the antithrombin III or 82-kDa component antibodies.

Interactions of Purified Thrombin, Antithrombin III, and Vitronectin—We wanted to determine whether the 82-kDa complex could be constructed from purified components and would exhibit the same properties as the serum-derived complex. When thrombin (Fig. 5, lane 1) and antithrombin III (Fig. 5, lane 2) were combined in the presence of heparin (used to facilitate complexing of thrombin and antithrombin III), a complex which moved with an Mₙ of 82 kDa in SDS-PAGE was obtained (Fig. 5, lane 5). Serum 82-kDa component for comparison with the thrombin-antithrombin III complex was obtained in partially purified form from heparin-Sepharose onto which serum vitronectin had been bound. Most of the 82-kDa component along with some of the 75-kDa vitronectin polypeptide eluted from heparin-Sepharose at 0.12 M NaCl (Fig. 5, lane 9), whereas the 65-kDa polypeptide of vitronectin was recovered by eluting with 0.5 M NaCl (not shown). The serum-derived complex and the complex we prepared from the individual components had the same Mₙ and both, if not boiled prior to SDS-PAGE, migrated at 69 kDa (Fig. 5, lanes 4, 6, and 8).

Properties of Vitronectin-Antithrombin III Interaction—As described above, antithrombin III from plasma did not bind to vitronectin-Sepharose, suggesting that it must be complexed with thrombin to show affinity to vitronectin. This hypothesis was tested with additional binding assays. When vitronectin was immobilized onto plastic microtiter wells and

![Figure 3](image1.png)

**Fig. 3. Reactivity of the 82-kDa component with anti-human antithrombin III.** Partially purified 82-kDa complex was prepared by fractionating serum on monoclonal antibody-Sepharose followed by heparin-Sepharose. A fraction rich in the 82-kDa complex was eluted from the heparin-Sepharose with 0.12 M NaCl. Forty micrograms was subjected to SDS-PAGE under reducing conditions. Following electrophoresis, the gel was blotted onto nitrocellulose and reacted with rabbit anti-human antithrombin III followed by visualization of the bands with peroxidase-labeled goat anti-rabbit IgG. The 82-kDa component was dissolved in SDS and 2-mercaptoethanol without boiling (lane 1) or boiled for 5 min (lane 2) or 90 min (lane 3). Lane 4, purified antithrombin III (10 µg) boiled for 5 min.

![Figure 4](image2.png)

**Fig. 4. SDS-PAGE and immunoblotting analysis of proteins bound to vitronectin-Sepharose from plasma and serum.** Proteins from serum or plasma were fractionated on vitronectin-Sepharose or anti-vitronectin-Sepharose. Samples of proteins bound to each column (and eluted with 8 M urea) and a sample of fibrinogen were electrophoresed under reducing conditions, and the gel was blotted onto two nitrocellulose filters for subsequent staining with anti-82 kDa (panel A) or anti-antithrombin III (panel B). Lane 1, molecular size markers; lane 2, fibrinogen; lanes 3 and 4, proteins bound to vitronectin-Sepharose from two different samples of plasma; lanes 5 and 6, protein bound to vitronectin-Sepharose from two different samples of serum; lane 7, proteins isolated on anti-antithrombin antibody-Sepharose from serum; lane 8, proteins isolated on anti-vitronectin antibody-Sepharose from plasma.
purified antithrombin III was incubated at various concentrations in the wells, little or no binding of antithrombin III to the vitronectin took place as detected with antibodies to antithrombin III. However, when the antithrombin III was first incubated with thrombin, significant binding of antithrombin III to the vitronectin-coated wells was seen (Fig. 6). Incubation of antithrombin III with heparin had no effect on the ability of antithrombin III to bind to vitronectin.

When purified plasma antithrombin III was radiolabeled with $^{125}$I and applied to a vitronectin-Sepharose column, 60% of the radioactivity bound to the column. The bound radioactivity resisted elution with 1 M NaCl but eluted with 8 M urea (Fig. 7). The binding was independent of the presence of divalent cations, as it occurred in 50 mM EDTA. Only 15% of the radioactivity was bound when the $^{125}$I-antithrombin III was applied to a BSA-Sepharose column under identical conditions. This result indicates that a major portion, if not all, of antithrombin III possesses the potential of binding vitronectin.

Antithrombin III also acquired affinity for vitronectin upon adsorption to plastic as demonstrated by binding of vitronectin in ELISA. Incubation of increasing concentrations of vitronectin in microtiter wells coated with antithrombin III showed saturation of vitronectin binding at a concentration which suggests a high affinity for the interaction (Fig. 8).

**DISCUSSION**

These studies demonstrate that vitronectin binds an 82-kDa protein complex which exists in serum but not in plasma. Several lines of evidence show that this 82-kDa complex represents the thrombin-antithrombin III complex formed during blood coagulation. The 82-kDa complex could be dissociated to 56- and 32-kDa components identical in their molecular sizes to antithrombin III and thrombin, respectively. The 82-kDa complex reacted with antibodies generated...
against antithrombin III, and antibodies generated against the 82-kDa complex reacted with purified antithrombin III. Comparison of the vitronectin-associated 82-kDa complex with a complex constructed from purified thrombin and antithrombin III revealed identical molecular sizes in SDS-PAGE. Moreover, both complexes exhibited the same dramatic shift in apparent molecular size to 69 kDa when the samples were not boiled prior to SDS-PAGE. The specificity of the association between vitronectin and thrombin-antithrombin III complex was illustrated by the fact that the thrombin-antithrombin III complex was the only component among plasma proteins which copurified with vitronectin through the purification procedure. Densitometric scanning of SDS gels where serum vitronectin preparations had been separated revealed an 82-kDa component to vitronectin ratio of up to 1:3. Since this is roughly the same as the ratio of the expected serum concentration of thrombin-antithrombin III complex (up to 1.3 μM) and that of vitronectin (4 μM), the amount of the 82-kDa component copurifying with vitronectin suggests that most of the thrombin-antithrombin III complex is associated with vitronectin in serum.

The thrombin-antithrombin III complex appears to bind to vitronectin through a cryptic site in the antithrombin III moiety. That antithrombin III must be modified before it will bind to vitronectin is suggested by the following evidence. Antithrombin III from plasma binds neither to a vitronectin-Sepharose column nor to a vitronectin-loaded anti-vitronectin antibody-Sepharose column, although antithrombin III from serum does. Furthermore, purified antithrombin III does not bind to vitronectin coated onto plastic in an ELISA; however, it will bind to vitronectin after it has been reacted with thrombin. Iodination of antithrombin III or its binding to plastic also rendered antithrombin III capable of binding vitronectin, showing that a binding site for vitronectin resides in antithrombin III and that thrombin is not essential for the binding. Moreover, the cryptic binding site in antithrombin III may be the only site interacting with vitronectin in the thrombin-antithrombin III complex, since thrombin blotted on nitrocellulose shows no affinity for vitronectin whereas blots of antithrombin III show such binding. All these data, therefore, suggest that exposure of a cryptic site in antithrombin III must take place before antithrombin III will bind to vitronectin and that the binding of the thrombin-antithrombin III complex to vitronectin depends on the activation of this site through the association of antithrombin III with thrombin.

The significance of the vitronectin-thrombin-antithrombin III interaction can only be speculated upon at this time. We know that vitronectin possesses a cell attachment site, a potentially mitogenic somatomedin B sequence, and a glycosaminoglycan-binding site (4). The presence of vitronectin in the thrombin-antithrombin III complex could, therefore, fundamentally alter the properties of the complex.

Thrombin-antithrombin III complexes can be taken up by endothelial cells through a receptor specific for the complex (26). In addition, many cells possess receptors for thrombin (27-30). The presence of vitronectin in the thrombin-antithrombin III complex would endow the complex with an additional cell-binding site. Many different types of cells possess the vitronectin receptor judging from their ability to attach to vitronectin-coated surfaces (31). Less is known about the distribution of the receptor for the thrombin-antithrombin III complex (26), but it is possible that vitronectin causes routing of the complex to a different cell type than would be the case otherwise. Hepatocytes have been shown to eliminate thrombin-antithrombin III complexes from the circulation (32). Involvement of vitronectin with such a function would be similar to the role proposed for plasma fibronectin in the elimination of tissue debris through fibronectin-mediated opsonization and uptake in the liver (33).

The effects of vitronectin in the cellular interactions of the thrombin-antithrombin III complex need not be limited to the elimination of the complex, however. The presence of vitronectin in the complex could bring the somatomedin B sequence of vitronectin into a juxtaposition with the cell surface creating a potentially mitogenic signal (but see also Heldin et al. (34) on the possibility that somatomedin B is not mitogenic). In addition the complex contains the mitogen thrombin (27), which while inactive when complexed with antithrombin III can be released in an active form from the complex (14). The potential mitogenic properties of the thrombin-antithrombin III-vitronectin complex appear worth investigating because this complex could be a source of cell growth-promoting activity at the site of hemorrhage and tissue damage.

Finally, vitronectin possesses a glycosaminoglycan-binding site which interacts strongly with heparin (2, 4). Heparin accelerates the rate of inactivation of thrombin by antithrombin III (7, 35, 36). It will be interesting to see what effect, if any, vitronectin might have on the thrombin-antithrombin III interaction and its modification by heparin. Our observations on the association of vitronectin with the thrombin-antithrombin III complex, therefore, suggest a number of new avenues of study which are likely to increase the understanding of phenomena related to blood clotting and may lead to delineation of the function of circulating vitronectin.

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