Biochemical and Functional Studies of a Novel Complement Inhibitor, CRIT, with Its Interaction Partners

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GENERAL SUMMARY

Complement C2 receptor trispanning (CRIT), a three transmembrane receptor, was first discovered on the surface of the parasite *Schistosoma haematobium* and formerly termed *Schistosoma* trispanning orphan receptor (Sh-TOR). This receptor acts as decoy C2-binding receptor to protect the parasite from classical pathway-mediated complement attack by competing with C4 for the binding of C2. The first extracellular domain (ed1) proved to be the hot spot for the binding of C2.

The first part of my work and the detailed results will be presented and discussed in Section I. Apart from *Schistosoma*, another parasite *Trypanosoma cruzi* and rat also express CRIT and show high sequence homology. The cloning of human CRIT was started by PCR with the templates from both genomic DNA and cDNA. The primers used were selected from the conserved region. The human CRIT nucleotide sequences from genomic DNA and cDNA are identical, implying that human CRIT is encoded by a single exon, and therefore an intronless gene. A phylogenetic analysis of the available CRIT genes giving a measure of their evolutionary distance shows that the parasite species are as far removed from their human host as is the rat sequence. This is thought to be an example of horizontal gene transfer (HGT) in terms of genetic transfer between host and parasite. In addition, the cellular distributions and localizations of human CRIT were determined by immonblotting and immunohistochemistry.

It has been showed 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 domain(s) involved in these bindings, however, has remained unknown. Since most of the von Willebrand factor-A (vWFA) domains are components of the extracellular matrix and very often are the sites for protein-protein interaction in cell adhesion protein, such as integrins, the vWFA domain of C2 was speculated to be the potential site of interaction with CRIT and C4. To further address this question, the vWFA domain (amino acids 224-437) of C2 was cloned and the first functional recombinant vWFA protein was expressed and purified. Based on the functional data, the vWFA domain of C2 is a potential binding site for both C4 and CRIT, and this forms the major aspect of Section II.

The major findings of the regulatory effect of CRIT on the alternative pathway are summarized in Section III. In view of the fact that the classical and alternative complement pathways have many functional and structural similarities, the regulatory effect of CRIT on the alternative pathway was further investigated. In this study, as 11 amino acid peptide (CRIT-H17) derived from the C-terminal part of CRIT-ed1 was used, as it was found to be a potent inhibitor of the classical pathway. The data presented indicates that CRIT-H17 also functions as a regulator of the alternative pathway. Besides binding C2, CRIT-H17 was shown to bind factor B and its two fragments, Ba and Bb, and C3b but not factor D. For this, interacting proteins were stabilized with a cross-linking agent and complexes detected by immunoblots. CRIT-H17 bound to FB, possibly at the junction of Ba and Bb near the factor D cleavage site, and blocked the factor D-mediated cleavage of FB. Unlike DAF, CRIT-H17 had no decay accelerating activity on

the C3bBb complex. Additional experiments showed that CRIT-H17 partially inhibited the factor I-mediated cleavage of C3b in the presence of factor H or CR1. These data indicate that the regulatory role of CRIT-H17 in the alternative pathway is complex, probably related to its dual binding to factor B and C3b.

GENERAL INTRODUCTION

Complement System

Complement is the name given to a triggered enzyme system of over 30 glycoproteins found in blood plasma and on body cell membranes [1]. Many of them are labeled with the letter *C* followed by a digit, with the further suffixes indicating the cleavage products of proteolysis, and with the letter *i* representing an inactive derivative. The complement system is part of the immune system and is a crucial component of innate immunity as well as adaptive immunity [2, 3], and mediates acute inflammatory and cytolytic reactions. It plays an important role in host defense and in the elimination of invading foreign pathogens. Three separate pathways can activate the complement cascade: classical pathway (CP), alternative pathway (AP), and mannose-binding lectin pathway (MBL-P), and a serial of host proteins which regulate the complement activation (Figure 1). Each pathway responds to different activators and activates the complement system in a sequential manner but all three pathways converge in the assembly of the C3 convertase and end in the formation of C5b-9, known as the membrane attack complex (MAC).

The CP is normally activated by antibody-antigen complexes on the pathogen surface, and also acute phase protein (CRP) and is considered predominantly to be an effector of the adaptive immune response, whereas the AP and MBL-P are activated directly by pathogen surface components and are considered effectors of the innate immune response.

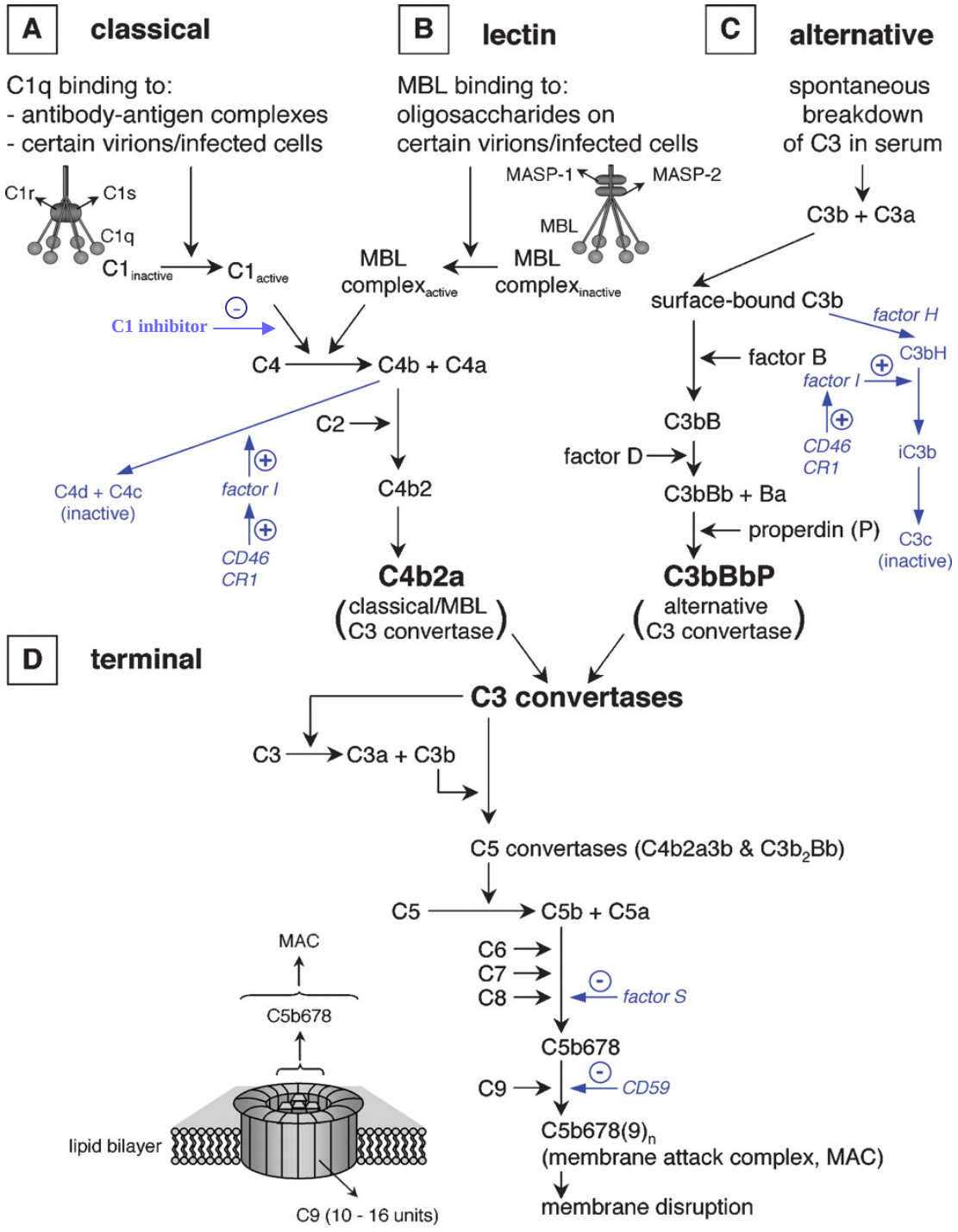

Figure 1 Complement pathways activation and the regulation of host proteins

The classical **(A)**, alternative **(B)** and lectin **(C)** pathways of complement activation are illustrated. Complement activation results in the initiation of the terminal complement pathway **(D)**, leading to the formation of membrane attack complexes. Host proteins that regulate the different steps of complement activation are indicated in blue [4].

The 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 C1s are cleaved. Activated C1s then cleaves C4 with formation of a major fragment C4b, which binds to native C2 in the presence of Mg^{2+} [5]. 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 [6].

The MBL-P is initiated by binding of mannose-binding lectin (MBL) specifically to mannose residues and to certain other sugar residues on the surface of bacteria, yeast, parasitic protozoa and viruses [7]. The concentration of MBL in normal plasma is very low and its production by liver is increased during the acute-phase reaction of the innate immune response. MBL is a collectin (C-type lectin with a collagen-like domain) which is similar to C1q. MBL associated serine protease (MASP)-1 and MASP-2 are similar to C1r and C1s in the CP, respectively [8]. Like C1s, MASP-2 can cleave C4 and leads to formation of MBL-P derived C3 convertase. MBL-P has structural and functional similarities to the CP. Besides the initiating factors, these two complement pathways are identical.

The activation of the AP is antibody-independent and generates a distinct C3 convertase, C3bBb. The AP is a self amplifying pathway and is important in the clearance and recognition of pathogens in the absence of antibodies. The activation of the AP is mainly initiated by the spontaneous hydrolysis of the internal thioester bond in plasma C3 to

form C3(H_2O), which has an altered conformation allowing the binding of factor B (FB) [9, 10]. The first step in the AP activation is the nucleophilic attack on the internal thioester bond in C3b by an amine or hydroxyl group on the pathogen surfaces, followed by the binding of FB to form the C3bB complex [11]. Binding of C3b to FB induces a conformational change in FB, which in turn becomes susceptible to the cleavage by factor D (FD). The FD-mediated cleavage of FB is the rate-limiting step for generating the AP C3 convertase, C3bBb.

Regulation of Complement Activation

Complement activation is an important but a potentially dangerous system. Inappropriate activation of the complement cascades is known to play a key role in various pathological conditions. Thus, there are several complement regulatory proteins that protect host cell from the potentially destructive effect of complement activation. Most of these proteins belong to the regulators of complement activation (RCA) gene family, which are encoded in the gene cluster on the long arm of chromosome one [12]. There is one common structural motif among the RCA gene family termed the short consensus repeat (SCR), which comprises the majority of the molecule and shares approximately 20-40 % homology to each other. The RCA proteins interact with C3b and either prevent the formation or promote a rapid irreversible dissociation of complement convertases. The RCA proteins, decay accelerating factor (DAF), factor H (FH) and complement receptor 1 (CR1) are responsible for the dissociation of complement C3 convertases and membrane cofactor protein (MCP), FH and CR1 act as cofactors in the factor I (FI)-mediated cleavage of C3b.

Besides the RCA proteins, there are several complement inhibitors developed, including recombinant forms of naturally occurring inhibitors, monoclonal antibodies to complement components, serine protease inhibitors and analogues of anaphylatoxins [13, 14]. The search for complement inhibitors continues and aims at the discovery of one or a few specific and potent complement inhibitors for clinical use. Table 1 shows various potential complement inhibitors, which may be available to the critical care physician.

Table 1 Potential complement inhibitors

Type of inhibitor	Target	Reference
Peptides; monoclonal antibody	C1q	[15]
Anti-MBL monoclonal antibody	MBL	[16, 17]
C1-inhibitor	CP; AP; MBL-P	[18, 19]
Anti-FD monoclonal antibody	FD (AP)	[20]
Anti-FB monoclonal antibody	FB (AP)	[21]
Soluble CR1	C3 and C5 convertases	[22]
Compstatin	C3	[23, 24]
Pexelizumab (monoclonal antibody)	C5	[25]

Complement C2 Receptor Inhibitor Trispanning, CRIT

Schistosomiasis (or bilharzias) is a human disease caused by infection from parasitic trematodes of the genus *Schistosoma*, and is the major source of morbidity and mortality for developing countries in Africa, South America, the Caribbean, the Middle East and Asia. Parasites use various strategies to evade the complement system. In 1999, a novel protein, Sh-TOR (*Schistosoma* trispanning orphan receptor) was isolated in the blood-dwelling *Schistosoma* parasite [26], which was mainly localized on the parasite surface with unknown function. The membrane topology of Sh-TOR was predicted by computer analysis (PHDhtm) [27] (Figure 2). A 27 amino acids synthetic peptide was made corresponding to the first extracellular domain (Sh-TOR-ed1). By affinity chromatography using a column of epoxy-activated Sepharose 6B covalently coupled with Sh-TOR-ed1, one protein in normal human serum was pulled down. By immunoblotting and N-terminal sequencing, this protein was confirmed as complement C2 protein [28]. In a hemolytic assay, Sh-TOR-ed1 could block the CP of complement activation. These data support that the Sh-TOR-ed1 region possesses a C2 binding site and that it functions as a complement inhibitor in the CP. Henceforth, Sh-TOR was re-named complement C2 receptor inhibitor trispanning (CRIT). A model of CRIT-mediated inhibition of CP C3 convertase formation was proposed (Figure 3). Expression of CRIT on the parasite surface acts a decoy C2 binding receptor to protect the parasite from CP-mediated complement attack by competing with C4 for the binding of C2.

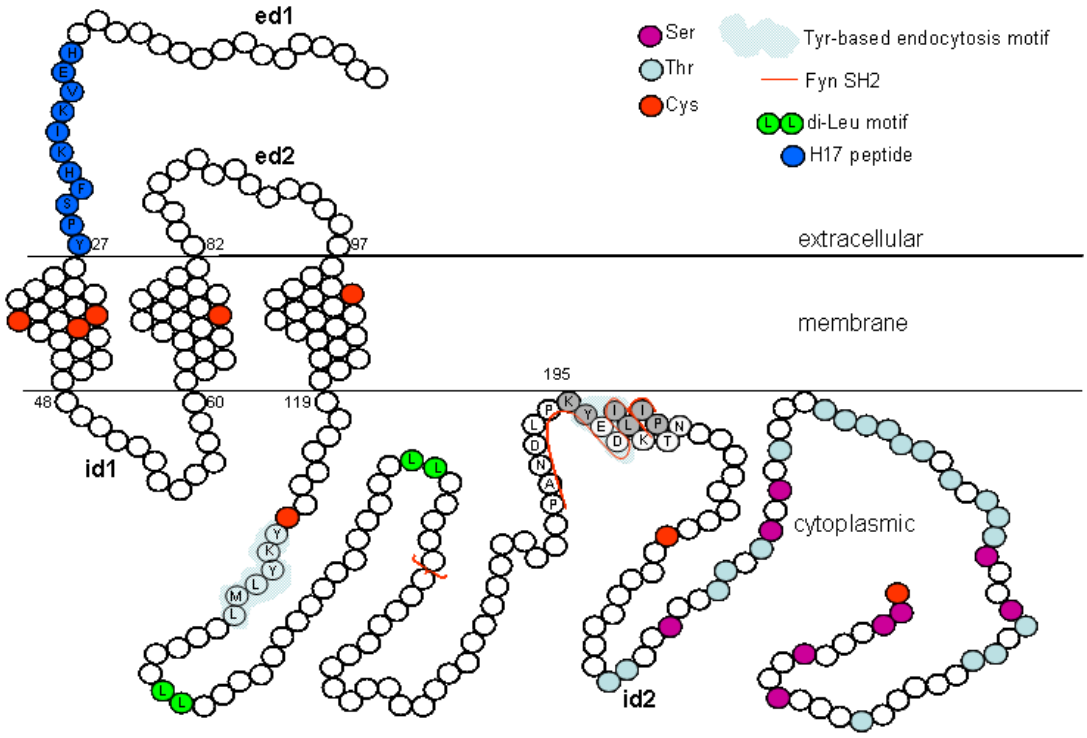

Figure 2 Schematic representation showing predicted membrane topology of CRIT

CRIT is a three transmenbrane receptor with two extracellular and two intracellular domains. An 11 amino acid peptide (CRIT-H17) is derived from the C-terminal region of CRIT-ed1, which is highlighted in blue. Some predicted functional motifs in the id2 are also highlighted [28].

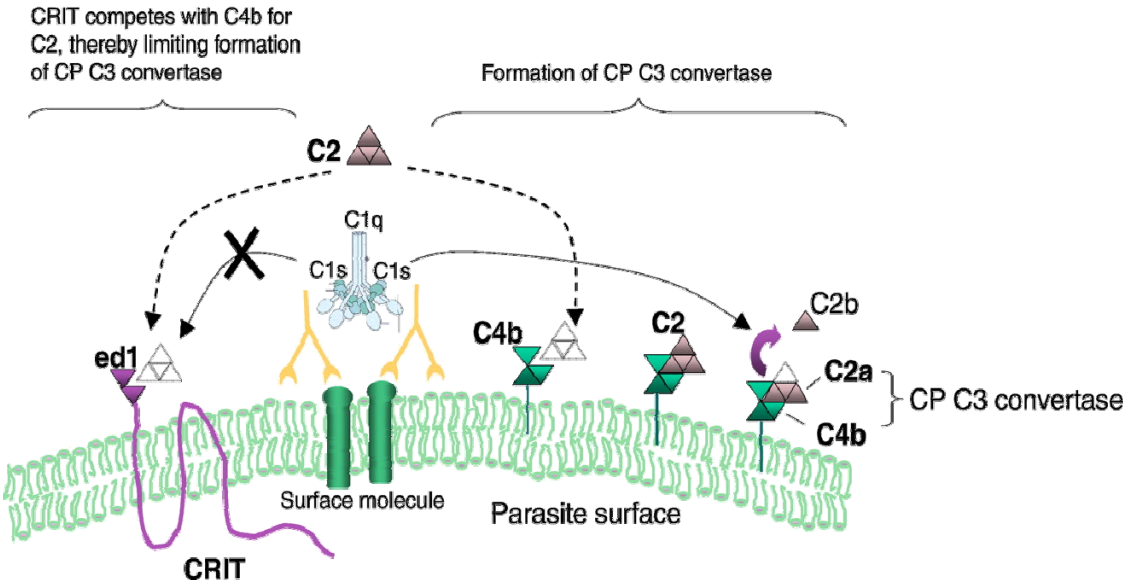

Figure 3 Model of CRIT-mediated inhibition of CP C3 convertase formation on parasite surface.

Antibody (in yellow) binds to the parasite, via some surface molecule, thus fixing C1q and associated C1s. C4b binds via a thioester bond to the surface and then associates with C2, which is cleaved by C1s to produce the CP C3 convertase, C4b2a. CRIT expressed on the parasite surface is able to compete with surface-bound C4b for C2 in the host serum, thereby reducing the extent of C3 convertase formation. As the C2 in the C2-CRIT complex cannot be cleaved by C1s, it is unable to function as a CP C3-like convertase [29].

Objectives

A proper control of complement activation is extremely important in many complement-mediated diseases. A potentially new and advancing area of therapeutics that may hold promise for the critically ill is inhibition of the complement system. Thus, the design or discovery of new potent complement inhibitor(s) would be valuable in the future for critical care medicine.

CRIT is a novel complement inhibitor of the CP which is presently being biochemically and functionally studied. However, as a prerequisite, it's necessary to check whether CRIT also expresses in other organisms, apart from parasites. In addition, to better understand its functional role, the cellular and tissue distribution of CRIT must be determined. To address these questions, the cloning of CRIT in rat, cod and human were performed. For the cell and tissue distribution of CRIT, total protein extracts of various cell lines and tissues were prepared for the Western blotting probed with anti-CRIT antibody. Also, fresh surgical tissues or tissue microarrays were used for the immunohistochemistry.

To dissect out more precisely the CRIT binding region on C2, vWFA domain of C2 was cloned and the recombinant C2 vWFA protein was expressed and purified. The recombinant C2 vWFA protein was also useful for studying the interaction between C2 and C4. Besides the protein-protein interaction studies, it could be used for future structural studies by X-ray crystallography. With all this empirical data, the mechanism of CRIT-mediated inhibition of the CP activation will be defined more clearly.

Besides the inhibitory effect on the CP activation, it was important to test whether CRIT also possesses any regulatory effect on the related AP. The ultimate goal of this study is to fully understand the regulatory role of CRIT in the complement system.

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SECTION I

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Complement C2 Receptor Inhibitor Trispanning: A Novel Human Complement Inhibitory Receptor

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ABSTRACT

The complement system presents a powerful defense against infection and is tightly regulated to prevent damage to self by functionally equivalent soluble and membrane regulators. We describe complement C2 receptor inhibitor trispanning (CRIT), a novel human complement regulatory receptor, expressed on hemopoietic cells and a wide range of tissues throughout the body. CRIT is present in human parasites through horizontal transmission. Serum complement component C2 binds to the N-terminal extracellular domain 1 of CRIT, which, in peptide form, blocks C3 convertase formation and complement-mediated inflammation. Unlike C1 inhibitor, which inhibits the cleavage of C4 and C2, CRIT only blocks C2 cleavage but, in so doing, shares with C1 inhibitor the same functional effect, of preventing classical pathway C3 convertase formation. Antibody blockage of cellular CRIT reduces inhibition of cytolysis, indicating that CRIT is a novel complement regulator protecting autologous cells.

INTRODUCTION

An overview of known human membrane regulators of complement activation (RCA) [1, 2] indicates that cell membranes have no equivalent to C1 inhibitor (C1-INH), a serine protease inhibitor that regulates the classical pathway (CP). C1-INH also inhibits the mannan-binding lectin pathway by inhibiting mannan-binding lectin-associated serine proteases [3]. *Schistosoma* complement C2 receptor inhibitor trispanning (CRIT; formerly, trispanning orphan receptor) like C1-INH inhibits C1s-mediated cleavage of C2, so preventing formation of the CP C3 convertase, C4b2a. However, their mode of action differs. C1-INH regulates the CP at the C1 level by binding stoichiometrically to the active sites on both C1r and C1s [4]. CRIT binds to C2 in a manner that inhibits its C1s-mediated cleavage. There is no enzymatic inhibition and CRIT is presumably protecting the C1s cleavage site on C2.

Homologs of complement regulator proteins are found on parasites such as trypanosomes and schistosomes living in the mammalian host vasculature [5]. Trypanosomes are protected by decay-accelerating factor (DAF) and CD59 homologs with decay-accelerating activity either for the alternative pathway (AP) [6] or else for both the CP and AP convertases [7], whereas schistosomes adsorb host DAF from erythrocytes [8]. *Schistosoma* CRIT having a putative complement-protecting function in schistosomes is found on the surface of the *Schistosoma* parasite [9]. We have now cloned a CRIT homolog from the *Trypanosoma* parasite, and found close homologs in human, rat, and cod.

We found human (Hu)-CRIT to be expressed on a wide range of tissues as well as on hemopoietic cells. We suggest that this novel receptor with three N-terminal

transmembrane (TM) domains represents a novel family of receptors and present several lines of evidence supporting CRIT as receptor for C2. The complement regulatory function of CRIT was suggested previously [10, 11] by using CRIT extracellular domain 1 (ed1) and its 11 amino acid C terminus (CRIT-H17) to inhibit CP-mediated hemolysis in the presence of normal human serum (NHS). We have now shown that, by blocking CRIT through preincubation with anti-CRIT-ed1, two CRIT-expressing human myeloid cell lines as well as monocytes, can be deprotected, resulting in increased susceptibility to Ab-dependent complement lysis in vitro. When C2 binds to CRIT on the cell surface, it does not form a variant-C3 convertase, because C2 cannot be cleaved by C1s. Having found this previously with synthetic CRIT-ed1 peptide, we now show this to be the case with native membrane-bound CRIT.

EXPERIMENTAL

Antibodies and peptides

Affinity-purified polyclonal anti-CRIT-ed1 and anti-C2 antibodies were described before [10]. A rabbit polyclonal antibody against *Schistosoma hematobium* (Sh)-CRIT-ed2 (NH_2-SSTSDIRLVIHTKTGPIYIKST-CO_2H (two conservative amino acid changes relative to human CRIT-ed2)) was made and affinity purified as described for anti-CRIT-ed1. Anti-CRIT-intracellular domain 2 (id2) mouse polyclonal antibody was obtained by a standard immunization of mice with the synthetic peptide (NH_2-KYEDILKIPTNAYAC-CO_2H) based on K195-A208 of the cytoplasmic tail of human CRIT, coupled through a C-terminal cysteine to KLH. HRP-conjugated secondary antibodies were obtained from Bio-Rad. All synthetic peptides were synthesized as described before [10]. The sequences were as follows: ed1 (NH_2-MSPSLVSDTQKHERGSHEVKIKHFSPY-CO_2H); ed2 (see above); H17 (NH_2-HEVKIKHFSPY-CO_2H); H17S (NH_2-EKFYHIHSKPY-CO_2H); C4$\beta_{212–232}$ (NH_2-FEVKKYVLPNFEVKITPGKPYCO_2H); and C4βS (NH_2-FYFNEPVEPKVLPKGVKYTKI-CO_2H).

Biotinylation, FITC labeling, and radioiodination od C2

C2 (50 µg) was biotinylated by using NHS-LC-biotin (Pierce) according to previously described methods [10], but using a lower biotin-to-protein ratio of 50 µg of biotin per milligram of protein. After dialysis against PBS or PBS containing Mg^{2+} (1 mM), the protein was assayed. For FITC labeling, C2 (10 µg) was incubated with FITC (Sigma-Aldrich) (6 µl of 0.1 mg/ml) and 1 ml of carbonate buffer at room temperature for 2 h in

the dark, followed by extensive dialysis against PBS. Radioiodination of C2 was conducted according to a standard protocol [12]. Essentially, 100 µg of protein in 0.5 ml was added to 100 µg of IodoGen (Pierce). Iodination was conducted by adding 1 mCi of $Na^{125}I$ (Amersham Biosciences). Unincorporated iodine was separated from iodinated C2 using a PD-10 gel filtration column (Amersham Biosciences) presaturated with 5 mg of BSA. The specific activity of ^{125}I-labeled C2 was 8×10^4 cpm/mg.

Human cells and cell lines

Lymphocytes were obtained from heparinized human blood diluted 1/1 with RPMI 1640 using Histopaque 1077 (Sigma-Aldrich). Erythrocytes and platelets were isolated according to a standard protocol [13]. The lymphocytes were washed and resuspended in RPMI 1640. Erythrocytes were recovered, and the pellet was washed with PBS. Monocytes were obtained from heparinized blood buffy coat by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich). Monocytes were purified by adherence separation [14]. Purified monocytes (2×10^5 cells/ml) were dispensed in 100 µl aliquots into the wells of a 96-well microtiter plate and stimulated, if necessary, with 10 U/ml final concentration of human IFN-γ (Sigma-Aldrich) and incubated overnight at 37°C in 5% CO_2. To remove the fibroblasts that proliferate on addition of IFN-γ, the monocytes were washed three times with RPMI 1640. The human carcinoma cell lines, Jurkat, Raji, THP-1, U937, ECV-304, HeLaS3, HS1, MRC5, T47D, and rat cell line, C58(NT) (all American Type Culture Collection) were maintained in RPMI 1640 medium with 10% FCS, glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C and 5% CO_2.

SDS-PAGE, Western and ligand (Far Western) blotting

Protein assays by dye binding using a Bio-Rad protein assay kit were conducted to ensure even loadings for SDS-PAGE. SDS-PAGE and immunoblotting analysis of proteins was conducted as described before [10]. Reduced samples (with 20 or 50 mM DTT) and nonreduced samples were prepared in SDS-PAGE loading buffer. Where necessary in experiments to study the covalent oligomerization of CRIT, cell lysis buffer included 10 mM iodoacetamide. For ligand blotting, the protein to be used as probe (usually C2) was biotinylated and detected as described above. For preincubation of biotinylated C2 ($C2^{bio}$) with peptides, before probing of a blot, a 10,000 M excess of peptide over $C2^{bio}$ was added at 4 °C for 2 h.

Tissue extraction for Western analysis was conducted by homogenizing 50 mg of tissue in a Dounce homogenizer in 150 µl of RIPA (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) in the presence of protease inhibitors. After centrifugation (14,000 rpm for 10 min), the supernatant was respun as above.

For lysates of mammalian cells, cells (typically 30 x 10^6) were washed twice in PBS and resuspended in 0.75 ml of lysis buffer (PBS with 1% (v/v) Nonidet P-40 and 5 mM EDTA (pH 8)). After adding protease inhibitors (0.2 mM PMSF, 4 mM aminoethylbenzenesulfonylfluoride (Pefabloc), 1 mM NaF, 5 µg/ml aprotinin, 4 mM benzamidine) (all Sigma-Aldrich), the cells were mixed gently and incubated on ice for 10 min. After low-speed centrifugation (250 *g*; 5 min), the supernatant was respun (25,000 *g*; 25 min), and the supernatant was aliquoted and stored (–80°C).

CRIT expression in *Escherichia coli* was induced with isopropyl-D-thiogalactopyranoside for 1 h. After harvesting, the bacteria were resuspended in 1/10 the original volume of buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 2 mM EGTA, protease inhibitor mixture as for mammalian cell lysate) and lysed with 0.1 mg/ml lysozyme, 0.1% Triton X-100, 10 mM $MgCl_2$, 50 µg/ml DNase, and 20 µg/ml RNase for 60 min at room temperature. Cell lysates were centrifuged (25,000 *g* for 30 min), and supernatants were used freshly.

Immunohistochemistry

Fresh surgical tissue (kidney and testis) was fixed in 10% neutral buffered formalin. Tissue was embedded in paraffin, and 4- to 5-µm sections were cut. Tissue for microarrays (BioCat) was similarly prepared. Blocking of endogenous peroxidase was conducted with 0.3% H_2O_2 in methanol for 30 min followed by three 5-min washes in distilled water and then one wash in PBS. Tissues were digested with 0.05% proteinase K in PBS for 2 min and then washed in PBS. Blocking of nonspecific staining was performed by incubating for 20 min in normal horse serum, excess serum then being blotted off the sections. The primary antibody was anti-CRIT-ed2 (1/100) incubated either overnight at 4 °C or for 1 h at room temperature. For detection, the liquid diaminobenzidine substrate-chromogen system (DakoCytomation) was used according to the manufacturer's instructions. Counterstaining was by hematoxylin Mayer, and dehydration by using decreasing alcohol concentrations. Finally, stained sections were mounted with permanent mounting medium (Eukitt/Fluka Chemical).

Immunohistological examination was performed using a Zeiss Axiophot microscope as described before [11].

Hemolytic assay

Essentially, in the hemolytic assay performed, the increasing concentration of CRIT-ed1 being added to NHS competes with C4b for binding to C2. The effect of this competition is measured in this case by the decreased CP C3 convertase assembly and resulting decrease of percent hemolysis. This standard procedure has been described previously [10].

Complement-dependent cytotoxicity on CRIT-expressing cells

Cells (0.25×10^6) were incubated with 100 µl of anti-human lymphocyte serum (1/30) or rabbit prebleed/normal rabbit IgG as a control followed by incubation with anti-CRIT-ed1. After 1 h at 4°C and two washes in isotonic veronal buffered saline with divalent cations (GVB^{2+}) [15], 100 µl of 10% NHS in GVB^{2+} was added for 30 min at 37°C. The percent lysis was determined as the percentage of cells permeable to trypan blue.

Flow cytometry

To detect the binding of C2 to the Jurkat cell surface, the C2 was biotinylated as described above. Cells were treated according to the manufacturer's instructions, for 5 min at room temperature, with an avidin/biotin blocking reagent (Vector Laboratories). Cells were then washed in RPMI 1640 (containing 1% BSA and 0.01% sodium azide), resuspended at a concentration of 1×10^6 cells/ml, and incubated on ice for 30 min with

50 µl of $C2^{bio}$ (2.75 µg/ml) made up to 100 µl with FACS buffer (1x PBS, 1% BSA, 0.02% sodium azide). After washes and incubation with 5-µl per reaction streptavidin-PE, the cells were fixed with 1% paraformaldehyde for 30 min at room temperature and analyzed on a FACScan flow cytometer (BD Biosciences). CellQuest software was used for data acquisition and analysis. A total of 10,000 events was analyzed. FITC-labeled C2 (25 µl of 10 µg/ml) was similarly incubated with 1.5×10^6 Jurkat cells.

To examine CRIT expression on the Jurkat cell surface, 1×10^6 cells incubated with avidin/biotin blocking reagent, as above, were treated as for labeling with $C2^{bio}$ but instead with biotinylated anti-CRIT-ed1 (20 µl of 50 µg/ml).

ELISA

To investigate the C2 binding to CRIT-ed1 or C4b, first CRIT-ed1 peptide (0.5 µg/well) or C4b (2 µg/well) in carbonate buffer (pH 9.6) (with or without 5 mM $MgCl_2$) were coated on the plate (overnight at 4°C). After washing in PBS (with 0.05% Tween 20) and blocking with 200 µl of PBS (containing 2% BSA), increasing amounts of $C2^{bio}$ (with or without 1 mM Mg^{2+}) were added. To see whether CRIT-ed1 can block C2-C4b binding in this system, C4b in carbonate buffer was coated on the plate and incubated with $C2^{bio}$ (150 ng) in the presence or absence of 1.2 µM ed1 (300 ng) in a final volume of 100 µl. Finally, the procedure to detect $C2^{bio}$ binding in our ELISA requiring incubation with streptavidin-HRP (1/2500) followed by development with *o*-phenylenediamine was essentially as described before [16]. A_{490} was measured using a microplate reader (spectroMAX 190; Molecular Devices).

Apparent dissociation constants ($K_{D(app)}$) were calculated using nonlinear regression, which was performed by plotting A_{490} against the concentration of C2 (rather than concentration of bound C2 against concentration of free C2 as required for Scatchard analysis and K_D calculations) and fitting with the one-binding class model (a better fit than the two-binding class model) using the GraphPad Prism software (GraphPad Software).

DNA isolation and southern blotting

Rat genomic DNA was purchased (Stratagene). Human genomic DNA for Southern blotting was prepared from 500 µl of packed Jurkat cells [17]. Southern blotting [17] using the full-length Sh-CRIT cDNA as a probe was conducted at low stringency by incubating at 45°C for 16 h, followed by two washes with 3x SSC and 0.1% SDS at room temperature, rinsing in 2x SSC. For higher stringency, blots were incubated at 65°C and then washed with 1x SSC and 0.1% SDS at 65°C. Blots were exposed to x-ray film with an intensifying screen for 48 h.

Cloning of CRIT homologs and in vitro transcription/translation

A rat homolog of Sh-CRIT was obtained by PCR from rat genomic DNA, using degenerate oligonucleotides based on the Sh-CRIT N-terminal and C-terminal nucleotide sequence and, where possible, based on a rat codon usage. The primers were as follows: RaF (sense), 5'-CGCGATGTC(C/T)CC(A/C/G/T)I(C/G)ICTIGTITC-3'; and RaR (antisense), 5'-GCGTTA(G/A)CAAGAIGA(C/G)TG(A/C/G/T)GC(A/G)TT-3'. The PCR was conducted at 94°C (30 s), 55°C (60 s), and 72°C (60 s) for 35 cycles. The purified

product (using GeneClean (Bio 101)) was then cloned into pGEM-T (Promega), according to the manufacturer's instructions, and fully sequenced in both strands. Human genomic DNA was extracted from whole blood using a blood and cell culture DNA mini-kit (Qiagen), and human testis and pancreas cDNA was purchased from Ambion. Based on the sequence alignment of *Schistosoma* and rat CRIT, oligonucleotides were designed with the introduction of a *Hin*dIII restriction site at the 5'-end and a *Kpn*I restriction site at the 3'-end to enable subcloning into the pcDNA3.1 vector in the correct orientation (HuF (sense), 5'-CGAAGCTTATGTCTCCAAGTCTAGTG3'; and HuR (antisense), 5'-CGGGTACCTTAGCAAGAGAGTGAGC-3'). Pfu ultra high-fidelity DNA polymerase (Stratagene) was used, and the amplified PCR product was digested with *Hin*dIII and *Kpn*I, and ligated into the pcDNA3.1 vector precut with the same enzymes. The ligation was transformed into DH5α competent cells [17]. In vitro transcription/translation from the pcDNA3.1-HuCRIT template was conducted using the TNT-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. The trypanosome and cod homologs were obtained similarly by PCR using oligonucleotides based on the Sh-CRIT sequence.

DNA sequencing

All sequencing was conducted on both strands of the pGEM-T clones using an ABI Prism BigDye terminators, version 3.0, cycle sequencing kit and detected on an ABI 3100 Avant Genetic Analyzer, automated DNA sequencer, according to the manufacturer's protocols (Applied Biosystems). Vector-specific primers SP6 and T7 were used, as well as primers based on the established insert sequence.

Assay for cleavage of C2 by C1s

This was essentially conducted as described previously [10]. To test for the effect of CRIT as expressed on the Jurkat cell surface (5×10^3 cells) as well as the CRIT-ed1 synthetic peptide (105 μM), on C1s cleavage of C2, they were preincubated with C2 (2 μg) in 20 mM Tris at 20°C for 30 min. Then, 0.2 μg of C1s was added and incubated for 1 h at 37°C before analysis by immunoblotting.

RESULTS

Human and parasite CRIT

CRIT is found on the larval and adult worm stage of the *Schistosoma* parasite [9]. Under the previous designation of "trispanning orphan receptor," it has also been found recently on schistosome eggs [18]. Schistosomes live in veins near the urinary bladder or intestines of the host and the CRIT receptor for C2 is able to limit CP C3 convertase formation on the parasite surface [10, 19-21]. Southern blotting of human genomic DNA under low stringency conditions, using the *Sh-CRIT* open reading frame (ORF) as a probe, revealed a *Hu-CRIT* homolog (Figure 1A). It was also found that the antibody against the N-terminal extracellular domain, ed1, of Sh-CRIT recognized a 32-kDa protein in various human cells and tissues (described below).

The partial sequence of the *CRIT* homolog in *Gadus morhua* (*Cod-CRIT*), and full-length sequences of *CRIT* in *Trypanosoma cruzi* (*Tc*)-*CRIT*, rat (*Ra*)-*CRIT*, and *Hu-CRIT* were obtained by PCR, the latter from testis and pancreas cDNA. Alignment at the amino acid level (Figure 1B) reveals near identical TM domains and ligand-binding ed1 in all species, the latter accounting for the cross-immunoreactivity, between schistosomes and humans in ed1. The high interspecies homology at 82% between full-length Hu- and Ra-CRIT also occurs in the G protein-coupled receptor (GPCR) family which can show 85–98% identity between species [22]. At the nucleotide level, as shown in the phylogenetic tree in Figure 1*C*, the parasite cluster (*Sh-CRIT* and *Tc-CRIT*) is as closely related to *Hu-CRIT* as rat, suggesting that these human parasites acquired the *CRIT* gene from their host. The appropriation of host genes by parasites accounts for the many genes with high degrees of identity between parasite (especially schistosome) and host [23, 24],

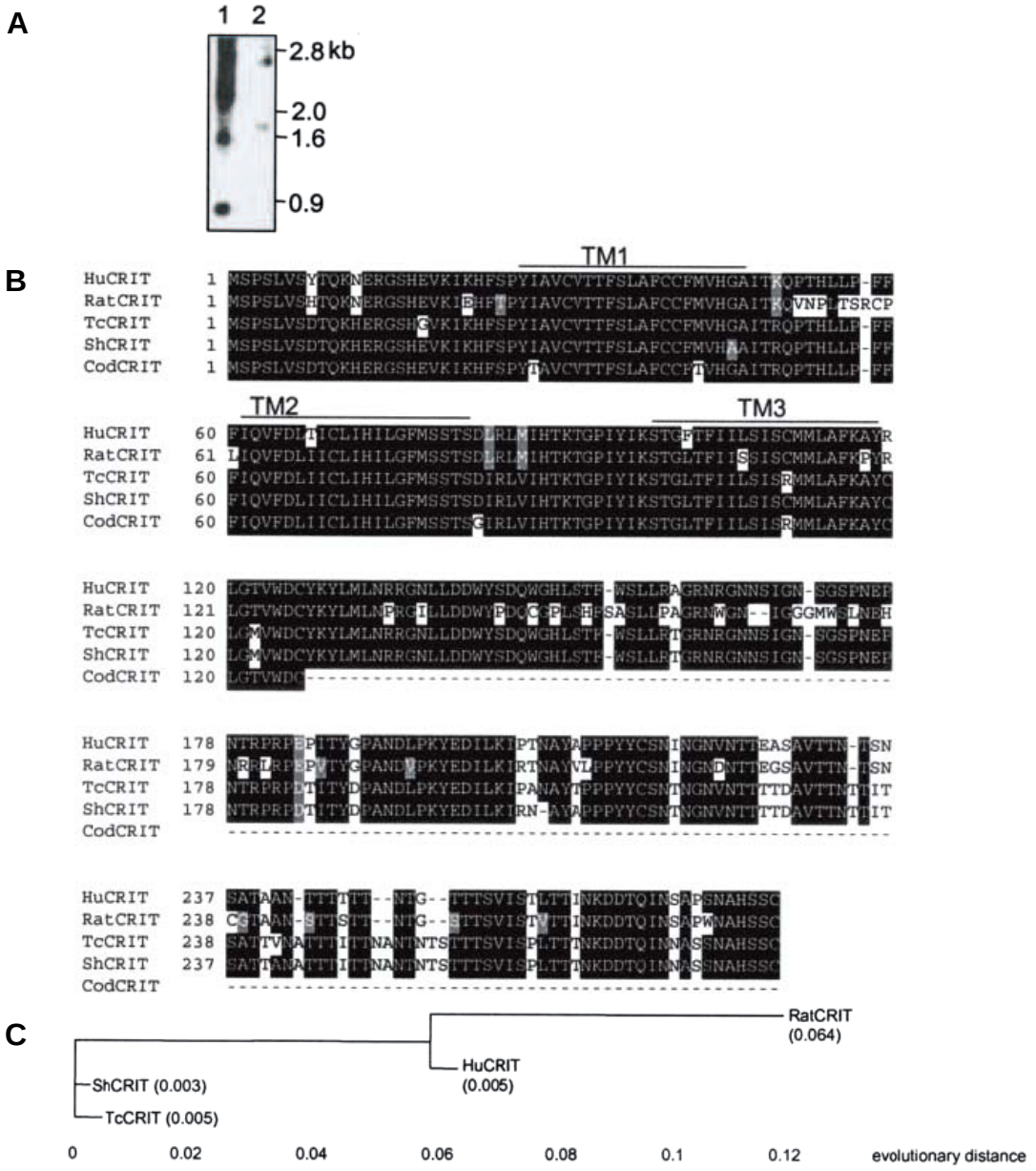


Figure 1 CRIT family genes

(A) Southern blot of human genomic DNA digested with: *lane 1*, *Eco*RI, and *lane 2*, *Pst*I, probed with Sh-CRIT full-length cDNA under low and high stringency conditions, respectively. **(B)** Amino acid sequence alignment of CRIT from human (Hu), rat (Ra), *T. cruzi* (Tc), *S. hematobium* (Sh), and cod. Amino acid identity or similarity is indicated by black or gray shading, respectively. The three TM domains are indicated by a bar above the sequence. **(C)** Unrooted phylogenetic tree based on analysis of nucleotides 1–846 of Hu-CRIT cDNA sequence with other CRIT cDNAs, using a topological algorithm (European Molecular Biology Laboratory-European Bioinformatics Institute Molecular Biology Server). Length of line segments, in brackets, is indicative of evolutionary distance.

and such molecular mimicry in which host proteins are expressed by parasites helps disguise the parasite as "self" in terms of the host immune system. We found the nucleotide sequence of the *Hu-CRIT* cDNA to be identical with that of the genomic sequence, implying that the ORF of *Hu-CRIT* is encoded by a single exon, although we cannot yet rule out an intron in the untranslated regions. In this study, we present the *Hu-CRIT* gene structure only as the predicted ORF based on that of *Schistosoma* CRIT, thus excluding 5' and 3' noncoding regions and other structures such as promoters and transcription regulatory elements. The presence of a *CRIT* gene in cod of which we present a partial sequence in Figure 1B, indicates that *CRIT* genes may have evolved from a common ancestral gene, at least present in the earliest teleosts.

CRIT membrane topology and sequence motifs

Fig. 1D shows how, for five of six topology algorithms, Hu-CRIT, representative of the other CRIT sequences, is expected to lie in the membrane. Empirical evidence for the internal/external orientation of CRIT has been described before [9] and is supported by the fluorescence staining of CRIT-positive cells incubated with anti-CRIT-ed1 (Figure 2A). The majority of substitutions between the species occur within the first 60 residues of the 161-residue cytoplasmic tail, id2, proximal to the third TM domain. Within the remainder of the cytoplasmic tail, as illustrated in Figure 1D, and shared by parasite and mammalian sequences, are three predicted tyrosine-based YXXØ endocytosis motifs (where X is any amino acid and Ø is a strongly hydrophobic amino acid), two di-leucine endocytosis motifs, and, illustrated schematically in Figure 1D, an amphipathic α-helix

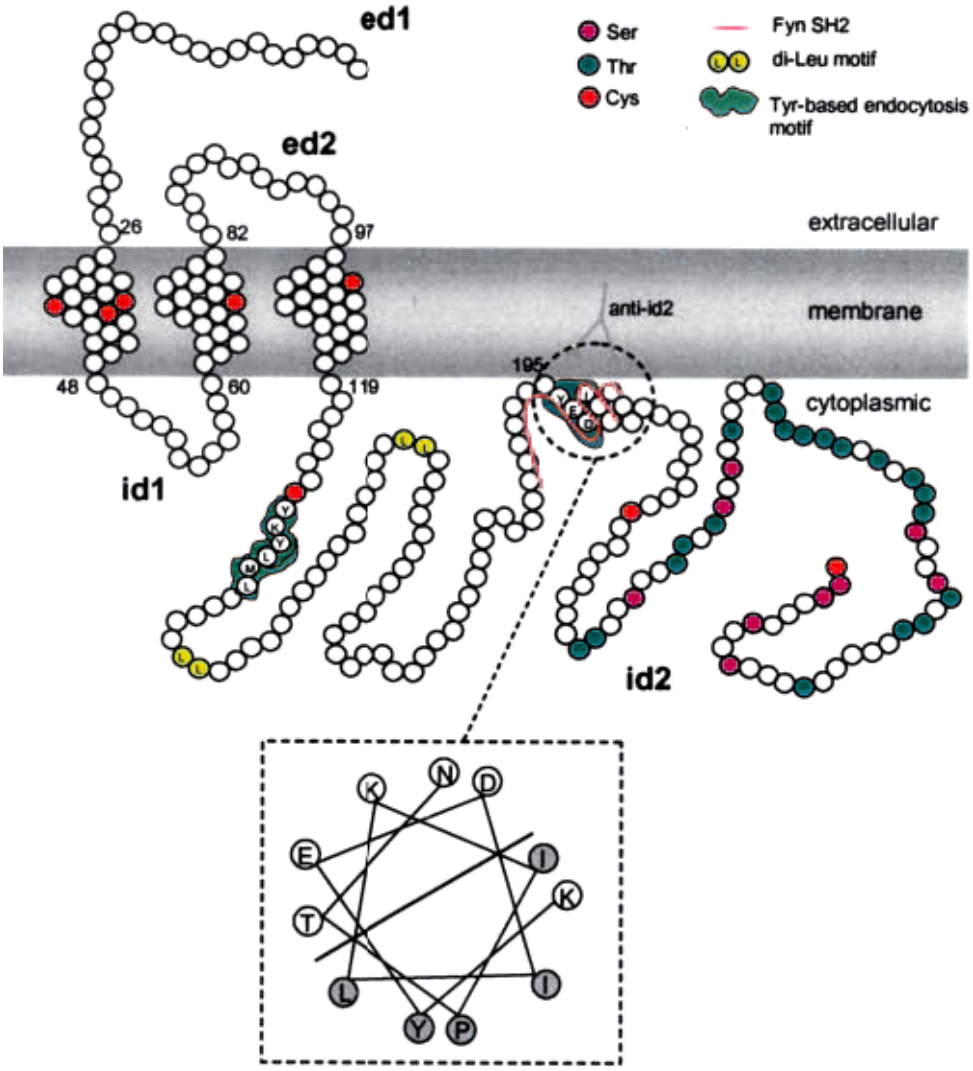


Figure 1D Membrane topology

Schematic of representative Hu-CRIT, showing membrane topology and important sequence motifs, common to all sequences presented, including, in the cytoplasmic tail, three Tyr-XX-Ile/Leu endocytosis motifs, the third lying within an amphipathic helix (in which hydrophobic residues are within shaded circles), as well as two di-leucine endocytosis motifs and a serine/threonine rich region. The extracellular domains (ed1 and ed2) and intracellular domains (id1 and id2) are also indicated. The region to which the anti-CRIT-id2 Ab was designed is illustrated.

(putative sorting signal toward degradation) [25]. Also within the cytoplasmic tail, there is a consensus binding motif for the Src homology 2 domain of the Src family cytoplasmic tyrosine kinases [26], in particular, Fyn, Fps/Fes, and Syk, as well as consensus phosphorylation sites for various tyrosine kinases.

CRIT forms covalently linked dimmers

Having first detected CRIT receptor by flow cytometry on the surface of Jurkat cells (Figure 2A), we found, by immunoblotting of a cell lysate, that CRIT exists as a dimer under nonreducing conditions (B). Comparison of a nonreduced Jurkat extract (NR, lane 1) with a reduced extract (R, lane 2) shows, under reducing conditions, the disappearance of the dimeric form and a slight increase in intensity (which was frequently more prominent) of the monomeric form. The presence of the sulfhydryl trapping agent iodoacetamide (10 mM) in the cell lysis buffer did not prevent the appearance of the covalently linked dimeric CRIT (not shown), meaning that disulfides were not formed artifactually as a result of prevailing non-denaturing and oxidizing conditions during cell lysis. At this stage, we cannot categorically say whether homo- or heterodimerization is occurring. However, because dimers occurred in Jurkat cells that do not make C2, regardless of whether they were maintained in complete or serum-free medium, it appears that the dimerization is constitutive and not dependent on ligand. Furthermore, heterodimerization could conceivably occur between CRIT and a molecule other than C2. To resolve this, studies are ongoing to cotransfect cells with CRIT possessing different tags to see whether (homo-)dimers can be detected with both tag-specific antibodies. If

homodimerization is occurring, then disulfide bonds could be formed between any of the three cysteines in TM1, one each in TMs 2 and 3 and three in the cytoplasmic tail.

CRIT cellular and issue distribution

We expressed Hu-CRIT both in *E. coli* (Figure 2C) and by in vitro transcription translation (D) as a ~31- to 32-kDa protein. Full-length $CRIT_{1–280}$ was also detected in Jurkat cell lysates using anti-ed1 or anti-ed2 as well as an antibody, anti-id2, against the cytoplasmic tail of CRIT, specifically against a peptide based on the region K195–A208. Anti-ed1 also revealed the rat CRIT homolog as a ~31- to 32-kDa protein in the rat T cell line, C58 (NT). Comparing CRIT sequences available, the predicted size of human CRIT is not dissimilar to CRIT from *T. cruzi*, *S. hematobium*/*S. mansoni*/*S. japonicum*, or rat. Compared with CRIT monomer electrophoresed under nonreducing conditions (Figure 2D), 1 mM DTT decreased the electrophoretic mobility of CRIT monomer (as viewed on an 8% SDS-PAGE gel to maximize the resolution of proteins between 20 and 40 kDa), indicating a likely reduction of an intramolecular disulfide bond.

Western blotting (Figure 2E) showed expression of CRIT in various human hemopoietic cell lines including Jurkat (T lymphocyte), Raji (B lymphocyte), THP-1 (myeloid), U937 (myeloid), and ECV304 (endothelial), as well as several nonleukocyte cells (Table 1) such as T47D (epithelial breast ductal) and weakly on HeLaS3 (epithelial) and MRC5 (fibroblast). In addition, CRIT was detected by immunoblotting in human platelets, monocytes, dendritic cells, and lymphocytes, but not in erythrocytes, neutrophils, nor HS1 (liver fibroblast-like cell line). CRIT was also found (Figure 2E and Table I) in testis, tonsil, kidney, thymus, and liver (latter not shown), but not in colon.

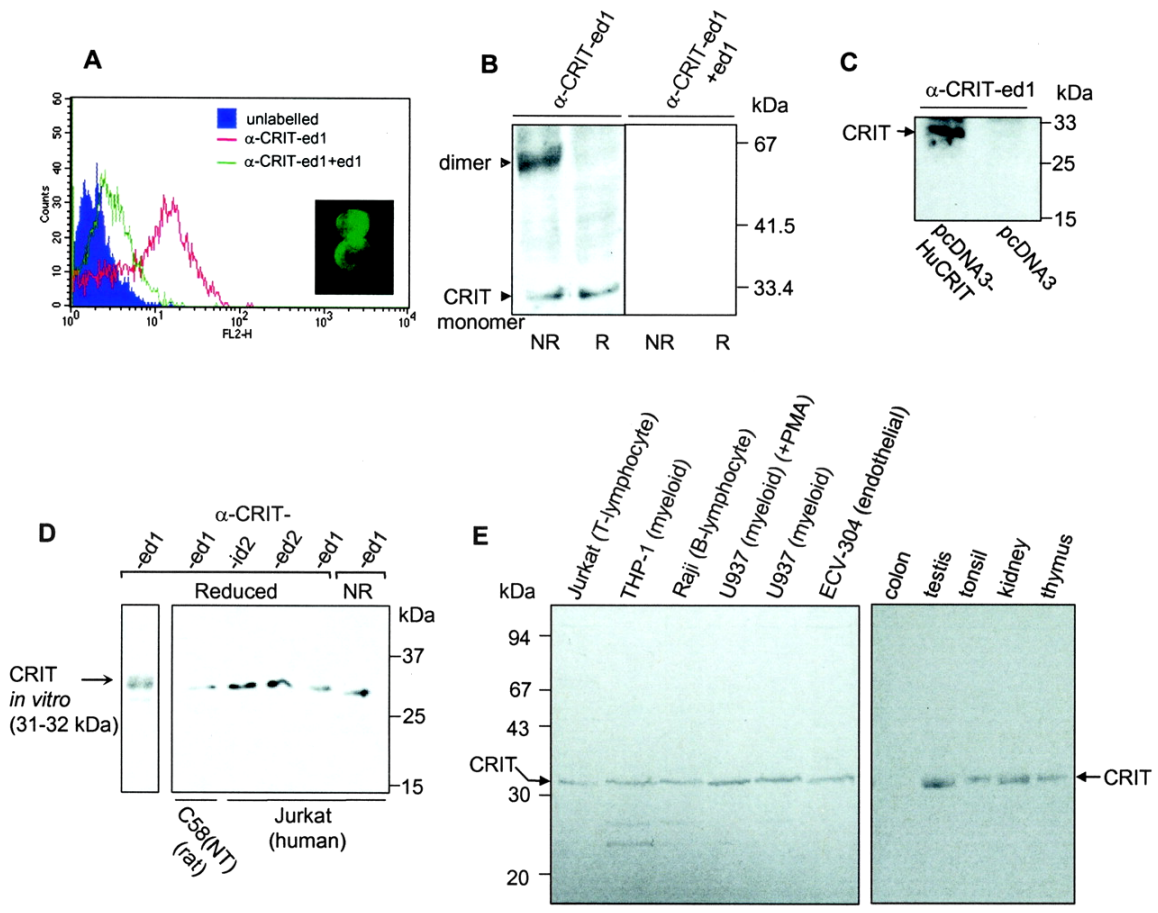


Figure 2 CRIT cell/tissue distribution and oligomer formation

(A) Recognition of CRIT on Jurkat cell surface by FACS analysis and immunofluorescence microscopy (*inset*). **(B)** Immunoblot of Jurkat cell lysate run under nonreducing (without DTT) and reducing conditions (+50 mM DTT) on a 12% SDS-PAGE gel shows dimer formation. The signal, which was absent after incubation with anti-CRIT-ed1 preabsorbed with ed1 peptide was also absent after incubation with preimmune serum (latter not shown). Cell lysates made in the presence of iodoacetamide (50 mM) also showed CRIT dimers under nonreducing conditions. **(C)** Expression in *E. coli* of 32-kDa Hu-CRIT (1–282 aa). **(D)** CRIT ORF cDNA (nt 1–846) expressed in a cell-free in vitro transcription/translation system and resulting protein probed in Western blot with anti-CRIT-ed1. Full-length Ra-CRIT$^{1–282}$, is detected in a rat T cell line, C58(NT), with anti-ed1 on an 8% gel and in a human T cell line (Jurkat) lysate, Hu-CRIT$_{1–280}$ is detected with several antibodies: CRIT is detected in Jurkat cells with anti-id2 antibody, against the region K195-A208 of the cytoplasmic tail, as well as anti-ed2. CRIT monomer is also detected under reducing conditions with anti-ed1 at a slightly lower electrophoretic mobility than the nonreduced monomer. **(E)** Western blots probed with anti-CRIT-ed1 of total protein extracts of various human hemopoietic cell lines and human tissues, run under reducing conditions.

Table 1 Cell and tissue distribution of Hu-CRIT

Cell Type / Tissue	CRIT
T47D (epithelial breast ductal carcinoma)	+
MRC5 (fibroblast, passage 31)	+/–
HS1 (liver fibroblast)	–
HeLa S3 (cervix uteri epithelial carcinoma)	+/–
Monocytes	+
Macrophages	–
Lymphocytes	+
Neutrophils	–
Dendritic cells	+
Platelets	+
Erythrocytes	–
Liver	+

Localization of CRIT in 38 normal, fresh (biopsy material) human tissues was determined by immunohistochemistry (summarized in Figure 3) using anti-CRIT-ed2, which unlike anti-CRIT-ed1, worked well in staining of paraffin-embedded sections. We were able to establish the following distribution for the CRIT receptor: CRIT was found to be strongly to moderately positive in almost all tissues in smooth muscle cells (SMCs) and related cells (pericytes) in vessels. According to the literature, complement regulators DAF, CD59, membrane cofactor protein (MCP), and Crry are expressed at the mRNA level by vascular SMCs in rat [27], whereas C3 and C4 are found in human SMCs [28]. Interestingly, CRIT stained strongly in glandular epithelial cells in the endometrium (Figure 3A) during the proliferating but not secretory phase. Likewise, MCP, DAF, and CD59 are expressed on an endometrial epithelial cell line [29] and, together with complement receptor 1 (CR1), expressed in normal endometrial tissue in all phases of the menstrual cycle [30]. Other CRIT-positive cells include pancreatic islet cells (Figure 3B) (which, according to their percentage and distribution, are suggestively insulin-producing β cells), cells which also express CD59 and MCP [31]. Keratinocytes in the esophagus (which express DAF, MCP, and CD59) [32] also stained positive for CRIT (Figure 3C). In the esophagus, SMCs were also clearly CRIT positive (Figure 3D). In the kidney, anti-CRIT-ed2 showed a strong reaction with podocytes in kidney glomeruli (Figure 3E) (as for CR1) [33] as well as endothelia and SMCs of blood vessels (F) but not in fibrocytes. However, DAF, MCP, and CD59 are expressed on glomerular epithelial, endothelial, and mesangial cells [34]. In the testis (Figure 3G and H), there was a positive reaction of scattered and partly elongated cells attached to the basement membrane of the testicular tubules, which most likely are Sertoli cells. CRIT was also found in the myoepithelia of

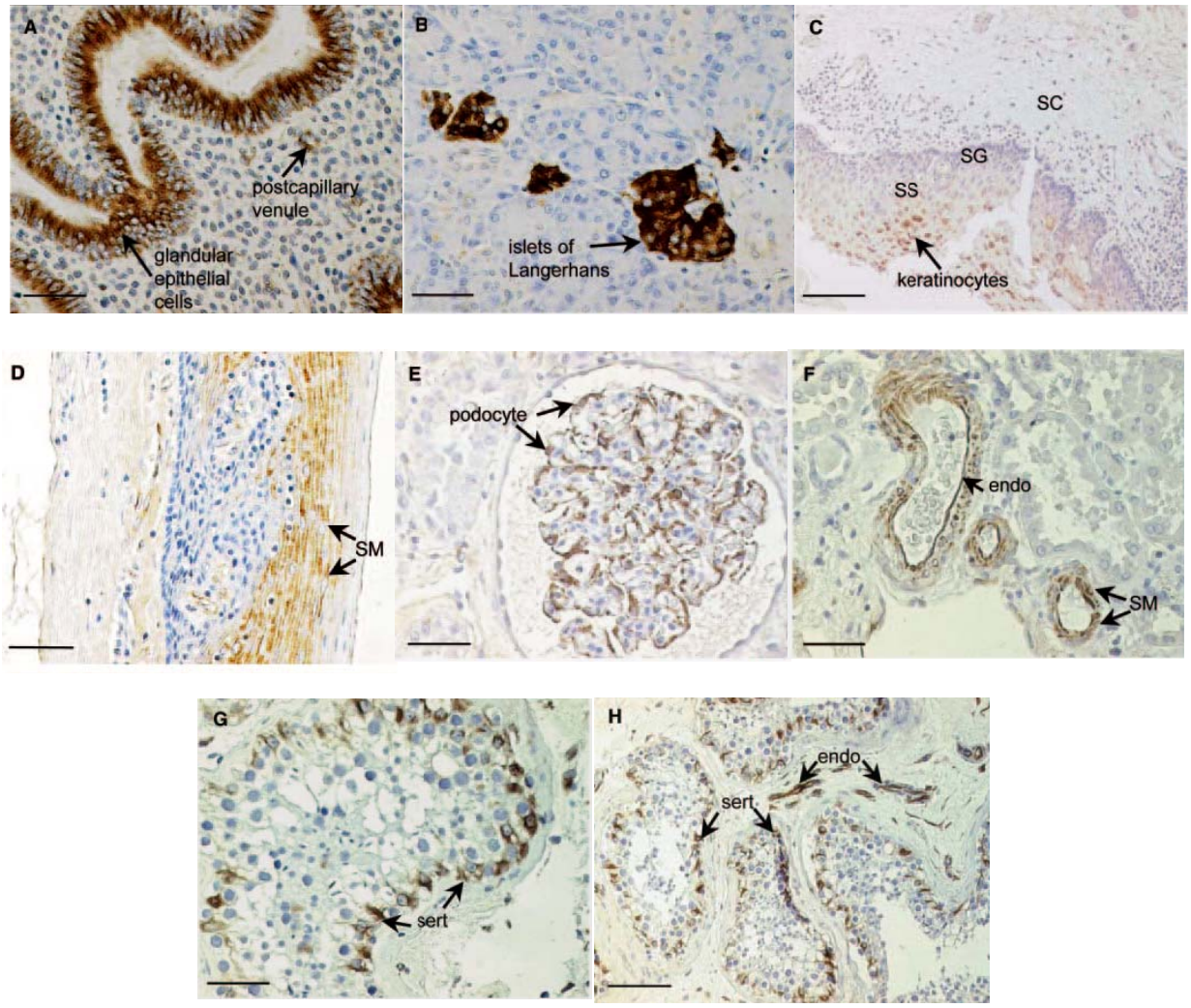


Figure 3 Cellular localization of CRIT by immunohistochemistry in various human tissues probed with anti-CRIT-ed2

Proliferating endometrium **(A)**; pancreas **(B)**; esophagus **(C and D)**; kidney **(E)**; venules/arterioles in kidney **(F)**; testis **(G and H)**. Scale bars, **F** and **H**, 100 µm; **A-E** and **G**, 50 µm. SS, Stratified spinosum; SG, stratified germinativum; SC stratified corneum; SM, smooth muscle; sert, Sertoli cells. Controls that consistently gave no staining (not shown) included omission of primary antibody (anti-ed2) as well as primary antibody absorbed with ed2 peptide. Identical staining patterns in kidney were obtained from at least 10 fresh tissue samples.

some exocrine glands (breast) and in stromal cells in breast and prostate gland. Finally, in the placenta, there were groups of decidual cells weakly positive for CRIT.

CRIT binds complement C2

We previously demonstrated C2 binding to Sh-CRIT-ed1 by affinity purification of C2 from NHS using the Sh-CRIT-ed1 peptide [19]. Furthermore, by using C2 hemolytic assays in which limiting amounts of C2 (just sufficient to restore complement activity) were added to C2-deficient serum, we showed that preincubation of C2 with CRIT-ed1 peptide inhibited complement activation [10]. We now confirm the C2 interaction with Hu-CRIT by ligand blotting. For this, total protein lysates of Jurkat cells, immunoblotted onto nitrocellulose, were probed with $C2^{bio}$ (Figure 4A). C2, like anti-ed1, bound to monomeric and dimeric CRIT. C2 still bound when preincubated with either CRIT-ed2 peptide or CRIT-H17S peptide. However, CRIT-ed1 peptide abrogated C2 binding. Identical results were obtained with ^{125}I-labeled ligand. In work to be reported in detail elsewhere, we showed that a recombinant von Willebrand factor A (vWFA) domain of C2, binds CRIT via ed1, and that this binding can be blocked by prior incubation of vWFA with the C2 monoclonal antibody Hyb-5050 [35]. In this study, we show that the vWFA domain of C2 expressed and purified from *E. coli*, binds in vitro-expressed CRIT. After stabilizing the protein interaction by cross-linking, both unbound CRIT and CRIT-vWFA C2 are detected by anti-CRIT-ed1 in Western blots (Figure 4B). The binding of biotinylated C2 or FITC-labeled C2 (not shown) to Jurkat cells via Hu-CRIT-ed1 was also shown by FACS analysis (Fig. 4C) (and immunofluorescence microscopy). Binding

could be eliminated by prior incubation of the cells with anti-CRIT-ed1 blocking antobody.

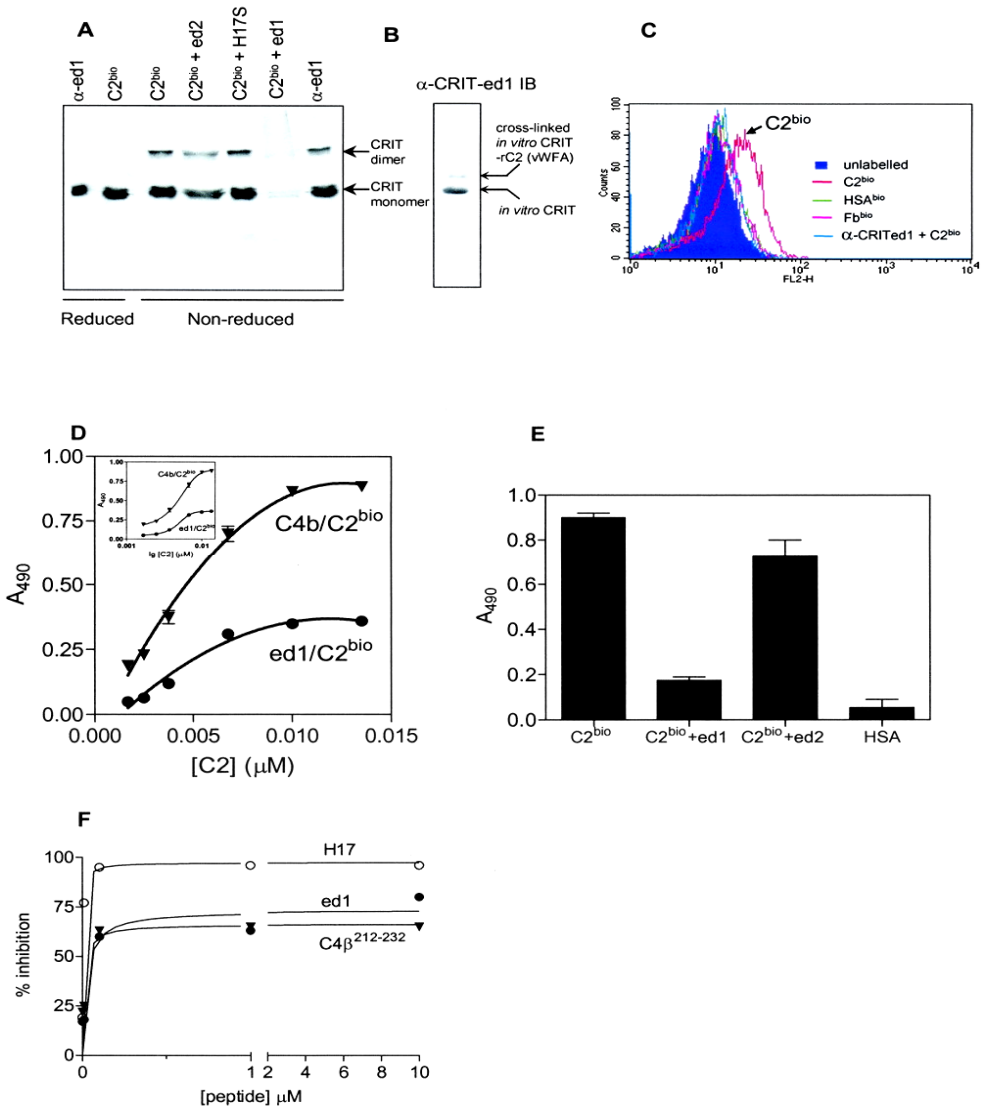
The dependence on $C2^{bio}$ concentration for the ELISA signal of $C2^{bio}$ interacting with C4b or CRIT-ed1 and that this interaction is a saturable phenomenon are shown in the graph of binding data or A_{490} (y) as a function of ligand concentration (X) (Figure 4D). Analysis of the results indicated that the one-binding class model was a better fit than the two-binding class model. This dose dependency and saturable binding is also shown in the graph *inset*. Nonlinear regression of the equation describing one-site binding of ligand to receptor that follows the law of mass action ($y = B_{max}X/K_{D(app)} + X$; Equation 1), where B_{max} is the maximal binding, and $K_{D(app)}$ is the concentration of ligand required to reach half-maximal binding) onto the data shown in Figure 4D gave an estimate for $K_{D(app)}$ of 0.0152 µM for C4b/$C2^{bio}$. This is in close agreement with the K_D estimate of 0.015 µM for C4b/C2 by plasmon surface resonance [36] and indicates that the biotinylation of C2 had not compromised its protein-binding ability. In our study, the K_D from a Scatchard analysis plot of concentration of C2 bound (v_{bound}) against concentration of free C2, was not calculated.

Although we have no data for the affinity of C2 for native CRIT, we have been able to estimate the affinity for the ligand-binding region, CRIT-ed1 in the form of a synthetic peptide. Comparison of data for $C2^{bio}$ binding CRIT-ed1 or C4b (Figure 4D) indicates that an ELISA reading for CRIT-ed1/$C2^{bio}$ binding ~3-fold lower than for C4b/$C2^{bio}$ corresponds to a $K_{D(app)}$ for CRIT-ed1/$C2^{bio}$ (0.022 µM) 2-fold lower than between C4b/$C2^{bio}$ (0.0152 µM) (K_D of 0.015 µM [36] (Table II). According to our estimations, C2 has an equal to 2-fold lower affinity for CRIT than for C4b and therefore

could not prevent assembly of the CP C3 convertase but is more likely reducing excessive activation on a target. The concentration of C2 (ligand) in plasma is 0.25 μM. This is ~10 times the average (using two different methods; Table 2) equilibrium dissociation constant of C2-CRIT receptor complexes, $K_{D(app)}$ of 0.0235 μM, implying (Equation 1) that C2 will occupy 90% of CRIT receptors at equilibrium.

Again, maximum blockage of the C4b/$C2^{bio}$ interaction was possible (Figure 4E) by preincubation of $C2^{bio}$ with a concentration of ed1 (1.2 μM) ~80 times that of the K_D for C4b/C2 (0.015 μM) and ~51 times the K_D for CRIT-ed1/C2 (0.0235 μM). This is in agreement with predictions for ligand/receptor associations that the ligand concentration needs to be 99 times the K_D to occupy 99% of the receptors at equilibrium. C4b/$C2^{bio}$ binding could not be inhibited by preincubating $C2^{bio}$ with ed2 (Figure 4E).

As an additional test of the binding of C2 to CRIT-ed1, it was possible to inhibit the binding of C2 to C4 in the assembly of the CP C3 convertase with increasing concentrations of CRIT- or C4-based peptides. Plotting percent inhibition of hemolysis, as a measure of the extent of binding of CRIT- or C4-based peptides to C2, resulted in Figure 4F. Similar nonlinear regression calculations of the data gave estimates of $K_{D(app)}$ for CRIT-ed1/C2 of 0.025 μM and $C4\beta_{212–232}$/C2 (where $C4\beta_{212–232}$ is the C4β-chain peptide described before [10] of 0.011 μM. These data are in good agreement with other empirical data summarized in Table II, and are not compromised by the many proteins in NHS, because the CRIT-ed1/C2 vs C4b/C2 interactions are highly specific. CRIT-H17 representing the 11 amino acid C-terminal part of CRIT-ed1 has a 10-fold order of magnitude greater affinity for C2 than CRIT-ed1. Interestingly, in preliminary unreported



Fifure 4 Complement component C2 binds Hu-CRIT on the cell surface via extracellular domain 1

(A) Ligand blotting showing binding of $C2^{bio}$ to the dimeric and monomeric forms of Hu-CRIT in Jurkat cells. Blocking is successful by preincubation of C2 with a 10,000 M excess (100 μM) of CRIT-ed1 peptide only. **(B)** CRIT expressed in an in vitro reticulocyte lysate system binds to rC2 (vWFA domain). The proteins are then cross-linked as described before [10] and detected by immunoblotting (IB) with anti-CRIT-ed1. **(C)** Binding of $C2^{bio}$ to Jurkat cell surface in FACS analysis. Binding is blocked by preincubating cells with anti-CRIT-ed1. **(D)** ELISA for binding of $C2^{bio}$ to C4b or CRIT-ed1. **(E)** Blockage of ELISA signal for C4b/$C2^{bio}$ interaction with CRIT-ed1, but not CRIT-ed2 peptide. **(F)** Percent inhibition of hemolysis (as a measure of the extent of binding of CRIT- or C4-based peptides to C2) with increasing peptide concentration.

Table 2 Dissociation constant ($K_{D(app)}$) for C2 binding to CRIT-ed1/-H17 and $C4\beta_{212-232}$/C4b[a]

Assay	C2-Interacting Protein		
	CRIT-ed1	CRIT-H17	$C4\beta_{212-232}$
Hemolytic assay	0.025	0.0033	0.01
Surface plasmon resonance [36]			0.015 (C4b/C2) (K_D)
ELISA	0.022		0.0152 (C4b/C2)

[a]Micromolar concentration. Mean of four independent experiments.

results before the study of Inal and Schifferli [10], CRIT-H17 alone, but not CRIT-ed1, gave significant reductions in various parameters of complement-mediated inflammation in the reversed passive Arthus reaction in mice [11]. In all current experiments, C2 binding to Hu-CRIT-ed1 was not Mg^{2+} dependent.

CRIT inhibits CP-mediated complement cytolysis

To ascertain whether CRIT is able to protect cells from complement-mediated attack, cells sensitized with antibodies against human lymphocytes (or whole human serum) were incubated with increasing concentrations of anti-CRIT antibody, before exposure to NHS as a source of complement. A titration was first conducted with increasing NHS (Figure 5A) to show that lysis was being achieved with this system and to find a convenient serum concentration (chosen to be 10%) to be able to see easily any increase in percent lysis of cells. The results (Figure 5B) show a significant increase, from 8% lysis without antibody to 28% lysis with 12.5 μg/ml anti-CRIT-ed1 in the promonocytic cell line, U937. With the macrophage-like THP-1 cells (Figure 5C), and monocytes (D), there were significant increases from 10 and 15% lysis, respectively, to 43 and 52%, respectively, with 10 μg/ml. As noted previously, macrophages are negative for CRIT (see Table I). Monocytes treated with IFN-γ, thereby expressing lower levels of CRIT, showed a nonsignificant increase in cytolysis from ~20% without antibody to 34% with 10 μg/ml anti-CRIT-ed1. To control for a possible activation of complement through the presentation of additionally bound anti-CRIT and so additional Fc by the neutralizing anti-CRIT antibody, we incubated cells with increasing anti-CRIT-ed1 but without prior sensitization with the complement-fixing anti-lymphocyte serum. Under these conditions

shown in Figure 5B and C (▲), there was no increase in cytolysis with increasing anti-CRIT-ed1 concentration, indicating that the blocking antibody did not activate complement.

C2 bound to CRIT is rendered resistant to C1s cleavage

We and others have shown previously that CRIT-ed1 inhibits the complement-mediated lysis of sheep erythrocytes [10, 19-21]. Having also shown that cleavage of C2 by C1s was inhibited in the presence of ed1 peptide, we now wanted to see whether this inhibition could be provided by native CRIT as found on the surface of Jurkat cells. As illustrated in Figure 5E, CRIT-ed1 peptide completely inhibited C1s cleavage of C2. This is in contrast to cleavage to C2a and C2b by C1s in the presence of sham peptide (C4βS). Interestingly, in the presence of Jurkat cells (and therefore CRIT), there is almost no cleavage of C2. When the receptor is blocked with anti-CRIT-ed1, the inhibition by CRIT of C1s-mediated cleavage of C2 is lifted.

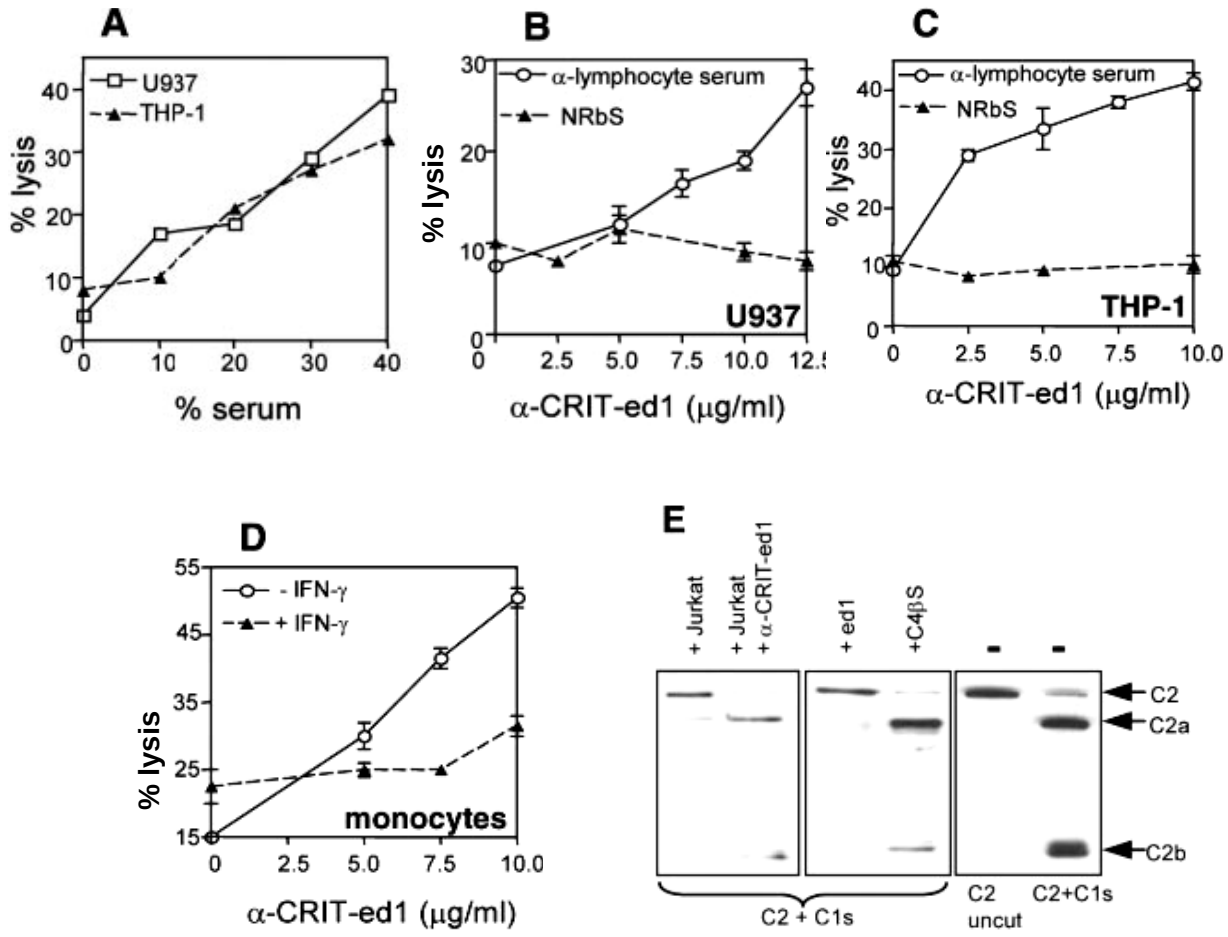


Figure 5 Complement-dependent cytotoxicity is suppressed by CRIT on CRIT-expressing cells

(A) Mean effect of increasing NHS on the percent lysis of antibody-sensitized (using anti-lymphocyte serum) human U937 and THP-1 carcinoma cells after 37°C incubation for 30 min. Effect of increasing anti-CRIT-ed1 on the percent lysis in the presence of 10% NHS, after 37°C incubation for 30 min, of antibody-sensitized U937 cells **(B)**, THP-1 cells **(C)**, and IFN-γ-differentiated monocytes **(D)**, vs untreated monocytes. As a control both for probable activation of complement by the blocking anti-CRIT-ed1 and for showing that CP is being considered, in **B** and **C**, the mean effect of increasing anti-CRIT-ed1 on THP-1 and U937 cells, pretreated with normal rabbit prebleed or rabbit IgG, was also detected in the same assay. The results presented are the mean of three experiments. **(E)** Cleavage of C2 (2 μg) by C1s (0.2 μg) in the presence of the following: *lane 1*, 5 x 10^3 Jurkat cells; *lane 2*, as for *lane 1* but cells preincubated with anti-CRIT-ed1 (20 μg/ml) for 30 min at 20°C; *lane 3*, 150 μM CRIT-ed1 peptide; *lane 4*, 150 μM control sham peptide (C4$^{\beta}$S) [10]; *lane 5*, uncut C2; *lane 6*, C2 cleaved with C1s. Throughout, C2 is detected by immunoblotting using anti-C2 antibody [10].

DISCUSSION

The complement system is a rapidly activated and self-amplifying system that, to avoid extensive consumption and self-depletion, requires control at several levels. Interestingly, there is often symmetry of function between inhibitors of complement in the fluid phase and on cell surfaces [37], although there is an exception: C1-INH is a major regulator of C4 and C2 cleavage in the fluid phase, preventing excessive formation of the C3 (C4b2a) convertase.

Hu-CRIT was cloned from both genomic and cDNA. Thus, although the 5' and 3' untranslated regions may yet be found to possess introns, the high level sequence identity suggests that the coding region of Hu-CRIT could be coded for within a single exon and therefore be intronless, as for example are many *GPCR* genes. Whether the entire *CRIT* gene is shown to possess introns or not, we have presented evidence that it encodes a functional receptor (of a size equivalent to that predicted from the ORF) that has affinity for the human complement serum protein, C2. Using an antibody raised against the N-terminal extracellular domain, CRIT was found on the plasma membrane in a wide variety of human cell types. We show that the *Schistosoma* (and *Trypanosoma*) parasite and its human host share a receptor for C2, and we postulate that they share a complement regulatory function. The high sequence homology between the mammalian (human and rat)/parasite (*Schistosoma* and *Trypanosoma*) and cod *CRIT* genes is also indicative that they may have evolved from a common ancestral gene.

CRIT regulates the formation of the CP C3 convertase by reducing the association of C2 with C4b. The $K_{D(app)}$ of CRIT-ed1/C2 is of the same order as the K_D for C4b/C2 (0.015 μM) [36] and also CR1 for C3b dimers (0.01–0.02 μM) [38, 39]. Overall, CRIT-

ed1 appears to compete with C4b for binding to C2 with an apparent moderate affinity, but we do not yet know whether CRIT accelerates the decay of the C4b2a convertase. In this study, we have found that anti-CRIT-ed1 blockage of the CRIT receptor increases the complement-mediated killing of two human myeloid cell lines (U937 and THP-1) and of monocytes. However, the relative contribution of CRIT to protecting against complement-dependent cytotoxicity in the THP-1 cells, for example, cannot be known, because certain other complement regulatory proteins are expressed, such as CD59 [40]. Once C2 is bound to CRIT (unlike C2 bound to C4b), it cannot be cleaved by C1s to yield C2a and C2b and thus no longer partakes in C3 convertase formation. Of the complement regulators that control the C3 convertase (C4b2a) and C5 convertase (C4b2a3b), three of them, DAF, CR1, and C4BP, compete with C2 for binding to C4b and also bind to C3 or C5 convertases, causing C2 dissociation. These proteins are composed mainly or only of complement control protein repeats, which contain the C4b (and C3b) binding sites and are all encoded by genes in the RCA cluster on chromosome 1q31–32. CRIT, like CD59, is another member of the complement control family of proteins that lacks structural resemblance to the RCA group of proteins.

In humans, CRIT is widely distributed. It is present on many hemopoietic cells, but not neutrophils, and notably, like MCP, not on erythrocytes. CRIT is found in many tissues, and interestingly, like DAF [41], its expression appears to be hormonally regulated during the menstrual cycle. CRIT is also found on endothelial and epithelial cells. Besides its complement-regulating function concomitant with its wide distribution in the body, we speculate that its particular expression in highly differentiated cell types,

such as glomerular podocytes [42], Sertoli cells [43], and keratinocytes [44, 45], could suggest additional roles.

Complement results in the release of the anaphylatoxins C3a and C5a, which in turn bring about the proinflammatory responses of complement, including an increase in vascular permeability, mast cell degranulation, and smooth muscle contraction. C5a is then able to further increase the proinflammatory response by inducing chemotaxis of macrophages and neutrophils and by the activation of neutrophils. These inflammatory responses are a natural reaction of host tissue to injury. However, an unregulated inflammatory response may injure host tissues and is important in many complement-mediated diseases [46]. As complement and its activation products, in particular, C5a, can aggregate, stimulate, and cause degranulation of neutrophils, any anti-inflammatory agents should, in certain diseases, preferably interrupt the complement cascade at an early stage. In myocardial infarction, for example, it might be important to block CP and/or lectin pathway but leave the AP and lytic pathway intact. By competing with C4b for binding to C2, and inhibiting its activation, CRIT prevents the formation of the CP C3 convertase and is thus an obvious target for diminishing the proinflammatory response of unregulated complement activation. A soluble form of CRIT in the form of the CRIT-ed1 peptide has been shown to inhibit CP-mediated hemolysis by human serum. Indeed, recombinant soluble forms of the membrane complement inhibitors have been proven to inhibit complement-mediated inflammation [46]. If soluble CRIT in the form of the CRIT-ed1 peptide or the smaller CRIT-H17 derivative peptide is not antigenic in humans, then it could potentially be used as a therapeutic agent against certain complement-mediated human diseases. The first promising tests of the in vivo efficacy of CRIT-H17

in blocking immune complex-mediated tissue injury in the classical inflammation model, the reversed passive Arthus reaction [11], and in Forsman shock [20] should be further addressed.

It is well documented that conformational changes can occur within the secondary structure of a receptor upon reduction [47, 48]. Essentially, reduction of the intramolecular disulfide bond(s) of a protein, as we believe occurs with CRIT, results in its unfolding from a globular state to a more rod-like conformation, which migrates more slowly in an SDS-PAGE gel. Whether CRIT folding or dimerization affects its ability to bind ligand, as for example with the TNFR, BAFF-R [49], will be the subject of future work.

If the dimerization of CRIT occurs through the formation of intermolecular disulfide bonds then candidate cysteine residues would be $Cys^{31,40,41}$ of TM1, Cys^{69} of TM2, Cys^{110} of TM3, or $Cys^{126,214,280}$ of the cytoplasmic tail. Disulfide linkages are not usually found in the cytoplasm because of the reducing environment there. Because there are no cysteines in the extracellular domains, it seems probable that the cysteines in the TM domains are involved in dimerization. Disulfide-bonded dimers, mediated through TM domains have been reported for some receptors such as CD44 [50] and the insulin receptor [51]. Although reducing agents can dissociate receptor homodimers suggesting disulfide-bonded dimerization, other intermolecular hydrophobic interactions, between TM domains, have been implied, at least for the GPCRs [52]. We previously noted that the C2-binding CRIT-H17 motif [F/H]EVK$X_{4/5}$P, which in the C4β chain occurs contiguously, separated by a β-turn, in CRIT, occurs only once, and also speculated that CRIT might be found to exist as a dimmer [10]. Our current study has shown Hu-CRIT to

form dimers (and that C2 binds to both CRIT monomers and dimers). If these dimers are shown to be homodimers, then it is conceivable that two CRIT-H17 motifs (forming part of two ed1 regions) could be brought into close proximity for C2 binding, as in the C4β chain. Whether dimeric CRIT binds C2 with greater affinity and represents the functional form of the receptor we cannot say, but already the work of Oh et al. [20] indicates that a recombinant homodimeric CRIT-H17 peptide has an increased binding capacity for C2 and 5-fold higher anti-complementary activity.

In addition to genetic abrogation and mutagenesis experiments in cells, which together with structural studies are revealing details of the CRIT-ed1 interaction with C2, it is envisaged that *CRIT* gene knockout experiments will place the relative contribution of CRIT toward complement regulation in context with other CP regulators.

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SECTION II

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Expression of Functional Recombinant von Willebrand Factor-A Domain from Human Complement C2: A Potential Binding Site for C4 and CRIT

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ABSTRACT

CRIT (complement C2 receptor inhibitor trispanning) 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 C4α, 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 C4β-chain. In a hemolytic 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.

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 hemopoietic 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-amino acid 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 C4β 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 Bade 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	HEVKIKHFSPY	-CO_2H
H17	NH_2-	HEVKIKHFSPY	-CO_2H
H17-2	NH_2-	HEVKIKHFSPYHEVKIKHFSPY	-CO_2H

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. Ni^{2+}-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 $MgCl_2$ 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. GVB^{2+} buffer was prepared by mixing 200 ml GVB with 1 ml of 30 mM $CaCl_2$ and 2 ml of 100 mM $MgCl_2$.

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 Ni^{2+}-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 Ni^{2+}-NTA resin (Qiagen, Switzerland) at room temperature for 1 h with gentle shaking, which was equilibrated with 40 ml Ni^{2+}-NTA chromatography buffer, with addition of 30 mM imidazole. After incubation, the Ni^{2+}-NTA resin was washed thoroughly with Ni^{2+}-NTA chromatography buffer supplemented with 30, 60, and 90 mM imidazole respectively, until the absorbance A_{280} became zero. Then, the recombinant vWFA-214 was eluted with Ni^{2+}-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 μl 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 μg of purified recombinant vWFA-214 was diluted in 50 μl binding/wash buffer included in the kit and mixed with 20 μl 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 μg of pure C4 protein (diluted in 50 μl binding/wash buffer) or 50 μl 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.

Hemolytic 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 x 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 μg) were mixed with different volumes of diluted NHS (1:100 dilution; 0, 20, 40, 60,

80, 100 and 120 µl) and made up to 150 µl with freshly prepared GVB^{2+} buffer, which was then pre-incubated for 30 min at room temperature. After the pre-incubation, 50 µl EA was added and incubated for 30 min at 37 °C. Background control was obtained by incubating NHS with GVB^{2+} 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 C4α, 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 µl 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 µl was added to each well (1 µg per well) and incubated at RT for 1 h. Next, 100 µl 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 μl 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 μl 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 μl of 2 N H_2SO_4 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 μg) was mixed with CRIT peptides (1.5 μg) 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 μg) 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.

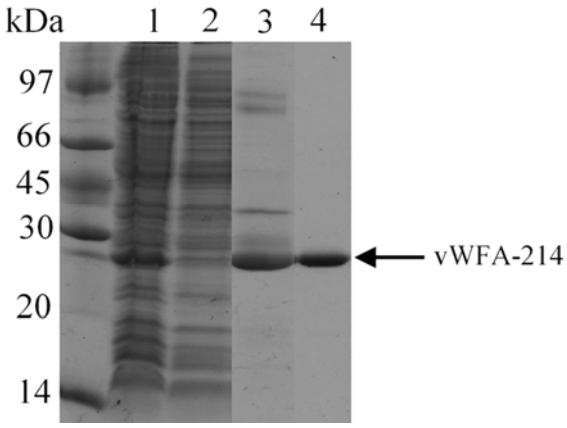
RESULTS AND DISCUSSION

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 Cys^{267} is absent, the yield and solubility of C2 vWFA domain cannot be improved by introducing a $Cys^{267} \rightarrow$ Ser mutation [14]. Even though there is another free Cys (residue 241) in the C2 vWFA domain, Cys^{241} 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 Ni^{2+}-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.

A



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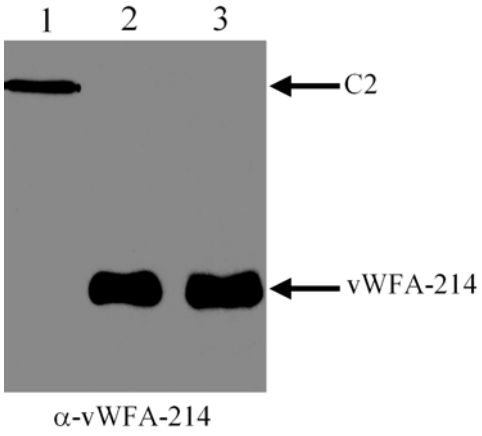

Figure 1 Expression and purification of the recombinant vWFA-214 domain

(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 Ni^{2+}-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 μg 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).

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 C4α 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 C4α chain into C4d and α4 [23].

In using C2 deficient serum (obtained from a patient with type I complement C2 deficiency) in the hemolytic assay, vWFA-214 could not restore the hemolytic 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 hemolytic assay using NHS, the recombinant vWFA-214 domain was able to inhibit the hemolysis by competing with intact C2 in NHS for C4b binding in a dose dependent manner.

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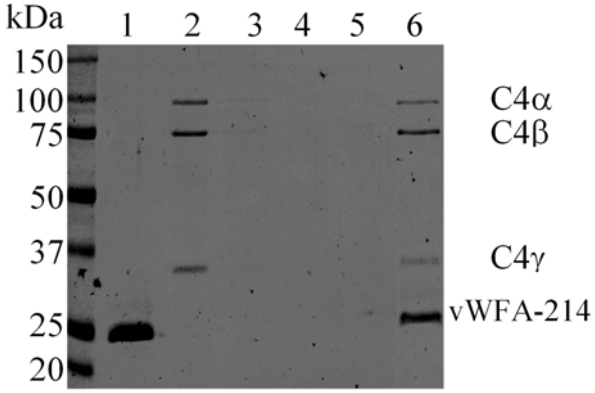

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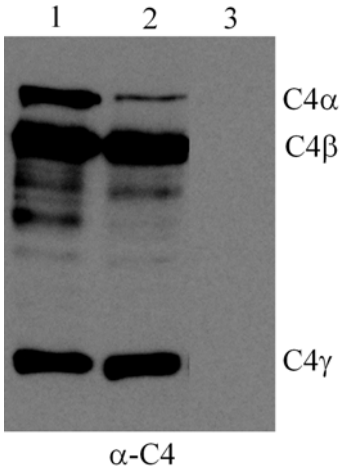

Figure 2 Interaction of C4 with the recombinant vWFA-214 by MagneHis Ni-Particles pull-down assay.

(A) 3 μg 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 μl of elution from vWFA-214-NHS pull down assy. Lane 3, 20 μl of elution from negative control, pull-down assay using NHS without vWFA-214.

In the presence of 2.5 μg vWFA-214, there was minimal inhibition of hemolysis (data not shown). 8.26 % of inhibition was observed with 5 μg vWFA-214 and this increased to 20.54 % and to a maximum 44.93 % with 10 and 20 μg vWFA-214 respectively.

By site-directed mutagenesis, Horiuchi *et al* [9] showed that Cys^{241} 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 Cys^{241}. 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 C4β 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 C4α, 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 C4α and C4γ chains, there is only negligible interaction with the vWFA-214 domain.

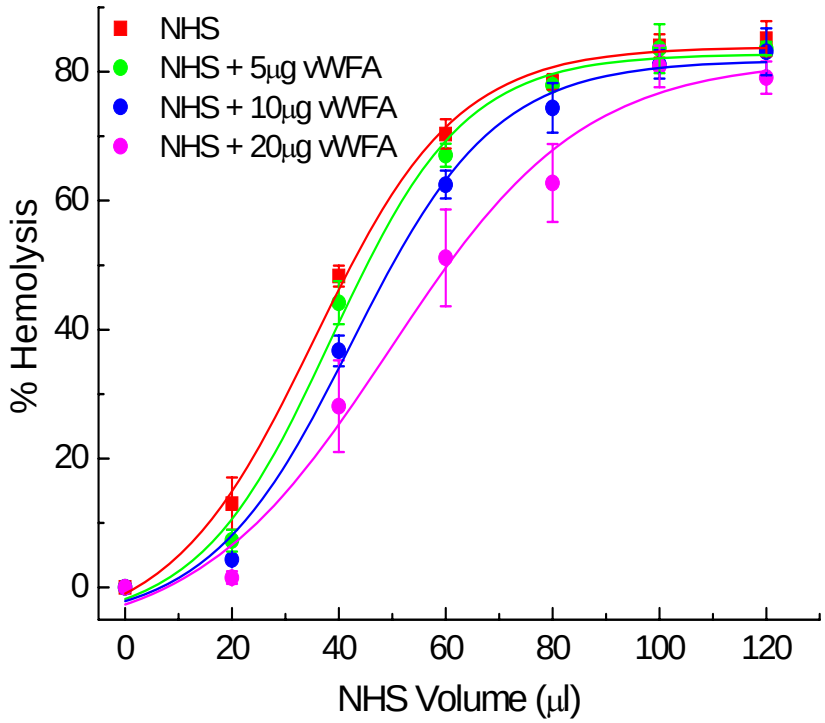

Figure 3 Inhibition of classical complement pathway activation by the recombinant vWFA-214 protein.

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 ± standard deviation (SD) of three independent experiments each of duplicate measurements.

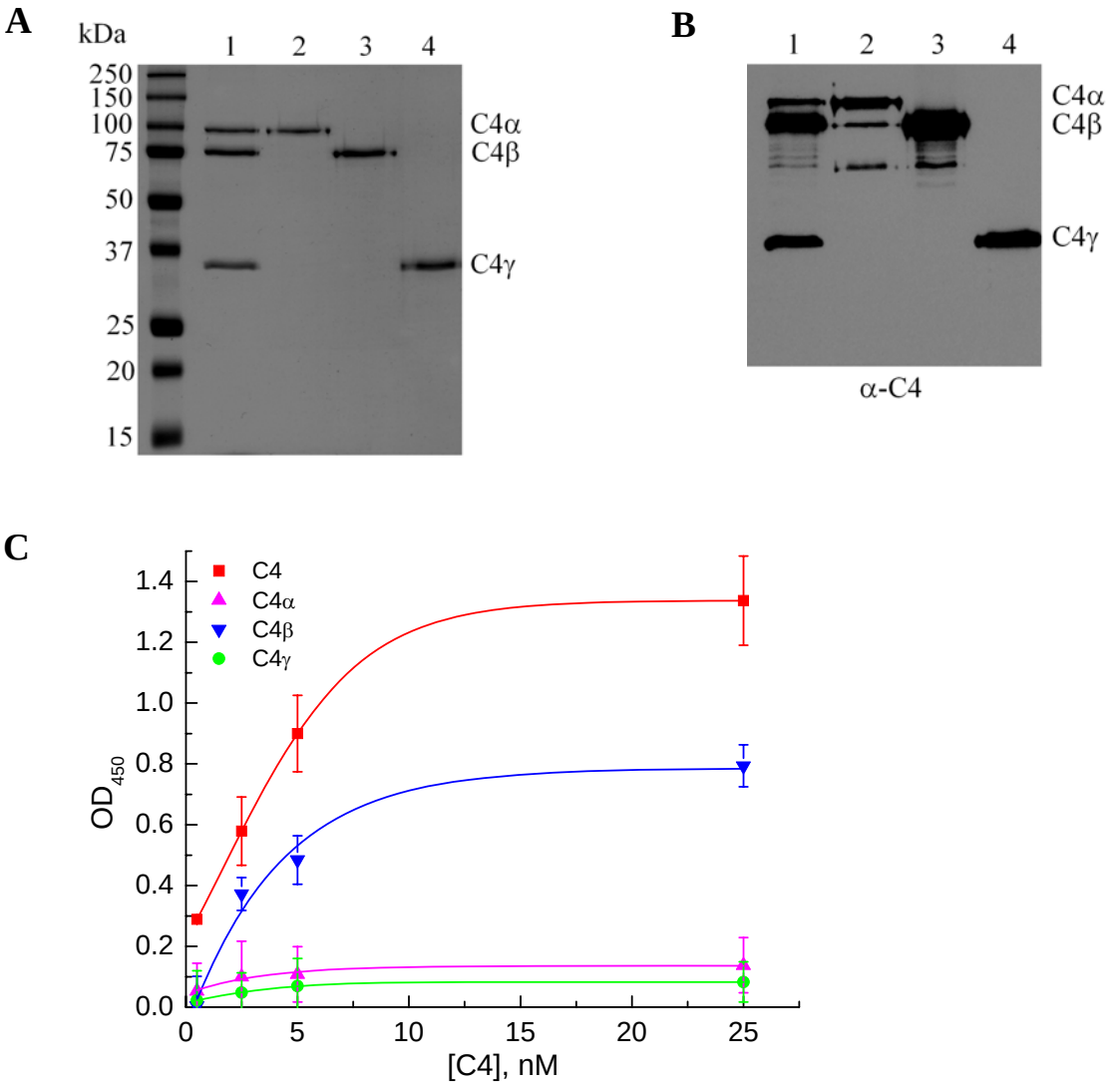

Figure 4 Sandwich ELISA of C4 and purified C4 chains with the recombinant vWFA-214 protein.

(A) C4 protein was separated into C4α, C4β and C4γ 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 C4α, C4β and C4γ 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 C4α, C4β and C4γ 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 C4β chain and vWFA-214 domain. The results show the mean ± SD of three independent experiments each of duplicate measurements.

By contrast, C4β 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 C4β chain only. Although these experiments do not allow an evaluation of the possible contribution of additional binding sites, the interaction between vWFA and the C4β 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

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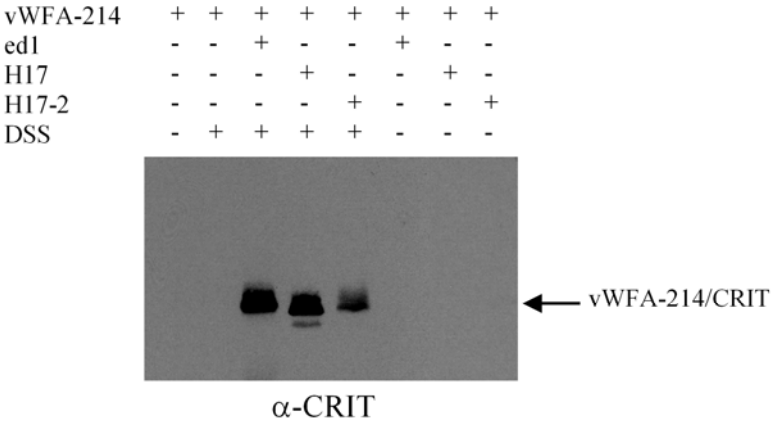

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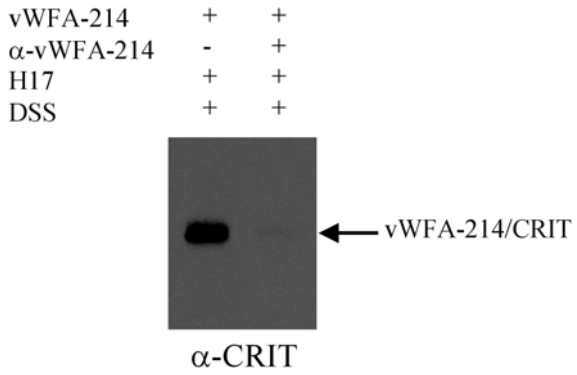

Figure 5 Ligand blot of the recombinant vWFA-214 and CRIT peptides showing the presence of a CRIT binding site on vWFA-214.

(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 corsslinker. **(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).

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 C4β 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 Cys^{241} of the C2 vWFA domain. In view of this, there is a high possibility that both CRIT and the C4β 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/C4β 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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SECTION III

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CRIT Peptide Regulates The Alternative Complement Pathway

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ABSTRACT

Complement C2 receptor inhibitor trispanning (CRIT) inhibits the classical pathway (CP) C3 convertase formation by competing with C4b for the binding of C2. The C terminal 11 amino acid of the first CRIT-extracellular domain (CRIT-H17) have a strong homology with a sequence in the C4β chain, which is responsible for the binding of C2. Since the CP and alternative pathway (AP) C3 convertases have many functional and structural similarities, we further investigated the effects of CRIT-H17 on the AP. The cleavage of factor B (FB) in the presence of C3b and factor D (FD) was blocked by CRIT-H17 but not by control peptides. By immunoblots using a cross-linking agent, CRIT-H17 was shown to bind FB and its two fragments, Ba and Bb, and C3b but not FD. The binding to FB was three fold higher than to C3b by ELISA. These data suggested that CRIT-H17 lies across the junction of the FD cleavage site. CRIT-H17 had no decay activity on the C3bBb complex as compared to decay-accelerating factor. In a hemolytic assay using C2 deficient serum, CRIT-H17 inhibited partially complement activation by the AP. Additional experiments showed that CRIT-H17 inhibited slightly the factor I-mediated cleavages of C3b in the presence of factor H or CR1 indicating that the regulatory role of CRIT-H17 is complex, probably related to its dual binding to FB and C3b. These findings reemphasize the analogies between both the AP and CP C3 convertases formation, and should help us to define the exact molecular contacts.

INTRODUCTION

The complement system consists of about 30 proteins, and is an important component of both innate and acquired immunity [1, 2], which mediates acute inflammatory and cytolytic reactions. It plays an important role in host defense and in the elimination of invading foreign pathogens. There are three separate pathways to activate the complement system; classical, alternative, and lectin pathways and several regulatory proteins to control the complement activation. Each pathway responds to different activators and activates the complement system in a sequential manner but all three pathways converge in the assembly of the C3 convertase and end in the formation of C5b-9, known as the membrane attack complex.

The classical pathway $(CP)^3$ relies on antibody to initiate activation of the C1 molecule and the mannan-binding lectin pathway is initiated by binding of mannan-binding lectin to sugar residues on pathogen surfaces. Besides the initiating factors, these two pathways are identical. The activation of the alternative pathway (AP) is Ab-independent and generates a distinct C3 convertase, C3bBb. The AP provides an immediate line of defense to foreign particles due to the spontaneous hydrolysis of the internal thioester bond in plasma C3 to form $C3(H_2O)$, which has an altered conformation allowing the binding of factor B (FB) [3, 4]. The first step in AP is the nucleophilic attack on the internal thioester bond in C3b by an amine or hydroxyl group on the pathogen surfaces, followed by binding of FB which forms the C3bB complex [5]. The Ba fragment of FB is involved in the initial interaction with C3b [6, 7] and both the von Willebrand factor-A (vWFA) domain and the serine protease (SP) domain of the Bb fragment interact with C3b [8, 9]. Binding to C3b induces a conformational change in FB

which in turn becomes susceptible to the cleavage by factor D (FD). The FD-mediated cleavage of FB is the rate-limiting step for generating the AP C3 convertase, C3bBb. However, the exact mechanism of assembling this bimolecular complex, C3bBb, is still under investigation even 50 years of the discovery of the AP [10].

As a protective mechanism, there are several complement regulatory proteins that protect self from the potentially destructive effects of complement activation. These proteins belong to the regulators of complement activation (RCA) gene family, which are encoded in the gene cluster on the long arm of chromosome one[11]. The RCA proteins control complement activation by either promoting an irreversible dissociation of complement convertases or acting as cofactors in the factor I (FI) mediated cleavage of C3b. The RCA proteins, decay accelerating factor (DAF), factor H (FH) and complement receptor 1 (CR1) are responsible for the dissociation of complement convertases and membrane cofactor protein (MCP), FH and CR1 act as cofactors in the FI-mediated cleavage of C3b. There is one common structural motif among the RCA gene family termed the short consensus repeat (SCR), which comprises approximately 60 amino acids and shares 20-40 % homology with each other.

Previously, we described a novel human complement CP inhibitor, complement C2 receptor inhibitor trispanning (CRIT), which is expressed on hemopoietic cells and in a wide range of tissues [12]. The first extracellular domain (ed1) is a binding site of C2. After binding, C2 is protected by CRIT from the cleavage by activated C1s. Synthetic peptides corresponding to CRIT-ed1 and to the C-terminal 11 amino acid of CRIT-ed1 (CRIT-H17) were shown to have a strong inhibitory effect on the CP of complement activation [13, 14]. This inhibition is best explained by competition of CRIT-ed1/CRIT-

H17 with C4 for C2 binding, and by the blockade of C2 cleavage by C1s. Later, we expressed the recombinant C2 vWFA domain and further demonstrated that there is a CRIT binding site on the C2 vWFA domain [15].

Since there are many common features between the complement CP and AP and many proteins in the CP function in a manner analogous to those of the AP, we further studied the regulatory effects of CRIT-H17 in the AP. In the AP, C3b, FB and FD serve as functional analogues to that of C4b, C2 and C1s of the CP, respectively. We started the studies with a hypothesis that CRIT-H17 interacts with FB and possibly inhibits the FD-mediated cleavage of FB. This report describes the novel regulatory properties of CRIT-H17 in the AP.

EXPERIEMENTAL

Reagents

The complement proteins FI and FH and the anti-Ba and anti-Bb monoclonal antibodies were purchased from Quidel (San Diego, CA). FB and C3b protein were obtained from Juro Supply AG (Lucerne, Switzerland). Recombinant soluble human CR1 was kindly provided by T-cell Science (Cambridge, MA). Recombinant DAF was a gift from Dr. I.W. Caras (Genentech, San Francisco). FD was purified from the peritoneal fluid of patients with end stage renal failure on chronic ambulatory peritoneal dialysis [16]. Polyclonal rabbit anti-CRIT antibody was derived as described previously [13, 15]. Rabbit erythrocytes (Erab) were from Bade Behring (Marburg, Germany). The C2-deficient serum (C2-D) was obtained from a patient with type I complement C2 deficiency. Disuccinimidyl suberate (DSS) cross-linking reagent was obtained from Perbio Science (Lausanne, Switzerland). ProteoBlue staining solution was purchased from Qbiogene (Basel, Switzerland). The CRIT synthetic peptides: ed1 (MSPSLVSDTQKHERGSHEVKIKHFSPY), H17 (HEVKIKHFSPY), H17S (scramble peptide of H17) (EKFYHIHSKPY) and H17-2 (HEVKIKHFSPYHEVKIKSPY) and human C4β chain peptides: Hu-C4β chain F^{212} (FEVKKYVLPN) and Hu-C4β chain F^{222} (FEVKITPGKPY) were described elsewhere [13]. All other analytical grade reagents were purchased from Sigma or Fluka Biochemika (Buchs, Switzerland).

Buffers

GVB (gelatin/veronal buffer) was prepared by mixing 10 ml of 10 % (w/v) gelatin, 200 ml of 5x VB (veronal buffer, containing 727 ml NaCl, 9 mM sodium barbitone and

3.1 mM diethylbarbituric acid, pH 7.4) and 790 ml of water. GVB-Mg^{2+}-EGTA buffer was prepared by mixing 50 ml 100 mM EGTA, 35 ml 200 mM $MgCl_2$, 104 ml GVB, 311 ml 5 % (w/v) D-glucose and adjusted to pH 7.4.

Electrophoresis and immunoblotting

Electrophoresis was conducted using a mini-gel system from Bio-Rad (Hercules, CA). Proteins were separated by SDS-PAGE on 10 % gels under reducing or non-reducing condition. Immunoblotting on to nitrocellulose membrane (Amersham Biosciences, Bucks, UK) was performed as described previously [13].

FB cleavage

FB was cleaved by incubating 500 ng FB, 200ng FD and 200 ng C3b at 37 °C for 60 min. The reaction was stopped by adding SDS-PAGE loading buffer. In some experiments, different amount (10-100 μg) of either CRIT or C4β chain peptides were pre-incubated with FB for 30 min at RT before adding other components. After the incubation, the reaction mixtures were analyzed by SDS-PAGE under non-reducing condition and the gel was stained with ProteoBlue staining solution.

AP components-CRIT ligand blotting

AP components FB (500 ng), FD (500 ng), C3b (500 ng), mixture of FB and C3b (500 ng each) and FB cleavage reaction mixture were mixed with CRIT-H17 (1.5 μg) in PBS and incubated at RT for 30 min. DSS cross-linker was dissolved in DMSO and added to the reaction mixtures to a final concentration of 2 mM. The reaction mixtures were then

further incubated at RT for 30 min. 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 AP components-CRIT-H17 complexes were analyzed by SDS-PAGE under reducing condition and probing with anti-CRIT Ab in Western blot.

Hemolytic assay

The inhibition of AP-based hemolysis was conducted using the standard method described in the literature [17, 18]. In brief, Erab (2 x 10^8 cells/ml) were prepared in GVB-Mg^{2+}-EGTA buffer. 10 μM of CRIT peptides (ed1, H17 and H17-2) were mixed with different volumes of diluted C2-D (1:20 dilution; 0, 20, 40, 60, 80, 100 and 120 μl) and made up to 150 μl with freshly prepared GVB-Mg^{2+}-EGTA buffer, which was then pre-incubated at RT for 30 min. After the pre-incubation, 50 μl of Erab was added and incubated at 37 °C for 30 min. Background control was obtained by incubating C2-D with GVB-Mg^{2+}-EGTA buffer, and 100 % lysis was determined by adding distilled water to Erab. After 5 min centrifugation at 1000 *g*, the degree of complement hemolysis was determined by measuring the absorbance of the supernatant at 414 nm on a SpectraMax 190 microtitre plate reader (Molecular Devices, Sunnyvale, CA).

ELISA of CRIT-H17 with FB and C3b

The microtitre plate was coated with either 55 nM FB or C3b, prepared in 0.1 M bicarbonate buffer pH 9, at 4 °C overnight. The plate was emptied and washed five times with washing 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. 100 μl of CRIT-H17

(5, 10, 20 and 40 μM) was added and incubated for 1 h. A 1:100 dilution of anti-CRIT antibody was prepared and 100 μl was added and incubated for 1 h. Then, the anti-rabbit HRP secondary antibody (1:3000) was added to each well and incubated for 45 min. The plate was washed thoroughly, and 100 μl of tetramethylbenzidine (TMB) substrate reagent set (BD Biosciences, San Diego, CA) was added to each well and incubated for approximately 5-10 min, until the wells turned blue. A 50 μl volume of 1 M H_2SO_4 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 RT, and after each incubation step, the plate was washed at least five times with washing buffer. The samples and antibodies were prepared in blocking buffer. The plate coated with 2 μg BSA and incubated with 40 μM CRIT-H17 was used as control.

Generation and decay of C3bBb-Ni^{2+} complexes

For the generation of C3bBb-Ni^{2+} complexes, C3b-coated (500 ng) microtitre wells were incubated with 50 ng FB and 3 ng FD in reaction buffer (phosphate buffer supplemented with 5 mM $NiCl_2$, 25 mM NaCl, 4 % BSA and 0.05 % Tween 20) at 37 °C for 2 h [6, 19]. After the formation of C3bBb-Ni^{2+} complexes, the plate was washed five times with reaction buffer and further incubated either with reaction buffer or CRIT-H17 (50 μg) or DAF (5 and 10 ng) at RT for 30 or 60 min. After the incubation, C3bBb-Ni^{2+} complexes were detected by ELISA employing anti-Bb monoclonal antibody (1:5000 dilution).

FI-mediated cleavage of C3b

The cleavage reaction was performed by incubating C3b (500 ng) with FH (500 ng) or CR1 (500 ng) and FI (100 ng) at 37 °C for 2 h. In some experiments, C3b was pre-incubated with CRIT peptides (10, 50 and 100 μg) at RT for 30 min before addition of FI and cofactor FH or CR1. The cleavage products were analyzed by SDS-PAGE under reducing condition and the gel was stained with ProteoBlue staining solution.

RESULTS

Sequence alignments between CRIT-H17 and the Hu-C4β chain peptides

In previous studies, the CRIT-H17 has been demonstrated to be an effective inhibitor of the CP [13]. The blockade of the CP was more efficient with CRIT-17 than with CRIT-ed1, suggesting that it corresponds to the active sequence. Two sequences showing homologies with CRIT-17 in the β chain of C4 have been previously identified. 10 and 11 aa peptides corresponding to these sequences are both as potent inhibitors of the CP as CRIT-H17: C4β F^{212} and C4β F^{222} share 45 % identity and have 45 % and 55 % identity with CRIT-H17 respectively (Figure 1). In 2001, Laich et al. [20] demonstrated with surface plasmon resonance that compared to the affinity between C2 and C4b, FB interacts only weakly with immobilized C4b. Thus, these two C4β chain peptides were included in the studies on the regulation of the AP. CRIT-H17S is a peptide with amino acid identical to CRIT-H17 but in a scrambled sequence, (Figure 1), which does not inhibit the CP. CRIT-H17S peptide was used as a control.

Inhibition of FD-mediated cleavage by CRIT-H17 and weakly by C4β F^{212}

FB binds to C3b and is immediately cleaved by FD into Ba and Bb fragments, with Bb remaining attached to C3b forming the AP C3 convertase, whereas Ba is released. When CRIT-H17 was pre-incubated with FB before the addition of FD and C3b, FB cleavage was inhibited and less Ba and Bb fragments were formed. This inhibiton was dose dependent as shown in Figure 2A, with a complete inhibition at the highest concentration of CRIT-H17 used. The control peptide CRIT-H17S, which shares only 18 % identity and 55 % similarity with CRIT-H17, had no inhibitory effect on the FD-mediated

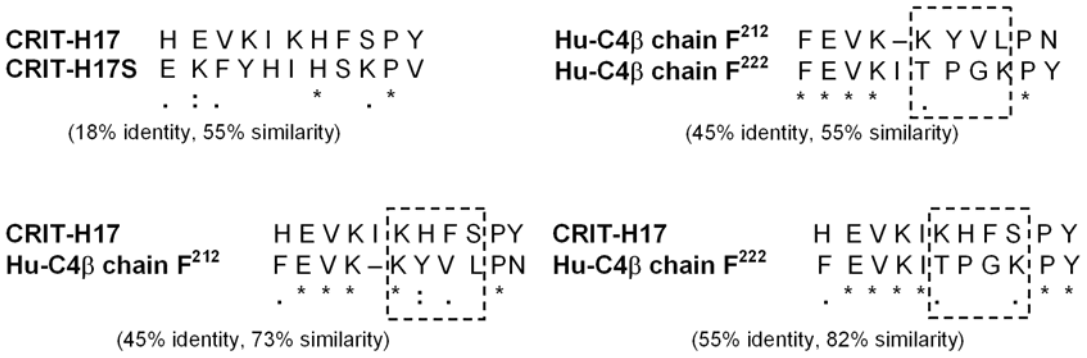


Figure 1 Amino acid sequence alignments of CRIT-H17 and human C4β chain peptides showing similarities between different peptides.

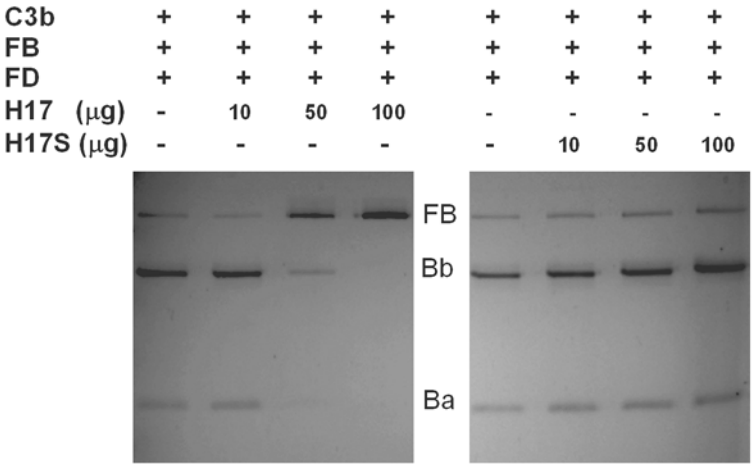
Alignments were made using the MAFFT program. Periods indicate similar residues and asterisks indicate identical residues. The dash line box shows the potential region responsible for blocking FD-mediated cleavage of FB.

cleavage of FB.

Since CRIT-H17 and the two C4β chain peptides showed high sequence homology and comparable inhibitory effect in the CP activation, we were intrigued to see whether these peptides would interfere with the cleavage of FB as well. As shown in Figure 2B, the C4β chain F^{212} peptide showed inhibition of FB cleavage, although only a partial inhibition at the highest concentration tested, whereas the C4β chain F^{222} peptide did not have any measurable inhibitory effect. However, these data indicated that peptides derived from CRIT and one of its homologous region in the C4β chain were capable to interfere with the formation of both, the CP and AP C3 convertases.

Referring to Figure 1, we were surprised that the C4β chain F^{222} peptide, with no inhibitory activity, had even higher sequence homology with CRIT-H17 than the C4β chain F^{212} peptide. The dash line box highlights the region showing the major differences between these two C4β chain peptides and CRIT-H17. In addition, it highlights the potential region responsible for blocking the FD-mediated cleavage of FB. At close scrutiny, it appears that the additional proline residue in the C4β chain F^{222} peptide may induce a change from cis- to trans-conformation or vice versa [21, 22]. Also, this proline residue may possess certain degrees of steric hindrance in the interaction between FB and the C4β chain F^{222} peptide. Thus, this C4β chain F^{222} peptide loses its inhibitory effect on the FD-mediated cleavage of FB.

A



B

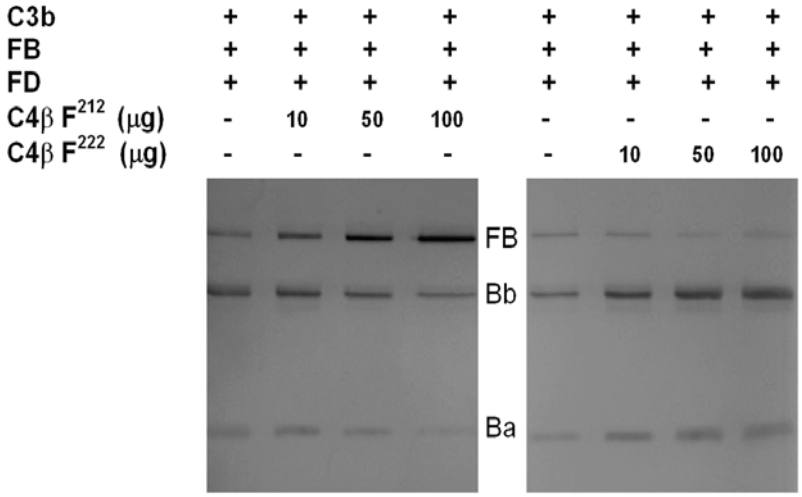


Figure 2 CRIT-H17 and C4β F^{212} peptides but not CRIT-H17S and C4β F^{222} peptides inhibit the FD-mediated cleavage of FB.

FB (500 ng) was pre-incubated with either CRIT or C4β peptide for 30 min at RT before adding FD (200 ng) and C3b (200 ng). The mixtures were then further incubated for 60 min at 37 °C. The effect of increasing concentrations (10 – 100 μg) of CRIT **(A)** and C4β **(B)** peptides on the cleavage of FB by FD were analyzed by SDS-PAGE (10% gel) under non-reducing conditions, stained with ProteoBlue staining solution.

CRIT-H17 peptide interacts with FB and C3b but not FD

From the analogy to the CP, we predicted that CRIT-H17 would bind to FB, with at least one binding site of the vW domain of Bb, since CRIT-H17 binds C2 and has at least one binding site on the vW domain of C2. First the binding was tested using a cross-linking agent, DSS and immunoblots to reveal the bound CRIT-H17 to its target protein. As shown in Figure 3, in various mixtures of C3b, Factor B and FD, there was a consistent binding of CRIT-H17 to intact FB and its two main fragments Bb and Ba, but also to C3b. There was no binding to FD. This unusual binding to two proteins involved in a mutual contact did not allow us to define the precise site involved in inhibition of AP C3 convertase by CRIT-H17, since any one or more of these 3 bindings demonstrated might be involved. However the binding to Ba and Bb suggested that the small peptide CRIT-H17 may well bind FB at the site of FB cleavage by FD. Indeed, CRIT-H17 binds C2 near or at the site of C2 cleavage by C1s.

To confirm these results, we performed a sandwich ELISA, fixing FB or C3b on the plates, followed by incubation with CRIT-H17 and detection of the peptide bound to FB or C3b with the anti-CRIT antibody. Again, CRIT-H17 bound to both FB and C3b in a dose dependent manner, with no binding of CRIT-H17 to albumin as a control, indicating specific bindings (Figure 4). Of interest was that the binding to FB appeared to be superior to that of C3b although a direct comparison remains difficult, not knowing how many binding sites are involved.

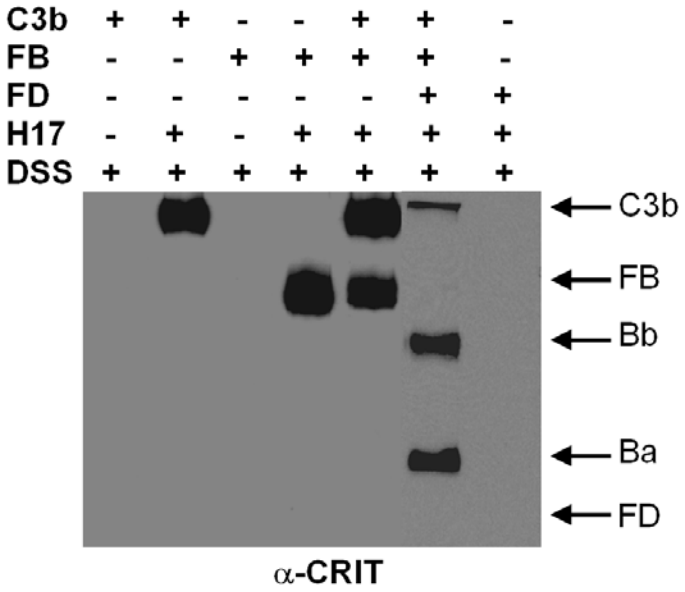


Figure 3 Ligand blot showing CRIT-H17 interaction with C3b and FB but not FD.

CRIT-H17 (1.5 μg) was incubated with C3b (500 ng) or FB (500 ng) or FD (500 ng) or mixture of C3b (500 ng) and FB (500 ng) or mixture of C3b (200 ng), FB (500 ng) and FD (200 ng) pre-incubated for 60 min at 37°C. The resulting complexes were cross-linked with DSS. After cross-linking, the complexes were detected by immunoblotting probing with polyclonal anti-CRIT antibody.

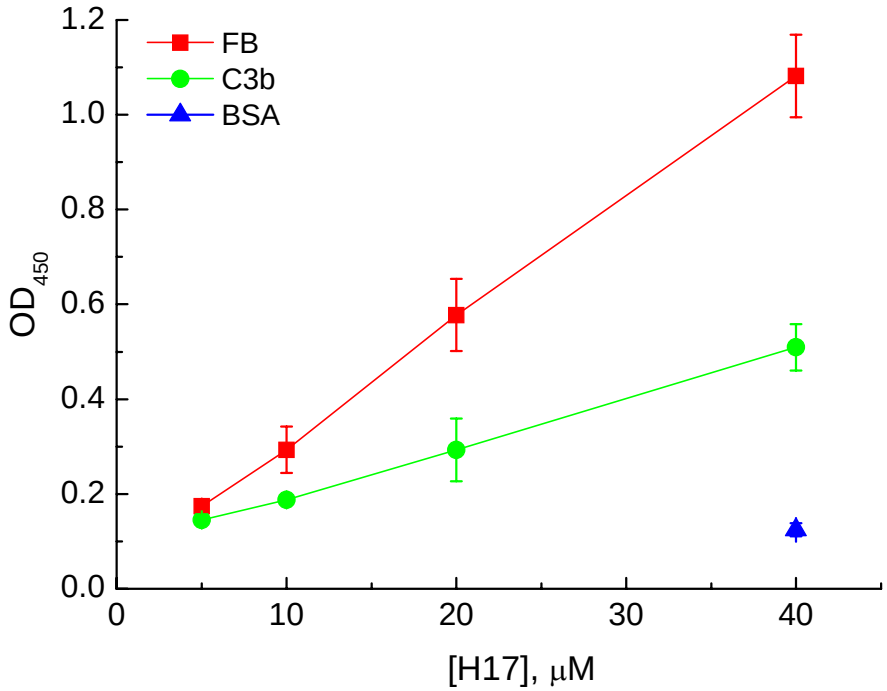


Figure 4 ELISA of CRIT-H17 with FB and C3b protein.

Either 55 nM FB or C3b was coated on the ELISA plate and incubated with increasing concentration of CRIT-H17 (from 5 to 40 μM). BSA was used as control. The affinity of CRIT-H17 for FB is almost three times higher than C3b. Results are means±S.D. for three independent experiments, each with duplicate measurements.

Inhibition of AP activation by CRIT peptides in serum

We have shown that CRIT-H17 could block the FD-mediated cleavage of FB in an assay using purified proteins, and similarly that it binds to FB and C3b when added to soluble mixtures. Whether these interactions would have a biological significance in a more physiological context was our next question. Three different peptides derived from CRIT were studied for their potential inhibition of the AP in a hemolytic assay specific for the AP and using C2 deficient serum, so as to be sure that the effects observed were not due to C2 inhibition. The inhibitory effects of CRIT peptides on the AP are shown in Figure 5. In the presence of 10 μM peptides CRIT-H17 was the best inhibitor reducing the lysis by 22.1% whereas the CRIT-ed1 peptide was less potent, and the peptide expressing the CRIT-H17 sequence twice had almost no inhibitory activity.

Decay activity of CRIT-H17 on C3bBb complexes

The complement regulatory protein DAF accelerates the decay of the C3bBb convertase and has been shown to interact with both FB and C3b [23, 24]. Since CRIT-H17 bound to both FB and C3b as well, we tested the hypothesis that CRIT-H17 would have the same property of dissociating C3bBb. The convertase was formed on a solid phase by binding first C3b, adding B in the presence of Ni^{2+} and Factor D. After washing, buffer alone, CRIT-H17 (50 μg) or DAF (5 and 10 ng) were added and the decay of the C3bBb complexes measured after 30 and 60 min. The results clearly showed that, unlike DAF, CRIT-H17 does not possess any decay activity on the dissociation of C3bBb complexes (Figure 6). Thus CRIT-H17 appears to have its main activity of the formation of the AP C3 convertase.

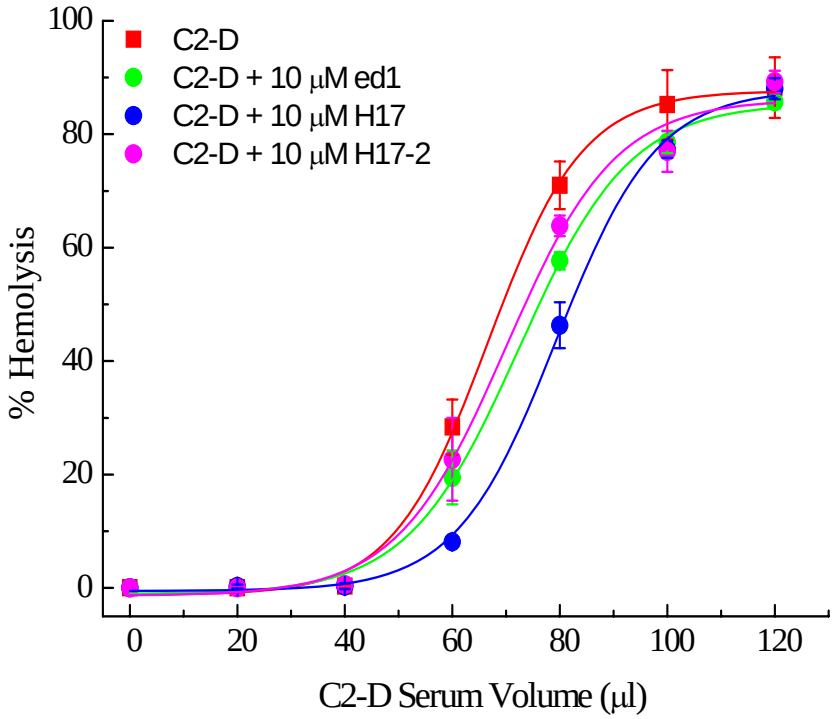


Figure 5 Inhibition of the AP activation by CRIT peptides.

10 μM CRIT peptides were pre-incubated with C2-D for 30 min at RT before adding to the Erab. The CRIT peptides inhibited the AP C3 convertase formation by binding to FB or C3b or both and decreased the percentage of hemolysis. Results are means±S.D. for three independent experiments, each with duplicate measurements.

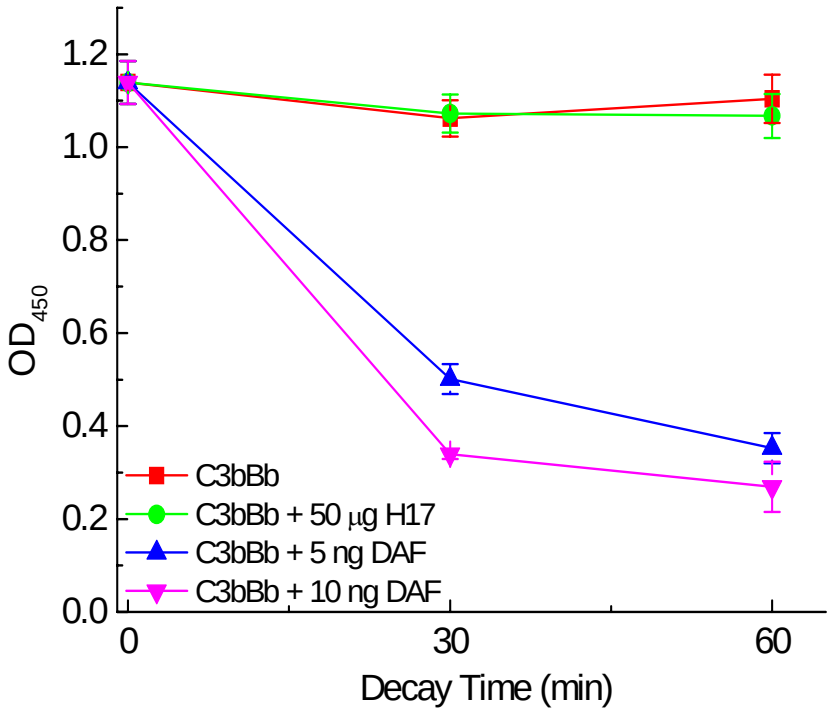


Figure 6 Effect of CRIT-H17 on Ni^{2+} dependent C3bBb complexes.

C3bBb complexes were formed by incubating 50 ng FB and 3 ng FD in the presence of 5 mM Ni^{2+} for 2 h at 37°C in C3b-coated microtiter wells. After the formation of C3bBb complexes, the wells were washed five times with reaction buffer and further incubated with buffer or CRIT-H17 or DAF for the indicated time at RT. C3bBb complexes were then detected by ELISA employing anti-Bb monoclonal antibody. Unlike DAF, CRIT-17 does not have any decay activity. Results are mean±S.D. for three independent experiments, each with duplicate measurements.

CRIT-H17 reduces the FI-mediated cleavage of C3b

As mentioned, the binding of CRIT-H17 to C3b was unexpected, but suggested that other aspects of the biological properties of C3b might be modified. C3b is further cleaved to C3bi then C3dg by FI in the presence of the correct cofactor. In Figure 7A, a schematic representation of C3b shows the FI cleavage sites in the presence of the cofactors, FH and CR1. FI cleaves C3b at the first and second cleavage sites in the presence of cofactor FH or CR1 to generate the 64.3 and 39.5 kDa fragments. The appearance of a smaller 24.3 kDa fragment and C3dg indicates that FI cleaves C3b at a third site, this only in the presence of CR1. The pre-incubation of CRIT-H17 with C3b inhibited the FI-mediated cleavage in the presence of FH (Figure 7B). At the lowest dose (10μg), CRIT-H17 showed a small reduction in the cleavage but completely blocked it at the doses of 50 and 100 μg. CRIT-H17 could only weakly block FI cleavage of C3bi to generate the C3dg and 24.3 kDa fragments in the presence of CR1 (Figure 7C). CRIT-H17S, the scramble peptide of H17, had no inhibitory effects. The two C4β chain F^{212} and F^{222} peptides, showing high sequence homology to CRIT-H17, had no inhibitory effect either (data not shown).

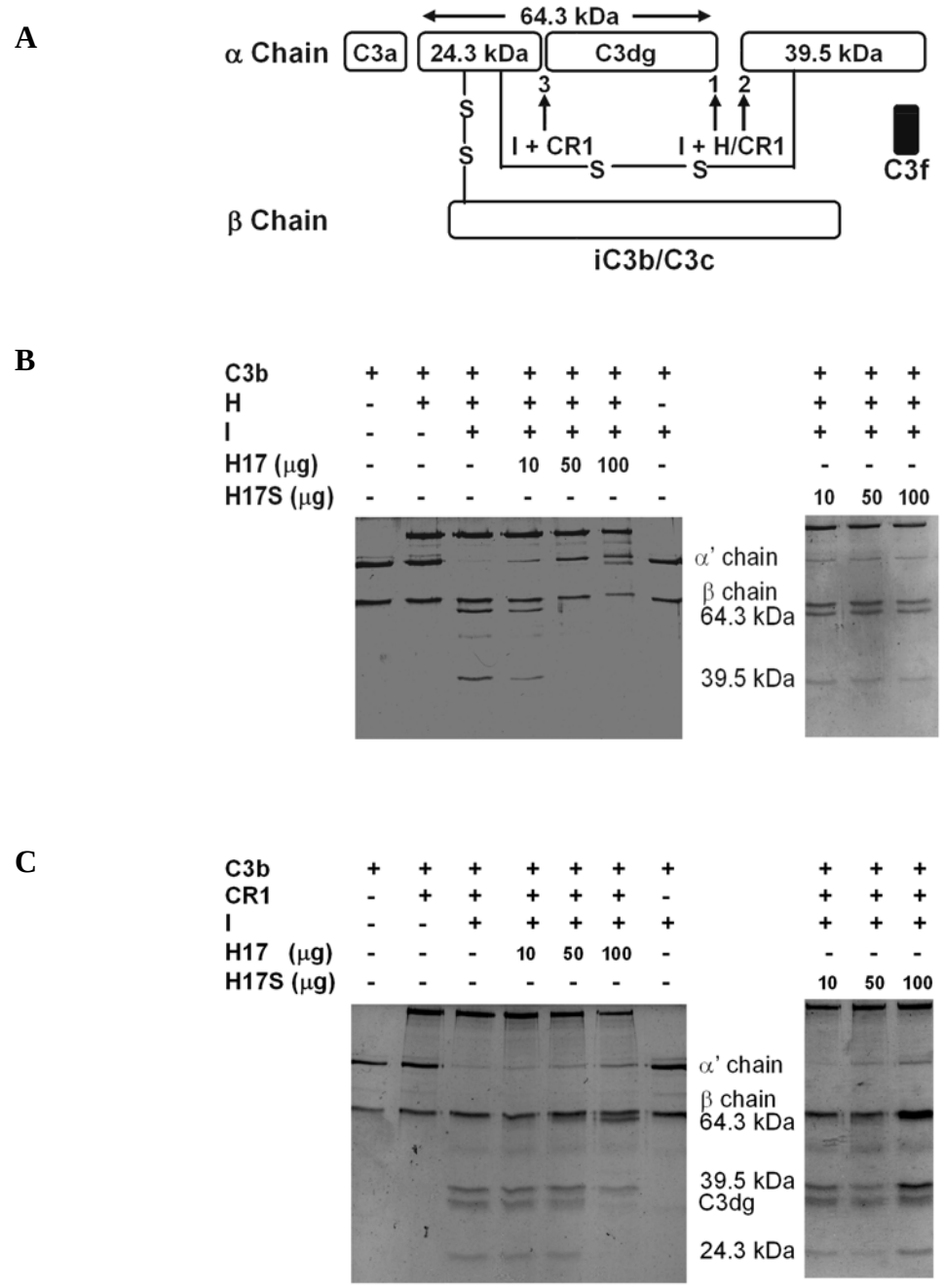


Figure 7 Inhibition of FH and CR1 cofactor activities by CRIT-H17.

(A) Schematic representation of C3b showing the FI cleavage sites in the presence of FH and CR1. Different amounts of either CRIT-H17 or CRIT-H17S peptides (10-100 μg) were pre-incubated with 500 ng C3b for 30 min at RT. After the pre-incubation, 100 ng FI and either 500 ng FH **(B)** or 500 ng CR1 **(C)** were added for an additional 2 h incubation at 37 °C. The inhibition effect of CRIT-H17 and CRIT-H17S peptides on the cleavage of C3b by FI were analyzed by SDS-PAGE (10 % gel under reducing condition) and the gel was stained with ProteoBlue staining solution.

DISCUSSION

CRIT, a three transmembrane receptor first discovered on the *Schistosoma* parasite surface, acts as a decoy receptor for C2 to protect the parasite from the assembly of C4bC2 and the following C2 cleavage, thus preventing complement attack by the classical pathway [13]. This receptor was later shown to be expresses in cod fish, rat and human [12]. CRIT-H17 peptide proved to be the region interacting with C2 or more specifically with the C2 vWFA domain [15]. It is as potent inhibitor of the CP in vitro and in vivo [13, 14].

Because of the many similarities between the CP and AP C3 convertases, and the fact that many regulators of complement interact with both convertases, we postulated, that the CRIT-H17 peptide might have inhibitory effects on the formation the C3bBb convertase as well. Our major findings (Figure 8) were that CRIT-H17 inhibited the cleavage of FB by FD, probably by binding to FB, and reduced AP function. However additional finding were unexpected: CRIT-H17 bound also to C3b, and reduced the inactivation of C3b by FI whether this cleavage occurred in the presence of FH or CR1.

CRIT-H17 is known to bind the vWFA domain of C2, and inhibits its cleavage by C1s probably because the cleavage site is not any more exposed in a correct way. CRIT-H17 bound to B, both to the Ba and the Bb fragments, suggesting that the interaction occurs over a region covering the FD cleavage site on FB (see Figure 8.). Of particular interest was that a peptide corresponding to the homologue of CRIT-H17 on the C4β chain blocked FB cleavage as well, although less potent than CRIT-H17. This finding strengthened the hypothesis, that there are strong analogies between the

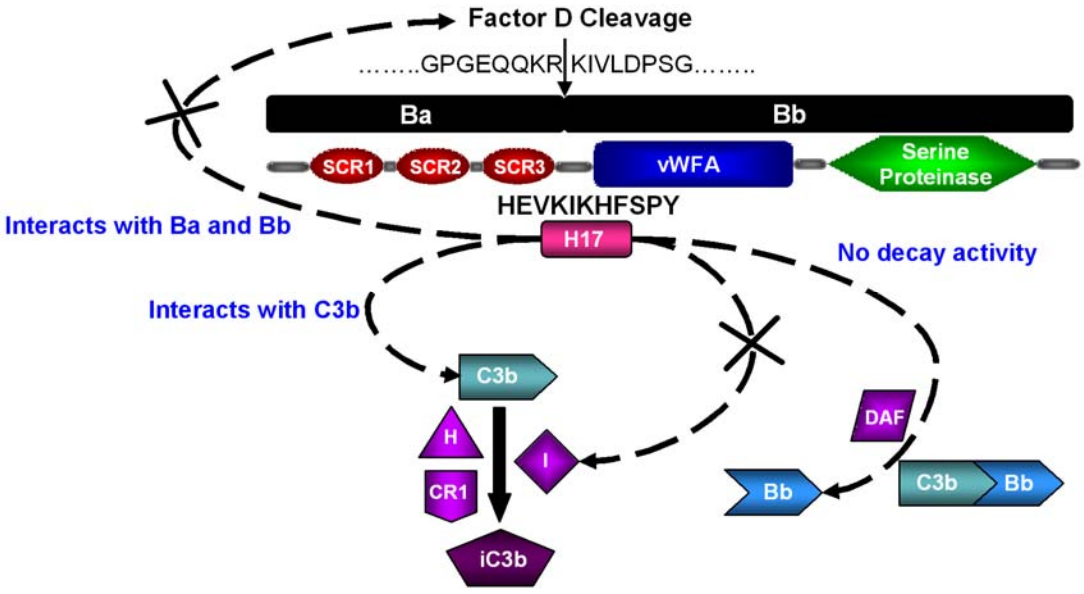


Figure 8 Schematic representation summarizing CRIT-mediated regulations of the AP C3 convertase formation.

CRIT-17 interacts with both Ba and Bb fragments of FB, which in turn blocks the FD-mediated cleavage. It's highly possible that CRIT-H17 binds to the junction of the FD cleavage site. In addition, CRIT-H17 interacts with C3b and slightly inhibits the FI-mediated cleavage of C3b in the presence of both cofactors FH and CR1. Furthermore, CRIT-H17, unlike DAF, does not have any decay activity of C3bBb complexes as DAF.

mechanisms leading to the formation of both CP and AP C3 convertases. The 4 amino acid (K, H, F and S) in CRIT-H17 peptide highlighted in Figure 1 could be important for FB binding. Referring to the two C4β chain peptides used in this study, both of them have been shown to block CP activation effectively and to the same extent [13]. However, only C4β chain F^{212} peptide was able to slightly inhibit the FD-mediated cleavage of FB. Any of these 4 amino acid highlighted in Fig. 1 (K, Y, V and L) in C4β chain F^{212} peptide could be the important amino acid residue(s) responsible for the FB binding, which are also the major differences between these two C4β chain peptides. We postulate that these 3 amino acid residues E, V and K, in CRIT-H17 and C4β chain peptides are responsible for C2a binding and these 4 amino acid residues K, H, F and S in CRIT-H17 peptide are possibly responsible for the FB and C3b binding. In future, alanine positional scanning may help to further dissect out the key residue(s) responsible for the FB binding. Modification(s) of CRIT-H17 peptide would eventually create a peptide, which is more specific only towards FB, a more potent inhibitor in the AP activation. Referring to the sequence alignment between CRIT-H17 and C4β chain F^{222} peptides in Figure 1, one interesting aspect is the additional proline residue in C4β chain F^{222} peptide, which may induce conformational change of the peptide, *cis/trans* isomerization [21, 22]. In case, we change the amino acid H→P in CRIT-H17 peptide, it will be interesting to test whether this modified CRIT-H17 peptide will lose its ability to inhibit the FD-mediated cleavage of FB.

The binding of CRIT-H17 to C3b appeared to be specific, since in a mixture of CRIT-H17, FB, C3b and FD, CRIT-H17 cross-linked only to C3b and FB. However it might be that the association of FB and C3b allowed some CRIT-H17 to come into near

contact with C3b before the cross linking agent was added. However the direct ELISA assay suggested that there was some affinity of CRIT-H17 for C3b, although the binding was much lower than for FB. Whether this binding may influence the reaction between C3b and FB remains unclear, since the binding of CRIT-H17 to FB might in itself modify the association of C3b and FB. Further experiments will be required to answer this point. C1-inhibitor has been shown to interfere with the activation of the AP as well by binding to C3, and blocking the formation of the C3bB complex. However C1-inhibitor does not bind FB [25].

DAF regulates complement activation by accelerating the dissociation of the C3 convertases. The mechanism of decay acceleration of DAF is still unknown. In 1996, Kuttner-Kondo et al. [26] proposed a molecular model and mechanism of action of DAF. In this model, DAF and Ba fragment of FB share a common binding site on C3b. FB binds to C3b and the Ba fragment of FB is released from the complex after FD cleavage. After the release of Ba fragment from C3b, DAF binds to the Ba binding site on C3b. Upon the binding of DAF, Bb fragment is destabilized and released. CRIT-H17 did not modify the decay of the C3bBb convertase, despite its binding to purified Bb and C3b. Thus the mechanism by which CRIT-H17 inhibits the AP in serum does not include the decay of the convertase, and indicates interactions with C3b and Bb very different from DAF.

The FI mediated cleavage of C3b to iC3b then to C3dg requires the binding of a cofactor to C3b (FH or CR1). CRIT-H17 reduced the activity of both cofactors, suggesting that the observed binding to C3b was not without consequences, although alternatives cannot be excluded: direct inhibition of FI, or interference with CR1 or FH

function. Many more studies have to be done to answer this question, but the main aspect worth underlining is that CRIT-H17 had properties in vitro suggesting multiple interactions regulating the AP. The assay of CRIT-H17 in whole serum suggested that the sum of the different observations made here lead to inhibition of the AP. This inhibition was however much lower than the blockade of the activation of the CP [13].

To study CRIT-H17-Ba/Bb and CRIT-H17-C3b interactions more precisely, structural studies by NMR or X-ray crystallography should be carried out. The structural data will enable us to firmly predict which residues are more likely to be involved in these protein-protein interactions and further understand the mode of regulatory actions of CRIT-H17 peptide in the AP activation. Dissecting out the interaction region of CRIT-H17-Ba/Bb, the SCR 3 region of Ba and the vWFA domain of Bb will be the best place to start with. Kuttner-Kondo et al. [26] has predicted with his model that the SCR 3 of Ba would be an important region for interacting with C3b. Previously, we have shown that a CRIT binding site presents in C2 vWFA domain [15]. Because of the sequence homology between C2 vWFA and FB vWFA domains, we predict CRIT-H17 peptide interacts with Bb fragment of FB via the vWFA domain. In addition, binding of CRIT-H17 peptide to FB could inhibit the FD cleavage. Thus, CRIT-H17 peptide should bind to the region near to the FD cleavage site. The SCR 3 of Ba and vWFA domain of Bb should be a right guess. For CRIT-H17-C3b interaction, it's more difficult to assign the potential region of interaction. However, since CRIT-H17 peptide could inhibit the FI-mediated cleavage of C3b in the presence of FH and CR1, we can assume CRIT-H17 peptide binds to the C3α chain, the region close to the first, second and third FI cleavage sites. Expressing the recombinant proteins of Ba (or SCR 3), the vWFA domain of Bb and C3α chain can be

used for functional studies or complexed with CRIT-H17 peptide for structural studies by X-ray crystallography

In summary, the data present evidences that CRIT-H17 peptide, proved to be a potent inhibitor of the CP, also functions as a regulator in the AP. The AP has been proved to be important in various diseases. Developing a specific AP inhibitor should be therapeutically important in the future. Alanine positional scanning and structural studies will provide an insight and invaluable information about the mode of regulatory actions of CRIT in the AP. The most important issue will be the opportunity to modify and create a more specific and potent AP inhibitor.

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CONCLUSION AND FUTURE PERSPECTIVES

The novel complement regulator, CRIT, previously described in the *Schistosoma* parasite was cloned and the partial sequence of cod CRIT and full-length sequences of rat and human CRIT were obtained by PCR [1], as well as that of *Trypanosoma cruzi*. The human CRIT nucleotide sequences from genomic DNA and cDNA are identical, implying that human CRIT is encoded by a single exon, and is intronless gene. When comparing the human CRIT nucleotide sequence with rat, it shows 82 % homology. Western blotting showed expression of human CRIT in various hemopoietic cells as well as platelets, monocytes, dendritic cells and lymphocytes. The distribution of CRIT receptor in various human tissues was determined by immunohistochemistry and CRIT was found in pancreas, proliferating endometrium, esophagus, kidney, and testis.

The expression of the recombinant vWFA domain of C2 was proved difficult, and the use of similar strategies as had been used to express its functional homologue, FB, has not worked well [2]. After many unsuccessful trials, we managed to express the first functional recombinant vWFA domain of C2. This recombinant C2 vWFA protein provides an ultimate chance to further dissect out the fine interaction between C2a and CRIT or C4b. The results presented in Section II clearly demonstrate that there is one CRIT binding site on the C2 vWFA domain [3]. In addition, by sandwich ELISA and pull-down assay, we further confirm that C2 vWFA domain contains a C4 binding site. C4 binds to C2 vWFA domain mainly via its β chain. However, further studies need to be

performed to clearly elucidate whether CRIT and C4 share the same binding site on C2 vWFA domain.

CRIT inhibits the CP C3 convertase formation by competing with C4 for the binding of C2, which in turn, blocks the C1s cleavage of C2 [4]. Besides the inhibitory effect on the CP, CRIT also functions as a regulator in the AP by binding to FB and in turn blocking cleavage of FB. However, the inhibitory effect of CRIT on the AP is much weaker than on the CP. As a result of this study, an unexpected observation was made. Besides binding to FB, CRIT also bound to C3b. In addition, CRIT inhibits partially the FI-mediated cleavage of C3b in the presence of both cofactors CR1 and FH. We postulate that CRIT binds to the C3α chain, the region close to the FI cleavage sites, and in turn blocks the FI cleavage or it interferes with the ability of cofactors to bind C3b. Compared to the relative binding affinity between CRIT-FB and CRIT-C3b, based on the ELISA data, the binding to FB is almost three-fold higher than to C3b. Even though the binding of CRIT to C3b is weak, the influence of CRIT-C3b binding on FB and on the inhibition of the AP C3 convertase formation remains unknown. The overall CRIT-mediated regulations of both the CP and AP are summarized in Figure 1.

Besides the initiating factor, the CP and MBL-P are identical. We also tested whether CRIT would have any effect on the MBL-P. Based on the unpublished data (Figure 2), CRIT could block the MBL-P, with similar potency as the CP, in an ELISA-based hemolytic assay. Since MASP-2 is structurally and functionally similar to C1s, the binding of CRIT to C2 could probably block the MASP-2 cleavage of C2, in the same

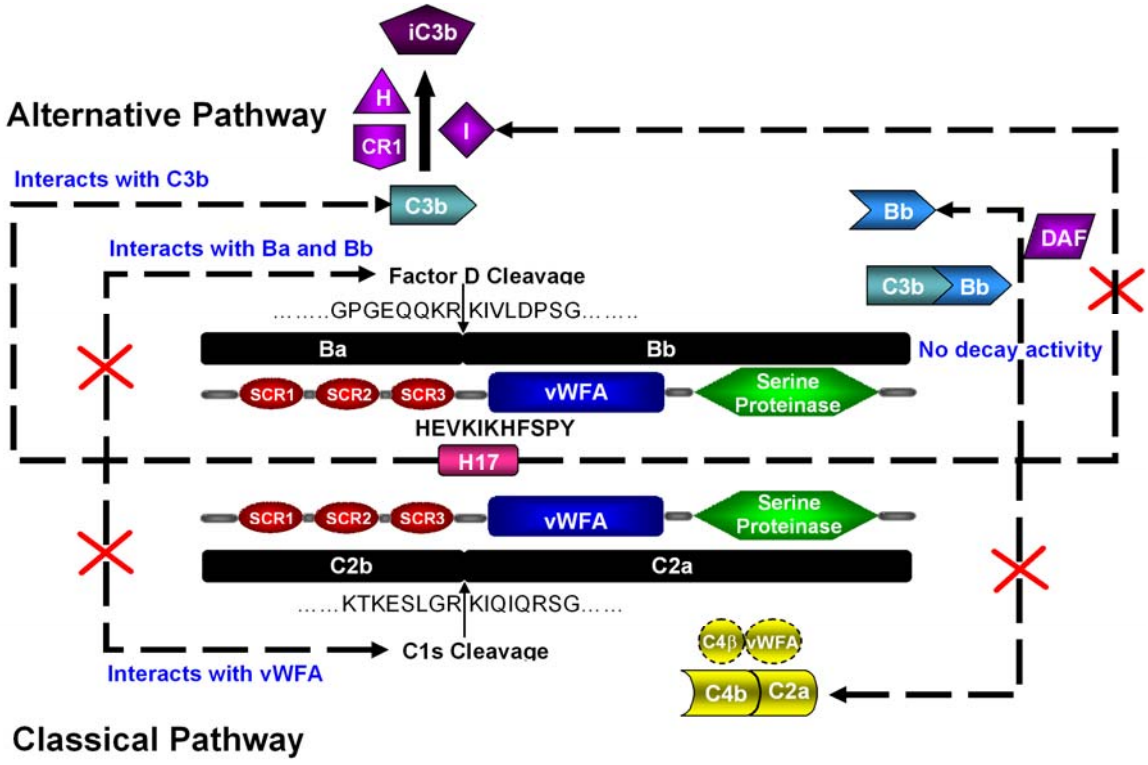

Figure 1 Schematic representation summarizing CRIT-mediated regulations of both the CP and AP C3 convertases formation.

In the AP, CRIT-H17 peptide interacts with FB, both Ba and Bb fragments, which in turn blocks the FD-mediated cleavage. In addition, it interacts with C3b and slightly inhibits the FI-mediated cleavage of C3b in the presence of cofactors FH and CR1. Unlike DAF, CRIT-H17 doesn't have any decay activity of C3bBb complex. In the CP, CRIT-H17 binds to C2, mainly to vWFA domain, and blocks the C1s cleavage, which in turn blocks the assembly of the CP C3 convertase, C4bC2a. C2 vWFA domain contains both C4 and CRIT binding site(s) and C4 binds to C2 vWFA mainly via its β-chain. The red crosses mean inhibitory effect of CRIT-H17 on the CP or AP.

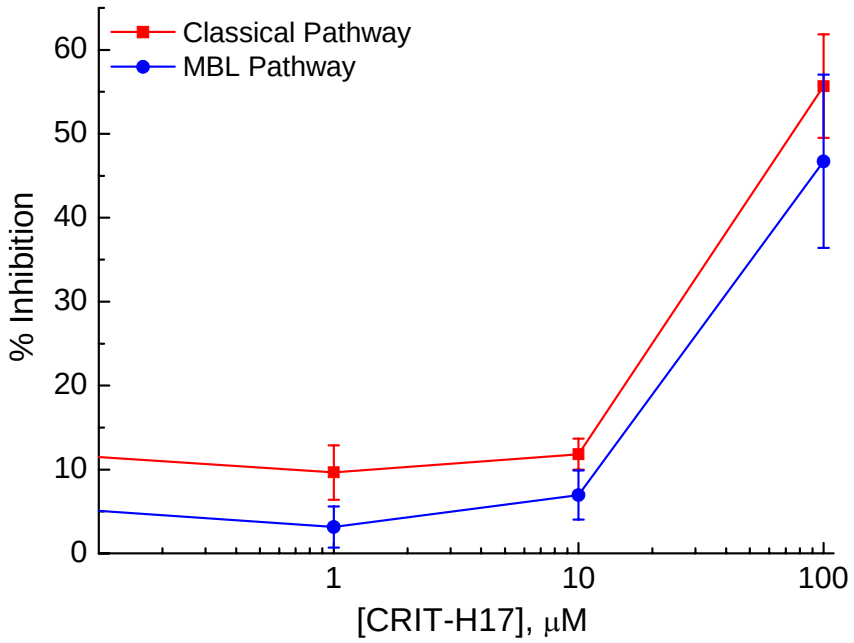

Figure 2 Comparison between the inhibitory effect of CRIT-H17 on the CP and MBL-P

Different amounts of CRIT-H17 were pre-incubated with normal human serum for 30 min at RT before adding to the ELISA well (CP; coated with IgM, MBL-P; coated with mannan). The inhibitory effect of CRIT-H17 on C5b-9 complex was detected with a specific alkaline phosphate labeled antibody to the neoantigen expressed during MAC formation.

way as C1s in the CP. Thus, CRIT can block the MBL-P derived C3 convertase formation. However, further experiments need to be done to confirm this result.

Since CRIT interacts with more than one complement components, it will be interesting to perform the kinetic analysis of protein-protein interactions between CRIT and C2 or FB or C3b, both parallel and competitive reactions, by surface plasmon resonance biosensors [2, 5, 6]. The kinetic data should give an insight into the mode of regulation of CRIT in the complement systems.

WFA (often called an I domain in integrins) domain are a family comprising molecules of approximately 200 amino acids with a highly conserved MIDAS (metal-ion-dependent adhesion site) motif. Most of the well-characterized vWFA domains are found in extracellular matrix (ECM) [7] and are the sites of protein-protein interaction in cell-adhesion protein, such as integrins [8, 9]. All integrin β subunits and nine known integrin α subunits (α1, α2, α10, α11, αD, αE, αL, αM and αX) contain vWFA domain [7, 10]. The MIDAS motif is involved in integrin-ligand interaction [11] with a mutual coordination of metal ion [12]. Integrins play an important role in cell-cell and cell-ECM interaction, which for example mediate cell growth and differentiation, tumor growth and metastasis. Integrin inhibitors, such as disintegrins and α2β1 integrin inhibitor, play an important role in the treatment and prevention of thrombosis and cancer [13]. Since CRIT interacts with the C2 vWFA domain and the interaction point is predicted to be close to the MIDAS motif, this raises one interesting question: will CRIT interact with integrin(s) via the vWFA domain or the highly conserved MIDAS motif? If the answer is YES, it

will be interesting to test in the near future to see whether CRIT, besides acting as complement regulator, can also function as an integrin inhibitor.

CRIT receptor expresses in a wide range of cells and tissues and even though the latest studies indicate that CRIT is involved in signal transduction and endocytosis from the cell surface [14], very little is known about its other functional properties. The expression of CRIT on platelets and endothelial cells may play an important role in the prevention of thrombus formation, if CRIT can bind to collagen exposed at the site of damaged endothelial cell surface and in turn, prevent blood platelets from adhering to collagen and subsequent activation of blood platelets. In addition, the expression of CRIT on monocytes may play a role in the differentiation of monocytes into macrophages.

In the future, functional studies should be carried out to further characterize this novel membrane receptor, CRIT. Yeast two-hybrid and phage display library screening should help to fish out any potential interaction partners. Structural studies and alanine positional scanning will provide an insight into the site of interaction between CRIT and the complement components, C2, FB and C3b. Furthermore, it provides invaluable information on the design and modifications of CRIT peptide so as to create a more specific and potent complement inhibitor.

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CURRICULUM VITAE
Of
KWOK MIN HUI

PREVIOUS POSITIONS

PhD Student
University Hospital Basel, Department of Research, Immunonephrology
Aug 2002 – Oct 2005

- Conducted research on biochemical and functional studies of a novel complement inhibitor, CRIT, with its interaction partners
  - cloned and confirmed the presence of CRIT gene in human and cod
  - studied the inhibitory effects of CRIT on complement activation
  - expressed and purified recombinant C2/FB vWFA domains for the protein interaction studies with CRIT peptides
  - performed protein crystallization of recombinant vWFA domains or the complexes C2/FB vWFA with/without CRIT peptides

Research Assistant
Dept. of Biochemistry, Hong Kong University of Science and Technology
Oct 2000 - March 2001

- Assisted the research fellow to conduct the fusion PCR and DNA sequencing

MPhil Student
Dept. of Biochemistry, Hong Kong University of Science and Technology
Sept 1998 - June 2000

- Conducted research to isolate and characterize benzodiazepine receptor ligands from *Scutellaria baicalensis* Georgi
  - prepared the herbal extracts with ethanol, methanol, dichloromethane and water
  - performed radio-receptor binding assay to locate the active fractions
  - purified several active benzodiazepine receptor ligands from the herbal extracts with preparative column (silica gel or LH-20)
  - conducted qualitative analysis of the purified compounds by HPLC method
  - performed several animal tests (elevated-plus maze, holeboard test, horizontial wire test) to confirm the purified compounds exert anxiolytic effect without the association of sedative and muscle relaxing side effects

Research Project Student
Dept. of Biochemistry, Hong Kong University of Science and Technology
Sept 1997 - May 1998

- Conducted quantitative and qualitative analysis of Nifedipine and its related compounds in dosage form by HPLC method

Maintenance Helper
Computer Center, Hong Kong University of Science and Technology
July 1996 - Aug 1998

- Maintained the computers and printers in good working condition, as well as assisted the users in computer center

Part-time Administrative Assistant
Yip's Children Choral
June 1990 - Mar 1992

- Performed general clerical works and prepared documents for the manager

EDUCATION AND TRAINING

PhD in Medical-Biological Research, University of Basel
2002 - 2005
PhD Thesis: "Biochemical and functional studies of a novel complement inhibitor, CRIT, with its interaction partners".

MPhil in Biochemistry, Hong Kong University of Science and technology
1998 - 2000
MPhil Thesis: "Isolation and characterization of benzodiazepine receptor ligands from *Scutellaria baicalensis* Georgi".

BSc in Biochemistry Second Class Honors Division I, Hong Kong University of Science and Technology
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PAPERS

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Huen M.S., **Hui K.M.,** Leung J.W., Sigel E., Baur R., Wong J.T., Xue H. (2003). Naturally occurring 2'-hydroxyl-substituted flavonoids as high-affinity benzodiazepine site ligands. *Biochem. Pharmacol.* 66:2309-23407.

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Hui K.M., Orriss G.L., Schirmer T., Magnadottir B., Schifferli J.A., Inal J.M. (2005). Expression of functional recombinant von Willerbrand factor-A domain from human complement C2: potential binding site for C4 and CRIT. *Biochem. J.* 389:863-868.

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SCIENTIFIC MEETINGS AND ABSTRACTS

Society for Neuroscience's 29th Annual Meeting, October 23-28, 1999, Miami Beach, USA. (Poster Presentation).
Hui K.M., Wang X.H., Xue H. (1999) Interactive benzodiazepine site flavones from *Scutellaria baicalensis* Georgi. *Soc Neurosci. Abstr.* 25:965.

9th European Complement Workshop, September 6-9, 2003, Trieste, Italy. (Poster Presentation).
Inal J.M., **Hui K.M.,** Schifferli J.A. (2003) CRIT-based and C4β-chain but not α- nor γ-chain peptides inhibit classical pathway complement activation. *Mol. Immunol.* 40:201.

10th European Meeting on Complement in Human Disease, September 9-13, 2005, Heidelberg, Germany. (Oral Presentation).
Prize: *"Recognition of excellent contribution on complement of human disease"*
Hui K.M., Orriss G.L., Schirmer T., Magnadottir B., Schifferli J.A., Inal J.M. (2005). Expression of functional recombinant von Willerbrand factor-A domain from human complement C2: potential binding site for C4 and CRIT.

US PATENT

Xue H., Wang H.Y., Huen S.Y.M., **Hui K.M.** Methods of treating benzodiazepine site associated syndromes using 2'-hydroxyflavonoids. Patent No. 6,740,677.

NUCLEOTIDE SEQUENCES

Ramirez M., Inal J., **Hui K.M.** (2003). Accession No. AY464185.

Inal J.M., **Hui K.M.**, Miot S., Lange S., Ramirez M.I., Schneider B., Krueger G., Schifferli J.A. (2005). Accession No. AY464186.

Inal J.M., **Hui K.M.**, Miot S., Lange S., Ramirez M.I., Schneider B., Krueger G., Schifferli J.A. (2005). Accession No. AY464184.

ADDITIONAL RESEARCH SKILLS

Basic techniques in preparing DRG neurons, cell culture, micro-injection, RNA extraction, RT-PCR, two electrodes voltage clamp and basic techniques in patch clamp and protein crystallization.