

Expression of functional recombinant von Willebrand factor-A domain from human complement C2: potential binding site for C4 and CRIT

Kwok-Min HUI*‡, George L. ORRISS†, Tilman SCHIRMER†, Bergljót MAGNADÓTTIR§, Jürg A. SCHIFFERLI*, Jameel M. INAL*‡

*University Hospital Basel, Immunonephrology, Department of Research, Hebelstrasse 20, CH-4031 Basel, Switzerland, †Division of Structural Biology, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland, and §Institute for Experimental Pathology, University of Iceland, Keldur v. Vesturlandsveg, IS-112 Reykjavík, Iceland

‡Corresponding authors:

Kwok-Min Hui, University Hospital Basel, Immunonephrology, Department of Research, Hebelstrasse 20, CH-4031 Basel, Switzerland. E-mail: kwok-min.hui@unibas.ch

Jameel M. Inal, University Hospital Basel, Immunonephrology, Department of Research, Hebelstrasse 20, CH-4031 Basel, Switzerland. E-mail: jameel.inal@unibas.ch

Running title: C4 and CRIT interacting with C2 vWFA-214 domain

Abbreviations used: vWFA, von Willebrand factor type A; SP, serine protease; NHS, normal human serum; CRIT, complement C2 receptor inhibitor trispanning.

SYNOPSIS

Complement C2 receptor inhibitor trispanning (CRIT) is a newly described transmembrane molecule capable of binding C2 via its first extracellular domain (ed1). CRIT competes with C4b for the binding of C2. Previous experiments have suggested that a major binding site for C2 is located on short almost identical peptide sequences of CRIT-ed1 and the β -chain of C4. The C2 domains involved in these bindings, however, remain unknown. We cloned the von Willebrand factor-A (vWFA) domain of C2 as it is a region likely to be involved in interactions with other proteins, and were able to functionally express the 25 kDa human complement C2 vWFA domain (amino acid 224-437). The recombinant vWFA protein fixed on MagneHis Ni-particles bound C4 in normal human serum. The C4 α , β and γ chains were separated by SDS-PAGE and purified separately by electro-elution. The purified C4 chains were then used in a sandwich ELISA, which showed the vWFA to bind C4 only via the C4 β -chain. In a haemolytic assay, the recombinant vWFA protein inhibited complement activation by the classical pathway in a dose dependent manner by competing with native C2 for binding to C4b. vWFA bound the ed1 peptide of CRIT as well, and specifically the 11 amino acids peptide fragment of ed1 known to interact with whole C2. These findings show that the vWFA domain is centrally involved in the C2/CRIT and C2/C4b bindings. The cloned vWFA domain will allow us to dissect out the fine interactions between C2 and CRIT or C4b.

Key words: complement C2, recombinant vWFA domain, CRIT, expression, classical pathway, haemolytic assay.

INTRODUCTION

In general the classical pathway (CP) of complement is activated by the formation of immune complexes that bind C1 and allow a conformational change to occur in the C1 macromolecular complex so that C1r and then C1s are cleaved. Activated C1s then cleaves C4 with formation of a major fragment C4b. This fragment is endowed with a novel property, which is to bind native C2 in the presence of Mg^{2+} . In turn C1s cleaves C2 into C2a and C2b, with the larger fragment, C2a remaining attached to C4b. When bound to C4b, C2a is a newly formed enzyme capable of cleaving C3. C4bC2a is the CP C3 convertase. In humans the early activation of the CP is regulated by C1 inhibitor in the fluid phase, but until recently no inhibitor preventing the formation of the CP C3 convertase was known to be present on cell surfaces. Complement C2 receptor inhibitor trispanning (CRIT) is a novel human complement inhibitor expressed on haemopoietic cells and a wide range of tissues [1]. The denomination trispanning was chosen because CRIT has 3 transmembrane domains with one end of the molecule being extracellular. This extracellular domain, called extracellular domain 1 (ed1) is a binding site for C2. After binding, C2 is protected by CRIT from cleavage by activated C1s. Synthetic peptides of CRIT corresponding to ed1 and to an 11-mer peptide derived from the C-terminal end of ed1 were shown to have a strong inhibitory effect on the CP of complement activation [2, 3]. This inhibition is best explained by competition of ed1 of CRIT with C4b for C2 binding, and by the blockade of C2 cleavage by C1s.

Very little is known about the topology and structure of the sites of C2, which lead to the assembly of the C4b-C2 and the CRIT-C2 complexes. Human C2 is a 102 kDa serum glycoprotein, 39% identical to its functional homologue factor B (FB) [4]. The single polypeptide chain C2 is folded into three globular domains that were identified by electron microscopy [5]. The three complement control protein modules (CCP1, CCP2 and CCP3) forming the N-terminal domain correspond to C2b. The larger C2a portion of the molecule is composed of a von Willebrand factor-A (vWFA) domain and a serine protease (SP) domain at the C-terminal end. The initial binding of C2 to C4b occurs via two low affinity sites [6]. The first site located on the C2b domain is Mg^{2+} independent [7, 8], whereas the second site on the vWFA domain of C2a is Mg^{2+} dependent [9]. Nothing is known about the site(s) of C2 interaction with CRIT, although it might be speculated that the vWFA domain is responsible.

vWFA domains are a family comprising molecules of approximately 200 amino acids, most of which are components of the extra-cellular matrix and very often are the sites for protein-protein interaction in cell adhesion proteins, such as integrins [10, 11]. There is a highly conserved metal ion-dependent adhesion site (MIDAS) motif among 46% of all vWFA domains, which is involved in ligand binding [11, 12]. In 1998, Schultz *et al* [13] compiled the SMART database (<http://coot.embl-heidelberg.de/SMART>), which lists all the known vWFA domains; 601 vWFA domains in 452 proteins. Through the intensive research of vWFA domains over the past six years, there has been a drastic increase in the number of vWFA domains and now 2863 vWFA domains in 2230 proteins are listed in the SMART database. Fifty-three structures containing vWFA domains have been solved as the important widely expressed vWFA domain has been drawing an increasing degree of attention.

The recombinant expression system of the vWFA domain from human complement FB was successfully established in 1999 [14] and its crystal structure, which reveals an integrin-like open conformation solved in 2004 [15]. However, unlike its functional homologue, FB, recombinant expression of C2 or its fragment has proved difficult and use of similar expression strategies (as for FB) has not worked well. Purification of C2 from human serum using antibody affinity chromatography [16] can only produce a reasonable amount of pure C2 for functional studies. Unfortunately, that quantity and quality of purified C2 from human serum will never be sufficient for structural studies, such as NMR and X-ray crystallography.

Study of the biological function of the vWFA domain in C2 requires the use of a suitable recombinant protein. In the present study, we report a successfully developed recombinant expression system for a functional vWFA domain in C2, which can aid in more precise studies of C2/C4 and C2/CRIT interactions [1, 2]. The most important issue will be the opportunity to perform the structural studies of C2 vWFA domain complexed with peptides corresponding to the binding site on C4 (a known specific sequence on C4 β chain) or on CRIT (ed1 or H17) [2].

EXPERIMENTAL

Material

The complement proteins C2 and C4 and the anti-C4 polyclonal antibody were purchased from Juro Supply AG (Switzerland). Sheep erythrocytes and Amboceptor were from Bode Behring (Marburg, Germany). MagneHis protein purification system, Wizard plus minipreps kit and restriction enzymes were purchased from Promega (Switzerland). The human liver total RNA was from Ambion (Huntingdon, UK). Superscript III reverse transcriptase and AccuPrime *Pfx* DNA polymerase were obtained from Invitrogen (Switzerland). The EDTA-free protease inhibitor cocktail tablets came from Roche (Penzberg, Germany). The CRIT synthetic peptides:

ed1	NH ₂ - MSPSLVSDTQKHERGS	<u>HEVKIKHFSPY</u>	-CO ₂ H
H17	NH ₂ -	<u>HEVKIKHFSPY</u>	-CO ₂ H
H17-2	NH ₂ -	<u>HEVKIKHFSPYHEVKIKHFSPY</u>	-CO ₂ H

were described elsewhere [2]. Underlined is the 11 amino acids sequence known to interact with C2. This sequence is double in H17-2. All other analytical grade reagents were purchased either from Sigma or Fluka Biochemika (Switzerland).

Buffers

TEDP buffer contained 20 mM Tris-HCl (pH7.6), 1 mM EDTA, 1 mM DTT and 0.001% PMSF. Ni²⁺-NTA chromatography buffer contained 20 mM Tris-HCl (pH 8), 400 mM NaCl and 2 mM β-mercaptoethanol. HiLoad 16/60 Sephadex 75 gel-filtration chromatography buffer made up with 20 mM Tris-HCl (pH 8), 100 mM NaCl, 5 mM MgCl₂ and 2 mM DTT. GVB buffer (gelatin/veronal buffer) was prepared by mixing 10 ml of 10 % (w/v) gelatin, 200 ml of 5X VB buffer (containing: 727 mM NaCl, 9 mM Na barbitone and 3.1 mM diethylbarbituric acid; pH 7.4) and 790 ml water. GVB²⁺ buffer was prepared by mixing 200 ml GVB with 1 ml of 30 mM CaCl₂ and 2 ml of 100 mM MgCl₂.

Electrophoresis and immunoblotting

Electrophoresis was conducted using mini-gel systems (Bio-Rad, Hercules, CA) under reducing conditions. Proteins were separated by SDS-PAGE on 12 % gels. Immunoblotting to nitrocellulose membrane (Amersham Bioscience, UK) was performed as described previously [2].

Construction, expression and purification of vWFA-214

Oligonucleotides were designed to flank the region of interest incorporating an *Nde*I restriction site and 6X His-tag at the 5'-end and *Eco*RI restriction site at the 3'-end for cloning and purification purposes. 1 µg total human liver RNA was used to synthesize the first-strand cDNA using Superscript III reverse transcriptase with oligo(dT)₂₀ following the manufacturer's recommended protocol. The synthesized cDNA was used to amplify the region of interest by PCR with the above synthetic oligonucleotides. The amplified PCR product (672 bp) was digested with *Nde*I and *Eco*RI and ligated into the pRUN vector (derived from pBR32), which was pre-digested with the same restriction enzymes. The ligation product was then transformed into XL1-Blue competent cells. The DNA sequence of the positive clone was verified by automated DNA sequencing, which was carried out on an ABI PRISM 3100 genetic analyzer using the ABI PRISM dye terminator ready reaction cycle sequencing kit (version 3, Applied Biosystems, Switzerland) in both directions. Since this recombinant product contained 214 residues, it was denoted as vWFA-214.

vWFA-214 was expressed in the *E. coli* strain C41 (DE3) and pre-cultured at 25 °C overnight. The overnight culture was diluted approximately 1:10 with fresh Luria broth medium containing 100 µg/ml ampicillin and the cells were grown at 18 °C until an absorbance A_{600} of 0.9 was attained. The cultures were then induced overnight with 0.2 mM isopropyl β-D-thiogalactoside at 16 °C. Cells were harvested in a Sorvall GS-3 rotor at 6000 g for 10 min and frozen at -20 °C until required.

Cell pellets from 1.2 L culture were thawed and resuspended in 30 ml ice-cold TEPD buffer with addition of one protease inhibitor cocktail tablet. The cells were then lysed by three passages through a French Pressure Cell (SLM Instrument, Urbana IL, USA) and the lysate was centrifuged in a Sorval SS34 rotor at 40,000 g for

30 min. Streptomycin sulphate (10%, w/v) was added dropwise to the lysate to a final concentration of 1 % and stirred on ice for 20 min. The suspension was cleared by centrifugation at 40,000 *g* for 30 min. The supernatant was dialyzed against the Ni²⁺-NTA chromatography buffer at 4 °C overnight. The next day, the dialyzed sample was centrifuged at 40,000 *g* for 30 min to remove debris. The supernatant was incubated with 8 ml Ni²⁺-NTA resin (Qiagen, Switzerland) at room temperature for 1 h with gentle shaking, which was equilibrated with 40 ml Ni²⁺-NTA chromatography buffer, with addition of 30 mM imidazole. After incubation, the Ni²⁺-NTA resin was washed thoroughly with Ni²⁺-NTA chromatography buffer supplemented with 30, 60, and 90 mM imidazole respectively, until the absorbance A₂₈₀ became zero. Then, the recombinant vWFA-214 was eluted with Ni²⁺-NTA chromatography buffer supplemented with 150 mM imidazole and collected in 1.5 ml fractions. The pooled fraction was dialyzed against the HiLoad 16/60 Sephadex 75 gel filtration chromatography buffer at 4 °C overnight. After dialysis, the partially purified recombinant vWFA-214 was centrifuged at 40,000 *g* for 30 min and concentrated using a Millipore Centricon 10 unit at 4 °C, to 1 ml. The concentrated sample was loaded onto a calibrated FPLC HiLoad 16/60 Sephadex 75 gel-filtration column (Amersham Bioscience, UK). The partially purified vWFA-214 was further purified by gel filtration at a flow rate of 1 ml/min and collected in 2 ml fractions. As assessed by SDS-PAGE, the fractions containing pure vWFA-214 were pooled and stored at -20 °C.

Antibodies

Polyclonal rabbit anti-CRIT-ed1 antibody was derived as described before [2]. 200 µg of KLH-CRIT-ed1 in PBS, emulsified with CFA was administrated on day 21 and with IFA on day 28, and was followed by subsequent injections with 100 µg of KLH-CRIT-ed1 on days 35, 50 and 60. At day 60, rabbit polyclonal anti-CRIT-ed1 serum was affinity-purified using an ed1 epoxy-activated Sepharose 6B (Sigma-Aldrich, Switzerland) column.

Polyclonal anti-vWFA antibody was produced in mouse ascitic fluid according to a method described elsewhere [17, 18]. In brief, 10 µg of purified vWFA-214 was electrophoresed on SDS-PAGE and the gel was stained with 0.01 %

Coomassie blue for 30 min. After staining, the gel was washed four times in distilled water for 15 min each. The band containing vWFA-214 was excised and emulsified in 200 μ l 1X PBS by passing ten times through a 1 ml syringe and finally several times through a 21 G needle. The emulsified gel was injected intraperitoneally into Balb/c mice.

MagneHis Ni-Particles pull-down assay

MagneHis Ni-Particles pull-down assays were performed according to the manufacturer's protocol (Promega, Switzerland) with some modifications. Briefly, 3 μ g of purified recombinant vWFA-214 was diluted in 50 μ l binding/wash buffer included in the kit and mixed with 20 μ l MagneHis Ni-Particles at room temperature for 30 min with gentle shaking. After incubation, the tube was placed in a magnetic stand for 1 min to allow the MagneHis Ni-Particles to be captured by the magnet and the supernatant was removed. The MagneHis Ni-Particles were washed three times with the binding/wash buffer. 5 μ g of pure C4 protein (diluted in 50 μ l binding/wash buffer) or 50 μ l of freshly prepared normal human serum (NHS; 1:50 dilution) was added and incubated for 20 min. After washing three times, the vWFA-214-C4 complex was eluted, subjected to SDS-PAGE, and detected by Western blotting with polyclonal antibody against C4.

Haemolytic assay

The inhibition of CP based haemolysis was conducted as described elsewhere [19, 20]. In brief, sheep erythrocytes were washed three times with ice-cold GVB/10 mM EDTA. The antibody sensitized sheep erythrocytes (EA, 2×10^8 cell/ml) were prepared by mixing equal volume of washed sheep erythrocytes and Amboceptor (1:40 dilution), which was incubated for 30 min at 37 °C. Different amounts of purified vWFA-214 (5, 10, and 20 μ g) were mixed with different volumes of diluted NHS (1:100 dilution; 0, 20, 40, 60, 80, 100 and 120 μ l) and made up to 150 μ l with freshly prepared GVB²⁺ buffer, which was then pre-incubated for 30 min at room temperature. After the pre-incubation, 50 μ l EA was added and incubated for 30 min at 37 °C. Background control was obtained by incubating NHS with GVB²⁺ buffer

and 100 % lysis was determined by adding distilled water to EA. The degree of complement haemolysis was determined by measuring the absorbance of the supernatant at 414 nm on a SpectraMAX 190 microtitre plate reader (Molecular Devices, Sunnyvale, CA).

Purification of C4 chains

Pure C4 protein was separated by SDS-PAGE on a 12 % gel under reducing conditions. After electrophoresis and destaining, the bands containing C4 α , β and γ chains were excised separately. The C4 chains were eluted from the gel with the Millipore Micro-Electro-eluter according to the manufacturer's protocol. Electro-elution was carried out using the Millipore Centricon YM-10 at 150 V for two hours. The concentration of the eluted C4 chains was estimated by the Bradford reagent (Bio-Rad, Hercules, CA).

Sandwich ELISA

The microtiter plate was coated with 100 μ l diluted anti-vWFA antibody (1:1000 in 0.1 M Bicarbonate buffer, pH 9) overnight at 4 °C. The plate was emptied and washed five times with wash buffer (0.05 % Tween-20 in PBS). The unoccupied sites were blocked with blocking buffer (PBS with 0.2 % Tween-20 and 2 % BSA) for 1 h. vWFA-214 was diluted in blocking buffer and 100 μ l was added to each well (1 μ g per well) and incubated at RT for 1 h. Next, 100 μ l diluted C4 or purified C4 chains (0.5, 2.5, 5 or 25 nM) was added and incubated for 1.5 h. A 1:5000 dilution of anti-C4 antibody was prepared and 100 μ l was added and incubated for 1 h. Then, the anti-goat HRP secondary antibody (1:3000) was added to each well and incubated for 45 min. After this step, the plate was washed thoroughly and 100 μ l tetramethylbenzidine substrate (TMB substrate reagent set, BD Bioscience, San Diego, CA) was added to each well and incubated for approximately 5-10 min, until the wells turned blue. 50 μ l of 2 N H₂SO₄ was added to each well to stop the reaction. The binding activity was determined by measuring the absorbance at 450 nm. All the incubations were carried out at room temperature and after each incubation step, the plate was washed at least five times with washing buffer. The samples and antibodies were diluted with

blocking buffer. A background control was included either without vWFA-214 or native C4/C4 chains.

vWFA-214-CRIT ligand blotting

vWFA-214 (2 μ g) was mixed with CRIT peptides (1.5 μ g) in PBS and incubated on ice for 2 h. Disuccinimidyl suberate (DSS) cross-linker (Perbio Science, Switzerland) was dissolved in DMSO and added to the reaction mixtures to a final concentration of 2 mM. The reaction mixtures were incubated for a further 2 h on ice. At the end of the incubation, 1 M Tris-HCl, pH 7 was added to a final concentration of 40 mM to quench the reaction. The vWFA-214-CRIT complexes were analyzed by probing with anti-CRIT antibody in Western blots. For the blocking experiment, vWFA-214 (2 μ g) was pre-incubated with anti-vWFA antibody (1:500 dilution) on ice for 2 h and the ligand blot was carried out as described above.

RESULTS AND DISCUSSION

Expression and purification of the vWFA-214 domain of C2

Recombinant expression of C2 or its fragment proved to be difficult. Unlike its functional homologue FB, in which Cys²⁶⁷ is absent, the yield and solubility of C2 vWFA domain cannot be improved by introducing a Cys²⁶⁷ → Ser mutation [14]. Even though there is another free Cys (residue 241) in the C2 vWFA domain, Cys²⁴¹ was shown to be essential for the normal assembly and decay of C3 convertase [9, 21, 22]. The expression of vWFA-214 at 37 or 30 °C, resulted in the serious problem of inclusion body formation. To overcome this problem, we expressed vWFA-214 at 16 °C and the result was a good level of expression (6-8 mg per liter of bacterial culture). The solubility of vWFA-214 was very good, with no aggregation or precipitation occurring during short-term storage at 4 °C or long-term storage at -20 °C (1 mg/ml or 15 mg/ml).

The recombinant vWFA-214 (214 amino acids) domain was expressed as a His-tag fusion protein. The predicted molecular mass was 25 kDa. By matrix-assisted laser adsorption ionization (MALDI) MS, vWFA-214 showed a prominent single peak in clean spectra with mass of 25244, which was in good agreement with the predicted mass of 25252. Figure 1A shows the SDS-PAGE analysis of vWFA-214 after both Ni²⁺-NTA and gel filtration chromatography. The yield was estimated using both the Bradford reagent and the absorbance at 280 nm at 6-8 mg per liter of bacterial culture. The purified vWFA-214 protein was used to raise the polyclonal antibody in mouse for the functional studies. Figure 1B shows the specificity of this anti-vWFA antibody against C2 and the recombinant vWFA-214.

Functional binding of vWFA-214 to C4

The recombinant vWFA-214 domain provided a unique opportunity to study its functional activity as work on the isolated vWFA domain had not previously been possible. The interaction between vWFA-214 domain and C4 was first investigated by a pull down assay using MagneHis Ni-particles. The vWFA-214 immobilized onto the MagneHis Ni-particles interacted with C4 and was successfully pulled down either

pure C4 (Figure 2A) or C4 in NHS (Figure 2B). In order to show that C4 was present in the elution from vWFA-214-NHS pull-down assay, Western blotting was performed. After probing with anti-C4 antibody it was clearly shown that C4 in NHS interacted with and was pulled down by the immobilized vWFA-214. The negative control, pull-down assay with MagneHis Ni-particles and NHS without vWFA-214, showed that C4 in NHS did not interact with MagneHis Ni-particles (Figure 2B, lane 3). The low intensity of the band corresponding to the C4 α chain (Figure 2B, lane 2) is probably due to degradation by Factor I during incubation times. In the presence of Factor H or C4-binding protein in NHS, Factor I degrades the C4 α chain into C4d and α 4 [23].

In using C2 deficient serum (obtained from a patient with type I complement C2 deficiency) in the haemolytic assay, vWFA-214 could not restore the haemolytic activity because of the absence of the serine protease domain [6, 24]. Figure 3 shows the inhibitory effect of vWFA-214 on the CP of complement activation. In the haemolytic assay using NHS, the recombinant vWFA-214 domain was able to inhibit the haemolysis by competing with intact C2 in NHS for C4b binding in a dose dependent manner. In the presence of 2.5 μ g vWFA-214, there was minimal inhibition of haemolysis (data not shown). 8.26 % of inhibition was observed with 5 μ g vWFA-214 and this increased to 20.54 % and to a maximum 44.93 % with 10 and 20 μ g vWFA-214 respectively.

By site-directed mutagenesis, Horiuchi *et al* [9] showed that Cys²⁴¹ in the C2 vWFA domain is important for C4b binding and predicted the presence of a C4b binding site within the vWFA domain region around Cys²⁴¹. Although site-directed mutagenesis is a powerful tool to study protein-protein interaction, substitution of important amino acid(s) may result in conformational change of the binding site, which eventually loses its binding activity. In the current study, we clearly demonstrate the presence of a C4 binding site in the isolated C2 vWFA-214 domain. Further experiments may be needed to delineate the exact binding site of C4 in the vWFA-214 domain.

vWFA-214/C4 interaction through C4 β chain

Having confirmed the presence of a C4 binding site on the functional recombinant vWFA-214 domain, we were curious to define which chain(s) of C4 mediate(s) the vWFA-214/C4 interaction. To study this, C4 was separated into C4 α , β and γ chains by SDS-PAGE and each C4 chain was purified by electro-elution. Figure 4A shows the SDS-PAGE of the purified C4 chains. Western blotting, probed with anti-C4 antibody, was also performed to confirm that the anti-C4 antibody recognized all three purified C4 chains (Figure 4B). Sandwich ELISA was then performed to investigate the interaction between vWFA-214 and the purified C4 chains using pure C4 as a positive control. Figure 4C shows the binding curves generated from the sandwich ELISA data. For C4 α and C4 γ chains, there is only negligible interaction with the vWFA-214 domain. By contrast, C4 β chain has significant binding with vWFA-214 domain, which saturated similarly to the native C4. On the basis of this, we conclude that the vWFA-214 domain most probably interacts with C4 via its β chain.

Since there are other demonstrated or suggested C4b binding sites on C2 (respectively on C2b) [7-9] and SP domains of C2a [25, 26], we compared the binding of intact C2 and recombinant vWFA-214 protein for C4/C4 chains. The binding curves for C2 were however almost identical to those observed for recombinant vWFA-214 with binding to intact C4 and the C4 β chain only. Although these experiments do not allow an evaluation of the possible contribution of additional binding sites, the interaction between vWFA and the C4 β chain appears to be central to explain the binding of C2 to C4b at equilibrium. The structural study of vWFA-214 domain should help to better define this binding.

Functional binding of vWFA-214 to CRIT peptides

Previously we have described that CRIT binds to the C2a portion of C2 and inhibits the formation of the CP C3 convertase [2]. We tested whether the recombinant vWFA-214 domain bound to CRIT using cross-linking agent (DSS). CRIT peptides of different sizes but including the C2 binding site on ed1 (ed1, H17 or H17-2) were first

incubated with vWFA-214. Then the complexes formed were cross-linked with DSS, immunoblotted and probed with anti-CRIT antibody. Since this antibody was raised against whole ed1, it reacted against all three peptides. As shown in Figure 5A, the CRIT peptides (ed1, H17 and H17-2) bound to vWFA-214 and the signals were specific. In a separate set of experiments, the binding of CRIT/vWFA-214 could be blocked by pre-incubation with polyclonal anti-vWFA antibody (Figure 5B). These results indicated that the vWFA domain of C2 binds the peptide sequence of CRIT known to interact with C2.

The amino acid sequence of CRIT-ed1 and CRIT-H17 show 35 % and 55 % identity with a specific sequence in the C4 β chain (S206-Y232) respectively [2]. In addition, Horiuchi *et al* [9] showed by site-directed mutagenesis that there is evidence for a C4b binding site around residue Cys²⁴¹ of the C2 vWFA domain. In view of this, there is a high possibility that both CRIT and the C4 β chain act on the same or similar region within vWFA-214 domain, possibly the perfectly conserved MIDAS motif. The structural study by X-ray crystallography with co-crystallization of vWFA-214/CRIT and vWFA-214/C4 β chain peptide complexes should give an insight into their common region of interaction. In addition, the ligand-binding function of C2 vWFA domain can now be included in the SMART database of the vWFA domain superfamily.

In conclusion, the vWFA-214 domain of C2 appears to play a central role in the interactions of C2 with C4 and CRIT. The use of this recombinant domain may help to further clarify the fine molecular interactions between these proteins, a better understanding of which may help to develop a synthetic inhibitor capable of blocking activation of complement by the classical pathway. Such a reagent might be of great clinical utility in diseases mediated by the formation of immune complexes.

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FIGURE LEGENDS

Figure 1 Expression and purification of the recombinant vWFA-214 domain

(A) SDS-PAGE analysis of different stages in the expression and purification of the recombinant vWFA-214 domain. Lane 1, total *E. coli* cell lysate after overnight induction at 16 °C with 0.2 mM isopropyl β -D-thiogalactoside. The major band at approximately 25 kDa represents the His-tag vWFA-214 fusion protein. Lane 2, loss of the 25 kDa His-tag vWFA-214 fusion protein after incubation with Ni²⁺-NTA resin. Lane 3, His-tag vWFA-214 fusion protein eluted with 150 mM imidazole. Lane 4, purified vWFA-214 fusion protein after additional HiLoad 16/60 Superdex 75 gel filtration chromatography. (B) Western blot showing that the antibody raised against vWFA-214 (1:1000 dilution) detects intact C2 and the recombinant vWFA-214 in an *E. coli* cell lysate. Lane 1, 1 μ g intact C2. Lane 2, total *E. coli* cell lysate (lane 1 in Figure 1A). Lane 3, 500 ng purified vWFA-214 fusion protein (lane 4 in Figure 1A).

Figure 2 Interaction of C4 with the recombinant vWFA-214 by MagneHis Ni-Particles pull-down assay.

(A) 3 μ g purified His-tag vWFA protein was bound to the MagneHis Ni-Particles. Pure C4 protein interacted with vWFA protein and was retained on the MagneHis Ni-Particles. The pull-down assay results were analyzed by SDS-PAGE, under reducing conditions, stained with Coomassie Blue. Lane 1, 500 ng vWFA protein. Lane 2, unbound C4 in the flow through. Lanes 3-5, three times 50 mM imidazole washing. Lane 6, elution of C4 from vWFA-214-C4 pull-down assay. (B) C4 of NHS was retained on MagneticHis Ni-particles. Western blotting probed with anti-C4 antibody confirming that C4 present in the elution from vWFA-214-NHS pull-down assay using NHS. Lane 1, 500 ng C4 protein. Lane 2, 20 μ l of elution from vWFA-214-NHS pull down assay. Lane 3, 20 μ l of elution from negative control, pull-down assay using NHS without vWFA-214.

Figure 3 Inhibition of classical complement pathway activation by the recombinant vWFA-214 protein.

Different amounts of recombinant vWFA-214 proteins were pre-incubated with NHS for 30 min at room temperature before adding to the sensitized sheep erythrocytes. The recombinant vWFA-214 protein competed with C2 in NHS for C4 binding and decreased the % hemolysis in a dose dependent manner. The results show the mean \pm standard deviation (SD) of three independent experiments each of duplicate measurements.

Figure 4 Sandwich ELISA of C4 and purified C4 chains with the recombinant vWFA-214 protein.

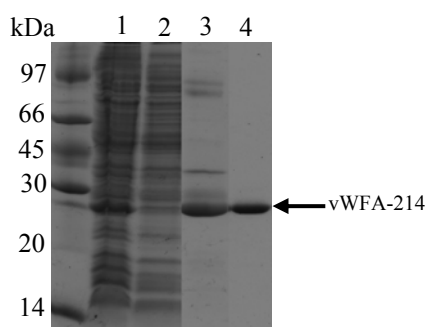
(A) C4 protein was separated into C4 α , C4 β and C4 γ chains by 12 % SDS-PAGE, under reducing conditions. After electrophoresis, the gel was stained with Coomassie Blue and the separated C4 chains were excised and purified by electro-elution. Lane 1, 500 ng pure C4 protein. Lane 2-4, each loaded with 500 ng purified C4 α , C4 β and C4 γ chain respectively. (B) Western blotting confirming that anti-C4 antibody recognizes each of the purified C4 chains. Lane 1, 500 ng pure C4 protein. Lane 2-4, each loaded with 500 ng purified C4 α , C4 β and C4 γ chain respectively. (C) Sandwich ELISA to investigate the binding of vWFA-214 to C4/C4 chains shows the presence of a C4 binding site on the vWFA-214. The binding affinity of intact C2 and recombinant vWFA-214 protein to C4/C4 chains was nearly the same (data not shown). It was also revealed that the interaction between C4 and C2 was mediated through the C4 β chain and vWFA-214 domain. The results show the mean \pm SD of three independent experiments each of duplicate measurements.

Figure 5 Ligand blot of the recombinant vWFA-214 and CRIT peptides showing the presence of a CRIT binding site on vWFA-214.

(A) The recombinant vWFA-214 protein was incubated with CRIT peptides and the resulting complexes were cross-linked with or without DSS. Those complexes not cross-linked with DSS were used as a control. After cross-linking, the complexes were detected by immunoblotting and probing with polyclonal anti-CRIT antibody which recognizes ed1, H17 and H17-2. The results showed that the recombinant vWFA-214 protein interacted with CRIT ed1 and H17 peptides. It also interacted weakly with H17-2 peptide. The negative control included CRIT peptides and vWFA-214 protein without DSS crosslinker. (B) The interaction between vWFA-214 and CRIT H17 could be blocked by the pre-incubation of recombinant vWFA-214 protein with anti-vWFA antibody (1:500 dilution).

Figure 1

A



B

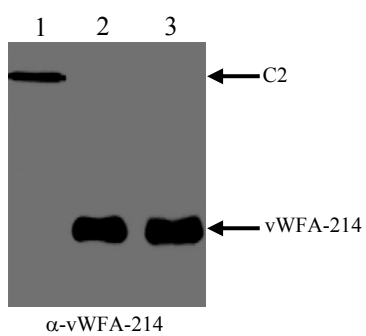
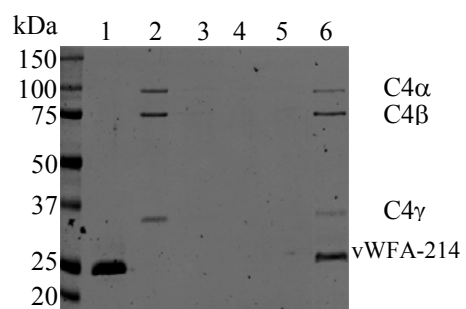


Figure 2

A



B

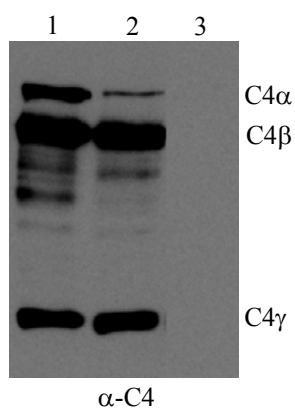


Figure 3

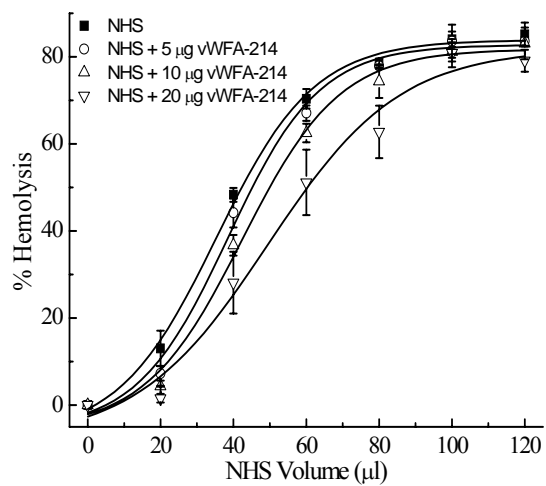
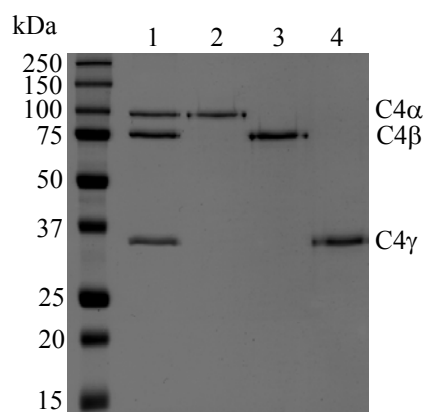
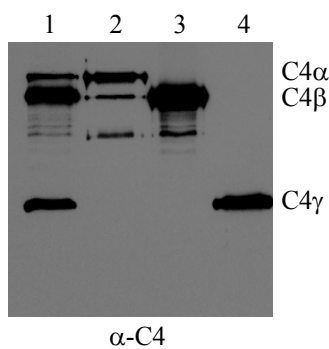


Figure 4

A



B



C

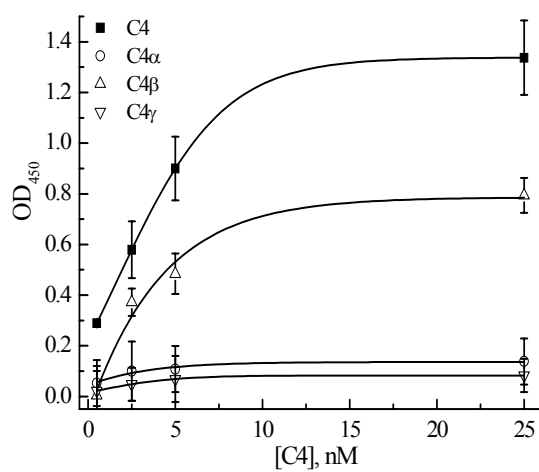
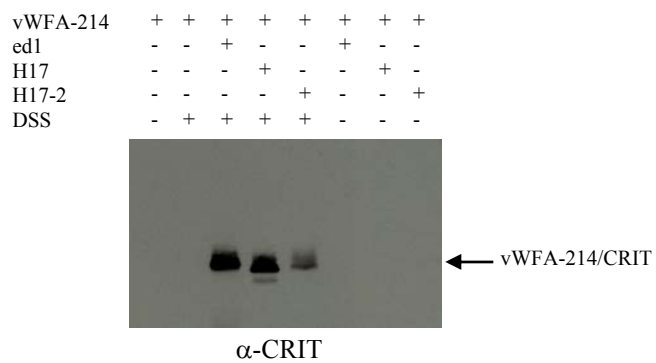


Figure 5

A



B

