A Single High-Affinity Binding Site for von Willebrand Factor In Collagen III,
Identified Using Synthetic Triple-Helical Peptides.

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VWF BINDING SITE IN COLLAGEN III

Scientific Section:  HEMOSTASIS, THROMBOSIS, AND VASCULAR BIOLOGY

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Abstract

The essential event in platelet adhesion to the injured blood vessel wall is the binding to sub-endothelial collagen of plasma von Willebrand factor (VWF), a protein which interacts transiently with platelet glycoprotein (GP) Ibα1-3, slowing circulating platelets to facilitate firm adhesion through collagen receptors, including integrin α2β1 and GPVI. To locate the site in collagen that binds VWF, we synthesized 57 overlapping triple-helical peptides comprising the whole triple-helical domain of collagen III. Peptide #23 alone bound VWF, with similar affinity to that of native collagen III. Immobilized peptide #23 supported platelet adhesion under static and flow conditions, processes blocked by an antibody which prevents collagen from binding the VWF A3 domain. Truncated and alanine-substituted peptides derived from #23 either strongly interacted with both VWF and platelets, or lacked both VWF and platelet binding. Thus, we identified the sequence RGQGVMGF (O is hydroxyproline) as the minimal VWF-binding sequence in collagen III.
Introduction

The interaction of collagen with von Willebrand Factor (VWF) requires unique structural properties in both proteins. Optimal haemostatic function requires multimerisation of up to fifty VWF monomers in circulating plasma; higher-order multimers bind collagen more tightly than smaller assemblies of VWF\(^4\). Several collagens occur in the vessel wall, of which collagens I and III are considered most important in supporting platelet adhesion to the damaged vasculature\(^5\). We have identified the residues in the VWF A3 domain that bind collagen III, using site-directed mutagenesis guided by the crystal structure of the VWF A3 domain in complex with a monoclonal antibody (RU5) that inhibits its interaction with collagen\(^6,7\). Nishida et al. mapped the collagen-binding mode of the A3 domain by NMR, and confirmed results by site-directed mutagenesis\(^8\). However, the VWF-binding site(s) in collagen are unknown, although progress in understanding how collagen interacts with integrin $\alpha_2\beta_1$ and GPVI has been made using short synthetic triple-helical peptide analogues of collagen\(^9,10\), including the Collagen III Toolkit\(^11\).

We used the same approach to identify the high-affinity VWF-binding site in human collagen III, information which may help to develop the collagen–VWF interaction as an anti-thrombotic target\(^12,13\).
Materials and Methods

Peptide synthesis

The synthesis and characterisation of the fifty-seven overlapping triple-helical peptides of the Collagen III Toolkit (Supplementary Table S1) is detailed elsewhere\textsuperscript{11}. The same approach was used to synthesise and verify derivative peptides (Supplementary Table S2). The sequence of Peptide #23 is GPC(GPP)\textsubscript{5}-GPOGPSGPRGQOGVMGFOGPKGNDGAO-(GPP)\textsubscript{5}GPC-NH\textsubscript{2}, and of the minimal VWF-binding derivative peptide, GPC(GPP)\textsubscript{5}-GPRGQOGVMGFO-(GPP)\textsubscript{5}GPC-NH\textsubscript{2}

Static platelet-binding assay

Blood was obtained from the antecubital vein of informed volunteers, in accordance with the Helsinki protocol, into 0.105M citrate Vacutainers\textregistered. Platelet-rich plasma was prepared from after 2 spins for 1 min at 1200\texttimes\textit{g}. 10\% (v/v) of ACD buffer (39 mM citric acid, 75 mM tri-sodium citrate, 135 mM D-glucose, pH 4.5) and prostaglandin E\textsubscript{1} (280 nM final concentration) were added, and the platelets were pelleted for 12 min at 700\texttimes\textit{g}, then resuspended in 6 ml of buffer (5.5 mM D-glucose, 128 mM NaCl, 4.26 mM Na\textsubscript{2}HPO\textsubscript{4}, 7.46 mM NaH\textsubscript{2}PO\textsubscript{4}, 4.77 mM tri-sodium citrate, 2.35 mM citric acid, 0.35\% bovine serum albumin (BSA), pH 6.5). Prostaglandin E\textsubscript{1} was added as above, and the platelets were spun for 6 min at 700\texttimes\textit{g}. Platelets were resuspended to 1.25 \times 10\textsuperscript{8} platelets/ml in adhesion buffer (0.05 M Tris-HCl, 0.14 M NaCl, 0.1\% BSA, pH 7.4), and the adhesion of platelets from 100 \textmu l portions was determined colorimetrically, as described\textsuperscript{14}. Peptides (1\textmu g/well) were coated onto Immulon-2-HB plates, conditions supporting maximal platelet binding to peptide #23. The $\alpha_{\text{IIb}}\beta_{3}$
antagonist, GR144053F (2 µM; Calbiochem), or antibody RU5 (2 µg/ml) was pre-
incubated for 15 min with platelets, where indicated.

**Solid phase binding assays**

Ninety-six well plates were coated with peptides (1 µg/well) or collagen III (10
µg/well, Sigma, St. Louis, MO), to measure binding of VWF\(^\text{15}\) (purified from
Haemate P, Aventis-Behring, Hattersheim am Main, Germany).

**Platelet binding under flow conditions**

Peptides (0.5 µg/cm\(^2\)) or collagen III (6.5 µg/cm\(^2\)) were spray-coated onto
Thermanox® coverslips, then blocked with 1% human albumin in PBS and perfused
at 22°C with citrated whole blood for 5 min at a shear rate of 300 s\(^{-1}\) using a single-
pass perfusion chamber\(^\text{16}\), and platelet adhesion was evaluated\(^\text{17}\).

**Surface plasmon resonance**

A Biacore 2000 system (Biacore AB, Uppsala, Sweden) was used for surface plasmon
resonance analysis (SPR). Peptides were immobilized on a CM5 sensor chip via the
cysteine free thiols in the peptide N- and C-termini, using the manufacturer's
procedures. Binding to a channel coated with control peptide, GPC-(GPP)\(_{10}\)-GPC was
used to correct the binding of VWF or A3 domain to collagen- or other peptide-coated
channels, and VWF concentration used to calculate affinities was based on that of the
monomer, measured by ELISA.
Results and Discussion

Mapping the VWF-binding site within collagen III

A single Collagen III Toolkit peptide, #23, bound plasma-derived human VWF (figure 1A), a process that resembled VWF binding to collagen: 1) the interaction was abolished by a monoclonal antibody, RU5⁶, directed against the collagen-binding site of VWF A3 domain (figure 1B); 2) dysfunctional recombinant variants of VWF, delta A3 VWF¹⁸ which lacks the A3 domain, and His1023Ala VWF⁶ in which a crucial A3 amino acid is substituted, showed severely reduced binding to peptide #23 (figure 1B); 3) VWF had similar affinity for peptide #23 as for full-length collagen III measured in a solid-phase assay (figure 1C); 4) peptide #23 bound washed human platelets in static assays, most likely reflecting the presence on the platelet surface of residual plasma-derived VWF or VWF inevitably secreted during platelet preparation, and supported platelet deposition from flowing whole blood at low shear rate (300 s⁻¹; figure 1D). The high density (20-fold that of native collagen) of VWF-binding sites on the peptide-coated surface may be responsible for the static binding activity, and the complete blockade of these events by RU5 shows the fundamental role of VWF. Under static conditions, antagonism of either integrin αIbβ₃ or GpVI slightly impaired platelet binding to peptide #23, consistent with minor involvement of other receptors but with GPIb being the main VWF receptor (data not shown). A non-helical derivative of peptide #23, flanked by GAP rather than GPP repeats, bound neither VWF nor platelets (data not shown), indicating the same requirement for triple-helical structure for the recognition of collagen by VWF as for α2β₁ and GPVI⁹,¹⁰.
Identification of the minimal collagen sequence required for VWF binding

A set of truncated triple-helical derivatives of peptide #23 was synthesised, including an alanine-scanned set, peptides 7 -14 (Supplementary Table S2). Peptides either bound VWF (figure 2A) and platelets (figure 2B) strongly, or completely lacked affinity for both VWF and platelets. Thus we identified RGQOGVMGF as the minimum sequence within collagen III required to bind VWF. Residues R, O, V, and F appear crucial for VWF binding, but not Q and M. The triple-helical peptide GPC(GPP)₅-GPRGQOGVMGFO-(GPP)₅GPC-NH₂, its parent peptide #23, and full-length collagen bound VWF in a solid-phase assays with similar affinity (data not shown). SPR allowed more detailed kinetic analysis. A recombinant VWF A3 domain¹⁹ bound the immobilized peptides with modest affinity (Kₐ 1.8 µM for peptide #23, Kₐ 2.5 µM for GPC(GPP)₅-GPRGQOGVMGFO-(GPP)₅GPC-NH₂, data not shown), whereas full-length, plasma-derived VWF displayed much higher affinity, attributable to its multimeric nature (Kₐ 2.1 nM for peptide #23, Kₐ 2.5 nM for GPC(GPP)₅-GPRGQOGVMGFO-(GPP)₅GPC-NH₂, figure 2C). These affinity constants are consistent with those observed for A3 and VWF binding to full-length collagen¹⁵,²⁰, and imply a single VWF-binding site within collagen III.

We previously reported putative VWF-binding sites in collagen III²¹, but VWF bound weakly to these synthetic triple-helical peptides compared with full-length collagen. These peptides were short stretches of cyanogen bromide fragments of bovine collagen, and so lacked the intact high-affinity binding site, which was cleaved in the parent collagen by cyanogen bromide digestion at the methionine residue within the sequence that we now report.
The triple-helical peptide GPRGQOQVMGF at 500µg/ml (~40µM) inhibited binding of VWF, or platelets from whole blood, to immobilized collagen III under flow conditions almost completely (figure 2D).

These results confirm the sequence RGQOQVMGF as the major, high-affinity VWF-binding site, and exclude the prominent role for the VWF A1 domain in collagen binding postulated previously\textsuperscript{22}. We cannot exclude the possibility that the residual binding of the A3-domain mutant, His1023Ala, reflects weak binding of VWF to Peptide #23 through A1, nor that binding of A3 is required to render A1 competent to bind collagen at a separate site.

Our data suggest that inhibition of VWF binding to collagen, by peptides, antibodies or other antagonists that target this nonapeptide collagen sequence, may be a useful therapeutic strategy. Current anti-platelet therapy addresses platelet activation through amplification pathways including purinergic receptors and thromboxane generation, or the up-regulation of the fibrinogen receptor integrin α\textsuperscript{IIb}β\textsubscript{3}. Previous use of in vivo models suggested that inhibition of the collagen–VWF interaction with a monoclonal antibody or a naturally-occurring collagen-binding protein, by targeting primary platelet adhesion to the arterial sub-endothelium, has a better risk/benefit ratio than inhibition of platelet-platelet interaction\textsuperscript{12,13}, and might provide a valuable alternative to the anti-platelet therapies in current clinical practice, recently reviewed\textsuperscript{23}.

The sequence RGQOQVMGF occurs in only one other human protein, collagen II. A peptide, GPC-(GPP)\textsubscript{5}-GAOGEDGROGPQGARGQQOQVMGF-(GPP)\textsubscript{5}-GPC, amino acids 510-536 from collagen II, bound VWF with high affinity (data not shown). This may be irrelevant to haemostasis, since collagen II is not found in vascular sub-endothelium, being largely restricted to cartilage and vitreous humour.

The sequence RGQOQVMGF is 100% conserved in collagen III from mouse, rat,
cow, and chicken. Interestingly, in human collagen I, a heterotrimer comprising two \( \alpha_1 \) and one \( \alpha_2 \) chains, a closely-related sequence occurs in the \( \alpha_1 \) chain, differing by a single amino acid (RGQAGVMGF). This O-to-A substitution prevented the VWF-binding activity in our homotrimeric synthetic peptide from the Ala-scanned set. In this region, the sequence RGQAGVMGF of the human \( \alpha_1(\text{I}) \) chain aligns with the sequence RGEOGNIGF of the \( \alpha_2(\text{I}) \) chain, suggesting that the essential O in position 4 of the VWF-binding homotrimer, although substituted with an A in the \( \alpha_1(\text{I}) \) chain, is provided by position 4 of the \( \alpha_2(\text{I}) \) chain sequence, so that VWF may bind collagens I, II and III in identical manner. Modelling experiments, shown in Supplementary Data (File S3), support this conclusion.
References


14. Onley DJ, Knight CG, Tuckwell DS, Barnes MJ, Farndale RW. Micromolar Ca\(^{2+}\) is essential for Mg\(^{2+}\)-dependent binding of collagen by the integrin \(\alpha_2\beta_1\) in human platelets. J Biol Chem. 2000;275:24560-24566.


Figure 1. **Identification of a single peptide containing 27 amino acids of collagen III sequence that specifically binds VWF.** A) The peptides (10µg/ml) of the Collagen III Toolkit (supplementary Table S1) were each immobilized on a 96 well plate and the adhesion of plasma-derived VWF (1 µg/ml) was determined. B) Immobilized peptide #23 was incubated with plasma-derived VWF (1 µg/ml) in presence or absence of monoclonal antibody RU5 (1 µg/ml), or with recombinant wild-type, delta-A3, or His1023Ala VWF (all at 1 µg/ml). In A and B, bound VWF was detected as described in Materials and Methods, and the mean ± SD is shown from a representative of three independent experiments, each performed in duplicate. C) Immobilized peptide #23 or immobilized collagen III (100 µg/ml) were incubated with increasing concentrations of VWF, detected as above. D) Peptide #23 or vehicle
was coated in a 96-well plate (10 µg/ml) or sprayed onto a coverslip (0.5 µg/cm²) and incubated with washed platelets or perfused with whole blood for 5 minutes at a shear rate of 300 s⁻¹ in presence or absence of RU5 (1 µg/ml). Uncoated wells or coverslips served as controls. The adhesion of platelets bound in the static assay (filled bars) and the surface coverage of platelets (open bars) were each determined as described in Materials and Methods. Shown is the mean ± SD of three independent experiments each performed in triplicate.
Figure 2. Identification of the minimal collagen sequence required for VWF binding. A) The truncated and alanine-modified peptides derived from peptide #23 (see Figure 2 and Supplementary data, Table S2) were immobilized on a 96-well plate, and incubated with plasma-derived VWF (1 µg/ml), and bound VWF was detected as in Figure 1. B) The same peptides were spray-coated onto Thermanox® coverslips (0.5 µg/cm²), and perfused with whole blood at a shear rate of 300s⁻¹ for 5 minutes, then surface coverage was measured as described for Figure 1. C) Peptide #23 and peptide GPC-(GPP)₅-GPRGQOGVMGFO-(GPP)₅-GPC-NH₂ were immobilized onto a Biacore CM5 sensor chip via their free cysteine residues. The equilibrium binding capacity of the peptides was determined for different concentrations of plasma-derived VWF. D) Collagen (100 µg/ml) was immobilized on a 96-well plate or a Thermanox® coverslip and incubated with purified VWF (1
µg/ml) or perfused with whole blood in the presence or absence of different concentrations of peptide GPC-(GPP)₅-GPRGQOVMGFO-(GPP)₅-GPC-NH₂. VWF binding and platelet deposition were evaluated as indicated above. Shown is a representative of three independent experiments, each performed in duplicate (A), triplicate (B and D) or as single determinations (C).
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