Defining Sites of Interaction in the α-chain of C3 for Factor H, Membrane Cofactor Protein (MCP), and Complement Receptor 1 (CR1)

by

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Abstract

Thesis title: **Defining Sites of Interaction in the α-chain of C3 for Factor H, Membrane Cofactor Protein (MCP), and Complement Receptor 1 (CR1).**

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The goal of this thesis was to identify sequences in human complement component C3 that contribute to its interaction with several of its regulatory molecules. Using mapping approaches which included studying the effects of blocking antibodies, proteolytic fragment binding studies and peptide mimetic binding studies, several groups have implicated residues within the 727-767 segment at the N-terminus of C3b α'-chain as being important for its interactions with the regulatory molecules factor H and complement receptor 1 (CR1), as well as the alternative pathway component factor B. Since there are significant inherent limitations in the above-mentioned approaches, this lab has been using the complementary approach of site-directed mutagenesis to corroborate and further define candidate binding sites, or in some cases, to disprove them. Aiko Taniguchi-Sidle of this laboratory had previously conducted a mutagenic scan of the charged residues within the 727-737 target segment and identified residues 736^{E} and 737^{E} as being important for both factor B binding and CR1 binding, but mutation of these residues had little or no effect on factor H binding. To identify residues within the remainder of the candidate segment that might play a role in the binding of factor H, I conducted a charged residue to alanine mutagenic scan between residues 741 and 767.

Recombinant molecules were transiently expressed in COS-1 cells and were examined for their interaction with factor H using a factor I-mediated cleavage assay that was dependent upon prior complex formation between the C3b-equivalent molecule C3(H_2O) and the I-cofactor, in this case factor H. This approach identified the negatively charged side chains of residues 744^{E} and 747^{E} as being important for the binding interaction with factor H. These observations supported the prediction from peptide mimetic studies that the region spanning residues 745-754 in C3 would provide a contact surface for the binding of factor H. Since CR1 and membrane cofactor protein (MCP), like factor H, also have I-cofactor activity, the 727-767 series of mutants were further examined for their interactions with soluble forms of CR1 and MCP. With respect to CR1, in addition to confirming that residues $736,737^{EE}$ play a role in this interaction, I have further identified residues 747^{E} and $754,755^{ED}$ as being similarly important for the interaction with CR1. Residues 744^{E} and $757,758^{KE}$ also appeared to contribute to this interaction, although to a lesser extent than the other residues mentioned above. Thus, although the factor H and CR1 sites of interaction within the 727-767 segment appeared to overlap, the negatively-charged residue contacts required for CR1 binding appeared to be more extensive and to extend over a wider portion of this segment than was the case for factor H. None of the mutations had any effect on the interaction with MCP, thereby strongly suggesting that the N-terminal α'-chain segment plays no role in this binding interaction.

Acknowledgements

I would like to acknowledge my supervisor, David, for assigning me a challenging project that was both technically and intellectually stimulating. I have learned many useful things during my term in the laboratory, honing skills and establishing habits that will no doubt prove to be immensely useful in my future pursuits. It has been an enlightening experience.

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I would like to express my best wishes to Rusty Jones and Qun Pan in their future endeavours as they continue their work in David's lab. I hope that my input was useful in anyway.

Finally, I would like to thank my parents and my brother for being understanding and supportive these past two years. It has been a difficult trek but it is finally over.

This chapter is done but the book is far from complete...

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Abbreviations

BSA: bovine serum albumin

CR1, CR2: complement receptors type 1 and 2, respectively.

CVF: cobra venom factor

DAF: decay accelerating factor

DMEM: Dulbecco's modified eagle's media

EAC4b2a; EAC423b: hemolysin sensitized sheep erythrocytes coated with complement components C4b and ^{oxy}C2a; C4b, ^{oxy}C2a, and C3b, respectively.

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

FCS: fetal calf serum

HBSS: Hanks' buffered salt solution

HSA: human serum albumin

MAC: membrane attack complex

MBL: mannose binding lectin

MCP: membrane cofactor protein

PBS: phosphate buffered saline

RCA: regulators of complement activation

RIA: radioimmunoassay

RT: room temperature

SCR: short consensus repeat

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

VBS, veronal bufferd slaine; GVB, VBS containing gelatin; GVBE, GVB containing EDTA; SVB, low ionic strength VBS containing sucrose; SGVB, SVB containing gelatin.

Chapter 1

Introduction

1.1 Complement: Background

The immune system in higher vertebrates possesses several mechanisms through which the host is capable of coping with a plethora of endogenously- and exogenously-borne factors that if left unchecked, pose a serious risk to host survival. The system itself can be simplified into two general modes of action: an innate and an adaptive immune response, both of which act to recognize and destroy foreign biotic and/or abiotic material. These two facets of the immune system are not however entirely independent of one another and synergistic responses between the two can be enlisted through a variety of mediators. One example of such bridging can be seen through the diverse actions of a subsystem of proteins called complement. Originally thought to play a role in the innate defense of a pre-immune individual, the complement system has also been shown more recently to play a role in the humoral responses of the adaptive immune system as well, specifically by playing a co-stimulatory role in the activation of B cells in the presence of antigen (Fischer *et al.*, 1996).

Complement represents a relatively complex and highly conserved system of interacting blood-borne and surface-bound proteins and its importance in maintaining health can be inferred from clinical cases of different complement deficiencies (for a review, see Colten and Rosen, 1992). Clinical symptoms as a consequence of such deficiencies range from increased susceptibility to recurrent infections by certain pathogenic microorganisms to autoimmune diseases and nephritic diseases that result from impaired immune complex clearance.

Most of the key players in the complement system are secreted into the blood and circulate as inactive zymogens. These zymogens can then be activated either by cleavage of the pro-molecule or by protein-induced conformational changes brought about through one of three pathways of activation, namely the classical, the lectin, and the alternative pathways. Each pathway represents a cascade of reactions whereby different complement components are activated in a consecutive order. The principle players, the zymogens, become activated serine proteases that go on to cleave various complement components. Some of the products from these proteolytic reactions go on to mediate other activities through cognate ligand/receptor interactions. The reactions themselves are potentially self-perpetuating and so, at different points along the pathway, the progression is tightly regulated by factors which include serine proteases, protease inhibitors and regulatory binding molecules, some of which are members of a family of proteins called regulators of complement activation (RCA).

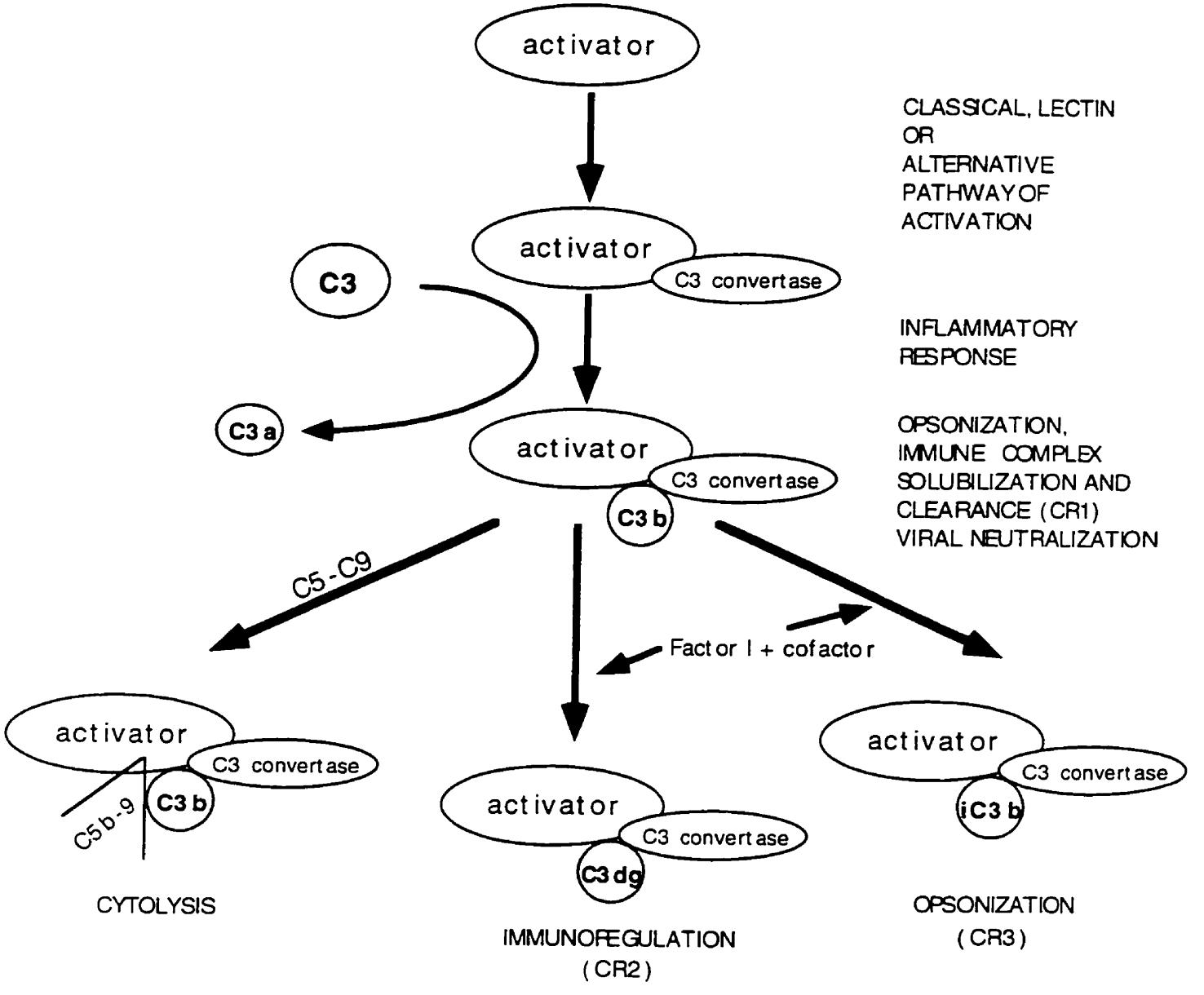
The primary goal of the complement cascade is the formation and surface-deposition onto foreign cells and antigens of the enzyme complex known as C3 convertase. As its name implies, the complex's role is to proteolytically convert complement component C3 into its active state (Volanakis, 1989). The significance of C3 in the complement cascade derives from the multiple modes of action of its various degradation products (see Figure 1.1). The functions of C3 activation products include virus particle neutralization (C3b), inflammation (C3a), improving the clearance and solubilization of immune complexes (C3b), vasopermeability and smooth muscle contraction (C3a), regulating the proliferation and/or differentiation of lymphocytes and leukocytes (C3dg), and aiding in the clearance of pathogenic microorganisms through complement-mediated lysis and opsonization (C3b,iC3b). Further evidence of its multifunctionality can be seen in clinical cases of C3 deficiencies which result in recurrent infections, immune complex diseases and

impaired immune responses (reviewed by Bitter-Suerman and Burger, 1989; Singer *et al.*, 1994).

Given the central role of C3 in the complement system, it is both interesting and important to elucidate the mechanisms through which C3 fulfills its different functions. Although my project deals with a particular set of interactions between C3b and several of its functional regulators, in the remainder of the introduction I endeavour to provide the reader with sufficient background information on the biochemistry and immunobiology of C3 and its interactions to place in context the studies which I have undertaken.

Figure 1.1 The central and multifaceted role of C3.

Activation of native C3 by a C3 convertase that has been assembled on an activator surface results in its cleavage into C3a and C3b. C3a is an anaphylatoxin that is bound by a specific cellular receptor and mediates an inflammatory response. Nascently activated C3b has the transient ability to transacylate onto nucleophilic acceptor surfaces where it has many biological consequences. First, deposition of C3b aids in immune complex solubilization and viral neutralization. Second, C3b can bind and modulate C5, allowing its activation and leading to assembly of C5b-9 in a membrane attack complex that can effect cytolysis. Third, C3b can be degraded by serine protease factor I in the presence of appropriate cofactor proteins into iC3b and C3dg. C3b and iC3b are the primary C3-derived ligands for complement receptors type 1 (CR1) and type 3 (CR3), respectively, and fulfill important opsonic functions, whereas C3dg is recognized by complement receptor type 2 (CR2), which plays an immunoregulatory role.


activator
CLASSICAL, LECTIN OR ALTERNATIVE PATHWAY OF ACTIVATION
activator
C3 convertase
C3
INFLAMMATORY RESPONSE
C3 a
activator
C3 convertase
C3 b
OPSONIZATION, IMMUNE COMPLEX SOLUBILIZATION AND CLEARANCE (CR1) VIRAL NEUTRALIZATION
C5-C9
Factor I + cofactor
activator
C3 convertase
C5b-9
C3 b
activator
C3 convertase
C3 dg
activator
C3 convertase
iC3 b
CYTOLYSIS
IMMUNOREGULATION (CR2)
OPSONIZATION (CR3)

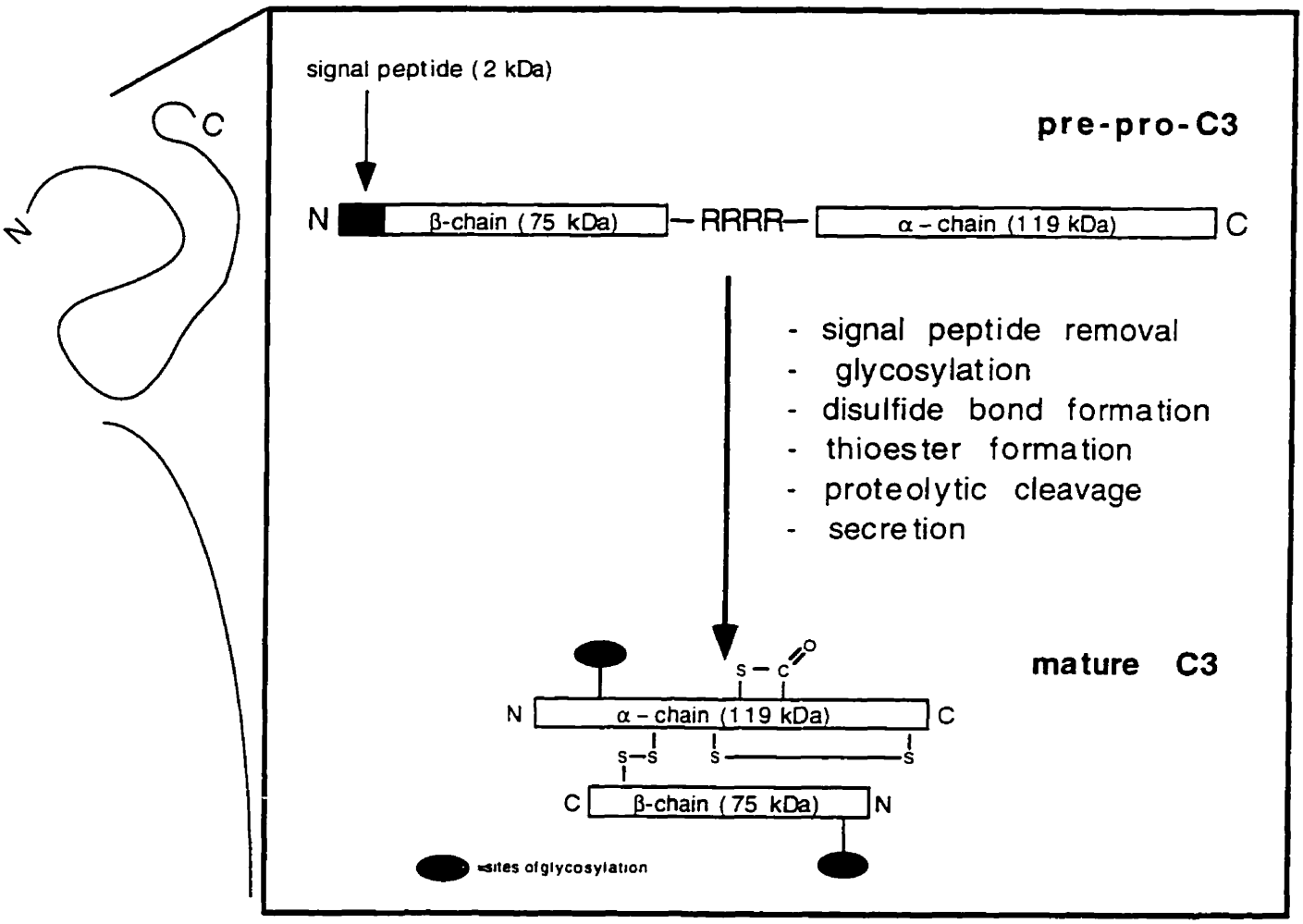
1.2 Complement Component C3

Of all the complement proteins, C3 is the most abundant component in plasma (~1.3mg/ml)(Barnum *et al.*, 1989) with the main site of production being liver hepatocytes (Alper *et al.*, 1969). To a lesser extent C3 is also biosynthesized by circulating macrophages (Celada *et al.*, 1989), neutrophils (Botto *et al.*, 1992), T lymphocytes (Pantazis *et al.*, 1990), and natural killer cells (Finberg *et al.*, 1992). The gene encoding C3 in humans is found on chromosome 19 (Whitehead *et al.*, 1982) and is up-regulated by the pro-inflammatory cytokine IL-1 (Juan *et al.*, 1993).

The human C3 gene encodes a 1663 amino acid (a.a.) precursor protein consisting of a N-terminal 22 a.a. signal sequence, a 645 a.a. β-chain, a 'linking' peptide of four arginine residues, and finally the remaining 992 a.a. α-chain at its C-terminus (de Bruijn and Fey, 1985). C3 also harbours an intramolecular thioester bond which is formed between the sulphydryl group of cys^{1010} and the γ-carbonyl of gln^{1013}. Though the mechanism of thioester synthesis remains unclear, the structure plays an important role in the conformational and functional dynamic of C3. With the signal sequence cleaved and the appropriate sugars added, the so-called pro-C3 protein is translocated from the endoplasmic reticulum to the golgi apparatus where the endoprotease furin cleaves at the arginine-rich linking peptide to form the α and β -chains of 119 kDa and 75 kDa respectively (Misumi *et al.*, 1991). The now mature C3 is secreted into the blood (see Figure 1.2).

Figure 1.2 Biosynthetic processing of human complement component C3.

C3 is synthesized as a single chain pre-pro-C3 precursor molecule. Within the endoplasmic reticulum, the signal peptide is removed, two high-mannose oligosaccharides are added, and inter- and intra-chain disulfide bonds as well as an intramolecular thioester bond are formed. The tetra-arginine linker is then removed within the Golgi complex and mature C3, composed of a 119 kDa α-chain and a 75 kDa β-chain, is secreted.


signal peptide (2 kDa)
pre-pro-C3
N
β-chain (75 kDa)
RRRR
α - chain (119 kDa)
C
- signal peptide removal
- glycosylation
- disulfide bond formation
- thioester formation
- proteolytic cleavage
- secretion
mature C3
N
α - chain (119 kDa)
C
C
β-chain (75 kDa)
N
sites of glycosylation

1.3 The Intramolecular Thioester bond

The native conformational state of secreted C3, and thus the precursor functional state of the molecule, is dependent upon the integrity of the thioester bond in the molecule (Isenman *et al.*, 1981). Cleavage of mature C3 by a C3 convertase produces the 77 residue C3a peptide from the N-terminus of the α-chain and leaves behind C3b, which is composed of the now shortened α'-chain, disulfide linked to the β-chain. This cleavage results in conformational changes within C3b (Isenman and Cooper, 1981) such that the internal thioester bond becomes susceptible to nucleophilic attack either with water, creating a fluid-phase molecule, or, with some surface nucleophile, usually a free hydroxyl group, resulting in a surface-bound form. However, some spontaneous hydrolysis of the internal thioester, in the absence of any peptide cleavage, occurs naturally and produces a species referred to as C3(H_2O), the significance of which is described in greater detail below.

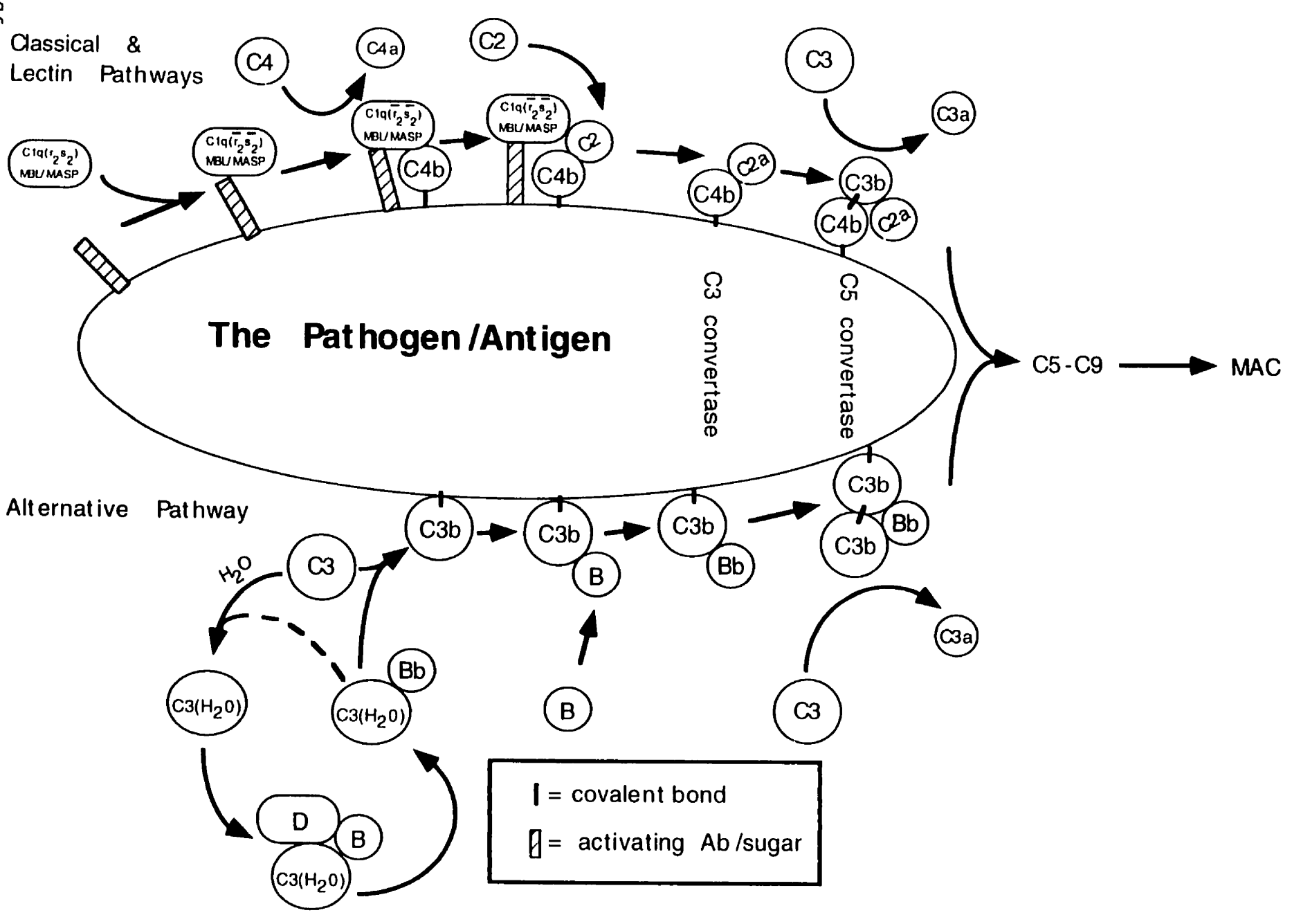
1.4 The Classical Pathway of Complement Activation

This particular pathway is initiated primarily in an antibody-dependent manner and requires the presence of complement components C1, C4 and C2 to form the C4b2a classical pathway C3 convertase (see Figure 1.3). Complement component C1 is a 740 kDa multisubunit complex composed of one C1q subcomponent non-covalently associated with two C1r and C1s proteins. Activation of C1 typically occurs when the globular heads of C1q bind to the Fc portions of activating immunoglobulin isotypes, causing a distortion down C1q's stem regions (Burton, 1993). It should be noted that C1 can also be activated in this way by binding to DNA, mitochondrial membranes, and some extracellular matrix proteins that result from tissue damage (reviewed by Volanakis *et al.*, 1990).

Figure 1.3 The major pathways of complement activation.

The classical pathway of complement activation can be initiated by both antibody-dependent and antibody-independent mechanisms, and is a cascade of reactions involving complement components C1, C4, and C2. The lectin pathway of complement activation is initiated in an antibody-independent manner (usually surface sugars) that involves MBL/MASP, C4 and C2. The alternative pathway is initiated in an antibody-independent manner, and involves complement factor D, factor B and C3, which fulfill roles that are analogous to their classical pathway counterparts C1s, C2 and C4, respectively. All three pathways result in the formation of their respective C3 convertase complexes, C4b2a and C3bBb, which cleave C3 into C3a and C3b. This activated C3b fragment can then bind and modulate C5 to allow its cleavage by C2a or Bb into C5a and C5b. Activation of C5 initiates assembly of the terminal complement components, C6-9, into the membrane attack complex (MAC). Shaded regions denote active serine proteases. The nature of the covalent attachment by C4b/C3b is through a transacylation event of its internal thioester to some surface nucleophile forming either an amide or ester linkage.

9b


Classical & Lectin Pathways
C4
C4a
C2
C3
C3a
C1q(r2s2) MBL/MASP
C4b
C2a
C3b
The Pathogen /Antigen
C3 convertase
C5 convertase
C5-C9
MAC
Alternative Pathway
H2O
Bb
B
D
C3(H20)
= covalent bond
= activating Ab /sugar

The interaction between C1 and the activating molecule induces one of the zymogen C1r subunits of C1 to change conformation into a $C1r^{*}$ intermediate, this representing the unsplit form of the zymogen in which the catalytic site has essentially been formed. This intermediate can then go on to cleave the second C1r subunit creating active $\overline{C1}r$. This $\overline{C1}r$ cleaves and activates both C1s subunits to give $\overline{C1}r_2\overline{C1}s_2$ (reviewed by Colomb *et al.*, 1984; Schumaker *et al.*, 1986; Schumaker *et al.*, 1987).

Now the $\overline{C1}s$ subcomponent of the activated C1 complex goes on to cleave C4 at the N-terminal end of its α-chain, releasing the 74 a.a. C4a polypeptide. C4, like C3, possesses an intramolecular thioester bond and undergoes a conformational change upon cleavage (Isenman and Kells, 1982). This change in structure exposes the thioester to the surrounding media allowing for a transacylation event in the C4b molecule to occur onto an acceptor hydroxyl or amino group on the antigen surface.

The 102 kDa C2 molecule then non-covalently associates with the C4b molecule before it is cleaved by $\overline{C1}s$, resulting in the release of a 34 kDa C2b fragment and leaving behind a C4b-bound 70 kDa C2a active serine protease. So long as the C2a portion remains associated with the C4b subunit of the enzyme, this surface bound C4bC2a complex functions as the classical pathway C3 convertase that can then go on to cleave mature C3 into C3a and C3b.

1.5 Lectin Pathway of Complement Activation

The most recent addition to our understanding of complement activation has come about with the discovery of a lectin that shares structure and function with component C1q. Mannose binding lectin (MBL) is a complement-dependent bacteriacidal lectin which is present in the sera of a wide variety of vertebrates (as reviewed by Turner, 1996). In

humans, MBL is composed of four identical 32 kDa subunits, each with a long collagenous stem ending with a C-terminal cluster of three lectin domains. Like C1q, these four subunits associate to form a bouquet-like structure that allows MBL to bind to mutiple and widely different polysaccharides expressed on the cell surface, including those found on the surface of various pathogenic bacteria. The collagenous regions are known sites of interaction for a pro-serine protease which has limited structural homology (38-39%) to components C1r and C1s. Called MBL-associated serine protease (MASP), this 100 kDa protein once activated acts as $C\overline{1}s$ does by cleaving both C4 and C2 and thereby generating C4bC2a complexes with C3 convertase activity (Matsushita and Fujita, 1992). C1 and MBL appear to play complementary functional roles in complement activation, with activation triggered respectively by binding to immune complexes (C1) and the bacterial polysaccharides (MBL)(see Figure 1.3).

1.6 The Alternative Pathway of Complement Activation

Like the classical pathway, the alternative pathway involves a cascade of reactions with proteins that are analogous to their classical pathway counterparts, only this time in an antibody-independent manner. Here, complement factor D (the $C\overline{1}s$ analogue), factor B (the C2 analogue) and C3 (the C4 analogue) interact to form the C3bBb alternative pathway C3 convertase (see Figure 1.3). This pathway is thought to predate on an evolutionary time-scale the classical pathway (Farries and Atkinson, 1991) based on the presence of complement activation in the absence of antibody in invertebrates (Day *et al.*, 1970).

Unlike the classical or the lectin pathway, there is no formal 'recognition' molecule in the alternative pathway. Rather, this pathway is constitutively active and is self-initiating. This 'activity' is a consequence of the fact that ~1% of total serum C3 undergoes spontaneous hydrolysis of its internal thioester bond. Thioester hydrolysis leads to

conformational changes in C3 such that it attains a C3b-like structure, as seen, for example in near UV circular dichroism comparison studies with C3b (Isenman *et al.*,1981). This so-called C3(H_2O) molecule can interact with the soluble zymogen factor B and be acted upon by the blood-borne serine protease factor D. The result is the formation of a fluid-phase C3(H_2O)Bb C3 convertase which like its surface-bound counterpart described above, can go on to cleave other C3 molecules and form C3a and C3b. This newly released C3b can in turn bind with other factor B molecules and be cleaved by factor D to form the alternative pathway C3 convertase, initially in the fluid-phase and then some of the nascently cleaved C3b molecules can transacylate to a surface nucleophile forming the surface-bound form of the enzyme. So long as the Bb active serine protease subunit remains associated to either C3b or the C3b-like C3(H_2O), more C3 convertase will form until all the available pool of C3 is depleted (see Figure 1.4). Not surprisingly, therefore, both the fluid-phase and surface-bound convertases are tightly regulated, principally through the action of the serine protease, factor I, and its cofactors.

1.7 Mechanisms and Consequences of C3-directed Regulation

There are 3 principle mechanisms through which complement regulatory molecules act to block the self-feeding cycle of the alternative pathway. The first is the inhibition of association of C3b/C3(H_2O) with factor B by the regulator; factor B can only be cleaved to its active serine protease form (Bb) once associated with C3b/C3(H_2O). The second is the destabilization of the C3bBb convertase so that it dissociates into its component parts (so called decay accelerating activity). The third is irreversible cleavage of the C3b/C3(H_2O) molecules into subfragments which can no longer associate with factor B (specifically iC3b/iC3(H_2O) and C3dg) and, therefore, no longer partake in complement activation. Given its central role in complement-mediated effects, it is no wonder that C3 is the target of such tight regulation (see Figure 1.4).

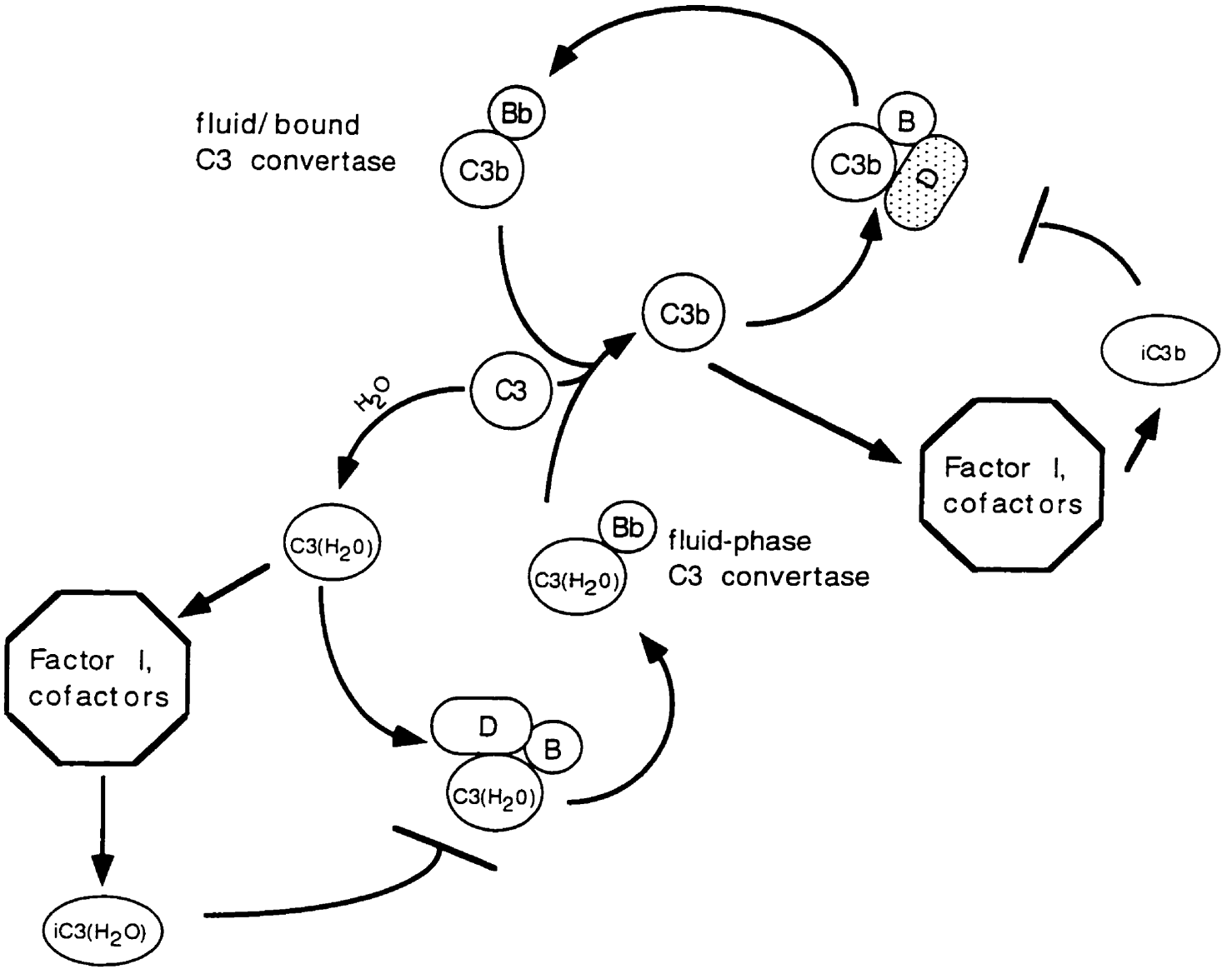
Figure 1.4. Model of the alternative pathway activation and its irreversible deactivation.

(i) Initiation occurs in the fluid-phase when a thioester-hydrolyzed C3 molecule, $C3(H_2O)$, binds and modulates a molecule of factor B to allow its cleavage by factor D. This results in the formation of a fluid-phase alternative pathway C3 convertase, $C3(H_2O)Bb$.

(ii) Random deposition of C3b occurs when nascent C3b that has been generated by the fluid-phase $C3(H_2O)Bb$ complex transacylates onto a nucleophilic acceptor surface.

(iii) The C3b can then go on to interact with factor B and after cleavage by factor D form surface-bound alternative pathway C3 convertase.

(iv) Propagation is regulated by the serine protease factor I which in combination with the appropriate cofactor can degrade C3b into subfragments iC3b/$iC3(H_2O)$ and C3dg that can no longer participate in the self-feeding cycle.


fluid/bound
C3 convertase
Bb
C3b
B
C3b
D
C3b
iC3b
C3
H_2O
Factor I,
cofactors
$C3(H_2O)$
Bb
$C3(H_2O)$
fluid-phase
C3 convertase
Factor I,
cofactors
D
B
$C3(H_2O)$
$iC3(H_2O)$

These regulators (to be discussed in greater detail below) exist either as soluble proteins in blood or as surface molecules on diverse tissues and cells of the body. Some have both soluble and membrane-associated forms. Through their action, the host organism is protected against alternative pathway-mediated destruction of its cells. In contrast, many pathogenic microorganisms do not express such regulatory molecules or do not possess a surface biochemistry that is conducive to recruit blood-borne regulators of complement activation. For example, it is possible to convert 'protected' sheep red blood cells into alternative pathway 'activators' by removing sialic acid from their surfaces. In the absence of a polyanionic surface, one potent down-regulator of complement, factor H, can no longer associate with surface-deposited C3b (Meri and Pangburn, 1990; Pangburn and Müller-Eberhard, 1978). These so-called alternative pathway 'activators' are now prone to unmitigated C3 convertase deposition on their surfaces.

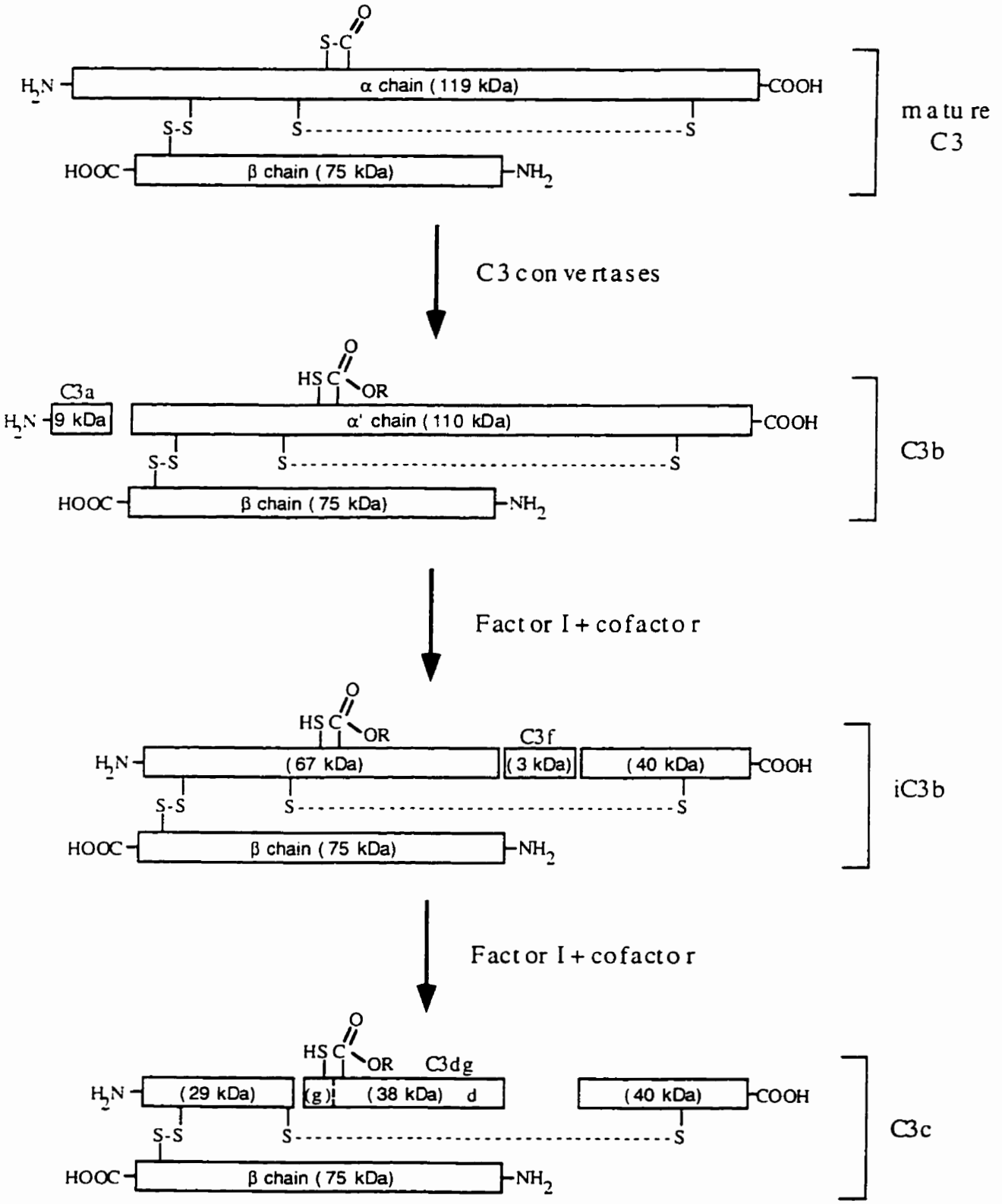
1.8 Factor I-mediated Degradation/Regulation of the Complement Cascade

So far we have seen that the formation of C3 convertases is potentially self-perpetuating and is regulated by proteolytic cleavage of the C3b component into subfragments that can no longer participate in the cycle. The principle mediator of this irreversible form of regulation is a blood-borne serine protease called factor I (reviewed by Sim *et al.*, 1993). This 88 kDa protein is capable of 'inactivating' C3b (and C4b) by degrading the molecule into subfragments iC3b and C3dg (see Figure 1.5). But purified factor I alone cannot cleave C3b. Cofactors have been identified to play a role in the activity of the enzyme. The cofactors are members of a family of proteins called regulators of complement activation (RCA) and include factor H, C4 binding protein (C4bp), complement receptors type 1 & 2 (CR1,CR2), decay acceleration factor (DAF) and membrane cofactor protein (MCP). The amount and extent of expression of these RCA proteins varies but all act to protect host tissue from complement-mediated destruction. In

terms of structure, RCA proteins are composed of varying numbers of a 60 a.a. motif known most commonly as the short consensus repeat (SCR); the SCR has been shown to be the primary structural motif of all the RCA members (see Figure 1.6) and confers specificity towards complement proteins, primarily C3b and C4b. ^{1}H-NMR analysis showed that this SCR formed a compact globular domain which was stable even at pH 3.3 and had approximate dimensions of 3.8 x 2.0 x 2.0 nm. It contained no α-helical regions but was composed of one triple-stranded and two double-stranded anti-parallel β-sheets held together by disulphide bridges between cys^{5} and cys^{47}, and cys^{33} and cys^{58}. Further analyses revealed that when 2 factor H-derived SCRs were expressed as a tandem pair they adopted the same conformation as when they were individually expressed (Barlow *et al.*, 1993). Given the high degree of sequence similarity between SCRs of different RCA members, it was postulated that these same members recognized and interacted with similar or overlapping sequences in C3. Factor H, CR1 and MCP are of special significance to this thesis and are discussed in greater detail below. It is the specificity of these three cofactors that in part explains the high degree of substrate specificity to C3b and C4b shown by factor I.

Figure 1.5 Activation and proteolytic degradation of complement component C3.

Activation of native C3 by a C3 convertase releases the C3a peptide from the N-terminus of the α-chain, and produces C3b, which is composed of the α'-chain disulphide-linked to the β-chain. Factor I, in the presence of an appropriate cofactor, cleaves the α'-chain of C3b to release the 3 kDa C3f peptide and produce iC3b. Factor I can further degrade iC3b into C3dg (38 kDa) and C3c. Note that all of C3b, iC3b and C3dg can remain deposited on a target surface after thioester-mediated transacylation of C3b onto nucleophilic acceptor groups. C3(H_2O) would be similar in chain structure to native C3 except the thioester would be hydrolyzed. iC3(H_2O) would be similar in chain structure to iC3b except that the N-terminal C3a fragment would still be present.


S-C
O
H2N
α chain (119 kDa)
COOH
S-S
S
S
HOOC
β chain (75 kDa)
NH2
mature C3
C3 convertases
HS C
O
OR
C3a
H2N
9 kDa
α' chain (110 kDa)
COOH
S-S
S
S
HOOC
β chain (75 kDa)
NH2
C3b
Factor I + cofactor
HS C
O
OR
C3f
H2N
(67 kDa)
(3 kDa)
(40 kDa)
COOH
S-S
S
S
HOOC
β chain (75 kDa)
NH2
iC3b
Factor I + cofactor
HS C
O
OR
C3dg
H2N
(29 kDa)
(g)
(38 kDa) d
(40 kDa)
COOH
S-S
S
S
HOOC
β chain (75 kDa)
NH2
C3c

1.9 Factors H, Complement Receptor Type 1 and Membrane Cofactor Protein

Factor H (reviewed by Vik *et al.*, 1989) is a 150-155 kDa serum protein consisting of 20 SCR domains. It interacts with C3b and inhibits the binding of factor B and C5 to C3b. Additionally, through its decay accelerating activity, factor H decreases the half-life of any pre-formed C3bBb complexes, and finally, it serves as a cofactor in the factor I-mediated proteolysis of C3b (Whaley and Ruddy, 1976; Weiler *et al.*, 1976; DiScipio, 1981; Fischer and Kazatchikne, 1983; Ito and Tamura, 1983). All 3 activities act to down regulate the progression of the complement cascade, principally the alternative pathway.

With the aim of elucidating the structure/function relationships within factor H, deletion mutants of the full length protein were made in order to determine where co-factor and C3b binding activity resided. The results of these experiments suggested that factor H has at least 3 sites that can bind to C3b, located within SCRs 1-4, 6-10 and 16-20 (Sharma and Pangburn, 1996). Furthermore, functional assays using these deletion mutants ascribed factor I cofactor activity to SCRs 1-4. As mentioned earlier, factor H often requires a polyanionic surface in order to associate with surface-bound C3b. In one study, the polyanionic binding site on factor H was localized by tryptic cleavage, peptide mapping and photoaffininty labeling to SCR13, which contains many positively charged amino acids and is located outside all of the above mentioned C3b binding sites (Pangburn *et al.*, 1991). However, more recent studies also point to a major polyanionic binding site in SCR 7 of factor H (Sharma & Pangburn, 1996). The manner in which binding of polyanions to factor H affects its interaction with C3b remains unknown.

Complement receptor type 1 (CR1; CD35) is a 205 kDa integral membrane protein comprised of 30 SCRs that regulates complement progression in two ways: i) it has decay accelerating activity for both the classical and alternative pathway C3 convertases, and ii) it acts as a cofactor, like H, for factor I-mediated cleavage of C3b to iC3b and subsequently to C3dg (also C4b into C4c and C4d). Though CR1's tissue distribution is not as wide as other surface-expressed complement regulatory molecules, it is expressed on erythrocytes, neutrophils, macrophages, B, and T cells (Fearon, 1980; Dobson *et al.*, 1981; Wilson *et al.*, 1983). It has been shown, however, that soluble versions of CR1 are shed from lymphocytes into the blood (Pascual *et al.*, 1993) and we have obtained for the purposes of our study a functional recombinant source of soluble CR1 (sCR1).

Deletion mutants of CR1 and site-directed mutations within particular SCRs were generated and assessed for their ability to associate with C3b and C4b in order to help localize sites of interaction. C3b-binding activity was lost when SCRs 8 to 21 were removed whereas C4b-binding activity was ablated when SCRs 1 to 5 were removed (Klickstein *et al.*, 1988). More refined deletions/mutations have localized the principle C4b binding site to SCRs 1 and 2 and have identified C3b binding sites in SCRs 8-9 and 15-16 (Krych et al., 1994)(see Figure 1.6). Despite considerable *in vitro* analysis of the regulatory activities of CR1 and the identification of these binding sites, the physiological significance of CR1 to the regulation of complement is still unclear. However it is now strongly believed that the primary physiological role of CR1 on blood cells, particularly erythrocytes of primates, is to mediate clearance of C3b-coated immune complexes to hepatic and splenic macrophages, which in turn use their own CR1 and CR3 to enhance antibody-dependent cellular cytotoxicity (ADCC), phagocytosis and antigen presentation. It should be mentioned that macrophages also express Fcγ receptor type I (CD64), II (CD32), and III (CD16) which recognize the Fc region of IgG (reviewed by Ravetch, 1991). As it turns out, aggregated IgG-C3b complexes were cleared more efficiently in

mice than complexes made up of aggregated IgG alone. It was proposed, therefore, that the presence of both IgG and C3b on a particle might facilitate phagocytosis simply by increasing the avidity of interaction on $FcR^{+}CR1^{+}$ phagocytic cells (Ehlenberger and Nussenzweig,1977; Mehta *et al.*, 1986; Fries *et al.*, 1987). Thus the C3b/C4b regulatory activity of CR1 is probably a secondary function of the molecule.

Membrane cofactor protein (MCP; CD46) is a widely expressed protein that serves as a cofactor for I-mediated cleavage of C3b/C4b to iC3b/iC4b, but unlike CR1, it does not accelerate the decay of either fluid-phase or surface-bound C3 convertase. Instead decay accelerative activity on host tissue expressing MCP is mediated by DAF (decay accelerating factor), another RCA member. MCP's effects, moreover, are restricted to the cell on which it is expressed (Seya & Atkinson, 1985). It has a wider cell distribution than CR1 and is believed to play an important role in the regulation of complement activation. MCP was first cloned from the U937 promonocytic cell line and its cDNA encodes a protein consisting of 4 SCRs, followed by a serine/threonine rich O-glycosylated stretch, an additional 13 residues, a transmembrane region and a variable length cytoplasmic tail (Lublin *et al.*, 1980).

MCP is expressed primarily as four isoforms that arise by alternative splicing of a single gene (reviewed by Atkinson *et al.*, 1991). These isoforms tend to differ in degree of their O-glycosylation as well as the length of their cytoplasmic tails (16-23 residues in length). Interestingly, the isoforms with larger O-glycosylation domains were better protectors against the complement cascade (Liszewski and Atkinson, 1996). Cofactor and C3b/C4b binding activity of MCP resides in SCRs 2-4 (Adams *et al.*, 1991). Recently, MCP was demonstrated to be the receptor for measles virus (Dorig et al, 1993) and to be involved in the adherence of group A *Streptococcus pyogenes* to epithelial cells (Okada *et al.*, 1995).

Figure 1.6 The regulators of complement activation (RCA) family.

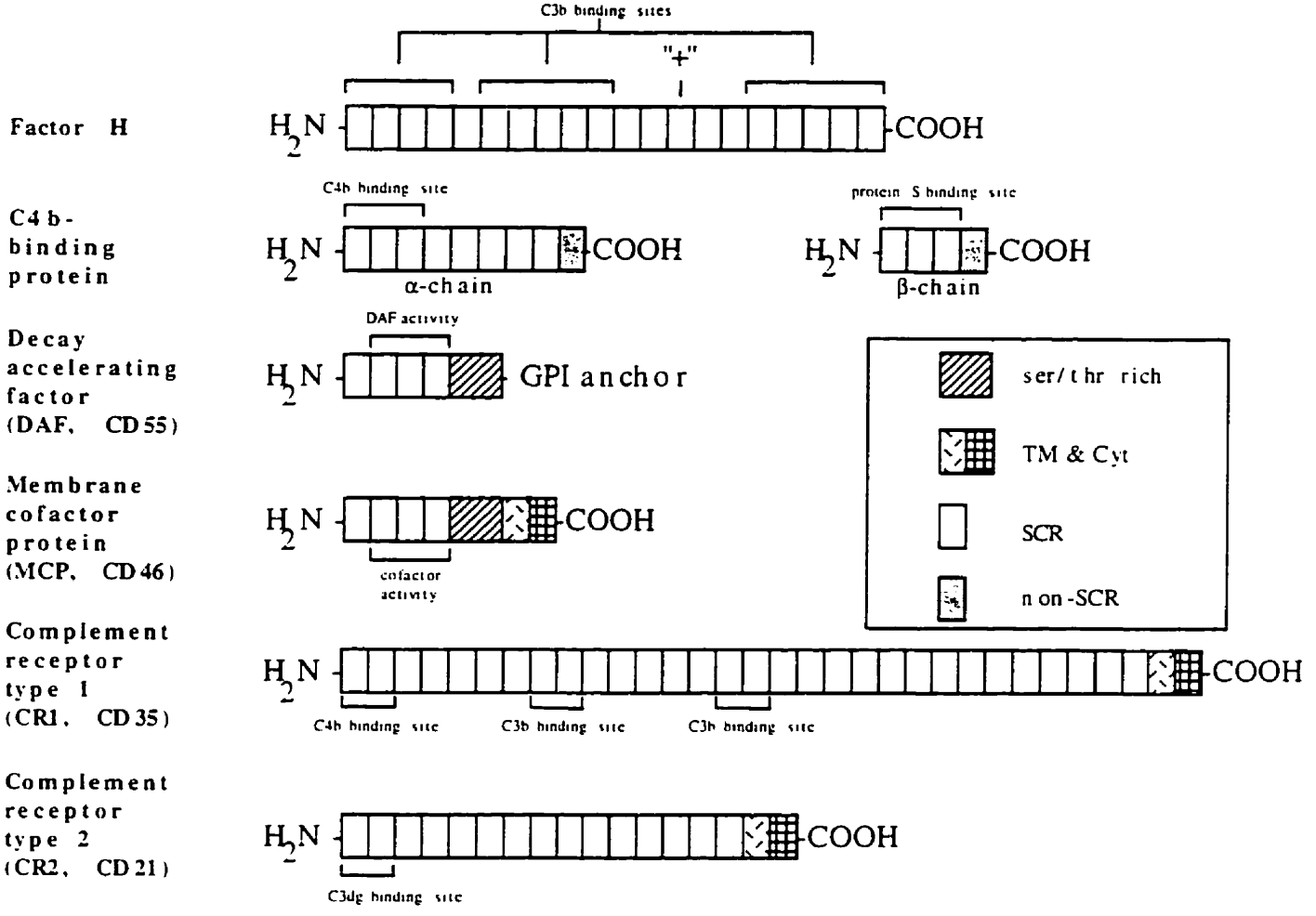
The regulators of complement activation (RCA) family are composed primarily of short consensus repeat (SCR) modules, and include the following C3b/C4b-bindng proteins: All primary sites of interaction identified thus far are indicated.

Factor H is a single chain plasma protein composed only of SCRs.

C4b-binding protein (C4bp) is another plasma protein, composed of α and β chains, which consist of 8 and 3 SCRs, respectively, at their N-termini, followed by 60 amino acid domains that are not SCRs.

DAF and MCP are cell-bound proteins that are composed of 4 SCRs at their N-termini, followed by O-glycosylated serine/threonine-rich stretches. DAF is membrane anchored by a glycophosphatidylinositol (GPI) tail whereas MCP has transmembrane and cytoplasmic domains.

CR1 and CR2 are also type I membrane proteins with transmembrane regions and cytoplasmic tails.



The Short Consensus Repeat (SCR) motif:

.C..PP.I.NG.I.....Y..GE.V.Y.C..GY.. G...I.C...G.WS...P.C.. (60 a.a.)

1.10 Complement-mediated Responses Through C3 and its Degradation Fragments

Upon association with either surface-deposited or fluid-phase C3 convertase, C3 is cleaved to release the anaphylatoxin C3a and a C3b fragment that is metastable because of an exposed internal thioester which can either be hydrolyzed by water or transacylate onto a surface-bound nucleophile. As mentioned above, C3b can be cleaved further by the regulatory serine protease, factor I, into the smaller proteins iC3b and C3dg in the presence of appropriate cofactors (see Figure 1.5). Both C3b and their subsequent proteolytic degradation fragments can then participate in a variety of receptor-ligand interactions whose interplay results in responses that are not restricted simply to deactivation of the complement cascade. Beyond immunoregulation, these receptor-ligand interactions exhibit a multitude of roles including enhancement of phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), leukocyte homing, antigen presentation, and the release of cytokines and cytotoxic mediators.

The pinnacle of the complement cascade for both the classical and the alternative pathways is the formation of the membrane attack complex (MAC) which is initiated by C3b and its association with the C3 convertase creating the C5 convertase; the membrane attack complex represents a complex of proteins that contribute to the destruction of a cell by creating protein-lined channels in the plasma membrane. Without the necessary complement regulatory molecules to protect themselves (reviewed by Frank, 1992), pathogenic microorganisms are susceptible to C5 convertase and MAC assembly on their surfaces.

Certain viruses and bacteria can directly activate both the classical and alternative pathways of complement, but the consequences of C3b deposition on the surface of these

pathogens may be both harmful as well as beneficial to the infected host. In the ideal situation, C3b, as a subunit of the C5 convertase, can recruit the remaining C5-C9 complement proteins, form the membrane attack complex, and lyse the pathogen. More often, C3b acts to block virus uptake by masking coat structures normally used for infection (reviewed by Cooper, 1988). Yet certain pathogens have evolved ways to take advantage of surface-deposited C3b by using its degradation products or complement regulatory molecules as an anchoring and/or uptake mechanisms through which they infect susceptible cells.. A recent example has been described with the human immunodeficiency virus-1 (HIV-1) which has been shown to recruit factor H as means of protecting itself from complement-mediated destruction and bind to DAF on cells to improve tissue infection (Stoiber *et al.*, 1996).

Deposition of C3b onto immune complexes has been suggested to play a role in solubilization of otherwise insoluble antigen-antibody complexes. It is believed that C3b acts to destabilize Fc-Fc and antigen-antibody interactions by intercalating within the structures. Moreover, such C3b-embedded complexes can be cleared from the circulation by binding to CR1 on erythrocytes, which transport them to the liver for disposal by splenic and hepatic macrophages(reviewed by Petersen *et al.*, 1985; Medof, 1988; Ng and Schifferli, 1993).

In summary, C3 is an abundant serum protein made in a variety of locations by a variety of cell types. It plays a central and multifunctional role in the complement cascade but in a tightly regulated manner. In its absence, the ability of a host to affect clearance of pathogen and even to mount a humoral immune response to particular pathogenic microorganisms is severely curtailed. Not surprisingly, therefore, the elucidation of the mechanisms by which C3 accomplishes its multitude of functions has been the focal point of much research.

1.11 Defining Binding Sites in C3 for its Ligand and Receptors

Despite the absence of a 3-dimensional structure for the entire C3 protein, many independent groups have nonetheless gone on to use a variety of indirect approaches to elucidate those regions within C3 that contribute to its association with its receptors and ligands. The approaches have included sequence analyses, especially xenogenic comparisons, blocking antibodies against C3, and the combined use of proteolytic fragmentation and synthetic peptides derived from C3 as functional mimetics of the intact physiologic fragment. For example, the sequence of human C3 was compared to C3 of other species and the C3 molecules of various species were then analyzed for their ability to bind to various human complement components following the rationale that those non-human C3 molecules which could bind to a particular human protein would exhibit sequence similarity to the candidate binding site in human C3 (Alsenz *et al.*, 1992).

Polyclonal and monoclonal antibodies have been generated against segments of C3. It has been shown that the binding of these antibodies to C3/C3b do in some instances lead to the abrogation of one or more of C3's activities. By mapping the sites of antibody binding, it has been possible to propose candidate binding sites in C3 for some of its ligands and receptors (reviewed by Alsenz *et al.*, 1989a; Garred *et al.*, 1989). Similarly, C3-derived peptides have been used in competitive or direct binding assays as an approach in identifying regions involved in a particular interaction of the intact molecule (reviewed by Lambris and Müller-Eberhard, 1986; Lambris *et al.*, 1993a; Geysen *et al.*, 1984; Fishelson, 1991).

The ability of factor H to bind to C3b-coated erythrocytes or C3b-zymosan was blocked by the use of either polyclonal or monoclonal antibodies against both C3c and C3d, suggesting the presence of at least two sites of interaction on C3 (Burger *et al.*, 1982;

Koistinen *et al.*, 1989; Becherer *et al.*, 1992). Another study proposed the presence of a factor H binding site in the N-terminal region of the α'-chain of C3b because synthetic peptides spanning amino acids 727-767/8 (mature C3 numbering), or antibodies raised against these segments, could block binding of factor H to C3b-coated erythrocytes or C3b coated onto microtitre wells (Ganu and Müller-Eberhard, 1985; Becherer *et al.*, 1992). This region was further suggested to encompass residues 745-754 as a result of a study involving the use of a series of overlapping hexameric and heptameric peptides corresponding to C3 residues 727-767 that assessed their ability to directly bind factor H (Fishelson *et al.*, 1991). It should be noted, however, that another contact site for factor H was suggested to involve residues 1187-1214 and 1234-1249. This non-continuous site within C3dg (see Figure 1.5) was proposed based on experiments measuring the interaction of factor H to microtire plate-fixed peptides as well as those using these peptides to inhibit both binding of factor H to microtitre plate-fixed C3d and cofactor H-dependent factor I-mediated cleavage of C3b to iC3b (Lambris *et al.*, 1988). Yet another H binding site was described in the β-chain of C3 (Wörner *et al.*, 1989). So at the very least, it can be said that there appears to be multiple binding sites for factor H within the C3b molecule.

Some binding sites for CR1 in C3 have been elucidated. It was suggested that CR1 may interact with C3b in a similar manner to factor H because several monoclonal antibodies against C3c (see Figure 1.5) and polyclonal antibodies against the 727-768 C3 peptide as well as the peptide itself inhibited the interaction of C3b with both factor H and CR1 (Becherer and Lambris, 1988; Becherer *et al.*, 1992). Furthermore, factor H can inhibit the binding of CR1, CR2, and MCP to C3b (Farries *et al.*, 1990b). Most recently, a site directed mutagenesis approach taken by Aiko Taniguchi-Sidle, a previous graduate student in the lab, further implicated the N-terminus of the α'-chain in the binding of C3b to CR1 and factor B, and iC3b to CR3. Her studies pointed to an essential role for the

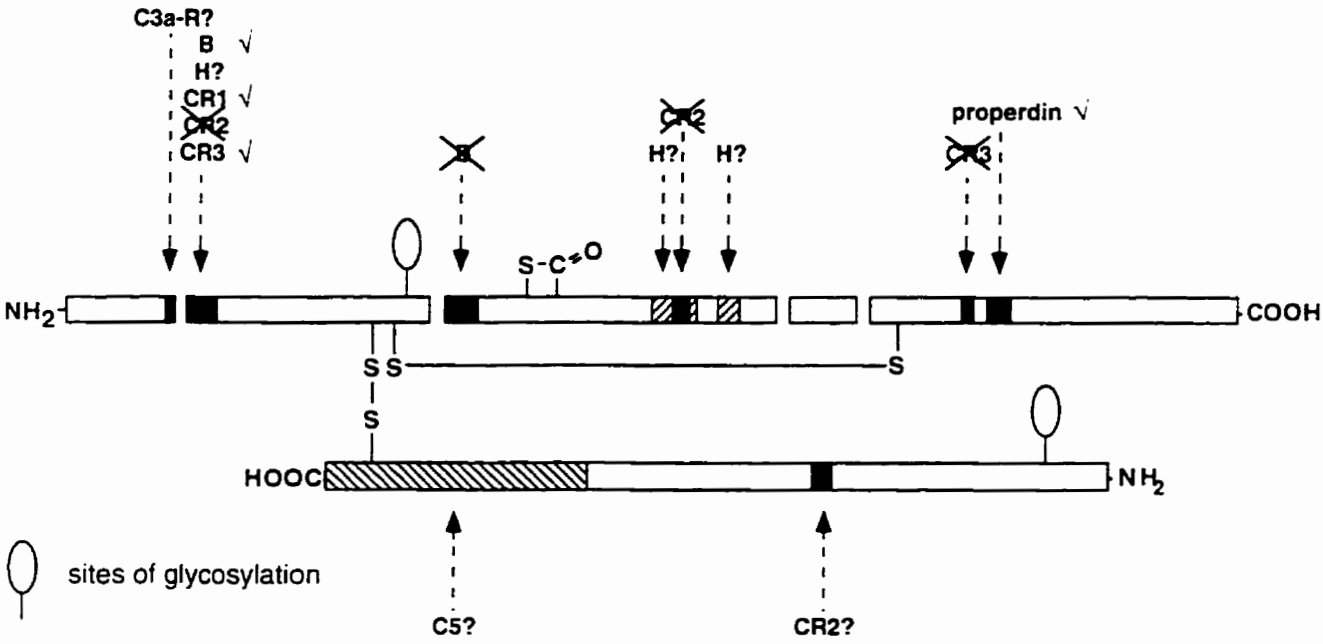


Figure 1.7 Proposed and tested binding sites in human complement component C3.

Schematic representation of C3 α- and β-chains, indicating the fragments generated by C3 convertases and factor I in the presence of appropriate cofactors, as well as the location of the intramolecular thioester bond, sites of glycosylation and disluphide bonds which link α- and β-chains, and α'-29 and α-40 fragments. The shaded and hatched boxes delineate particular regions or sequences in C3 that have been predicted by a variety of approaches to be binding sites for the indicated ligands and receptors of C3. Check marks indicate sites of interaction supported by site-directed mutagenic studies done in this lab. Crosses represent sites of interaction argued against by site-directed mutagenic studies in this lab.

negative charge on residues 736,737^{EE} and to a lesser extent in residues 730,731^{DE} in mediating these interactions (Taniguchi-Sidle and Isenman, 1994).

Based on the data accumulated thus far from these and other indirect studies one can draw a tentative map describing the putative location of C3 interaction sites with its ligands and receptors (see figure 1.7). Until the day a lab manages to crystallize and obtain a 3-dimensional image of C3 and its major physiologic fragments (ideally co-crystallized with the ligand), we must rely on these various indirect techniques to map out sites of interaction. For reasons described in the next section, our laboratory has used site-directed mutagenesis as a complementary approach to examine within the context of the physiologically relevant fragment of C3, the effect on a particular activity after altering specific amino acids that have been proposed to serve as contact residues with C3 ligands and receptors. Whereas concordance between results obtained with the site-directed mutagenesis approach and one or more of the other approaches provides strong support for a candidate interaction site, discordance can in most cases rule out a candidate site. As summarized in figure 1.7, the location of certain sites have been supported (check marks) and argued against (crosses) by previous studies in the laboratory using the site-directed mutagenesis approach. As denoted by the question mark, the binding site for factor H requires further investigation and there are some interactions (e.g. with MCP) for which candidate sites have yet to be proposed.

1.12 Project Rationale

Despite all the evidence that has accumulated over the years concerning the presence of the various interactive sites on C3, there are certain experimental limitations that must be taken into consideration when viewing these results.

It has been shown (Spangler, 1991) that synthetic peptides do not necessarily adopt a conformation analogous to what is seen for their corresponding sequences in the native protein. Moreover, in the situation of a discontinuous binding site, a linear peptide will not be an accurate reflection of the true nature of association between a protein and its ligand. Another matter for contention is the effect that fixation/adsorption of the peptide has on the peptide's conformation (Spangler, 1991); it has been demonstrated that monoclonal antibodies that normally recognize fluid-phase C3 degradation products, do not recognize immobilized forms of the same proteins (Nilsson *et al.*, 1990). It could be argued, therefore, that any study pertaining to elucidation of binding sites on C3 should be done in the context of physiologically relevant conditions and not to C3 absorbed to microtitre wells or microspheres (Alsenz *et al.*, 1992). Finally, assays using antibodies to indicate the presence or absence of sites of interaction must be taken with some circumspection as the effect may be due to steric hindrance or antibody-induced allosteric effects in the native structure. Clear examples of the latter can be seen in studies whereby monoclonal antibodies that recognize different integrins have been shown to potentiate the affinity of the integrins for their ligands as a consequence of induced long-range conformational changes (reviewed by Hogg *et al.*, 1993; Diamond and Springer, 1994).

In light of the above discussed weaknesses of the peptide and antibody blocking approaches, the complementary approach that I have chosen to use is site-directed mutagenesis. Specifically I initially set out to use this technique to examine the particular C3 sequences suggested by the other experiments to interact with the C3b regulatory molecule factor H. By 'functional extension' the same series of mutants were also examined for their ability to interact with soluble forms of CR1 and MCP. The main advantage of the site-directed mutagenesis approach is that any changes made could be examined in the context of the appropriate physiologic form of the C3 protein. With the observation that the interactions between RCA proteins and their ligands are strengthened

under low ionic strength conditions, suggesting a role for ionic forces, the charged residues of the target region were initially replaced with alanines. Alanine was chosen as the 'substituting' residue because it has the a small apolar side chain that is tolerated in both hydrophilic and hydrophobic environments and does not alter the main chain conformation, or impose extreme electrostatic or steric effects (Cunnigham and Wells, 1989).

In this thesis, mutations have been generated within the 742-767 segment of human complement component C3 (mature C3 numbering) that had been suggested by the Fishelson (1991) hexapeptide experiments to contain a factor H interaction site. The series of mutants were assessed for their ability to interact with complement factors H, soluble CR1 and soluble MCP using factor I cofactor assays as an indirect reporter of the binding interaction. The conformational "integrity" of the mutant C3 molecules was also assessed.

Chapter 2

Materials & Methods

2.1 Buffers

Veronal buffered saline (VBS) and sucrose veronal buffered (SVB) solutions of varying sorts were used in the complement assays. Proteins were dialyzed in a 1 x VBS solution consisting of 4 mM sodium barbital, pH 7.2, 150 mM NaCl, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$. GVB is VBS containing 1 g/l gelatin; GVBE is GVB containing 10 mM EDTA; SGVB is SVB (low ionic strength VBS containing 112 mM sucrose, and 30 mM NaCl) with 1 g/l gelatin. Phosphate buffered saline (PBS) is 10 mM NaH_2PO_4, pH 7.3, 150 mM NaCl.

2.2 Purified Complement Components and Antibodies

$C\bar{1}s$ (Gigli *et al.*, 1976), C2 (Nagasawa and Stroud, 1977a), C3 and C5 (Tack and Prahl, 1976), C4 (Bolotin *et al.*, 1977), factor B (Götze and Müller-Eberhard, 1971), factor D (Lesavre *et al*, 1979), factor H (Pangburn *et al.*, 1977), and factor I (Nagasawa *et al.*, 1980) were purified from human plasma using previously established methods by previous members in the lab and frozen at - 80 °C. C6-9 reagent used in hemolytic assays was obtained from guinea pig serum (GIBCO, Oakville, Ontario) treated with potassium isothiocyanate and hydrazine hydrate followed by dialysis in VBS (Cooper and Müller-Eberhard, 1970). C1 euglobulin, needed for classical pathway C3 convertase formation, was precipitated from whole human serum (Cooper and Müller-Eberhard, 1968).

Rabbit polyclonal IgG against human C3c (Sigma Chemical Co., St. Louis, MO), goat antiserum against human C3 (Quidel, San Diego, CA) and alkaline-phosphatase conjugated antibody against goat IgG (Jackson Immuno Reasearch Laboratories, West Grove, PA) were purchased from the indicated suppliers. Mouse monoclonal IgG_1 4C2 against C3dg was a gift from Dr. V. Koistinen (Helsinki, Finland). Mouse monoclonal IgG1 against C3c was a gift from Dr. D. Dobbie (Dominion Biologicals Ltd., Truro, Nova Scotia). Radioiodination of purified complement component C3 involved the use of [^{125}I]-Na (Amersham Canada, Oakville, Ontario) and lactoperoxidase (Sigma) as described by Marchalonis (1969). In short, up to 250 µg of C3 in 50 µl, stored in 0.1 M sodium phosphate, pH 6.5, was mixed with lactoperoxidase (80:1 protein: lactoperoxidase w/w), 200 µCi [^{125}I]-Na (100 mCi/ml) and 15 µl 1 M NaH_2P0_4, pH 7.3. Radiolabelling was initiated upon the addition of 3 µl H_2O_2 (1/4000 dilution in H_2O of a 30% w/w stock) and after 10 minutes at RT the reaction was stopped by adding 500 µl of PBS containing 0.02% NaN_3 and 5 µl of 0.1 M KI. Separation of unincorporated [^{125}I]-Na was achieved by rocking the solution in 1 gram of BSA-saturated PBS-washed Dowex-1 beads (Sigma) for 20 minutes at RT in a 1.5 ml microfuge tube (Starstedt). After perforating the bottom of the tube once with a needle, the solution was separated from the beads by centrifugation into another microfuge tube. In order to determine the fraction of protein bound ^{125}I, 1 µl of the iodinated protein was mixed with 50 µl BSA (1 mg/ml) and precipitated with 500 µl of 8% trichloroacetic acid (TCA) solution. This technique, by in large, gives greater than 95% TCA precipitable counts and specific activities of 0.2-2.0 x 10^6 cpm/µg protein.

Tissue Culture Methods:

2.3 Inactivation of bovine factor I in fetal calf serum with K76-monocarboxylic acid

K76-monocarboxylic acid (K76-COOH) was a gift from Otsuka Pharmaceutical Co. (Tokushima, Japan) and was used to irreversibly inactivate the bovine factor I present in the fetal calf serum (FCS)(Hong *et al.*, 1981). This treatment allowed me to perform human factor H and I cofactor-dependent cleavage assays of biosynthetically labeled recombinant human C3 directly in the culture medium, without interference from the bovine factor H and I present in the culture medium. As was the case in all cell culture media, the FCS was first heat-inactivated for 1 hour at 56 °C. The process of factor I inactivation required the pH of the serum to be raised to 9.0 with 1 N NaOH, after which solid K76-COOH was added (2 mg/ml). The pH was then readjusted to 9.0 with 1N NaOH and the serum was incubated at 37 °C for 2-3 hours. An equal volume of 0.1 M $Na_2B_4O_7$, pH 9.2 was added and the mixture was cooled on ice. $NaBH_4$ (4 mg/10 ml FCS initial volume) was dissolved in 0.01 N NaOH and added to the FCS with stirring for 1 hour. The solution was dialyzed extensively against 1 x VBS, filter sterilized (0.2 micron) and then stored at -20 °C. It should be noted that the treatment of FCS with K76-COOH partially compromises the media's ability to promote the growth of cells. For this reason, a media supplement was added (see below).

2.4 Cell lines

All cell lines were maintained at 37 °C with 5% CO_2 in a humidified tissue culture incubator (Johns Scientific).

African green monkey kidney COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (henceforth, referred to as complete media). Murine myeloma J558L cells that were stably transformed with pKG5-En-C3, a vector encoding human C3 and harbouring a neomycin resistance gene, were selected and maintained in complete media containing 0.6 mg/ml active G418-sulfate (Geneticin, Gibco).

C3 cofactor binding assays required that these cells be washed in Hanks' buffered salt solution (HBSS, GIBCO) and then grown overnight in total media in which the 10% FCS was replaced with 4% K76-COOH-treated FCS and supplemented with 1% Nutridoma-HU, a media supplement to help the growth of cells in growth factor-deficient media (Boehringer Mannheim Biochemicals, Montreal, Quebec).

2.5 DNA methods

The principal vectors used in all the studies were pBST-C3B and pSV-C3. These vectors had been previously generated by Dr. Aiko Taniguchi-Sidle. In short, pBluescript (pBST) was digested with *Hind* III producing a site into which a full-length C3 cDNA (as a *Hind* III fragment) was inserted in one of two orientations; these insertions generated pBST-C3A (top strand is C3 coding strand) and pBST-C3B (bottom strand is C3 coding strand). pSV is a pBluescirbe M13+ (Stratagene) plasmid which contains a SV40 promoter, a three enzyme cloning site (*Hind* III-*Bam*H I-*Xho* I) and a SV40 polyadenylation signal in lieu of the original polylinker of pBluescribe M13+. The very same *Hind* III cDNA fragment of C3 cDNA was blunt ended and inserted into a blunt-ended *Bam*HI site of pSV in an orientation that would permit C3 expression, thereby producing the plasmid pSV-C3.

Generation of Mutant C3 Recombinant Molecules:

2.6 Polymerase Chain Reaction-mediated site directed mutagenesis

Site-directed mutations were generated by the overlap-extension variation of polymerase chain reaction-based mutagenesis (Ho *et al.*, 1989), as illustrated in figure 2.1.

The technique involves the use of four different oligonucleotide primers, specifically two so-called 'flanking' primers and two overlapping 'mutagenic' primers. One of each pair was copied from the sense strand of the DNA sequence, while the other of was copied from the antisense strand, with the exception of the appropriate nucleotide substitutions in the mutagenic primers. The construction of each primer followed certain rules. All were about 20 to 22 base-pairs (bp) in length with a calculated melting temperature (T_m) greater than 60 °C. Moreover, the 3' end of each primer was made to end with at least a pair of G/C nucleotides. Each pair of mutagenic primers overlapped at least 90% with one another. All primers were "screened" for non-specific binding to other sites in the human C3 sequence using the MacMolly sequence comparison software to give a rough indication of potential PCR background problems. In addition to the above rules, the mutagenic primers were constructed such that the mutated bases were more or less in the centre of each oligonucleotide with at least 6 perfect match nucleotides on either end.

In terms of the location of the oligonucleotides, the 5'-flanking primer (sph 1.2) was situated upstream of a unique restriction enzyme site for *Sph* I. The other flanking primer (pml 1.2) was found downstream of another unique restriction enzyme site (*Pml* I). The upstream flanking primer orientation was sense with respect to the coding sequence whereas the downstream flanking primer orientation was antisense. Another important

Figure 2.1 Site-directed mutagenesis by overlap extension using PCR.

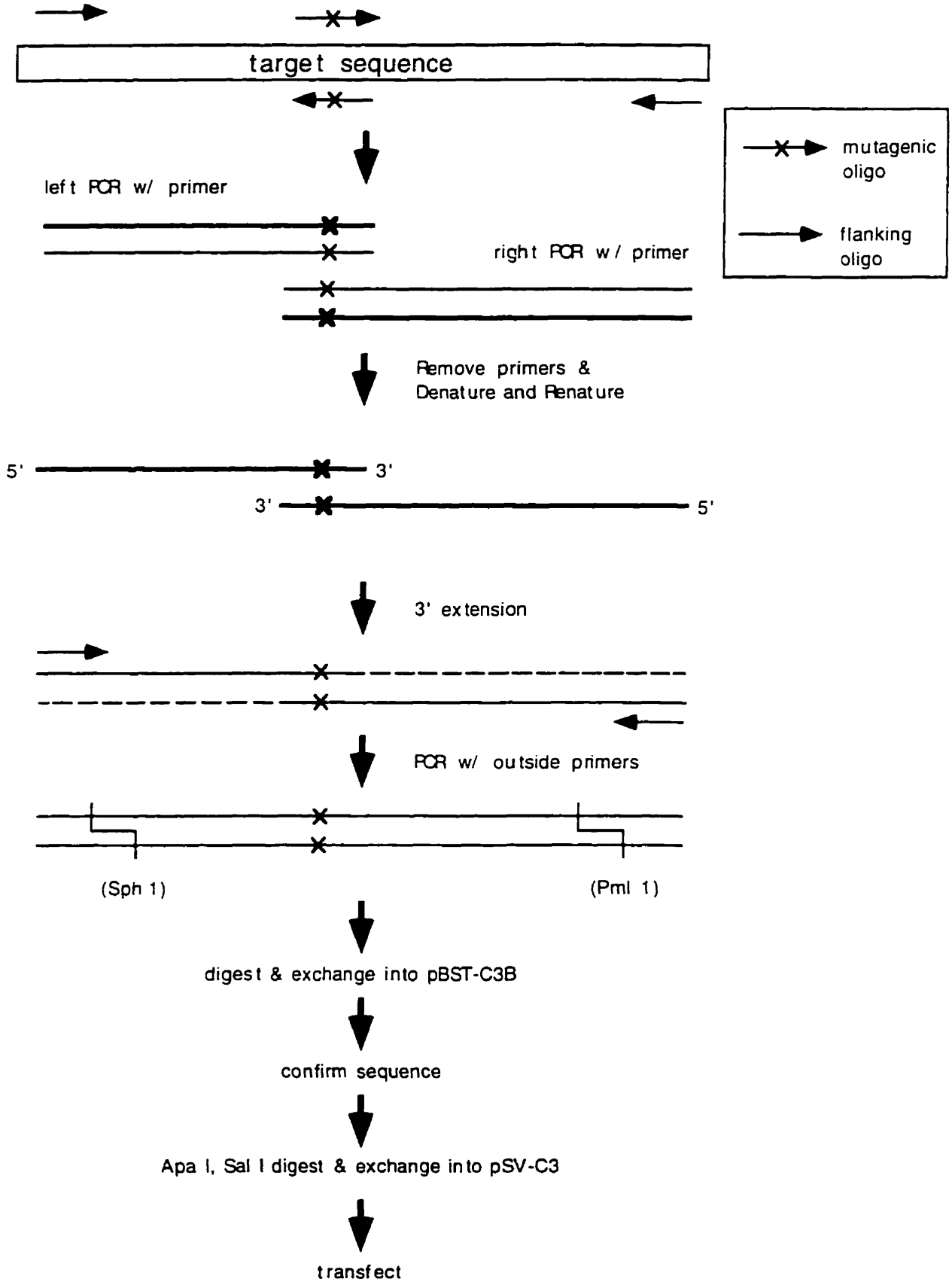
1. Primary reaction: One flanking primer is paired with one mutagenic primer of the opposite orientation.

2. The primary PCR products are purified, mixed together, denatured, and re-annealed.

3. Secondary reaction: 3' ends of each reaction are allowed to extend before the flanking primers are added, thereby amplifying the full-length sequence.

4. The secondary products are purified, digested with SphI and PmlI and subcloned first into pBST-C3B, and then form pBST-C3B into the pSV-C3 expression vector before transfection.

Mutagenesis & Expression of human C3



point is that the flankers were designed to be positioned at specific distances from all the sites to be mutated such that the size of the PCR fragments generated were sufficiently large enough (ie. >200 bp) to i) resolve from the primers and 'failed' products on a 1.5% (w/v) agarose gel, and ii) isolate using Geneclean II (BIO 101, Vista, CA).

The procedure involves two sets of PCR reactions, i) the primary reaction - where the partially overlapping left-side and right-side fragments are generated with the mutations being in the overlapping regions, and ii) the secondary reaction - where the overlapping fragments from i) are allowed to anneal and form a larger double-stranded fragment with the mutant nucleotide(s) on both strands (see Fig. 1.2).

The primary PCR products (ie. the left-side and right-side fragments) are generated in the presence of template (either pBST-C3B or pSV-C3) by taking a flanking primer and pairing it with its opposing-orientation mutant primer. That is, the upstream sense strand flanker is placed in a PCR reaction mix with the antisense (3'-5') mutagenic primer. Likewise, the downstream flanker (antisense) is placed in a PCR reaction mix with the sense mutagenic primer. These are done in separate reaction mixes. Specifically, 10 ng of pBST-C3B/pSV-C3 was mixed with 100 pmol of each primer of a pair, 6 µl dNTPs (2.5 mM), 10.0 µl 10 x Vent polymerase buffer (10 mM KCL, 10 mM $(NH_4)_2SO_4$, 2 mM Mg_2SO_4, 0.1% Triton X-100, 20 mM Tris-HCL, pH 8.8) and 1 U of Vent DNA polymerase (New England Biolabs, Inc., Beverly, MA) to a total volume of 100 µl. The primary polymerase chain reactions were then performed in a Perkin Elmer 2400 PCR machine (Perkin Elmer, California) using 30 cycles of denaturation at 94 °C, annealing at 52 °C, and extension at 72 °C for 30 seconds each. The primary PCR products were then separated on an agarose gel and isolated from the gel slice using Geneclean II (BIO101), as described by the manufacturer. Recovery was usually estimated from ethidium staining

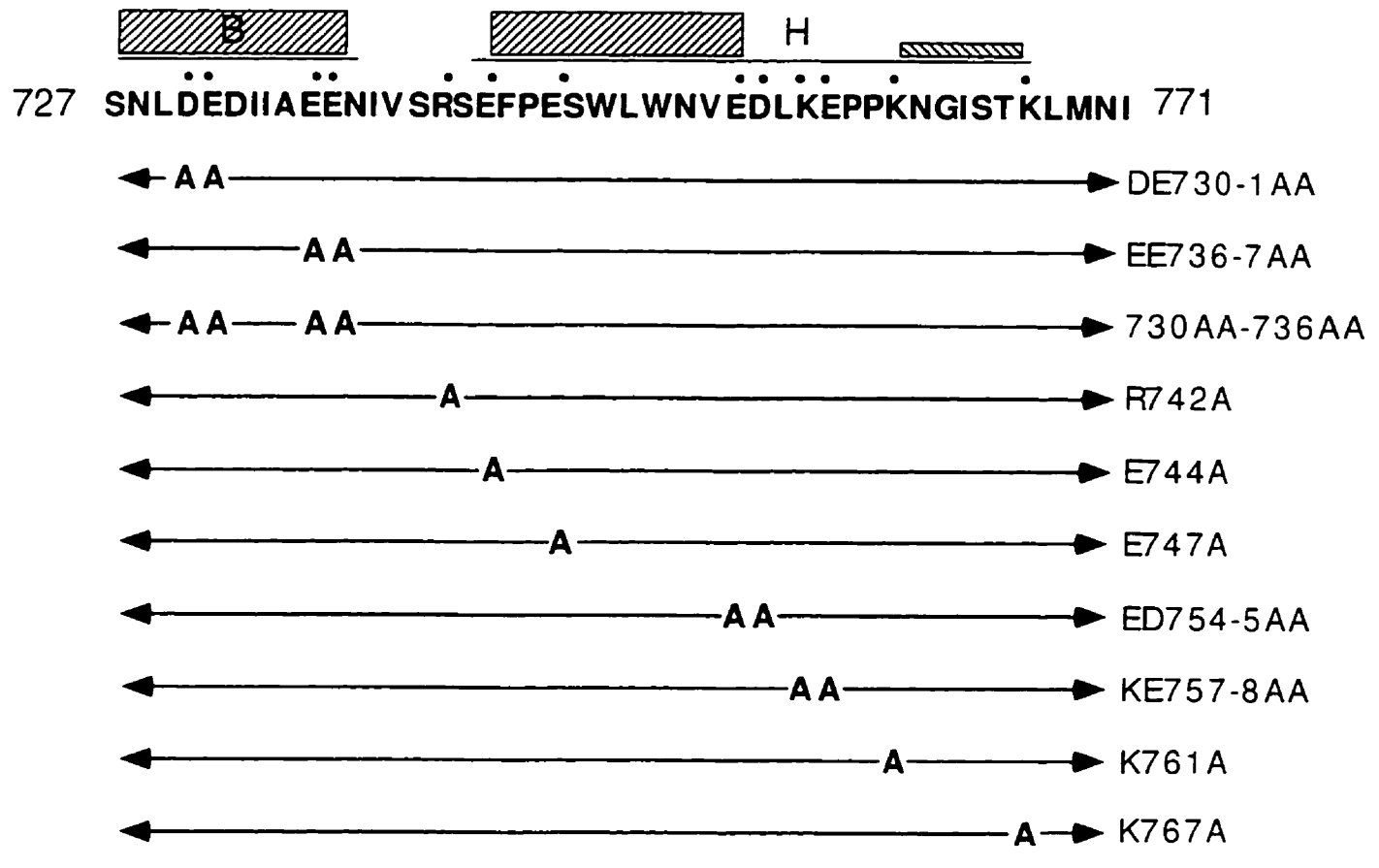
intensity of an aliquot on an agarose gel electrophoresis in comparison with size markers of known quantity.

Purified primary PCR products were then used in the secondary PCR reaction. Specifically, 10 ng of each fragment was mixed with 100 pmol of each flanking primer, 6 µl dNTPs (2.5 mM), 10 µl 10 x Vent polymerase buffer, and 1 U Vent DNA polymerase in a total volume of 100 µl. The conditions of the second polymerase chain reaction were as described above for the primary reaction. The final product has a size of 612 bp, corresponding to the distance between the 5' end upstream flanker sph1.2 and the 5' end of the downstream flanker pml 1.2. These secondary PCR products were then digested with the restriction enzymes *Pml* I and *Sph* I to generate a 312 bp fragment that contained the coding sequence mutation(s). This fragment was first subcloned into a *Pml* I-*Sph* I digested pBST-C3B plasmid. Once the presence of the mutation and absence of unwanted changes was confirmed by sequencing of the 312 bp 'exchange' fragment, it was then digested with *Apa* I and *Sal* I, thereby generating a 1300 bp fragment which was subcloned from pBST-CB into the *Apa* I-*Sal* I digestion vector fragment of the expression plasmid pSV-C3. The presence of a successful insertion of mutant fragment was confirmed by sequencing. The intermediate vector step was required because *Sph* I was not a unique digestion site in pSV-C3.

Figure 2.2 Mutants assessed in this study.

The appropriate substitutions were made at each of the charged residues indicated above. Isosteric amide substituents shown to have an effect with regard to factor B binding (Fishelson, 1991), as indicated in the hatched box above, were also assayed. All charged residues between 742 and 767, the region suggested to bind to factor H (Ibid, 1991), were converted to alanine.

N-Terminal Mutants of the Human C3α' Chain



Expression & Quantitation of Recombinant C3:

2.7 Transfection of COS-1 cells

The DEAE-Dextran method (Oprian *et al.*, 1987) was used in the transfection of COS-1 cells with the wild type and mutant pSV-C3 expression vectors. Freshly split COS-1 cells were allowed to adhere to 35 mm or 60 mm tissue culture plates and grow to a density of 50% +/- 10% confluency prior to transfection (seeded at 0.1 x 10^6 cells per 35mm plate; 0.5 x 10^6 cells per 60 mm plate). Cells were then washed with HBSS two times and then incubated in the presence of FCS-free DMEM containing 0.5 mg/ml DEAE-Dextran (Sigma), 50 mM Tris-HCl, pH 8.0, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and pSV-C3 (5-10 µg) for 40 minutes at 37 °C. The supernatant was aspirated and replaced with DMEM containing all of the above except in lieu of DEAE-Dextran and Tris-HCl, chloroquine and FCS were added to a final concentration of 100 µM and 10 %, respectively. The cells were incubated in this slightly toxic media for 2-3 hours. The supernatant was then removed carefully by aspiration, the adhering cells were washed two times with HBSS, and then complete media was added. The media was changed after 48-72 hours to DMEM containing 4% K76-COOH-treated FCS and 1% Nutridoma in place of the 'standard' 10% FCS. The cells were cultured overnight in this media prior to metabolic labeling as described below. All supernatants were harvested 72-96 hours after transfection and dialyzed against 1 x VBS.

2.8 Metabolic Labeling of C3

Prior to radiolabeling, the transfected cell lines were washed 2x with sterile PBS to remove residual media. To these cells, methionine-, and cysteine-depleted DMEM containing 4% K76-treated FCS and 1% Nutridoma was added (1 ml per 35 mm plate; 1.5

ml per 60 mm plate). The cells were cultured for 1 hour in the incubator. The cells were then 'pulse-labeled' with 100-200 μCi of [^{35}S]-Translabel (ICN Biochemicals) for 6 hours. After 6 hours, an equal volume of Met$^-$Cys$^-$K76$^-$Nu$^-$ DMEM was added and the 'chase' incubation was continued overnight.

2.9 Quantitative measurement of secreted recombinant C3

In order to determine the amounts of recombinant C3 secreted by our transfections, both unlabelled and [^{35}S]-labeled, a competitive solid-phase radioimmunoassay (RIA; Harlow and Lane, 1988) was performed. Non-flexible polystyrene microtitre wells (Packard, Meridan, CT) were coated with rabbit polyclonal IgG anti-human C3c (10 μg/ml in 0.01 M $NaHCO_3$ pH 9.8; 200 μl/well) for 2 hrs at 37 °C. The wells were flicked dry and then blocked with Blotto (5% skim milk in PBS-Tween 20, 0.5%; 200 μl/well) for 30 minutes at 37 °C. The wells were washed 3x with PBS-Tween and flicked dry. C3 that had been purified from human serum and radioiodinated (10-30,000 cpm/well) was allowed to compete with known concentrations of unlabelled, purified C3 and with unknown concentrations of recombinant C3 for binding to rabbit polyclonal IgG against human C3c. Following washing, the amount of bound radioactivity was determined using an automated TopCount^{tm} system (Packard). From these data, a standard curve was plotted and the concentration of each recombinant C3 in the culture media was determined by the interpolation of the standard curve.

2.10 Metabolic Processing of C3

The supernatants were harvested and divided into 2 aliquots. One aliquot was incubated for 1 h at 37 °C with fluid-phase C3 convertase (C4b^{oxy}C2a), which was prepared by treating C4 at 37 °C with active $\overline{C1s}$ (1.5 %, w/w) for 1 hour followed by

addition of iodine-oxidized C2 (25 %, w/w) for 3 min. Four C4 µg equivalents of $C4b^{oxy}2a$ were used per sample and the C3 was then immunoprecipitated as described below. Iodine-oxidized C2 ($^{oxy}C2$) was prepared by adding a stock solution of a KI/I_2 mixture to the C2 protein solution such that the final solution was 1.3 mM KI, 0.024 mM I_2. Prior treatment of C2 with this oxidizing soltuion greatly enhances the lifetime of the classical pathway C3 convertase (Polley & Müller-Eberhard, 1967).

2.11 Immunoprecipitation of C3

Prior to immunoprecipitation, dialyzed supernatants containing the metabolically-labeled recombinant C3 were treated with one tenth volume of 10x detergent concentrate (5% SDS, 2.5% deoxycholate, 5% Triton-X 100, 10 mM EDTA in 1x PBS). The protease inhibitors iodoacetamide and phenylmethylsulfonylfluoride were added to the solutions at final concentrations of 1 mM and 2 mM, respectively. 10 µl of formalinized *Staph. aureus* suspension (10% Protein A suspension; Sigma) was added to clear non-specific binding proteins from the supernatants; the samples were rocked for 1 hour at 4 °C. The samples were then spun down and the supernatants recovered, to which 20-30 µg of rabbit anti-human C3c polyclonal antibody (IgG fraction) was added. A specificity control using rabbit anti-human C4 polyclonal was included in the experiment. These antibodies were allowed to bind overnight at 4 °C. The next day the samples were treated with 10 µl of Protein A suspension for 1 hour with rocking at 4 °C. The samples were again spun down, the pellet was recovered (Protein A-IgG-C3 complexes) and washed at least 3x with 1x detergent buffer. The pellets were then resuspended in 30 µl of 1x sample buffer (5x stock contains 0.309M Tris-HCl, pH 6.8, 50 % v/v glycerol, 10% SDS, 0.15 mg/ml bromophenol blue) and dithiothreitol was added to a final concentration of 40 mM. The samples were finally boiled for 3 minutes to incorporate the SDS and reduce the disulfide bonds. The *Staph. aureus* particles were pelleted and the supernatants were then loaded on

a 9% SDS polyacrylamide minigel (BioRad apparatus). Following electrophoresis, the gels were fixed in a methanol:acetic acid: water (5:1:5) solution with rocking at 65 °C for 10 minutes. The gels were then washed in water with rocking at 65 °C for 10 minutes followed by a 10 minute soak at 65 °C in 1 M sodium salicylate, a compound that fluoresces when excited by β-particle radiation. The gels were dried and exposed to Kodak XAR film at -70 °C for at least 2 days before development. Semi-quantitative analysis of band intensities was performed by scanning the film on an Epson ES-1000C color scanner (ScanTasticCentral^{tm} software, Adobe) and quantitating the scanned image using the program NIH Image v1.58 (NIH, Bethesda, Maryland).

Functional and Conformational Analyses of Recombinant C3:

2.12 Preparation of C3-coated sheep erythrocytes

Sheep red blood cells (SRBC)(PML microbiologicals, Tualatin, Oregon) were treated with anti-SRBC IgM antibody (Sigma) and then with C1 euglobulin, purified C4 and ^{oxy}C2 to produce C4b^{oxy}2a-coated erythrocytes (Polley and Müller-Eberhard, 1967; Cooper and Müller-Eberhard, 1968; Cooper *et al.*, 1970).

2.13 Classical Pathway-dependent Hemolytic assays

The above C3-convertase bearing cells, EAC4b^{oxy}2a (1.5×10^7) were incubated for 1 hour at 37 °C with 500 µl of dialyzed culture supernatants containing varying amounts of C3 per assay, as determined by RIA. The resulting EAC423b cells were washed in GVBE and then incubated with human C5 (1 µg) and guinea pig C6-9 reagent (1/50 dilution in GVBE, 1 ml). These cells were then incubated at 37 °C until a suitable degree of lysis developed in the positive controls (usually between 15-60 minutes). After spinning down

unlysed cells, the degree of lysis in each tube was determined by measuring absorbance of the supernatant at 412 nm. The degree of lysis was converted to 'Z' units where Z = [-ln(1-fractional lysis)] and where fractional lysis equals the "specific" absorbance at 412 nm of a particular test sample divided by the absorbance at 412 of an equal number of erythrocytes lysed in an equal volume of distilled water. The specific absorbance has been corrected for spontaneous lysis in a control incubated with dialyzed media that does not contain C3. The physical meaning of Z is that it corresponds to the number of hemolytically effective molecules of C3 per target cell (Rapp and Borsos, 1970).

2.14 Cleavage of [^{35}S]-C3(H_2O) by factor I and cofactors H, sCR1 and sMCP

The biosynthetically-labelled C3-containing supernatants were collected and then treated for 4 hours at 37 °C with an equal volume of 4 M KBr in order to deliberately hydolyze the thioester and convert native C3 to the C3b-like C3(H_2O) molecule. The supernatants were dialyzed extensively in 1 x VBS at 4 °C. Equal concentrations of [^{35}S]-C3(H_2O) (100 ng), as determined by RIA, were treated with 2 variable amounts of either purified human factors H, sCR1, or sMCP, and a single amount of purified factor I for 2 hours at 37 °C; exact quantities added are given in various figure legends (see *Results*). A control sample in each case had factor I added without any cofactor. Afterwards, the samples were immunoprecipitated using rabbit polyclonal IgG against C3c in conjunction with formalized *Staph. aureus* (Sigma) as described above. These samples were then treated with SDS-PAGE sample buffer under reducing conditions, separated on a 9% or 11% SDS-PAGE, and analyzed by autofluorography (Kodak XAR-5 film) using 1 M Sodium Salicylate as the gel-impregnating fluor. A variant of this assay done with H as the I cofactor involved adding to the KBr-treated supernatants fresh fetal calf serum (10% final concentration) as a source of heterologous (ie. bovine) factors H and I.

2.15 Enzyme-linked immunoabsorbant assay of native/denatured C3

96-well polystyrene microtitre plates (Starstedt) were coated in separate wells with varying dilutions of purified human C3 either treated or not treated with SDS ($[]_{final}$ = 10% v/v) in 1 x PBS for 2 hours at 37 °C. A starting concentration of 30 µg/ml was used and a 3-fold dilution series was setup (diluted in PBS; 200 µl/well). C3 was allowed to coat the wells for 2 hours at 37 °C and flicked dry. The wells were then blocked with 5% skim milk in PBS (no Tween) for 30 minutes at 37 °C, washed 3x with PBS, and flicked dry. Two different primary mouse monoclonal antibodies were used in the experiment, one being against a C3c epitope and the other against a C3dg epitope (1:1000 dilution in 5% skim milk in PBS; 200 µl/well). A polyclonal rabbit anti-human C3c Ab (1:1000 dilution in 5% skim milk in PBS; 200 µl/well) was used as a control for the presence of C3/C3(H_2O) on the plates. These antibodies were allowed to adhere for 2 hours at 37 °C. Following washing, the alkaline phosphatase conjugated secondary antibodies, donkey anti-mouse IgG in the case of the mouse monoclonals and goat anti-rabbit IgG in the case of the polyclonal primary antibody, were then added (1:5000 dilution in 5% skim milk in PBS; 200 µl/well) and allowed to bind for 2 hours at 37 °C before washing and drying as described above. 200 µl of 0.5 mg/ml p-nitrophenylphosphate in alkaline phosphatase buffer (0.1 M diethanolamine, 0.5 mM $MgCl_2$, 0.02% NaN_3, pH 9.8) was added to each well and the absorbance at 405 nm (Microplate Reader Model 450, Biorad) was measured after approximately 30 minutes at room temperature. Negative controls included no coat (i.e. no C3) and no primary antibody.

2.16 Enzyme-linked immunoabsorbant assay of recombinant C3 (Antibody Capture Version)

96-well polystyrene microtitre plates (Starstedt) were coated in separate wells with one of three antibodies: rabbit polyclonal IgG anti-human C3c, mouse monoclonal IgG anti-human C3c and mouse monoclonal IgG anti-human C3dg (1:1000 dilution in 0.01 M $NaHCO_3$, pH 9.8; 200 µl/well). After a blocking with Blotto (5% skim milk powder in 1 x PBS with 0.05% Tween 20), the wells were incubated with equal concentrations of the recombinant C3 molecules for 2 hours at 37 °C (200 µl/well). The primary sandwiching antibody, goat anti-human C3 (Quidel), was then added followed by the detecting antibody (or secondary), alkaline phosphatase conjugated rabbit anti-goat IgG_{1-4} (Jackson), 2 hours at 37 °C for each incubation. From the blocking stage onwards, the wells were washed with PBS-Tween (no milk) between each incubation and flicked dry. 200 µl of 0.5 mg/ml p-nitrophenylphosphate in alkaline phosphatase buffer (0.1 M diethanolamine, 0.5 mM $MgCl_2$, 0.02% NaN_3, pH 9.8) was added to each well and the absorbance at 405 nm (Microplate Reader Model 450, Biorad) was measured after approximately 30 minutes at room temperature. Negative controls included no coat (i.e. no capture Ab added), no C3, and no primary antibody.

Chapter 3

Results

A charged residue to alanine scan was performed through the target segment of C3 residues 742-767, this segment having been implicated as contributing to the binding interactions of C3b with factor H (Fishelson, 1991). However prior to assessing the various recombinant C3 molecules in the cofactor-dependent cleavage assays, it was felt that some measurement of general conformational integrity was in order. Four different assays were used to directly or indirectly address this issue, these being levels of secretion of the recombinant molecule, epitope recognition by conformationally-sensitive monoclonal antibodies, C3 dependent hemolytic activity measurements and cleavability by fluid phase classical pathway C3 convertase.

3.1 Recombinant C3 (rC3) molecules are secreted into the supernatants

The supernatants from unlabeled transfectants were harvested 3 to 4 days after transfection and the secreted C3 levels were measured using a radioimmunoassay (see Materials & Methods). In preliminary experiments it was determined that the level of protein varies as a function of the amount of each pSV-C3 plasmid used in the transfection, but peaked at about 200 ng/ml using 5-10 μg of plasmid DNA per 35 mm plate. The transfection optimization data for each of the 15 plasmids used in this study are presented in Table I. Excess plasmid in all cases inhibited the level of C3 expression. "Optimal" amounts of either 5 or 10 μg of a given plasmid were used in subsequent transfections to generate stock recombinant C3 for the subsequent functional/conformational analyses. In each subsequent transfection, the level of production by the various mutants was never significantly different from the wild type transfectants. This suggested that there was no selective intracellular retention or degradation of the mutant C3 molecules due to misfolding of the molecule.

Table I

Amount of C3 produced as a function of amount of DNA transfected

Recombinant	Amount Protein Secreted (ng/ml) with Varying Transfecting DNA Vector Amounts[a]			
	1 μg	5 μg	10 μg	30 μg
Wild type	60 ±16	190 ±32	174 ±43	111 ±41
742^{RA}	43 ±18	183 ±24	162 ±38	106 ±36
744^{EA}	56 ±18	194 ±36	140 ±33	122 ±21
747^{EA}	61 ±31	212 ±34	268 ±61	134 ±28
761^{KA}	52 ±29	245 ±28	155 ±51	116 ±23
767^{KA}	47 ±17	206 ±36	162 ±36	143 ±24
$744,747^{EA.EA}$	56 ±21	221 ±43	148 ±46	120 ±46
$754,755^{EA.DA}$	52 ±11	214 ±36	182 ±62	119 ±52
$757,758^{KA.EA}$	51 ±14	251 ±28	128 ±38	109 ±62
$744,7\text{-}754,5^{AA\text{-}AA}$	53 ±17	201 ±37	158 ±51	113 ±40
744^{EQ}	50 ±14	196 ±41	146 ±63	107 ±34
747^{EQ}	49 ±17	197 ±33	132 ±49	98 ±41
$730,731^{DN.EQ}$	62 ±24	185 ±42	145 ±52	102 ±27
$736,737^{EQ.EQ}$	57 ±15	189 ±33	138 ±45	109 ±41
$730,1\text{-}736,7^{NQ\text{-}QQ}$	53 ±12	198 ±51	140 ±37	121 ±37

[a] as determined by RIA from supernatants 3 days post-transfection of unlabelled COS-1 cells in 35mm plates

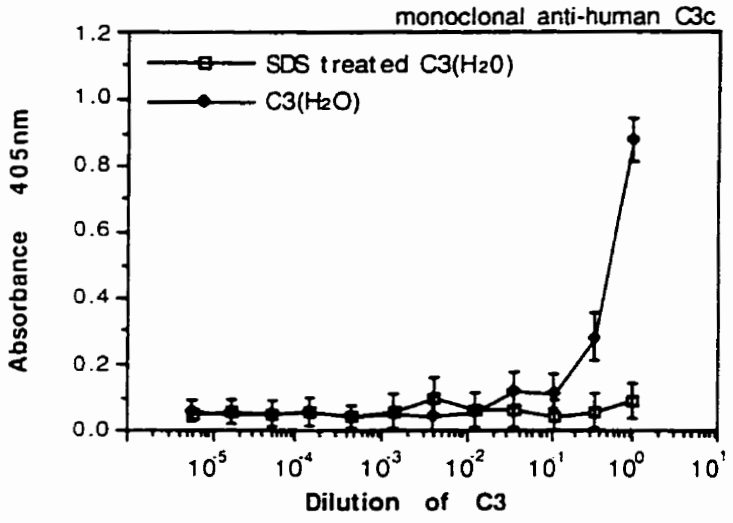
3.2 Two antibodies recognizing C3c and C3d respectively, are specific for native conformational epitopes

Two monoclonal antibodies, one specific for an epitope in C3c and the other for an epitope in C3dg, were employed as conformational probes. To demonstrate the conformational dependence of the antibodies, their respective binding to native and SDS-denatured purified C3 or C3(H_2O), the latter being the C3b-like thioester-hydrolyzed form of C3 used in all of the subsequent cofactor cleavage assays, was determined. Purified human C3 or C3(H_2O), with or without prior SDS denaturation, was serially 3-fold diluted and allowed to coat microtitre wells. The level of binding of each of the monoclonals to their ligands was then assessed in an ELISA assay using an alkaline phosphatase-conjugated anti-mouse IgG detection reagent. Additionally, the presence of both denatured and native C3 molecules on the plate was confirmed using a rabbit polyclonal anti-C3c which would be expected to recognize the denatured molecule. Detection in this case employed an alkaline phosphatase-conjugated anti-rabbit IgG. The results of these experiments are shown in figure 3.1. It can be seen that in the case of C3(H_2O), both antibodies bind only when the protein was in its native conformation. For intact C3, the anti-C3dg monoclonal also only binds to the non-denatured protein, however, while the same is true at high concentrations of antigen for the binding of the anti-C3c monoclonal to intact C3, the SDS denatured, but 27-fold dilution sample of intact C3 also registered significant binding of the anti-C3c monoclonal. The reason for this anomalous data point is unclear, but it may reflect the re-acquisition of the native epitope as the SDS was diluted out from the initial 10% to ~0.9% in the 27-fold diluted sample. In any event, since the cofactor assays all employ the C3b-like C3(H_2O) molecule, it is this species whose conformation we were primarily interested in probing and for it, both monoclonals show conformationally-sensitive binding.

Figure 3.1 Conformation-dependent binding of mouse monoclonals to epitopes in human C3 and C3(H_2O).

Starting from a concentration of 30 mg/ml serial 3-fold dilutions of purified human C3/C3(H_2O), either untreated or SDS-treated (10% w/v), were allowed to coat microtitre wells. The wells were incubated with either mouse anti-human C3c mAb (A) or mouse anti-human C3dg mAb (B) was added. The presence of C3/C3(H_2O) on the plate was tested using a rabbit polyclonal anti-C3c antibody (C). The degree of binding by the antibodies to C3/C3(H_2O) was measured using an ELISA-based approach as described in the Materials & Methods. All measurements were made in duplicate and the values presented are representative of at least three independent experiments.

A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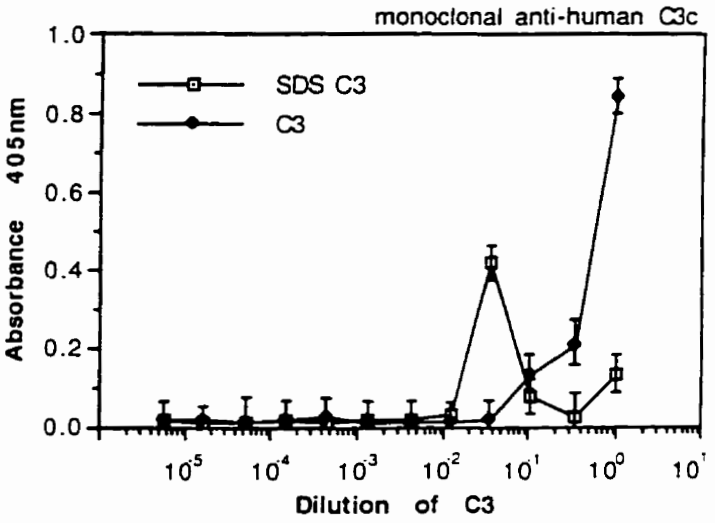
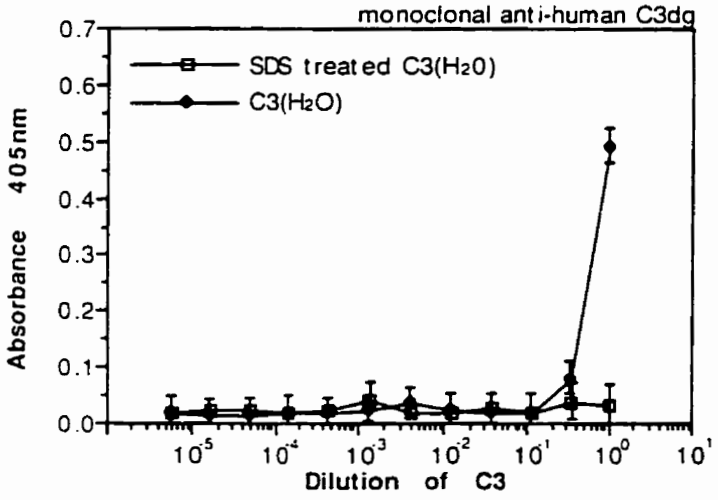
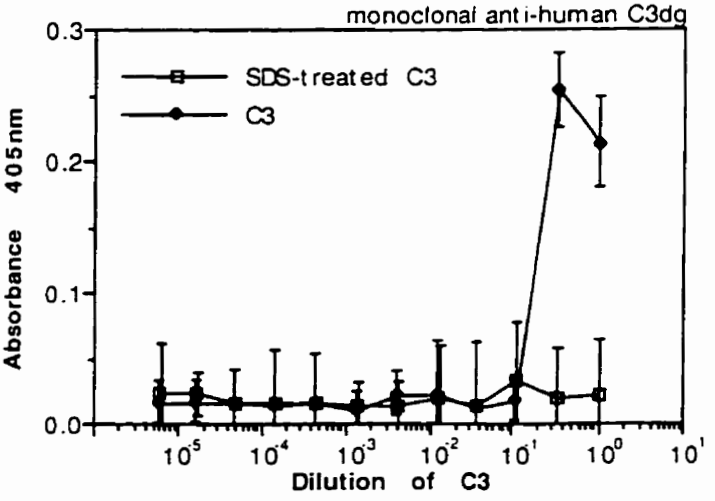
SDS treated C3(H2O)
C3(H2O)
Absorbance 405nm
Dilution of C3

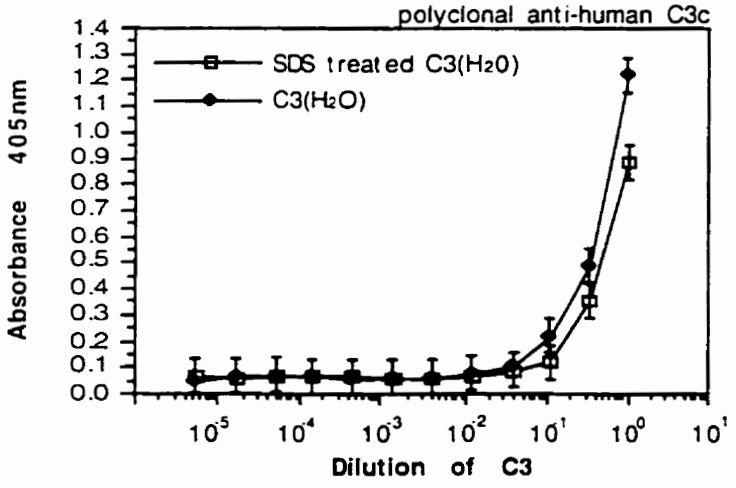

monoclonal anti-human C3c
SDS C3
C3
Absorbance 405nm
Dilution of C3

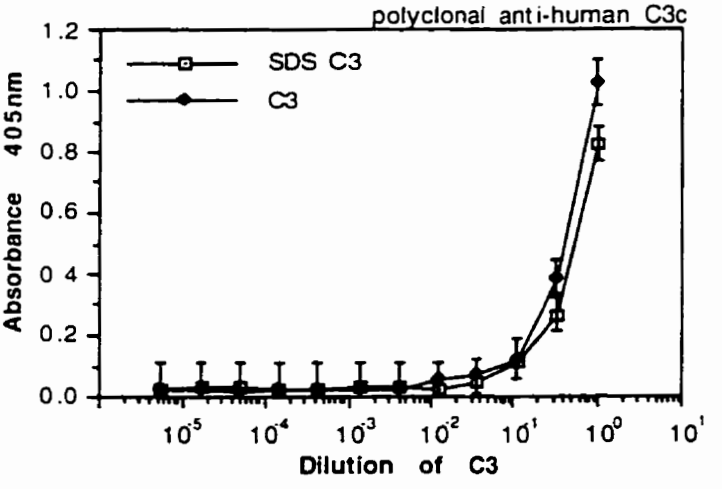
B


monoclonal anti-human C3dg
SDS treated C3(H2O)
C3(H2O)
Absorbance 405nm
Dilution of C3


monoclonal anti-human C3dg
SDS-treated C3
C3
Absorbance 405nm
Dilution of C3

C


polyclonal anti-human C3c
SDS treated C3(H2O)
C3(H2O)
Absorbance 405nm
Dilution of C3


polyclonal anti-human C3c
SDS C3
C3
Absorbance 405nm
Dilution of C3

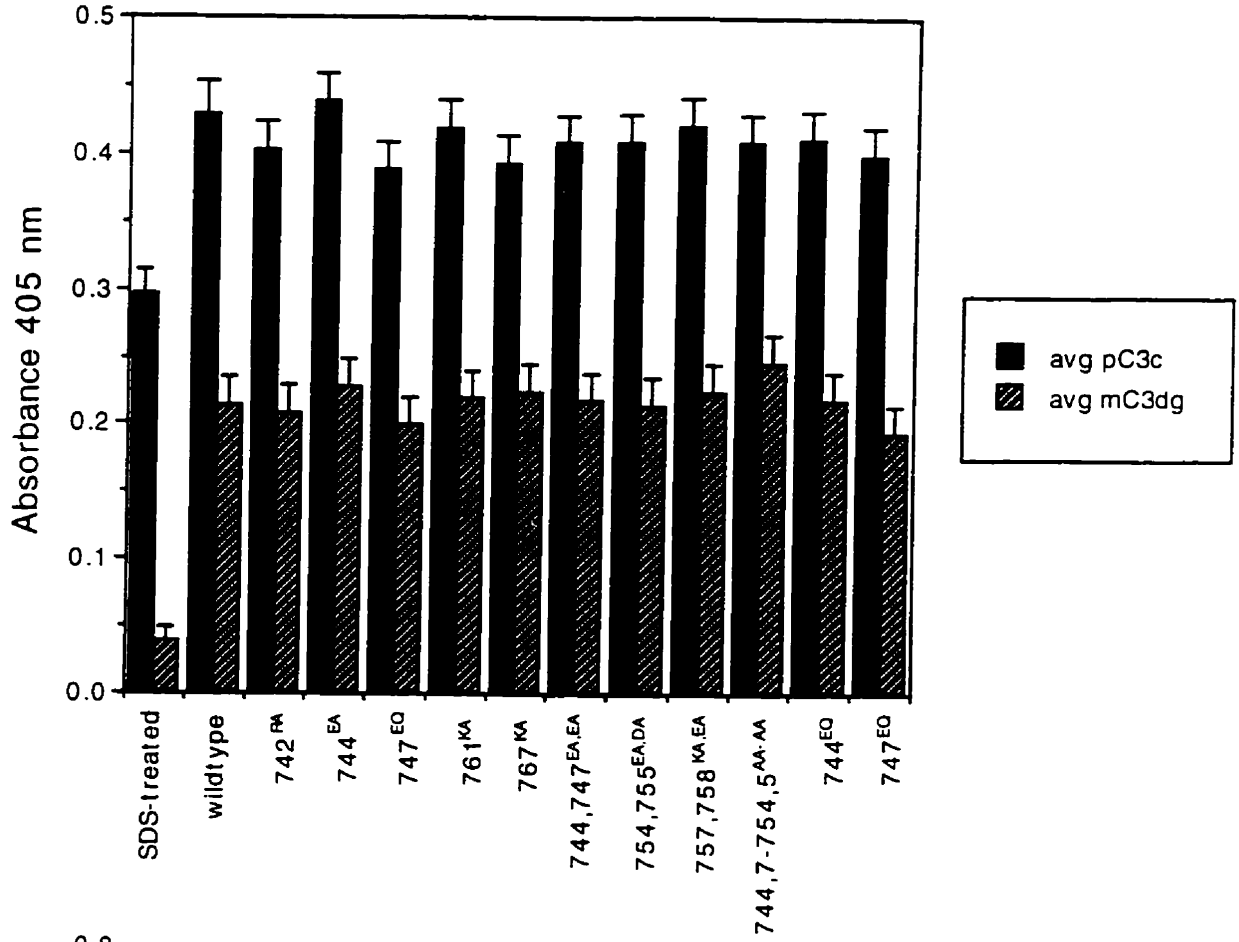
3.3 rC3 structures are not significantly perturbed versus wild type C3

As mentioned above, one could argue that normal secretion of C3 provides indirect evidence that the structure of the molecule is not severely disrupted. A more direct method, which employed the above described conformation-dependent monoclonals, was also used to assess the effect of the various mutations on the conformation of the various C3 mutants. In this approach, the two monoclonals, as well as the polyclonal anti-C3c, were individually coated on wells as capture antibodies. Binding of the various C3(H_2O) molecules to the three capture antibodies was then detected using a common polyclonal anti-C3 "sandwiching" antibody (see *Materials & Methods*). A representative experiment indicating the level of binding of the polyclonal anti-C3c and the monoclonal anti-C3dg to each recombinant molecule is shown in figure 3.2A. The results from such assays were then expressed as the ratio of a given monoclonal to polyclonal absorbance for each recombinant tested. These ratios were then compared to the ratios obtained using the wild type recombinant C3(H_2O) molecule. It can be seen from the data presented in figure 3.2B that the ratios were essentially constant among all of the mutants and further that they did not differ from the ratios observed with the wild type molecule. Thus, the conformationally-sensitive epitopes recognized by both the monoclonal anti-C3dg and monoclonal anti-C3c were unaffected by the various substitutions introduced in the course of this study.

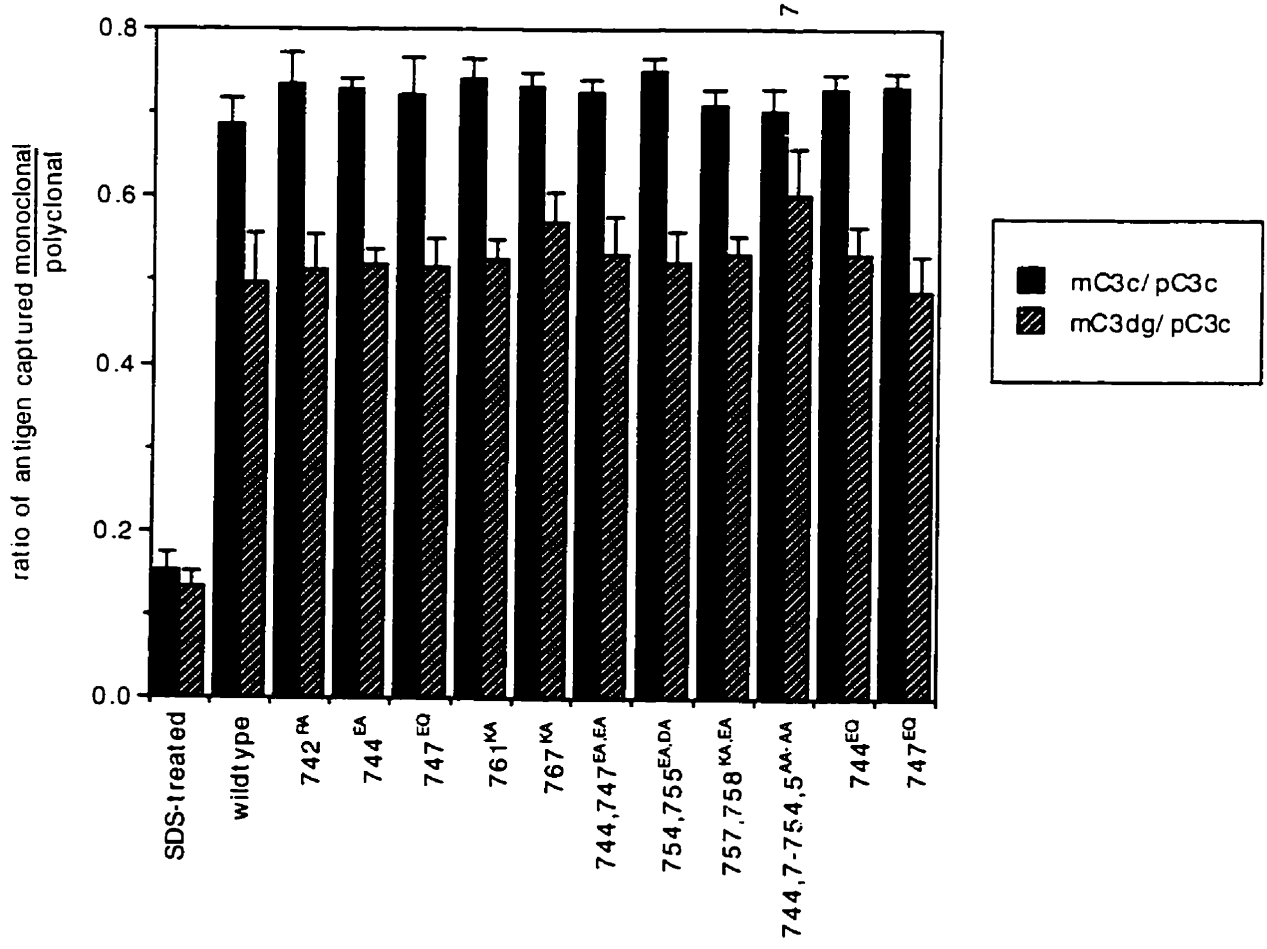
Figure 3.2 Binding of wild type and mutant rC3(H_2O) to two conformationally sensitive monoclonal antibodies.

Plates were coated with either mouse anti-human C3c mAb (mC3c), mouse anti-human C3dg mAb (mC3dg), or rabbit polyclonal anti-human C3c (pC3c). 100 ng of the various recombinant C3(H_2O) molecules were added to the wells and the level of binding was assessed using a goat polyclonal anti-human C3c "sandwiching" antibody, which was in turn detected with an alkaline phosphatase conjugated rabbit anti-goat IgG. A sample ELISA is shown in panel A. For each recombinant molecule, the ratio of antigen capture by the monoclonal versus the polyclonal is presented in panel B. These results are representative of three independent experiments using duplicate measurements each time.

A


Absorbance 405 nm
0.0
0.1
0.2
0.3
0.4
0.5
SDS-treated
wildtype
742RA
744EA
747EQ
761KA
767KA
744,747EA,EA
754,755EA,DA
757,758KA,EA
744,7-754,5AA-AA
744EQ
747EQ
avg pC3c
avg mC3dg

B


ratio of antigen captured monoclonal / polyclonal
0.0
0.2
0.4
0.6
0.8
SDS-treated
wildtype
742RA
744EA
747EQ
761KA
767KA
744,747EA,EA
754,755EA,DA
757,758KA,EA
744,7-754,5AA-AA
744EQ
747EQ
mC3c/ pC3c
mC3dg/ pC3c

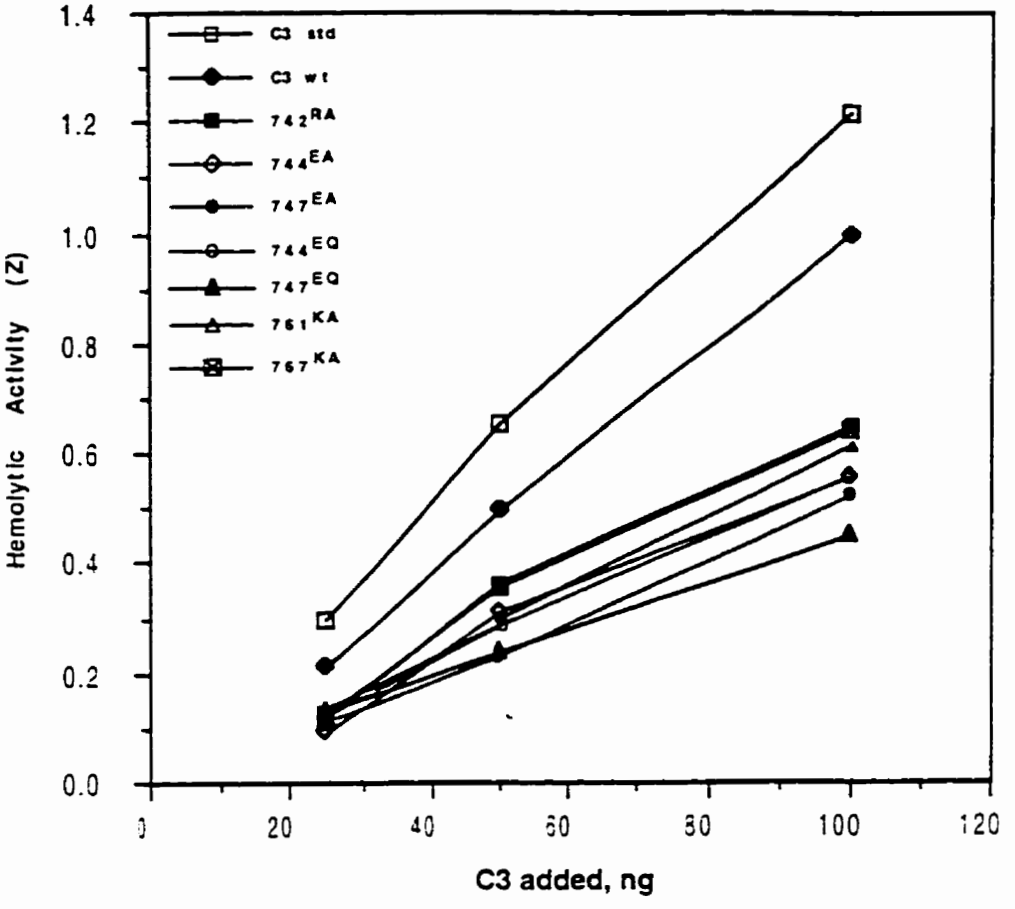
3.4 Hemolytic activities of the rC3 molecules

As a further control for conformation, I measured the relative ability of a C3 mutant molecule to become a subunit of a classical pathway C5 convertase on the surface of a sheep red blood cell. In the presence of excess C5 and C6-9, such C5 convertase-bearing cells (EA4b^{oxy}2a3b) will be lysed. Accordingly, increasing amounts of rC3 were added to equal numbers of EAC4b^{oxy}2a cells and lysis was developed upon addition of C5 and C6-9 reagents to the washed cells. The results of these C3-dependent hemolytic assays are shown in figure 3.3A for the single amino acid substituents and in figure 3.3B for the multiple amino acid substituents. Relative to the recombinant wild type C3, most of the mutants show some level of hemolytic defect ranging from about 50% to 70% of the wild type control (comparison made on basis of ng/0.5 Z units of activity). This level of defect is similar to that seen in a series of mutants generated just N-terminal to the present target region (Taniguchi-Sidle and Isenman, 1994). Since the hemolytic assay reflects a complex read-out dependent upon at least six C3 properties, these relatively minor defects in hemolytic activity suggest that the substitutions introduced have not grossly compromised the structure of the various mutant molecules. Another point that is evident from figure 3.3 is that the hemolytic activity of recombinant wild type C3 is about 70% that of purified serum-derived C3. This likely reflects the fact that whereas the non-recombinant C3 is composed entirely of mature 2-chain C3, the COS cell-derived recombinant C3 shows less than complete processing to the hemolytically active 2-chain form. Additionally, the amount of the C3 convertase-resistant, C3(H_2O) present in the sample is likely to be greater in the case of recombinant C3 due to the prolonged incubation at 37 °C. To determine whether the extent of either post-translational conversion of pro-C3 to mature C3, or the C3 convertase-mediated cleavability of the mutant molecules differed from the patterns observed in wild type C3, metabolically-labeled C3 derived form the entire series of transfected COS cells was immunoprecipitated with anti-C3, with and without prior

treatment with either the fluid-phase C3 convertase $C4b^{oxy}2a$. The SDS-PAGE analysis of this experiment is shown in figure 3.4 and it can be seen that the substitutions introduced have little or no effect on the processing of pro-C3 to mature 2-chain C3. In contrast, the cleavability of all of the mutants by $C4b^{oxy}2a$ was somewhat impaired relative to the wild type control. In another experiment, using a larger amount of $C4b^{oxy}2a$, the differences in cleavage susceptibility between mutant and wild type C3 were less apparent (data not shown). Collectively these data suggest that the mutations have an effect on susceptibility to cleavage by the classical pathway C3 convertase which ranges from 25 to 60% of wild type and that this may account for the partial defects seen in the hemolytic activities of the mutant C3 molecules analyzed in this study.

Figure 3.3 Classical Pathway-dependent hemolytic activities of wild type and mutant rC3.

EA423b were prepared with various amounts of recombinant C3 produced by transient transfections of COS-1 cells. Measurement of classical pathway-dependent hemolysis, as described in Materials & Methods, was done in duplicate. These results are representative of at least 4 independent experiments. Background lysis, determined using media that contained no rC3, was subtracted in all cases and was never more than 6% (+/- 4%) of maximal lysis produced by addition of H_2O to the cells.


C3 std
C3 wt
742 RA
744 EA
747 EA
744 EQ
747 EQ
761 KA
767 KA
Hemolytic Activity (Z)
C3 added, ng
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
20
40
60
80
100
120

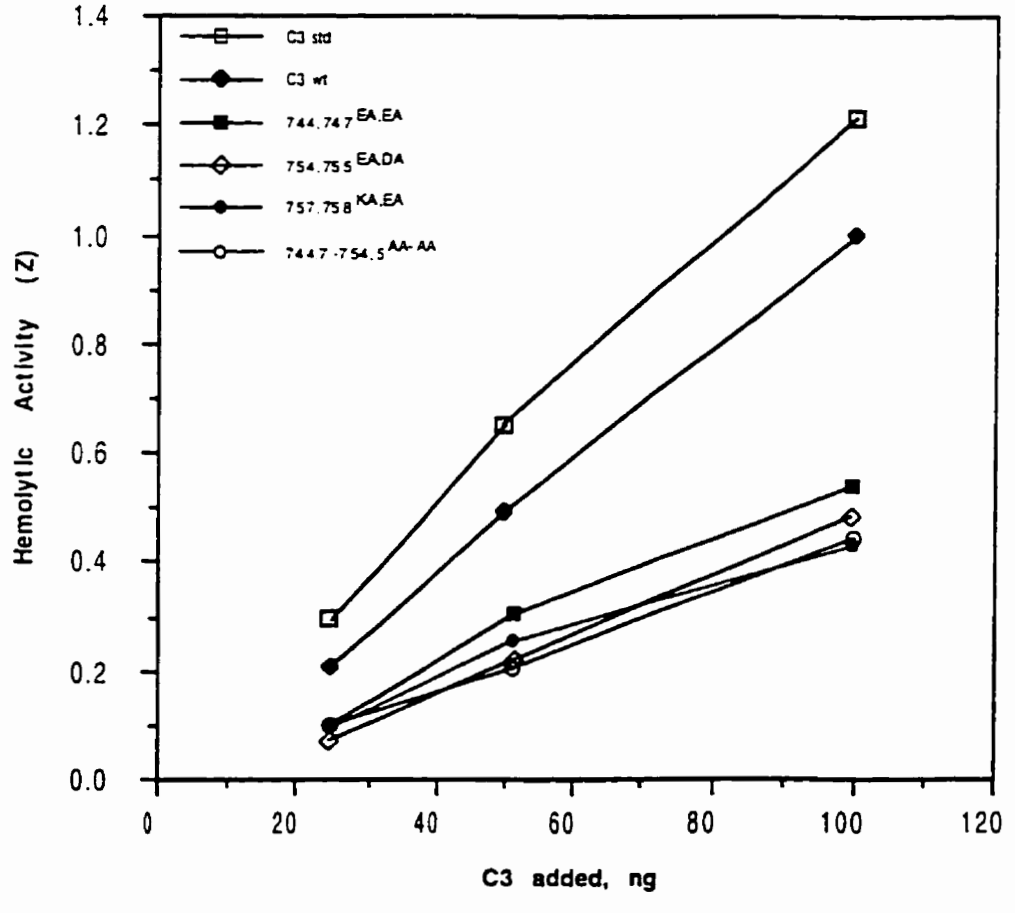
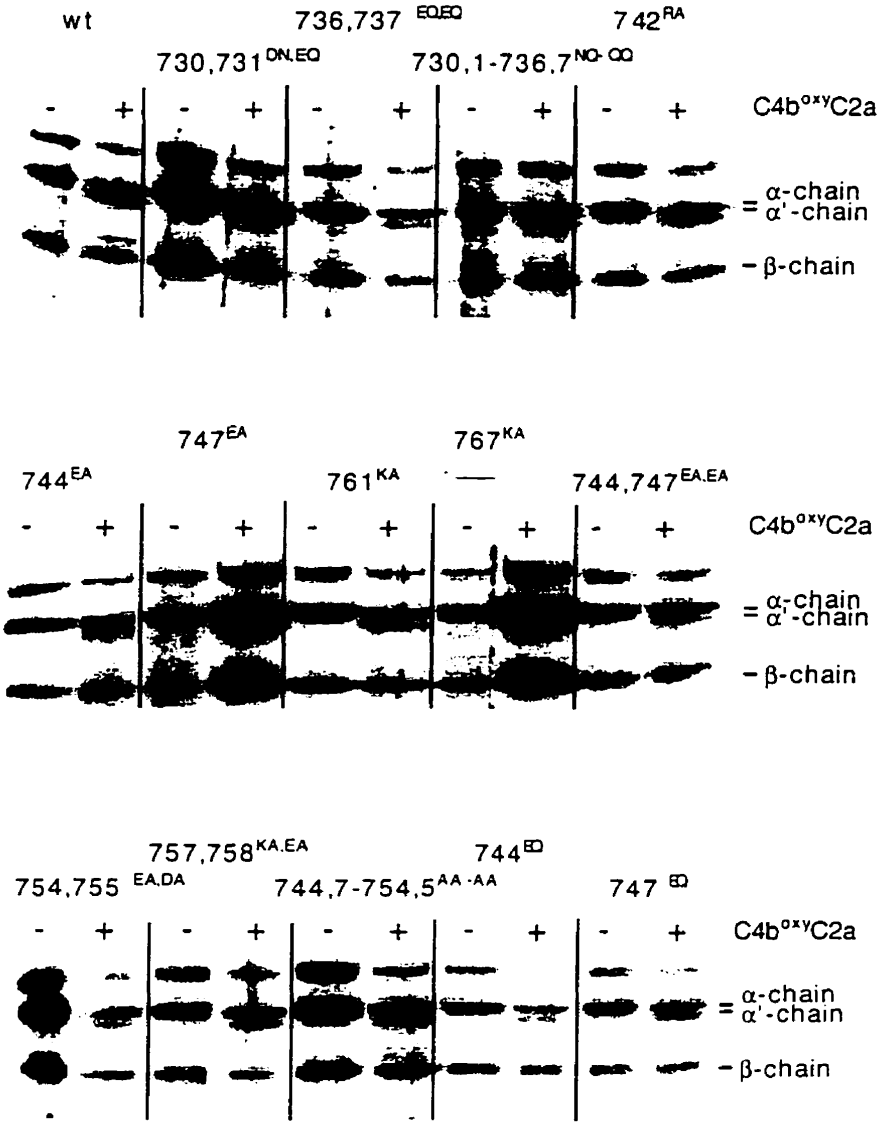

C3 std
C3 wt
744,747 EA,EA
754,755 EA,DA
757,758 KA,EA
744,747-754,755 AA-AA
Hemolytic Activity (Z)
C3 added, ng
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
20
40
60
80
100
120

Figure 3.4 Biosynthetic processing and C3 convertase cleavability of recombinant wild type and mutant C3.

Autoradiograph of 8% SDS-PAGE (reducing conditions) of C3 immunoprecipitated from the culture supernatants of metabolically labeled COS-1 cells that transiently express wild type and mutant C3. Prior to immunoprecipitation, fluid-phase C3 convertase, $C4b^{oxy}C2a$, was either added (+) or not added (-) to the supernatants as indicated to cleave the native C3 α-chain to the α'-chain of C3b.


wt
736,737 EQ,EQ
742 RA
730,731 DN,EQ
730,1-736,7 NQ-QQ
- + - + - + - + - +
C4b oxy C2a
α-chain
= α'-chain
- β-chain
747 EA
767 KA
744 EA
761 KA
744,747 EA,EA
- + - + - + - + - +
C4b oxy C2a
α-chain
= α'-chain
- β-chain
757,758 KA,EA
744 ED
754,755 EA,DA
744,7-754,5 AA-AA
747 ED
- + - + - + - + - +
C4b oxy C2a
α-chain
= α'-chain
- β-chain

3.5 Rationale and optimization of the cofactor-dependent, Factor I -mediated cleavage assay

In order to measure the effect of the various alanine scan mutations on cofactor binding, I have employed a sensitive, although somewhat indirect assay that makes the measurement of cofactor interaction possible with the amounts of protein attainable in transient transfections of COS-1 cells. This assay takes advantage of the fact that factor I-mediated cleavage of C3b to iC3b, with the excision of the small C3f fragment, is absolutely dependent upon the binding of C3b to one of the three I cofactors i.e. H, CR1 or MCP (see Fig. 1.5 for chain diagram of C3b to iC3b conversion). The C3b-like $C3(H_2O)$ molecule undergoes the same I-mediated cleavage in a cofactor-dependent manner to form the product $iC3(H_2O)$. In this case, however, the large α-chain cleavage product has the same molecular weight as β-chain, because the N-terminal C3a fragment is still covalently attached. CR1 can also act as a cofactor for the so-called "third" cleavage of the α-chain by factor I which produces the C3dg fragment plus an N-terminal α-chain fragment (see Figure 1.5). Using [^{35}S]-biosynthetically labeled culture supernatants as the source of the recombinant C3, all of these cleavages are observable on SDS-PAGE autofluorographic analysis if the appropriately treated supernatants are first subjected to immunoprecipitation with a polyclonal anti-C3 antibody.

Based on the evidence in the literature there appears to be more than one site for cofactor H binding in $C3b/C3(H_2O)$ (summarized in Figure 1.7). If the mutations introduced in this study had a partial effect on only one of the sites at which H was bound, the effect might be missed in the cofactor-dependent cleavage assay if a saturating amount of H was employed. To establish a concentration range over which H was subsaturating in the assay, metabolically-labeled wild type $C3(H_2O)$ was treated with a constant amount of factor I and a range of factor H concentrations. It can be seen in figure 3.5 that the extent

of α-chain conversion to α-75 and α-40 was dependent upon the concentration of H up to 100 ng/ml, after which the assay was saturated with respect to factor H. Also shown in figure 3.5 are the concentration profiles for the factor I cleavage assay when using soluble CR1 (sCR1) and soluble membrane cofactor protein (sMCP) as the I-cofactors. For sMCP, which yields the same end-state cleavage products as does factor H, the assay is subsaturating with respect to cofactor up to 400 ng/ml. For sCR1, by 200 ng/ml there is essentially quantitative conversion of α-chain into α-75 and α-40. However, the further cleavage of α-75 into the 38kDa C3dg fragment and the N-terminal fragments of approximately the same molecular weight only became apparent at the 400 ng/ml concentration of sCR1. Since it was desirable to simultaneously assay all of the mutants, there was a practical need to restrict the number of samples to be handled. Consequently, the entire cofactor concentration range shown in figure 3.5 could not be reproduced for each of the 15 recombinant C3 molecules analyzed in this study. Thus, in addition to the zero cofactor control, one cofactor concentration giving an intermediate level of cleavage and one giving near total cleavage with wild type C3(H_2O) was employed in each case. These concentrations corresponded to 100 ng/ml and 200 ng/ml for each of factor H and sMCP. For sCR1, the two concentrations chosen were 100 ng/ml and 400ng/ml, the latter being chosen in order to see whether the mutations had an effect on the cleavage to C3dg/α-37.

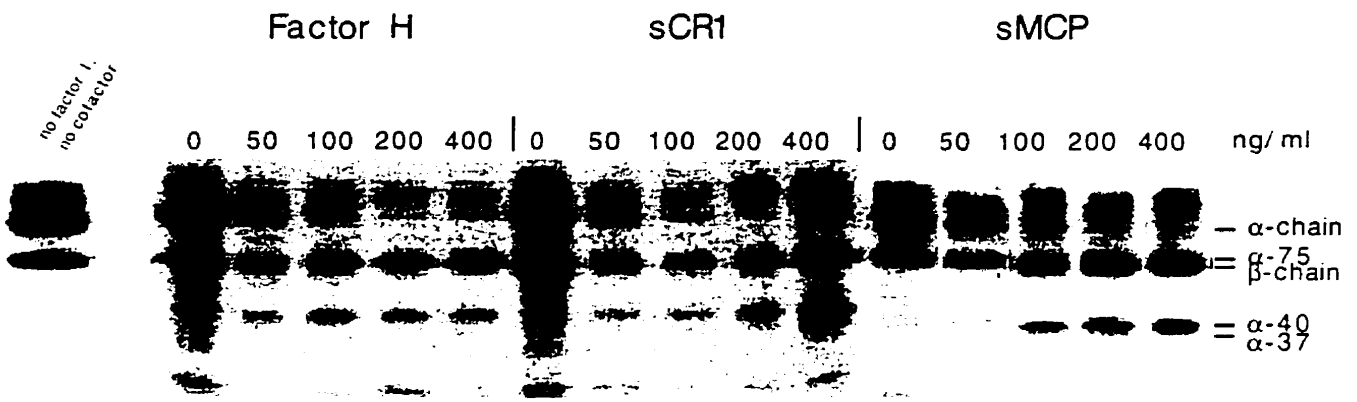


Figure 3.5 Cofactor concentration dependence of the Factor I-mediated cleavage assay using wild type C3(H_2O).

Metabolically-labeled culture supernatants from stably-transfected J588L cells containing wild type human [^{35}S]-C3(H_2O)(diluted to 100 ng/ml) were treated with increasing concentrations of cofactor in the indicated amounts plus 500 ng/ml of factor I. Digestion products were immuno-isolated using rabbit anti-human C3c and resolved on a 9% SDS-PAGE gel under reducing conditions, as described in detail in the Materials & Methods.

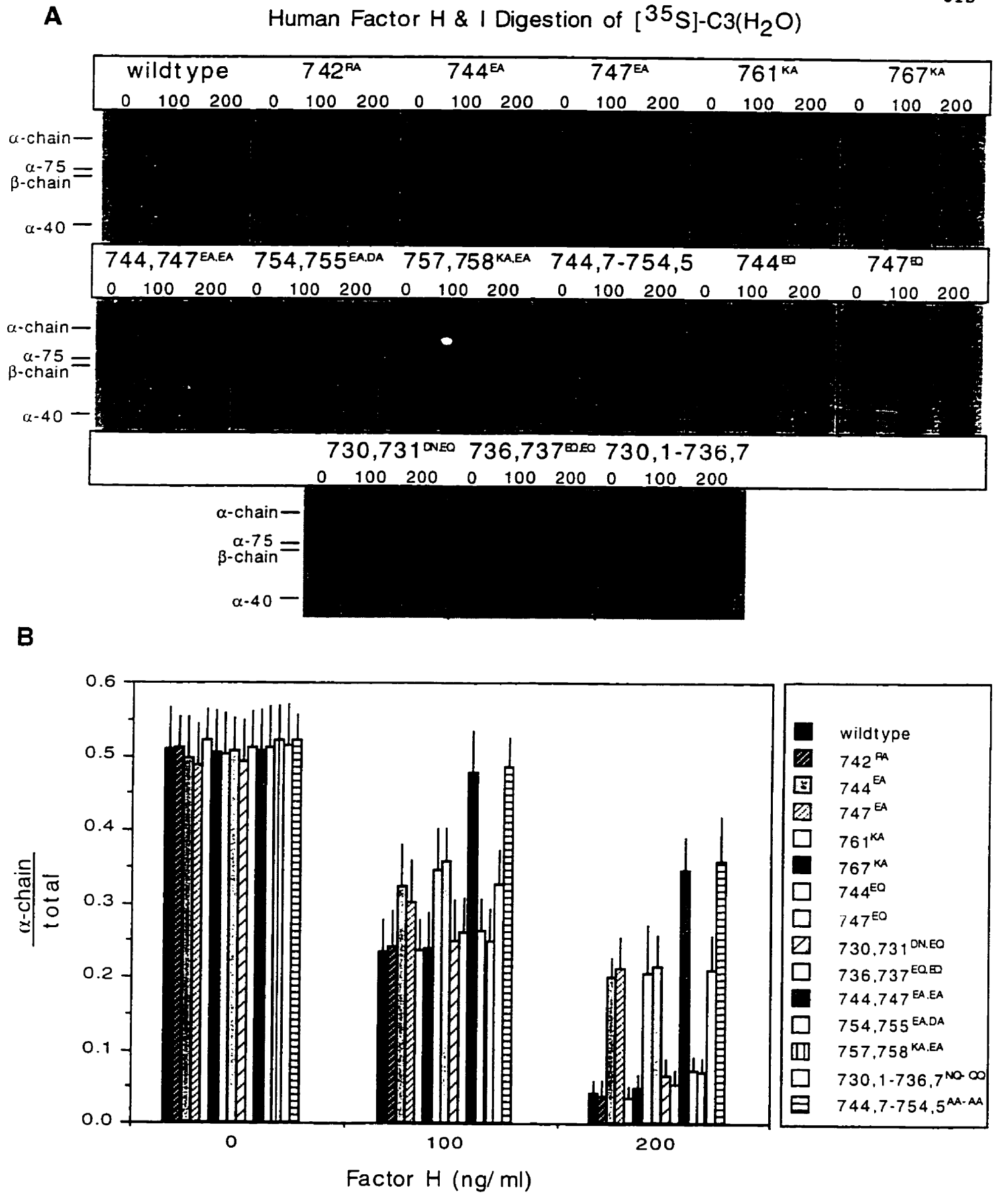
3.6 Mutation of the residues 744^{E} and 747^{E} have an effect on factor I-mediated cleavage in the presence of cofactor H

Shown in figure 3.6A is the H cofactor-dependent cleavage assay for wild type C3 and for the 11 charged residue mutants which I constructed within the 742-767 target region. Visual inspection of the autoradiogram reveals that alanine scan mutants $744^{E.A}$ and $747^{E.A}$ show a significant impairment in H-dependent cleavage by factor I that is apparent at both 100 and 200 ng/ml of the cofactor. At least an equal degree of impairment was also observed in mutants harbouring alanine substitutions of both residues 744^{E} and 747^{E}. However, mutations at any of the other 7 charged residues in the 742-767 target segment were without effect in this assay. In order to determine whether the negatively-charged side chains at residue 744^{E} and 747^{E} were required the glutamic acid was replaced by the isosteric amide glutamine and the functional activities of these two point mutants were determined. It can be seen in figure 3.6A that the isosteric amide 744^{EQ} and 747^{EQ} mutants showed the same impairment as did the equivalent alanine substituents, thereby implying the negative charge *per se* is important for mediating binding of factor H.

Three previously constructed mutants (Taniguchi-Sidle and Isenman, 1994), $730,731^{DN.EQ}$, $736,737^{EQ.EQ}$ and $730,1\text{-}736,7^{NQ\text{-}QQ}$ were also examined in this assay. These mutants which were generated just upstream of the site that I am examining and which were shown to play a role in Factor B binding were previously examined in a H cofactor-dependent cleavage assay. While no significant impairment in cleavage relative to the wild type control was seen at the time, it is now clear that the assay had been carried out using saturating concentrations of factor H. When reexamined using my more sensitive assay, only the $730,1\text{-}736,7^{NQ\text{-}QQ}$ tetra-mutant showed an effect that was on par with that seen with either $744^{E.A}$ or $747^{E.A}$ alone.

Figure 3.6 Factor I-mediated cleavage of recombinant C3(H_2O) molecules using H as the cofactor.

100 ng of [^{35}S]-rC3(H_2O) molecules (as determined by RIA) were digested with the indicated amounts of cofactor and 500 ng/ml factor I. Radiolabeled digestion products were immunoisolated using rabbit anti-human C3c and resolved on an 9% SDS-PAGE under reducing conditions (A). Autofluorographs from at least three independent experiments were analyzed using densitometry to quantify the major bands. The ratio of the α-chain band pixel intensity over the combined signals from the α-chain, α-75, α-40 and β-chains (total) was calculated and plotted as indicated (B). The order of the bars is the same as the order in the legend.

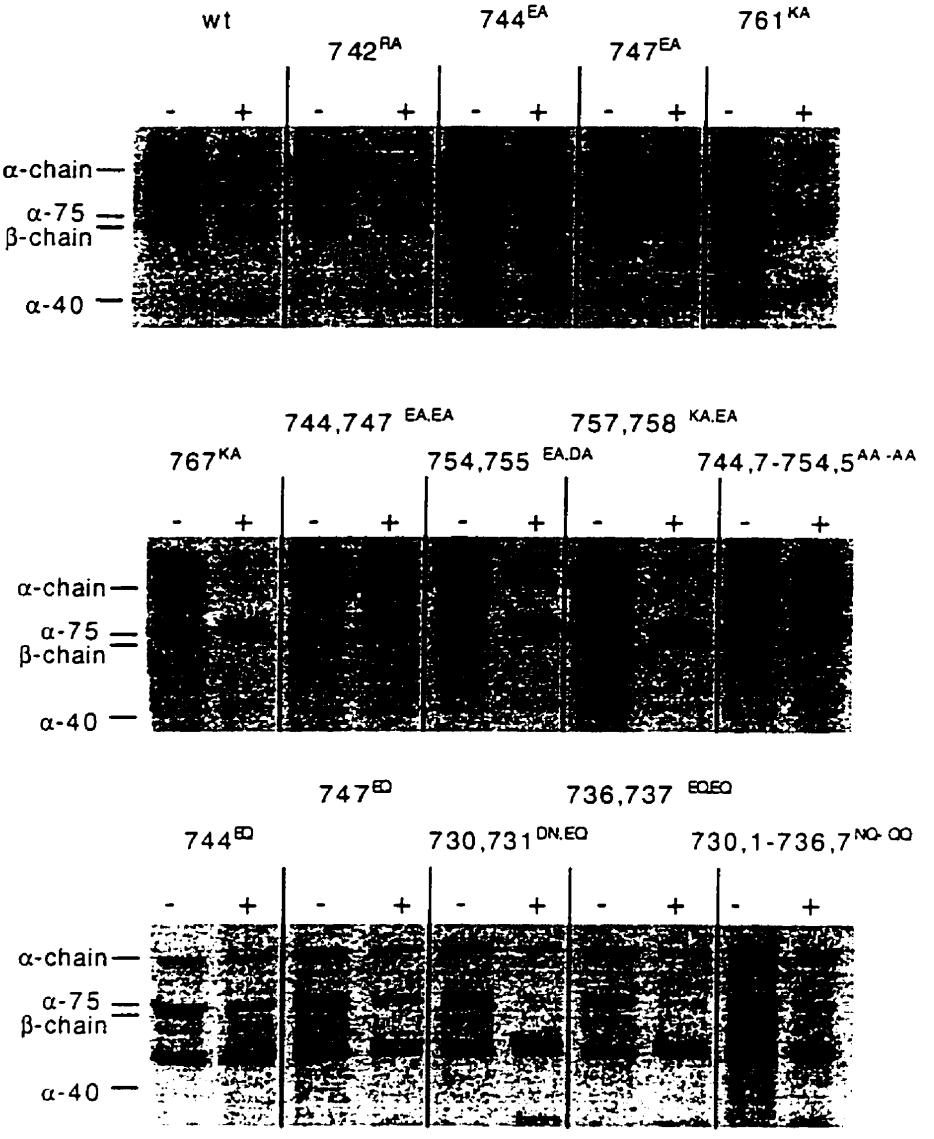

A
Human Factor H & I Digestion of [35S]-C3(H2O)
wildtype
742RA
744EA
747EA
761KA
767KA
0 100 200 0 100 200 0 100 200 0 100 200 0 100 200 0 100 200
α-chain
α-75
β-chain
α-40
744,747EA,EA
754,755EA,DA
757,758KA,EA
744,7-754,5
744ED
747ED
0 100 200 0 100 200 0 100 200 0 100 200 0 100 200 0 100 200
α-chain
α-75
β-chain
α-40
730,731DN,EQ
736,737ED,EQ
730,1-736,7
0 100 200 0 100 200 0 100 200
α-chain
α-75
β-chain
α-40
B
α-chain / total
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
100
200
Factor H (ng/ml)
wildtype
742RA
744EA
747EA
761KA
767KA
744EQ
747EQ
730,731DN,EQ
736,737EQ,ED
744,747EA,EA
754,755EA,DA
757,758KA,EA
730,1-736,7NQ-QQ
744,7-754,5AA-AA

In order to assess the reproducibility of the assay, and to determine whether the effects of the mutations at 744^{E} and 747^{E} were cumulative, this assay, as well as two other independent replicates were subjected to densitometric analyses (see Materials & Methods). For each lane the intensity of the α-chain was expressed as a fraction of the summed intensities arising from α-chain, α-75, β-chain and α-40. The means and standard deviations of fractional α-chain intensity for each recombinant at each factor H concentration are shown in bar graph form in figure 3.6B. As judged from the size of the error bars, the assays were quite reproducible. The bar graph presentation of the data, especially for the 200 ng/ml factor H concentration, readily identifies molecules having substitutions at either 744^{E} or 747^{E} (or both) as being impaired with respect to factor H binding. There is also some suggestion that the effects on H binding of the individual mutations at residues 744 and 747 are cumulative, although definitely not additive.

As a second approach to assessing subtle defects in H binding activity produced by the various mutations, a version of the cofactor dependent cleavage assay was employed in which 10% non-K76-COOH-treated fetal calf serum was used as a source of heterologous factors H and I. The logic was that the interaction between the human C3(H_2O) derivatives and the bovine H would be less strong than with its autologous counterpart and that therefore subtle defects or cumulative effects that were difficult to detect when using the autologous components, even under non-saturating assay conditions, may be observable using the heterologous components. The results of this experiment are shown in figure 3.7 and they essentially mirror those obtained using the autologous components under non-saturating conditions. In this case, however, the cumulative effect of having residues 744^{EA} and 747^{EA} both mutated to alanine is more readily apparent.

Figure 3.7 Susceptibility of wild type and mutant C3(H_2O) to cleavage by bovine factors H and I.

Autoradiograph of 9% SDS-PAGE under reducing conditions of C3(H_2O) immunoprecipitated by polyclonal anti-C3c from the culture supernatants of metabolically labeled COS-1 cells that transiently express wild-type and mutant C3. Before immunoprecipitation, equal amounts of C3(H_2O) (100 ng/ml), as determined by RIA, were treated with 10% non-K76-treated FCS for 1 hour, as indicated.


wt
742RA
744EA
747EA
761KA
- + - + - + - + - +
α-chain
α-75
β-chain
α-40
744,747 EA.EA
757,758 KA.EA
767KA
754,755 EA.DA
744,7-754,5AA-AA
- + - + - + - + - +
α-chain
α-75
β-chain
α-40
747EQ
736,737 EQ.EQ
744EQ
730,731DN.EQ
730,1-736,7NQ-QQ
- + - + - + - + - +
α-chain
α-75
β-chain
α-40

3.7 Mutations affecting the interaction with sCR1

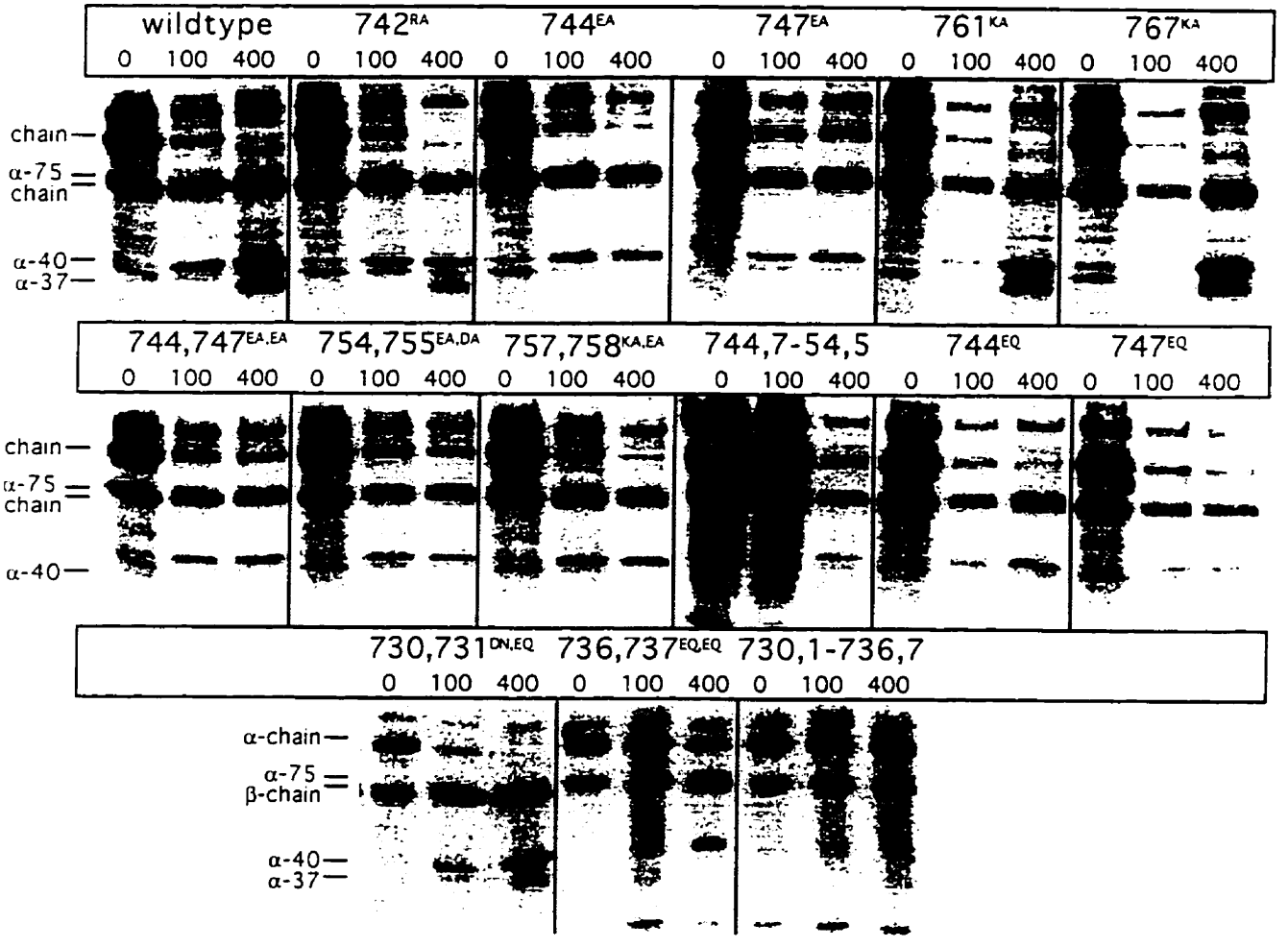
Previous work from this lab showed that replacement of the glutamic acids at C3 residues 736 and 737 by glutamine resulted in a marked loss in the ability of the mutant C3b to mediate binding to CR1 on neutrophils, as assessed in a rosette type of assay. It followed, therefore, that these two residues played an important role in the interaction between C3b and CR1 (Taniguchi-Sidle and Isenman, 1994). Soluble CR1 was not available at the time and thus the contribution to the "quality" of binding with CR1 required for the latter to act as a cofactor for factor I-mediated cleavages, remained to be determined. Accordingly sCR1-dependent I-cofactor cleavage assays were performed on the 3 mutants covering the 730-737 segment, as well as the series of mutants that I engineered within the 742-767 target region. The results of a representative cleavage experiment are shown in figure 3.8A and as a more quantitative bar graph from replicate experiments in figure 3.8B. In accordance with the CR1-dependent rosette assay results, mutant $736,737^{EQ.EQ}$ shows markedly impaired conversion of $C3(H_2O)$ to $iC3(H_2O)$ and no evidence of the third cleavage converting α-75 to C3dg and the 37 kDa N-terminal fragment (α-37). Although mutation of residues 730 and 731 on their own has no effect in this assay, the combined tetra-mutant $730,1-736,7^{NQ-QQ}$ shows the same cleavage defect as was observed for the $736,737^{EQ.EQ}$ mutant. In examining the 742-767 series of mutants in this assay, it became apparent that those mutants showing impaired cleavage fell into two categories: those affecting the first I-mediated cleavage and those affecting only the third cleavage. For example, whereas mutant 747^{EA} showed significantly impaired CR1-dependent conversion of $C3(H_2O)$ to $iC3(H_2O)$ and no evidence of the third cleavage at the high concentrations of sCR1, mutant 744^{EA} showed no impairment in $C3(H_2O)$ to $iC3(H_2O)$ conversion but nevertheless was resistant to the sCR1-dependent third cleavage.

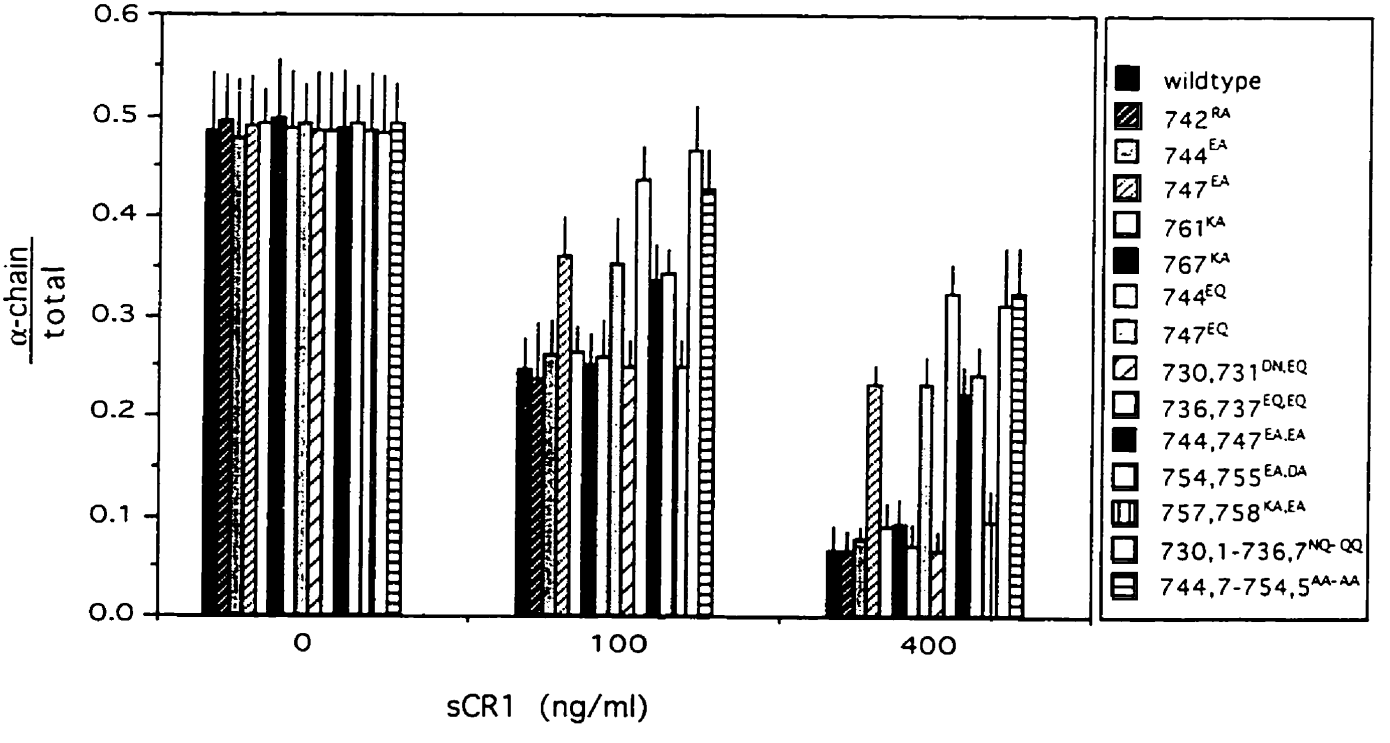
Impairments of similar magnitude in C3(H_2O) to iC3(H_2O) conversion was also seen with the $754,755^{EA,DA}$ mutant. Although there was no impairment in this conversion for neighboring $757,758^{KA,EA}$ mutant, there was impairment of the third cleavage. Combination mutants showed the phenotype of the individual mutations, and at least in this range of sCR1 concentrations, there was no obvious indication of a cumulative effect. Finally, as was the case for the interaction with factor H, isosteric amide substitutions at residues 744 and 747 did not restore wild type activity. Thus for these two residues at least, one can conclude that the negative charge *per se* is important to the binding with CR1.

Figure 3.8 Factor I-mediated cleavage of recombinant C3(H_2O) molecules using sCR1 as the cofactor.

100 ng of [^{35}S]-rC3(H_2O) molecules were digested with the indicated amounts of cofactor and 500 ng/ml of factor I. Samples were immunoisolated using rabbit anti-human C3c and resolved on 9% SDS-PAGE under reducing conditions (A). Autofluorographs from at least three independent experiments were analyzed using densitometry of the major bands. The ratio of the α-chain band pixel intensity over the combined signals from the α-chain, α-75, α-40, α-37 and β-chains was calculated and plotted as indicated (B). Note, α-37 represents the N-terminal half of α-75 that is produced as result of the third factor I-mediated cleavage of C3(H_2O). The C-terminal half corresponds to the similar mass C3dg fragment, however, this fragment is not immunoprecipitated by the anti-C3c antibody. Therefore in cases where this cleavage occurs, the denominator of the ratio will be somewhat underestimated.

Human sCR1 & I Digestion of ^{35}S-C3(H_2O)





3.8 There is no observable defect in factor I-mediated cleavage of rC3(H_2O) molecules using sMCP as the cofactor.

Given that like factor H and CR1, MCP is also a cofactor for the factor I-mediated cleavage of C3b to iC3b (or C3(H_2O) to iC3(H_2O)) and further that as of yet no binding site for MCP has been delineated, the complete series of 730-767 segment mutants were analyzed in a factor I cleavage assay in which a soluble form of MCP acted as the I cofactor. The results of a representative experiment are shown in figure 3.9A and as a bar graph from replicate experiments in figure 3.9B. In contrast to what was observed with both factors H and sCR1 as I cofactors, there appears to be no definitive impairment in any of the mutant molecules on the cleavage of C3(H_2O) to iC3(H_2O) when sMCP was used as the I cofactor.

3.8 There is no observable defect in factor I-mediated cleavage of rC3(H_2O) molecules using sMCP as the cofactor.

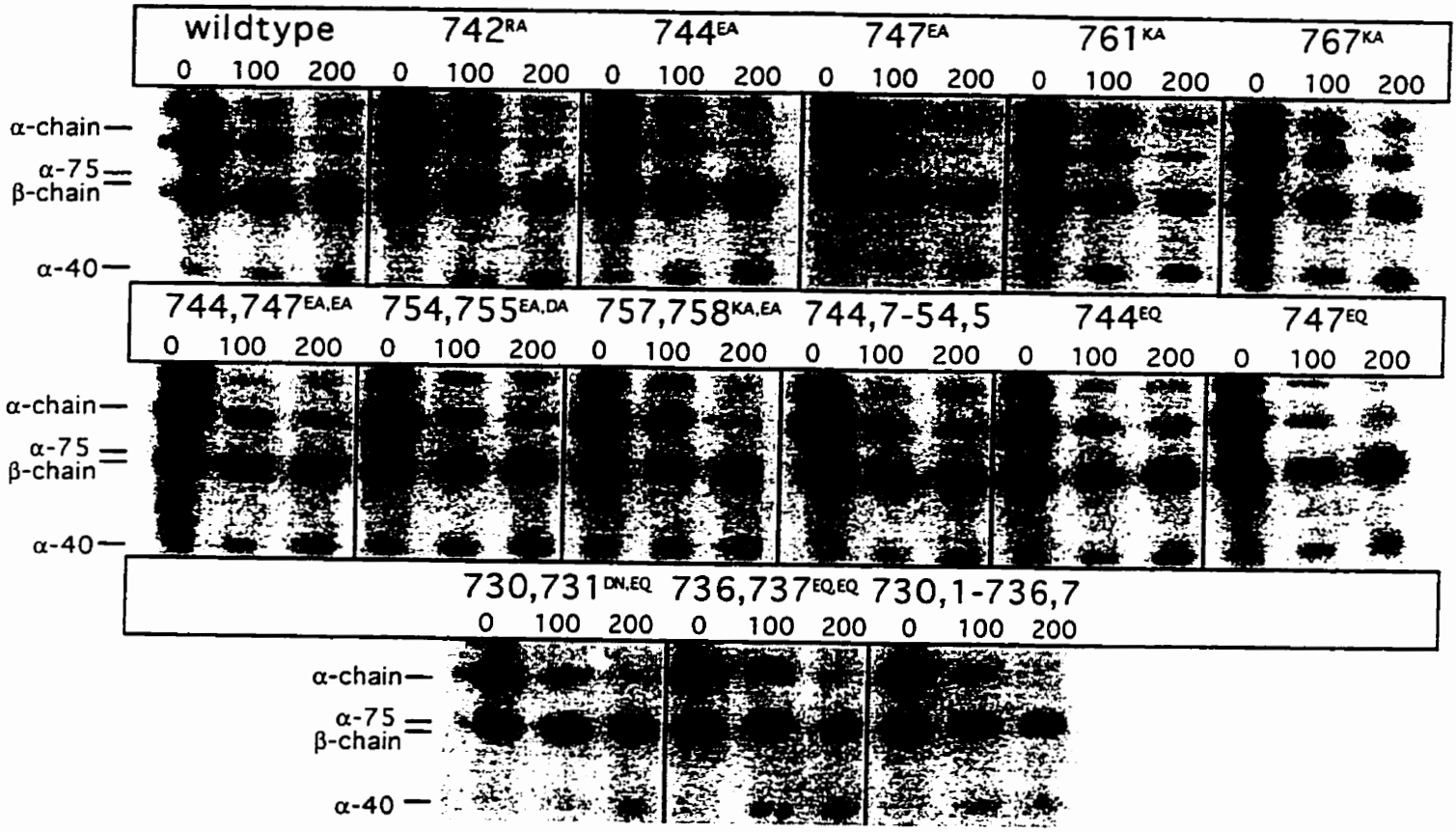
Given that like factor H and CR1, MCP is also a cofactor for the factor I-mediated cleavage of C3b to iC3b (or C3(H_2O) to iC3(H_2O)) and further that as of yet no binding site for MCP has been delineated, the complete series of 730-767 segment mutants were analyzed in a factor I cleavage assay in which a soluble form of MCP acted as the I cofactor. The results of a representative experiment are shown in figure 3.9A and as a bar graph from replicate experiments in figure 3.9B. In contrast to what was observed with both factors H and sCR1 as I cofactors, there appears to be no definitive impairment in any of the mutant molecules on the cleavage of C3(H_2O) to iC3(H_2O) when sMCP was used as the I cofactor.

Figure 3.9 Factor I-mediated cleavage of recombinant C3(H_2O) molecules using sMCP as the cofactor.

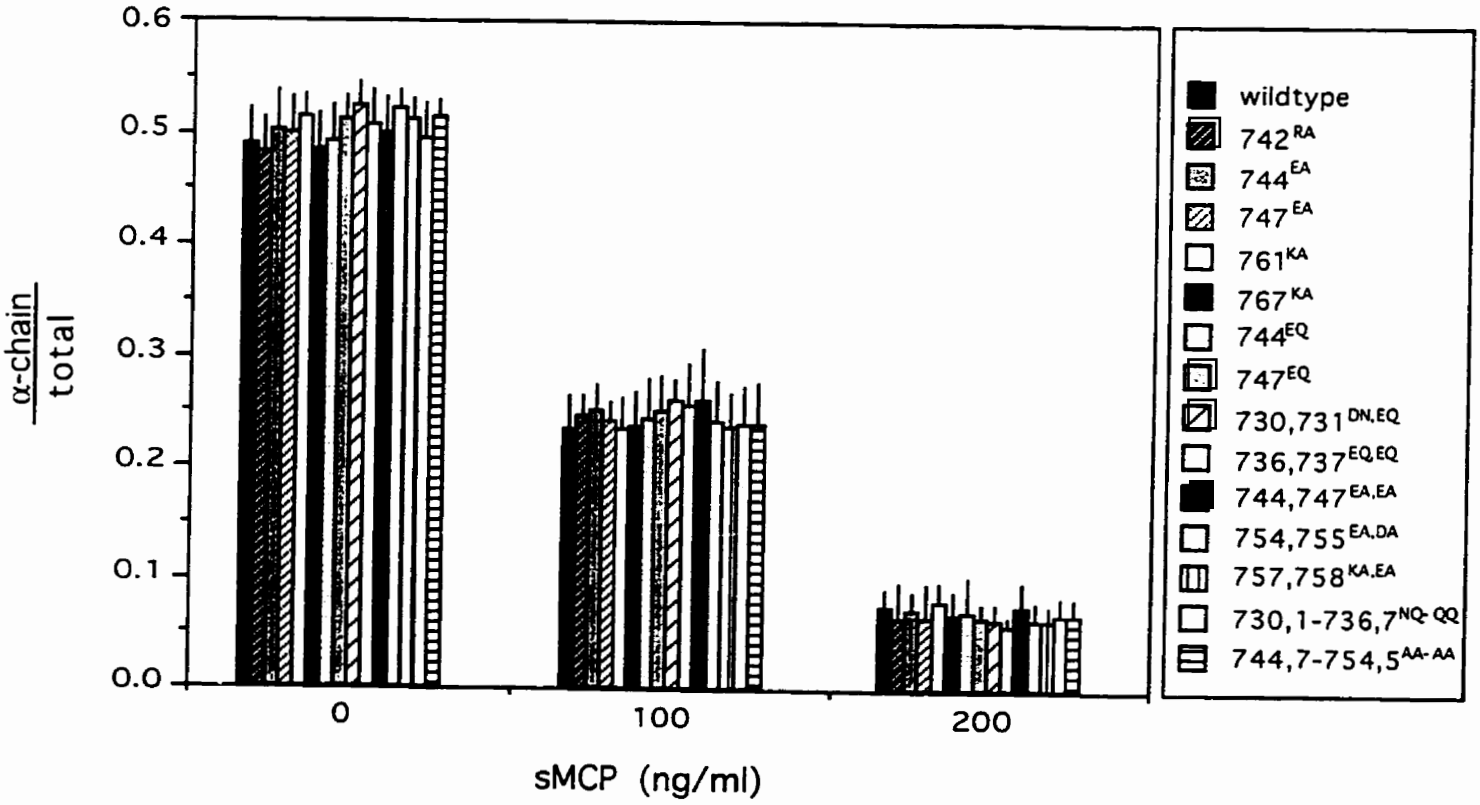
100 ng of [^{35}S]-rC3(H_2O) molecules were digested with the indicated amounts of cofactor and 500 ng/ml of factor I. Digestion products were immunoisolated using rabbit anti-human C3c and resolved on 9% SDS-PAGE under reducing conditions (A). Autofluorographs from at least three independent experiments were analyzed using densitometry of the major bands. The ratio of the α-chain band pixel intensity over the combined signals from the α-chain, α-75, α-40 and β-chains was calculated and plotted as indicated (B).

A

Human sMCP & I Digestion of [^{35}S]-C3(H_2O)



B



Chapter 4

Discussion & Future Directions

4.1 Discussion

In this study, I have used site-directed mutagenesis to examine candidate binding sites in C3b/C3(H_2O) for the family of I cofactors, namely H, CR1, and MCP. Loss of function as a consequence of such site-specific substitutions would argue in favour of the role of that particular site in the interaction under study. Conversely, mutagenesis of a targeted region with no associated functional consequences would suggest that the sequence was not involved in the interaction under study. As mentioned earlier, mutagenic studies have sometimes (Taniguchi-Sidle & Isenman, 1994) but not always (Diefenbach & Isenman, 1995) corroborated findings derived from other mapping techniques. When there is agreement between the mutagenic approach and the other approaches, our understanding of the chemistry of interaction is further refined as a result of the identification of specific residues important for the interaction.

The initial objective of this study was to examine the role of the charged residues in the region of human complement component C3 previously proposed to be involved in the interactions with factor H (Fishelson *et al.*, 1991). The region of interest is located in the C-terminal two-thirds of a 42 amino acid polar stretch near the N-terminus of the α'-chain of C3b. It is logical that this end of the α'-chain ought to play a part in the binding of C3 with its ligands as it is a highly polar region that becomes surface accessible as a result of the removal of the C3a activation peptide and/or the conformational change resulting from internal thioester cleavage (Becherer *et al.*, 1992). Other groups have previously noted that the binding site for H may overlap other binding sites, including that for CR1 with which it shares both I cofactor and decay acceleration activity. Consequently, each C3 mutant generated for H was subsequently tested for its ability to interact with CR1. Since candidate binding sites in C3b for MCP have not yet been formally proposed, but since this molecule is also an I cofactor, it was of interest to determine whether a binding site for

MCP also existed within this region. Although both CR1 and MCP are normally membrane-associated molecules, their functions are fully retained in the recombinant soluble forms of these proteins. Because we were able to obtain sCR1 and sMCP, the same type of I cofactor cleavage assay could be used for all three molecules as an indirect measure of their binding interaction with the C3b-like molecule C3(H_2O).

As summarized in table II, my experiments confirmed that the N-terminus of the α'-chain is indeed involved in contributing to the binding interactions with factor H and with CR1. In contrast, I was unable to show any defect in the association of MCP with any of my mutant C3 recombinants.

Several important points can be raised based on the observations made. First, although factor H and sCR1 sites of interaction appear to overlap in this region, the charged residue contacts required by CR1 extend over a wider portion of the N-terminal α'-chain segment than is the case for factor H. In particular, whereas mutations of residues 744^{E} and 747^{E} have the most dramatic impairment effect in proteolytic assays done with cofactor H, for CR1 there were 3 clusters of charged residues, namely $736,737^{EE}$, 747^{E} and $754,755^{ED}$, that had approxiamately equal effects on the CR1 dependent cleavage of C3(H_2O) to iC3(H_2O) upon replacement of the negative charge by a neutral residue. Additional contacts with negatively charged residues 744^{E} and the $757,758^{KE}$ pair appeared to be required for CR1 to act as a cofactor for the third factor-I mediated cleavage. This requirement of what would appear to be a higher affinity interaction for a cofactor, in this case CR1, to facilitate the third factor I cleavage of α-chain is reminiscent of previous results using factor H as the cofactor for the third cleavage. Whereas H is not a cofactor for this cleavage at physiologic ionic strength, it becomes one at low ionic strength, suggesting an important role for additional ionic bonds in this interaction. Furthermore, the tetra-mutant $730,731\text{-}736,737^{NQ\text{-}QQ}$, which at non-limiting concentrations of human factor

Table II

Summary of activities[a] of mutant C3 molecules examined in this study

recombinant	Factor H $C3(H_2O)$ to $iC3(H_2O)$	sCR1 $C3(H_2O)$ to C3dg	sMCP $C3(H_2O)$ to $iC3(H_2O)$
Wild type	+++	+++	+++
742^{RA}	+++	+++	+++
744^{EA}	++	+++*	+++
747^{EA}	++	+	+++
761^{KA}	+++	+++	+++
767^{KA}	+++	+++	+++
$744,747^{EA.EA}$	+	+	+++
$754,755^{EA.DA}$	+++	+	+++
$757,758^{KA.EA}$	+++	+++*	+++
$744,7\text{-}754,5^{AA\text{-}AA}$	+	+	+++
744^{EQ}	++	+++*	+++
747^{EQ}	++	+	+++
$730,731^{DN.EQ}$	+++	+++	+++
$736,737^{EQ.EQ}$	+++	+	+++
$730,1\text{-}736,7^{NQ\text{-}QQ}$	++	±	+++

[a]Percentage of activity relative to wild type based on disappearance of α-chain:
± <25%; + 25-50%; ++ 50-75%; +++ 75-100%
* no cleavage by I of $iC3(H_2O)$ to C3dg

H showed no impairment in the C3(H_2O) to iC3(H_2O) conversion (this mutant did show defective cleavage at the more limiting H concentrations used in the present study), was unable to undergo the third cleavage, even at very high concentrations of factors H and I (Taniguchi-Sidle and Isenman, 1994).

Interpretation of any mutagenic study is dependent upon the conformational integrity of the molecule not being significantly perturbed by the substitutions introduced. This issue was addressed by making a number of conformationally-sensitive measurements and, for the most part, the behaviour of the mutants was fairly similar to that of the wild type molecule. For example, secretion of protein was on par with levels seen for the wild type transfectants, arguing against any major structural changes which would likely induce intracellular digestion of unstable or improperly folded proteins. The second test was to use mAbs specific for conformationally-sensitive epitopes in different regions of C3 (C3c/C3dg) and determine if the level of binding was different from that of wild type. The level of binding to each C3 mutant was in fact indistinguishable from wild type for both monoclonal antibodies employed, further arguing against severe conformational changes. To be sure, using only 2 monoclonal antibodies as conformational probes does not constitute an extensive conformational epitope mapping of the molecule, but these mAbs were the only ones readily available to us. Using the C3-dependent hemolytic assay as a further conformational probe revealed that most of the mutants displayed some degree of impairment relative to wild type activity. The 50-70% of wild type hemolytic activities observed for the mutants could either reflect the summation of fairly minor perturbations at a number of the functionalities contributing to the overall hemolytic activity, or the defects could be restricted to one or two specific functions. For example, if processing of pro-C3 to mature 2-chain C3 was less complete in the mutants, this would affect hemolytic activity since pro-C3 is hemolytically inactive. However, as was evident in all of the gels shown in this thesis, there was no difference in the extent of processing seen between mutant and

wild type protein. On the other hand, under conditions of limiting $C4b^{oxy}2a$, the mutant molecules showed partial impairment of susceptibility to cleavage by the convertase. Assuming that this step would also be limiting in the hemolytic assay, the somewhat greater resistance of the mutant C3 molecules to C3 convertase-mediated cleavage could by itself account for the defects observed in the hemolytic assay. The corollary to this is that functionalities downstream of the cleavage event, such as transacylation to the target and C5 binding activity, were essentially unperturbed by the mutations. Given that the sessile bond at which $C4b^{oxy}2a$ acts is fairly close in primary sequence to the target region of the mutations, one might expect an effect on cleavage. It is noteworthy, however, that there is no correlation between the extent of the hemolytic defect (or $C4b^{oxy}2a$ cleavage susceptibility) and whether or not a given substitution had an effect in the three cofactor activity assays employed. This, therefore, argues that with respect to these functions, the overall conformational integrity of the mutant molecules can be considered intact.

The observation that residues 744^{E} and 747^{E} are crucial for the binding of factor H to $C3(H_2O)$ is fully consistent with the prediction of the Fishelson (1991) overlapping hexapeptide study which suggested that C3 residues 745-754 contribute to the binding of factor H. The only other mutant in which H binding appeared to be compromised was the $730,731\text{-}736,737^{NQ\text{-}QQ}$ tetra-mutant molecule. Since the magnitude of impairment is the same as that seen with either 744^{EQ} or 747^{EQ} alone, it would suggest that either the contributions of the $730,731^{NQ}$ and $736,737^{QQ}$ charged pairs on their own to the binding of factor H were too small to be detectable in our assay, or more likely, there is sufficient local conformational distortion caused by the loss of 4 negative charges over a space of 7 amino acids to have had an effect on the nearby residue 744^{E}, this residue making a very definitive contribution to H binding.

Residues 736,737^{EE} in C3b were previously shown to be important for mediating the interaction between red cell-bound C3b and neutrophil-associated CR1 (Taniguchi-Sidle and Isenman, 1994). These same residues were identified in the present study as being important for the interaction between sCR1 and iC3(H_2O) using the I-cofactor assay. The fact that the same residues were identified by both assays validates the use of the I cofactor cleavage assay as a surrogate binding assay for the interaction between C3b/C3(H_2O) and the ligands H, CR1 and MCP.

The fact that there is an overlap between the H and CR1 sites within the 727-767 target segment is consistent with a previous report suggesting that these activities were both inhibited by an antibody against this peptide segment and by the peptide itself (Becherer *et al.*, 1992). Our results are also partly in keeping with a recent study by Lambris and colleagues (Lambris *et al.*, 1996) in which the 728-764 segment was either deleted from human C3 or was replaced by the corresponding amino acid segment from trout or *Xenopus* C3 or from cobra venom factor (a C3-related molecule), none of which bind to human factor H. When the 728-764 segment was deleted, interactions with H and sCR1, as measured in a cofactor assay, were lost. However, interpretation of the results with the deletion mutant is somewhat clouded by the fact that this molecule has absolutely no hemolytic activity. The chimeric molecules, which retain native-like hemolytic activity and reactivity with a panel of monoclonal antibodies, show no impairment in an I-cofactor assay employing factor H. With sCR1 as the I cofactor, there is no impairment of the conversion of intact α-chain of thioester-cleaved C3 to α-75 and α-40, but there is impairment of the third cleavage to C3dg and α-37. Since the chimeric segments all show a considerable degree of sequence similarity to the corresponding human segment, this may account for the lack of effect on the interaction with H and the partial effect seen with CR1. Alternatively, the conditions of their assay, especially with respect to factor H, may not have been sufficiently limiting to detect the partial defects that I was able to detect with my

series of substitution mutants. Finally, the observation that MCP cofactor-dependent cleavage of C3 was unaffected by any of the mutations of the 727-767 target segment examined in this study is consistent with the Lambris *et al.* (1996) report where even the 728-764 deletion variant of C3 was efficiently cleaved by factor I in the presence of sMCP.

Thus despite the functional homology between MCP and the other two molecules which act in concert with factor I to degrade C3b/C3(H_2O), the evidence is now fairly conclusive that the mode of binding of MCP to C3b/C3(H_2O) must be different from that of CR1 and factor H. Although MCP does not share with H and CR1 the binding site at the N-terminus of the α'-chain, the possibility remains that other contact points within the C3 molecule may be shared by the three cofactors.

4.2 Concluding remarks and Future Directions

In this site-directed mutagenesis study, I have shown that a cluster of negatively charged residues located in the 42 amino acid N-terminal hydrophilic stretch of the α'-chain play a role in the interaction of C3 with factor H and (soluble) CR1 but not (soluble) MCP. In terms of the putative contact surface in C3b for these ligands, it may be noteworthy that substitution of 4 positively charged side chains by alanine was without affect on any of the 3 binding functions assessed. It should be mentioned that according to the Fishelson study (Fishelson, 1991), there may be a potential binding role for a stretch of hydrophobic amino acids lying between residues 747 and 754. It would be interesting to see if mutations within this hydrophobic patch do adversely affect the interaction of at least factor H to C3b. It would also be interesting to determine whether SCRs 1-5 within factor H (Sharma and Pangburn, 1996), the region that harbours I cofactor activity, is the structure that physically associates with the 742-767 region. Given the important role of negatively charged residues on the C3b side of the interaction, it would be logical to ask whether a

complementary region of positively charged residues exist within any one of the SCRs that show C3b/C4b cofactor activity in factor H. Unfortunately, all the SCRs harbour positively charged residues dispersed throughout the sequence that may or may not be involved in binding. Clearly, fine mapping studies within SCRs 1-5 of factor H need to be carried out in order to identify critical residues contributing to this side of the C3b:H interaction.

It may be possible to determine whether residues identified within a given SCR of factor H as contributing to the interaction with C3b form a patch on the surface of the domain that is complementary to residues in C3b by "threading" the sequence into the NMR-determined structure of a prototypical pair of SCR domains. Such an approach has recently been employed to visualize the relative disposition of segments within SCRs 1 and 2 of CR2 that had been identified by a combination of mapping approaches to be important for mediating the interaction between CR2 and C3dg (Molina *et al.*, 1995).

Given that none of the mutants tested exhibited the complete loss of any one function, other regions may play an important role for the binding of H, CR1 and MCP. One region that remains to be more fully investigated is the C3d region which blocking mAb, C3 proteolytic fragmentation, and peptide studies have suggested to be involved in the interaction with factor B, factor H and CR1 (Koistinen *et al.*, 1989; Lambris *et al.*, 1988).

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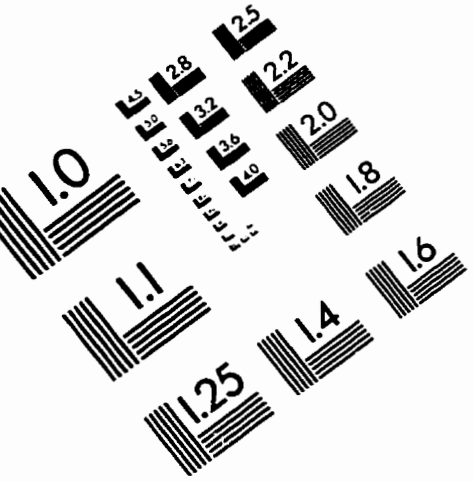
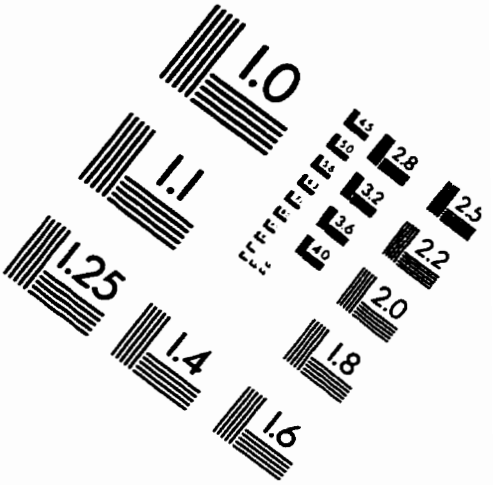
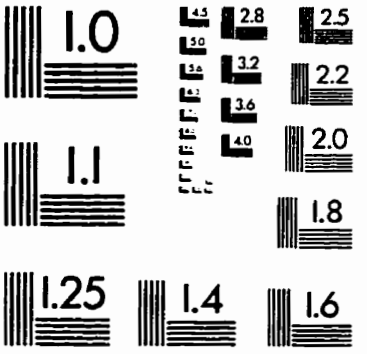
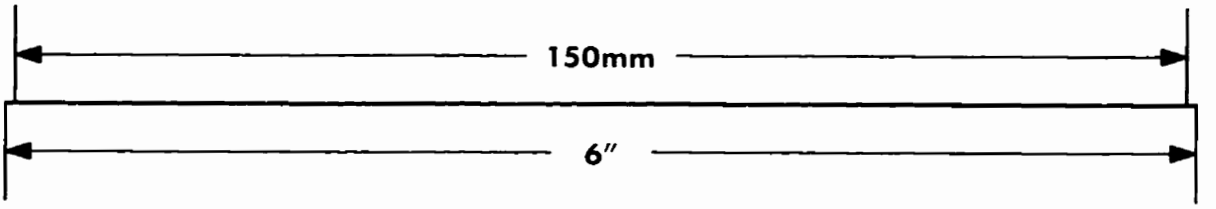
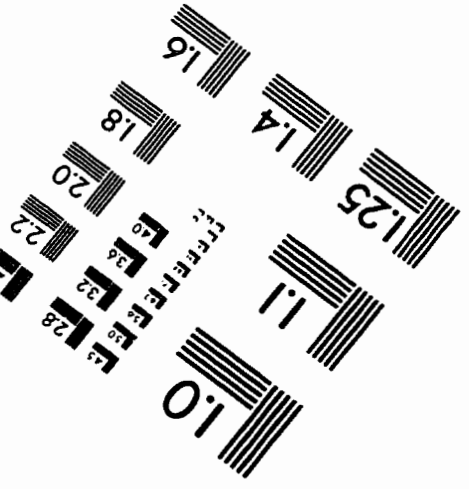
IMAGE EVALUATION TEST TARGET (QA-3)

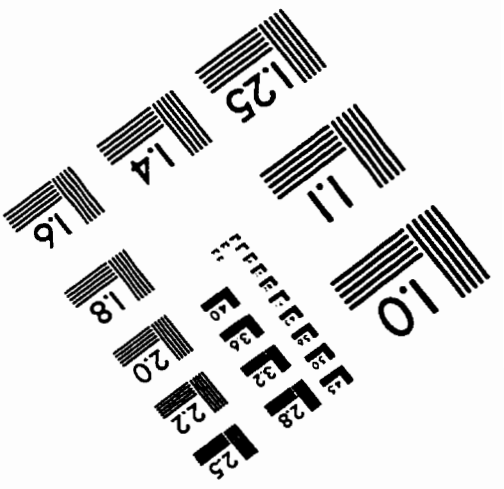












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