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CRIT peptide interacts with factor B and interferes with alternative pathway activation

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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) has 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 factor D-mediated cleavage of factor B (FB) was blocked by CRIT-H17. By ELISA and immunoblot, CRIT-H17 was shown to bind FB. CRIT-H17 had no decay activity on the C3bBb complex as compared to decay-accelerating factor. Binding of CRIT-H17 to FB did not interfere with the assembly of C3bB complex. In a haemolytic assay using C2-deficient serum, CRIT-H17 interfered with AP complement activation.

Keywords: Alternative pathway; Factor B; Complement C2 receptor inhibitor trispanning; C3 convertase; Haemolytic assay; Factor D

The complement system consisting of about 30 proteins 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 lectins, as well as several regulatory proteins to control complement activation. Each pathway is initiated by 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) 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, converging to form the C4bC2a C3 convertase. 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 against 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]. As a result of this spontaneous so-called "C3 tick-over," C3b is deposited on cell surfaces of microorganisms [5,6]. 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 [7]. The Ba fragment of FB is

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involved in the initial interaction with C3b [8,9] and both the von Willebrand factor-A (vWFA) domain and the serine protease (SP) domain of the Bb fragment interact with C3b [10,11]. 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 of generating the AP C3 convertase, C3bBb. However, the exact mechanism of assembling this bimolecular complex, C3bBb, is still under investigation even 50 years after the discovery of the AP [12].

Previously, we described a novel human complement CP inhibitor, complement C2 receptor inhibitor trispanning (CRIT), which is expressed on haemopoietic cells and in a wide range of tissues [13]. The first extracellular domain (ed1) is a binding site for 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 both in vitro [14] and in vivo [15,16]. 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 [17].

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 of C4b, C2, and C1s of the CP, respectively. We started the studies with the 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.

Materials and methods

Materials. FB and C3b proteins 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 [18]. 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 (HEVKIKHF SPY), H17S (scrambled peptide of H17) (EKFYHIHSKPY), H17-2 (HEVKIKHFSPYHEVKIKHFSPY), and human C4β chain peptides: Hu-C4β chain F^{212} (FEVKKYVLPN) and Hu-C4β chain F^{222} (FEV KITPGKPY) were described elsewhere [14]. 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 5× 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 of 100 mM EGTA, 35 ml of 200 mM $MgCl_2$, 104 ml GVB, and 311 ml of 5% (w/v) D-glucose and adjusted to pH 7.4.

Antibodies. The monoclonal anti-Bb antibody was purchased from Quidel (San Diego, CA). Polyclonal rabbit anti-CRIT antibody was derived as described previously [14,17]. Polyclonal anti-Bb antibody was produced in mouse ascetic fluid according to a method described elsewhere [19,20]. In brief, 5 μg FB, 1 μg FD, and 1 μg C3b were incubated at 37 °C for 1 h and then subjected to SDS/PAGE. 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 the Bb fragment was excised and emulsified in 200 μl PBS by passing 10 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.

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 conditions. Immunoblotting onto nitrocellulose membrane (Amersham Biosciences, Bucks, UK) was performed as described previously [14].

FB cleavage. FB was cleaved by incubating 500 ng FB, 200 ng FD, and 200 ng C3b at 37 °C for 1 h. The reaction was stopped by adding SDS/PAGE loading buffer. In some experiments, different amounts (10–100 μg) of either CRIT or C4β chain peptides were pre-incubated with FB for 30 min at room temperature (25 °C) before adding other components. After the incubation, the reaction mixtures were analyzed by SDS/PAGE under non-reducing conditions and the gel was stained with ProteoBlue staining solution.

FB-CRIT ligand blotting. FB (500 ng) was mixed with CRIT-H17 (1.5 μg) in PBS and incubated at room temperature 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 room temperature 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 FB-CRIT-H17 complexes were analyzed by SDS/PAGE under reducing conditions and probed with anti-CRIT antibody by Western blot. For the blocking experiment, FB (500 ng) was pre-incubated with polyclonal anti-Bb antibody (1:500 dilution) at room temperature for 30 min, and the ligand blot was carried out as described above.

Haemolytic assay. The inhibition of AP-based haemolysis was conducted using the standard method described in the literature [21,22]. In brief, Erab (2×10^8 cells/ml) were prepared in GVB–Mg^{2+}–EGTA buffer. Ten micromolar 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 room temperature for 30 min. After the pre-incubation, 50 μl of Erab was added and incubated at 37 °C for 30 min. A 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 haemolysis was determined by measuring the absorbance of the supernatant at 414 nm on a SpectraMax 190 microtiter plate reader (Molecular Devices, Sunnyvale, CA).

ELISA of CRIT-H17 with FB. The microtiter plate was coated with 55 nM FB, 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. One hundred microliters 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 room temperature, 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) microtiter wells were incubated with 50 ng FB and 5 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 [8,23]. 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 room temperature for 30 or 60 min. After the incubation, C3bBb–Ni^{2+} complexes were detected by ELISA employing monoclonal anti-Bb antibody (1:5000 dilution).

Assembly of C3bB complexes. The C3b-coated (500 ng) microtiter wells were incubated with 55 nM FB alone (used as a reference, in which 100% of C3bB complexes were formed) or with 55 nM FB and different amount of H17 (6.75–54 μM) or FH (31.25–250 nM) or CR1 (31.25–250 nM) at room temperature for 1 h. The ELISA was performed as described above and the C3bB complexes were detected by monoclonal anti-Bb antibody (1:5000 dilution).

Results

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

In previous studies, CRIT-H17 has been demonstrated to be an effective inhibitor of the CP [14]. The blockade of the CP was more efficient with CRIT-H17 than with CRIT-ed1, suggesting that it corresponds to the active sequence. Two sequences showing homologies with CRIT-H17 in the β chain of C4 have been identified previously. Peptides of 10- and 11-amino-acids, 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 (Fig. 1). In 2001, Laich et al. [24] 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 composed of identical amino-acid residues to CRIT-H17 but in a scrambled sequence (Fig. 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. The Bb fragment remains 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 inhibition was dose dependent as shown in Fig. 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 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 Fig. 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 regions in the C4β chain were capable of interfering with the formation of both the CP and AP C3 convertases.

Referring to Fig. 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 box with a dashed outline 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 [25,26]. 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.

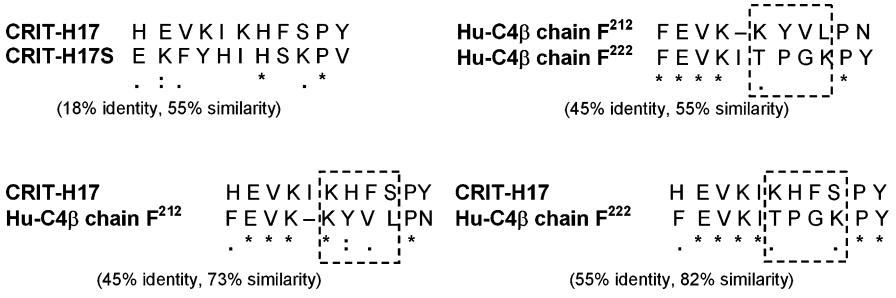

Fig. 1. Amino acid sequence alignments of CRIT-H17 and human C4β chain peptides. Alignments were made using the MAFFT program. Periods indicate similar residues and asterisks indicate identical residues. The box with a dashed outline shows the potential region responsible for blocking FD-mediated cleavage of FB.

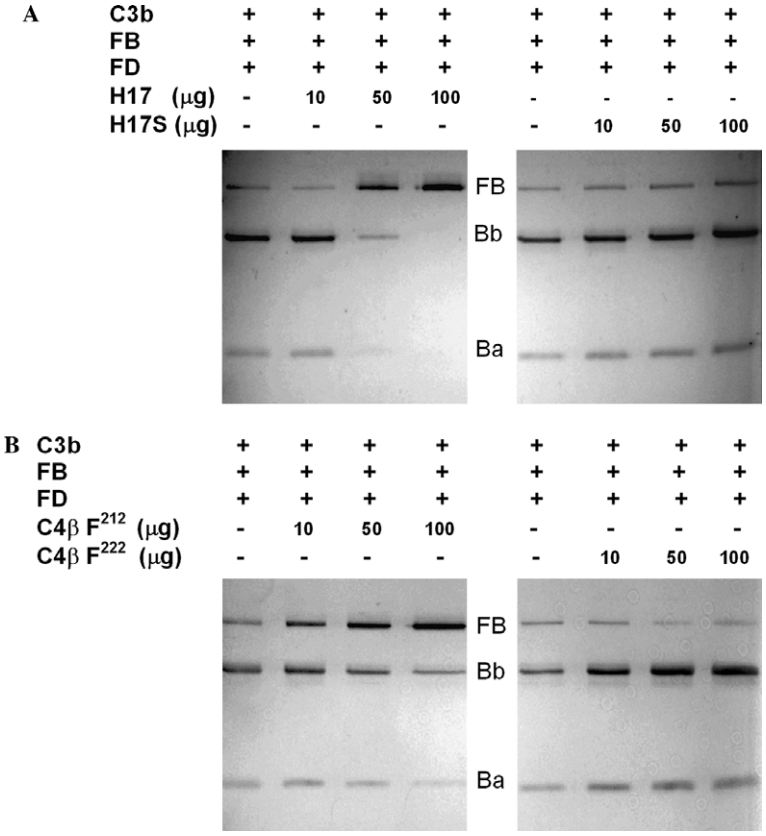

Fig. 2. CRIT-H17 and C4β F^{212} peptides but neither CRIT-H17S nor C4β F^{222} peptides inhibit the FD-mediated cleavage of FB. FB (500 ng) was pre-incubated with either CRIT or C4β peptide for 30 min at room temperature before adding FD (200 ng) and C3b (200 ng). The mixtures were then further incubated for 1 h 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 was analyzed by SDS/PAGE (10% gel) under non-reducing conditions, stained with ProteoBlue staining solution.

CRIT-H17 peptide interacts with FB

From the analogy to the CP, and since CRIT-H17 binds to C2 and has at least one binding site on the vWFA domain of C2, we predicted that CRIT-H17 would bind similarly to FB, through at least one binding site of the vWFA domain of FB. First, the binding was tested by sandwich ELISA, fixing FB on the plates, followed by incubation with CRIT-H17 and detection of the peptide bound to FB with the anti-CRIT anitbody. Fig. 3A shows that CRIT-H17 bound to FB in a dose-dependent manner, with no binding to BSA as a control, indicating specific binding. Next, to confirm this result, ligand blotting was performed using the cross-linking agent, DSS, and probed with the anti-CRIT antibody. As shown in Fig. 3B, CRIT-H17 bound to FB and this binding could be blocked by pre-incubation of FB with polyclonal anti-Bb antibody.

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 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 haemolytic 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 Fig. 4. In the presence of 10 μM peptides CRIT-H17 was the best inhibitor reducing the haemolysis by 22.1% whereas the CRIT-ed1 peptide was less potent, and CRIT-H17-2 had almost no inhibitory activity.

Effect of CRIT-H17 on the dissociation of C3bBb and on the assembly of C3bB complexes

The complement regulatory protein DAF accelerates the decay of the C3bBb convertase. Since CRIT-H17 bound to FB, we continued to test whether CRIT-H17 would have the same property of dissociating C3bBb convertase. The convertase was formed on a solid phase by binding first C3b, adding FB in the presence of $Ni^{2+}$and FD. After washing, buffer alone, CRIT-H17 (50 μg) or DAF (5 and 10 ng) was added and the decay of the C3bBb complexes was measured after 30 and 60 min. The results clearly showed that, unlike DAF, CRIT-H17 does not possess any decay activity resulting in the dissociation of C3bBb complexes (Fig. 5).

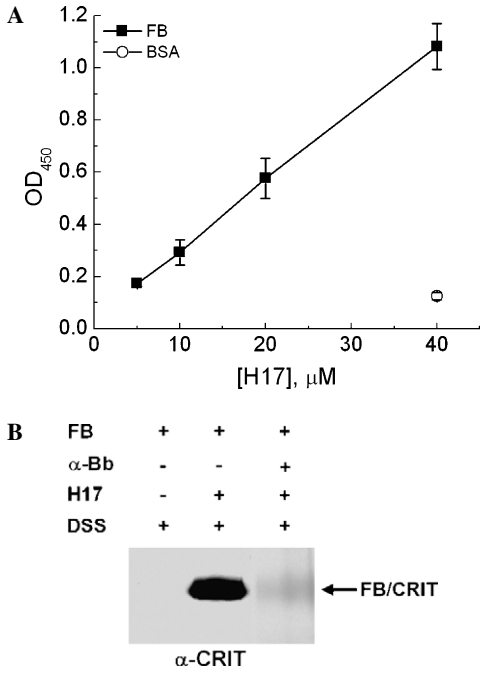

Fig. 3. ELISA and ligand blot showing that CRIT-H17 interacts with FB. (A) Fifty-five nanomolar FB was coated on the ELISA plate and incubated with increasing concentrations of CRIT-H17 (from 5 to 40 μM). BSA was used as control. The result showed that CRIT-H17 interacted with FB but not the control protein BSA. Results are means ± SD for three independent experiments, each with duplicate measurements. (B) The ligand blot showing that CRIT-H17 interacted with FB. CRIT-H17 (1.5 μg) was incubated with FB (500 ng). The resulting complexes were cross-linked with DSS. After cross-linking, the complexes were detected by immunoblotting and probing with polyclonal anti-CRIT antibody. The interaction between CRIT-H17 and FB could be blocked by the pre-incubation of FB with polyclonal anti-Bb antibody (1:500 dilution).

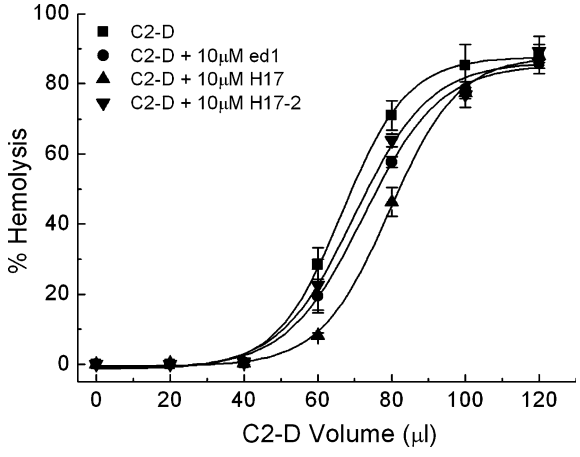

Fig. 4. Inhibition of the AP activation by CRIT peptides. Ten micromolar CRIT peptides were pre-incubated with C2-D for 30 min at room temperature before adding to the Erab. The CRIT peptides inhibited the AP C3 convertase formation by binding to FB and decreased the percentage of haemolysis. Results are means ± SD for three independent experiments, each with duplicate measurements.

Binding to FB without any interference on the dissociation of C3bBb, it would be interesting to further study the effect of CRIT-H17 on the assembly of C3bB. A competitive ELISA was performed on the C3b-coated microtiter plate. In Fig. 6A, it was clearly demonstrated that CRIT-H17, unlike FH and CR1, did not interfere with the assembly of C3bB. FH and CR1 bound to C3b which blocked the binding of FB or dissociated the formed C3bB complexes as DAF. Thus, in the presence of FH or CR1, the amount of assembled C3bB was dropped to approximately 5% at a concentration of 250 nM (Fig. 6B).

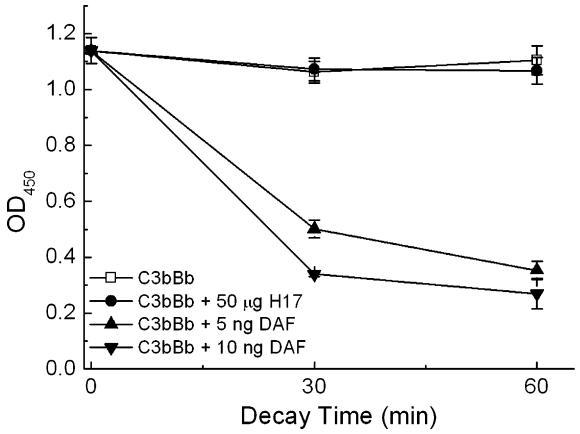

Fig. 5. Effect of CRIT-H17 on Ni^{2+} dependent C3bBb complexes. C3bBb complexes were formed by incubating 50 ng FB and 5 ng FD in the presence of 5 mM Ni^{2+} for 2 h at 37 °C in C3b-coated microtiter wells. After the formation of C3bBb complexes, the wells were washed five times with reaction buffer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

Discussion

CRIT is a three transmembrane receptor first discovered on the *Schistosoma* parasite surface and later described in cod, rat, and human [13]. It acts as a decoy receptor for C2 to protect cells from the assembly of C4bC2 and the following C2 cleavage, thus preventing complement attack by the CP [14]. CRIT-H17 proved to be the region interacting with C2 or more specifically with the C2 vWFA domain [17]. Furthermore, the CRIT peptide is as a potent inhibitor of the CP in vitro [14] and in vivo [15,16].

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 CRIT-H17 might have inhibitory effects on the formation of the C3bBb convertase as well. Our major findings were that CRIT-H17 bound to FB which inhibited the FD-mediated cleavage of FB and reduced AP function. The binding of FB-CRIT-H17 could be blocked by the pre-incubation with polyclonal anti-Bb antibody. This indicated that CRIT-H17 might bind to the Bb fragment of FB, most likely within the vWFA domain. Binding of CRIT-H17 to FB neither interfered with the dissociation of C3bBb nor the assembly of C3bB. These results implied that CRIT-H17 and C3b bound to different regions on FB.

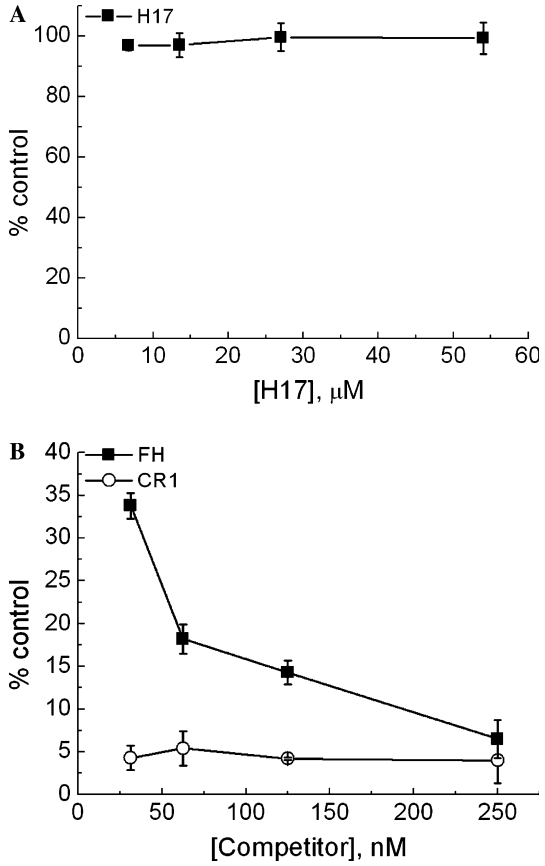

Fig. 6. Effect of CRIT-H17 on the assembly of C3bB complexes. FB alone (used as reference) or FB with CRIT or FH or CR1 was incubated in C3b-coated microtiter wells at room temperature. (A) CRIT-H17 showed no interference on the assembly of C3bB. (B) FH and CR1 competed with FB for the binding of C3b or dissociated the formed C3bB and thus, the amount of C3bB formed decreased to approximately 5% at the highest concentration tested (250 nM). Results are means ± SD for three independent experiments, each with duplicate measurements.

However, further experiments are needed to confirm all these predictions.

CRIT-H17 is known to bind the vWFA domain of C2 and inhibit its cleavage by C1s probably because the cleavage site is no longer exposed in a correct way. We believe that CRIT-H17 bound to FB and blocked the FD-mediated cleavage in a similar way. Of particular interest was that a peptide corresponding to the homologue of CRIT-H17 on the C4β chain partially blocked FB cleavage as well, although less effectively than CRIT-H17. This finding strengthened the hypothesis, that there are strong analogies between the mechanisms leading to the formation of both CP and AP C3 convertases. These 4-amino-acids (K, H, F, and S) in CRIT-H17 peptide, highlighted in Fig. 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 [14]. However, only C4β chain F^{212} peptide was able to slightly inhibit the FD-mediated cleavage of FB. Any of these 4-amino-acids 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, these also representing the major differences between these two C4β chain peptides. We postulate that the 3-amino-acid residues E, V, and K in CRIT-H17 and C4β chain peptides are responsible for C2a binding and the 4-amino-acid residues K, H, F, and S in CRIT-H17 peptide are probably responsible for the FB 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 could eventually create a peptide, which is more specific for FB, and a more potent inhibitor in the AP activation. Referring to the sequence alignment between CRIT-H17 and C4β chain F^{222} peptides in Fig. 1, one interesting aspect is the additional proline residue in the C4β chain F^{222} peptide, which may induce a conformational change of the peptide, *cis*/*trans* isomerization [25,26]. If we make the amino-acid change H → P in CRIT-H17 peptide, it will be interesting to test whether this modified peptide will lose its ability to inhibit the FD-mediated cleavage of FB.

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. [27] proposed a molecular model and mechanism of action of DAF. In this model, DAF and the 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, the Bb fragment is destabilized and released. CRIT-H17 did not modify the decay of the C3bBb convertase, despite its binding to FB, probably the Bb fragment. Thus, the mechanism by which CRIT-H17 inhibits the AP in serum does not include the decay of the convertase and indicates interaction with FB would not induce any conformational change or destabilize the C3bBb convertase.

To study CRIT-H17–FB/Bb 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 likely to be involved in these protein–protein interactions and further understand the regulatory role of CRIT-H17 in AP activation. Dissecting out the interaction region of CRIT-H17-FB/Bb, the vWFA domain of Bb will be the best place to start with. Previously, we have shown a CRIT binding site within C2 vWFA domain [17]. Because of the sequence homology between C2 vWFA and FB vWFA domains, we predict CRIT-H17 peptide to interact with the Bb fragment of FB via the vWFA domain. In addition, binding of CRIT-H17 to FB could inhibit its cleavage by FD. Thus, CRIT-H17 peptide should bind to the region near to the FD cleavage site. Expressing the recombinant vWFA domain of Bb or the whole Bb fragment can be used for functional studies, or complexed with CRIT-H17 for structural studies by X-ray crystallography.

In summary, the data present evidence that CRIT-H17 peptide, a proven inhibitor of the CP, is capable to interact with FB and interfere with the AP. The AP has been proven to be important in various diseases and developing a specific AP inhibitor will be therapeutically important for 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, but the most important issue will be the opportunity to modify and create a more specific and potent AP inhibitor.

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