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†

J. David Becherer,‡,§ Jochem Alsenz,‡ Inmaculada Esparza,‡ C. Erik Hack,∥ and John D. Lambris*,‡,⊥

Basel Institute for Immunology, Grenzacherstrasse 487, 4005-Basel, Switzerland, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Plesmanlaan 125, 1006 CX, Amsterdam, The Netherlands, and Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

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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 ($C3^{727-768}$) inhibited factor H binding. Second, antibodies raised against this peptide inhibited binding to CR1, factor H, and 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 pathogens 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 C4b (Reid et al., 1986; Holers et al., 1985; Fearon & Ahearn, 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 predited that a series of such structures would form a semirigid, elongated 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 H, 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 C1r and C1s and the noncomplement proteins β2-glycoprotein 1, IL-2 receptor, and factor XIII.

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 (Alsenz et al., 1990), we hypothesized that similar or adjacent sites in C3 are recognized by the members of this family of

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* To whom correspondence should be addressed at the Department of Pathology and Laboratory Medicine.

‡ Basel Institute for Immunology.

§ Present address: Glaxo Research Institute, 5 Moore Dr., Research Triangle Park, NC 27709.

∥ Central Laboratory.

⊥ Department of Pathology.

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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 NH_2 terminus of 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 κ) was purchased from American Type Tissue Culture, and the IgG from ascites fluid was purified on a protein A Sepharose column. The anti-C3c 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-C3-9 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 haemocyanin 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 $(NH_4)_2SO_4$ 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-$C3^{727-768}$ 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-$C3^{727-768}$ antibody (affinity purified on C3b-Sepharose) for 30 min at 22 °C. The binding of anti-$C3^{727-768}$ 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 lysing buffer (1% Nonidet P-40 in PBS containing 5 mM ethylenediaminetetraacetic acid, 2 mM phenylmethanesulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin, and 2 mM diisopropyl fluorophosphate) for 1 h at 4 °C at a concentration of 5×10^8 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-methylbenzylhydrylamine resin with *tert*-butyloxycarbonyl protected amino acids, dicyclohexylcarbodiimide couplings, 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-pressure liquid chromatography, and amino acid analysis using an Applied Biosystems 420A derivatizer with an on-line 130A analyzer. All peptides were resuspended and dialyzed in 1000 M_r cut-off dialysis membranes (Spectrum) against PBS, pH 7.2, before use. The synthetic peptides $C3^{727-768}$ (SNLDEDIIAEENIVSRSEFPESWLWNVEDLKEPPKNGISTKL), $C3^{1187-1214}$ (KFLTTAKDKNRWEDPGKQLYNVEATSYA), $C3^{894-923}$ (VYHHFISDGVRKSLKVVPEGIRMNKTVAVR), and $C3^{1402-1435}$ (GVDRYISKYELDKAFSDRNTLIIYLDSVSHSEDD) have been used previously and are described elsewhere (Becherer & Lambris, 1988; Lambris et al., 1985).

Preparation of C3 Fragments and Other Complement 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 [90 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 equilibrating 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 C3c or C3d fragments. Iodination of the C3b fragment was performed using the iodogen method (Fraker & Speck, 1978), resulting in a specific activity of 7×10^5 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). $(1–3) \times 10^{10}$ 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

with ice-cold gelatin veronal saline buffer, pH 7.3 (GVBS-Ni^{2+}; 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 1×10^9 cells/mL in GVBS-Ni^{2+} and warmed to 37 °C, and 45 μg of factor B and 120 ng of factor D were added per 1×10^9 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 Ni^{2+}. Generally, $(1-3) \times 10^4$ 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. 1×10^6 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 two times with GVBS. The cells were resuspended in 100 μL of GVBS containing tonsil lysates (the equivalent of 1×10^7 tonsil 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 ^{125}I-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 Ni^{2+} was used instead of GVBS. Th properdin assay was performed as previously described (Lambris 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 mS). 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 ^{125}I-C3b (7×10^5 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 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 ^{125}I-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 ^{125}I-C3b. Cleavage of ^{125}I-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 (Lambris 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, ^{125}I-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 ^{125}I-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-$C3^{727-768}$ and Zymosan-Activated or EDTA-Treated Serum. The following ELISA was developed to determine if an antibody (in this case anti-$C3^{727-768}$) recognizes an epitope in C3 that is expressed only after cleavage to C3b and/or iC3b. Fifty microliters of anti-$C3^{727-768}$ (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 wtih 1% BSA for 30 min. Serum that was treated with zymosan in the presence or absence of 20 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 Laemmli (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/H_2O (2.8:2.0:1.0:4.2, 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

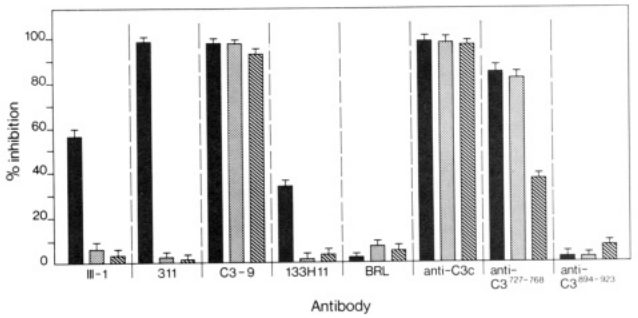

FIGURE 1: Inhibition of CR1, factor H, and factor B binding to EC3b by monoclonal anti-C3c and anti-C3d and antipeptide antibodies. 1×10^6 EC3b cells were incubated with 10 μg (100-fold molar excess) of the appropriate protein A or affinity-purified antibody for 20 min at 37 °C in GVBS. After the cells were washed, the appropriate radiolabeled ligand [factor H (solid bars) and factor B (cross-hatched bars)] or receptor [CR1 (stippled bars)] was added, and specifically bound material was detected as described under Experimental Procedures. The results represent the average of three experiments, and the amount of antibody used was greater than or equal to the amount that gave maximal inhibition. The anti-C3c antibody was a rabbit polyclonal antibody.

Table I: Summary of Antibody Inhibition Studies on the Binding of C3 Fragments to CR1, Factor H, and Factor B

		inhibition of EC3b binding to			
antibody	specificity	CR1	factor H	factor B	properdin
III-1	C3d	–	+	–	ND
311	C3d	–	++	–	ND
C3-9	C3c	++	++	++	–
133H11	C3c	–	+	–	–
BRL	C3c	–	–	–	–
anti-C3c	C3c	++	++	++	++
anti-$C3^{727-768}$	C3c	++	++	+	–
anti-$C3^{894-923}$	C3c	–	–	–	–

were tested for their ability to inhibit these interactions. Figure 1 shows that the antibodies anti-C3-9, anti-$C3^{727-768}$, 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-$C3^{727-768}$ 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-$C3^{727-768}$, 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 ^{125}I-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).

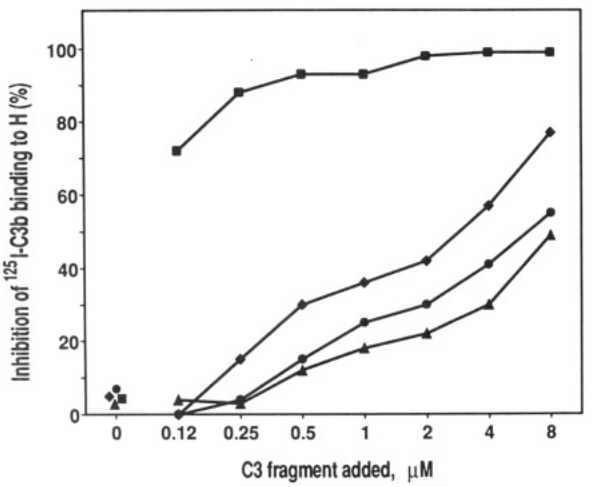

FIGURE 2: Inhibition of C3b binding to factor H by fluid-phase C3 fragments. Factor H-coated microtiter wells were preincubated with 2-fold dilutions of C3b (■), C3c (▲), C3d (●), or C3c + C3d (◆) prior to the addition of ^{125}I-C3b. ^{125}I-C3b bound to C3b was determined, and the percent inhibition was calculated relative to the binding observed in the absence of competitor.

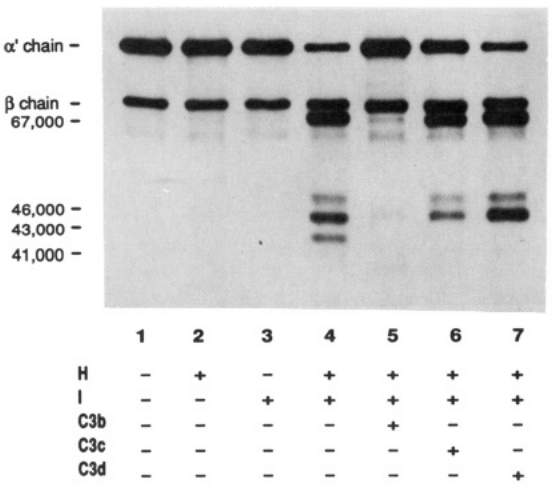

FIGURE 3: Inhibition of H cofactor activity for fluid-phase C3b by C3 fragments. Factor H was preincubated with C3b, C3c, or C3d before addition of ^{125}I-C3b and factor I. The molar ratio of iodinated C3b to C3 fragments was 1:400. The cofactor activity was monitored by SDS–PAGE and autoradiography. The 46 000-Da band indicates cleavage of C3b at the first factor I cleavage site, the 43 000-Da band at the second, and the 41 000-Da band at the third. The 27 000-Da NH_2-terminal α'-chain fragment which is generated when C3b is cleaved at positions 1, 2, and 3 is not visible due to its poor labeling by the ^{125}I.

Mapping of the Epitopes in C3 Recognized by the Anti-C3 Antibodies that Inhibit CR1, H, and B Binding. Since the fator H binding site in C3d has been identified (Lambris et al., 1988b), efforts were directed toward identifying the epitopes recognized by those anti-C3c antibodies that inhibited H binding to C3b. The anti-$C3^{727-768}$ 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-$C3^{727-768}$, a sandwich ELISA was developed. Here, microtiter wells were coated with affinity-purified anti-$C3^{727-768}$. Fluid-phase fragments of C3, either purified or in 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-$C3^{727-768}$ recognizes neoepitopes

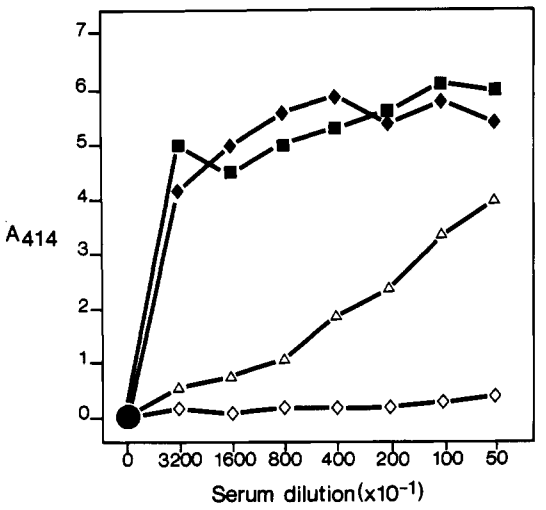

FIGURE 4: Binding of native C3 or its fragments to anti-C3$^{727-768}$ or polyclonal anti-C3c antibodies. Three picomoles of affinity-purified anti-C3$^{727-768}$ (open symbols) or protein A-purified rabbit anti-C3c (closed symbols) was fixed to microtiter plates overnight. The wells were than saturated with 1% BSA in PBS for 30 min. Normal human serum was treated either with zymosan (1 mg/mL) in the presence (◇, ◆) or absence (△, ■) of 10 mM EDTA for 30 min. After centrifugation, the serum samples were added to the microtiter plate in serial dilutions, and bound C3 or its fragments (primarily iC3b) were detected using a monoclonal anti-C3c antibody followed by peroxidase labeled goat anti-mouse Ig.

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-C3$^{727-768}$ were performed. Using purified components, anti-C3$^{727-768}$ recognizes fluid-phase C3b, iC3b, and C3c but not C3d or C3 (Figure 5A). However, when C3 is fixed to microtiter wells, the anti-C3$^{727-768}$ 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-C3$^{727-768}$ antipeptide antibody described above. However, anti-C3-9 MoAb did not compete with anti-C3$^{727-768}$ for binding to C3b, failed to recognize the peptide C3$^{727-768}$, and apparently recognizes a conformational epitope. Characterization of enzymatic digests of C3c implicated Glu^{737} 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 C3c, 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-C3$^{727-768}$ 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

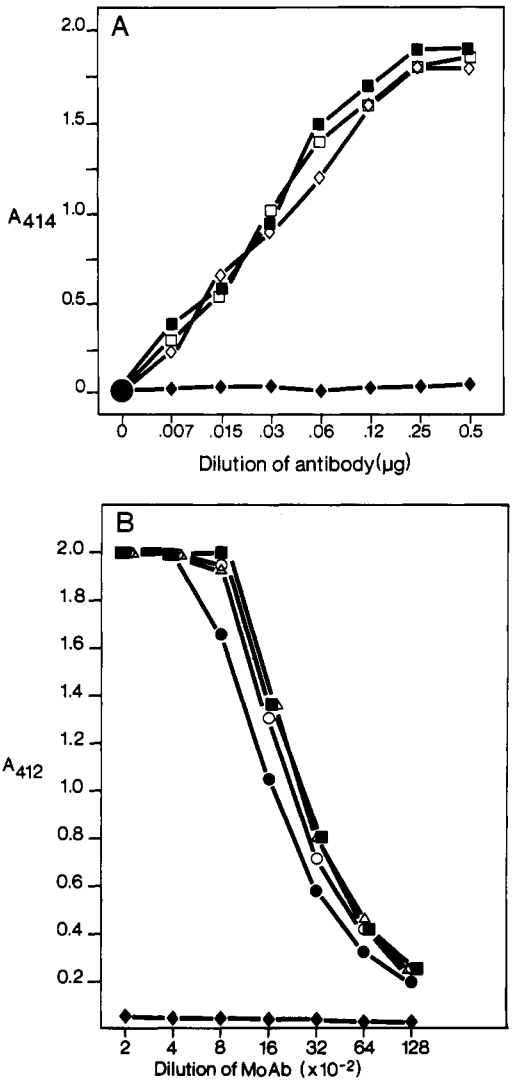

FIGURE 5: Binding of anti-C3$^{727-768}$ and anti-C3-9 to C3 fragments. (A) Two picomoles of C3b (■), iC3b (□), C3c (◇), or C3d (◆) was fixed to microtiter wells. After saturation, affinity-purified anti-C3$^{727-768}$ was serially diluted, and bound antibody was detected using peroxidase-conjugated goat-anti-rabbit Ig. Anti-C3$^{727-768}$ exhibited identical binding specificity when the purified fluid-phase fragments of C3 were tested. (B) Binding of anti-C3-9 to C3 (■), C3b (○), iC3b (△), C3c (●), and C3d (◆) was performed as in panel A except that the serial dilutions of the culture supernatant of MoAb anti-C3-9 were made.

to microtiter-fixed C3b. The results depicted in Figure 6 demonstrate that the synthetic peptides C3$^{727-768}$ and C3$^{1189-1214}$ 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 C3$^{1189-1214}$ agrees with previous results (Lambris et al., 1988), and the ability of C3$^{727-768}$ 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-C3$^{727-768}$ 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-

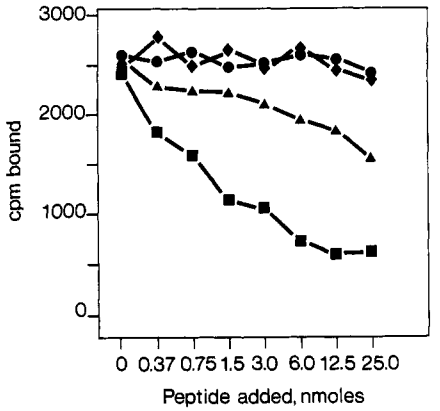

FIGURE 6: Inhibition of factor H binding to C3b by synthetic peptides. C3b (2 pmol) was fixed to microtiter wells overnight and then saturated with 1% BSA. ^{125}I factor H (1×10^5 cpm; 25 ng), preincubated with serial dilutions of synthetic peptides, was then added to the C3b-coated wells. After 30 min the plates were washed, and bound factor H was determined. Peptides tested: $C3^{727-768}$ (■), $C3^{1187-1214}$ (▲), $C3^{894-923}$ (●), and $C3^{1402-1435}$ (◆). When peptides $C3^{727-768}$ and $C3^{1187-1214}$ were incubated together with factor H, no further inhibition than that exhibited by $C3^{727-768}$ (■) was observed.

servations led us to suspect that the binding of these two proteins to C3b is mediated via sites that are proximal or share structural elements. This may also be true for other members in this family of C3b-binding proteins and is based on several observations. First, factor H and CR1, as well as CR2, DAF, MCP, C4bp, and C2, and factor B, are structurally related, being comprised, to varying degrees, of short consensus repeating units (SCRs). Secondly, all of the above proteins are involved in complement control and bind to C3b. In particular, both factor H and CR1 have the same functions in regulating the complement cascade in that they serve both as decay accelerating factors and as cofactors in the factor I-mediated inactivation of C3b. Finally, several reports have demonstrated that some of these proteins compete with one another for binding to C3b (Discipio, 1981; Pangburn et al., 1978; Pangburn, 1986; Farries et al., 1990). Since the binding sites for CR1 and H have been localized to distinct domains in C3, we hypothesized that there may be multiple sites of interaction in C3 for these molecules and that some of these sites may be common to the binding of both molecules. Such a hypothesis would explain the ability of these molecules to compete for binding to C3b. In addition, it would also explain previous reports describing (1) that antibodies from the C3c and C3d domain inhibit factor H (Tamerius et al., 1982; Burger et al., 1982; Lambris & Ross, 1982; Nilsson & Nilsson, 1987) and factor B binding (Burger et al., 1982; Koistinen et al., 1989), (2) that two sites exist in CR1, factor H, and factor B for binding to C3b (Klickstein et al., 1988; Discipio, 1981; Horstmann et al., 1985; Ueda et al., 1987; Alsenz & Lambris 1988; Pryzdial & Isenman, 1987), and (3) that C3b binds with higher affinity to CR1 than C3c (Becherer & Lambris, 1988).

To test this hypothesis, we used a panel of anti-C3c and anti-C3d MoAbs and an antipeptide antibody for inhibition studies of CR1, factor H, and factor B binding to C3b. MoAbs have been routinely used to identify functionally important sites in proteins due to their remarkable specificity. Although MoAb inhibition of C3 binding to a given ligand is a good starting point for identifying the ligand-interaction sites in C3, such inhibitory effects may also be due to steric or allosteric effects. In the present work, several interesting findings were observed from the antibody inhibition studies of CR1, factor H, and factor B binding to C3b. Of the antibodies tested, only the anti-C3c MoAb anti-C3-9 and the anti-$C3^{727-768}$ inhibited 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 neoepitope 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 Klickstein 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 ($K_a = 1 \times 10^5$). Discounting steric or allosteric affects, 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 by anti-$C3^{727-768}$. The inhibition of H binding 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 ^{125}I-C3b by factor I in the presence of H is completely inhibited by C3b and to a lesser degree by C3c. The

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 $C3^{727-768}$, 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 (Lambris et al., 1988) and is borne out by the observation that neither $C3^{727-768}$ nor $C3^{1187-1214}$ 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 & Nussenzweig, 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 (Lambris 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 NH_2 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., iC3, C3b, iC3b, 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-$C3^{727-768}$. 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 $C3^{727-768}$ contains the CR1 site, binding of factor B within this domain would explain the partial inhibition observed by anti-$C3^{727-768}$ (Figure 1) as well as the regulatory role in CR1 in the decay and dissociation of the C3 convertase (C3b,Bb). The ability of peptide $C3^{727-768}$ 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 $C3^{727-768}$. While the NH_2-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 (Alsenz 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 C3b-binding proteins.

Registry No. C3, 80295-41-6; factor H, 80295-65-4; factor B, 80295-62-1; properdin, 11016-39-0.

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Role of Hydrophobic Forces in Bilayer Adhesion and Fusion†

Christiane A. Helm,‡ Jacob N. Israelachvili,*,§ and Patty M. McGuiggan§

Institut für Physikalische Chemie, Johannes Gutenberg-Universität, Jakob-Welder Weg 11, D-6500 Mainz, Germany (FRG), and Department of Chemical and Nuclear Engineering and Materials Department, University of California, Santa Barbara, California 93106

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ABSTRACT: With the aim of gaining more insight into the forces and molecular mechanisms associated with bilayer adhesion and fusion, the surface forces apparatus (SFA) was used for measuring the forces and deformations of interacting supported lipid bilayers. Concerning adhesion, we find that the adhesion between two bilayers can be progressively increased by up to two orders of magnitude if they are stressed to expose more hydrophobic groups. Concerning fusion, we find that the most important force leading to *direct* fusion is the hydrophobic attraction acting between the (exposed) hydrophobic interiors of bilayers; however, the occurrence of fusion is not simply related to the strength of the attractive interbilayer forces but also to the internal bilayer stresses (intrabilayer forces). For all the bilayer systems studied, a single basic fusion mechanism was found in which the bilayers do not "overcome" their short-range repulsive steric-hydration forces. Instead, local bilayer deformations allow these repulsive forces to be "bypassed" via a mechanism that is like a first-order phase transition, with a sudden instability occurring at some critical surface separation. Some very slow relaxation processes were observed for fluid bilayers in adhesive contact, suggestive of constrained lipid diffusion within the contact zone.

The traditional view of the fusion of two amphiphilic layers (e.g., two vesicles) is that the two surfaces first overcome their short-range repulsive electrostatic and/or hydration forces so that they can come into close contact (Chernomordic et al., 1987; Westerhoff, 1985; Rand & Parsegian, 1986). However, the relation between interbilayer forces, adhesion, and fusion is still far from clear. There are examples of fusion being induced without apparently altering the interbilayer forces simply by stressing bilayers or by creating an osmotic gradient across them (Chernomordic et al., 1987; Papahadjopoulos et al., 1977; Cohen et al., 1980, 1982; Akabas et al., 1984a,b;

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* Author to whom correspondence should be addressed.
‡ Johannes Gutenberg-Universität.
§ University of California, Santa Barbara.

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