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Structure and biology of complement protein C3, a connecting link between innate and acquired immunity

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Summary: Complement protein C3 is a central molecule in the complement system whose activation is essential for all the important functions performed by this system. After four decades of research it is now well established that C3 functions like a double-edged sword: on the one hand it promotes phagocytosis, supports local inflammatory responses against pathogens, and instructs the adaptive immune response to select the appropriate antigens for a humoral response; on the other hand its unregulated activation leads to host cell damage. In addition, its interactions with the proteins of foreign pathogens may provide a mechanism by which these microorganisms evade complement attack. Therefore, a clear knowledge of the molecule and its interactions at the molecular level not only may allow the rational design of molecular adjuvants but may also lead to the development of complement inhibitors and new therapeutic agents against infectious diseases.

Introduction

The complement system is an integral participant in the innate mechanisms of immunity, in which the third component of complement, C3, plays a central role. C3 supports the activation of all the three pathways of complement activation i.e. the classical, alternative, and lectin pathways. Among the complement proteins, it is probably the most versatile and multifunctional molecule identified to date, having evolved structural features that allow it to interact in a specific manner with at least 25 different proteins (1). C3 is an ancient molecule; its identification in echinoderms (2) and tunicates (3) suggests that it emerged at least 700 million years ago, long before the appearance of immunoglobulins.

Studies of C3 began during the early part of the 20th century. By that time, it was accepted that complement activation proceeds in a sequential manner and that cellular intermediate products are formed during immune hemolysis. C3 activity was then considered to be due to a single component. However, in 1958, Rapp (4) demonstrated through modified fractionation methods that the hemolytic reaction of C3 must involve more than one component. After the advent of chro-

***Table 1.* Properties of human C3**

Serum concentration (mg/ml)	1.2
Molecular weight	
Apparent[a]	185,000
Calculated[b]	184,342
Electrophorectic mobility	β-2
Subunits (mol. wt[a])	α chain (110,000)
	β chain (75,000)
Amino acids	
Signal peptide	22
Mature protein	1,637
Carbohydrate (~%w/w)	1.5
Isoelectric point	5.7
Extinction coefficient (280 nm, 1%, 1 cm)	9.7
S20, w (Svedberg units)	~8.3
Diffusion coefficient (10^{-7} cm^2/s)	4.0
Thioester hydrolysis rate (%/min)	0.005
Genetic locus	19p13.3

[a] Determined by SDS-PAGE.

[b] Based on the primary sequence of the plasma protein.

matographic techniques it was discovered that C3 activity, as defined by old assays, was actually a mixture of six individual proteins (C3, C5, C6, C7, C8, and C9).

In the latter part of the 20th century, C3 was primarily studied in the context of its function within the complement system. It was shown to interact with other complement proteins and with cell-surface receptors and to serve as a modulator of complement-dependent leukocyte functions. Its role in mounting an effective antibody response, though reported (5), was overlooked. The recent availability of C3 knockout mice has allowed us to revisit this potential role for C3. The data obtained from studies of these mice have provided strong supporting evidence for the paradigm that C3 forms a connecting link between the complement system and the acquired immune response that helps trigger and modulate the acquired immune response.

Although the interaction of C3 with other complement proteins and receptors is important for the host defense, the interactions of this complement component with proteins from foreign pathogens provide a mechanism by which these microorganisms can evade complement neutralization. In addition, unregulated activation of C3, and in turn the complement system, leads to host cell damage. Thus, elucidation of the molecular features of C3 that are important for C3–ligand interactions is of great importance. We have devoted considerable research effort to defining the regions of the C3 protein that are expressed at the various stages of its proteolytically controlled life cycle and that determine the various functional roles of the C3 molecule. In this review we describe our work on the C3–ligand interactions, C3 evolution and diversity, C3-related viral molecular mimicry, and identification of C3-based complement inhibitors. We have also included a section on basic C3 structure and biochemistry.

C3 structure, activation, and regulation

Human C3, the most abundant complement protein in serum (1.2 mg/ml), is comprised of α and β chains (M_r 110,000 and 75,000, respectively) (*Table 1*) that are connected covalently by a single disulfide bond and associated by non-covalent forces (*Fig. 1*). The protein is encoded by a 41 kb gene located on chromosome 19 (6). The C3 gene is composed of 41 exons (ranging in size from 52–213 bases), of which 16 encode the β chain and 25 encode the α chain. The primary structure of C3 as deduced from the cDNA sequence (7) consists of 1,663 amino acids, including a 22-amino acid signal peptide. C3 is synthesized as a single-chain pre-pro-molecule with α and β chains linked by a tetra-arginine sequence, which is removed by a furin-like enzyme (8) during post-translational modification; the order of the subunits is β–α. Following translocation through the endoplasmic reticulum to the Golgi, *N*-linked high-mannose carbohydrate moieties are attached at residues 917 of the α chain ($Man_8GlcNac_2$+$Man_9GlcNac_2$) and 63 of the β chain ($Man_5GlcNac_2$+$Man_6GlcNac_2$) (*Fig. 1*) and together account for 1.5% of the molecular weight of C3 (9, 10). A complete disulfide bridge pattern for C3 has been determined (11, 12) (*Fig. 1*): the α and β chains are linked by a single disulfide bridge. Three linkages are found in C3a and a single bridge is present in the β chain as well as the C3d portion of the α chain. The N- and C-terminal regions of the α chain are connected with each other via a disulfide linkage. Of interest is the fact that six linkages are clustered in the 46 kDa C-terminal peptide of the α chain. With the exception of the C3a (11) and C3d (13) regions of C3, the three-dimensional structure of C3 is not yet available. Although methylamine-treated C3 and C3b have been crystallized (14), data could be collected only at 7.7 Å resolution.

One of the most intriguing features of C3 is its ability to attach covalently to acceptor molecules on cell surfaces (15) via ester or amide linkages (16). This property of C3 is derived from the presence of an intramolecular thioester bond within the C3d region. The thioester bond is formed during post-translational modification as a result of intramolecular transacylation between the thiol group of cysteine and the

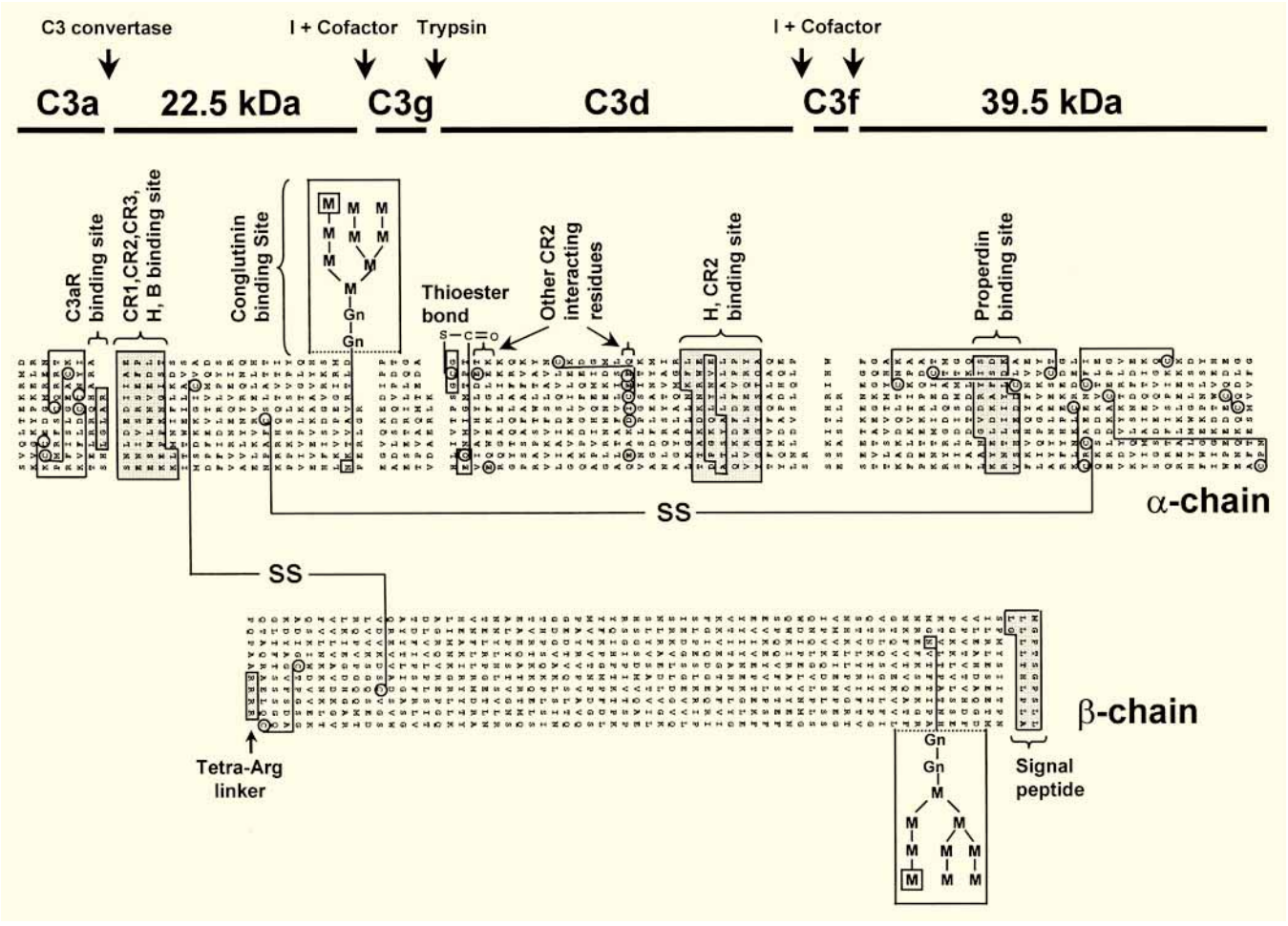

***Fig. 1.* Schematic representation of human C3.** The molecule consists of an α- and β-chain structure linked by a disulfide bond. Proteolytic cleavage sites, disulfide linkages and binding sites for receptors (C3aR, CR1, CR2, CR3), regulatory proteins (H and P), and binding proteins (factors B and conglutinin) are shown. N-linked high-mannose carbohydrates are located at residues 63 and 917.

γ-amide group of the glutamine within the C3 sequence Gly-Cys^{988}-Gly-Glu-Gln^{991}-Asn (*Fig. 1*) (17). Interestingly, the highly conserved thioester domain is encoded by class 1-1 exons, the type most commonly reshuffled and duplicated (18).

It is believed that in native C3 the thioester bond is protected within a hydrophobic pocket and is exposed only in the C3b fragment upon cleavage of C3 by C3 convertases. This belief is supported by the fact that the rate of spontaneous hydrolysis of the thioester bond is very slow under physiologic conditions and has been estimated to be 0.005%/min (19). It has been estimated that, at any given time, 0.5% of the total C3 present in fresh human plasma is present in its hydrolyzed form ($C3_{H_2O}$) (20). Once C3 is cleaved to C3b, the transiently exposed thioester bond in C3b (half life ~100 μs) participates in a transacylation reaction with nucleophilic groups present on cell surfaces, complex carbohydrates, or immune complexes (16, 21–24). In many biological systems the majority of C3b is linked via an ester bond indicating a strong preference for the hydroxylated targets.

Until recently, attachment of C3b to various acceptors had been considered a non-specific reaction; however, recent studies have clearly demonstrated that C3b displays a high degree of specificity in reacting with targets such as carbohydrates, C3b, C4b, and IgG (21, 22, 25, 26), and that this specificity can be an important factor in complement activation (21). Analysis of its reactivity with sugars and alcohols has clearly revealed that thioesters of C3 show a preference for the primary OH in the 6 position and the OH group next to the less bulky group. In addition, in human IgG_1 and C4b, Thr^{144} and Ser^{1213} respectively have been identified as the major, if not the only, sites with which C3b reacts (21, 22,

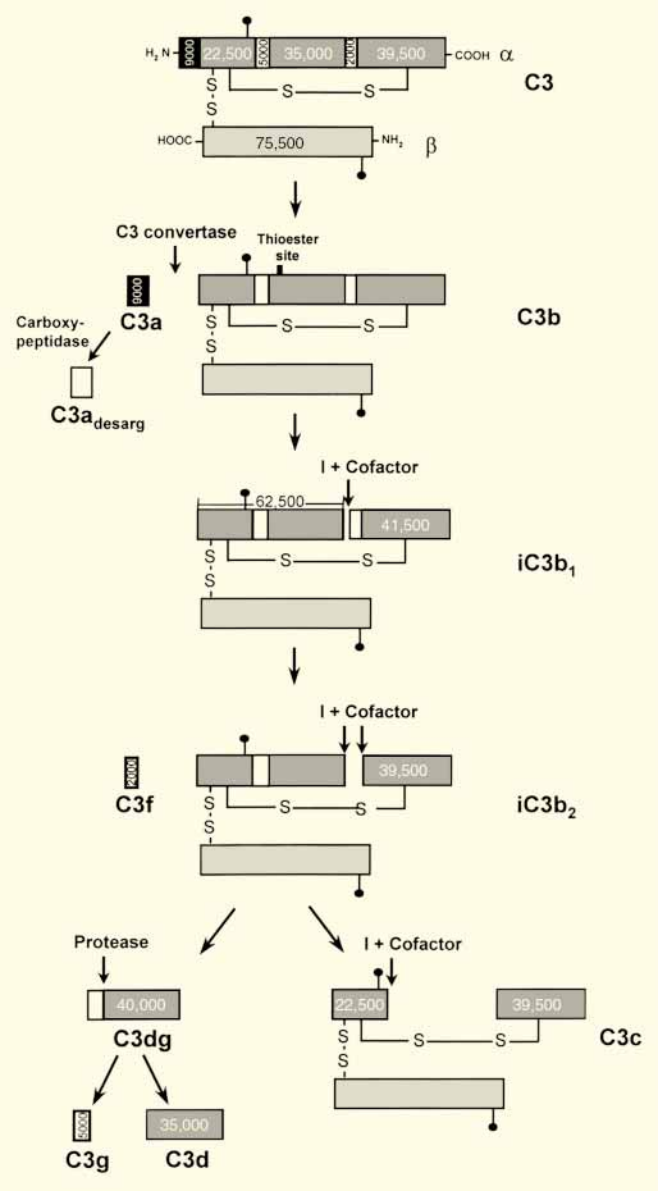

***Fig. 2.* Activation and degradation of C3.** The cleavage sites are indicated by arrows. The location of *N*-linked carbohydrate sites are indicated with closed balloons. The molecular weights of the polypeptides are calculated on the basis of their deduced amino acid sequences (7).

25). These findings demonstrate the selection by C3b of specific OH groups and specific residues on proteins. Thus, the belief that metastable C3b reacts randomly or non-specifically is apparently not correct. Having said this, it should be pointed out that although C3b shows a preference for certain hydroxyl groups, it does not have the ability to discriminate between self and non-self. Thus, in the absence of regulators, it can bind equally well to host cells, thereby causing damage to these cells. The attachment of C3b to acceptor molecules is necessary to initiate the formation of the membrane attack complex (MAC), phagocytosis of foreign particles, enhancement of humoral responses to antigens, and probably elimination of self-reactive B cells (27).

Proteolytic activation of native C3 by either the classical/lectin (C4b,2a) or alternative (C3b,Bb) pathway C3 convertases leads to cleavage between residues 726 and 727 (Arg–Ser) and generation of C3b (M_r 176,000) and C3a (M_r 9,000) (*Figs* 1 & 2). In contrast to native C3, C3b expresses multiple binding sites for other complement components, including C5; properdin (P); factors H, B, and I; complement receptor 1 (CR1); and the membrane co-factor protein (MCP) (28). Binding of these proteins to C3b leads either to amplification of the C3 convertase (by B and P in the presence of factor D) and initiation of the MAC (C5b-9) or to the inactivation of C3b (by factor I in the presence of H, CR1, and MCP) (1). Whether amplification or inactivation occurs depends on the nature of the surface to which the C3b is attached.

The downregulation of C3b by factor I proceeds in three steps (29) and requires one of the several co-factor molecules (MCP, CR1 or H) (*Figs* 1 & 2). Cleavage of the α′ chain of C3b occurs in two places: first between residues 1281 and 1282 (Arg–Ser) to generate $iC3b_1$ and then between residues 1298 and 1299 (Arg–Ser) to liberate the C3f fragment (Mr 2,000) and yield $iC3b_2$ (30–32). A third factor I cleavage site, with CR1 or factor H serving as the co-factor (30, 31), has been reported to exist at residues 932–933 (Arg–Glu) of the α chain of C3; cleavage here generates the C3c and C3dg fragments (32). It was previously thought that the first two cleavages are necessary to inactivate C3b, but recently our laboratory has shown that the first cleavage by itself is sufficient to inactivate C3b (33).

Studies of C3–ligand interactions

C3 is a large molecular weight protein (M_r 185,000) that interacts with at least 25 different soluble and membrane-bound proteins. Thus, identification of its binding sites for various ligands is a challenging task. Our laboratory has chosen a multiattack approach. 1) The first strategy involved localization of binding sites on C3 by studying the molecule at the protein level through degradation of C3 either enzymatically or chemically, analysis of protein fragments for binding to various C3-binding proteins, identification and characterization of the fragments, of interest and validation of the data using synthetic peptides and analogs. 2) The second approach originated at the DNA level and involved construction of an expression minilibrary (~200–300 bases) from

the cDNA spanning the entire coding sequence of C3 (34). The library was screened with anti-C3 antibodies known to block specific interactions and also with various proteins that bind C3. The reactive clones were sequenced, allowing the mapping of specific sites. 3) The third approach involved studies of the evolution and conservation of binding sites within the C3 molecule from various species and other homologous proteins, such as C4, C5, and α_2-macroglobulin. Here, comparison of the C3 amino acid sequences among species and correlation of this information with the ability of these C3s to bind various ligands and receptors were used to design site-directed mutagenesis and chimeric molecule experiments (29). A similar approach, referred to as the INDEL approach, was used by Ogata and colleagues to identify the functionally important regions of C3 and C5 (35, 36). Using the strategies outlined above, we have successfully identified the binding sites on C3 for properdin (37), bovine conglutinin (38), factor H (39), CR1 (40), and CR2 (41).

The plasma glycoprotein properdin stabilizes C3 convertase and allows rapid amplification of surface-bound C3b. Our efforts to localize the properdin-binding site in C3b were greatly assisted by a phylogenetic analysis of C3. Initial studies showed that properdin binds to both C3b and C3c (42), and subsequent studies have placed the properdin binding site within residues 1385–1541 of C3 (43). Comparison of the amino acid sequences of human, mouse, and rabbit C3 (all of which bind to human properdin) with those of human and mouse C4, C5, and α_2-macroglobulin (homologous but non-properdin-binding proteins) identified a region (residues 1402–1435 of the human C3 sequence) that was conserved and therefore a possible candidate for the properdin-binding site. We have shown that a synthetic peptide ($C3^{1402–1435}$) corresponding to this segment of C3 binds to properdin, inhibits properdin binding to C3, and inhibits the activation of the alternative pathway by rabbit erythrocytes (37). Our data reinforce the belief that properdin stabilization of the C3 convertase is necessary for efficient amplification of the enzyme cascade during alternative pathway complement activation and are in agreement with studies of patients with properdin deficiency, which have indicated that properdin is essential for optimal complement activation (44).

To date the N-terminal region (residues 727–768) of the α' chain of C3b is considered to be one of the most important regions of C3 because it is known to interact with multiple proteins. Using C3 fragments, synthetic peptides, and anti-peptide antibody, we and others (45, 46) have shown that this region encompasses the binding sites for CR1, factor H, and factor B. To further probe the importance of this region, in a subsequent study we have constructed chimeric molecules in which residues 727–768 were substituted with a corresponding segment of C3 from a species whose C3 either binds or does not bind to a specific ligand. In addition, we also engineered and expressed a deletion mutant in which residues 727–768 were deleted, and then asked: do the co-factor molecules (CR1, H, and MCP) bind to multiple sites on C3 in order for factor I to cleave C3b at multiple sites? Do the co-factor molecules (CR1, H and MCP) share the same binding sites on C3? Does factor B bind to the N-terminal region (residues 727–768) of the α'-chain of C3b?

A careful analysis of the data led us to conclude the following. 1) A major binding site for CR1 and factor H is located in the N-terminal region of the α' chain of C3b, and both proteins interact with C3b via a second site. This view is consistent with a recent report demonstrating involvement of different regions of C3 in factor H binding (47). 2) MCP protein binding sites in C3b differ from those for CR1 and H, thus supporting the idea that binding sites on C3b are different for some co-factors. 3) Although site-directed mutagenesis studies performed by others (46) have suggested an involvement of charged residues of the N-terminal region in factor B binding, other residues must also contribute significantly to factor B binding (29).

Recently, Oran & Isenman (48) confirmed our conclusion that residues 727–768 are not involved in the interaction with MCP. They also identified the residues of the N-terminal region that are important for factor H and CR1 interactions. Their study demonstrated that the factor H interaction involved residues E^{744} and E^{747}, whereas the CR1 interaction involved two classes of residues: The first class, consisting of the E^{736}/E^{737} pair, E^{747}, and the E^{754}/D^{755} pair, was found to be important for the first two factor I cleavages (that result in generation of $iC3b_2$); the second class, consisting of residue E^{744} and the pair K^{757}/E^{758}, were important for the third factor I cleavage (resulting in the generation of C3dg) (48).

In recent years the interaction of complement receptor 2 (CR2) with its ligand C3dg, a proteolytic fragment of C3, has received considerable attention not only from the scientists in the complement field but also from the researchers outside the field who are interested in B-cell biology. CR2 is a 140 kDa glycoprotein expressed on B cells, on some T cells, and on follicular dendritic cells (49–51). The involvement of complement in the humoral immune response was first observed more than 2 decades ago (5); however, this report did not receive much scientific attention. The recent availability of C3 knockout mice has allowed complementologists to revisit this issue. C3 knockout mice have been found to produce

an impaired antibody response to the T-cell-dependent ϕX 174 antigen, with a response characterized by a reduced number and size of germinal centers (52). Similarly, mice with a disrupted *CR2* locus showed impaired antibody response to ϕX 174 (53) and sheep red blood cells (54). In addition, administration of anti-CD21 antibody or soluble CD21 suppressed the *in vivo* immune response (55–57).

In general, invading pathogens, antigens, or immune complexes activate complement pathways, leading to covalent attachment of complement C3 to these pathogens/antigens/immune complexes (21, 22). Whether the antibody-enhancing function of complement is mediated primarily by attachment of complement C3 to antigen was elegantly investigated by Fearon and his colleagues (58). They coupled hen egg lysozyme (HEL) to multiple copies of C3d (a proteolytic fragment of C3dg) and injected this model antigen into mice without adding adjuvant. HEL attached to two or three copies of C3d was 1,000- or 10,000-fold more immunogenic, respectively, than HEL alone (58). Thus, it became clear from their study that C3d acts as a "natural adjuvant"

Previously we localized a CR2-binding site to residues 1201–1214 of the C3d region of C3 (41). Using a site-directed mutagenesis approach, Diefenbach & Isenman analyzed the involvement of eight of the 11 residues of this region (62). They found that substitution with alanine at positions 1999–1200 (ED), 1203–1204 (KQ) or 1207–1208 (NV) had no effect on the binding of C3 mutants to CR2, whereas a triple mutant containing substitutions at all six residues showed a 20% reduction in binding as compared to that for wild-type C3. From these data they inferred that this region is not involved in CR2 binding. However, several other studies point toward the involvement of this region in CR2 binding. a) A synthetic peptide corresponding to this region binds to Raji cells in a CR2-dependent manner (41). b) The CR2-binding site in Epstein–Barr virus (EBV) gp350/220 (a viral protein that binds to CR2) is homologous to these residues, and mutation in this region of the intact EBV gp350/220 molecule abolishes the binding to CR2 (61). c) In the study by Diefenbach & Isenman (62), a mutant that is substituted at six residues (ED, KQ and NV) with alanine displayed a 20% reduction in binding to CR2. d) It has been demonstrated that C3d peptide K^{1195}-A^{1210} when coupled to anti-idiotype antibody, induces a strong idiotype and antigen-specific response (59).

Using human/trout as well as trout/human C3dg chimeras, we recently found that both the N- and C-terminal halves of human C3dg are important for CR2 binding (63), and that a number of weak interactions may have a cumulative effect on C3dg binding to CR2. Very recently, Clemenza et al. (64) studied the role of charged residues in C3dg in the interaction with CR2. Their data suggested that mutation at charged residues E^{1008}/E^{1010} pair, E^{1131}, D^{1134} and E^{1137}, and I^{1135} results in a significant loss in CR2 binding. It should be noted that the human/trout chimera, which contains all these residues, binds only at low ionic strength, suggesting that residues other than these six must be involved. We believe that detailed structural design of C3d mutants based on the crystal structure and species specificity data, together with analysis of the X-ray structure of informative mutants, as well as the development of technologies that will allow measurement of the dynamics of protein–protein interactions should give us a global picture of the C3d–CR2 interaction.

C3 origin, evolution, and diversity

A thorough understanding of any biological system requires detailed knowledge of its origin, evolution, and diversity. Since C3 is essential for activation of all three complement pathways, it is not unreasonable to expect that understanding its origin would shed light on the origin of the complement system.

At present, a prevailing hypothesis is that complement proteins C3, C4, and C5 were generated by gene duplication from α_2-macroglobulin. According to this hypothesis, one of the duplicated α_2-macroglobulin genes was modified to form a *C3/C4/C5* ancestral gene, which was then duplicated a second time to form the *C4* and *C3/C5* genes. Finally, the *C3/C5* gene was duplicated to produce the *C3* and *C5* genes. This hypothesis is based on the sequence similarity and exon–intron organization of these proteins (65–67). It is, however, not known at what point in evolution the first gene duplication took place. Thus, probing the molecular structure and functions of these proteins at various stages of the phylogenetic tree should lead to a detailed understanding of the origin of these proteins in particular and of the complement system in general.

To study the evolution of C3 and to probe the origin of C3 we characterized C3 from a number of animal species. Research in this direction suggested that in lower vertebrates, complement may compensate for primitive adaptive response by providing greater diversity (68). Over the course of 15 years, we have purified C3 molecules from pig, rabbit, mouse, chicken, cobra, *Xenopus*, axolotl, sea bream, trout, and sea urchin and have studied their biochemical and functional properties. In addition, we have cloned the genes encoding chicken (69) and *Xenopus* C3 (70) as well as three trout (*Salmo gairdneri*) isoforms of C3 (C3-1, C3-3 and C3-4) (71).

During the process of cloning the gene encoding trout C3 (now named C3-1), we obtained a PCR product whose deduced amino acid sequence was only 50% similar to the corresponding sequence of trout C3-1. These results prompted us to hypothesize that trout contain multiple forms of C3s/C3-related proteins. This finding was particularly noteworthy because all the functionally active C3 molecules that had been isolated from various species until that time were thought to be the products of single genes. Our effort to isolate these molecules revealed the presence of two new functionally active isoforms of C3 (C3-3 and C3-4) in trout (72). Efforts in other laboratories led to the identification of an additional isoform, C3-2, which was functionally inactive and showed a tryptic map that differed by 20% from that of C3-1 (73).

In the carp (*Cyprinus carpio*) at the DNA level, as many as eight different polymerase chain reaction clones showing 85–90% amino acid identity with each other and homology to C3 from other species have been characterized (74). Although it is not certain at this point whether these are allelic variants or products of different genes, all the clones were derived from a cDNA library derived from a single fish. Trout and carp are tetraploid fish; therefore, it appeared that the presence of multiple forms of C3 might be unique to the tetraploid condition. However, multiple forms of C3 were also found in the gilthead sea bream (*Sparus aurata*) and medaka fish (*Oryzias latipes*), both of which are diploid teleost fish, suggesting that this phenomenon is not unique to the tetraploid fish. This finding also suggests that genes encoding for these isoforms must have been fixed into the genome of these animals. We would like to point out here that the presence of multiple forms of proteins in these fish is not restricted to C3, since two forms of factor B/C2 molecules have been identified in trout (75). It would be interesting to study the mechanism through which these multiple forms of complement proteins have been generated.

Since C3 is present in multiple forms in fish, the obvious question is: do these C3s differ functionally? Analysis of the binding of these C3 isoforms to various complement-activating surfaces has shown that these C3s bind with different efficiencies to various surfaces. For example, in the trout, all three C3 isoforms bind to various erythrocyte surfaces and to *E. coli* to varying degrees, but only C3-1 binds to zymosan. Similarly, in the gilthead sea bream, C3-1 and C3-2 bind to zymosan, whereas C3-3, C3-4, and C3-5 fail to bind (76). These results suggest that the specificity of the thioester bond in these C3s varies considerably. Such a precedent has already been established in the case of human C4 isotypes: human C4A shows strong reactivity with amino groups, whereas C4B displays a preference for hydroxyl groups (77, 78). Analysis of the binding mechanism at the molecular level has indicated that C4B uses a two-step mechanism.Upon C4B activation, H^{1106} attacks the internal thioester to form an acyl–imidazole bond. The released thiol then acts as a base to catalyze the transfer of the acyl group from the imidazole to the hydroxyl of the acceptor molecule. Such reactions are also known to occur in the case of C3 (79).

Alignment of trout C3 isoforms with various C3s and human C4B has shown that the His^{1126} residue (equivalent to H^{1106} of human C4B) is conserved in C3-1 and C3-3 but is substituted by Thr in C3-4. Thus, it is possible that the presence of the His residue in C3-1 and C3-3 would generate a preference for hydroxyl groups. Recently it has also been shown that the Glu residue located two amino acids downstream from the His^{1126} forms a hydrogen bond with H^{1126}, which renders H^{1126} a stronger nucleophile. This situation may increase the rate of acyl–imidazole formation in C3 and promote its specificity for hydroxyl groups, in comparison to C4B (H^{1126}, Ser^{1128}) (13). Alignment of trout C3s with human C3 has shown that although this residue is conserved in trout C3-1, it is substituted by Thr and Ser in C3-3 and C3-4, rendering C3-1 more like human C3. Whether these differences in the isoforms indeed correlate with differences in thioester reactivity among the various trout C3 molecules requires further investigation.

It is now clear that both tetraploid and diploid fish contain multiple forms of C3, a situation which has led us to ask why fish demonstrate such structural and functional C3 diversity. As previously mentioned, structural diversity in C3 allows these molecules to recognize a broader range of activating surfaces. Thus, it is reasonable to presume that this diversity would allow C3 to react with a wider repertoire of microorganisms and would therefore enhance fish's defense against pathogens (68). This diversity could also play a vital role in the survival of the animals, because in fish the antibody response is represented only by IgM antibodies. Moreover, these antibodies are of low affinity and limited heterogeneity, and their response is impaired during the winter. In contrast, the complement system in these animals can be activated in temperatures as low as 4°C (80).

Virus–C3 interactions

Unlike other pathogens, viruses have to depend on their hosts for their replication. Thus, in order to survive and multiply they must overcome the formidable defense mechanisms of

their hosts, including the complement system, which is one of the major defense mechanism against viruses. The complement system serves as both an innate and an acquired immune defense against viral infection. Activation of complement in the presence or absence of antibodies can lead to virus neutralization, phagocytosis of C3b-coated viral particles, lysis of infected cells, and generation of inflammatory and specific immune responses. During the co-evolution of the viruses with their hosts over several million years, viruses have not only developed mechanisms to control the complement system but have also turned these interactions to their own advantage. Since C3 is a common denominator in the activation of all three pathways of the complement system, it became the obvious target for viruses (81). Important examples of viruses that have developed mechanisms against C3 include vaccinia, herpes simplex virus (HSV) type 1, and type 2 (HSV-2).

Vaccinia virus encodes a protein that is homologous to human complement control proteins. This protein, named the vaccinia virus complement control protein (VCP) (82), is one of the two major proteins secreted by vaccinia virus-infected cells. VCP received scientific attention when it was discovered that an attenuated mutant of vaccinia virus does not secrete this protein. Sequence analysis revealed that it is structurally related to members of the RCA family (82). VCP, which is encoded by the *C3L* open reading frame (ORF) of the vaccinia genome, is a 27 kDa protein that is composed entirely of four tandemly repeating domains called short consensus repeats (SCRs) or complement control protein repeats (CCPs) (83). VCP apparently protects the infected cells and released virions from attack by host complement. Evidence for this theory has come from experiments in which VCP was shown to abrogate complement-mediated antibody-dependent neutralization of vaccinia virions (84). In addition, studies using recombinant vaccinia viruses that do not express VCP have clearly shown that these viruses are attenuated *in vivo* (84). A culture medium containing secreted VCP has been shown to inhibit complement-mediated lysis of sheep erythrocytes, to bind to C3b and C4b, and to accelerate the decay of the classical as well as the alternative pathway C3 convertases (83, 85).

To understand the detailed mechanisms by which VCP inactivates complement, our laboratory has generated a recombinant form of VCP (33). A comparison of its effect on complement to that of human factor H and sCR1 has revealed that the recombinant VCP is less effective than CR1 in inhibiting the classical and alternative pathways of complement and less effective than factor H in inhibiting the alternative pathway. However, it is noteworthy that on a molar basis this VCP was four times more effective than factor H in inhibiting the classical pathway, possibly because of its dual action on C3 and C4. Our study also demonstrated that VCP supports the factor I-mediated cleavages of C3b and C4b. It is known that CR1 and factor H support the factor I-mediated cleavage of C3b between $Arg^{1281}$and Ser^{1282} (site 1), $Arg^{1298}$and Ser^{1299} (site 2), and $Arg^{932}$and Glu^{933} (site 3) (*Figs* 1 & 2) (1). Analysis of VCP-supported cleavages of C3b showed that it primarily serves as a co-factor for the first site, leading to the generation of C3b through a single cleavage ($iC3b_1$) (33). The factor I co-factor activity of VCP for C4b was similar to that of CR1. Purification and functional analysis of the VCP-generated $iC3b_1$ showed that it was unable to interact with factor B to form the alternative pathway C3 convertase (C3b,Bb) (33). These results demonstrate that the interaction of VCP with C3 is different from that of all the other factor I co-factors characterized to date.

Recent studies have shown that extracellular vaccinia virus incorporates host complement control proteins into its envelope (86), suggesting that apart from VCP it possesses additional mechanisms to evade complement attack. Like VCP, proteins that have four SCRs and are also homologous to complement control proteins have been found in various members of the poxvirus family, including smallpox virus (87, 88), herpesvirus saimiri (HVS) (89, 90), Kaposi's sarcoma-associated herpesvirus (HHV-8) (91), and murine γ-herpesvirus 68 (92). Currently, our laboratory is attempting to decipher the role of complement control protein homologs of HVS and HHV-8 in immune evasion.

Viruses have also developed proteins that are structurally different from CCP but also function to inactivate complement. Examples include glycoprotein C (gC) molecules of HSV-1 and HSV-2, which are expressed on the virion envelope and on the surface of infected cells (93–96). Glycoprotein C of HSV-1 (gC-1) and HSV-2 (gC-2) are highly glycosylated proteins with numerous O-linked oligosaccharides and nine and seven potential sites for N-linked glycosylation, respectively (97). Both proteins bind to C3b when they are expressed on the surface of transfected cells or as purified proteins (98). It is interesting to note that unlike other regulators of complement activation (factor H, MCP, decay acceleration factor (DAF) and CR1), which interact with C3b, both gC-1 and gC-2 bind to native C3 (99).

Studies on the mechanism of gC interaction with complement have shown that gC-1, like other regulators of complement activation (factor H, CR1, and DAF), accelerates the decay of a bimolecular C3 convertase (C3b,Bb) into its subunits;

however, in contrast to factor H and CR1, it does not mediate the proteolytic inactivation of C3b by factor I (100). It has also been shown that gC-1, but not gC-2, can inhibit the binding of C5 to C3b and destabilize C3 convertase by inhibiting the binding of properdin to C3b (101). Structure–function analysis of the gC-1 molecule has shown that the C5 and P-blocking domain is located near the amino terminus, while the C3-binding domain is found in the central region of the molecule (99, 101). Recent *in vivo* studies on the role of complement-interacting domains of gC-1 has clearly shown that the C3 binding domain is more important than the C5/P-blocking domain and is a major contributor to complement evasion (102).

In addition to the above-mentioned mechanisms, some viruses have also developed ways to mimic C3 and interact with complement receptors to aid their entry into cells (103). One of the important and well-studied examples of this group is the EBV, which infects B cells and epithelial cells through CR2 (104). The protein on EBV that is responsible for binding to CR2 is gp350/220; both C3d (a natural ligand of CR2) and gp350/220 interact with first two SCRs of CR2 (105). As described before, one of the binding sites in C3d is located between residues 1201 and 1214 (PGKQLYNVEATSYA) (41). A sequence similar to this C3d sequence has also been identified in gp350/220, suggesting that the C3d and EBV binding sites on CR2 are either identical or conformationally related. A series of studies using monoclonal antibodies, purified proteins, and site-directed mutagenesis of the proposed sequence has confirmed that the binding site for EBV on CR2 is located within the C3dg-like sequence of gp350/220 (61). Other viruses that use complement receptors for cellular entry include measles, echo, and West Nile viruses. These viruses use membrane cofactor protein, DAF, and complement receptor type 3, respectively, to initiate infection (103, 106, 107).

C3-based inhibitors

Complement proteins that are involved in the activation process, including C3, do not have the ability to discriminate between the self and the non-self. Thus, they have the potential to destroy any cells to which they bind, including those of the host. As a means of preventing this destructive activity, the complement system is tightly controlled by regulatory proteins such as C1 inhibitor, factor H, C4bp, CR1, DAF, MCP, carboxypeptidase N, and CD59. Therefore, it is not surprising that unregulated activation of the complement system leads to host cell damage (108, 109).

At present many basic research scientists and applied scientists in the biotechnology field and larger pharmaceutical firms are interested in developing complement inhibitors that would block various steps of its activation. We have focused our attention on C3, since inactivation of this protein would lead to inhibition of C3a and C5a generation and of C5b-9 formation, all of which are implicated in complement-mediated damage to host cell (110). Although others are developing high molecular weight complement proteins (CR1, DAF, and MCP) to block complement-mediated damage, we are interested in developing small molecular weight inhibitors because they are cost-effective, have better tissue penetration, and can be developed for oral use.

In our initial efforts we used combinatorial peptide libraries to identify C3-interactive peptides, with the goal of identifying C3-binding peptides that would functionally mimic other C3-regulating proteins. Our approach led to the identification of a novel 13-residue cyclic peptide (111), later named compstatin. Unlike natural inhibitors of complement that act on C3b, compstatin binds to native C3 and inhibits its cleavage by C3 convertase (111). Most importantly, this inhibition is not caused by sterically hindered access to the C3a/C3b cleavage site (111). Recently, an independent study has confirmed our results (112). We have observed that the kinetics of compstatin binding to native C3 do not follow a 1:1 Langmuir binding model; instead, the binding data fits well to a two-state conformational change model. Whether such a binding mechanism is important for compstatin's activity needs further investigation (113).

Thus far, compstatin has been tested in three different clinically relevant models. 1) Hyperacute rejection in discordant kidney xenotransplantation has been studied *ex vivo* in a porcine-to-human perfusion model. In this model, compstatin significantly prolonged the survival of the kidneys (114, 115). 2) The effect of compstatin has also been tested in models of extracorporeal circulation (116), where it effectively inhibited the generation of C3a and sC5b-9 and the binding of C3/C3 fragments to a polymer surface. As a result of the inhibition of complement activation, the activation of polymorphonuclear leukocytes (as assessed by the expression of CD11b) and the binding of these cells ($CD16^+$) to the polymer surface were almost completely lost (116). 3) Most recently, compstatin has been tested *in vivo* in primates to examine its effect on complement activation induced by a heparin-protamine complex; here it effectively inhibited complement activation (117).

Structure-based rational design of peptidomimetics and crafting of small molecule inhibitors requires knowledge of the complete 3D of the peptide inhibitor and the target protein. We have achieved the first step towards this goal by de-

termining the 3D structure of a major conformer of compstatin in solution by two-dimensional NMR (118). Although the peptide in its current form is effective *in vivo*, the structural information obtained for compstatin is being used for the rational design of a small-molecule inhibitor that can be administered orally.

Concluding remarks

As described above, a wealth of information has been obtained regarding the structure of C3 and its interactions with ligands. Nevertheless, the information obtained is essentially one-dimensional, in part because C3 is a large molecular weight protein and, with the exception of C3a and the C3d region of C3 its three-dimensional (3D) structure is still not available. The studies conducted thus far have utilized a variety of approaches to define the binding sites on C3, including generation of synthetic peptides corresponding to various regions of C3, development of monoclonal and site-specific antipeptide antibodies against C3, site-directed mutagenesis, and expression of chimeric molecules. Each of these methods has its limitations. Therefore, conclusions concerning the involvement of a particular region in ligand binding can be drawn only if the various methods point in a single direction. We believe that the identification of the functional domains of C3 by the methods discussed above, together with a determination of their 3D structure and the development of technologies that will allow determination of the dynamics of protein–protein interactions are needed if we are to gain a complete understanding of C3–ligand interactions. Although this approach is ambitious, it is the only way to obtain 3D structural information regarding a large, architecturally complex and multifunctional protein such as C3.

Because C3 emerged millions of year ago, it is reasonable to hypothesize that it may participate in "non-immune" mechanisms. For example, it has recently been shown to be expressed in the regenerating limb blastema cells of urodeles (119), suggesting that it plays a role in regenerative processes. In an another study, C5 has been implicated in liver regeneration (120). Further studies in this direction may shed light on "new" functions of C3 in particular and of the complement system in general.

References

1. Lambris JD, Sahu A, Wetsel R. The chemistry and biology of C3,C4, and C5. In: Volanakis JE, Frank M, eds. The human complement system in health and disease. New York: Marcel Dekker Inc; 1998. p. 83–118.
2. Al-Sharif WZ, Sunyer JO, Lambris JD, Smith LC. Sea urchin coelomocytes specifically express a homologue of the complement component C3. J Immunol 1997;**160**:2983–2997.
3. Nonaka M. Phylogeny of the complement system. In: Volanakis JE, Frank MM, eds. The human complement system in health and disease. New York: Marcel Dekker Inc; 1998. p. 203–215.
4. Rapp HJ. Mechanism of immune hemolysis: recognition of two steps in the conversion of $EAC'_{1,4,2}$ to E*. Science 1958;**127**:234–236.
5. Pepys MB. Role of complement in induction of antibody production *in vivo*. Effect of cobra factor and other C3-reactive agents on thymus-dependent and thymus-independent antibody responses. J Exp Med 1974;**140**:126–145.
6. Whitehead AS, Solomon E, Chambers S, Bodmer WF, Povey S, Fey G. Assignment of the structural gene for the third component of complement to chromosome 19. Proc Natl Acad Sci USA 1982;**79**:5021–5025.
7. De Bruijn MHL, Fey GH. Human complement component C3: cDNA coding sequence and derived primary structure. Proc Natl Acad Sci USA 1985;**82**:708–712.
8. Misumi Y, Oda K, Fujiwara T, Takami N, Tashiro K, Ikehara Y. Functional expression of furin demonstrating its intracellular localization and endoprotease activity for processing of proalbumin and complement pro-C3. J Biol Chem 1991;**266**:16954–16959.
9. Hase S, Kikuchi N, Ikenaka T, Inoue K. Structures of sugar chains of the third component of human complement. J Biochem (Tokyo) 1985;**98**:863–874.
10. Hirani S, Lambris JD, Müller-Eberhard HJ. Structural analysis of the asparagine-linked oligosaccharides of human complement component C3. Biochem J 1986;**233**:613–616.
11. Huber R, Scholze H, Paques EP, Deisenhofer J. Crystal structure analysis and molecular model of human C3a anaphylatoxin. Hoppe Seylers Z Physiol Chem 1980;**361**:1389–1399.
12. Dolmer K, Sottrup-Jensen L. Disulfide bridges in human complement component C3b. FEBS Lett 1993;**315**:85–90.
13. Nagar B, Jones RG, Diefenbach RJ, Isenman DE, Rini JM. X-ray crystal structure of C3d: a C3 fragment and ligand for complement receptor 2. Science 1998;**280**:1277–1281.
14. Dolmer K, Thirup S, Andersen GR, Sottrup-Jensen L, Nyborg J. Crystallization of human methylamine-treated complement C3 and C3b. Acta Crystallogr 1994;**D50**:786–789.
15. Müller-Eberhard HJ, Dalmasso AP, Calcott MA. The reaction mechanism of β1c-Globulin (C′3) in immune hemolysis. J Exp Med 1966;**123**:33–54.

16. Law SKA, Dodds AW. The internal thioester and the covalent binding properties of the complement proteins C3 and C4.
Protein Sci 1997;**6**:263–274.
17. Tack BF, Harrison RA, Janatova J, Thomas ML, Prahl JW. Evidence for presence of an internal thiolester bond in third component of human complement.
Proc Natl Acad Sci USA 1980;**77**:5764–5768.
18. Fong KY, Botto M, Walport MJ, So AK. Genomic organization of human complement component C3.
Genomics 1990;**7**:579–586.
19. Pangburn MK, Schreiber RD, Müller-Eberhard HJ. Formation of the initial C3 convertase of the alternative pathway: acquisition of C3b-like activities by spontaneous hydrolysis of the putative thioester in native C3.
J Exp Med 1981;**154**:856–867.
20. Hack CE, Paardekooper J, Van Milligen F. Demonstration in human plasma of a form of C3 that has the conformation of "C3b-like C3".
J Immunol 1990;**144**:4249–4255.
21. Sahu A, Kozel TR, Pangburn MK. Specificity of the thioester-containing reactive site of human C3 and its significance to complement activation.
Biochem J 1994;**302**:429–436.
22. Sahu A, Pangburn MK. Covalent attachment of human complement C3 to IgG: identification of the amino acid residue involved in ester linkage formation.
J Biol Chem 1994;**269**:28997–29002.
23. Sahu A, Pangburn MK. Tyrosine is a potential site for covalent attachment of activated complement component C3.
Mol Immunol 1995;**32**:711–716.
24. Sahu A, Pangburn MK. Investigation of mechanism-based inhibitors of complement targeting the activated thioester of human C3.
Biochem Pharmacol 1996;**51**:797–804.
25. Kim YU, et al. Covalent binding of C3b to C4b within the classical complement pathway C5 convertase: determination of amino acid residues involved in ester linkage formation.
J Biol Chem 1992;**267**:4171–4176.
26. Kinoshita T, Takata Y, Kozono H, Takeda J, Hong K, Inoue K. C5 convertase of the alternative complement pathway: covalent linkage between two C3b molecules within the trimolecular complex enzyme.
J Immunol 1988;**141**:3895–3901.
27. Carroll MC. The role of complement and complement receptors in induction and regulation of immunity.
Annu Rev Immunol 1998;**16**:545–568.
28. Lambris JD. The multifunctional role of C3, the third component of complement.
Immunol Today 1988;**9**:387–393.
29. Lambris JD, Lao Z, Oglesby TJ, Atkinson JP, Hack E, Becherer JD. Dissection of CR1, factor H, MCP, and factor B binding and functional sites in third complement component.
J Immunol 1996;**156**:4821–4832.
30. Ross GD, Lambris JD, Cain JA, Newman SL. Generation of three different fragments of bound C3 with purified factor I or serum. I. Requirements for factor H vs CR1 cofactor activity.
J Immunol 1982;**129**:2051–2060.
31. Medicus RG, Melamed J, Arnaout MA. Role of human factor I and C3b receptor in the cleavage of surface-bound C3b.
Eur J Immunol 1983;**13**:465–470.
32. Davis AEI, Harrison RA. Structural characterization of factor I mediated cleavage of the third component of complement.
Biochemistry 1982;**21**:5745–5749.
33. Sahu A, Isaacs SN, Soulika AM, Lambris JD. Interaction of vaccinia virus complement control protein with human complement proteins: factor I-mediated degradation of C3b to $iC3b_1$ inactivates the alternative complement pathway.
J Immunol 1998;**160**:5596–5604.
34. Nilsson B, Grossberger D, Nilsson Ekdahl K, Riegert P, Becherer D, Lambris JD. Conformational differences between surface-bound and fluid-phase complement-component-C3 fragments. Epitope mapping by cDNA expression.
Biochem J 1992;**282**:715–721.
35. Low PJ, Ai R, Ogata RT. Active sites in complement components C5 and C3 identified by proximity to indels in the C3/4/5 protein family.
J Immunol 1999;**162**:6580–6588.
36. Ogata RT, Ai R, Low PJ. Active sites in complement component C3 mapped by mutations at indels.
J Immunol 1998;**161**:4785–4794.
37. Daoudaki ME, Becherer JD, Lambris JD. A 34-amino acid peptide of the third component of complement mediates properdin binding [published erratum appears in J Immunol 1988;141:1788].
J Immunol 1988;**140**:1577–1580.
38. Hirani S, Lambris JD, Müller-Eberhard HJ. Localization of the conglutinin binding site on the third component of human complement.
J Immunol 1985;**134**:1105–1109.
39. Lambris JD, Avila D, Becherer JD, Müller-Eberhard HJ. A discontinuous factor H binding site in the third component of complement as delineated by synthetic peptides.
J Biol Chem 1988;**263**:12147–12150.
40. Becherer JD, Lambris JD. Identification of the C3b receptor-binding domain in third component of complement.
J Biol Chem 1988;**263**:14586–14591.
41. Lambris JD, Ganu VS, Hirani S, Müller-Eberhard HJ. Mapping of the C3d receptor (CR2)-binding site and a neoantigenic site in the C3d domain of the third component of complement.
Proc Natl Acad Sci USA 1985;**82**:4235–4239.
42. Chapitis J, Lepow IH. Multiple sedimenting species of properdin in human and interaction of purified properdin with the third component of complement.
J Exp Med 1976;**143**:241–257.
43. Lambris JD, Alsenz J, Schulz TF, Dierich MP. Mapping of the properdin-binding site in the third component of complement.
Biochem J 1984;**217**:323–326.
44. Braconier JH, Sjoholm AG, Soderstrom C. Fulminant meningococcal infections in a family with inherited deficiency of properdin.
Scand J Infect Dis 1983;**15**:339–344.
45. Fishelson Z. Complement-C3 – a molecular mosaic of binding sites.
Mol Immunol 1991;**28**:545–552.
46. Taniguchi-Sidle A, Isenman DE. Interactions of human complement component C3 with factor B and with complement receptors type 1 (CR1, CD35) and type 3 (CR3, CD11b/CD18) involve an acidic sequence at the N-terminus of C3 α-chain.
J Immunol 1994;**153**:5285–5302.
47. Jokiranta TS, Hellwage J, Koistinen V, Zipfel PF, Meri S. Each of the three binding sites on complement factor H interacts with a distinct site on C3b.
J Biol Chem 2000;**275**:27657–27662.
48. Oran AE, Isenman DE. Identification of residues within the 727–767 segment of human complement component C3 important for its interaction with factor H and with complement receptor 1 (CR1, CD35).
J Biol Chem 1999;**274**:5120–5130.
49. Ross GD, Polley MJ. Specificity of human lymphocyte complement receptors.
J Exp Med 1975;**141**:1163–1180.

50. Reynes M, et al. Human follicular dendritic cells express CR1, CR2, and CR3 complement receptor antigens. J Immunol 1985;**135**:2687–2694.
51. Fischer E, Delibrias C, Kazatchkine MD. Expression of CR2 (the C3dg/EBV receptor, CD21) on normal human peripheral blood T lymphocytes. J Immunol 1991;**146**:865–869.
52. Fischer MB, et al. Regulation of the B cell response to T-dependent antigens by classical pathway complement. J Immunol 1996;**157**:549–556.
53. Ahearn JM, et al. Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. Immunity 1996;**4**:251–262.
54. Molina H, et al. Markedly impaired humoral immune response in mice deficient in complement receptors 1 and 2. Proc Natl Acad Sci USA 1996;**93**:3357–3361.
55. Heyman B, Wiersma EJ, Kinoshita T. *In vivo* inhibition of the antibody response by a complement receptor-specific monoclonal antibody. J Exp Med 1990;**172**:665–668.
56. Hebell T, Ahearn JM, Fearon DT. Suppression of the immune response by a soluble complement receptor of B lymphocytes. Science 1991;**254**:102–105.
57. Kinoshita T, et al. Characterization of murine complement receptor type 2 and its immunological cross-reactivity with type 1 receptor. Int Immunol 1990;**2**:651–659.
58. Dempsey PW, Allison MED, Akkaraju S, Goodnow CC, Fearon DT. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. Science 1996;**271**:348–350.
59. Lou D, Kohler H. Enhanced molecular mimicry of CEA using photoaffinity crosslinked C3d peptide. Nat Biotechnol 1998;**16**:458–462.
60. Ross TM, Bright RA, Robinson HL. C3d enhancement of antibodies to hemagglutinin accelerates protection against influenza virus. Nat Immunol 2000;**1**:127–131.
61. Tanner J, Whang Y, Sample J, Sears A, Kieff E. Soluble gp350/220 and deletion mutant glycoprotein block Epstein–Barr virus adsorption to lymphocytes. J Virol 1988;**62**:4452–4464.
62. Diefenbach RJ, Isenman DE. Mutation of residues in the C3dg region of human complement component C3 corresponding to a proposed binding site for complement receptor type 2 (CR2, CD21) does not abolish binding of iC3b or C3dg to CR2. J Immunol 1995;**154**:2303–2320.
63. Irani VR, Lambris JD. Studies on the interaction of C3dg with CR2. FASEB J 1999;**13**:A282–A282.
64. Clemenza L, Isenman DE. Structure-guided identification of C3d residues essential for its binding to complement receptor 2 (CD21). J Immunol 2000;**165**:3839–3848.
65. Vik DP, et al. Structural features of the human C3-gene – intron/exon organization, transcriptional start site, and promoter region sequence. Biochemistry 1991;**30**:1080–1085.
66. Carney DF, Haviland DL, Noack D, Wetsel RA, Vik DP, Tack BF. Structural aspects of the human C5-gene – intron/exon organization, 5′-flanking region features, and characterization of two truncated cDNA clones. J Biol Chem 1991;**266**:18786–18791.
67. Ogata RT, Rosa PA, Zepf NE. Sequence of the gene for murine complement component C4. J Biol Chem 1989;**264**:16565–16572.
68. Sunyer JO, Zarkadis IK, Lambris JD. Complement diversity: a mechanism for generating immune diversity? Immunol Today 1998;**19**:519–523.
69. Mavroidis M, Sunyer JO, Lambris JD. Isolation, primary structure, and evolution of the third component of chicken complement and evidence for a new member of the α_2-macroglobulin family. J Immunol 1995;**154**:2164–2174.
70. Lambris JD, et al. The third component of *Xenopus* complement. cDNA cloning, structural and functional analysis, and evidence for an alternate C3 transcript. Eur J Immunol 1995;**25**:572–578.
71. Zarkadis IK, Sarrias MR, Sfyroera G, Sunyer JO, Lambris JD. Cloning and structure of three rainbow trout C3 molecules: a plausible explanation for their functional diversity. Dev Comp Immunol 2001;**25**:11–24.
72. Sunyer JO, Zarkadis IK, Sahu A, Lambris JD. Multiple forms of complement C3 in trout, that differ in binding to complement activators. Proc Natl Acad Sci USA 1996;**93**:8546–8551.
73. Nonaka M, Irie M, Tanabe K, Kaidoh T, Natsuume-Sakai S, Takahashi M. Identification and characterization of a variant of the third component of complement (C3) in rainbow trout (*Salmo gairdneri*) serum. J Biol Chem 1985;**260**:809–814.
74. Nakao K, Obo R, Mutsuro J, Yano T. Sequence diversity of cDNA encoding the third component (C3) of carp (*Cyprinus carpio*). Dev Comp Immunol 1997;**21**:144.
75. Sunyer JO, Zarkadis I, Sarrias MR, Hansen JD, Lambris JD. Cloning, structure, and function of two rainbow trout Bf molecules. J Immunol 1998;**161**:4106–4114.
76. Sunyer JO, Tort L, Lambris JD. Structural C3 diversity in fish – characterization of five forms of C3 in the diploid fish *Sparus aurata*. J Immunol 1997;**158**:2813–2821.
77. Isenman DE, Young JR. The molecular basis for the difference in immune hemolysis activity of the Chido and Rodgers isotypes of human complement component C4. J Immunol 1984;**132**:3019–3027.
78. Law SKA, Dodds AW, Porter RR. A comparison of the properties of two classes, C4A and C4B, of the human complement component C4. EMBO J 1984;**3**:1819–1823.
79. Gadjeva M, Dodds AW, Taniguchi-Sidle A, Willis AC, Isenman DE, Law SK. The covalent binding reaction of complement component C3. J Immunol 1998;**161**:985–990.
80. Saha K, Dash K, Sahu A. Antibody dependent haemolysin, complement and opsonin in sera of a major carp, *Cirrhina mrigala* and catfish, *Clarias batrachus* and *Heteropneustes fossilis*. Comp Immunol Microbiol Infect Dis 1993;**16**:323–330.
81. Sahu A, Sunyer JO, Moore WT, Sarrias MR, Soulika AM, Lambris JD. Structure, functions, and evolution of the third complement component and viral molecular mimicry. Immunol Res 1998;**17**:109–121.
82. Kotwal GJ, Moss B. Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. Nature 1988;**335**:176–178.
83. Kotwal GJ, Isaacs SN, Mckenzie R, Frank MM, Moss B. Inhibition of the complement cascade by the major secretory protein of vaccinia virus. Science 1990;**250**:827–830.

84. Isaacs SN, Kotwal GJ, Moss B. Vaccinia virus complement-control protein prevents antibody-dependent complement-enhanced neutralization of infectivity and contributes to virulence. Proc Natl Acad Sci USA 1992;**89**:628–632.
85. Mckenzie R, Kotwal GJ, Moss B, Hammer CH, Frank MM. Regulation of complement activity by vaccinia virus complement-control protein. J Infect Dis 1992;**166**:1245–1250.
86. Vanderplasschen A, Mathew E, Hollinshead M, Sim RB, Smith GL. Extracellular enveloped vaccinia virus is resistant to complement because of incorporation of host complement control proteins into its envelope. Proc Natl Acad Sci USA 1998;**95**:7544–7549.
87. Massung RF, et al. Potential virulence determinants in terminal regions of variola smallpox virus genome. Nature 1993;**366**:748–751.
88. Shchelkunov SN, et al. Structural-functional organization of the smallpox virus genome. 1. Cloning of viral-DNA HINDIII and XHOI fragments and sequencing of HINDIII fragment-M, fragment-L, and fragment-I. Mol Biol 1992;**26**:731–744.
89. Albrecht JC, Fleckenstein B. New member of the multigene family of complement control proteins in herpesvirus saimiri. J Virol 1992;**66**:3937–3940.
90. Fodor WL, et al. The complement control protein homolog of herpesvirus saimiri regulates serum complement by inhibiting C3 convertase activity. J Virol 1995;**69**:3889–3892.
91. Russo JJ, et al. Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). Proc Natl Acad Sci USA 1996;**93**:14862–14867.
92. Virgin HW, et al. Complete sequence and genomic analysis of murine gammaherpesvirus 68. J Virol 1997;**71**:5894–5904.
93. Friedman HM, Cohen GH, Eisenberg RJ, Seidel CA, Cines DB. Glycoprotein C of herpes simplex virus 1 acts as a receptor for the C3b complement component on infected cells. Nature 1984;**309**:633–635.
94. Spear PG. Antigenic structure of herpes simplex viruses. In: van Regenmortel MVH, Neurath AR, eds. Immunochemistry of viruses. The basis for serodiagnosis and vaccines. Amsterdam: Elsevier Science Publishers; 1985. p. 425–446.
95. McNearney TA, Odell C, Holers VM, Spear PG, Atkinson JP. Herpes simplex virus glycoproteins gC-1 and gC-2 bind to the third component of complement and provide protection against complement mediated neutralization of viral infectivity. J Exp Med 1987;**166**:1525–1535.
96. Friedman HM, et al. Immune evasion properties of herpes simplex virus type 1 glycoprotein gC. J Virol 1996;**70**:4253–4260.
97. Johnson DC, Spear PG. O-linked oligosaccharides are acquired by herpes simplex virus glycoproteins in the Golgi apparatus. Cell 1983;**32**:987–997.
98. Tal-Singer R, et al. Interaction of herpes simplex virus glycoprotein gC with mammalian cell surface molecules. J Virol 1995;**69**:4471–4483.
99. Kostavasil I, Sahu A, Friedman HM, Eisenberg RJ, Cohen GH, Lambris JD. Mechanism of complement inactivation by glycoprotein C of herpes simplex virus. J Immunol 1997;**158**:1763–1771.
100. Fries LF, Friedman HM, Cohen GH, Eisenberg RJ, Hammer CH, Frank MM. Glycoprotein C of herpes simplex virus 1 is an inhibitor of the complement cascade. J Immunol 1986;**137**:1636–1641.
101. Hung SL, et al. The interaction of glycoprotein C of herpes simplex virus types 1 and 2 with the alternative complement pathway. Virology 1994;**203**:299–312.
102. Lubinski J, Wang L, Mastellos D, Sahu A, Lambris JD, Friedman HM. *In vivo* role of complement-interacting domains of herpes simplex virus type 1 glycoprotein gC. J Exp Med 1999;**190**:1637–1646.
103. Cooper NR. Complement evasion strategies of microorganisms. Immunol Today 1991;**12**:327–331.
104. Nemerow GR, Houghten RA, Moore MD, Cooper NR. Identification of an epitope in the major envelope protein of Epstein–Barr virus that mediates viral binding to the B lymphocyte EBV receptor (CR2). Cell 1989;**56**:369–377.
105. Molina H, et al. Analysis of Epstein–Barr virus-binding sites on complement receptor 2 (CR2/CD21) using human–mouse chimeras and peptides. At least two distinct sites are necessary for ligand–receptor interaction. J Biol Chem 1991;**266**:12173–12179.
106. Bergelson JM, Chan M, Solomon KR, Stjohn NF, Lin HM, Finberg RW. Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. Proc Natl Acad Sci USA 1994;**91**:6245–6249.
107. Manchester M, Liszewski MK, Atkinson JP, Oldstone MB. Multiple isoforms of CD46 (membrane cofactor protein) serve as receptors for measles virus. Proc Natl Acad Sci USA 1994;**91**:2161–2165.
108. Lambris JD, Holers VM. Therapeutic interventions in the complement system. Totowa: Humana Press Inc; 2000.
109. Sahu A, Lambris JD. Complement inhibitors: a resurgent concept in anti-inflammatory therapeutics. Immunopharmacology 2000;**49**:133–148.
110. Sahu A, Morikis D, Lambris JD. Complement inhibitors targeting C3, C4, and, C5. In: Lambris JD, Holers VM, eds. Therapeutic interventions in the complement system. Totowa: Humana Press Inc; 2000. p. 75–112.
111. Sahu A, Kay BK, Lambris JD. Inhibition of human complement by a C3-binding peptide isolated from a phage displayed random peptide library. J Immunol 1996;**157**:884–891.
112. Furlong ST, et al. C3 activation is inhibited by analogs of compstatin but not by serine protease inhibitors or peptidyl α-ketoheterocycles. Immunopharmacology 2000;**48**:199–212.
113. Sahu A, Soulika AM, Morikis D, Spruce L, Moore WT, Lambris JD. Binding kinetics, structure–activity relationship, and biotransformation of the complement inhibitor compstatin. J Immunol 2000;**165**:2491–2499.
114. Fiane AE, et al. Compstatin, a peptide inhibitor of C3, prolongs survival of *ex vivo* perfused pig xenografts. Xenotransplantation 1999;**6**:52–65.
115. Fiane AE, et al. Prolongation of *ex vivo*-perfused pig xenograft survival by the complement inhibitor compstatin. Transplant Proc 1999;**31**:934–935.
116. Nilsson B, et al. Compstatin inhibits complement and cellular activation in whole blood in two models of extracorporeal circulation. Blood 1998;**92**:1661–1667.
117. Soulika AM, et al. Inhibition of heparin/protamine complex-induced complement activation by compstatin in baboons. Clin Immunol 2000;**96**:212–221.

118. Morikis D, Assa-Munt N, Sahu A, Lambris JD. Solution structure of compstatin, a potent complement inhibitor.
Protein Sci 1998;**7**:619–627.

119. Del Rio-Tsonis K, Tsonis PA, Zarkadis IK, Tsangas AG, Lambris JD. Expression of the third component of complement, C3, in regenerating limb blastema cells of urodeles.
J Immunol 1998;**161**:6819–6824.

120. Mastellos D, Papadimitriou J, Franchini S, Tsonis PA, Lambris JD. A novel role of complement: mice deficient in the fifth component of complement (C5) exhibit impaired liver regeneration.
J Immunol (In press).