A Consensus Tetrapeptide Selected by Phage Display Adopts the Conformation of a Dominant Discontinuous Epitope of a Monoclonal Anti-VWF Antibody That Inhibits the von Willebrand Factor-Collagen Interaction*

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Monoclonal antibody (mAb) 82D6A3 is an anti-von Willebrand factor (VWF) mAb directed against the A3-domain of VWF that inhibits the VWF binding to fibrillar collagens type I and III in vitro and in vivo. To identify the discontinuous epitope of this mAb, we used phage display, mutant analysis, and peptide modeling. All 82D6A3-binding phages displayed peptides containing the consensus sequence SPWR that could be aligned with P981W982 in the VWF A3-domain. Next, the binding of mAb 82D6A3 to 27 Ala mutants with mutations in the A3-domain of VWF revealed that amino acids Arg961, Pro981, Asp1009, Arg1016, Ser1020, and Trp982 in the three-dimensional structure of the A3-domain demonstrated that these residues are close together in space, pointing out that the structure of the SPWR consensus sequence might mimic this discontinuous epitope. Modeling of a cyclic 6-mer peptide containing the consensus sequence and superposition of its three-dimensional structure onto the VWF A3-domain demonstrated that the Ser and Arg in the peptide matched the Ser1020 and Arg1016 in the A3-domain. The Pro residue of the peptide served as a spacer, and the side chain of the Trp pointed in the direction of Trp982. In conclusion, to our knowledge, this is the first report where a modeled peptide containing a consensus sequence could be fitted onto the three-dimensional structure of the antigen, indicating that it might adopt the conformation of the discontinuous epitope.

Platelet adhesion to subendothelial structures, more specifically to the thrombogenic compound collagen, is one of the first steps in a sequence of reactions that can lead to arterial thrombosis. Platelets interact with collagen both in a direct manner and via their collagen receptors (e.g., αIIbβ3, (1, 2) and glycoproteins IV (3) and VI (4, 5)) and indirectly with VWF, forming the bridge between collagen and its platelet receptor glycoprotein Ib/IX/V (6). Binding via both αIIbβ3 and VWF is necessary to sustain platelet adhesion under high shear forces (7–9); VWF-mediated interaction results in rolling of the platelets over the collagen surface (10), upon which the collagen receptors can interact with the damaged vessel wall, leading to firm adhesion. This is the result of platelet activation by the signal-transducing glycoprotein VI (11), leading to a gain-in-affinity of αIIbβ3 (12) and activation of αIIbβ3 with platelet aggregation as a consequence.

Both αIIbβ3 and VWF bind to collagen through their I-domains, in VWF known as A-domains (13–19). A-domains form independent globular modules of some 200 amino acid residues. In VWF three such domains have been identified. The A1-domain contains the binding site for glycoprotein Ib (20, 21), sulfatides (22), heparin (23), and collagen VI (24, 25), which constitutes the main reactive collagen in the extracellular matrix of endothelial cells. The A2-domain has no clear binding function but is sensitive to protease ADAMTS13-mediated enzymatic degradation (26, 27), whereas the A3-domain (residues 920–1111) contains the main binding site for fibrillar collagens such as type I and III (19, 28). Recombinant A3-domain also binds to collagen (28), whereas deletion of A3 domain in VWF results in a VWF that binds 40 times less to collagen (19). By using synthetic triple helical collagen-related peptides, the VWF-binding site has been localized to residues 541–558 of the α1CB4(III) fragment of collagen type III (29). Recently, we identified the collagen binding site by cocrystallization of the A3-domain with an inhibitory anti-A3 antibody, RU5, which was confirmed by showing that especially an H1023A mutant abolished binding of VWF to collagen (30). This study was further extended by the analysis of a series of 27 VWF-A3 mutants, which defined the collagen binding site of the VWF-A3-domain to the “front” face of the domain (31), an observation confirmed by Nishida et al. (32).

We raised a monoclonal antibody (mAb), 82D6A3, against human VWF that prevents the binding of VWF to collagen (24) and that is antithrombotic in a baboon arterial thrombosis model (33). Since a previous effort to determine the epitope of 82D6A3 using phage display, was not successful (34, 35), we repeated this study using less stringent selection criteria. Us-

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ing phage display together with mutagenesis studies, we now identified the epitope of mAb 82D6A3. Moreover, we demonstrate that the structure of the consensus sequence in a selected cyclic 6-mer peptide mimics the discontinuous epitope in the VWF A3-domain. To the best of our knowledge, this is the first report where modeling of a consensus sequence in a cyclic 6-mer peptide indicates that it indeed adopts the conformation of the antigen and thereby can represent the discontinuous epitope.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human placental collagen type I and III were purchased from Sigma. The collagen was solubilized in 50 mmol/liter acetic acid and subsequently dialyzed against phosphate-buffered saline PBS (48 h, 4 °C) to obtain fibrillar collagen. A phage display library with random hexapeptides flanked by cysteine residues was obtained from Covaris (Gent, Belgium), and a pentadecamer phage display peptide library was a kind gift of Dr. G. Smith (University of Missouri, Columbia, MO). VWF was purchased from the Red Cross Belgium. mAb and phages were biotinylated using N-hydroxysuccinimide-LC-Biotin (Pierce) according to the manufacturer's instructions.

**Preparation of 82D6A3 and Its Fab Fragments**—82D6A3, raised against human VWF, was purified from murine ascites by Protein A chromatography. 82D6A3-Fab was prepared by digestion with papain. Briefly, 5 mg of mAb was digested with 50 μg of papain (Sigma) in the presence of 10 mmol/liter cysteine and 50 mmol/liter EDTA (37 °C, overnight). The Fab fragments were purified by protein A affinity chromatography (Amersham Biosciences), and purity was checked by SDS-PAGE.

**Binding of 82D6A3 to Different Forms of VWF**—A 96-well plate (Greiner, Frickenhausen, Germany) was coated overnight with VWF (Red Cross), A3-domain of VWF, or A3-VWF (10 μg/ml in PBS) and blocked with 3% milk powder solution. A dilution series of 82D6A3 (in 0.3% milk powder solution) was added for 1.5 h, bound mAb was detected for 1 h with goat anti-mouse IgG labeled with horseradish peroxidase (GAM-HRP) (Sigma) (1:10,000 in PBS, 0.3% milk powder), and visualization was performed using H2O2 and ortho-phenylenediamine (Sigma). The reaction was stopped with 4 mol/liter H2SO4, and absorbance was determined at 490 nm. In between each incubation step, the plates were washed 3–9 times with PBS, 0.1% Tween 20 (PBST).

**Inhibition of VWF Binding to Collagen by 82D6A3 and Its Fab Fragments**—A 96-well plate (Greiner, Frickenhausen, Germany) was coated overnight with human collagen type I (25 μg/ml in PBS) and blocked with 3% milk powder solution. Purified human VWF (0.5 μg/ml final concentration) was preincubated with a dilution series of 82D6A3 or its Fab fragments during 30 min before the addition to the collagen-coated plate. After a 90-min incubation, bound VWF was detected with a polyclonal anti-VWF-Ig solution conjugated with horseradish peroxidase (Dako, Glostrup, Denmark) (1:3000 in PBS, 0.3% milk powder), and visualization was performed as described above. In between each incubation step, the plates were washed 3–9 times with PBST.

**Flow Experiments**—Flow experiments were performed in a parallel plate flow chamber with a slit height of 0.4 mm as described (36). Briefly, blood was taken from healthy volunteers, who had not taken aspirin or analogues for the last 10 days, on 0.33% citrate. Theramox coverslips (Nunc, Rochester, NY) were coated with human fibrillar collagen type III (1 mg/ml, 100 μl coverslip). The perfusion chamber and tubings were blocked with 1% bovine serum albumin, 0.1% glucose in PBS, and 3% bovine serum albumin solution) was added for 1.5 h, bound mAb was detected for 1 h with goat anti-mouse IgG labeled with horseradish peroxidase (GAM-HRP) (Sigma) (1:10,000 in PBS, 0.3% milk powder), and visualization was performed using H2O2 and ortho-phenylenediamine (Sigma). The reaction was stopped with 4 mol/liter H2SO4, and absorbance was determined at 490 nm. In between each incubation step, the plates were washed 10 times with PBST. When using the linear pentadecamer library, in the first panning round, bound phages were eluted with 0.1 mol/liter glycine, pH 2.2, and the eluate was immediately neutralized with 1 mol/liter Tris-HCl, pH 8. After amplification of the phages, two additional rounds of panning were performed where bound phages were eluted using the recombinant A3 domain of VWF (20 μg/ml). Using the cyclic hexamer library, four panning rounds were performed, and each time a non specific elution with glycine was performed. After all panning rounds, phages were amplified by infection of Escherichia coli TG1 cells and partially purified from the supernatant by polyethylene glycol precipitation. Enrichment of 82D6A3 binding phages over the panning rounds was tested in an enzyme-linked immunosorbent assay (see below). Phage DNA was prepared by phenol/chloroform extraction, and sequencing reactions were performed using the Sequenase version 2.0 DNA Sequencing Kit (Amersham Biosciences) using [35S]dATP according to the manufacturer's instructions. For the linear pentadecamer phages, primer 5’-CTCATAGCCTAACG-3’ was used, and for the cyclic hexamer phages, 5’-CCGTGATGTTAGCGTAACG-3’ was used.

**Measurement of Phage Binding to 82D6A3**—A 96-well plate was coated overnight with purified 82D6A3 (10 μg/ml in PBS). After 2 h of blocking with a 2% milk powder solution, a dilution series of either a phage preparation obtained after the different rounds of panning or the individual phage clones selected after the final panning round were added to the wells (all in PBS with 0.2% milk powder), and phages were incubated at room temperature for 90 min. Bound phages were detected after a 1-h incubation with a polyclonal anti-M13-HRP-conjugated antibody (Amersham Biosciences), and visualization was performed as described above. After all incubation steps, plates were washed with PBST, 0.1% Tween 20.

**Specificity of Phage Binding to 82D6A3**—A 96-well plate was coated overnight with purified 82D6A3 (10 μg/ml in PBS). After blocking with 2% milk powder for 2 h, a dilution series of VWF or recombinant A3-domain in PBS, 0.2% milk powder was added. After a 30-min preincubation, a constant amount of phages (PBS, 0.2% milk powder) was added to the VWF/A3-containing wells. After 90 min, bound phages were detected as described above.

Competition between different phage clones for binding to 82D6A3 was analyzed as above, except that 2·1010/ml biotinylated phages of clone 1 were mixed with various concentrations of phages from clone 2, after which bound biotinylated phages were detected with streptavidin-HRP and ortho-phenylenediamine.

**Immunoblotting of Phages**—Purified phages (2·1011/ml) were analyzed on a 10% SDS-PAGE gel under nonreducing and reducing conditions and blotted to a nitrocellulose membrane (Schleicher and Schuell). After blocking the membrane with a 4% milk powder solution, the membrane was incubated with 82D6A3 (2 μg/ml in PBS, 0.4% milk powder) during 90 min, followed by a 1-h incubation with GAM-HRP (Sigma). The membrane was developed using the ECL detection system (Amersham Biosciences). After each incubation step, the membrane was washed with PBS containing 0.05% Tween 80.

**Expression, Purification, and Characterization of VWF**—The construction and purification of VWF mutants with point mutations in the A3-domain has been described (30, 31). Briefly, VWF was stably expressed in murine fibroblast BHK cells and purified by immunoaffinity using monoclonal antibody R8. VWF concentration was determined by a sandwich enzyme-linked immunosorbent assay, and its multimeric structure was analyzed by agarose gel electrophoresis followed by Western blotting.

**Binding of 82D6A3 to VWF Mutants**—Microtiter plate wells (Costar, Cambridge, MA) were coated with a 2.5 μg/ml concentration of a polyclonal antibody directed against the D- and D3-domains of VWF (37) in 50 mm carbonate buffer, pH 9.6, and blocked with 3% bovine serum albumin in PBS, 0.1% Tween 20. Wells were incubated with expression medium diluted with PBS, 0.1% Tween 20, 3% bovine serum albumin to a final VWF concentration of 100 μg/ml for 60 min at 37 °C. Next, 5 μg of 82D6A3 was added, and bound antibodies were detected with HRP-conjugated rabbit anti-mouse antibodies (DAKO, 1:2500 in PBS, 0.1% Tween 20, 3% bovine serum albumin). *orthophenylendiamine* was used as substrate for detection as described above. Between each incubation step, wells were washed with PBST.
SER and ARG residues were used as anchors to superimpose the fitted onto the VWF A3-domain. The main and side chain atoms of the cyclic peptide. The lowest energy structure was selected and manually simulation were performed to explore the conformational space of the free bondng. Next, 20,000 steps of unconstrained molecular dynamics simulating package (38). First, the linear sequence was generated and during of the cyclic peptide CMTSPWRC was modeled using the Brugel mod-
amino acid sequence SPWR.

of two sequences: GDCFFGFLN sequence was determined, which resulted in the identification binding site of the antibody and could thus represent an demonstrating that the phages were binding to the antigen A3-domain binding (not shown) to 82D6A3 to the same extent, types of phage clones inhibited VWF binding (Fig. 3 A). G8, G2, and C2 had a similar affinity for 82D6A3, clone C5 had to 82D6A3. Seven phage clones were positive, of which clones individual clones were grown and tested for their ability to bind to 82D6A3, of which 13 were positive. All of these phage clones inhibited the VWF binding to 82D6A3. Eight of the 13 clones displayed the sequence CMTPWRC (C6H5), four displayed the sequence CRTSPWRC (C6G12), and one had a CYESPWRC (C6A12) sequence. These sequences thus also contained the SPWR sequence, and indeed the L15G8 and C6H5 phage did compete with each other for binding to 82D6A3 (Fig. 4). Furthermore, 82D6A3 was only able to recognize the L15G8 and C6H5 phage when blotted under nonreducing conditions and not when the disulfide bond was reduced, pointing out that the two cysteines present in both clones are forming a disulfide bridge and that the structure of the conformational constrained peptide is thus necessary for recognition by 82D6A3 (Fig. 4, inset).

All of the selected peptide sequences were aligned to the VWF-A3 sequence using ClustalW using all of the program default values (39) (available on the World Wide Web at www. ebi.ac.uk/clustalw/). With this program, progressive multiple sequence alignments are performed, and the alignment is built up in stages where a new sequence is added to an existing alignment through sequence weighting, position-specific gap penalties, and weight matrix choice (39). Multiple sequence alignment of all of the peptide sequences with the VWF A3-domain sequence or sequence alignment of each peptide sequence separately with the VWF A3-domain sequence always resulted in positioning of the peptide sequences at the PW sequence (positions 981 and 982) in the VWF A3-domain (Table I). This particular PW sequence is only present once in the VWF A3-domain. However, more similarity was not identified, as expected, since 826A3 does not have a linear epitope. Thus, the SPWR consensus sequence might mimic the epitope, presumably bearing similarity to its three-dimensional structure.

**Epitope Mapping of 82D6A3 by Mutant VWF Analysis**—To
further unravel the epitope of 82D6A3, binding of 82D6A3 to different VWF mutants was analyzed. The VWF mutants containing mutations in the A3-domain were previously constructed to identify the collagen-binding region within the A3-domain (30, 31). Both 82D6A3 (Fig. 5) and RU5 bind to recombinant wild type VWF and not to ΔA3-VWF and compete with each other for binding (30).

Next, binding of 82D6A3 to these 27 VWF mutants was studied, and a number of residues important for antibody recognition were identified (Fig. 5). The mutant P981H no longer bound to 82D6A3; however, mutant P981A bound normally. 82D6A3 bound less (residual binding of less than 75% binding) to VWF with Ala mutations of residues Arg963, Asp 1009, Arg1016, Ser1020, Met1022, and His1023 (Fig. 5).

Interestingly, the effects of the various mutants on 82D6A3 binding correlates well with the effect on collagen binding. We previously demonstrated that of these 27 VWF mutants used, R963A, I975A, T977A, D979A, P981H, V997A, E1001A, R1016A, S1020A, and H1023A all reduced collagen binding (30, 31). Comparison of the effects of the various mutants on 82D6A3 binding and on collagen binding revealed that of these mutants, R963A, P981H, R1016A, S1020A, and H1023A also had a reduced binding to 82D6A3 (Fig. 6).

Modeling of a Peptide Containing the SPWR Consensus Sequence—Identification of the residues important in 82D6A3 recognition by mutant analysis suggested that the SPWR consensus sequence identified by phage display might represent amino acids Ser1020, Pro981, Trp982, and Arg1016 in the VWF A3-domain. Inspection of these residues in the three-dimensional structure of the A3-domain demonstrated that these residues are indeed close together in space (Fig. 7).

The SPWR consensus sequence in the selected peptides is in boldface type, the PW sequence is identified by alignment between the peptide sequences and the VWF A3 domain sequence, and is boldface and underlined.

| Peptide | Sequence |
|---------|----------|
| L15G8   | GDCFGFLNSPWRVC |
| L15C5   | RSSYVYSPWRFSR |
| C6H5    | CMTPSPWR   |
| C6G12   | CRTBSPWR   |
| C6A12   | CYR8SPWR   |
| VWF A3 domain | ^67VSVLQYGSIITIDVP^SPWRPEKAH<300 |

![Fig. 3](image3.png) Selection of phages interacting with the antigen binding pocket of 82D6A3. A, dose-dependent binding of phage clone L15G8 (○) and L15C5 (○) to 82D6A3. Bound phages were detected with polyclonal anti-M13-HRP antibodies. B, inhibition of the binding of phage clones L15G8 (○) and L15C5 (○) to 82D6A3 by VWF. Phages (final concentration: 8*10^10 phages/ml) and a dilution series of VWF were preincubated for 30 min before the addition to the 82D6A3-coated plate. Bound phages were detected with polyclonal anti-M13-HRP antibodies.

![Fig. 4](image4.png) Characterization of the L15 and C6 phages. Binding of biotinylated C6H5-phages to 82D6A3 was inhibited by L15G8 phages. C6H5 phages were used at a final concentration of 2*10^10/ml. Bound biotinylated C6H5 phages were detected with streptavidin-HRP. Inset, phages (2*10^10) were analyzed in SDS-PAGE using a 10% gel under nonreducing (lane 1) and reducing (lane 2) conditions. Proteins were transferred to a nitrocellulose membrane, and detection was performed using 82D6A3 followed by the addition of GAM-HRP.

![Fig. 5](image5.png) Binding of 82D6A3 to wild type VWF, ΔA3-VWF, and VWF with the indicated mutations in the A3-domain. VWF and its mutants were captured with polyclonal anti-VWF antibodies, 5 μg/ml 82D6A3 was added, and bound mAb was detected with rabbit anti-mouse antibodies conjugated with HRP. Values are expressed as percentage of binding to wild type VWF (mean ± S.D., n = 3).

![Table 1](image1.png) Alignment of selected peptide sequences with part of the VWF A3 domain sequence

| Peptide | Sequence |
|---------|----------|
| L15G8   | GDCFGFLNSPWRVC |
| L15C5   | RSSYVYSPWRFSR |
| C6H5    | CMTPSPWR   |
| C6G12   | CRTBSPWR   |
| C6A12   | CYR8SPWR   |
| VWF A3 domain | ^67VSVLQYGSIITIDVP^SPWRPEKAH<300 |

![Table 2](image2.png) Alignment of selected peptide sequences with part of the VWF A3 domain sequence

| Peptide | Sequence |
|---------|----------|
| L15G8   | GDCFGFLNSPWRVC |
| L15C5   | RSSYVYSPWRFSR |
| C6H5    | CMTPSPWR   |
| C6G12   | CRTBSPWR   |
| C6A12   | CYR8SPWR   |
| VWF A3 domain | ^67VSVLQYGSIITIDVP^SPWRPEKAH<300 |
peptide superimpose with A3 residues Ser$^{1020}$ and Arg$^{1016}$, with the Pro residue serving as a spacer that correctly orients the Ser and Arg residues. The side chains of the Trp in the cyclic peptide and Trp$^{982}$ in VWF could be mapped, provided the latter is rotated in such a way that it becomes exposed to the solvent (Fig. 8). This indicates that the linear SPWR sequence actually mimics the conformation of the epitope.

**DISCUSSION**

The anti-VWF mAb 82D6A3 and its Fab fragments are potent inhibitors of the VWF-collagen interaction under both static and flow conditions. We recently demonstrated that inhibiting the VWF-collagen interaction by 82D6A3 results in an effective antithrombotic therapy when tested in baboons using a modified Folts model in the femoral artery (33), thereby confirming that the VWF-collagen interaction has a relevant physiological role.

82D6A3 binds to the A3-domain of VWF but not to denatured and reduced VWF, pointing out that the mAb does not recognize a linear epitope. To determine the epitope of 82D6A3 more accurately, we used phage display technology, mutagenesis, and computer modeling. Selection of antibody-binding phages from a pentadecamer and cyclic hexamer phage display library resulted in phages that bind to 82D6A3 in a dose-dependent manner. Moreover, VWF and the recombinant A3-domain were able to inhibit phage binding to the mAb, indicating that the phages bind at or near the antigen-binding site of 82D6A3. Sequence comparison of the phage-displayed peptides revealed a consensus SPWR sequence in all phages selected. Using the ClustalW program, the SPWR-sequence was aligned to the PW

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**Fig. 6.** Correlation between the effects of VWF mutations on 82D6A3 binding and collagen binding. Percentage of binding to 82D6A3 and to collagen type III of ΔA3-VWF (○) and VWF A3-domain mutants (●) is represented. Binding to wild type VWF (△) was set as 100%.

**Fig. 7.** Location of dominant 82D6A3 epitope amino acid residues within the VWF-A3-domain. Coordinates of the crystal structure of the VWF A3-domain were taken from Protein Data Bank entry 1ATZ.

**Fig. 8.** Superposition of the modeled cyclic CMTSPWRC onto the structure of VWF-A3. Right panel, the cyclic CMTSPWRC peptide (blue, with Arg, Ser, and Trp in dark blue) was modeled using the Brugel modeling package and was subsequently fitted onto the VWF-A3-structure (red). Amino acid residues Arg$^{1016}$ and Ser$^{1020}$ within the VWF A3-domain are in bold red. The left panel shows the “exposed form” of Trp$^{982}$ in close vicinity and orientation of the cyclic Trp in the cyclic hexapeptide.
Glu1001). This demonstrates that 82D6A3 interacts with amino acids with long peptides (two half-cystine residues at fixed positions is present, or library epitopes, constrained libraries, where a disulfide bond between so obvious, since it is difficult to mimic such epitopes with a phage display to identify the original epitope. The use of phage display to be aligned with the primary sequence of the antigen, thus phages are selected that display consensus sequences that can dimensional structure of the epitope.

A Peptide Representing a Discontinuous Epitope in VWF peptide CMTSPWRC and superimposed its SPWR consensus peptide compares well with those of the Ser 1020 and Arg 1016. However, Nishida introduction of a much larger histidine side chain at this position overlaps then with the Trp in the peptide (Fig. 8, Å). Only a few reports are available where the consensus sequence (amino acids 981 and 982) within the A3-domain. sequence (amino acids 981 and 982) within the A3-domain. 1001). This demonstrates that 82D6A3 interacts with amino acids in the VWF A3-domain critical for collagen binding and that 82D6A3 does not inhibit the VWF collagen interaction through steric hindrance.

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