von Willebrand factor binds to fibrillar type I collagen in a rapid, temperature-independent, reversible, specific, and saturable manner. Evaluation of binding isotherms by Scatchard-type analysis demonstrated that 6–18 µg of von Willebrand factor bind per mg of collagen, with K_d between 2 and 8 × 10^8 M^{-1}. Five distinct tryptic fragments, purified under denaturing and reducing conditions and representing over 75% of the molecular mass of the von Willebrand factor subunit, were tested for their capacity to inhibit the von Willebrand factor-collagen interaction. Complete inhibition was obtained with a 52/48-kDa fragment at a concentration of ~1 µM. The location of this fragment in the subunit was established to be between Val-449 and Lys-728. Fifteen monoclonal antibodies against the 52/48-kDa fragment inhibited von Willebrand factor binding to collagen. Six antibodies against other portions of the von Willebrand factor subunit had no inhibitory effect. The tryptic fragment was a competitive inhibitor of von Willebrand factor binding to collagen and, therefore, recognizes the same interaction site as the intact molecule. These studies precisely define a domain in the von Willebrand factor subunit that interacts with type I collagen.

von Willebrand factor (vWF) plays an essential role in primary hemostasis as one of the adhesive proteins that mediate platelet plug formation at sites of vascular injury (1–4). Its activity is expressed through the interaction with receptors on the platelet surface (5–7) as well as with components of the vessel wall (4, 8–11). Thus, vWF acts as an essential anchoring molecule that initiates platelet deposition at the wound site. The physiological importance of this function is demonstrated by the fact that platelet adhesion to exposed subendothelium is severely impaired in patients with von Willebrand disease, who lack vWF (1–3).

A significant amount of experimental work, performed over the past several years, has led to the demonstration that vWF binds to both collagen and noncollagenous components of the vessel wall (12–21). More recent studies have attempted to define the structural basis for the interaction with collagen and have provided preliminary information on discrete fragments of vWF that appear to subserve this function (22–24).

In this report we describe the isolation and characterization of a high affinity collagen binding domain of human vWF by using a combination of different techniques, including limited proteolysis, partial NH2-terminal sequence analysis of proteolytic fragments, immunoinhibition, and direct inhibition binding studies, we demonstrate here that this functional domain of vWF resides in a 52/48-kDa tryptic fragment of the constituent subunit that begins at amino acid residue Val-449 and extends through Lys-728. This is the same fragment of vWF that contains the binding domain for the platelet membrane glycoprotein (GP) Ib, as recently demonstrated (25).

**Materials and Methods**

**Purification of vWF.**—Purified vWF for trypsin cleavage studies was obtained from commercial factor VIII concentrate (the generous gift of Armour) by immunoadsorbent chromatography followed by high-pressure liquid chromatography (HPLC), as recently described in detail (25). Purified vWF for binding studies was isolated from cryoprecipitate (the generous gift of American Red Cross, Bethesda, MD) following a procedure previously described (7). The latter material had multimeric structure and ristocetin cofactor activity comparable to vWF in plasma, as demonstrated in previous publications (7, 26).

**Preparation and Characterization of Monoclonal Antibodies against vWF.**—The methodology involved in establishing marine hybridoma cultures producing antibodies against vWF has been previously reported (27). Three different immunogens were used, namely intact vWF, reduced and S-carboxymethylated vWF; and the isolated tryptic fragment, 52/48 kDa in size, described in this report as containing the collagen binding domain of vWF. The reactivity of all monoclonal antibodies was tested against each of the immunogens described above using a solid-phase enzyme-linked immunoassay (27). Moreover, the epitope specificity of the 21 antibodies used in these studies was established by a dot blotting procedure, using epithelial purified fragments obtained by trypsin digestion of vWF (see below) or the cyanogen bromide fragments comprising the entire vWF subunit (28). These latter fragments were supplied by Dr. K. Tizzi (Department of Biochemistry, University of Washington, Seattle, WA). The dot blotting procedure was performed using 0.1-µm pore nitrocellulose paper (pH 7.9, Schleicher and Schuell). The paper was first soaked in phosphate-buffered saline (0.01 M mono- and disodium phosphate, 0.14 M NaCl, 0.02% NaN3, pH 7.4) and air dried, and then 1 to 2 µl of the fragments tested were applied and dried in the air. The paper was subsequently treated with the "Blotto" solution described by Johnson et al. (29) in order to saturate unreacted binding sites. After this, the paper was soaked in a suitable dilution of the monoclonal antibody being tested for 4 h, then washed with buffer, and blocked overnight in a solution of 1%–3% labeled rabbit anti-mouse IgG. Both incubations were at 22–25 °C. After extensive washing with buffer and "Blotto," the paper was finally dried and an autoradiogram
obtained at -70 °C with a Kodak XRP-1 film using a Cronex Light
ning Plus screen (Du Pont). Purified IgG of the monoclonal antibodies was prepared from ascitic fluid by chromatography on DEAE-Affi-
Gel blue (Bio-Rad) following a method previously published (30).

Trypsin Cleavage and Separation of Fragments—The methods used here have been described in detail elsewhere (25). Trypsin digestion was performed by mixing 1 mg of vWF with 2500 units of trypsin (bovine pancreatic Type I; 15,000 units/mg; Sigma) and incubating for 2 h at 37 °C, pH 7.0. The fragments generated were separated by HPLC size exclusion chromatography in three main fractions (denominated A, B, and C) as previously described (25). Isolation of the 52/48-kDa fragment was performed by HPLC chromatofocusing, salt gradient elution, and size exclusion chromatography, all in the presence of 6 M urea, as recently reported (25). Reduction and S-carboxymethylation of proteins was achieved by treatment with dithiothreitol, in equal amount (w/w) to protein, for 1 h at 37 °C, followed by treatment with a 2.7-fold excess (w/w) of iodoacetamide for 30 min at room temperature (22–25 °C) and in the dark. All samples were finally dialyzed extensively against a buffer composed of 0.05 M Tris, 0.15 M NaCl, pH 7.3, containing by ultrafiltration (Amicon) and dialyzed again before storage at
-70 °C. The tryptic fragments of vWF were analyzed by polyacryl-
amide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) using 5–15 or 10–15% linear gradient gels under reduc-
ring and nonreducing conditions (31). The gels were stained with Coomassie Brilliant Blue R (Bio-Rad). NH₂-terminal sequence analy-
ysis of the purified fragment C was performed using a gas-
phase sequenator (model 470A, Applied Biosystems). Identification of the phenylthiohydantoin column (Du Pont) following the manufacturer's in-
structions.

Binding of vWF to Collagen—vWF for these studies was radio-
abeled with 125I using the technique described by Fraker and Speck (32) using IODO-GEN (Pierce Chemical Co.). The radiolabeled 125I-
vWF retained its native multimeric structure, as previously described in four par.

RESULTS

Characterization of vWF Binding to Collagen—Preliminary ex-
periments were performed to establish the optimal conditions for measuring vWF binding to collagen. The time course of the interaction demonstrated apparent equilibrium after 10 min of incubation, with minimal effect of varying the temperature between 4 and 37 °C (Fig. 1). Maximal binding was observed with pH between 6.5 and 7.5 and with NaCl concentration between 0.05 and 0.2 M in the buffer system described under “Materials and Methods.” Excess unlabeled vWF inhibited the binding of 125I-vWF when added at the same time and displaced the bound 125I-vWF when added after apparent equilibrium of binding had been reached (Fig. 2). These experiments established that the binding of vWF to collagen was saturable and reversible. Moreover, it was also found that the labeling procedure did not alter the affinity of the interaction (Fig. 3). The amount of vWF bound varied as a function of the collagen concentration in the mixture. The latter was kept constant, as indicated under “Materials and Methods,” at a concentration selected to have a sufficient number of counts bound when 125I-vWF was used, with concentrations up to 10 μg/ml. The relevant binding parameters for the vWF-collagen interaction were derived from Scatchard-type analysis of binding isotherms, which suggested the existence of a single class of noninteracting binding sites (Fig. 4). The results of three such experiments are summarized in Table I.

The Effect of Monoclonal Antibodies on the Binding of vWF to
Collagen—Twenty-one monoclonal antibodies directed against defined epitopes of vWF were tested for their ability to block the vWF-collagen interaction. Of these, 15 inhibited

![Fig. 1. Time course and temperature dependence of vWF binding to collagen. The collagen concentration was 83 μg/ml, and the 125I-vWF was added at 2 μg/ml. After incubation for 20 min at the temperature indicated for each curve, bound 125I-vWF was separated from free ligand by centrifugation of the insoluble collagen microfibrils through a layer of 20% sucrose. The tip of the microcentrifuge tube containing the pelleted collagen and bound 125I-vWF was cut and counted in a γ-scintillation spectrometer. The amount of 125I-vWF bound was calculated on the basis of its specific activity.](image-url)
the binding of 125I-vWF to collagen, and six had no inhibitory effect (Fig. 5). The binding was actually increased in the presence of five of these antibodies (Fig. 5). All the inhibitory antibodies reacted with the 52/48-kDa tryptic fragment, whereas all the noninhibitory antibodies reacted with other fragments of the vWF subunit (see legend to Fig. 5). In particular, three antibodies (RG 51, EG 3, and RG 38) that reacted with a 55-kDa tryptic fragment corresponding to the middle region of the vWF subunit (see below) were among those showing no inhibitory effect. Three control monoclonal antibodies were also tested. Two were directed against distinct platelet membrane glycoproteins, GP Ib and GPIIb-IIIa complex, and one was directed against thyroglobulin. They all showed no inhibitory effect.

The Effect of Proteolytic Fragments on the Binding of Intact vWF to Collagen—Three fractions containing the larger fragments derived from tryptic digestion of vWF (see also Ref. 25 for additional details) were tested for their inhibitory effect on the binding of intact vWF to collagen. This experiment was performed using the native preparations with intact disulfide bonds (Fig. 6). The fraction designated B completely blocked the binding, whereas fractions A and C had minimal or moderate inhibitory activity, respectively (Fig. 6).

The major component of fraction B had a molecular mass, under nonreducing conditions, of approximately 120 kDa (Fig. 6). After reduction of disulfide bonds, a closely spaced doublet of 52/48 kDa was seen in fraction B along with several other smaller fragments. The 52/48-kDa doublet was purified to homogeneity and was found to inhibit completely the vWF-collagen interaction (Fig. 7). Other tryptic fragments of vWF, with molecular mass of 13, 22, 41, and 55 kDa, were also purified from fractions B and C under reducing and denaturing conditions and tested for their inhibitory activity. The effect of these other fragments was minimal at concentrations corresponding to those of the 52/48-kDa fragment that completely inhibited vWF binding to collagen (Fig. 8). All the fragments, however, caused partial inhibition of binding when tested at higher concentrations (Fig. 8). The NH2-terminal sequence of each of the purified polypeptides was determined.

FIG. 2. Saturability and reversibility of vWF binding to collagen. Top panel, in this experiment, unlabeled vWF (at the final concentrations indicated) was added to the tubes containing the collagen (83 μg/ml) at the same time as 125I-vWF (2 μg/ml). After incubation for 20 min at 22–25°C, bound 125I-vWF was quantitated as indicated in the legend to Fig. 1. Lower panel, in this experiment, 125I-vWF (2 μg/ml) was incubated with collagen (83 μg/ml) for 15 min at 22–25°C. At this point (indicated as time 0 on the abscissa) unlabeled vWF was added at a 100-fold excess (curve with open circles) or the same volume of Tris buffer was added as a control (curve with closed circles). The incubation was then continued for the time indicated and the 125I-vWF bound was quantitated as indicated in the legend to Fig. 1.

FIG. 3. Relative binding of labeled and unlabeled vWF to collagen. The total concentration of vWF was maintained constant in this experiment (2 μg/ml), but the relative proportions of labeled and unlabeled ligand varied, inversely, between 0 and 100% as indicated on the abscissa. After incubation for 20 min at 22–25°C, the amount of radioactivity bound to the collagen was determined and expressed as a percentage of the value observed when only 125I-vWF was added to the mixture (100% binding). The line was fitted to the experimental points by linear regression analysis and gave a slope very close to the theoretical value of 1, expected when labeled and unlabeled ligand bind with the same affinity.

FIG. 4. Saturation curve of vWF binding to collagen. Increasing amounts of 125I-vWF, as indicated on the abscissa, were added to collagen (83 μg/ml) and incubated for 20 min at 22–25°C. The amount of 125I-vWF bound to the collagen was then determined as indicated in the legend to Fig. 1. The values shown in this graph represent total binding. The inset shows a Scatchard-type plot of the experimental data obtained using the computer-assisted program LIGAND (33), and the points represent specific binding. The best fit for the experimental points was represented by a straight line suggesting the existence of a single class of noninteracting binding sites. The relevant binding parameters were, for this experiment: 125I-vWF bound at saturation = 0.68 μg/mg of collagen; Kd = 7.76 x 10^8 M^-1 (calculated per vWF subunit assuming a molecular mass of 275 kDa); nonsaturable (nonspecific) binding = 0.
TABLE I
Relevant parameters of $^{125}$I-vWF binding to collagen

The binding parameters were obtained by Scatchard-type analysis using the microcomputer program LIGAND (33). The values of $K_0$ are calculated per vWF subunit assuming a molecular mass of 275 kDa.

| Experiment | $^{125}$I-vWF bound | $K_0$ microg/mg collagen | $K_0$ M$^{-1}$ |
|------------|----------------------|--------------------------|--------------|
| 1          | 5.67                 | 7.76 x 10^9             |              |
| 2          | 6.90                 | 6.58 x 10^9             |              |
| 3          | 18.11                | 2.32 x 10^9             |              |

DISCUSSION

We have characterized a high affinity interaction between vWF and type I collagen that involves a discrete portion of the vWF subunit. The present studies have located this col-
is not required for expressing the binding function of this domain, at least when isolated from the vWF molecule.

Several lines of evidence indicate that the fragment we have identified is likely to represent the main vWF interaction site with the type I collagen used in these studies. Four other tryptic fragments located in the amino-terminal, middle, and carboxyl-terminal regions of the vWF subunit exhibited limited inhibitory function and only at very high concentrations. At present, we cannot rule out the possibility that other collagen binding sites exist in the vWF subunit. Their function might be more susceptible to the denaturing conditions employed for the purification of discrete portions of the vWF subunit and thus be less apparent than that of the 52/48-kDa fragment. Against this hypothesis, however, is the observation that only the fraction containing the 52/48-kDa polypeptide, of the three main fractions derived from a tryptic digest of vWF, exhibited a marked inhibitory effect on vWF binding to collagen even when tested under native conditions. It is also important to consider that the 52/48-kDa fragment acted as a competitive inhibitor of vWF binding to collagen, thus demonstrating that the intact molecule and the reduced and alkylated tryptic fragment interact with the same site on collagen.

Studies with monoclonal antibodies provide additional support for the concept that the 52/48-kDa tryptic fragment represents the main vWF domain interacting with the type I collagen used in these studies. All antibodies directed against the 52/48-kDa polypeptide had an inhibitory effect on the vWF-collagen interaction, whereas none of those directed against other regions of the vWF subunit had any inhibitory effect. The observation that several antibodies not directed against the 52/48-kDa fragment actually increased the binding of vWF to collagen may, in fact, indicate the existence of regulatory mechanisms which are mediated by other regions of the vWF subunit and result in decreased binding to collagen.

In previous studies (22, 34) other investigators have concluded that the epitope of a monoclonal antibody inhibiting vWF binding to collagen resides on a fragment of 48 kDa (unreduced) and 58 kDa after reduction and alkylation. This fragment corresponds to the one indicated as 55 kDa in the present study, isolated from fraction C (Fig. 6). We have tested three monoclonal antibodies reacting with this fragment and found that none of them inhibited the vWF-collagen interaction. The native fraction C, however, and the purified 55-kDa fragment had a partial inhibitory effect on vWF binding to collagen. It is possible, therefore, that a second collagen binding domain resides in this part of the vWF subunit, although its role appears secondary as compared to that of the domain in the 52/48-kDa fragment, at least under the experimental conditions used in the present work.

The collagen-vWF interaction, as characterized in these studies, is a rapid process and is temperature independent, saturable, reversible, and specific. Type I collagen appears to have a single class of binding sites that interact with a discrete domain of the vWF subunit with high affinity. Under the conditions employed in these experiments, therefore, the contribution of other putative binding sites to the vWF-collagen interaction was not apparent. While we have demonstrated that the multimeric structure of vWF is not required for the interaction, we have not addressed in this work the nature of the structural requirements of collagen for binding to occur. Although previous studies (18) suggest that collagen quaternary structure, rather than collagen type per se, is the important factor determining vWF binding, additional work is necessary to elucidate at a molecular level the complex inter-

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**Fig. 8. Inhibition of intact vWF binding to collagen by four different purified tryptic fragments of vWF.** The fragments used are identified by their molecular mass, and their location in the sequence of the vWF subunit is reported under "Results." Binding is expressed as percent of that observed in the absence of competing fragment. The final concentrations used for each fragment are indicated. Note that the 52/48-kDa fragment inhibited binding greater than 90% at concentrations above 1 µM (see Fig. 7).

**Fig. 9. Competitive inhibition of intact vWF binding to collagen by the purified 52/48-kDa tryptic fragment of vWF.** In this experiment, the binding of varying concentrations of 125I-vWF to collagen was determined either in the presence (closed circles) or in the absence (open circles) of purified 52/48-kDa fragment (final concentration, 0.5 µM). The experimental conditions are as indicated in the legend to Fig. 4. The Kd of the interaction (calculated per vWF subunit assuming a molecular mass of 275 kDa) was 6.52 × 10-10 M-1 in the absence of competing fragment and 1.36 × 10-9 M-1 in the presence of it. The amount of 125I-vWF bound at saturation was 6.9 and 13.3 µg/mg, respectively, as calculated by Scatchard-type analysis performed with the program LIGAND (33). Non-specific binding was assumed to be 0 in both cases, as calculated for the curve in the absence of inhibitor.

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The collagen binding domain in a 52/48-kDa tryptic fragment that begins at amino acid residue Val-449 and extends through Lys-728 of the 2050 residues constituting the vWF subunit. This fragment retained the ability of completely inhibiting the binding of intact vWF to collagen when fully reduced and S-carboxymethylated, and 50% inhibition occurred at concentrations between 0.3 and 0.5 µM. Thus, native conformation
action of vWF with the different types of collagen.

The 52/48-kDa tryptic fragment that contains the high affinity collagen binding domain described here was previously found by us to mediate the interaction of vWF with the platelet membrane receptor GPIb (25). In agreement with our findings, other investigators have provided indirect evidence, obtained with monoclonal antibodies, that the GPIb binding domain collagen binding domain described here was precisely the structural basis for these functions of vWF. Additional experimental work will be necessary to define more precisely the structural basis for these functions of vWF.

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