The Journal of Immunology

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J Immunol 2002; 168:5213-5221; ;
doi: 10.4049/jimmunol.168.10.5213
http://www.jimmunol.org/content/168/10/5213

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Complement C2 Receptor Inhibitor Trispanning and the β-Chain of C4 Share a Binding Site for Complement C2[1]

Jameel M. Inal[2] and Jürg A. Schifferli

Complement C2 receptor inhibitor trispanning (CRIT) of the *Schistosoma* parasite binds human C2 via the C2a segment. The receptor in vivo functions as C2 decoy receptor by directly competing with C4b for binding to C2. As a result, CRIT is able to limit the extent of classical pathway (CP) C3 convertase formation. We report that the CRIT-extracellular domain 1 (ed1) peptide inhibits CP-mediated complement activation with an ICH_{50} of ~0.1 μM, the C-terminal 11 aa of CRIT-ed1, named H17, even more effectively. The β-chain region F222–Y232 of C4 shares 55% identity and 73% similarity with H17. Peptides based on this region also inhibit CP in a dose-dependent manner. As further evidence of C2 binding we showed CRIT-ed1 peptides and homologous C4 β-chain peptides to inhibit complement in C2 hemolytic assays. We have predicted C4 β-c F222–Y232 as a C2 binding site which we have termed the CRIT-ed1 domain, and the sequence [F/H]EVK$X_{4/5}$P as a consensus C2-binding sequence. Anti-CRIT-ed1 cross-reacts with the C4 β-chain and F222EVKITPGKPY232 appears to be the key epitope recognized by this Ab. Furthermore, anti-CRIT-ed1 was found to inhibit CP activation in a total hemolytic assay. We believe that *Schistosoma* CRIT-ed1, as well as C4 β-chain peptides based on the CRIT-ed1 domain, function as interface peptides. These peptides, based on C2-binding sequences in CRIT, or C4, competitively inhibit the binding of C2 to C4b and thus limit the activation of C. The C4 peptides, unlike CRIT-ed1, did not inhibit the cleavage of C2 by C1s. *The Journal of Immunology*, 2002, 168: 5213–5221.

Complement protein C3 is the central component of the complement system, and the proteolytic cleavage products of C3 are its biologically active forms. Cleavage is achieved by the alternative pathway (AP)[3] C3 convertase C3bBb (1) and the classical pathway (CP) C3 convertase C4b2a (2), the latter assembled from the binding of C4b with C2 and subsequent cleavage of C2 by C1s. The assembly of the CP C3 convertase occurs in three steps. Initially C1s cleaves a 9-kDa fragment, the anaphylatoxin C4a, from the N terminus of the α-chain of C4, the remaining C4b binding to the activator surface via the exposed thioester bond. As a result of this cleavage, binding sites for various proteins (C2, C3b, C5, CR1, C4BP, membrane cofactor protein, decay-accelerating factor, and factor I) are exposed. Initial binding of C2 to C4b is dependent on Mg^{2+} and occurs via two low-affinity sites: one on C2b, which is Mg^{2+} independent (3–5), and the other on the von Willebrand factor type A (vWFA) domain of C2a, which is Mg^{2+} dependent (6). Subsequent cleavage of C2 by C1s results in the release of C2b in the fluid phase. The transient conformational change in the vWFA site on C2 results in binding of C2a with an increased avidity to C4b, this interaction being Mg^{2+} dependent. Recently, a third C4b binding site within C2 has been postulated to lie within the serine protease (SP) domain (7, 8).

C2 is a serum glycoprotein of 102 kDa, 39% identical to its AP homolog, factor B (9). C2 is made up of three globular domains. The N-terminal domain, which constitutes the C2b segment, is made up of three short consensus repeats (SCRs). The remaining C2a segment of this single-chain polypeptide, consisting of a vWFA domain and then a SP domain, makes up the C-terminal end. Human complement C4 is a heterotrimer composed of a 93-kDa α-chain, a 75-kDa β-chain, and a 32-kDa γ-chain (10), the β- and γ-chains being linked to the α-chain by disulfide bonds. Very little is known about the binding site(s) between C4 and C2. Much information about CP C3 convertase (C4b2a) formation has in fact been gleaned from empirical data of the binding sites used between factor B and C3 to form the AP C3 convertase (11, 12). The first direct evidence of C4 and C2 binding sites came from a mAb able to block the interaction of C2 (and C4BP) to C4b (13). That the α-chain of C4 might be involved in interaction with C2 came from the identification of a C4BP binding site on C4b. This was found by means of an anti-C4 mAb able to block the C4b-C4BP interaction. The epitope of this mAb was mapped to a region in the N terminus of the C4 α′-chain, residues 738–808 of the mature protein (14, 15), homologous with a functionally conserved binding site in C3 for factor B (residues 727–793).

When considering a region(s) on C3/C4 previously suggested as being involved in protein-protein interactions, it has been useful to compare the sequence concerned in various species to identify conserved residues which may therefore be important for protein interaction (16). To assess the importance of these regions for binding, they may then be either deleted or substituted by the homologous region from other species (17). To implicate specific residues within a ligand binding site, site-directed mutagenesis has been used (18, 19). More recently, two clusters of acidic residues E744ED and D749EDD within the C4 α′-chain were shown by site-directed mutagenesis and by using synthetic peptide mimetics and anti-peptide blocking strategies to contribute to C2 binding with C4b (20). Although this is a useful confirmatory tool, a drawback that should be borne in mind is that, in addition to a local effect, amino acid substitutions may also result in conformational

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Received for publication January 15, 2002. Accepted for publication March 15, 2002.

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[1] This work was supported by grants from the Swiss National Foundation and the Roche Research Foundation.

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[3] Abbreviations used in this paper: AP, alternative pathway; C2D, C2 deficient; CFD, complement fixation diluent; CP, classical pathway; EA, Ab-sensitized sheep erythrocyte; ed1, extracellular domain 1; KLH, keyhole limpet hemocyanin; RT, room temperature; SCR, short consensus repeat; DSS, disuccinimidyl suberate; SP, serine protease; vWFA, von Willebrand factor type A; CRIT, complement C2 receptor inhibitor trispanning.

0022-1767/02/$02.00

changes in more distant parts of the protein that may be also involved in ligand binding. Relating such changes to functional effects may then be misleading.

Previously we described the trispanning orphan receptor found on the surface tegumental plasma membrane and tegumental surface pits of adult *Schistosoma* worms (21). We later showed that trispanning orphan receptor is a receptor for the human complement protein C2 (22) (now called complement C2 receptor inhibitor trispanning (CRIT)). CRIT does not bind the closely related factor B protein (22). By binding C2 we speculate that CRIT is able to prevent C2 binding to C4b on the parasite surface and forming the CP C3 convertase. CRIT has two extracellular domains. The first extracellular domain (ed1) at the N terminus of the protein is made up of 27 residues. This constitutes the ligand binding domain of the receptor. In this study we show that, within the C2 binding ed1 region, an 11-aa segment (H17–Y27), henceforth called H17, constitutes the ligand binding site. Furthermore, we indicate a C2 binding site on C4 by showing that a homologous region within the β-chain of C4 (F222–Y232), which we called the CRIT-ed1 domain, is also able to bind C2, via the C2a segment.

Materials and Methods

Peptide synthesis

Synthetic peptides (Table I) were from Primm (Milan, Italy). A solid-phase peptide synthesis method with F-moc chemistry was used. Peptides were purified by RP-HPLC to >90% purity and lyophilized. PBS or deionized water was then added to give the required concentration (usually 2000 μM). All peptides were freely soluble in deionized water or PBS. Peptides in solution were stored at −80°C. The peptide ed1 was also synthesized with a cysteine at the N terminus. This was coupled to keyhole limpet hemocyanin (KLH). The KLH-ed1 conjugate was used for rabbit immunizations.

Abs and complement components

Rabbit anti-ed1 Abs were derived by injection of 200 μg of KLH-ed1 in PBS, emulsified with CFA on day 21 and with IFA on day 28, and subsequently injected with 100 μg of KLH-ed1 on days 35, 50, and 60. The titer obtained on day 60 for the obtained serum against the preimmune serum was 1/10,000 in an ELISA against immobilized ed1 peptide. The Ab was then affinity-purified according to the protocol described below.

Polyclonal goat Abs against complement proteins C2, C3, C4, and C5 were purchased from Calbiochem (La Jolla, CA). Complement protein C2 was either kindly provided by Dr. R. B. Sim (Oxford, U.K.) and purified according to a protocol described elsewhere (23), or else purchased from Calbiochem, as were complement proteins C3, C4, and C5. C1s was purchased from Sigma-Aldrich (St. Louis, MO).

Affinity purification of polyclonal anti-ed1 Ab

Essentially ed1 peptide (6 mg/ml in PBS) was coupled to epoxy-activated Sepharose 6B (Sigma-Aldrich). The column was prepared by blocking excess active groups with ethanolamine, followed by two washes in high and low pH buffer (0.1 M bicarbonate buffer with 0.5 M NaCl (pH 8.3) and 0.1 M acetate buffer with 0.1 M NaCl (pH 4), respectively) and then equilibration in PBS. Rabbit polyclonal anti-CRIT-ed1 serum was then applied to the ed1 column. After extensive washing with PBS (10-column volumes), elution was conducted with 0.1 M glycine-HCl (pH 2.8), fractions being neutralized with 1/20 volume 1 M Tris-HCl (pH 8.5). Finally, the Ab was dialyzed extensively against PBS.

Table I. *Synthetic peptides used in this study*

Peptide	Amino Acid Sequence
ed1	MSPSLVSDTQKHERGSHEVKIKHFSPY
H17	HEVKIKHFSPY
H17S[a]	EKFYHIHSKPY
C4$\beta^{212-232}$	FEVKKYVLPNFEVKITPGKPY
C4$\beta^{212-221}$	FEVKKYVLPN
C4$\beta^{222-232}$	FEVKITPGKPY
C4βS$^{212-232}$[b]	FYFNEPVEPKVLPKGVKYTKI
ed2	SSTSDIRLVIHTKTGPIYIKST

[a] Scrambled sequence for peptide H17.
[b] Scrambled sequence for peptide C4$\beta^{212-232}$.

Buffers

Complement fixation diluent (CFD; 5×; pH 7.3) contained 728 mM NaCl, 9 mM Na barbitone, 4.13 mM $MgCl_2 \cdot 6H_2O$, 1.26 mM $CaCl_2 \cdot 2H_2O$, and 3.1 mM diethylbarbituric acid.

Hemolytic assays

Ab-sensitized sheep erythrocytes (EA; 1 ml of 1×10^9 cells/ml; bioMérieux, Charbonnier les Bains, France) were prepared after washing twice each in cold 0.9% NaCl and cold CFD, by resuspending in 1 ml CFD and mixing with 25 μl Amboceptor (1/40 dilution anti-SRBC, IgM; Dade Behring, Marburg, Germany) at 37°C for 20 min. Finally, EA was washed in CFD and resuspended to 2×10^8 cells/ml. To test the inhibitory capacity of synthetic peptides a CP hemolytic assay was set up. A total of 100 μl of titered normal human serum, typically 1/100–1/150, which gave 70–85% control hemolysis, preincubated for 30 min at room temperature (RT) with the particular peptide or anti-CRIT-ed1 Ab in CFD buffer, was incubated with 50 μl EA for 30 min at 37°C. Hemolysis was determined by measuring the absorbance of the supernatant at 414 nm. Sepharose-ed1 prepared as described above was used as an alternative to peptide alone at 1 μl (~4 μM) or 5 μl (~20 μM).

For C2 hemolytic assays, 0.125 μg C2 was added to a 1/25 dilution of serum (100 μl) from a patient with type I complement C2 deficiency (24) (C2D serum) to restore hemolytic activity. After incubation with 50 μl EA at 37°C for 30 min, the absorbance of the supernatant at 414 nm was determined as a measure of hemolysis. Inhibitory peptides made up in 50 μl PBS were preincubated with C2 at RT for 30 min before adding to C2D serum.

Measurement of C2 and C4 cleavage by C1s

C2 (2 μg) was incubated with 10-fold serial dilutions of peptide in 10 μl of 1× CFD for 30 min at RT. C1s (2 μg/ml) was then added for 1 h at 37°C. Uncleaved C2 was always included in each experiment. Samples were then probed in a Western blot with either polyclonal anti-C2 or, when biotin-labeled C2 was used, streptavidin-HRP. For monitoring the effect of ed1 peptide coupled to Sepharose on C1s cleavage of C2, the reaction was as above but with 7 μl Sepharose-ed1 (50% v/v) added. The Sepharose-ed1 was prepared as described above for affinity purification of C2. To test whether CRIT-ed1-based peptides were interacting and thereby inhibiting C1s activity (and therefore C) directly, peptides were preincubated with C1s in 10 μl 1× CFD for 30 min on ice before adding 0.4 μg of biotin-labeled C4. Incubation was continued at 37°C for 1 h and cleavage was monitored by immunoblotting.

Electrophoresis and immunoblotting

Electrophoresis and immunoblotting were conducted as described previously (22). Essentially, proteins were separated (1 h/150 V) by SDS-PAGE on 10 or 12% gels using mini-gel systems (Bio-Rad, Hercules, CA) under either reducing or nonreducing conditions. Immunoblotting to nitrocellulose (Amersham Pharmacia Biotech, Piscataway, NJ) was performed at 380 mA for 1 h. Blots were blocked for 1 h at RT or overnight at 4°C in PBST (PBS with 0.1% (v/v) Tween 20) plus 6% nonfat milk and then incubated in rabbit or goat primary Ab (1/1000) or streptavidin-HRP conjugate (1/2000) for 1 h at RT. Where required the second Ab used was either an anti-rabbit-HRP or anti-goat-HRP (1/3500). After 1 h at RT, blots were washed six times in PBST for 10 min each at RT. Detection was by chemiluminescence using the ECL substrate (Amersham Pharmacia Biotech) and exposure to Kodak Biomax MR film (Kodak, Rochester, NY).

Biotinylation of proteins

To the protein to be biotinylated (200 μg in 500 μl PBS), NHS-LC-biotin (Pierce, Rockford, IL) was added (200 μg/mg protein). After incubation for 1 h at RT with constant mixing, free biotin was removed by extensive dialysis against PBS at 4°C or else by filtration through a Microsep microconcentrator (with a 10-kDa cutoff membrane; Pall Life Sciences, Ann Arbor, MI), pretreated with 10% (v/v) glycerine overnight at RT, and rinsed with deionized water to avoid nonspecific adsorption. After two washes with PBS, the biotinylated protein was eluted with 100 μl of PBS, aliquots being stored at −80°C. Purification of biotinylated peptides (~3 kDa) was conducted by equilibrating a Sephadex G25 column with PBS-1% BSA and then eluting the sample in 1-ml fractions with PBS.

Chemical cross-linking of proteins

C2 or C2a and C2b as acceptor proteins were mixed with biotinylated peptide (10 μM) in CFD buffer to a total volume of 20 μl. After incubation for 15 min at 37°C, 50 μM of the cross-linker disuccinimidyl suberate (DSS), dissolved in DMSO, was added. After 30 min at RT the reaction was stopped by adding SDS-PAGE sample buffer.

Results

Sequence homology between CRIT and the β-chain of complement C4

CRIT from the *Schistosoma* parasite was identified previously as binding C2 via its first extracellular domain, ed1 (22). The 27-aa CRIT-ed1 sequence (Fig. 1*A*) shows 35% identity with a homologous region of the β-chain of C4 (S206–Y232). The terminal 11 aa of CRIT-ed1 (which we have termed CRIT-H17) share 55% identity and 73% similarity with the C4 β-chain (F222–Y232). Based on this homology we have termed this sequence within C4 the CRIT-H17 motif (Fig. 1*A*). Immediately N-terminal to this we find the motif repeated (F212–N221). The two contiguous CRIT-H17 motifs in C4 we have called the CRIT-ed1 domain. The consensus CRIT-H17 motif is thus [F/H]EVK$(X)_{4/5}$P and Fig. 1*B* shows an alignment of the two contiguous CRIT-H17 motifs in the C4 β-chain. Fig. 1*C* shows each of these two CRIT-H17 motifs from the C4 sequence aligned with the terminal 11 aa of CRIT-ed1. We also identified a 16-aa stretch of the 27-aa long second extracellular domain of CRIT (Fig. 1*D*), which shares a 56% identity with the C4 γ-chain, although each sequence involves a gap to make the alignment.

In the current study total hemolytic assays as well as C2 hemolytic assays were performed to further delineate the C2 binding site. We used synthetic peptides (Table I) derived from the CRIT-ed1 sequence and the homologous region (CRIT-ed1 domain) from the C4 β-chain (Fig. 1*A*).

Anti-CRIT-ed1 Ab binds to β-chains of C4 and C3 and unreduced C4 but not unreduced C3 or C5

In view of the homology between CRIT-ed1 and the C4 β-chain it was important to see whether the affinity-purified polyclonal Ab against the 27-aa CRIT-ed1 region, highlighted in Fig. 1*A*, could recognize the C4 β-chain. The proteins C3 and C5 have a high degree of homology with C4 within the CRIT-ed1 domain, as illustrated in the alignment in Fig. 2*A*. Therefore, we looked for anti-CRIT-ed1 recognition of C3 and C5 as well. C4 was run under partially reducing conditions such that the C4α, -β, -γ, -$\alpha\beta$, and -$\alpha\gamma$ chains as well as nonreduced C4 were visible when probed in a Western blot with anti-C4 (Fig. 2*B*). A nonreduced C4 was similarly probed with anti-CRIT-ed1. When C4 was partially reduced the C4 β-chain was strongly recognized. There was also a weaker recognition of the unreduced C4 and a very faint recognition of the disulfide-linked C4 $\alpha\beta$ chains. As anti-CRIT-ed1 recognizes the β-chain of the reduced C4, it would appear that the epitope for this Ab is linear, not requiring the disulfide bridging of the α- and β-chains. Because anti-CRIT-ed1 recognition of this C4 β-chain can be blocked by prior incubation of the Ab with the ed1 peptide and more specifically the H17 peptide (Fig. 2*C*), it appears that the epitope may lie within the region F222–Y232 of the C4 β-chain, the region of sequence homologous to CRIT-H17 (H17–Y27). In summary, the sequence homology between the C-terminal part of CRIT-ed1 (H17–Y27) and C4β F222–Y232 suggested the latter as a possible C2 binding site. Therefore, it was interesting that a polyclonal Ab against the 27-aa ed1 peptide of CRIT, the *Schistosoma* receptor for human complement C2, cross-reacts with C2-binding human complement component C4. However, this Ab did not recognize the closely related C3 protein except under reducing conditions whereupon the β-chain was recognized. This means that, unlike in C4, the CRIT-ed1 domain epitope in C3 is hidden. By contrast, anti-CRIT-ed1 did not cross-react with C5, either unreduced nor reduced (data not shown).

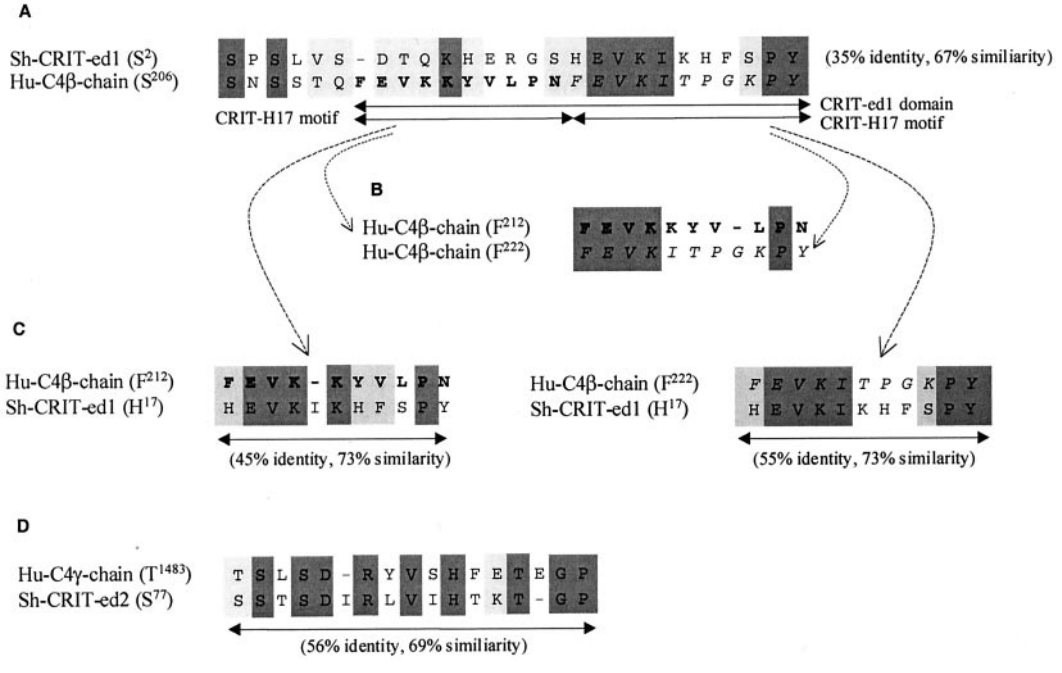

FIGURE 1. Amino acid sequence alignments of *Schistosoma* CRIT-ed1 and ed2 with homologous regions, C4β(S206–Y232) and C4γ(T1483–P1498), respectively, in human C4. Alignments were made using the CLUSTAL W program. Identical residues are shown with dark shading and similar residues are shown with light shading. Gaps are indicated by dashes. Sequence numbering is based on that of the mature CRIT and C4 proteins.

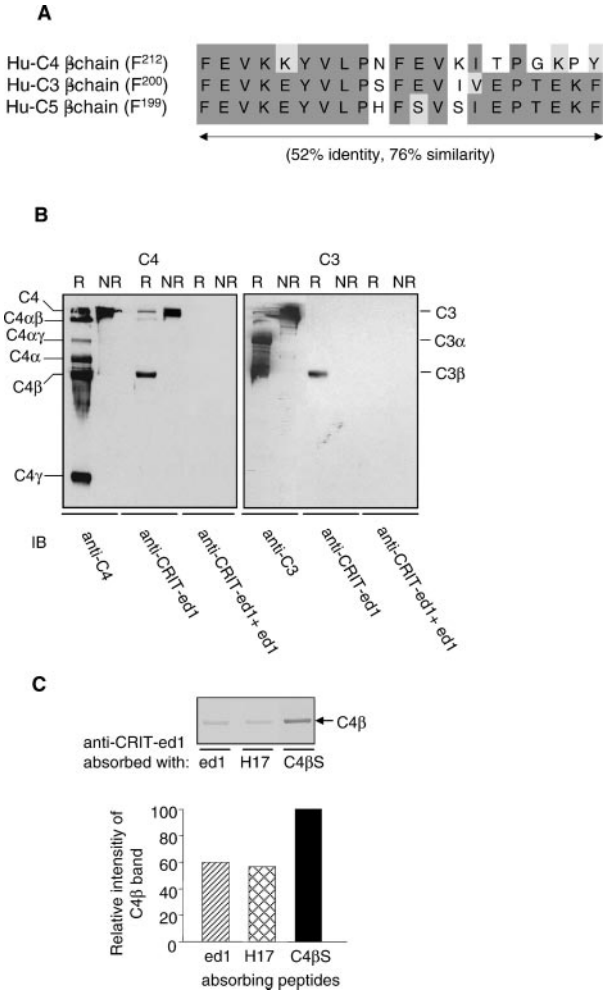

FIGURE 2. Monospecific CRIT-ed1 Ab recognizes C4 (via the β-chain) and the β-chain of reduced C3 but not unreduced C3 nor C5. *A*, Sequence alignment of the CRIT-ed1 domain regions of C3, C4, and C5. Identical residues have dark shading and similar residues have light shading. *B*, Immunoblot (IB) of human C3 and C4 separated by SDS-PAGE on a 10% gel under reducing (R) or nonreducing (NR) conditions probed with anti-C3, anti-C4, or anti-CRIT-ed1. *C*, Compared with the signal obtained by preabsorption of the anti-CRIT-ed1 Ab with the control C4βS peptide, both ed1 and H17 greatly diminish the recognition of the C4 β-chain by anti-CRIT-ed1.

Inhibition of complement by CRIT- and C4-based peptides

Peptides were first used in a total hemolytic assay (Fig. 3, *A* and *B*). The 27-residue long CRIT-ed1 peptide inhibited complement in a dose-dependent manner, giving an ICH_{50} (concentration of peptide required to reduce the hemolytic activity of serum to 50% of the control activity in the absence of peptide) of 100 nM, and the 21-aa long $C4\beta^{212–232}$ an ICH_{50} of 8 nM. The smaller H17 and homologous $C4\beta^{222–232}$ peptides also inhibited complement, both with ICH_{50} values of ~10 nM, but CRIT-ed2 up to a concentration of 10 μM did not inhibit CP activity. The CRIT-ed1 peptide bound to Sepharose compared with the C4βS control peptide was also found to inhibit CP activity (Fig. 3*C*), although only at an estimated 20 μM. We were also able to specifically block C4 at the β-chain with anti-CRIT-ed1, 50 μg causing a reduction in hemolysis from 70–38%.

CRIT-ed1 and $C4\beta^{212–232}$ bind C2 via a site in C2a

To show that this inhibition of the CP was due to an interaction with C2, C2 hemolytic assays were performed in which the inhibitory peptides were preincubated with a limiting concentration of C2, enough to reconstitute complement when added to C2D serum. A 30-min preincubation of the peptides with C2 had the following effects on C activity: considering CRIT-based peptides (Fig. 3*A*), hemolysis was reduced from 90% (without peptide) to 40% (0.1 nM ed1) and 0% (0.01 nM H17), indicating that H17 bound to C2 and more effectively prevented formation of the CP C3 convertase than ed1. The C4 β-chain peptides (Fig. 3*B*) also gave a dose-dependent inhibition of C-mediated hemolysis, showing a reduction in hemolysis from 85 to 20% for $C4\beta^{212–232}$. The N-terminal half of this peptide, $C4\beta^{212–221}$, in the C2 hemolytic assay showed a reduction from 85 to 25% at 0.1 nM. Just as the C-terminal region, H17, was more effective than the full-length ed1 peptide, $C4\beta^{222–232}$ was more effective than the full-length $C4\beta^{212–232}$, resulting in a 85–0% reduction in hemolysis at 0.01 nM.

These interactions with C2 were confirmed by allowing biotinylated ed1 peptide ($ed1^{bio}$) and $C4\beta^{(212–232)bio}$ to complex with C2, as acceptor protein, during a 15-min incubation at 37°C. Using a protocol similarly used to study the interaction of factor B and C3b (11), bound proteins were covalently cross-linked with DSS to stabilize the complexes formed and then detected by Western blotting. As shown in Fig. 4*C*, both $ed1^{bio}$ and $C4\beta^{(212–232)bio}$ bound C2 and the signals obtained for $ed1^{bio}$-bound C2 and $C4\beta^{(212–232)bio}$-bound C2 could be removed by prior incubation with an excess of unlabeled peptides. In addition, $ed1^{bio}$ and $C4\beta^{(212–232)bio}$, when incubated with C2a and C2b, specifically bound the C2a fragment. These interactions, which were performed in the presence of physiological $MgCl_2$ concentrations (0.8 mM) and 145 mM NaCl, also occurred in the presence of 2 mM EDTA without a major difference in degree of association (data not shown).

CRIT-ed1, unlike $C4\beta^{212–232}$, inhibits the C1s-mediated cleavage of C2

We have shown that CRIT-ed1 and $C4\beta^{212–232}$ bind C2. It is possible that such a competitive binding, interfering with the normal binding of C2 to C4b, could per se explain the complement inhibition due to these peptides in hemolytic assays. We then tested whether these peptides had any effect on C1s cleavage of C2 in addition to inhibition of C2-C4 binding. To maximize any inhibitory effect of the peptides, C1s digestion conditions were chosen such as to give limited cleavage of C2. The effects on C1s cleavage were monitored by immunoblotting with anti-C2. Neither $C4\beta^{212–232}$ (Fig. 5*A*) nor C4βS (Fig. 5*B*) had any effect on C1s-mediated cleavage of C2 at any of the concentrations tested. CRIT-ed1 at 0.01 and 0.1 nM (Fig. 5*C*) had no effect on C2 cleavage, but at concentrations ≥1 nM C2 cleavage was inhibited. Sepharose beads coupled with the ed1 peptide, as opposed to Sepharose-C4βS or uncoupled Sepharose, also gave a total inhibition of C1s-mediated cleavage of C2 (Fig. 5*D*). We then tested whether the $C4\beta^{212–232}$ or CRIT-ed1 peptides inhibited C1s-mediated cleavage by interfering with the action of C1s itself. For this we looked for inhibition of C1s-mediated cleavage of C4 to which the peptides do not bind (data not shown). Here, $C4\beta^{212–232}$, C4βS, and CRIT-ed1 up to a concentration of 1000 nM were not able to inhibit C1s cleavage of the C4 α-chain (93 kDa) to yield the α′-chain (87 kDa) (Fig. 5*E*).

Interspecies alignment of the C4β chain sequence reveals conservation within the CRIT-ed1 domain

In this study we have pointed to the CRIT-ed1 domain (F212–Y232) of the C4 β-chain as being a site of C2 interaction. Therefore, it is not surprising that on comparing this domain in five different species, using CLUSTAL W software (25), there is a high

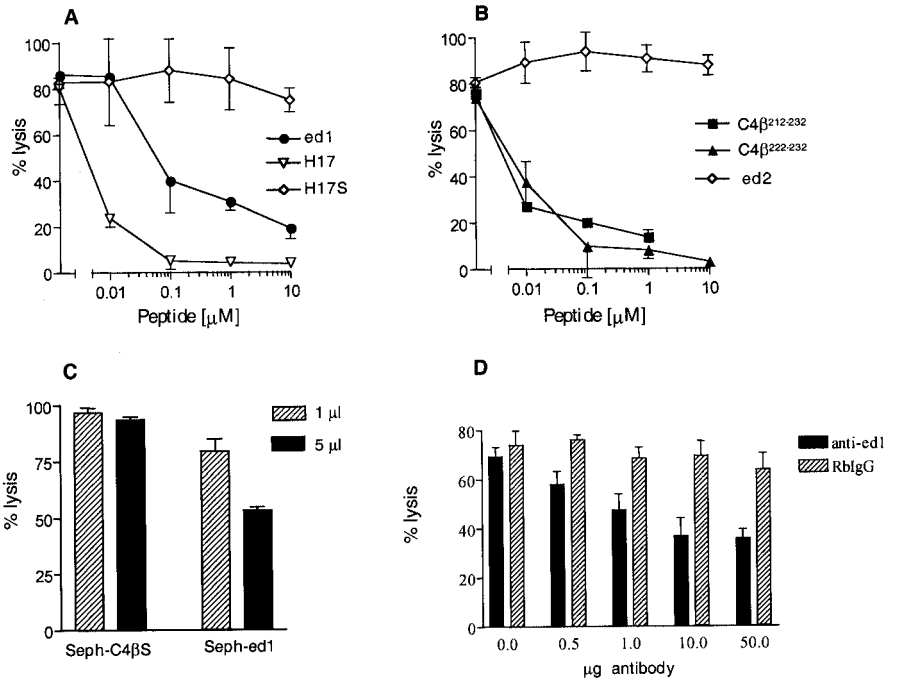

FIGURE 3. Inhibition of complement activation by CRIT- and C4-based synthetic peptides. CRIT-ed1-based peptides (*A*) and C4β-based peptides (and CRIT-ed2) (*B*) were tested in total hemolytic assays at a range of concentrations for their capacity to inhibit the CP of C activation. The percentage of lysis is based on the degree of lysis of SRBC, measured at A_{414} as hemoglobin released. *C*, CRIT-ed1 and control C4βS peptide bound to Sepharose beads were similarly tested for inhibition of hemolysis. *D*, Monospecific anti-CRIT-ed1 Ab was tested for CP inhibition.

degree of conservation (Fig. 6). In human, mouse, frog, carp, and chicken C4, across the 21 residues of the CRIT-ed1 domain, which is boxed, there is 52% identity and 62% similarity. Taking the adjoining N-terminal 44 aa and C-terminal 21 aa together there is only 6% identity and 31% similarity. The high degree of sequence identity within this region compared with the adjoining 65 residues points to the importance of this region. Within the CRIT-ed1 domain we have shown the residues that are identical in at least three species in bold (Fig. 6). Above this alignment lies the CRIT-H17 peptide, representing the terminal 11 residues of CRIT-ed1. This is shown as a repeat, to parallel the CRIT-ed1 domain in C4 that is made up of two contiguous CRIT-H17 motifs. By looking at amino acid differences across the species within the conserved CRIT-ed1 domain of C4 and CRIT-H17 we can predict residues that are most

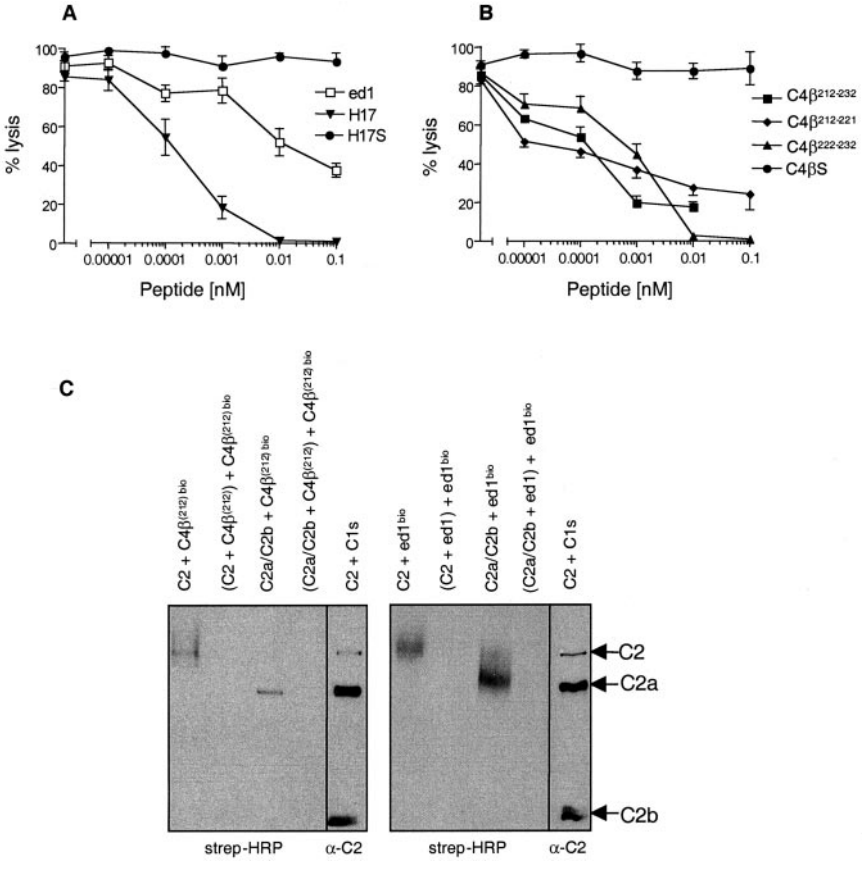

FIGURE 4. CRIT-ed1 and C4$\beta^{212-232}$ bind C2. *A* and *B*, The effect of the peptides in a C2 hemolytic assay was tested. *A*, Different amounts of CRIT-based peptides were preincubated for 30 min at RT with an amount of C2 that was just sufficient to restore hemolytic activity when added to a C2D serum. Hemolysis was measured as hemoglobin released. *B*, C4 β-chain peptides based on the CRIT-ed1 domain were similarly tested for their ability to specifically bind to C2. *C*, CRIT-ed1^{bio} and C4$\beta^{(212-232)bio}$ peptides were allowed to bind either to uncleaved C2 or to a mixture of C2a and C2b. Resulting complexes were cross-linked with DSS and then detected by immunoblotting and probing with streptavidin-HRP as described in *Materials and Methods*. In control lanes, on the *right side* of each panel this interaction is blocked by preincubation with an excess of unlabeled CRIT-ed1 or C4$\beta^{212-232}$ peptide. A partial digest of C2 with C1s, probed with polyclonal anti-C2 (α-C2), is included on the *right side* of each panel to indicate the relative migrations of C2, C2a, and C2b.

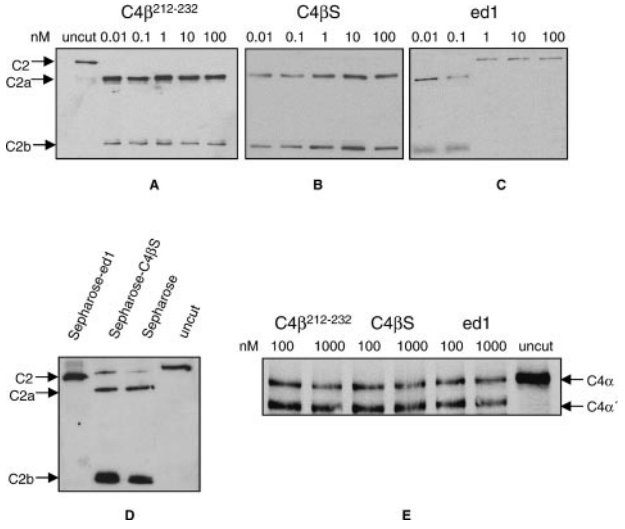

FIGURE 5. CRIT-ed1 but not C4$\beta^{212-232}$ inhibits the C1s-mediated cleavage of C2. The effect of increasing concentrations (10-fold serial dilutions from 0.01 to 100 nM) of C4$\beta^{212-232}$ (*A*), C4βS (*B*), and CRIT-ed1 (*C*) on the cleavage of C2 by C1s (2 μg/ml C1s for 1 h at 37°C) as monitored by Western blotting with anti-C2. *D*, The effect of ed1 coupled to Sepharose compared with Sepharose-C4βS and Sepharose alone on C1s-mediated cleavage of C2 was similarly monitored. *E*, To show that inhibition of C1s-mediated cleavage of C2 was not due to interference by CRIT-ed1-based peptides with C1s itself, it was found that these peptides had no effect on C1s-mediated cleavage of biotinylated C4 as monitored by the appearance of the C4 α'-chain.

likely important for C2 binding of a particular species. These residues, which are shown underlined in the human sequence (Fig. 6) include K215 (R in chicken), K216 (P and E in frog and carp, respectively), K225 (T and R in carp and chicken, respectively) and P231/Y232 (G and F, respectively, in chicken). Although the fully conserved residues are clearly crucial for this binding site, we can also suggest K215, K216, K225, P231, and Y232 as likely candidates for substitution in site-directed mutagenesis experiments which could alter the C2 binding capability of this region.

Our findings have indicated the CRIT-ed1 domain (F212–Y232) of C4 as a potential C2 binding site. We have also shown that anti-CRIT-ed1 recognizes the β-chain of C4, most likely via the CRIT-ed1 domain. As this Ab also reacts with the β-chain of C3, we were also interested to compare this sequence with the equivalent region in C3 which is overall ~29% identical in its amino acid sequence to C4. From an alignment of full C4 and C3 sequences, using the CLUSTAL W program, the CRIT-ed1 domain shows 57% identity and 71% similarity between C4(F212–Y232) and C3(F200–F220) (Fig. 6). There is only a 16% identity in the preceding 44 residues and 19% identity in the following 14 residues of both C3 and C4. This level of identity was also described for a 27-aa C2 binding region in the C4 α-chain and its equivalent factor B binding region in C3 (20). As C2 and its functional and structural homolog, factor B, are 40% identical and already known to share at least one binding site within equivalent regions of C4 and C3, respectively (20), it would be interesting to investigate C3(F200–F220), which according to our knowledge has not yet been done, as a potential factor B binding site.

Discussion

Parasites use various strategies to evade the C system. One is to prevent C activation on their surface. For example, paramyosin produced by both *Schistosoma mansoni* and *Taenia solium* binds C1q, thus blocking C1 activation on the parasite surface (26). The outer lipid envelope on *S. mansoni* adult worms possesses a 130-kDa C3 receptor protein (27). On activation of C, C3b and other C proteins are thus bound to the envelope, but by shedding the C-bearing envelope the worms are protected. Adult schistosome worms also express schistosome C inhibitory protein-1, a protein functionally and antigenically related to CD59 that in humans inhibits the C5b-9 complex by binding to C8 and C9 (28). In the chronic phase of infection with *Trypanosoma cruzi*, Abs directed at the parasite fix complement thereby lysing the blood-borne trypomastigote form and maintaining a latent infection. *T. cruzi* also expresses an ~90-kDa protein with decay-accelerating activity for both the CP and AP (29, 30) and possesses a C3b-binding protein which inhibits the formation of the AP C3 convertase (31). A fibronectin/collagen receptor of *T. cruzi*, gp58/68 (32), inhibits AP C3 convertase formation by preventing factor B from binding to surface-bound C3b.

We believe that CRIT protects the parasite from CP-mediated complement attack by acting as a decoy C2-binding receptor (Fig. 7). In the chronic phase of a schistosomal infection, Abs directed against the parasite surface fix C1q. The associated C1s cleaves C4 in the serum. The resulting C4b, covalently bound to the surface, binds serum C2 which is also cleaved by C1s, releasing C2b. The C2a segment remains tightly bound to C4b, and this constitutes the CP C3 convertase. The presence of CRIT in tegumental plasma membrane on the surface of the adult worm and within the tegumental surface pits and channels competes with C4b for the C2, thus limiting the extent of CP C3 convertase formation on the

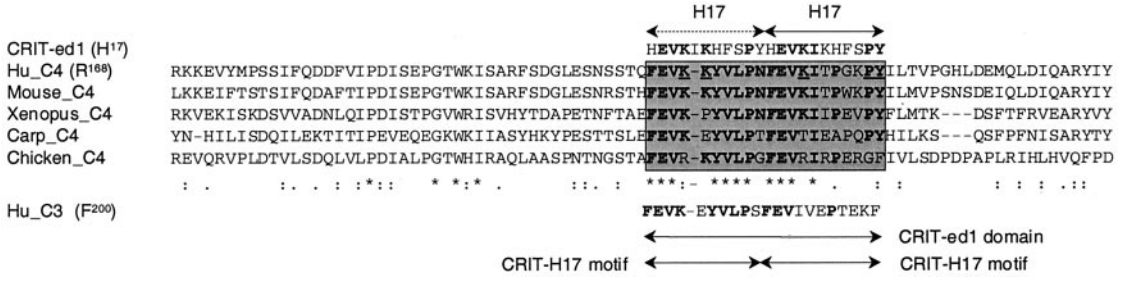

FIGURE 6. CLUSTAL alignment of human C4 β-chain R168–Y253 with equivalent region of C4 from four other species. The CRIT-ed1 domain (F212–Y232) within this region, which we describe as a C2 binding site, is boxed. Residues common to at least three of the five species considered within the CRIT-ed1 domain are in bold. *, Amino acid identity in all five species; : and . indicate conserved and nonconserved amino acid substitutions, respectively. Gaps introduced into the sequence to aid the alignment are illustrated by a dash. The five C4 sequences are also aligned with the CRIT-ed1 (H17) sequence shown *above* as a contiguous repeat. Residues in the CRIT-ed1 domain that are substituted in different species and that are discussed as potentially being involved in C2 interaction (and therefore likely candidates for specific substitution in mutagenesis experiments) are underlined in the human sequence.

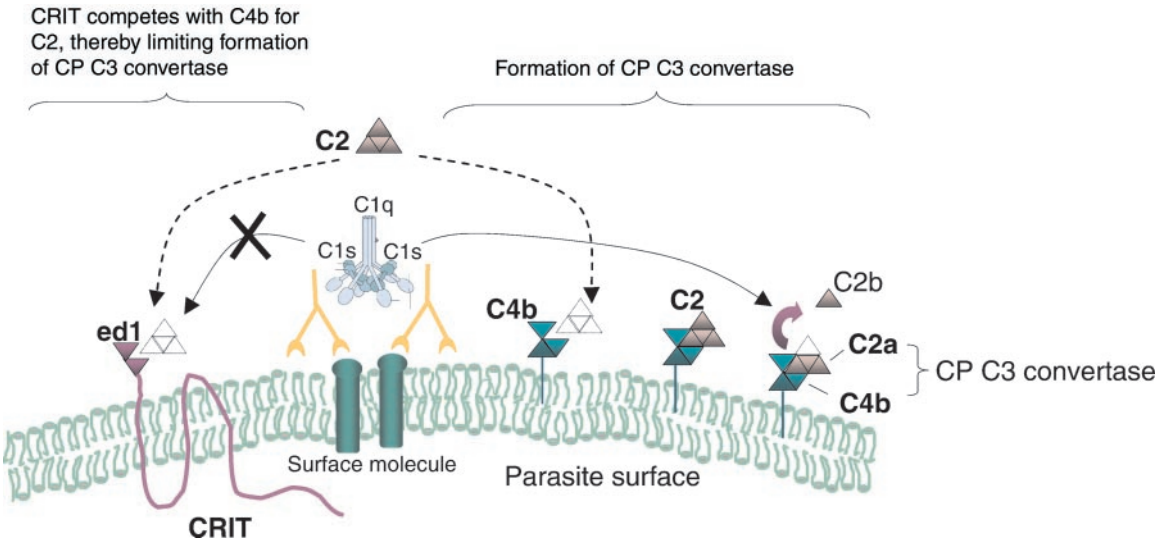

FIGURE 7. Model of CRIT-mediated inhibition of CP C3 convertase formation on parasite surface. Ab (in yellow) bound to the parasite surface, via some surface molecule, fixes C1q and associated C1s. C4b bound via a thioester bond to the surface then associates with C2, which is cleaved by C1s to produce 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.

parasite surface. Having described CRIT as a schistosomal receptor able to bind human C2, we sought to use the sequence homology between CRIT and human C4, which also binds C2, to try to define a binding site in C4.

Complement proteins C3, C4, and C5 are structurally related and belong to the α_2-macroglobulin superfamily (33). To date the only structural data on these proteins is for C3a (34) and C5a (35), anaphylatoxins released during C activation from C3 and C5, respectively, and the 35-kDa C3d segment of C3 (36). Although all three bind different proteins, certain similarities, most notably their sequence homology, suggest that they probably have similar three-dimensional structures. It may be that within a common structure certain regions may be predisposed to protein interaction and that the ability to bind to different proteins and therefore of having different functions may be due to unique sequences at these locations.

By virtue of the sequence homology of CRIT-ed1 with C4 β-chain F212–Y232 and because of the anti-CRIT-ed1 recognition of cross-reacting epitope(s) on the C4 β-chain, we consider CRIT-ed1 to be a C4-like peptide. In this study we used synthetic peptides, either free or coupled to Sepharose beads, based on the C2 binding ed1 region of CRIT itself or the homologous CRIT-ed1 domain within the C4 β-chain (F212–Y232), also predicted as binding C2. All were found to inhibit CP-mediated C activity. This suggested that the peptide was functioning as an interface peptide, inhibiting the association of C4 with C2. An interface peptide essentially comprises a sequence that corresponds to part of a protein interaction site which functions as a competitive inhibitor of binding between the proteins concerned, examples including those described by Sandoval et al. (37) and Babé et al. (38). Because the CRIT-ed1 sequence itself binds C2 (as we showed via C2a), we were able to test the assumption that the inhibition of C provided by the CRIT-ed1 interface peptide was due to its competitive binding to C2. For this, the C2 hemolytic assay was used to show that CRIT-ed1-based sequences, by binding C2, thus prevent C2 interaction with C4b and thereby inhibit CP C3 convertase activity. Our finding that the anti-CRIT Ab (which we had shown earlier as specifically recognizing the β-chain of C4) was able to block hemolysis in a dose-dependent manner further supported the CRIT-ed1 domain in C4 functioning as a C2 binding site, although we cannot rule out that any blocking of C2 binding to C4 by anti-CRIT-ed1 is due at least in part to steric hindrance. Whether this inhibition is due to a blocking of C3 (57% identical with C4 across the CRIT-ed1 domain) is not yet clear.

As we showed that CRIT-ed1 binds to C2, we wanted to confirm this and in so doing find out whether it binds via C2a or C2b. Biotin-labeled ed1 and C4$\beta^{212–232}$ peptides were allowed to react with a mixture of C2a and C2b and then were chemically cross-linked. From the size of the resulting biotin-labeled complex it was shown that both ed1 and C4$\beta^{212–232}$ bound to C2a. At this point we have no idea of exactly where on C2 lies the binding site with which CRIT-based and C4 β-chain peptides interact. As we mentioned in the introduction, it could be the already described site on the vWFA domain or that speculated as being on the SP domain.

Formation of the soluble complexes between C2 and either CRIT-ed1 peptide or C4$\beta^{212–232}$ (the CRIT-ed1 domain peptide from C4) was found to be Mg^{2+} independent. Unlike for the C4b-C2 and C3b-factor B interactions (15), Mg^{2+} may not be required to allosterically stabilize the CRIT-ed1-C2 or C4$\beta^{212–232}$-C2 complexes. Furthermore, as these complexes were formed in the fluid phase this may remove the need for Mg^{2+}, as is the case in the formation of soluble convertases (39). Finally, we do not know whether the peptides interact with the vWFA domain of C2a, maybe involving the metal ion-dependent adhesion site motif. In the formation of a surface-bound C3 convertase this interaction normally requires Mg^{2+}; however, the peptides may interact with the SP domain, also speculated as a C4b binding site on C2 (7, 8), and which lacks a metal ion-dependent adhesion site motif.

In this study we have identified a 21-aa peptide C4$\beta^{212–232}$ with C2 binding and C inhibitory activity. The 10-aa N-terminal region (C4$\beta^{212–221}$) and the 11-aa C-terminal region (C4$\beta^{222–232}$) retain the C2 binding and C inhibitory activity of the 21-aa parent peptide C4$\beta^{212–232}$. Together with the C2 binding site on CRIT, H17EVKIKHFSPY, we have derived a consensus sequence [F/H]EVKX$_{4/5}$P and called it the CRIT-H17 motif. In the C4 protein, the sequence C4$\beta^{222–232}$ is preceded by a β-turn and ends with a β-turn, the latter induced in all species (see Fig. 6) by a proline residue but in chicken by a glycine, as predicted using the

FIGURE 8. Secondary structure predictions for terminal 13-aa residues of CRIT-ed1 and for human C4β(F212–Y232).

self-optimized prediction method (40) (Fig. 8). Within the context of the 75-kDa β-chain in the native C4, these β-turns may actually present at least one CRIT-H17 motif as a loop (also predicted as an extended strand) for binding. Interestingly, within CRIT-ed1 the CRIT-H17 motif occurs only once, although as with many other receptors CRIT may be found to function as a dimer, in which case two CRIT-H17 motifs might be presented for ligand binding. As in C4, at the N terminus of CRIT-H17 there is a predicted β-turn (Fig. 8). In CRIT, as in C4, the motif ends with another β-turn, although in CRIT this is also the position where the first transmembrane domain begins. This 11-aa motif contains four residues, F, Y, and twice K, which are normally found either at the binding interface of proteins (K or F) (41) or else at binding hotspots (W, Y, or R) (42).

Insertions or deletions of amino acid residues, which occur at particular locations within a protein family, are known as indels (43). An important feature of indels is that they are normally found at the protein surface and form parts of β-hairpins or loop coils, structures often involved in intermolecular interactions (44). In studies of the regions proximal to indels in the C3/4/5 family aimed at defining likely sites of interaction in C3 and C5 and by inference in C4, a region N-terminal to indel 4 in the β-chain of C5 I220GYKNFKNFEITIK gave 50% inhibition of both hemolytic and bactericidal activities at 10 and 100 μM, respectively. Residues proximal to amino acids 150–200 were speculated to be important as likely sites of protein-protein interaction in C3 or C5 (45). The peptide Y222IYNEKGLEVTIT in the β-chain of C3 similarly had an ICH_{50} of >300 μM. The CRIT-H17 motif in C4 lies at position F222–Y232 of the mature protein. This is the same region of the molecule and is proximally N-terminal to the equivalent position in C3 and C5, identified in the work of Low et al. (45) as being indel 4.

Although the CRIT-ed2 peptide failed to inhibit C activity in the hemolytic assay, this does not necessarily mean that neither this sequence nor the corresponding region identified within the C4 γ-chain represents a binding site, if for example the site is in fact dependent on a conformation that is presented only in the nondenatured protein.

Despite the evidence that the inhibiting CRIT-ed1 sequence binds C2, it was conceivable that this binding per se was not the only factor abrogating C activity in the in vitro hemolytic assay. For example, ed1-bound C2 might in fact still be able to bind to C4b, in which case we hypothesized that the inhibition of complement might be provided at least in part by the prevention of the C1s-mediated cleavage of C2. We found that C4$\beta^{212-232}$ actually had no effect on C1s-mediated cleavage of C2. However, CRIT-ed1 as a free peptide or when coupled to Sepharose beads did inhibit C2 cleavage by C1s. This inhibition provided by CRIT-ed1 could be due to its binding site on C2a being proximal to the C1s cleavage site between R223 (of C2b) and K224 (of C2a). The binding of CRIT-ed1, unlike that of C4$\beta^{212-232}$, to this site on C2 might induce a conformational change in the structure of C2, thereby preventing the R223–K224 cleavage site from being presented to C1s. Alternatively, the bound CRIT-ed1 but not the C4$\beta^{212-232}$ peptides may provide a steric interference that prevents C1s-mediated cleavage.

Others have shown that alterations in the C-terminal SCR3 module of C2b at the junction of C2b/C2a (by substitution with the corresponding factor B module) abrogated or diminished hemolytic activity and also made the C2 less susceptible to C1s cleavage (46). They proposed that alteration of such a region close to the short, flexible, interconnecting segment between C2a and C2b, containing the C1s cleavage site, was able to change the conformation of this site. Another possibility was that the SCR3 module of C2b actually contained a second binding site for C1s. In previous work it was shown (6) that the region D240–S244 in the N-terminal region of the vWFA domain of C2a, just 16 residues from the C1s cleavage site, at least forms part of the C4b binding site, although it was not speculated as forming a C1s binding site. We do not believe that the CRIT-ed1 domain in C4 (C4$\beta^{212-232}$) or *Schistosoma* CRIT-ed1 represent a C1s binding site or a second C1s cleavage site, as neither of these sequences was found to inhibit the C1s-mediated cleavage of C4. Whatever the mechanism of CRIT-ed1 inhibition of C1s cleavage, we can assume that the CRIT-C2 complex would be unable to form a CP C3-like convertase, because for this the native C2 bound to CRIT would need to be cleaved by C1s to yield CRIT-C2a.

In summary, the sequence homology of a C2-binding human parasite receptor, CRIT, with the human complement component C4 alerted us to the possibility that the corresponding sequence in C4 may represent a C2 binding site. As a result of the current studies we believe that F212–Y232 indeed represents a C2 binding site in the β-chain of C4, as do the C-terminal 11 aa, F222–Y232. Current structural studies of the CRIT-ed1 domain will enable us to firmly predict which residues are most exposed and most likely to be involved in protein-protein interactions. These residues in the homologous CRIT-ed1 domain of the C4 β-chain will make the best candidates for site-directed mutagenesis in studies to inhibit interaction with C2.

Acknowledgments

We thank Dr. R. B. Sim for providing C2 protein and Prof. T. Schirmer for helpful comments about the manuscript.

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