Segment Spanning Residues 727–768 of the Complement C3 Sequence Contains a Neoantigenic Site and Accommodates the Binding of CR1, Factor H, and Factor B

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Received June 12, 1991; Revised Manuscript Received October 24, 1991

ABSTRACT: CR1, CR2, DAF, MCP, factor H, C4bp, factor B, and C3 are members of a family of structurally related molecules, the majority of which belong to the complement system. Several of these molecules also share functional features such as cofactor and decay/dissociation activity and compete with one another in binding to C3b. Since factor H appears to bind to multiple sites in C3, we investigated the relationship between the factor H- and CR1-binding sites in C3b. Factor H binding to C3b is inhibited by either the C3c or C3d fragments, and addition of both fragments together augments this inhibition. One monoclonal anti-C3c antibody, anti-C3-9, which recognizes a neoantigenic epitope expressed upon cleavage to C3 to C3b, inhibited both factor H and CR1 binding to EC3b cells. This monoclonal antibody (MoAb) also inhibited factor B binding to EC3b. Two observations further supported our hypothesis that these molecules bind to proximal sites in C3b. First, a synthetic peptide spanning this region of C3b (C3b727-768) inhibited factor H binding. Second, antibodies raised against this peptide inhibited binding to CR1, factor B to C3b. These data show that H binds to at least two sites in C3b: the site in the C3c fragment is within the identified CR1-binding domain while the site in the C3d fragment surrounds the CR2-binding site. Furthermore, the inhibition of H, CR1, and B binding to C3b by a single MoAb (anti-C3-9) while other MoAbs differentially inhibit the binding of these proteins to C3b suggests that (1) multiple sites exist in the C3 molecule for binding these C3b-binding proteins, (2) at least some of the sites are proximal or share structural elements, and (3) these features may explain observed affinity and specificity differences exhibited by these C3b-binding proteins.

The binding of numerous plasma and membrane proteins to the degraded products of C3 account for the molecule’s ability to mediate a variety of biological responses [for a review, see Lambris (1988) and Becherer et al. (1989)]. Fluid-phase and surface-bound fragments of C3 are generated during complement activation, both of which can bind to other complement components and the numerous regulatory molecules of the complement system. In addition, upon the differential binding of these fragments to their respective complement receptors, a variety of responses are elicited, with the fluid-phase fragments capable of migrating within their local environment and the surface-bound fragments capable of forming a bridge between the receptor-bearing cell and target. In addition, several pathways have been described to interact with C3, possibly as a means to escape complement-mediated neutralization, via proteins on their surface. To understand the numerous responses mediated by the degraded products of C3, a great deal of effort has been placed on understanding the structural features of C3 responsible for its multifunctionality.

C3 has been described to bind over 20 proteins found in serum or on the cell surface, and many of these proteins belong to a superfamily of structurally related molecules. Within the complement system, this family consists of the membrane proteins CR1 (Klickstein et al., 1987), CR2 (Weiss et al., 1986; Moore et al., 1987), DAF (Caras et al., 1987; Medof et al., 1987), and MCP (Lubin et al., 1988), and of the serum proteins factors B (Morely & Campbell, 1984) and H (Kristensen et al., 1986; Ripoche et al., 1988), C2 (Bentley, 1986), and C4bp (Chung et al., 1985). This group of proteins shares sequence homology and a common ability to interact with C3b or C3(c/d) (Reid et al., 1986; Hollar et al., 1986; Pauzen & Aeheim, 1989). These proteins all contain internal repeating units of approximately 60 amino acids that are characterized by the conservation of approximately 10–15 residues within each repeating unit. The repeating units are contiguous, starting at the N-terminus of the molecule, and it has been predicted that a series of such structures would form a semirigid, extended molecule that, in the case of surface receptors, could extend out from the membrane. Several of these molecules also share functional similarities in that they serve as cofactors for the factor I-mediated degradation of C3b or C4b (CR1, CR2, MCP, factor II, C4bp) or that they can accelerate the decay of the C3 convertases (CR1, DAF, C4bp, factor H). Other proteins that possess these repeating units but do not bind C3b or C4b are CR1 and C1s and the noncomplement proteins β2-glycoprotein 1, IL-2 receptor, and factor XII.

Due to these structural and functional similarities, to the ability of several of these molecules to compete for binding to C3b, and to previous reports of monoclonal antibodies (MoAbs) specific for different domains of C3 selectively inhibiting one or several of C3b’s interactions with these proteins (Alszenz et al., 1990), we hypothesized that similar or adjacent sites in C3 are recognized by the members of this family of proteins.
proteins. This was further supported by the recent observation that segments in C3 identified by their binding of CR1 and CR2 show amino acid similarity (Becherer et al., 1990; Esparza et al., 1991). In this study, we demonstrate that a monoclonal antibody and an antipeptide antibody inhibit the binding of CR1, H, and factor B to C3b, that the epitopes recognized by the antipeptide antibody are associated with the CR1-binding domain in the α-chain, and that a synthetic peptide previously shown to inhibit CR1 binding to C3b also inhibits factor H.

**EXPERIMENTAL PROCEDURES**

**Materials.** Trypsin and soybean trypsin inhibitor were purchased from Worthington. Porcine pancreatic elastase was obtained from Serva and concanavalin A from Vector Laboratories. Polyvinylidene difluoride (PVDF) membranes were from Millipore.

**Antibodies.** The hybridoma cell line producing MoAb 543 (anti-CR1, IgG1) was purchased from American Type Tissue Culture, and the IgG from ascites fluid was purified on a protein A Sepharose column. The anti-C3 MoAb BRL was purchased from Bethesda Research Laboratories. Anti-C3d MoAbs III-1 and 311 were provided by Dr. J. Tamerius (Cytotech). The MoAb 133H11 was produced by standard procedures (Kearney, 1984), and the Ig fraction from ascites fluid was purified by protein A affinity chromatography and found to react with the C3c fragment of C3. The MoAb anti-C3g-9 was produced and characterized previously (Hack et al., 1988). Peroxidase conjugated goat anti-mouse and goat anti-rabbit Ig were purchased from Bio-Rad. Antibodies against synthetic peptides were made in rabbits by subcutaneous injections of the synthetic peptides coupled to keyhole limpet hemocyanin by glutaraldehyde (Briand et al., 1985). The rabbit immunoglobulin was fractionated from other serum proteins by ammonium sulfate. The pellet was resuspended, dialyzed against PBS, pH 7.2, and the volume was adjusted to equal the starting volume of serum.

**Purification of Antipeptide Antibodies by Affinity Chromatography.** Synthetic peptides or C3b were coupled to CNBr-activated Sepharose (Pharmacia) in order to affinity purify the antipeptide antibodies generated by the above procedure. Coupling of ligand to CNBr-activated Sepharose was performed according to the manufacturer’s instructions. The affinity matrix was equilibrated in PBS and then incubated with the (NH4)2SO4, serum fraction, for 1-4 h at 4 °C. After the unbound protein was washed away with PBS, the adsorbed antibody was eluted with 0.1 M glycine/HCl, pH 2.5. The pH of the eluted fractions was immediately adjusted to pH 8.0 by the addition of 1.0 M Tris-HCl, pH 8.0. The antibody-containing fractions were pooled and dialyzed against PBS.

**Binding of Anti-C3**-**72-768 Antibody to C3 Fragments.** C3 fragments (8 μg/ml; 50 μL/well) were coated to the microtiter wells. After saturation with 1% bovine serum albumin in PBS, pH 7.2, wells were incubated with serially diluted anti-C372-768 antibody (affinity purified on C3b-Sepharose) for 30 min at 22 °C. The binding of anti-C372-768 was detected with peroxidase-conjugated goat anti-rabbit antibody. All other conditions and steps were carried out as described previously (Becherer & Lambris, 1988).

**Cells.** Tonsils were obtained from Bezirkspital, Rheinfelden, Switzerland. Cells were isolated from homogenized tissue and washed five times with PBS and then lysed with freezing buffer (1% Nonidet P-40 in PBS containing 5 mM ethylenediaminetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin, and 2 mM diisopropyl fluorophosphate) for 1 h at 4 °C at a concentration of 5 × 10^5 cells/mL. Insoluble material was removed by centrifugation, and the supernatants were stored at −70 °C prior to use.

**Synthetic Peptides.** Peptides representing particular stretches of amino acid residues of the C3 sequence were synthesized using an Applied Biosystems 430A synthesizer by the standard solid-phase procedure of Merrifield (Merrifield, 1963; Stewart et al., 1976). The synthesis was carried out on a 4-methylbenzhydrolamine resin with tert-butyloxycarbonyl protected amino acids, dicyclohexylcarbodiimide coupling, and trifluoroacetic acid (40%) for deprotection. The peptides were cleaved from the resin with anhydrous hydrogen fluoride in the presence of anisole as a scavenger (Becherer & Lambris, 1988). They were then washed with cold ether, and extracted with 10% acetic acid and lyophilized. Crude peptides were purified by gel filtration on a Sephadex G-15 column equilibrated with 4% acetic acid followed by reverse-phase high-performance liquid chromatography (HPLC) on a C-18 column (Vydac) using a 10–80% acetonitrile gradient containing 0.1% TFA. Purity and composition of the peptides were confirmed by thin-layer chromatography, reverse-phase high-performance liquid chromatography, and amino acid analysis using an Applied Biosystems 402A derivatizer with an on-line 130A analyzer. All peptides were reconstituted and dialyzed in 1000 M, cut-off dialysis membranes (Spectrum) against PBS, pH 7.2, before use. The synthetic peptides C372-768 (SNLDEEIAEENIVSRSEFPESWLNVEDLKEPPKNGISTKL), C371-724 (KFLTTAKDKNRWEDPKQLYNVEATSYA), C380-82 (VYHHFISDGVRKSLKTSPPGRIMKNTVAYR), and C3452-453 (GVRYSISKYELDKAFSDNRVTNLHEHLYDSVHSEDD) have been used previously and are described elsewhere (Becherer & Lambris, 1988; Lambris et al., 1985).

**Preparation of C3 Fragments and Other Component Components.** C3 was isolated from EDTA-plasma as previously described (Lambris et al., 1980) except that the C3 was passed over a Mono-Q HR10 column as a final step of purification. C3b was generated from C3 by limited digestion with trypsin (0 s at 37 °C with 1% enzyme/substrate [w/w]), and the reaction was stopped by the addition of 3% soybean trypsin inhibitor. C3c and C3d were generated by incubating C3 and 5% elastase (w/w) (Serva) for 6 h at 37 °C. Both C3b and C3c were purified immediately after cleavage by passage over a Mono-Q HR10 column (Pharmacia) attached to an FPLC system (Pharmacia). Separation was achieved by eluting the Mono-Q column in 20 mM Tris-HCl, pH 7.5, and eluting with 200 mL of a 0–500 mM NaCl gradient at a flow rate of 4 mL/min. C3c and C3d were free of C3d/C3b and C3c/C3b, respectively, as assessed by SDS-PAGE and by ELISA using MoAbs specific for either the C3a or C3d fragments. Identification of the C3b fragment was performed using the iodogen method (Fraker & Speck, 1978), resulting in a specific activity of 7 × 10^4 cpm/μg. Factors H and B were prepared as previously described (Ross et al., 1982; Lambris et al., 1980). Factor D was generously provided by Dr. J. Volanakis.

**Preparation of C3b-Coated Sheep Erythrocytes (EC3b).** The erythrocytes (E) from 10 mL of sheep blood were collected and washed and stored in Alsever’s solution until needed (maximum 2 weeks). (1–3) × 10^9 erythrocytes were washed with PBS and resuspended in purified C3 (1–2 mg of C3 per 1 × 10^10 E). The E–C3 suspension was warmed in a 37 °C water bath, and trypsin was added at 0.5% w/w ratio with C3. After 60 s, the reaction was stopped by washing three times
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with ice-cold gelatin veronal saline buffer, pH 7.3 (GVBS-Ni²⁺; Ross et al., 1982), containing 250 µg/ml SBTI and 0.15 mM NiCl and twice with the same buffer without the SBTI. The cells were resuspended at 1 x 10⁶ cells/ml in GVBS-Ni²⁺ and warmed to 37 °C, and 45 µg of factor B and 120 ng of factor D were added per 1 x 10⁶ EC3b and incubated for 3 min. Afterward, EDTA was added to a final concentration of 10 mM to prevent formation of fluid phase C3 convertases. C3 was added to give a final concentration of 60 µg/ml and incubated for 30 min at 37 °C. The cells were washed and resuspended in GVBS without Ni²⁺. Generally, (1-3) x 10⁴ C3b molecules/E were obtained using this method.

Inhibition of CR1 Binding to EC3b Cells by Monoclonal and Antipeptide Antibodies. The ability of a panel of antibodies to inhibit CR1's interaction with C3b-coated sheep erythrocytes was performed as follows. 1 x 10⁶ EC3b cells in GVBS were placed in round-bottom microtiter plates, and 10 µg of antibody was allowed to incubate for 20 min at 37 °C while shaking. The total reaction volume was brought to 100 µL by adding GVBS. The EC3b cells were then centrifuged at 1800 rpm for 5 min, the supernatant removed, and the cells were washed twice with GVBS. The cells were resuspended in 100 µL of GVBS containing tansil lysates (the equivalent of 1 x 10⁶ tansil cells/mL) and allowed to react for 30 min at 37 °C while shaking. The cells were again washed and resuspended as described above, and the bound CR1 was detected by incubating the EC3b cells with 125I-labeled MoAb 543 for 20 min at 30 °C. An 80-µL aliquot of the reaction mixture was layered on top of 400 µL of Lenzol (BDH) oil, and the bound MoAb 543 was separated from the unbound by centrifugation in a microcentrifuge. The tip of the microcentrifuge tube containing the EC3b pellet was cut with a razor blade into a test tube, and the cpm from the bound antibody were determined by a γ-counter. The percentage inhibition by the different anti-C3 antibodies was determined on the basis of the binding of CR1 to EC3b in the absence of antibody.

Inhibition of Factor H, Factor B, and Properdin Binding to C3b Cells by Monoclonal and Antipeptide Antibodies. The assay was done in a manner similar to that described for CR1 except that iodinated factor H was used instead of CR1. Likewise, for factor B binding to EC3b cells, iodinated factor B was used and GVBS containing 0.15 mM Ni²⁺ was used instead of GVBS. Th properdin assay was performed as previously described (Lambiri et al., 1984) except that the microtiter-fixed C3b was incubated with the various anti-C3 antibodies prior to addition of properdin.

Inhibition of C3b Binding to H by C3c and C3d. The binding of C3 fragments to H was assayed in an RIA. All steps were performed at room temperature by using phosphate buffered saline with low ionic strength (0.5 M). Flexible microtiter plates were coated with 50 µL of H (10 µg/mL) in PBS for 2 h. The plates were saturated with 200 µL of 1% bovine serum albumin/1% ovalbumin for 1 h. Serially diluted C3 fragments were then added to the wells and allowed to react for 30 min before addition of 125I-C3b (7 x 10⁵ cpm/well). The plates were washed three times with PBS/0.05% Tween 20, and bound C3b was detected by cutting out the microtiter wells and quantitating in a γ-counter.

Inhibition of H Cofactor Activity by C3c and C3d. The cofactor activity of H for fluid-phase C3b was measured as previously described (Alsenz et al., 1984) except that the assays were performed at normal ionic strength and that the samples were incubated for 30 min at 37 °C. Briefly, 5 µL of factor H (100 µg/mL) was preincubated with 20 µL of C3b, C3c, or C3d for 1 h. Five microliters of 125I-C3b (100 000 cpm = 0.14 µg) was then added followed by 2 µL of factor I (200 µg/mL). The reaction was stopped by boiling after the addition of SDS-PAGE sample buffer containing SDS and mercaptoethanol. C3b, C3c, and C3d were used at 400-fold molar excess over the concentration of 125I-C3b. Cleavage of 125I-C3b was analyzed by SDS-PAGE followed by autoradiography.

Inhibition of H Binding to C3b by Synthetic Peptides. The binding of H to C3 fragments was measured by an ELISA, and the method described below has been adapted from a similar one described previously (Lambiri et al., 1988b). Briefly, C3b (10 µg/mL; 50 µL/well) was coated to microtiter plates overnight at 4 °C. The wells were then saturated with 1% bovine serum albumin in PBS, pH 7.2. To determine the effect of the synthetic peptides on the binding of H to C3b, 125I-H was preincubated with the different synthetic peptides at various concentrations. This mixture was then transferred to the C3b-coated microtiter wells, and the bound H was detected, after washing, by cutting out the microtiter wells and quantitating 125I-H in a γ-counter.

Preparation of Zymosan-Activated Serum. One gram of zymosan A (Sigma) was added to 100 mL of 0.9% NaCl and boiled for 2 h under a reflux condenser. The mixture was centrifuged at 6500 g for 10 min, and the pellet was resuspended in 50 mL of saline and centrifuged twice more at 6500 g. This step was repeated twice. The pellet was resuspended in PBS to yield a 50 mg/mL solution and stored frozen at −70 °C. To activate serum zymosan is added to normal human serum at a final concentration of 1–2 mg of zymosan per milliliter of serum and incubated for 30 min at 37 °C. For controls, EDTA was added, at a final concentration of 10 mM, to inhibit zymosan activation of complement in serum.

Detection of Neoepitopes in C3 Using Anti-C3727–768 and Zymosan-Activated or EDTA-Treated Serum. The following ELISA was developed to determine if an antibody (in this case anti-C3727–768) recognizes an epitope in C3 that is expressed only after cleavage to C3b and/or C3c. Fifty microliters of anti-C3727–768 (affinity purified) or a polyclonal anti-C3 antibody (protein A purified) at 8 µg/mL was coated overnight at 4 °C to microtiter plates. Microtiter plates were saturated with 1% BSA for 30 min. Serum that was treated with zymosan in the presence or absence of 1% BSA to yield 10 mM EDTA was diluted 1/35 in 1% BSA in PBS containing 10 mM EDTA and centrifuged for 2 min at 10000 g. Fifty microliters of this diluted serum, serially diluted, was added to the antibody-coated microtiter plates for 30 min. Bound C3 or its fragments were detected using a monoclonal anti-C3c antibody (MoAb 133H11) that recognizes an epitope expressed in both native C3 and its fragments C3b and iC3b. The bound monoclonal antibody was detected using an anti-mouse Ig. All other conditions and steps were carried out as described in the ELISA above.

Polyacrylamide Gel Electrophoresis. Electrophoresis of the purified protein fragments was performed in the presence of sodium dodecyl sulfate (SDS) as described by Laemmi (1970). The samples were reduced with 2% mercaptoethanol. Protein bands were detected by staining with 0.1% Coomassie Blue R-250 (Bio-Rad) in 2-propanol/methanol/acetic acid/H₂O (2:8.2:0.1:0.4:2.4, by volume). The molecular weights were estimated using reference proteins.

RESULTS

Inhibition of C3b Binding to CR1 and Factors H and B by Anti-C3 Antibodies. To study the relationship of CR1, factor H, and factor B binding to C3b, various anti-C3 antibodies
were tested for their ability to inhibit these interactions. Figure 1 shows that the antibodies anti-C3-9, anti-C3727-768, and a polyclonal anti-C3c inhibit CR1 binding to EC3b while the other anti-C3c or anti-C3d antibodies tested had a marginal or no effect on this interaction. When the same panel of antibodies was tested for inhibition of factor H binding to EC3b cells, both anti-C3d and anti-C3c antibodies were inhibitory (Figure 1). These data support previously published results and suggest that more than one site is involved in C3b binding to factor H. The MoAb anti-C3-9 also completely inhibited factor B binding to EC3b, while anti-C3727-768 only partially inhibited this interaction (Figure 1). None of the other anti-C3c or anti-C3d antibodies tested, except for the polyclonal anti-C3c, inhibited factor B binding to EC3b cells. Table I summarizes the inhibition of CR1, factor H, and factor B by the anti-C3c and -C3d antibodies tested. Especially noteworthy are the MoAb anti-C3-9 and the antipeptide antibody anti-C3727-768, both of which inhibit CR1, factor H, and factor B binding to EC3b cells and suggest that the binding sites for these proteins are related to one another.

Inhibition of Factor H Binding and Cofactor Activity. Competition assays were developed to confirm the antibody inhibition results which suggested that sites within both C3c and C3d are capable of binding factor H. Figure 2 shows that purified C3c and C3d inhibit binding of 125I-C3b to microtiter-fixed H. This inhibition is augmented by the addition of both fragments together; yet it is still 100-fold less effective than C3b itself. The ability of factor H to function as a cofactor in the factor I-mediated cleavage of C3b to iC3b is also inhibited by either the C3c or C3d fragment of C3 (Figure 3).

Mapping of the Epitopes in C3 Recognized by the Anti-C3 Antibodies that Inhibit CR1, H, and B Binding. Since the factor H binding site in C3d has been identified (Lambert et al., 1988b), efforts were directed toward identifying the epitopes recognized by those anti-C3c antibodies that inhibited H binding to C3b. The anti-C3727-768 antiserum was affinity purified on a C3b-Sepharose matrix to obtain the fraction of Ig that recognizes C3b. To determine which fragments of C3 contain epitopes recognized by anti-C3727-768, a sandwich ELISA was developed. Here, microtiter wells were coated with affinity-purified anti-C3727-768. Fluid-phase fragments of C3, either purified in or serum treated with zymosan, were then added to the microtiter wells, and those fragments of C3 bound to the antipeptide antibody were detected by a MoAb which recognizes an epitope expressed in native C3, C3b, IC3b, and C3c. Figure 4 shows that, when the complement in serum is activated by zymosan, anti-C3727-768 recognizes neoeptopes.
expressed by activation fragments of C3 but not by native C3. Since iC3b is the predominant fluid-phase fragment in serum after complement activation, further specificity studies using the affinity-purified anti-C3727-768 were performed. Using purified components, anti-C3727-768 recognizes fluid-phase C3b, iC3b, and C3c but not C3d or C3 (Figure 5A). However, when C3 is fixed to microtiter wells, the anti-C3727-768 epitope(s) is expressed (data not shown). Therefore, the specificity of the antipeptide antibody for the different C3 fragments parallels that previously described for CR1 (Becherer & Lambris, 1988).

**Binding Specificity of MoAb Anti-C3-9 for C3 Fragments.**

With the idea that the MoAb anti-C3-9 recognizes a region of C3 involved in the binding of CR1, H, and B, attempts were made to define its binding specificity. This antibody, like CR1, binds to the C3, C3b, and C3c fragments of C3 when they are fixed to microtiter plates (Figure 5B). Previous reports have shown that the epitope recognized is not expressed in native C3 (Hack et al., 1988) but that when C3 is fixed to microtiter plates, it behaves like iC3. The specificity exhibited by the anti-C3-9 MoAb for the different fragments of C3 thus resembles that of CR1 and the anti-C3727-768 antipeptide antibody described above. However, anti-C3-9 MoAb did not compete with anti-C3727-768 for binding to C3b, failed to recognize the peptide C3727-768, and apparently recognizes a conformational epitope. Characterization of enzymatic digests of C3c implicated Glu737 as being important for anti-C3-9 binding (data not shown), but the inability to obtain a sequence from the three COOH-terminal fragments of C3b, and thereby exclude COOH-terminal nicking, prevented us from conclusively demonstrating this.

**Inhibition of H and B Binding to C3b by Synthetic Peptides.**

The above inhibitions by the monoclonal anti-C3-9 and anti-C3727-768 antibodies suggest that factor H and factor B may recognize a site within the domain previously identified to bind CR1. To further investigate this possibility, the synthetic peptide that inhibits CR1 binding to C3b was tested for its ability to inhibit iodinated factor H and factor B binding to microtiter-fixed C3b. The results depicted in Figure 6 demonstrate that the synthetic peptides C3727-768 and C31189-1214 inhibit factor H binding to C3b, while other peptides, including the peptide from C3 that blocks properdin binding, had no effect. The partial inhibition of H binding by C31189-1214 agrees with previous results (Lambris et al., 1988), and the ability of C3727-768 to also partially inhibit H binding confirms that a second site in C3b is involved in binding factor H. When both inhibitory peptides were added together, no synergistic or additive effects were observed. On the other hand, these same peptides did not interfere with factor B binding to C3b (data not shown), and it remains to be determined if the inhibition observed by MoAb anti-C3-9 and, to a lesser extent, anti-C3727-768 is due to steric or allosteric effects.

**DISCUSSION**

Although previous work has described two distinct sites in C3 involved in CR1 and factor H binding, a number of ob-
CR1 and factor B binding to EC3b cells. Neither of the two anti-C3d MoAbs tested inhibited CR1 or factor B binding. However, both of these MoAbs inhibited factor H binding to EC3b cells. In addition, the anti-C3c MoAb anti-C3-9, as well as the anti-C3$^{727-768}$, also inhibited factor H binding. The most interesting finding from the antibody inhibition studies was that the antipeptide antibody anti-C3$^{727-768}$ and the anti-C3c MoAb, anti-C3-9, inhibit the binding of CR1, factor H, and factor B to C3b. Despite not precisely determining the MoAb anti-C3-9 epitope, the fact that it blocks CR1, H, and B binding, but not properdin, to C3b agrees with the competition observed between these molecules.

The antipeptide antibody C3$^{727-768}$, with its predetermined specificity, was an excellent tool to study the conformational changes that occur within the CR1 binding domain during complement activation. CR1 fails to bind native C3, and one would expect that the anti-C3$^{727-768}$ would also fail to recognize native C3 if indeed this antibody recognizes an epitope near the CR1-binding site. The affinity-purified anti-C3$^{727-768}$ antibodies bound only to the C3b, iC3b, and C3c fragments but not to native C3 (Figures 4 and 5). The binding of anti-C3$^{727-768}$ to microtiter-fixed C3 indicates that upon immobilization of C3, its native conformation is disturbed, thereby exposing the anti-C3$^{727-768}$ epitope(s). This finding is similar to the observed binding of CR1 to C3 when it is fixed to microtiter wells. Furthermore, the binding of anti-C3$^{727-768}$ to the C3b, iC3b, and C3c fragments of C3 was inhibited by the synthetic peptide C3$^{727-768}$ (data not shown). Thus, the anti-C3$^{727-768}$ recognizes a neopeptide expressed after cleavage of C3 to C3b that is confined within the CR1-binding domain (residues 727-768) of the α-chain of C3b. This antibody, or a monoclonal antibody generated against this region, could therefore be used to detect C3 degradation fragments in biological fluids. This specificity is particularly important since one must detect the “activation” fragments of C3 without recognizing the native C3 which is usually present in high concentrations.

Several conclusions relating to the structural details of C3 can be drawn from these results when other reports from the literature are taken into account. First, only the anti-C3c MoAb anti-C3-9 and the anti-C3$^{727-768}$ inhibit CR1 binding to C3b. The CR1-binding site has been clearly defined (Becherer & Lambris, 1988), and the antibody inhibition data are not contradictory to those results. The work by Kückstein et al. (1988) using deletion mutants of recombinant CR1 showed that two C3b-binding sites exist in CR1. Therefore, a multivalent ligand–receptor interaction can be envisaged as a result of multiple molecules of C3b being deposited at the focal point of complement activation. Such a multivalent interaction would enhance the relatively low affinity of monomeric C3b for its receptor ($K_r = 1 \times 10^5$). Discounting steric or allosteric effects, the finding that an anti-C3d MoAb inhibits CR1 binding to C3b (Koistinen et al., 1989) suggests that there may be a second site in C3 capable of interacting with CR1.

The interaction of factor H to EC3b was inhibited by both anti-C3c and anti-C3d MoAbs and anti-C3$^{727-768}$. The binding of H to C3b by C3c and C3d confirmed that more than one domain in C3 is capable of interacting with factor H. These fragments of C3b degradation, when added together, resulted in a greater inhibition than either fragment alone; yet this failed to reconstitute the inhibition observed by C3b alone, being approximately 100-fold less effective. The cleavage of 1271-C3b by factor I in the presence of H is completely inhibited by C3b and to a lesser degree by C3c. The
Relationship between the CR1-, H-, and B-Binding Sites in C3

Biochemistry, Vol. 31, No. 6, 1992 1793

inhibitory effect of C3c is enhanced when C3d is added (data not shown). Interestingly, C3d, when added alone, inhibited only the third factor I-mediated cleavage of C3b. These results suggest that the cleavage of C3b by factor I at a specific site is associated with the binding of H to a specific domain. The finding that the synthetic peptide C372-768, which inhibits CR1 binding, also inhibited factor H binding to C3b clearly demonstrates that a second factor H-binding site exists in C3b. This was predicted from earlier results (Lambri et al., 1988) and is borne out by the observation that neither C372-768 nor C3118-1216 can completely inhibit H binding to C3b (Figure 6) and by the ability of C3c and C3d to inhibit the cofactor activity of factor H. In addition to its involvement in CR1 and H binding, this domain of C3 has also been identified as a site of interaction for factor B (Ganu & Müller-Eberhard, 1985) and, on one hand, would explain factor H’s ability to compete with factor B and CR1 for binding to C3b (Pangburn & Müller-Eberhard, 1978; Pangburn, 1986) and, on the other hand, would explain the ability of factor H to participate in the third factor I cleavage (Ross et al., 1982), a role more efficiently played by CR1. Since factor H, CR1, CR2, and C4bp are all cofactors for the factor I-mediated cleavages of C3b and since they are all structurally similar, one possibility is that multiple recognition domains within C3b and iC3b exist that are common, albeit with different affinities, to the different cofactor molecules. This supposition is reflected by C4bp’s weak affinity for C3b (Fujita & Nussenzeiw, 1979) and is circumstantially supported by the recent observation that there are stretches of internal sequence homology within C3 (Becherer et al., 1990; Esparza et al., 1991). One of these segments spans C3 residues 1199–1210 and comprises the CR2-binding site. As demonstrated previously (Lambri et al., 1988), the discontinuous factor H-binding site surrounds this region. A second region of C3 (residues 744–755) homologous to the CR2-binding site is found within the domain at the NH2 terminus of the α-chain predicted to bind CR1. In light of the sequence similarity between the domains containing the H-, CR1-, and CR2-binding sites and the recent finding that synthetic peptides from both these domains compete for binding to CR2 (Esparza et al., 1991), one could speculate that the CR2- and H-binding domain in C3d augments C3b binding to CR1. The exact contribution of the amino acids in this region of C3 with respect to CR1, CR2, and factor H binding remains to be determined and undoubtedly depends on the conformational state of C3 (i.e., iC3b, C3b, C3c, or C3d). Nonetheless, since all these molecules are cofactors for the factor I-mediated cleavage of C3b, it appears important for them to bind to common regions of the C3 molecule. This ensures that the conformation of the molecule is “locked” into the proper configuration, thus permitting factor I to cleave at precise positions within C3.

Factor B binding to EC3b cells was completely inhibited by anti-C3-9 and partially inhibited by anti-C372-768. These findings support two previous reports suggesting that the factor B-binding site is within the C3c fragment of C3 (Burger et al., 1982; Ganu & Müller-Eberhard, 1985). Since the region of C3 spanned by C372-768 contains the CR1 site, binding of factor B within this domain would explain the partial inhibition observed by anti-C372-768 (Figure 1) as well as the regulatory role in CR1 in the decay and dissociation of the C3 convertase (C3bBb). The ability of peptide C372-768 to inhibit factor B binding may be due to the assay systems employed in this study. Alternatively, inherent conformational properties may be such that the B-binding site is not properly expressed by the synthetic peptide C372-768. While the NH2-terminal end of the α-chain is involved in CR1, factor H, and factor B recognition (see also the review by Fishelson [1991]), binding experiments using C3b from different species (Alszen et al., 1992) suggest that these proteins bind to proximal, but not necessarily identical, sites within this region of C3. The synthesis of additional peptides or the expression of a recombinant C3 molecule should allow us to further investigate the relationship between the binding sites in C3 for CR1, CR2, factor H, factor B, and other members of this family of C3-binding proteins.

References


