Topography of the Human Factor VIII-von Willebrand Factor Complex

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Factor VIII circulates in noncovalent complex with von Willebrand factor (vWF). The topography of this complex was evaluated by fluorescence energy transfer using factor VIII subunits modified with N-(1-pyrenyl)maleimide (NPM; fluorescence donor) and vWF-derived fragments modified with 7-diethylamino-3-[4'-maleimidylphenyl]-4-methyl coumarin (CPM; fluorescence acceptor). Results from a previous study indicated an interfactor VIII subunit distance of 20 Å separating Cys1684 and Cys1685 in the factor VIII heavy and light chains, respectively (Fay, P. J., and Smudzin, T. M. (1989) J. Biol. Chem. 264, 14005-14010). Fluorophore modification of the vWF SPIII homodimer (residues 1-1365) indicated multiple attachment sites at Cys126/138,1360 as determined from sequence analysis of fluorescent tryptic peptides derived from the modified protein. Based upon donor quenching data, an interfluorophore distance of approximately 28 Å was calculated separating NPM-heavy chain or factor VIII reconstituted from NPM-light chain plus CPM-SPIII or CPM-SPIII-T4, energy transfer was observed with calculated interfluorophore distances of approximately 31 and 34 Å, respectively.

Levels of acceptor resulting in maximal donor quenching suggested an equimolar stoichiometry of factor VIII, with NPM-light chain paired with CPM-SPIII or CPM-SPIII-T4, energy transfer was also observed for the NPM-heavy chain/CPM-SPIII pairing. However, when NPM-heavy chain was reassociated with unmodified light chain prior to reaction with CPM-SPIII or CPM-SPIII-T4, energy transfer was observed with calculated interfluorophore distances of approximately 31 and 34 Å, respectively. Levels of acceptor resulting in maximal donor quenching suggested an equimolar stoichiometry of factor VIII (light chain)/vWF fragment in the reconstituted complexes. These results indicate a close spatial arrangement among the A3 domain of factor VIII light chain, the A2 domain of factor VIII heavy chain, and the NH2 terminus region of vWF in the factor VIII-vWF complex.

Factor VIII, a protein cofactor required for efficient activation of factor X by factor IXa, circulates in complex with vWF (1). Binding between the two proteins is noncovalent and shows characteristics of both electrostatic and hydrophobic interactions (2). The association of factor VIII with vWF stabilizes factor VIII activity in vitro (3) and that of recombinant factor VIII in cell culture media (4). Normal plasma levels of factor VIII are dependent upon complex formation with vWF in that vWF deficiency resulting from gene deletion is accompanied by significantly reduced levels of circulating factor VIII (5).

Factor VIII is synthesized as a single-chain precursor of 2351 amino acids (6) and is represented by the domain structure A1-A2-B-A3-C1-C2 (7). It is purified from plasma as a series of divergent metal ion-linked heterodimers (8-10) formed as a result of proteolysis at the B-A3 junction plus additional cleavages within the B domain (7). The factor VIII heavy chain is minimally represented by the A1-A2 domains but exhibits significant size heterogeneity resulting from the presence of some or all of the contiguous B domain. The light chain corresponds to the A3-C1-C2 domains derived from the COOH-terminal end of the precursor (residues 1649-2351). Factor VIII subunits are dissociated by chelating reagents, and isolated subunits reassociate in buffers containing Ca2+ or Mn2+ to regenerate the active heterodimers (11, 12). The factor VIII light chain has been reported to contain the binding domain for vWF (8, 13-16). Activation of factor VIII by thrombin dissociates factor VIII from vWF (17, 18) as a result of cleavage at residue 1689 (19), thus implicating the 41-residue NH2-terminal region of the light chain as containing the vWF binding domain. Localization of the binding region for an anti-factor VIII monoclonal antibody that inhibits factor VIII binding to vWF to within residues 1670-1684 suggests that this sequence is at or near the vWF-binding domain (20). vWF circulates as a population of multimers of molecular mass ranging from about 0.5 to 12 MDa (21). The protomeric unit of these multimers is a dimer of 270-kDa subunits (2050 residues) (22, 23). Dimers are linked by disulfide bonds near their COOH termini, while disulfide bonds near the NH2-terminal ends link protomers to form multimers (23-25). Limited proteolysis of vWF by Staphylococcus aureus V8 protease generates two disulfide-linked homodimeric fragments designated SPIII (residues 1-1365) and SPI (residues 1366-2050) (24). Digestion of SPIII with trypsin performed under nondenaturing conditions liberates the NH2-terminal residues 1-272 (fragment SPIII-T4) as a monomeric polypeptide (26).

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‡ The abbreviations used are: vWF, von Willebrand factor; RP-HPLC, reverse phase high pressure liquid chromatography; CPM, 7-diethylamino-3-[4'-maleimidylphenyl]-4-methyl coumarin; NPM, N-(1-pyrenyl)maleimide; Hepes, I-(%hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; MOPS, 4-morpholineethanesulfonic acid.
that has been shown to contain the factor VIII-binding domain (27, 28).

In a previous report, we employed fluorescence energy transfer to probe the topography of human factor VIII reconstituted from purified subunits (29). In this study, similar techniques are used to orient factor VIII subunits following binding to vWF-derived fragments. These data indicate a close spatial relationship between the A3 domain of the factor VIII light chain, the A2 domain of the factor VIII heavy chain, and the NH₂ terminus region of vWF in the human factor VIII-vWF complex.

MATERIALS AND METHODS

Reagents—Human factor VIII concentrate (Koate™) was a generous gift from the Cutter Division of Miles Laboratories. The murine monoclonal antibody used in the purification of factor VIII was prepared using the isolated factor VIII heavy chain as immunogen. Details of its preparation will be described elsewhere. Antibody was purified from ascites fluid using the Affi-Gel protein A MAPS II Kit (Bio-Rad) according to the manufacturer’s instructions. Purified IgG (18 mg) in 0.1 M MOPS, pH 7.5, was coupled to 4 ml of Affi-Gel 10 (Bio-Rad) following a 16-h incubation at 4°C. Coupling efficiency was approximately 95%; 1-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (bovine pancreas) was purchased from Sigma and further purified by RP-HPLC using a Vydac C₁₈ column (5 μm, 0.45 x 25 cm) developed with a linear gradient from 20–50% acetonitrile in 0.1% trifluoroacetic acid. S. aureus V8 protease was purchased from ICN Immunobiologicals. The fluorescent probes NPM and CPM were purchased from Molecular Probes. Urea (Sequana grade) was purchased from Pierce Chemical Co.

Proteins—Factor VIII and vWF were prepared from human factor VIII concentrates. Factor VIII and factor VIII subunits were purified as described previously (29). vWF was prepared from the unreduced polyethylene glycol supernatant fraction (29) following successive gel filtration runs on Sepharose 4B-CL (5 x 100 cm) in the absence and presence of buffer containing 0.25 M CaCl₂ (30). The vWF (Vₚ fraction) was devoid of factor VIII activity as determined by clotting assays. Fragments SPⅢ and SPⅠ were prepared from the S. aureus V8 protease digest of vWF and isolated by chromatography using a Mono Q column (HR5/5) according to the methods of Titani et al. (31). CPM-labeled or unmodified fragment SPⅢ-Ⅳ was prepared from a tryptic digest of (CPM-)SPⅢ (26). The digest mixture was treated with diisopropyl fluorophosphate (1 mM) and subjected to gel filtration using a Superose 12 (Pharmacia LKB Biotechnology, Inc.) column (1 x 40 cm) developed in 0.1 M Tris, pH 7.2, and 8 M urea. Protein-containing fragments SPⅢ-Ⅳ were localized by SDS-polyacrylamide gel electrophoresis and dialyzed into 0.02 M Hepes, pH 7.2, 0.15 M NaCl, and 0.02% NaN₃. The 100-kDa vWF propolypeptide peptide was purified from the unreduced polyethylene glycol supernatant fraction (29) following adsorption to an anti-vWF propolypeptide monoclonal antibody (25, 32) coupled to Affi-Gel 10. The vWF propolypeptide was eluted from the column in 0.01 M histidine, pH 6.0, 0.05 M NaCl, 0.01% Tween 20, and 50% (v/v) ethylene glycol. Further purification was achieved by chromatography on Mono Q (HR5/5) as described previously (33). Fig. 1 shows SDS-polyacrylamide gels of the purified factor VIII subunits and vWF-derived fragments. Protein concentrations were determined by the method of Bradford (34).

Reconstitution of Factor VIII from Isolated Subunits—Factor VIII was reconstituted from native or fluorophore-modified subunits in 0.02 M Hepes, pH 7.2, 0.4 M NaCl, and 0.03 M MnCl₂ as described previously (12, 29). Reactions employed a 2-fold excess of heavy chain (mean molecular mass = 136 kDa (12)) relative to light chain (mean molecular mass = 22 kDa) and were run for approximately 2 h at room temperature. Progress of factor VIII formation was followed using a one-stage clotting assay.

Fluorescent Labeling of Proteins—Factor VIII subunits were labeled with NPM as described previously (29). vWF fragment SPⅢ in 0.02 M Tris-HCl, pH 7.2, 0.2 M NaCl was reacted overnight at 4°C in the dark with a 20-fold excess of CPM to SPⅢ subunit (170 kDa; Ref. 24). Unreacted fluorophore was separated from the modified protein by gel filtration on a PD-10 column (Pharmacia) equilibrated in the above buffer and dialyzed for 24 h (three buffer changes). The molar ratio of probe bound to protein ranged from approximately 0.7 to 0.9 and 0.8 to 1.0 for NPM linked to factor VIII light and heavy chains, respectively, and from 1.3 to 2.8 for CPM linked to SPⅢ subunit, assuming extinction coefficients of 37,500 M⁻¹ cm⁻¹ and 343 nm for the NPM conjugate and 50,200 M⁻¹ cm⁻¹ at 387 nm for the CPM-conjugate.

Fluorescence Measurements—Fluorescence measurements were made using a SPEX Fluorolog 212 spectrophotometer. Samples were excited at 345 nm and emission spectra were scanned over 370–400 nm. Donor quenching was calculated as described elsewhere (32). The corrected emission spectra for the above samples were integrated from 370 to 400 nm. Donor quenching was calculated from the ratio of sample III to samples I + II. All reactions (0.3 ml) were carried out at room temperature in buffer containing 0.02 M Hepes (pH 7.2), 0.15 M NaCl, 0.003 M CaCl₂, and 0.01 M l-lysine HCl and were run for 3–5 h prior to fluorescence analysis.

Fluorescence energy transfer was determined as described by Marsh and Lowery (35). The distance between the fluorescence donor and the fluorescence acceptor was calculated from the following equation:

\[
 R_0 = \left( 8.79 \times 10^{-5} \right) \kappa'^{-1} n^{-1} N_0 D_{DO DA}
\]

where \( R_0 \), in Å², is the critical distance at which the efficiency of transfer is 50%; \( Q_0 \), the quantum yield of donor dye in the absence of acceptor was determined to be 0.11 as described previously (29). Values for \( n \), the refractive index of the medium, and \( k' \), an orientation factor between the donor and acceptor dyes, were assumed to be 1.33 and 95% respectively. Employing this value for \( k' \) introduces an uncertainty in the calculated distance \( R \) of less than 20% (36). \( D_{DO DA} \), the overlap integral of the absorbance spectrum of the acceptor and emission spectrum of the donor, was determined to be 7.67 x 10⁻⁴ nm² cm⁻¹ M⁻¹ as described previously (29). The efficiency \( E \) of energy transfer was determined from donor fluorescence intensities in the absence and presence of acceptor (35) and is related to the distance \( R \) between donor and acceptor by the following equation.

\[
 E = R_0^6 / (R_0^6 + R^6)
\]

Preparation of Tryptic Peptides from CPM-SPⅢ—CPM-SPⅢ (0.56 mg; 3.3 nmol of subunit) were denatured in 8 M urea, reduced, carboxymethylated, and digested with trypsin as described previously (29). Peptides were resolved by RP-HPLC using a Vydac C₁₈ column (5 μm, 0.45 x 25 cm) developed with a linear gradient of acetonitrile (7–60% in 150 min) in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Absorbance was monitored at 214 nm. Each peak was monitored for coumarin fluorescence (excited at 387 nm) from 420 to 520 nm. Fluorescent peptides were concentrated to approximately 100 μl in a Speed-Vac and stored frozen at −40°C.

Amino-terminal Sequencing of Tryptic Peptides—Peptides were subjected to pulsed liquid phase amino-terminal sequencing using an Applied Biosystems model 477A/120 Sequencer. Approximately 600
pmol of peptide was applied to a Polybrene-coated glass-fiber disc and the sequence program executed. The phenylthiohydantoin derivative residue from each cycle was identified by RP-HPLC on an Applied Biosystems model 120 chromatograph.

**Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed using the buffer system of Laemmli (37). Gels were cast on a mini-gel apparatus (Bio-Rad), and electrophoresis was for 1 h at 150 V. Bands were visualized following staining with silver nitrate (38).**

**RESULTS**

**Protein Modification and Sites of Probe Binding.—The vWf-SPIII homodimer was modified with CPM as described under "Materials and Methods." Covalent couplings employed a 20-fold excess of probe/170-kDa subunit and were performed under nondenaturing conditions. Levels of probe incorporation for several preparations of SPIII ranged from about 1.5 to 3 probes/SPIII subunit, suggesting reaction at multiple sites.

We have previously localized sites of probe attachment in the heavy (Cys169) and light (Cys150) chains of factor VIII (29). These sites were determined following sequence analysis of RP-HPLC-purified fluorescent peptides generated by trypsin cleavage of the modified proteins. This same approach was used to identify fluorophore-modified residues in CPM-SPIII. Fig. 2 shows the RP-HPLC chromatogram for tryptic peptides derived from CPM-SPIII. Two peptides, designated p59 and p70 based upon their elution positions, exhibited coumarin fluorescence, with p70 showing an approximate 3-fold greater fluorescence yield than p59 (data not shown). No coumarin fluorescence was detected in the unbound fraction, in column fractions beyond 40% acetonitrile, or following a wash cycle with 90% acetonitrile.

The two coumarin-labeled peptides were subjected to pulsed liquid phase amino-terminal sequence analysis (Table I). Sequences for p70 matched known vWf residues 121-143. No residues were identified beyond Arg143, indicating that p70 contained a single cysteine residue, Cys146, and thus represents the COOH terminus of SPIII. This fragment contains a single cysteine residue, which unambiguous identification of residues was no longer possible. Inspection of the known vWf sequence (31) allowed

**Fig. 2.** RP-HPLC of tryptic peptides derived from CPM-SPIII. RP-HPLC was performed using a C8 column developed in a linear acetonitrile gradient (7-60%; 150 ml) in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The solid line represents absorbance at 214 nm, and the dashed line indicates the acetonitrile gradient. Arrows identify peptides showing coumarin fluorescence.

**Fig. 3. Spectral relationship between NPM- and CPM-labeled proteins.** Corrected emission spectrum of NPM-factor VIII light chain excited at 343 nm (---) and excitation spectrum of CPM-SPIII assessed at 465 nm (----) were determined at 1-nm increments. Fluorescence intensity is in arbitrary units.

**Table I. Amino-terminal sequences of tryptic peptides from CPM-SPIII**

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*From Ref. 31.

**From Ref. 31.

( ), tentative assignment.

COOH-terminal residue of SPIII.

mapping of the two sequences to residues 855-869 and residues 1346-1358. No cysteine residues are contained within the sequences determined for either peptide. The peptide beginning Arg143, indicating that p70 was represented by the tryptic fragment Tyr190-Arg143. Two cysteine residues, Cys126 and Cys135, are contained within this fragment (31); however, residues from sequencing cycles 7 and 16 were tentatively identified as serine. Since phenylthiodyantoin-carboxymethylcysteine chromatographs near to the elution position of phenylthiodyantoin-serine, this result may suggest that CPM labeling at Cys135/136 was not quantitative, thus possibly accounting in part for the observed variability of probe incorporation into SPIII. However, the chromatographic behavior of the phenylthiodyantoin derivative of CPM-cysteine is not known.

Analysis of p59 indicated that two peptides were contained in this fraction. Fifteen cycles of sequence were obtained after which unambiguous identification of residues was no longer possible. Inspection of the known vWf sequence (31) allowed...
light chain and CPM-SPIII. Fig. 4 illustrates results obtained for a reaction mixture (0.3 ml) containing 0.5 µg of NPM-light chain and 4 µg of CPM-SPIII following a 3-h incubation at 23 °C. Donor quenching (26%) paralleled the sensitized emission of the acceptor. This extent of donor quenching was unchanged following an 18-h incubation (results not shown).

NPM-factor VIII subunits were titrated with CPM-SPIII to ensure an excess of acceptor relative to donor (Fig. 5). Donor quenching for the NPM-light chain/CPM-SPIII pairing was near maximal at an approximate weight ratio of 0.5 µg of light chain to 2 µg of SPIII and remained essentially unchanged at 12 µg of acceptor. Assuming molecular masses of 83 kDa for the factor VIII light chain and 170 kDa for the SPIII subunit, this result suggested a near stoichiometric equivalence of components in the light chain-SPIII complex.

No donor quenching was detected for the NPM-heavy chain/CPM-SPIII pairing over a range of acceptor concentrations up to a greater than 15-fold excess relative to donor, suggesting either no interaction between the two proteins or, if binding did occur, the resultant interfluorophore distance exceeded 70 Å (36).

Additional experiments were conducted using factor VIII reconstituted from an NPM-modified subunit plus its unmodified complement as the fluorescence donor (Fig. 5). Reconstitution reactions employed a 2-fold excess of heavy chain to light chain to ensure complete utilization of the latter subunit. Factor VIII formed from NPM-light chain and unmodified heavy chain yielded a similar extent of donor quenching (24%) following reaction with the CPM SPIII acceptor when compared with the NPM-light chain/CPM-SPIII pairing. This result suggested that the presence of heavy chain when bound to light chain did not contribute to the interaction between light chain and SPIII and was consistent with the above results, indicating no direct interaction between heavy chain and SPIII. Although the fluorescence of free NPM-heavy chain was not quenched by CPM-SPIII, donor quenching (14%) was observed for factor VIII reconstituted from NPM-heavy chain plus unmodified light chain. This result indicated that light chain was required to juxtapose the heavy chain subunit of factor VIII and SPIII.

Interfluorophore distances were calculated for the donor/acceptor pairings described above using values for maximal fluorescence quenching from Fig. 5. Distances of 27 Å (NPM-light chain), 28 Å (NPM-light chain plus unmodified heavy chain), and 31 Å (NPM-heavy chain plus unmodified light chain) were determined, separating the modified residues in these donors from those in CPM-SPIII. However, while factor VIII subunits were modified at unique sites, modification of SPIII occurred at two distinct regions, Cys136/135 near its NH2 termini and Cys1360 near its COOH termini. Furthermore, since SPIII is composed of two identical subunits, modification would occur on both subunits of the homodimer. To obviate the potential contribution to donor quenching from multiple probes on the acceptor, energy transfer experiments were performed using a tryptic fragment of SPIII, SPIII-T4, a monomeric polypeptide (34 kDa) containing vWF residues 1-272 (26). As shown in Fig. 6, preincubation of NPM-light chain with a 2-fold excess of either unmodified SPIII or SPIII-T4 blocked subsequent donor quenching following addition of CPM-SPIII, confirming that the factor VIII-binding region was common to both vWF-derived fragments. On the other hand, preincubation of NPM-light chain with an excess of either fragment SPIII, a homodimer of subunits containing vWF residues 1366-2050 (24), or the 100-kDa vWF propolyopeptide (32) failed to block interaction between light chain and SPIII.

Fluorescence energy transfer data using NPM-light chain or factor VIII reconstituted from NPM-heavy chain plus unmodified light chain as donors and CPM-SPIII-T4 as acceptor are shown in Fig. 7. For either donor, fluorescence quenching was saturable with maximal levels for 0.5 µg of light chain or 1 µg of reconstituted factor VIII observed with approximately 0.25 µg of SPIII-T4. This result was consistent with a factor VIII (light chain)-SPIII-T4 complex with components in equimolar stoichiometry. Extents of maximal fluorescence quenching observed for the modified light chain...
requirement for factor VIII light chain to juxtapose the heavy factor VIII heavy chain and the vWf fragments; and (iii) a binding of factor VIII light chain to NH$_2$-terminal-derived fragments of vWf, were consistent with prior studies using solid phase-binding assays (13-15, 27, 28, 39) and ultracentrifugation analyses (16,40). Furthermore, the observation that high molecular weight complexes containing predominant heavy chains of 146/155 kDa and 110/120 kDa, respectively, and a Stokes radius of approximately 34 Å separating Cys$^{505}$ in vWf, a value that was essentially unchanged following prior recombination of the modified light chain with unmodified factor VIII heavy chain. This relatively close spatial separation was compatible with the proposed site(s) of modification in the acceptor in that these residues are in close proximity to the sequence Thr$^{578}$-Thr$^{580}$ proposed to be at or near the factor VIII-binding site in vWf (39). Although no interactions between factor VIII heavy chain and the NH$_2$-terminal-derived vWf fragments were observed, when factor VIII was reconstituted from modified heavy chain plus unmodified light chain and used as the fluorescence donor, energy transfer was observed. The extent of donor quenching for this pairing indicated an interfluorophore distance of approximately 34 Å separating Cys$^{505}$ and Cys$^{105/115}$ in vWf. These results taken together with results from our prior study that indicated an interfactor VIII subunit distance of 20 Å separating Cys$^{505}$ (heavy chain) and Cys$^{488}$ (light chain) (29) provide for a geometrical assessment of interacting residues present in components of the factor VIII-vWf complex (see Fig. 8 for a schematic illustration).

Recent electron microscopy studies on porcine (41) or human$^8$ factor VIII have indicated structures containing a globular core region of about 14 nm in diameter. In the human protein, a rod-like tail extension of up to 50 nm in length also was observed, and its presence was correlated with the higher M$_r$ factor VIII heterodimers. Thus, the core structure likely contained the A and C domains of the factor VIII subunit with the tail represented by the B domain extension(s) present in the heavy chain. Gel filtration chromatography has indicated Stokes radii of 71 and 60 Å for human factor VIII heterodimers containing predominant heavy chains of 146/155 kDa and 110/120 kDa, respectively, and a Stokes radius of 41 Å for the disassociated light chain (10). Therefore, a spatial separation of 34 Å between the A2 domain of factor VIII heavy chain and the NH$_2$-terminal region of vWf would suggest aspects of a lateral orientation of heavy chain relative to light chain in the factor VIII-vWf complex rather than a stacked array with light chain sandwiched between heavy chain and vWf. This geometry would be compatible with results of Hamer et al. (17) which suggested a secondary

$^8$ W. E. Fowler, P. J. Fay, and V. J. Marder, submitted for publication.

Fig. 7. Effect of CPM-SPIII-T4 on NPM-factor VIII light chain and NPM-factor VIII modified in its heavy chain. Reactions (0.3 ml) contained 0.5 μg of (NPM-) factor VIII light chain (●) or 1 μg of (NPM-) factor VIII labeled in the heavy chain subunit (□) plus the indicated amounts of CPM-SPIII-T4. Reactions were run for 3 h prior to fluorescence analysis. Relative fluorescence was determined as described in the legend to Fig. 5. Data points represent the mean of at least three determinations.

Fig. 8. Geometry of the factor VIII-vWf complex. Interfluorophore distances separating the modified residues in factor VIII subunits and vWf represent values using SPIII-T4 as fluorescence acceptor. The interfactor VIII subunit distance is from Ref. 29. Solid lines indicate direct binding interactions between components. The dashed line indicates a requirement for factor VIII light chain (LC) to juxtapose factor VIII heavy chain (HC) and vWf.

donor (20%) and the heavy chain-modified factor VIII donor (11%) were somewhat less when SPIII-T4 served as acceptor compared with SPIII, a result consistent with multiple probes attached to the SPIIII acceptor. From results obtained using SPIII-T4 as fluorescence acceptor, interfluorophore distances of 29.3 and 33.6 Å were calculated separating Cys$^{126/135}$ in the acceptor from Cys$^{488}$ (factor VIII light chain) and Cys$^{808}$ (factor VIII heavy chain), respectively, in the reconstituted complexes.

DISCUSSION

The topography of the human factor VIII-vWf complex was studied by fluorescence energy transfer following reconstitution with fluorophore-modified components. Results indicating (i) binding of factor VIII light chain to NH$_2$-terminal-derived fragments of vWf; (ii) no direct interaction between factor VIII heavy chain and the vWf fragments; and (iii) a requirement for factor VIII light chain to juxtapose the heavy chain and vWf, were consistent with prior studies using solid phase-binding assays (13-15, 27, 28, 39) and ultracentrifugation analyses (16,40). Furthermore, the observation that high levels of vWf failed to inhibit the rate or extent of factor VIII subunit reassociation (12) suggested that vWf binding was localized to a single factor VIII subunit and not both.

Previous results from our laboratory suggested that factor VIII subunits were modified at unique sites, Cys$^{808}$ (A2 domain of heavy chain) and Cys$^{488}$ (A3 domain of light chain), following reaction with sulphydryl-specific fluorophores (29). However, reaction of vWf fragment SPIII with CPM resulted in modification at multiple sites. Sequence analysis of fluores-
binding interaction of heavy chain with vWF based upon retention of a 42-kDa factor VIII fragment (A2 domain) on immobilized vWF following dissociation of factor VIII.

Reaction of NPM-light chain with a molar excess of either unmodified SPIII or SPIII-T4 prior to addition of CPM-SPIII resulted in no donor quenching, indicating that energy transfer was the direct result of interaction between modified components and confirming that sequences common to SPIII and SPIII-T4 contain the factor VIII (light chain)-binding region. Fragment SPIII (residues 1366–2050) did not inhibit the light chain–SPIII interaction, consistent with earlier studies (27, 28). Furthermore, high concentration of the vWF propolypeptide relative to light chain did not inhibit energy transfer. Early results from our laboratory indicated copurification of this protein with factor VIII activity (30). The above result suggested that any potential interaction between these two proteins would not be mediated by those factor VIII sequences that interact with mature vWF.

Sedimentation velocity studies of Lollar et al. (40) using porcine proteins indicated the potential availability of approximately one factor VIII-binding site/vWF monomer. Based upon the plasma concentrations of factor VIII and vWF, these authors suggested that porcine vWF may be greater than the factor VIII-vWF complex (1). However, based upon the levels of fragment SPIII(T4) which resulted in maximal donor quenching, an availability of factor VIII-binding sites similar to that observed for the porcine system in multimerized vWF may be somewhat diminished compared with the fragments used in this study, possibly as a result of the presence of CPM-SPIII prior to addition of CPM-SPIII.

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REFERENCES

morrh. 27, 502–515
Topography of the human factor VIII-von Willebrand factor complex.
P J Fay and T M Smudzin


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