Production and functional activity of a recombinant von Willebrand factor-A domain from human complement factor B

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Factor B is a five-domain 90 kDa serine protease proenzyme which is part of the human serum complement system. It binds to other complement proteins C3b and properdin, and is activated by the protease factor D. The fourth domain of factor B is homologous to the type A domain of von Willebrand Factor (vWF-A). A full-length human factor B cDNA clone was used to amplify the region encoding the vWF-A domain (amino acids 229–444 of factor B). A fusion protein expression system was then used to generate it in high yield in *Escherichia coli*, where thrombin cleavage was used to separate the vWF-A domain from its fusion protein partner. A second vWF-A domain with improved stability and solubility was created using a Cys²⁶⁷ → Ser mutation and a four-residue C-terminal extension of the first vWF-A domain. The recombinant domains were investigated by analytical gel filtration, sucrose density centrifugation and analytical ultracentrifugation, in order to show that both domains

were monomeric and possessed compact structures that were consistent with known vWF-A crystal structures. This expression system and its characterization permitted the first investigation of the function of the isolated vWF-A domain. It was able to inhibit substantially the binding of ¹²⁵I-labelled factor B to immobilized C3b. This demonstrated both the presence of a C3b binding site in this portion of factor B and a ligand-binding property of the vWF-A domain. The site at which factor D cleaves factor B is close to the N-terminus of both recombinant vWF-A domains. Factor D was shown to cleave the vWF-A domain in the presence or absence of C3b, whereas the cleavage of intact factor B under the same conditions occurs only in the presence of C3b.

Key words: factor B, factor D, expression, mass spectroscopy, vWF-A domain.

INTRODUCTION

The complement system consists of a large number of soluble (plasma) and membrane-bound proteins that have a major role in innate immunity. Its function is to recognize foreign material and to facilitate its phagocytosis. Human complement factor B is a 90 kDa serine protease (SP) proenzyme present in plasma. When the complement system is activated, the major opsonin of complement, C3, is cleaved to form C3b. C3b forms a complex with factor B, whereupon factor D, a serine protease, cleaves factor B in the C3b–factor B complex, to form two fragments, Ba and Bb. Fragment Bb remains transiently bound to C3b, and the C3b–Bb complex itself contains an SP in the Bb fragment that activates more C3 [1,2]. The amplification of C3 activation, which occurs by this mechanism, is essential for opsonization of complement-activating materials.

When examined by electron microscopy, factor B presents a three-lobed globular structure [3]. The Ba fragment (approx. 30 kDa) consists of three complement control protein domains (also known as short consensus repeats) [4], each approx. 60 amino acids in length. The Bb fragment (60 kDa) consists of a C-terminal SP domain [2,5] and an N-terminal von Willebrand factor type A (vWF-A) domain [6,7]. vWF-A domains also occur in proteins of the extracellular matrix and in other proteins of the immune system (including complement C2, the leucocyte integrins CR3, CR4, LFA-1, and the VLA-1 and VLA-2 integrins) (reviewed in [6,7]). As this domain was first identified in von

Willebrand factor [8,9], it is most frequently known as the vWF-A domain, but it is also known as an A-domain or I-domain. A total of 75 sequences in 25 proteins have been analysed to show a relatively low degree of sequence conservation and no conserved disulphide bridges [7]. Its protein fold was predicted to resemble the doubly wound α/β fold of nucleotide binding proteins [10,11], and vWF-A crystal structures have been solved to confirm the prediction [12,13] (reviewed in [9]).

Factor B, C2, CR3 and CR4 all interact with degradation fragments of C3, known as C3b or iC3b, or with those of C4, a homologue of C3. Since the vWF-A domain is common to Factor B/C2 and CR3/CR4, it is possible that this domain mediates ligand binding [14]. The evidence for this is mostly indirect. Site-directed mutagenesis of C3 near the N-terminus of the α' -chain of C3b [15] resulted in mutant C3b proteins that were less able to support cleavage of factor B by factor D than normal C3b, implying a reduced binding with factor B, and mutant forms of iC3b likewise have reduced binding to CR3. As the only common region of CR3 and factor B is a vWF-A domain, this result implied that this domain is responsible for C3b and iC3b binding. Mutation of the divalent metal-ion-binding sites in the vWF-A domain of CR3 abolished binding to iC3b [16].

A model for the C3b–factor B complex involves interaction via two (or more) sites on factor B [17–22]. A C3b-binding site in the Ba fragment was identified using a monoclonal anti-Ba antibody that inhibited the binding of ¹²⁵I-labelled factor B to red-cell-

Abbreviations used: MALDI, matrix-assisted laser desorption ionization; SP, serine protease; vWF-A, von Willebrand factor type A.

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bound C3b by up to 50 % [18], and by the use of a cross-linking agent [17]. A C3b-binding site in the SP domain of factor B was identified by the direct binding of the isolated ^{125}I -labelled SP domain to C3b bound to zymosan in the presence of Mg^{2+} ions [21], and the SP domain also has Mg^{2+} -ion-independent binding affinity for fluid-phase C3b [20]. A C3b-binding site on the vWF-A domain was indirectly identified by a comparison of the Bb fragment and the SP domain in that study [20], and mutagenesis of recombinant factor B suggested that surface loops adjacent to the Mg^{2+} -site in the vWF-A domain influenced its C3b binding specificity [22]. As C2 and C4 are close homologues of factor B and C3 respectively, it is relevant that, in recombinant C2, mutations at residues 240–244 in the vWF-A domain affected the binding of C2a to C4b [23].

Study of the molecular function of the vWF-A domain in factor B requires the use of a suitable recombinant protein. Here we describe the development and characterization of two expression systems for isolated vWF-A domains. One of these has previously been used to study the vWF-A secondary structure by Fourier-transform IR spectroscopy [7]. Here, further evidence is presented to show that the recombinant vWF-A domain is folded and monomeric, and was therefore suitable for functional studies. The functional activity of the two recombinant vWF-A domains from factor B provide new insights into the role of vWF-A domains in complement activation.

MATERIALS AND METHODS

Materials

Glutathione-agarose beads (G-4510) and human thrombin (T-3010) were from Sigma (Poole, Dorset, U.K.), as were the constituents of the buffer used to lyse *E. coli* cells [DNase (D-7291), lysozyme (L-6876), Triton X-100 (T-6878) and benzamidine (B-6506)], as well as lactoperoxidase (L-8257) used for radiolabelling of factor B. Thiol-Sepharose 4B, T⁷ DNA sequencing kits and the expression vector pGEX-2T were from Pharmacia (Milton Keynes, Bucks., U.K.). Fresh-frozen plasma for the purification of complement components came from the Oxford Regional Blood Transfusion Service (John Radcliffe Hospital, Oxford, U.K.). The SP inhibitor Pefabloc-SC [4-(2-aminoethyl)benzenesulphonyl fluoride] was obtained from Pentapharm AG (Basel, Switzerland).

Vector construction

General procedures were as described previously [24]. The region encoding the vWF-A domain was amplified from a full-length factor B cDNA clone named pFB-2 [25] by PCR. The factor B cDNA clone was provided by Dr W. Schwaeble (Department of Microbiology and Immunology, University of Leicester, U.K.). The middle five exons of the factor B gene encode the vWF-A domain of factor B [5]. The first exon begins with Gly²²⁹ and the last ends with Ile⁴⁴⁴. Oligonucleotides were designed to flank the region of interest with the introduction of a *Bam*H1 restriction site at the 5'-end and an *Eco*R1 restriction site at the 3'-end to enable sub-cloning into the pGEX-2T vector in the correct orientation (5' oligonucleotide CG GGA TCC GGG GAA CAA CAG AAG CGG AAG; 3' oligonucleotide GGA ATT CTA GAT CAT TTG GTA GAA AAC ATC TTC). The amplified cDNA insert (663 bp) produced was digested with *Eco*R1 and *Bam*H1 and ligated into the pGEX-2T vector pre-cut with the same enzymes. The ligation mixture was used to transform MC1061 competent cells. Individual bacterial clones containing cloned cDNA were identified by the production of correct-sized PCR products using the oligonucleotides described above from

DNA mini-preps [26]. Sub-cloned DNA from a large-scale plasmid purification [27] was sequenced using a 'T⁷ Sequencing Kit' following the manufacturer's protocol. The sequence was found to be identical with that found in the pFB-2 clone from which the fragment was amplified. The modifications to the ends of the amplified product were found to be as intended. As this recombinant product contained 218 residues, it was denoted vWF-A-218.

Expression and purification of vWF-A-218

vWF-A-218 was expressed and purified from *E. coli* by a combination of the methods described in [24,28]. Overnight cultures of *E. coli* transformed with the pGEX-2T constructs were diluted 1:10 with fresh Luria broth/ampicillin (100 mg/l) and grown for a further 1 h with shaking. At this point isopropyl β -D-thiogalactoside (Calbiochem-Novabiochem, Nottingham, U.K.; A10007) was added to 1 mM. After a further 6 h of growth the cells were pelleted by centrifugation (20 min; 4000 g) and frozen at -80°C overnight. Samples were then thawed and resuspended in lysis buffer (1/50 of the starting volume) and left on ice for 1 h. This was followed by sonication for 3×20 s on ice using an MSE Ultrasonic Processor (375 W) and a 6.5 mm tapered microtip. The lysis buffer was a hypertonic PBS (MTPBS) (150 mM NaCl/16 mM Na_2HPO_4 /54 mM NaH_2PO_4 , pH 7.2) containing 0.2 mg/ml lysozyme, 1 $\mu\text{g}/\text{ml}$ DNase, 0.1 % (v/v) Triton X-100, 1 mM Pefabloc-SC, 5 $\mu\text{g}/\text{ml}$ iodoacetamide, 50 mM benzamidine and 5 mM EDTA. After centrifugation (10000 g; 20 min) the supernatant was incubated with glutathione-agarose beads (10 ml per litre of culture) at 4°C overnight using a rotary stirrer. The beads were recovered by centrifugation (5 min; 500 g) and washed five times with 2 vol. of MTPBS. To allow separation of vWF-A-218 from the glutathione S-transferase carrier protein, the fusion protein was cleaved directly on the beads with human thrombin. The fusion protein bound to 10 ml of packed resin (suspended in two volumes of MTPBS in a 50 ml conical centrifuge tube) was digested with 50 μg of human thrombin for 4 h at 37°C with gentle mixing. The beads were then spun down (5 min; 1000 rev./min) and the supernatant containing the soluble expressed domain was pooled with four subsequent washes of the resin, each with 10 ml of MTPBS. The expressed domain was then concentrated 3-fold by partial freeze-drying and separated from contaminating *E. coli* proteins by gel filtration on an FPLC Superose-12 HR10/30 column (Pharmacia). Gel filtration at a flow rate of 0.3 ml/min was done using PBS (137 mM NaCl/2.6 mM KCl/8.2 mM Na_2HPO_4 /1.5 mM KH_2PO_4 , pH 7.3) that was prepared from Dulbecco 'A' tablets (Oxoid, Unipath Ltd, Basingstoke, U.K.). The pooled fractions containing purified vWF-A-218 [as assessed by SDS/PAGE on 10 % (w/v) polyacrylamide gels] were stored at -20°C with the addition of iodoacetamide to 2 mM. The absorption coefficient at 280 nm (1 cm, 1 %) was calculated from the sequence [29] as 15.6 in order to determine protein concentrations and yields.

SDS/PAGE and N-terminal sequence analysis

For SDS/PAGE [30], vWF-A-218 samples were prepared for electrophoresis under reduced or unreduced (alkylated) conditions, and gels were stained using Coomassie Blue [31]. Samples for N-terminal sequence analysis were run on SDS/PAGE under reducing conditions and then electroblotted onto a Problott membrane (Applied Biosystems) in a Bio-Rad mini Trans-Blot electrophoretic transfer cell. The blots were stained with Coomassie Brilliant Blue and the bands corresponding to the protein of interest were excised. The N-terminal amino acid sequences

Table 1 Primers used for the mutation of the vWF-A-218 cDNA to vWF-A-222 by the 'QuickChange' method

The mut1/antimut1 and mut2/antimut2 sequences correspond to the Cys²⁶⁷ → Ser mutation and the four-residue C-terminal extension respectively. Characters in bold represent alterations from the original vWF-A cDNA sequence. The underlined TAG motif represents the stop codon required for termination of translation. The melting temperature T_m was calculated according to the Stratagene QuickChange manual. $T_m = 81.5 + 0.41(\%GC) - 675/N - \% \text{ mismatch}$

Identification	Amino acid and DNA sequences	Direction	Length (N)	GC	T_m (°C)
mut1	T G A K K S L V N C ACA GGA GCC AAA AAG TCA CTA GTC AAC	Forward	28	13	74
antimut1	GTT GAC TAG TGA CTT TTT GGC TCC TGT G	Reverse	28	13	74
mut2	V F Y Q M I D E S Q Stop GTT TTC TAC CAA ATG ATC GAC GAG AGC CAG TAG AAT TCA TCG TGA CTG	Forward	48	21	60
antimut2	CAG TCA CGA TGA ATT CTA CTG GCT CTC GTC GAT CAT TTG GTA GAA AAC	Reverse	48	21	60

were obtained using an Applied Biosystems 470A protein sequencer and Applied Biosystems 120A analyser.

Expression and purification of vWF-A-222

A second recombinant vWF-A domain, denoted vWF-A-222, was prepared by site-directed mutagenesis of the pGEX-2T plasmid containing the vWF-A-218 clone using the quick-change site-directed mutagenesis kit (QuickChange 200518, Stratagene, Cambridge, U.K.). Two cycles of mutagenesis were employed (Table 1). In the first, Cys²⁶⁷ was replaced by Ser and an *Spe*I restriction site (A/CTAGT) was introduced in the mut1 sequence. In the second, the C-terminus was extended by the next four residues of the factor B sequence (DESQ), and one *Bfa*I restriction site (C/TAG) was lost in the mut2 sequence. Oligonucleotide primers were synthesized at Perkin-Elmer Applied Biosystems (Warrington, Cheshire, U.K.). The mutant plasmids were ligated into Epicurian Coli XL1-Blue supercompetent cells for selection. These were identified by the modified restriction enzyme sites which gave unique digestion fragments. The restriction enzyme *Bfa*I was from New England Biolabs (Hitchin, Herts., U.K.), and the enzyme *Spe*I was from Promega (Southampton, U.K.). The double mutant plasmid was then transformed into *E. coli* MC1061/P3-competent cells (Invitrogen, Groningen, The Netherlands) for protein expression. vWF-A-222 was purified in a similar manner to vWF-A-218, except that iodoacetamide was not now required. The calculated vWF-A-222 absorption coefficient at 280 nm (1 cm, 1%) was 15.2 [29].

Hydrodynamic analyses of vWF-A-218 and vWF-A-222

In order to estimate the diffusion coefficient of vWF-A-218, its elution relative to those for standards was studied on the Superose 12 column described above. Diffusion coefficients of these standard proteins were plotted against their elution volumes (human ovalbumin, $7.8 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$; soya bean trypsin inhibitor, $9.05 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$; hen egg lysozyme, $11.2 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ [32]). The diffusion coefficient yields the Stokes' radius a [33]:

$$a = kT/6\pi\eta D,$$

where k is the Boltzmann constant ($1.4 \times 10^{-16} \text{ erg/}^\circ\text{C}$), T is the temperature (293 K), η is the viscosity of water at 20 °C (0.01 centipoise) and D is the diffusion coefficient at 20 °C in water.

The sedimentation coefficient of vWF-A-218 was estimated by linear sucrose-density gradients (12 ml) of 5–40% sucrose in 10 mM Tris/HCl, pH 7.4 [32]. vWF-A-218 (50 μl ; 300 $\mu\text{g/ml}$) in PBS was loaded onto the gradient. Centrifugation was carried out in a Beckman SW40 Ti rotor at 37000 rev./min for 12 h at

4 °C. Gradients were fractionated by peristaltic pumping from the base of the gradient, and fractions were analysed by measuring the absorbance at 280 nm and also by SDS/PAGE on 10% (w/v) polyacrylamide gels. The sedimentation coefficient was calculated by comparing the mobility of vWF-A-218 with those of standard proteins run under identical conditions (thyroglobulin, 19.2 S; bovine liver catalase, 11.2 S; BSA, 4.22 S; myoglobin, 2.04 S) [34]. Knowledge of the Stokes' radius a and the sedimentation coefficient gives the relative molecular mass M_r [35]:

$$M_r = (6\pi\eta Na s^{\circ}_{20,w})/(1 - \bar{v}\rho)$$

where N is Avogadro's number, $\bar{v}$ is the partial specific volume (calculated to be 0.739 ml/g from [29]) and ρ is the density of water at 20 °C. The frictional ratio f/f_0 was calculated from the ratio of Stokes radii a/a_0 , where a_0 is the radius of the sphere corresponding in volume to the hydrated volume of vWF-A-218 in which the hydration was 0.3 g of H₂O/g of protein.

Analytical ultracentrifugation of vWF-A-218 and vWF-A-222 was performed on a Beckman XLI instrument in which the sample was monitored using its absorbance at 280 nm and its refractive index measured by interferometry. Sedimentation equilibrium data were acquired over 48 h in six-sector cells with column heights of 3 mm at rotor speeds of 6000, 8500, 11000 and 15000 rev./min until equilibrium had been reached at each speed. The data were analysed on the basis of a single species within the Beckman software provided as an add-on to Origin Version 4.1 (Microcal Inc.), where $\bar{v}$ for vWF-A-222 was calculated to be 0.738 ml/g [29]. Sedimentation velocity data were acquired over 8 h at rotor speeds of 42000 rev./min in two-sector cells with column heights of 12 cm, where scans were recorded at 15 min intervals. Sedimentation coefficients were derived using the transport method, also provided as an add-on within the Origin program. To calculate the sedimentation coefficient of vWF-A from the crystal structure of the homologous vWF-A domain in CR3 (PDB code 1ido [12]), this structure was converted into spheres of the same total volume as vWF-A from factor B, using a grid with cubes of side 0.43 nm, then the model was hydrated by the addition of a surface layer of spheres using the HYPRO program [36] to give a total of 535–545 spheres, from which the frictional coefficient was calculated using the GENDIA method [36,37].

Assay of functional (C3b-binding) activity of vWF-A-218

An assay system was developed to study factor B binding to C3b, using C3b immobilized on thiol-Sepharose via the free SH group in C3b [38]. C3 was purified and converted into C3b [39]. Factor B was purified by dye-ligand affinity chromatography from

fresh-frozen human plasma [40]. Aliquots of purified factor B (100 μ g in 0.2–1 ml of PBS) were radioiodinated, using 0.5 mCi of Na^{125}I (Amersham International, Bucks., U.K.), by lactoperoxidase-catalysed iodination using the method of Marchalonis [41] to a specific activity of 1×10^6 c.p.m./ μ g.

C3b–thiol-Sepharose was produced as follows. Briefly, the resin was reduced by adding dithiothreitol (final concentration, 50 mM) to a 1:1 (v/v) slurry (i.e. 1 vol. of packed resin:1 vol. of buffer) in PBS. The mixture was then incubated for 30 min at 37 °C. The resin was then washed with at least five vol. of PBS before incubation with C3b. Purified C3b in 25 mM Tris/HCl/140 mM NaCl, pH 8.2, was incubated with pre-swollen thiol-Sepharose (0.5 mg C3b/ml of resin) for 2 h at room temperature using a rotary stirrer. The percentage of C3b bound was estimated by measuring the A_{280} of the original solution and three subsequent washes with PBS. Approx. 20–25 % of the C3b bound to the resin, in which the final content of C3b was routinely 100–125 μ g/ml of resin. Remaining free SH groups on the thiol-Sepharose resin were blocked by incubation with 5 mg of iodoacetamide per ml of resin for 1 h at room temperature. Non-specific binding sites on the C3b–thiol-Sepharose were blocked by incubation of the resin with 1 mg/ml BSA in PBS for 1 h at room temperature.

The effect of vWF-A-218 on the binding of radioiodinated factor B to C3b–thiol-Sepharose was assessed. In each assay, 50 μ l of C3b–thiol-Sepharose suspended in 50 μ l of binding buffer [10 mM Pipes (sodium salt), 30 mM NaCl, 1 mg/ml BSA, pH 7.0, containing 0.2 mM MgCl_2] was pre-incubated with serial two-fold dilutions of vWF-A-218 (200 μ l; maximum concentration 125 μ g/ml) for 2 h at 4 °C. ^{125}I -labelled factor B (4×10^5 c.p.m.) was then added and radioactivity bound to the C3b–thiol-Sepharose resin was measured after 1 h incubation at room temperature and five washes, each with 0.85 ml of binding buffer.

MS analyses

To measure the mass of vWF-A-218, matrix-assisted laser desorption ionization (MALDI) MS [42] was performed on a VG time-of-flight spectrometer. The sample was in 20 mM acetic acid and loaded in a 1:1 ratio with the matrix sinapinic acid (*trans*-3,5-dimethoxy-4-hydroxycinnamic acid) to aid in desorption. The instrument was calibrated externally using cytochrome *c* and insulin. To measure the mass of vWF-A-222, electrospray ionization MS was performed on a Finnigan Navigator spectrometer with a Waters Alliance liquid-chromatography system for sample delivery. The sample was in deionized water and was loaded at a 1:1 ratio with 50 % acetonitrile. The instrument was calibrated using water clusters. MALDI mass analysis was also performed with a PBS-1 mass analyser using a normal-phase chip surface (Ciphergen, Camberley, Surrey, U.K.). The vWF-A-218 and vWF-A-222 preparations (at 1 mg/ml in PBS containing 0.5 mM MgCl_2 ; Sigma, D-5773) were incubated with 2 % (w/w) recombinant factor D overnight at 37 °C and dialysed against distilled water before MS analysis. Each sample was mixed with an equal volume of matrix solution (50 % acetonitrile/1 % trifluoroacetic acid with sinapinic acid) before application to a normal-phase chip used in the mass analyser. The instrument was calibrated internally against bovine β -lactoglobulin A as standard. Recombinant factor D was a gift from Professor J. E. Volanakis (Department of Medicine, University of Alabama at Birmingham, AL, U.S.A.) [43]. Molecular masses were calculated from their amino acid sequences using averaged isotopic atomic masses in the PAWS MS program (<http://www.proteometrics.com>).

RESULTS

Expression and purification of the vWF-A-218 domain of factor B

The 218-residue vWF-A domain (denoted as vWF-A-218) was expressed as a fusion protein with glutathione S-transferase. The expected molecular masses from amino acid sequences were 51 kDa for the fusion protein, 26 kDa for the fusion partner glutathione S-transferase and 25 kDa for the vWF-A domain. Figure 1 shows from SDS/PAGE analysis that a major protein product was present with an apparent molecular mass of about 43 kDa in cell lysates, which has therefore migrated anomalously compared with its expected value. The fusion protein was stable even after 6 h of induction and showed no signs of degradation. Cleavage of the fusion protein bound to glutathione–agarose beads with thrombin resulted in the release of the recombinant domain into the supernatant, as confirmed by the 25 kDa band in Figure 1. The glutathione–agarose beads were suspended in 2 vol. of MTPBS before cleavage with thrombin, as smaller volumes resulted in protein precipitation in the supernatant. The yield was estimated as 7–10 mg/l of culture from the absorbance at 280 nm (see Materials and methods section). N-terminal sequence analysis of the recombinant product gave a homogeneous sequence $\text{NH}_2\text{-GS}^{229}\text{GEQKRRKIV} \dots$. The residues GS preceding the vWF-A domain sequence were derived from the thrombin-recognition site LVPRGS in the vector sequence. This sequence also contained the site at which factor D cleaves intact factor B between Arg-234 and Lys-235 [44]. MS of the product gave a molecular mass in agreement with the expected value for the vWF-A-218 domain (see below). On storage of the purified domain, a protein band was occasionally apparent at approx. twice the molecular mass of the recombinant domain when assessed by non-reducing SDS/PAGE on a 10 % (w/v) polyacrylamide gel. This band was not seen under reducing conditions

Figure 1 SDS/PAGE analysis on a 10 % (w/v) polyacrylamide gel of stages in the expression and purification of the vWF-A-218 domain under reducing conditions

Aliquots of 10 μ l were run on SDS/10%-PAGE and samples were stained with Coomassie Blue. Lane 1, total *E. coli* cell lysate after 6 h of induction with isopropyl β -D-thiogalactoside. The major band close to an apparent molecular mass of 43 kDa represents the glutathione S-transferase–vWF-A fusion protein, which has migrated anomalously compared with its expected value of about 50 kDa. Lane 2, total *E. coli* cell lysate after incubation with glutathione–agarose overnight at 4 °C to show that the fusion protein had been removed. Lane 3, fusion protein attached to the glutathione–agarose beads. Lane 4, material remaining bound to the glutathione beads after thrombin cleavage, where the major band at ~ 26 kDa is glutathione S-transferase. Lane 5, supernatant after thrombin cleavage that contains the soluble vWF-A-218 domain (~ 25 kDa).

and was therefore attributed to a disulphide-linked dimer of vWF-A-218, as there is a single free cysteine at position 267 [45]. Treatment with 2 mM iodoacetamide before storage prevented dimer formation.

For the large-scale production of vWF-A-218 for structural studies, the glutathione-agarose purification protocol was efficient, and over 90% purity was routinely obtained by thrombin cleavage. Since the solubility of the fusion protein was impaired if the *E. coli* cells were stored at -20°C between harvest and lysis, maximal yields were achieved by the growth of cells, their lysis and the binding to glutathione-agarose within 1 day. The use of 5 mM dithiothreitol as a reducing agent during lysis inhibited dimer formation. After binding the fusion protein to glutathione-agarose, the dithiothreitol was washed out using degassed buffer before the thrombin cleavage step. Since freeze-drying promoted vWF-A aggregation, protein concentration was achieved instead by using Amicon stirred cells under nitrogen pressure. To prevent dimer formation during storage at 4°C , the vWF-A-218 sample was exposed to 10 mM iodoacetamide for 1 min at 37°C . Despite this, minor aggregation and precipitation occurred during storage, and further purification to better than 95% by size-exclusion chromatography on a HiLoad 16/60 Superdex 75 column was successfully used to separate the monomer from other forms. The occurrence of these aggregates reduced the net yield of purified vWF-A-218 to 3 mg/l of culture during storage, and its solubility did not exceed 3 mg/ml.

Development of the vWF-A-222 expression system

To improve the yield and solubility of vWF-A, two modifications were made. (1) Since dimerization was attributable to the single Cys²⁶⁷ residue within vWF-A-218, the sequence alignment of the vWF-A superfamily [7] was examined to investigate the significance of Cys²⁶⁷. This was found not to be conserved in the superfamily, even though another free Cys residue is found in the vWF-A domain of complement C2 [7]. Whereas this thiol group in C2 was essential for the normal assembly and decay of the classical pathway C3 convertase, the function of Cys²⁶⁷ in native factor B was uncertain [45]. Thus a Cys²⁶⁷ → Ser mutation should eliminate dimerization with only a minor perturbation of the vWF-A structure, as both residues are polar and small. (2) The examination of vWF-A crystal structures showed that the two C-terminal residues of the vWF-A-218 domain were Met and Ile, both of which are hydrophobic and solvent-exposed. In addition, the C-terminal α -helix A12 was found to have been truncated (see Figure 1 of [11]). Since these may account for the reduced structural stability of vWF-A-218, the addition of the next four residues in factor B (DESQ) to vWF-A-218, all of which are hydrophilic, should assist α -helix formation and the stability and solubility of the vWF-A domain.

Primers were designed to adapt the vWF-A-218 domain to incorporate these two modifications in two steps, the final product being denoted vWF-A-222 (Table 1). The first and second mutations were verified by experimental observation of the expected changes in the restriction digestion pattern of the plasmid by *Eco*R1 and *Spe*I (first mutation) and by *Bfa*I (second mutation). In SDS/PAGE analysis of the vWF-A-222 cell culture, the cell lysate contained a major band at around 43 kDa. This fusion product was positive in a Western blot using factor B polyclonal antiserum. After purification (see Materials and methods section), both the cleaved vWF-A-218 and vWF-A-222 domains were again recognized in Western blot analyses using this polyclonal antiserum. By MS, both vWF-A-218 and vWF-A-222 showed a prominent single peak in clean spectra with masses of 24548 and 25002 respectively in MALDI and electro-

Figure 2 Sedimentation equilibrium data for the vWF-A-222 domain

The absorbance values at 280 nm are shown as a function of radial distribution at equilibrium for a loading concentration of 0.8 mg/ml at 20°C and a rotor speed of 15000 rev./min. The data are fitted to a monomer model which is shown as a line through the experimental data points ($\circ$). The corresponding distribution of the residuals in the upper panel ($\bullet$) is small and random, indicating that a good fit solution had been obtained.

spray experiments. These masses were in good agreement with predicted masses of 24562 and 25005 respectively (see Materials and methods section). In large-scale preparations of vWF-A-222, yields were improved to 10 mg/l of culture, no dimerization or precipitation was observed during storage at 4°C and the solubility was increased to about 8 mg/ml in PBS.

Hydrodynamic properties of the recombinant vWF-A-218 and vWF-A-222 domains

Given the occurrence of aggregation and precipitation during storage, it was necessary to demonstrate for the functional analyses below that the vWF-A domain was monomeric in solution. Based on the elution positions of three standard proteins in gel filtration (Materials and methods section), the diffusion coefficient for vWF-A-218 was found to be between 8.4×10^{-7} and $9.9 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$. From this, the Stokes' radius a of vWF-A-218 was found to be $2.4 \pm 0.2 \text{ nm}$. Using sucrose-density-gradient centrifugation (see Materials and methods section) [32], the sedimentation coefficient was found to be $2.4 \pm 0.2 \text{ S}$, based on comparison with four standard proteins. These data gave a vWF-A-218 molecular mass between 22 and 30 kDa, where the range reflects the errors in the determinations of a and $s_{20,w}^0$. The comparison with the expected molecular mass of 25 kDa from the sequence showed that vWF-A-218 was monomeric. The frictional ratio f/f_0 was calculated as 1.1 (see Materials and methods section); since this was close to 1.0 (the value for a sphere), this showed that vWF-A-218 possessed a compact structure.

To confirm quantitatively that the vWF-A-218 and vWF-A-222 domains were monomeric over the concentration range used for functional and structural studies, both were subjected to sedimentation equilibrium analysis (Figure 2). Curve fits on the

Figure 3 Effect of the recombinant vWF-A domain on the binding of ^{125}I -labelled factor B to C3b-thiol-Sepharose

In each tube 50 μl of C3b-thiol-Sepharose (containing 5 μg of C3b) was pre-incubated with serial 2-fold dilutions of intact unlabelled factor B ($\blacktriangle$), vWF-A domain ($\square$) or ovalbumin ($\bullet$) (200 μl ; maximum concentration 125 $\mu\text{g}/\text{ml}$) for 2 h at room temperature. The assays were all carried out in 10 mM Pipes (sodium salt)/0.2 mM MgCl_2 /30 mM NaCl/1 mg/ml BSA, pH 7.0. ^{125}I -labelled factor B (4×10^5 c.p.m.) was then added to each tube and the bound radioactivity was measured after a 1 h incubation at room temperature and five washes with the binding buffer.

basis of a single monomeric species in a concentration range between 0.3–0.9 mg/ml gave molecular masses of 24000 ± 2000 for vWF-A-218 and 27000 ± 2000 for vWF-A-222, both of which agree with the expected values of 24 500 and 25 000 respectively. Sedimentation velocity experiments gave an $s_{20,w}^0$ value of 2.5 ± 0.3 S for the vWF-A-218 and vWF-A-222 domains. Calculations of the sedimentation coefficient from the homologous vWF-A crystal structure from CR3 gave values of between 2.5 and 2.6 S. This comparison with the experimental values showed that the recombinant domains possessed monomeric structures that were similar to the CR3 crystal structure.

CD, Fourier-transform IR spectroscopy and NMR spectroscopy on the vWF-A-218 and vWF-A-222 domains demonstrated nearly identical results, which showed that both domains exhibited folded protein structures with α -helix and β -sheet [7]. This showed that the three-dimensional structure of vWF-A had not been perturbed in modifying the original expression vector.

Functional binding of the vWF-A-218 domain to C3b

The availability of a structurally characterized recombinant vWF-A-218 domain provided a unique opportunity to study its functional activity, as work on the isolated vWF-A domain had not previously been possible. Direct binding of ^{125}I -labelled vWF-A-218 to C3b-thiol-Sepharose was first investigated. Approx. 100 μg of purified vWF-A domain was radiolabelled to a specific activity of 1.2×10^6 c.p.m./ μg using the lactoperoxidase method, and a test assay was set up to investigate whether direct binding of the vWF-A domain to C3b-thiol-Sepharose could be demonstrated. No direct binding of ^{125}I -labelled vWF-A was

Figure 4 Representation of the factor D cleavage site in the recombinant vWF-A domain sequence

noted. Binding to C3b-thiol-Sepharose was no higher than to a control preparation of thiol-Sepharose which contained no C3b.

The potential interaction of the vWF-A-218 domain with C3b was then studied by a more sensitive indirect binding assay, based on the assessment of its ability to compete with ^{125}I -labelled factor B for binding to C3b attached to thiol-Sepharose. Using this, direct binding of ^{125}I -labelled factor B to C3b-thiol-Sepharose was observed in the presence of Mg^{2+} ions. Competition binding studies were carried out (Figure 3). The assay was validated using unlabelled factor B as a positive control and an irrelevant protein (ovalbumin) as a negative control. The maximum inhibition obtained with unlabelled factor B was close to 100% as expected. The recombinant vWF-A-218 domain was able to inhibit the binding of ^{125}I -labelled factor B to C3b-thiol-Sepharose to a maximum of 60%. This was achieved with 6.25 μg of vWF-A domain that was approx. an 8-fold molar excess over C3b. The same level of inhibition was achieved with approx. 4 μg of Factor B, a 1.7-fold molar excess over C3b.

Effect of factor D on vWF-A-218 and vWF-A-222

To test whether factor D could cleave the vWF-A-218 domain in the presence of C3b in the fluid phase, conditions were used in which factor D cleaves intact factor B. A mixture of vWF-A-218, C3b and factor D (in 10 mM Pipes/30 mM NaCl/0.2 mM MgCl_2 , pH 7.0) was placed in a 1.5 ml tube at a 50:25:1 ratio by mass in a final volume of 200 μl [vWF-A (100 μg)/C3b (50 μg)/factor D (2 μg)] and incubated for 4 h at 37 $^\circ\text{C}$. Cleavage of vWF-A-218 by factor D would result in the removal of an eight amino acid peptide (GSGEQQKR; Figure 4), so that the cleaved domain would have the new N-terminus KIVLDPSG. N-terminal sequence analysis (see Materials and methods section) was carried out on vWF-A-218 which had been incubated with C3b and factor D. Comparative sequencing was also carried out on vWF-A-218 which had been incubated with C3b only. The results obtained indicated that 10% of the vWF-A domain had been cleaved in the sample incubated with both C3b and factor D, whereas no cleavage was detected in the control in which factor D was omitted. These results suggest that factor D is capable of cleaving the vWF-A domain in the presence of C3b.

To test whether the vWF-A domain could be cleaved by factor D in the absence of C3b, vWF-A-218 and vWF-A-222 (50 μl at 1 mg/ml) were incubated with recombinant factor D (1 μl of factor D at 1 mg/ml) in PBS at 37 $^\circ\text{C}$ and pH 7.5 for 24 h. The products were dialysed into water, then mixed with matrix solution and applied to a mass analyser (see Materials and methods section). In both cases, the incubation resulted in the slow production of a cleaved vWF-A domain with a mass

Figure 5 Effect of factor D on vWF-A-218 and vWF-A-222 by mass analysis

Mass spectra from (a) the vWF-A-218 and (b) the vWF-A-222 domains are shown. Molecular mass values are reported for each peak, based on internal calibrations against bovine β -lactoglobulin A (18363 Da). In each of (a) and (b), the upper trace corresponds to the purified domain and the lower trace to that of the domain after treatment with factor D for 24 h. In both lower traces, a second peak with a reduced molecular mass is visible and corresponds to the cleaved vWF-A domain when each is compared with its respective upper trace.

reduction of 796 ± 73 (mean $\pm$ S.D.; three measurements) (Figure 5). This agreed well with the expected mass reduction of 871 based on the removal of the N-terminal eight-residue peptide in the recombinant domains (Figure 4). In both cases, peaks corresponding to the uncleaved domain were also visible (Figure 5). Even if it was assumed that the efficiency of desorption was equal for cleaved and uncleaved vWF-A-218 and vWF-A-222, this result suggested that cleavage had occurred, and that factor D was active against the free vWF-A domain in the absence of C3b.

DISCUSSION

In this study, a full-length cDNA clone for factor B was used to amplify the 218 residues encoding the vWF-A domain (residues 229–444) by PCR, and this resulted in expression with high yield. Expression was validated from SDS/PAGE molecular-mass determination and amino acid sequencing. This recombinant system corresponded to the exon structure of the gene and gave monomeric protein in gel filtration and sucrose-density centrifugation, but led to slow disulphide-linked dimerization and aggregation and precipitation during storage. The inspection of our alignment for 75 vWF-A sequences and our analysis of the vWF-A crystal structure [7,11–13] enabled us to develop rationally a modified recombinant system in which a Cys²⁶⁷ $\rightarrow$ Ser mutation had been performed and the C-terminus of the vWF-A-218 domain had been lengthened by four residues to incorporate a C-terminal α -helix. With these changes, the protein stability was improved, and monomeric protein in solution was obtained (Figure 2). In illustration of the improvement, crystallization trials with vWF-A-222 have yielded larger better-formed crystals compared with the large number of small crystals

obtained with vWF-A-218 (J. H. and S. J. P., unpublished work), and structural studies have been performed [46]. The observation of a compact solution structure is in agreement with the crystal structures of homologous vWF-A domains [12,13]. It is also consistent with the observation of three- and two-lobed structures for intact factor B and the Bb fragment by electron microscopy, since it can be assumed that the vWF-A domain would form one of these lobes. The production of eight contiguous vWF-A domains from the α -3 chain of collagen VI has been achieved using a eukaryotic expression system [47]. There, the structure of the expressed fragment was visualized to contain eight small globules using electron microscopy, which is consistent with the results on vWF-A domains and factor B.

The production of the vWF-A-218 domain enabled its functional binding to C3b to be studied. The studies carried out here agree with earlier suggestions for the role of the vWF-A domain in factor B [20]. Since the present work shows that the vWF-A domain can specifically inhibit the binding of factor B to C3b, it contains a binding site for C3b. Although the domain was unable to bind to C3b–thiol-Sepharose directly, it was able to compete with ¹²⁵I-labelled factor B for binding, the inhibition being maximally $\sim 60\%$ in the presence of 0.2 mM Mg²⁺. The negative results from the direct binding studies are best explained by postulating that the binding of the vWF-A domain to C3b is weak and that it dissociates during the washing steps. Interaction of Ba with C3b was detected by studying inhibition, by Ba, of formation of the convertase C3bBb, but direct binding of unmodified Ba to C3b was not observed [17]. These observations are characteristic of a complex formed by multiple weak binding interactions, not one formed by a single-site high-affinity interaction.

The availability of the recombinant vWF-A domain also provided insight into the cleavage of factor B by factor D. Factor D circulates in its active form as there is an apparent absence of a zymogen form in blood. The enzyme is also highly specific, as it cleaves its only known substrate, factor B, only when factor B is bound to C3b or C3(H₂O) [44]. Several hexapeptides corresponding to the factor B sequence surrounding the bond that is cleaved by factor D were studied [48]. The peptides were assessed for their ability to inhibit factor D enzymic activity and for their susceptibility to cleavage by several SPs including factor D. The peptides were all able to inhibit factor B cleavage by factor D, but were not substrates for factor D. Active-site mapping of factor D with peptide thioesters revealed some interesting features [19,49]. Factor D was able to express estero-lytic activity against some Arg thioesters, but its catalytic efficiency was found to be three or four orders of magnitude below that of trypsin and C1s. It was suggested on the basis of these results that the active site of factor D as it exists in serum has a zymogen-like conformation, which has an obstructed binding site. This view is supported by recent crystal structures for factor D which revealed a self-inhibitory loop in its structure [50]. The active conformation is then induced by its substrate the C3b–factor B complex.

Since the recombinant vWF-A domains contain factor D cleavage sites, the effect of factor D on vWF-A in the presence and absence of C3b was studied by two independent methods. It is significant that factor D was observed to cleave vWF-A slowly on its own in both cases, since this indicates that the free vWF-A domain is an adequate substrate for factor D. In contrast, if intact factor B was incubated with factor D in the presence of C3b, more than 95% of the factor B was efficiently converted into Ba and Bb. One possible explanation for the different extents of cleavage of free vWF-A domain and factor B is that the affinity of vWF-A for C3b is much less than that of intact

factor B, because of the existence of fewer contact points (Figure 3). This would mean that the concentration of the C3b-vWF-A complex is much lower than that of the C3b-factor B complex, so the cleavage of the C3b-vWF-A complex would be slower. Another possible explanation is based on the existence of two alternative conformations of the vWF-A domains. General evidence for these in the vWF-A superfamily is found from the distinct locations of mutation sites on the same vWF-A domain that corresponds to type 2B and 2M von Willebrand's disease [9], and several crystallographic studies of different structures in the presence and absence of metal in the vWF-A active site [13,51]. Thus, if the vWF-A domain of factor B has two conformations that depend on whether it is bound to C3b or to the remainder of the factor B structure, and only the C3b-bound vWF-A conformation is efficiently cleaved by factor D, the less efficient cleavage of the isolated vWF-A domain may correspond to the existence of both conformations of the isolated vWF-A domain when this is free in solution, only one of which is cleavable by factor D. Further kinetic and structural studies are required to resolve these questions.

The production of the isolated vWF-A domain from factor B has provided interesting insights into its previously uncharacterized function. Proteins that are members of the vWF-A domain superfamily have a wide variety of ligand-binding roles [6]. The vWF-A domain from factor B can now be included in the list of vWF-A domains that have ligand-binding functions.

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