Identification of the Collagen-binding Site of the von Willebrand Factor A3-domain*

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Von Willebrand factor (vWF) is a multimeric glycoprotein that mediates platelet adhesion and thrombus formation at sites of vascular injury. vWF functions as a molecular bridge between collagen and platelet receptor glycoprotein Ib. The major collagen-binding site of vWF is contained within the A3 domain, but its precise location is unknown. To localize the collagen-binding site, we determined the crystal structure of A3 in complex with an Fab fragment of antibody RU5 that inhibits collagen binding. The structure shows that RU5 recognizes a nonlinear epitope consisting of residues 962–966, 981–997, and 1022–1026. Alanine mutants were constructed of residues Arg963, Glu967, His990, Arg1016, and His1023, located in or close to the epitope. Mutants were expressed as fully processed multimeric vWF. Mutation of His1023 abolished collagen binding, whereas mutation of Arg963 and Arg1016 reduced collagen binding by 25–35%. These residues are part of loops α3β4 and α1β2 and α-helix 3, respectively, and lie near the bottom face of the domain. His1023 and flanking residues display multiple conformations in available A3-crystal structures, suggesting that binding of A3 to collagen involves an induced-fit mechanism. The collagen-binding site of A3 is located distant from the top face of the domain where collagen-binding sites are found in homologous integrin I domains.

Platelet adhesion to damaged vessel walls is the first step in the formation of an occluding platelet plug, which leads to the arrest of bleeding during normal hemostasis. Platelet adhesion can also cause thrombotic complications such as the occlusion of atherosclerotic arteries (1). The multimeric glycoprotein von Willebrand factor (vWF) plays an essential role in platelet adhesion under conditions of high shear stress (2, 3). In this process vWF serves as a molecular bridge that links collagen exposed by the damaged vessel wall to glycoprotein Ib located on the platelet surface. Collagens that act as binding sites for vWF include types I and III in perivascular connective tissue and type VI in the subendothelial matrix (3, 4).

Mature vWF consists of a 2050-residue monomer that contains multiple copies of so-called A, B, C, and D type domains and one CK (cystine knot) domain arranged in the order D9–D3–A1–A2–A3–D4–B1–B2–B3–C1–C2–CK (1, 3). Disulfide bond formation between N-terminal D3 domains and between C-terminal CK domains generates vWF multimers that consist of up to 80 monomers. The A1 domain contains the binding site for glycoprotein Ib (5). The A3 domain (residues 920–1111) contains the major binding site for collagen types I and III (6). The multimeric structure of vWF is essential for high affinity collagen binding (7). Multimeric vWF binds collagen with an apparent Kd of 1–7 μM (8), while a recombinant A3 domain has a much higher Kd of 2 μM (9). Deletion of the A2 and D4 domains, which flank the A3 domain, or deletion of the A1 domain do not decrease collagen binding of multimeric vWF (6, 8). These data show that a monomeric A3 domain contains a fully active collagen-binding site, the only requirement for tight binding to collagen being the presence of multiple A3 domains within one vWF multimer.

Integrin I-type domains are homologous to vWF A-type domains (10, 11). I domains of integrin α-chains α1, α2, α5, and α11 all possess collagen-binding sites. A crystal structure of the α2–I domain reveals binding of a collagen-like peptide to a groove in the surface of the “top” face of the domain (12). This groove contains a so-called metal ion-dependent adhesion site (MIDAS) (13, 14), which engages a glutamate residue of collagen.

The location of the collagen-binding site in the vWF-A3 domain is not known. Crystal structures of A3 do not display a collagen-binding groove in the top face, instead, the surface of A3 is rather smooth (15, 16). Although the MIDAS motif is partly conserved, binding of A3 to collagen does not require a metal ion (17, 18), and no metal ion is observed in crystal structures of A3. Moreover, point mutations in the MIDAS motif of A3 do not disrupt collagen binding (8, 16) showing that...

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The abbreviations used are: vWF, von Willebrand factor; CK, cystine knot; PBS, phosphate-buffered saline; BSA, bovine serum albumin; a.u., asymmetric unit; CDR, complementary determining region; ELISA, enzyme linked immunosorbent assay; for-BHK, baby hamster kidney cells overexpressing furin; MIDAS, metal ion dependent adhesion site; n.c.s., noncrystallographic symmetry; Sc-met, selenomethionine; wt, wild-type.

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the motif is not involved in collagen binding, at all. Site-directed mutagenesis studies of other residues in the top face of A3 have yielded conflicting results. Cruz et al. (19) reported in abstract form that alanine substitution D1069R, R1074D, R1090D, and E1092R resulted in a 50% reduction in binding of monoclonal A3 to collagen. Van der Plas et al. (8), however, observed normal collagen binding of fully processed multimeric vWF containing mutations D1069R, D1069A, or R1074A. In the same study, mutations V1040A/V1042A, D1046A, and D1066A also displayed normal collagen binding, suggesting that the collagen-binding site of vWF-A3 is not located in its top face.

The crystallographic study presented here was conducted to provide new clues on the location of the collagen-binding site of the vWF-A3 domain. We determined the structure of the A3 domain in complex with a Fab fragment of monoclonal antibody RU5, which inhibits binding of vWF to collagen. Site-directed mutagenesis of residues located in the epitope region show that the collagen-binding site is located distant from the top face of A3.

**EXPERIMENTAL PROCEDURES**

**Purification of vWF-A3 and RU5—**Recombinant selenomethionine (Se-Met) A3, comprising residues 920–1111 of human vWF, was expressed and purified as described before (15). For production of monoclonal antibody RU5 (IgG2a, clone RU5, GenBank accession number AF286587) (Fig. 1A). For determination of the sequence of the variable domain (VL) of the RU5 light chain, the same procedure was used as for the VH domain. For cloning of VL cDNA, one backward variable region consensus primer (PD1: GAT ATT GTG ATCC AAG CAG TCT (C/G)7T) and two forward variable-region consensus primers (PD2: CAG GAC GTA GAG CAG GAC TTC GTG ACC ACC CAG TCT CC, PD4: TGT AAA ACG ACC GCC AAT TCT AGG TGG GGA GAT GGA) were used. However, determination of the sequence was hampered by the abundance of mRNA encoding the light chain of the nonproducing myeloma fusion partner of the RU5 hybridoma cell, a known drawback of myeloma cell line P363Ag8.653 (22).

**Crystalization and Data Collection—**Crystals of the (Se-Met) A3-RU5 complex were grown by hanging-drop vapor diffusion at 4 °C using a protein concentration of 15 mg/ml and a precipitant solution consisting of 13% (v/v) isopropanol, 2.4 M ammonium sulfate, and 100 mM cacodylic acid, pH 5.3. The crystal used for structure determination had a size of 0.1 × 0.1 × 0.2 mm3 and was equilibrated in a cryoprotectant solution consisting of 8% isopropanol, 30% 2-methyl-2,4-pentanediol, 100 mM cacodylic acid, pH 5.3. X-ray diffraction data were collected on beam line BW7B of the synchrotron radiation facility at HASYLAB (Hamburg, Germany) with a MAR345 imaging plate (Mar, Evanston, IL). Data reduction, merging, and scaling were performed with DENZO and SCALEPACK (23). Diffraction data statistics can be found in Table I. Diffraction of the A3-RU5 crystal was anisotropic. For structure determination, anisotropic B-factors were applied with SCALFITE and SFTOOLS (24) to correct for the observed anisotropy.

**Structure Determination and Refinement—**A self-rotation function calculated with POLARRFN (24) and the unit-cell volume suggested the presence of three A3-RU5 complexes in the asymmetric unit (a.u.) with a Vₜₐₚ of 3.3 Å³/Da and a solvent content of 63%. Initial attempts to solve the structure by molecular replacement failed. Therefore, a rather weak anomalous signal (ΔFo-Fl/σ(Fl)) = 1.18) arising from the presence of two Se sites in A3 was used to locate Se atoms from an anomalous Patterson map. Six Se sites could be assigned to Met947, Met998, and Met1097. The positions of two A3 molecules in the a.u. allowed further molecular replacement to proceed in a straightforward manner. A Fab fragment of protein data bank entry 2MFA (25, 26) was oriented using the program AmoRe (27) followed by Patterson correlation refinement using the program CNS (28, 29). Cross-translation functions calculated with CNS identified the position of three Fab fragments. A third A3 molecule was placed on the basis of n.c.s. The a.u. finally contained three A3-RU5 complexes with an R-factor of 42.6% after rigid body refinement.

For model building, sequences of the constant domain of the light chain (Cl) and of the constant domain of the heavy chain (C₁H), of RU5 were taken from IgG2a—monoclonal antibody 4-4-20 (30, 31). The sequence of residues 1–110 of the Vₗ domain of RU5 (Fig. 1B) was derived from the electron density aided by a consensus sequence based on an alignment of 110 Fab sequences. For model refinement, cycles of rebuilding using O (32) and positional and B-factor refinement using CNS were performed until convergence. Cross-validation was used throughout using 5% of the set of reflections. Refinement was performed with the maximum-likelihood algorithm (33). Bulk solvent correction and anisotropic scaling of diffraction data were applied. During the first cycles of refinement n.c.s. restraints were used. Based on the behavior of the free R-factor, n.c.s. restraints were omitted in later stages of refinement. Water molecules were placed in difference electron density peaks with a peak height of at least 2.8 σ, a distance of 2.5–3.4 Å to a hydrogen bond donor/acceptor, and a B-factor smaller than 60 Å².

**Construction of vWF Point Mutants—**Point mutations were introduced in the vWF-A3 domain using the QuikChange method (Strategene, La Jolla, CA) and specific primers (Amersham Pharmaccia BioTech, Roosendaal, The Netherlands). First-strand cDNA was synthesized using Superscript II reverse transcriptase (Life Technologies, Inc., Rockville, MD) in the presence of 3.2 units/μl RNAse inhibitor RNaseOUT (Life Technologies, Inc.). cDNA, encoding the Vₗ domain, was amplified by polymerase chain reaction with Pwo DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany). A backward variable region consensus primer (B4: CCA GGG GAC GTG TAG ACG ACC TTG GTC GTC TGT TT) was used together with a forward primer (B3c: CGC ATG GAC CCC TCC (G/A)GCG TGG ACC (A/C)GAC GAC ACC TGA (C/T)G) that hybridizes with a consensus sequence in the Jₗ region (21). The polymerase chain reaction product was extended with a 3′-overhang using T4 Pol DNA polymerase (Promega, Madison, WI) and cloned into the pcR2.1-TOPO vector according to the manufacturer’s protocol (Invitrogen, Leek, The Netherlands). Nucleotide sequences of three clones were determined (GenBank accession number AF286587) (Fig. 1A). For determination of the sequence of the variable domain (Vₗ) of the RU5 45I light chain, the same procedure was used as for the Vₗ domain. For cloning of Vₗ cDNA, one backward variable region consensus primer (PD1: GAT ATT GTG ATC CAG CAC TCT (C/G)7T) and two forward variable-region consensus primers (PD2: CAG GAA GAC ATG ATC GAG CAC GTG ATC ACC ACC CAG TCT CC, PD4: TGT AAA ACG ACC GCC AAT TCT AGG TGG GGA GAT GGA) were used. However, determination of the sequence was hampered by the abundance of mRNA encoding the light chain of the nonproducing myeloma
Collagen-binding Site of vWF-A3 Domain

Amino acid sequences of RU5 VL and VH domains. CDRs (underlined) were defined according to Kabat et al. (23). Strict sequential numbering (9) is used throughout the text. Numbering according to the convention of Kabat et al. (23) (#K) is also shown. A, the amino acid sequence of VH was deduced from cDNA. Residues 1–7 could not be deduced precisely due to the use of a primer complementary to the 5'-coding region. Asn56 in CDRH2 is N-glycosylated. The nucleotide sequence of Vκ has been deposited in the GenBank* data base under accession number AF286587. B, the amino acid sequence of Vλ was deduced from the electron density of the A3 RU5 complex and an alignment of 110 Fab Vλ chains. Side chains of 13 amino acid residues that could not be determined unambiguously from the electron density and the consensus sequence are shown as lowercase characters.

The concentration of vWF was determined by a sandwich enzyme-linked immunosorbent assay according to Van der Plas et al. (9). Collagen was coated at 50 μg/ml instead of 100 μg/ml as used previously (8). A vWF concentration of 2.5 μg/ml was used in the binding experiments.

RESULTS

A3-RU5 Structure Determination and Refinement—The structure of the vWF (Se-Met) A3 domain in complex with an Fab fragment of RU5 was solved to 2.0-Å resolution. The structure was determined using the anomalous signal from selenium atoms in (Se-Met) A3 to position two A3 molecules and was completed by molecular replacement. The a.u. contains three A3-Fab complexes. The structure has been refined to an R-factor of 22.7% and a free R-factor of 26.4% (Table I).

The sequence of the RU5 Vλ domain could not be determined from cDNA and was therefore deduced from electron density aided by an alignment of 110 Fab Vλ sequences. The identity of 13 amino acid residues could not be determined unambiguously from the electron density and the consensus sequence are shown as lowercase characters.

The multiemer structure of vWF was analyzed by agarose gel electrophoresis followed by Western blotting as described by Larivi et al. (35).

Binding of vWF to monoclonal antibody RU5 was analyzed as follows. Microtiter-plate wells (Costar, Cambridge, MA) were coated with 2.5 μg/ml monomeric α-vWF (Dako, Glostrup, Denmark) for immobilization and detection, respectively (6). Normal pooled plasma from 40 healthy donors was used as a reference.

The overall structure of the (Se-Met) A3 molecule is the same as the structure of native A3 (15, 16). It consists of a central six-stranded β-sheet on both sides flanked by α-helices. The final model comprises amino acid residues 921–1110 of A3 molecules in complexes A and B and residues 920–1110 of the A3 molecule in complex C. Positions of the N and C termini of A3, including disulfide bond Cys923-Cys1109 that connects these termini, are poorly defined. The model of RU5 consists of residues 1–211 of the light chains and residues 1–129 and 136–216 of the heavy chains. Residues 130–135 of the heavy chains display very weak electron density and are excluded from the model. Disorder of this loop is a commonly observed feature in Fab structures (36). Asn56 of the Vκ domain is...
N-glycosylated. Electron density near Asn\textsuperscript{56} accounted for a GlcNAc(-Fuc)-GlcNAc moiety in complexes A and B and only a GlcNAc-Fuc moiety in complex C. The model contains one cacodylate ion from the crystallization solution.

**Crystal Packing and Differences between Complexes**—In the a.u. A3-RU5 complexes B and C form a tightly interacting anti-parallel dimer and are related by 2-fold n.c.s. A3-RU5 complex A forms a similar anti-parallel dimer with a crystallographically related complex A\textsuperscript{9}. Complexes within dimers B-C and A-A\textsuperscript{9} have large contact areas of 932 Å\textsuperscript{2} and 1454 Å\textsuperscript{2}, respectively (GRASP (37)). These large interaction surface areas suggest that the dimer of A3-RU5 complexes may also be stable in solution. Dynamic light-scattering measurements, however, clearly indicated that the A3-RU5 complex does not form dimers in solution. Therefore, dimers observed in the crystal are a result of crystal packing.

Superposition of C\textsubscript{\alpha} atoms of the three A3-RU5 complexes gives large root mean square coordinate differences ranging from 0.7 to 0.9 Å. When superpositions are limited to A3 molecules with bound V\textsubscript{H} and V\textsubscript{L} domains, root mean square coordinate differences of 0.5–0.6 Å are obtained. Visual inspection of these superpositions (Fig. 2) shows that the A3-RU5 interaction region is well conserved, whereas regions of A3 and the variable domains located further away from the interaction region superimpose less well. A more detailed inspection of the A3-RU5 binding region reveals that all side-chain conformations are very similar (data not shown). In conclusion, the A3-RU5 interaction region is well conserved among the three complexes in the a.u.

**The Epitope of RU5**—A3 interacts with RU5 through a non-linear epitope comprising residues in loop \(\alpha_1\beta_2\), loop \(\beta_3\alpha_2\) followed by helix \(\alpha_2\), and loop \(\alpha_3\beta_4\) (Table II). All three loops that contribute to the epitope are located in the bottom face of A3 (Fig. 3). The N and C termini of A3 that are also located in
the bottom face do not interact with RU5. This observation is in agreement with the fact that RU5 was raised against complete vWF in which the termini connect A3 to flanking A2 and D4 domains. Residues of RU5 that interact with loops \( a_1b_2 \) and \( b_3a_2 \) are located in CDR L1 and CDRL2 (Fig. 1). All six CDRs interact with residues in the contiguous segment formed by loop \( b_3a_2 \) and helix \( a_2 \). The A3\( \alpha_2 \)/RU5 interactions involve five hydrogen bonds and one salt bridge. The buried surface area of A3 molecules in the complex is 1200 Å\(^2\), which is about 7% of the total surface area of A3. Interestingly, the fucose moiety attached to residue Asn56 in CDRH2 of complex A interacts with Lys988-Ala989 of A3. The carbohydrate moieties attached to Asn56 in complexes B and C do not interact with A3.

Conformational Changes in A3—To analyze whether binding of RU5 causes conformational changes in A3, we compared models of A3 in the A3/RU5 complex with two structures of free A3\( \alpha_2 \) (15, 16). The two structures of free A3 were determined from different crystal forms with unrelated crystal-packing interactions. In both crystal forms, two molecules are present in the a.u. Because the A3/RU5 complex was obtained with (Se-Met) A3, we also included the structure of free (Se-Met) A3 in the comparison to detect possible structural differences caused by Se-Met.\(^3\) Root mean square coordinate differences after pairwise superimposing \( C_{\alpha} \) atoms of all eight models range from 0.24 to 0.81 Å. Large differences are restricted to three loops located within the RU5-binding site and to two loops that are located distant from the epitope (Fig. 3 and Table II).

The three conformational diverse loops located in the RU5 epitope are \( \alpha_1b_2 \), \( \beta_3a_2 \), and \( \alpha_3b_4 \) (Fig. 4A). Loop \( \alpha_1b_2 \) has a

\(^3\) E. G. Huizinga, unpublished results.
Phe939 is buried in the hydrophobic core of A3. The buried complex C and all models of free A3. In complexes A and B, mutation of His1023 (magenta) abolishes collagen binding almost completely. Mutation of Arg963 and Arg1016 (green) reduces collagen binding by 25–35%. Mutation of Glu987 and His990 (gray) does not have an effect on collagen binding. To illustrate the range of conformations of His1023 observed in different crystal forms of A3, two conformations of this residue and the backbone trace of flanking residues are shown. Residues that have been mutated in previous studies (8, 16, 19) are located in the top face of A3 and are indicated by black spheres. The diagram was generated with MOLSCRIPT (43) and RASTER3D (44).

DISCUSSION

The current study was aimed at locating the collagen-binding site of the vWF-A3 domain. For this purpose we solved the crystal structure of A3 in complex with a Fab fragment of RU5 that inhibits collagen binding. The structure of the complex shows that RU5 binds to residues within A3 sequences 962–966, 981–997, and 1022–1026. These residues are located in α-helix 2 and in loops α1β2, β3α2, and α3β4 at the bottom of one of the side faces of the A3 domain (see Fig. 3). Comparison of structures of A3 shows that RU5 binding does not induce long range conformational changes. This excludes a mechanism in which RU5-induced conformational changes inhibit collagen binding. It seems likely, therefore, that RU5 inhibits collagen binding by steric hindrance, which implies that the collagen-binding site is located at or close to the RU5 epitope.

To confirm the location of the collagen-binding site, we constructed five charged-to-alanine mutations of residues located in or close to the RU5 epitope. The multimer distribution of these mutants was similar to that of wild-type vWF. Therefore, observed differences in collagen binding are not caused by the known dependence of collagen binding on vWF multimer size (7). All five mutants bound normally to RU5, which shows that none of the mutated residues plays a dominant role in the A3-RU5 interaction and, more importantly, that the conformation of A3 in the neighborhood of the epitope and the collagen-binding site is not disturbed.

Mutation H1023A abolished collagen binding almost completely, residual binding being similar to that observed for ΔA3-vWF, a deletion mutant that lacks the entire A3 domain (6). Collagen binding of mutants R963A and R1016A was reduced by 25–35%, whereas collagen binding of mutants E987A and H990A was normal.

Collagen Binding of vWF Mutants—The effect of point mutations on vWF binding to collagen types I and III was investigated by enzyme-linked immunosorbent assay (Fig. 6). Similar results were obtained for both types of collagen. Mutation H1023A almost completely abolished collagen binding. The level of residual binding observed for this mutant was similar to binding observed for ΔA3-vWF, a deletion mutant that lacks the entire A3 domain (6). Collagen binding of mutants R963A and R1016A was reduced by 25–35%, whereas collagen binding of mutants E987A and H990A was normal.
served for loop $\beta_3\alpha_2$. Like His$^{1023}$, loop $\beta_3\alpha_2$ is located at the edge between the front and bottom faces of A3. Because we did not mutate residues in loop $\beta_3\alpha_2$, its involvement in collagen binding remains to be established. The observed flexibility of His$^{1023}$ suggests that collagen binding may involve an induced-fit mechanism in which significant conformational changes occur in loop $\alpha_3\beta_4$ upon binding of A3 to collagen.

The amino acid sequence of collagen that is recognized by vWF-A3 has not yet been identified. We hypothesized previously that negatively charged residues in A3 could interact with basic residues on collagen (15). Residues now implicated in collagen binding are positively charged. Therefore, interaction of A3 with negatively charged residues on collagen appears more likely.

In contrast to binding sites of other collagen binding domains, like the $\alpha_1$ and $\alpha_2$-I domains and the A domain of Staphylococcus aureus adhesin (12, 38), the collagen-binding region of A3 does not have a groove or trench that could accommodate a collagen triple helix. The front face of the domain, harboring Arg$^{1016}$, is rather flat. The bottom face, which contains Arg$^{663}$ is less smooth, but no groove is present. Docking of a collagen triple helix on the A3 domain is not straightforward. In particular, it is not obvious how His$^{1023}$, Arg$^{663}$, and Arg$^{1016}$ could simultaneously contact a triple helix in an extended conformation. To define the collagen-binding site more precisely, characterization of additional mutants will be necessary.

Previously, the collagen-binding site of A3 was proposed to be located at its top face (15, 19) similar to the homologous I domains of integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (12, 39–41). Point mutations introduced in the top face of an A3 monomer (19) and in multimeric vWF (8) gave conflicting results. Our results now show conclusively that the collagen-binding site is located close to the bottom face and not in the top face of A3.

Although our results rule out a role for the top face of the A3 domain in collagen binding, this side of the molecule may still be engaged in other interactions, such as binding of the A1 domain. Interaction between A1 and A3 has been suggested to play a role in activation of the A1 domain for binding to platelet receptor glycoprotein Ib (42). Interestingly in this respect are the buried and solvent-exposed conformation observed for residue Phe$^{939}$, which is located close to the vestigial MIDAS motif in buried and solvent-exposed conformation observed for residue receptor glycoprotein Ib (42). Interesting in this respect are the play a role in activation of the A1 domain for binding to platelet domain in collagen binding, this side of the molecule may still show conclusively that the collagen-binding site is located close multimeric vWF (8) gave conflicting results. Our results now tions introduced in the top face of an A3 monomer (19) and in

**Collagen-binding Site of vWF-A3 Domain**

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