

A Discontinuous Factor H Binding Site in the Third Component of Complement as Delineated by Synthetic Peptides*

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Factor H, a very important regulator of alternative pathway activation, exerts its effects by binding to the third component complement, C3. In this study we present evidence that factor H reacts with at least two sites in the third component of complement (C3), and we have mapped one of these sites within the C3d fragment of C3. By using direct binding assays of an anti-human H anti-idiotypic antibody ($\alpha\alpha$ H) and of H to C3 fragments, it was shown that both bound to the C3b and C3d (but not to C3c) fragments of C3. Cleavage of C3d by CNBr generated two major fragments with M_r values of 12,500 (residues 997-1107) and 8,600 (residues 1178-1252). Binding studies with these two fragments showed that only the M_r 8,600 fragment bound to both H and $\alpha\alpha$ H. Several synthetic peptides (A58, 1192-1249; P28, 1187-1214; P16, 1194-1209; P14, 1201-1214; B17, 1206-1222; J28, 1222-1249; and J16, 1234-1249) were synthesized according to the primary sequence of the M_r 8,600 fragment. Based on the differential binding of these synthetic peptides to H, their inhibitory effect on H binding to C3b or C3d, and their effect on H cofactor activity, we mapped the H binding site in C3 to a discontinuous site spanning residues 1187-1249 of the C3 sequence. By studying the inhibition of H binding to C3b or C3d by the different synthetic peptides, we also present evidence that a second binding site in C3b for H exists.

NH₂-terminal fragment and an M_r 46,000 COOH-terminal fragment. Then a second Arg-Ser bond (residue 1298-1299) near the NH₂ terminus of the M_r 46,000 fragment is cleaved, which liberates an M_r 2,000 fragment. Subsequently, a third factor I-mediated cleavage occurs between residues 932 and 933 (Arg-Glu), which generates the M_r 27,000 NH₂-terminal α fragment and an M_r 39,000 (C3dg) fragment (14). This third cleavage separates C3c from C3dg.

Several additional biological functions have been ascribed to H. Cell surface receptors for H have been found to be present on B-lymphocytes, neutrophils, and monocytes (15-17). Among the cellular responses described upon the binding of H to its receptor are: (a) release of factor I from lymphocytes (15), (b) respiratory burst of monocytes (17), (c) release of prostaglandin E and interleukin 1 by monocytes (18, 19), and (d) inhibition of B cell differentiation (20).

Transmission electron microscopy studies have shown that H is an elongated molecule $280 \times 30 \text{ \AA}$ with one globular end (21). Enzymatic degradation of H and use of monoclonal anti-H antibodies have localized the C3b binding site in H within an M_r 38,000 NH₂-terminal fragment (22, 23). Studies on H binding to surface-bound C3b have shown that C3b is heterogeneous with respect to H binding, having low and high affinity sites (24, 25).

In this study, using an anti-idiotypic anti-H antibody, CNBr fragments of C3, and synthetic peptides, we identified one of the H binding sites in C3 and found it to be a discontinuous site contained within residues 1187-1249 of the C3 sequence. We also present evidence for an additional H binding site in C3b.

EXPERIMENTAL PROCEDURES

Materials—Na¹²⁵I (carrier-free) was obtained from Amersham Corp. Trypsin and CNBr were purchased from Sigma. Chemicals for PAGE were from Bio-Rad. All chemicals for automated sequencing and synthesis were from Applied Biosystems. Acetonitrile, high performance liquid chromatography grade, was from J. T. Baker Chemical Co. Hydrogen fluoride was obtained from Merck.

Complement Components—Human C3, I, and H were prepared as previously described (12, 15). The C3 fragments C3b, C3c, and C3d were generated by trypsin digestion and purified by fast protein liquid chromatography using a Mono Q column (Pharmacia LKB Biotechnology Inc.) (26). The CNBr fragments of C3d were prepared as previously described (27). Protein iodination was performed as previously described (28).

Antibodies—The anti-idiotypic antibody to human H ($\alpha\alpha$ H) was prepared as previously described (29).

Synthetic Peptides—Seven peptides (see Fig. 3) were synthesized by use of either a BioSearch or an Applied Biosystems peptide synthesizer according to the Merrifield solid-phase method on a 4-methylbenzhydrylamine resin (30, 31). After cleavage with HF (HF/anisole/peptide resin; 10 ml:1 ml:1 g), the resin was washed with ether, and the peptide was extracted with 10% acetic acid. The peptides were purified by high performance liquid chromatography on a preparative C₁₈ reversed-phase column (Vydac) and analyzed,

Factor H¹ is a member of the family of C3b/C4b binding proteins which are encoded by tightly linked genes and which contain repeating homology units of 60 residues (1-3). Other members of this family are the C3b receptor (CR1) (4), C3d receptor (CR2) (5), C4 binding protein (C4bp) (6), factor B (B) (7), C2 (8), and decay accelerating factor (9, 10). All of these proteins share certain functional properties, and H, CR1, and CR2 serve as cofactors for the factor I-mediated degradation of C3b (11-13). The degradation of C3 by factor I proceeds in the following way. First, the α chain is cleaved at Arg-Ser (residues 1281-1282), generating an M_r 67,000

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¹ The abbreviations and trivial names used are: H, complement factor H; $\alpha\alpha$ H, anti-human H anti-idiotypic antibody; C3, third component of complement; CR1, CR2, and CR3, receptors for the C3b, C3d, and iC3b fragments of C3; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

after hydrolysis, in a Beckman or an Applied Biosystems automated amino acid analyzer. The amino acid sequence of the peptides was confirmed by Edman degradation (32) using a gas-phase Applied Biosystems 470 Sequencer connected on line with a model 120 PTH analyzer.

Binding of α H Antibody to C3 Fragments—The binding of α H antibody to C3 fragments was measured by an ELISA. Microtiter plates were first coated overnight at 4 °C with 50 μ l of C3 fragments (10 μ g/ml phosphate-buffered saline). After saturation with 1% bovine serum albumin in phosphate-buffered saline, wells were incubated with serially diluted α H antibody for 30 min at 22 °C. The binding of α H was detected with peroxidase anti-Ig antibody (Cappel).

Ligand-H Binding Studies—Two different assays were used: (i) The binding of H to C3 fragments was measured by an ELISA as previously described (22). Alternatively, the C3 fragments or synthetic peptides fixed to microtiter plates were reacted with 125 I-H for 30 min at 22 °C. The bound H was detected by cutting out the microtiter wells and quantitating 125 I in a γ -counter. To determine the effect of synthetic peptides on the H binding to C3b or C3d, the 125 I-H was preincubated with the different synthetic peptides (before addition to plates). (ii) To test the inhibition of the H cofactor activity by synthetic peptides, 1 pmol of 125 I-C3b was incubated with factors I (0.4 pmol) and H (4.25 pmol) in 50 μ l of phosphate-buffered saline in the presence or absence of 22.5 nmol of synthetic peptide. After 3 h at 37 °C the reaction was terminated by adding SDS-containing sample buffer and the cleavage products were analyzed by SDS-PAGE followed by autoradiography.

SDS-PAGE—Electrophoresis in 9% polyacrylamide gels was performed as previously described by Laemmli (33).

RESULTS

Localization of the H Binding Site Within the C3d Domain

The C3 fragments, C3b, C3c, and C3d were tested by an ELISA for their ability to bind H or α H antibody. This analysis revealed that both H and α H bound to C3b and C3d but not to C3c (Fig. 1). Similar results for the binding of H to C3b were obtained through the use of 125 I-H (data not shown). To further localize the H binding site in C3d, we analyzed CNBr fragments of C3d for their reactivity with H or α H. Fig. 2 shows that only the M_r 8600 fragment bound to both H and α H. The M_r 8600 fragment had earlier been identified to represent residues 1178–1252 of the C3 sequence (27).

Conformation Dependence of the H Binding Site in C3d

Since the H binding site and α H antigenic site are both located in the M_r 8600 fragment, several peptides, constructed according to the primary sequence of C3 (34), were synthesized and used to further define the H binding site. The amino acid sequences of these peptides are shown in Fig. 3. Direct

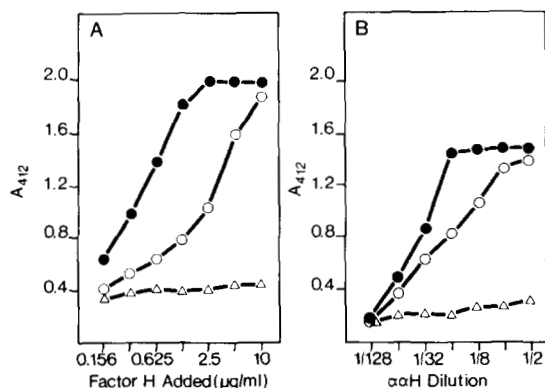


FIG. 1. **Binding of H and α H to C3 fragments.** 50 μ l of various dilutions of H (0.156–10 μ g/ml) (A) or α H (B) were applied to ELISA plates precoated with either C3b (●), C3c (Δ), or C3d (○). The bound H was detected with a goat anti-H antibody followed by a peroxidase-labeled rabbit anti-goat Ig. The bound α H was detected with a peroxidase-labeled goat anti-rabbit Ig.

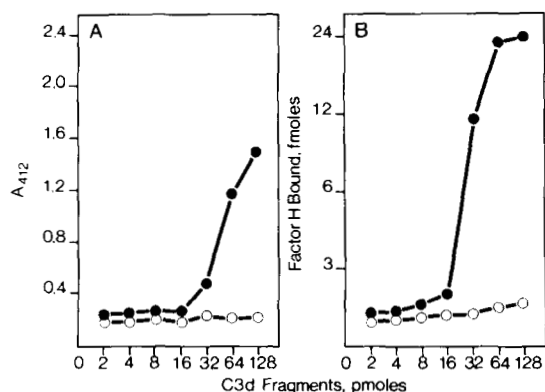


FIG. 2. **Binding of H and α H to CNBr C3d fragments.** Various amounts (2–128 pmol) of the M_r 8,600 (●) or 12,500 (○) CNBr fragments of C3d were fixed to microtiter plates. α H binding (A) to the fixed fragments was analyzed as in Fig. 1. To test for H binding (B) plates were saturated with 1% bovine serum albumin and then 5 pmol of 125 I-H were added. Bound 125 I-H was determined by cutting out each microtiter well and measuring its radioactivity.

binding of 125 I-H to synthetic peptides fixed to microtiter plates showed that 125 I-H bound to A58, J28, and J16 but not to P28, P16, P14, or B17 (Fig. 4). When the different synthetic peptides were tested for inhibition of H binding to C3b or C3d fixed to microtiter plates, it was found that P28 completely inhibited the binding of H to C3d but only partially inhibited the binding to C3b (Fig. 5). In contrast, the J28 and J16 peptides, which bind H when fixed to the plates, partially inhibited its binding to C3d but had no influence on its binding to C3b. The peptide J16 behaved similar to J28 (data not shown). The inhibitory effect of A58 was not tested due to its low solubility. These results suggest that the H binding site is discontinuous and comprises residues present in both P28 and J16. In addition, the partial inhibition of H binding to C3b by P28 and complete inhibition in case of the binding to C3d suggests that H binds to C3b via a second binding site. The observed differences between microtiter plate-fixed and fluid-phase P28, J28, and J16 peptides in their ability to bind or inhibit H binding to C3 fragments may be due to conformational and affinity differences between these two forms of the peptides.

Inhibition of H Cofactor Activity by Synthetic Peptides—To test the effect of peptides P28 and J16 on the cofactor activity of H, 125 I-labeled C3b was treated with H and I in the presence of these peptides (Fig. 6). Treatment of C3b (track 1) with H and I caused cleavage of the α chain into M_r 67,000, 46,000, and 43,000 fragments (track 5) and this cleavage pattern was observed when P16 was present (track 2). When C3b was treated in the presence of peptides P28 (track 3) or J16 (track 4), a complete inhibition of C3b cleavage was observed. Thus, both peptides P28 and J16 inhibit the cleavage of C3b by I at positions 1281–1282 and 1298–1299. The observed differences between peptide J28 and J16 in inhibiting H cofactor activity but not H binding to fixed C3b may be explained by the fact that H has a higher affinity for fixed rather than fluid-phase C3b.

DISCUSSION

The degradation of C3b by factor I is facilitated by several serum proteins and cell surface molecules. Among them are H (11), CR1 (35), CR2, (13) and gp45-70 (36). Since the degradation of C3b proceeds in three steps and requires the use of different cofactors, two questions are raised: 1) do these cofactor molecules bind to multiple sites in C3 in order for I to make three different cleavages, and 2) do these cofactor

PEPTIDE	C3 RESIDUE	SEQUENCE
A58	1192-1249	AKDKNRWEDPGKQLYNVEATSYALLALLQLKDFDFVPPVVRWLNQRYGGGGYSTQA
P28	1187-1214	KFLTTAKDKNRWEDPGKQLYNVEATSYA
P16	1194-1209	DKNRWEDPGKQLYNVE
P14	1201-1214	PGKQLYNVEATSYA
J28	1222-1249	KDFDFVPPVVRWLNQRYGGGGYSTQA
J16	1234-1249	LNEQRYGGGGYSTQA
B17	1206-1222	YNVEATSYALLALLQLK

FIG. 3. Amino acid sequences of the synthetic peptides used in this study.

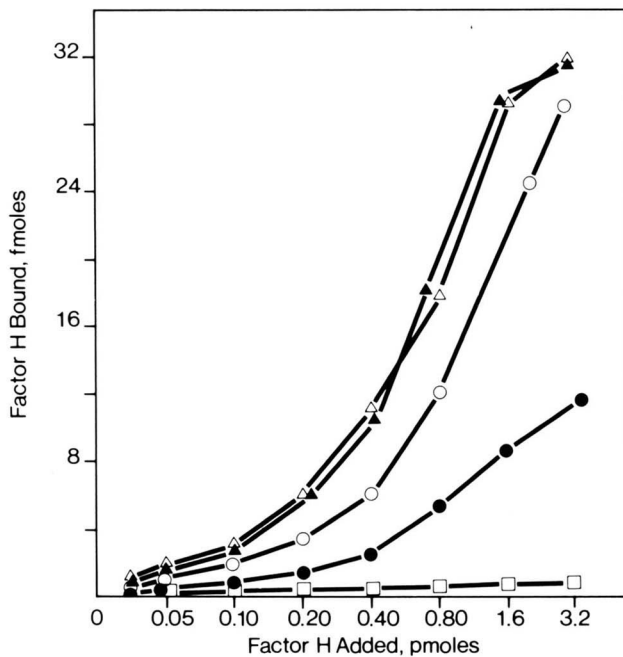


FIG. 4. Binding of H synthetic peptides to C3d. C3d (\blacktriangle), A58 (\triangle), J28 (\circ), J16 (\bullet), or P28, P16, P16 (\square) fixed to microtiter plates were incubated with serially diluted ^{125}I -H (0.1–12.8 pmol) for 30 min at 22 °C. Bound H was quantitated as in Fig. 2.

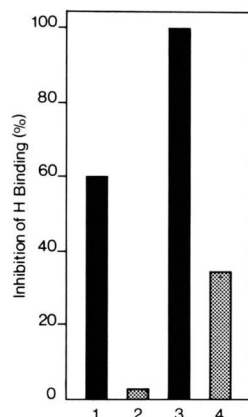


FIG. 5. Inhibition of H binding to C3b or C3d by synthetic peptides. 400 nmol of P28 (bars 1 and 3) or J28 (bars 2 and 4) were incubated with 12.8 pmol of ^{125}I -H (15 min at 22 °C) and then allowed to react with either C3b (bars 1 and 2) or C3d (bars 3 and 4) fixed to microtiter plates.

molecules share the same binding sites in C3. Experiments from different laboratories suggest that H binds to C3 via multiple binding sites. Monoclonal antibodies (37, 38) and two different anti-H anti-idiotypic antibodies (29, 39), with

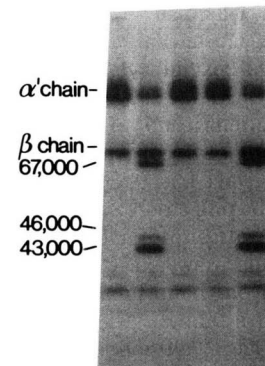


FIG. 6. Effect of the synthetic peptides on the H cofactor activity. 20 nM ^{125}I -C3b (track 1) was incubated for 3 h at 37 °C with I (8 nM) and H (85 nM) in the presence of 450 μM of P28 (track 2), J16 (track 3), or P16 (track 4). After incubation the mixtures were treated with SDS plus 2-mercaptoethanol and then analyzed by SDS-PAGE on a 9% gel and autoradiography.

specificities for either C3c or C3d, inhibited binding of H to C3b. The anti-idiotypic antibodies also mimic H in its cofactor activity and one displaces factor B from the C3bBb convertase. Binding studies of H to surface-bound C3b have shown a molar ratio of H to C3b of 0.32, in the case of zymosan-bound C3b (24), and 0.5 with erythrocyte-bound C3b (40), which may suggest multiple interaction sites in H for C3b.

In this study we have localized one of the H binding sites in C3b and present evidence that H interacts with at least two sites on the C3b molecule. The anti-H anti-idiotypic antibody and direct binding assays of H to C3 fragments were utilized to show that H binds to the M_r 8600 CNBr fragment of C3d which spans residues 1178–1252 of the C3 sequence. Inhibition of H binding to fixed C3b or C3d by fluid-phase C3b or C3d has shown that only the C3b is inhibitory (data not shown). This suggested that the H site in C3d is masked and is exposed upon its fixation to microtiter plates. To further localize the H binding site in C3d, several synthetic peptides were synthesized according to the primary sequence of the M_r 8600 fragment. Based on the reactivity of these peptides with H and their inhibitory effect on H binding to C3b or C3d, it is suggested that the H site in C3d is a discontinuous one and comprises amino acids present in both P28 and J16 peptides. This was confirmed by the inhibition of H cofactor activity, where both P28 and J16 inhibited the cleavage of C3b to iC3b. The inhibition of C3b cleavage by P28 or J16 peptides at both positions 1281–1282 and 1298–

1299 (Fig. 6) suggests that binding of H to C3b via the C3d site changes the conformation of C3b in a such a way that I can cleave C3b at both positions. Alternatively, since the I-mediated cleavage at positions 1281-1282 precedes that at positions 1298-1299 it may suggest that the C3d site is involved only in the cleavage at position 1281-1282. The binding of H to C3b via a second site is suggested by the ability of the P28 peptide to partially inhibit H binding to C3b and completely inhibit binding to C3d. The presence of two binding sites in C3 is in agreement with the inhibition of H binding to C3b by either anti-C3c or anti-C3d polyclonal antibodies (data not shown), as well as by monoclonal antibodies with specificities for either C3c or C3d (37, 38).

Previous studies have demonstrated that the area spanning residues 1205-1214 of the C3 sequence is involved in the binding of C3d to CR2 (27). In view of recent results showing that CR2 acts as a cofactor in the cleavage of C3b to C3c and C3dg (13) and since the binding sites on C3d for CR2 (residues 1205-1214) and H (residues 1187-1249) both lie within residues 1187-1249 of the C3 sequence, we suggest that, upon binding to C3b, H and CR2 induce similar conformational changes, thereby exposing the factor I cleavage sites. However, distinct differences exist in the cofactor activity of these two molecules and in their binding affinities for different fragments of C3. CR2, in contrast to H, has low affinity for C3b and high affinity for iC3b (41), which explains why CR2, as opposed to H, is more efficient in cleaving iC3b to C3c and C3dg. In view of our results it appears that the C3 segment spanning residues 1187-1249 in C3b is not fully accessible to CR2 but is accessible to H. Upon cleavage of C3b to iC3b, the conformation of C3 in this segment changes, thus leading to the preferential binding of CR2 as well as the binding of H to its second binding site. However, this does not exclude the possibility that H binds to C3b via the two binding sites simultaneously.

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