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Generation of Multiple Fluid-Phase C3b:Plasma Protein Complexes During Complement Activation. Functional Significance and Possible Implications in C3 Glomerulopathies

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Mahalakshmi Ramadass

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Mahalakshmi Ramadass

We, the dissertation committee for the above candidate for the

Doctor of Philosophy degree, hereby recommend acceptance of this dissertation.

Richard R. Kew, Ph.D., Dissertation Advisor
Associate Professor, Department of Pathology,
Stony Brook University

Martha Furie, Ph.D., Chairperson of Defense
Professor, Departments of Pathology and Molecular Genetics and Microbiology
Stony Brook University

Berhane Ghebrehiwet, DVM, DSc.
Professor, Departments of Medicine and Pathology
Stony Brook University

Antonius Koller, Ph.D.
Research Assistant Professor, Department of Pathology
Stony Brook University

Allen P. Kaplan, M.D.
Clinical Professor, Division of Pulmonary, Critical Care, Allergy and Immunology
Medical University of South Carolina

This dissertation is accepted by the Graduate School

Charles S. Taber
Interim Dean of the Graduate School

Abstract of the Dissertation

Generation of Multiple Fluid-Phase C3b:Plasma Protein Complexes During Complement Activation. Functional Significance and Possible Implications in C3 Glomerulopathies

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The complement system is tightly regulated in order to safeguard against tissue damage that results from unwanted activation. The key step of C3 cleavage to C3b is regulated by multiple mechanisms that control the initiation and extent of activation. This study demonstrated that C3b:plasma protein complexes form in the fluid-phase during complement activation, and complexes could function as a passive mechanism to intercept C3b from depositing on host cells. Several different plasma proteins displayed a discrete high molecular weight, SDS-resistant band when any of the three complement activating pathways were triggered in normal human serum or plasma. Serum depleted of individual complement proteins revealed that C3 and factors B and D were essential for complex formation. Inactivation of the thioester bond in C3 by hydroxylamine treatment also prevented formation of these complexes. In vitro, complexes could be generated using four purified proteins: C3, factor B, factor D and a target protein along with Mg^{2+} to allow

formation of the C3 convertase. These studies showed that the complexes consisted of a plasma protein covalently bound to C3b in a 1:1 molar ratio. Moreover, the C3b portion of the complexes was rapidly degraded by factors H and I, and complexes formed spontaneously in factor H and factor I depleted serum, indicating that loss of complement regulation facilitates complex formation. Thus, plasma samples from individuals with diseases of fluid phase complement dysregulation were examined. C3b:protein complexes were detected in the blood of patients with dense deposit disease (DDD) and to a lesser extent in C3 glomerulonephritis (C3GN) patients, but not in healthy controls. This finding supports the premise that these two C3 glomerulopathies are fluid-phase diseases of complement dysregulation. It is also possible that excessive generation and/or defective clearance of fluid-phase C3b:protein complexes contributes to the disease pathogenesis. Finally, in contrast to proteins in the native state, C3b more readily attaches to plasma proteins in their non-native unfolded state (chemically or thermally denatured), suggesting that C3 functions as an extracellular chaperone. Circulating C3b:protein complexes could be diagnostic and/or pathogenic in certain conditions of complement dysregulation.

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aHUS- atypical hemolytic uremic syndrome
AMD- Age-related macular degeneration
AP- Alternative Pathway
Apo E- Apolipoprotein E
APP- Amyloid precursor protein
Aβ 42- Amyloid β peptide (1-42)
Aβ- Amyloid β
BSA- Bovine serum albumin
C- complement
C1 inh- C1 inhibitor
C3G- C3 glomerulopathy
C3GN- C3 glomerulonephritis
C3Nef- C3 nephritic factor
C4 BP- C4 binding protein
CFHR- Complement factor H related
CP- Classical pathway
CR 1- Complement receptor type 1
CR 2- Complement receptor type 2
CR 3- Complement receptor type 3
CR 4- Complement receptor type 4
CUB- Complement protein subcomponents C1r/C1s, Urchin embryonic growth factor and Bone morphogenetic protein 1
CVF- Cobra Venom Factor
CVFAS- Cobra venom factor activated serum
DAF- Decay accelerating factor
DBP- Vitamin D binding protein
DDD- Dense deposit disease
Dpl serum- depleted serum
EAs- Antibody-coated sheep erythrocytes
fB- Factor B
fD- Factor D
fH- Factor H
FHL1- Factor H-like 1
fI- Factor I
fP- Factor P (Properdin)
GBM- Glomerular basement membrane
gC1qR- globular heads of the C1q receptor
GlcNAc- N-acetyl glucosamine
GuHCl- Guanidine hydrochloride
HAGG- Human aggregated IgG
HAGGAS- Human aggregated IgG activated serum
HK- High molecular weight kininogen

HSA- Human Serum Albumin
IgG- Immunoglobulin G
IVC- In vitro complex
LK- Low molecular weight kininogen
LP- Lectin pathway
LRP1- Low density lipoprotein receptor-related protein 1
LRP2- Low density lipoprotein receptor-related protein 2
MASP- MBL-associated serine protease
MBL- Mannose-binding lectin
MCP- Membrane cofactor protein
MeOSuc-AAPV-CMK- MeOSuc-Ala-Ala-Pro-Val Chloromethyl Ketone
MeOSuc-AAPV-pNA- N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide
MG8- Macroglobulin domain 8
MIRL- Membrane inhibitor of reactive lysis
NHS- Normal human serum
RCA- Regulators of complement activation
SAP- Serum Amyloid P
sCR1- soluble CR1
sCrry- soluble complement receptor-related protein y
SDS- Sodium dodecyl sulfate
TED- Thioester domain
TNS- 2-p-toluidinylnaphthalene-6-sulfonate
ZAS- Zymosan activated serum
α_1AG- Alpha-1 acid glycoprotein/ orosomucoid
α_1PI- Alpha-1 proteinase inhibitor/ alpha-1 antitrypsin
Δ Serum- 60 °C heated serum

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I dedicate this thesis to

my wonderful family for their constant support and unconditional love.

1. INTRODUCTION

1.1 COMPLEMENT SYSTEM: AN OVERVIEW

The complement system is the primary humoral component of the innate immune system that provides a first line of defense against invading microorganisms. The system consists of nearly 30 plasma proteins, which circulate as zymogens or inactive cofactors and are proteolytically cleaved on microbial surfaces by an enzymatic cascade **(Figure 1)**. This activation ultimately leads to products that tag the microbe for removal by phagocytic cells, as well as recruit and activate innate immune cells. Apart from the circulating plasma proteins, the complement system also consists of at least 12 cell surface proteins that act as either regulators or receptors for activated complement products.

The complement system was initially identified as a heat-labile protein that "complements" antibody-mediated killing of microbes. But extensive research has unraveled its functions that extend far beyond just microbial clearance. This system is also involved in immune surveillance, clearing pathogens, immune complexes, apoptotic cells and cellular debris and thus helping in maintaining homeostasis. The complement system also plays a key role in bridging the innate and adaptive immune responses. Its participation extends further into angiogenesis, mobilization of hematopoietic stem cells, synapse maturation, tissue regeneration and lipid metabolism (1,2).

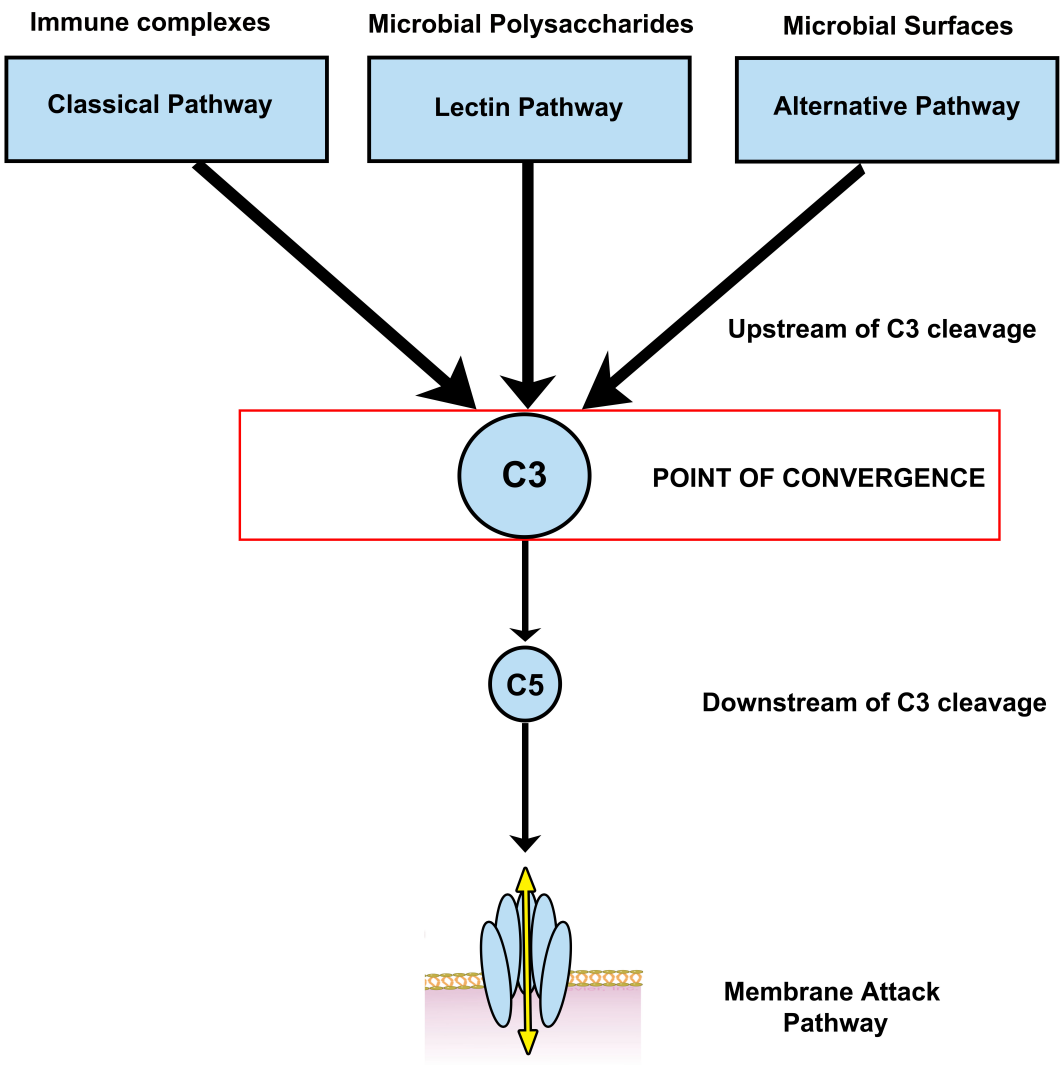


Figure 1: <u>Complement activation.</u>

Schematic figure showing the convergence of all three complement pathways at the step of C3 cleavage.

1.2. COMPLEMENT PATHWAYS

There are three major activation pathways for complement: the classical pathway, the lectin pathway and the alternative pathway. All three pathways converge at the key step of cleavage of C3, the central protein in the complement system.

1.2.a. Classical Pathway

The classical pathway (CP) is primarily activated by IgG and IgM immune complexes, and a wide variety of pathogenic microorganisms activate this pathway when they are bound to specific antibodies. The pathway is triggered upon binding of the complement C1 protein to the Fc regions of IgM and IgG (except IgG4) antibodies complexed with antigen. C1 binds immune complexes by its hexameric C1q subunit, and the affinity increases depending on the state of IgG aggregation (C1q binds monomeric IgG with 10,000 fold less affinity than IgG complexes). C1 can also be activated by other stimuli such as Gram-positive and Gram-negative bacteria, certain viruses, damaged cells, as well as several proteins (C-reactive protein and serum amyloid P component), carbohydrates and lipids (lipid A and cardiolipin). The C1 complex consists of C1q, C1r and C1s bound together by Ca^{2+} ions into a large macromolecular complex. C1q binds to its ligands by the globular head region, which causes a conformational change in the collagen-like tail region leading to auto activation of C1r, which then cleaves C1s (3). Activated C1s first cleaves C4 to C4a and C4b. In the C4b molecule, a thioester group is exposed which binds covalently to the bacterial surface and then binds C2 allowing it to be cleaved by C1s, leading to the formation of a C4b2a enzyme complex, the C3 convertase (C3 cleaving enzyme complex) of the classical pathway **(Figure 2)**.

1.2.b. Lectin Pathway

The lectin pathway (LP) involves carbohydrate binding by pattern recognition receptors such as mannose-binding lectin (MBL) and ficolins. MBL consists of a collagen-like tail domain and a carbohydrate recognition head domain, while the ficolins contain a collagen-like tail domain and a fibrinogen-like head domain. The prominent ligands for MBL are mannose and N-acetyl-glucosamine (GlcNAc), whereas carbohydrates on mammalian surfaces such as galactose and sialic acid have no affinity for MBL as they do not fit the steric requirement (4). L-ficolin is a versatile recognition protein able to bind acetylated molecules and neutral carbohydrates through different binding sites, whereas H-ficolin has a single binding site with a more restricted specificity for neutral carbohydrates (5). The lectin pathway of complement activation is initiated upon binding of collectin- or ficolin-MASP complex to target structures. Three MBL-associated serine proteases (MASP-1, MASP-2, and MASP-3) and two nonenzymatic proteins, MAp19 (sMAP) and MAp44 (MAP-1) have been described. MASP-2 is believed to be the key enzyme responsible for LP activation, while other proteins of the MASP family play up- or downregulatory roles. MASP-2 acts in a fashion similar to that of C1s to lead to the formation of the C3 convertase enzyme, C4b2a **(Figure 2)**. Though these pathways are initiated by different stimuli, they all converge at the step of C3 cleavage to C3a and C3b.

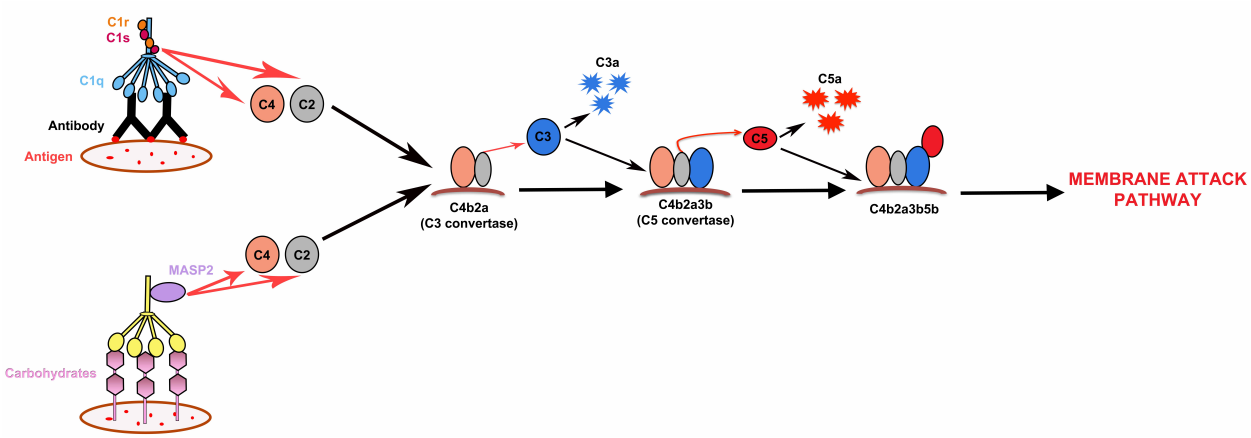


Figure 2: <u>Classical and Lectin pathways</u>.

Schematic figure showing the events occurring during classical and lectin pathway activation. Red arrows indicate proteolytic cleavage reactions.

1.2.c. Alternative Pathway

In contrast to the specific protein:protein and protein:carbohydrate interactions that initiate the classical and the lectin pathway, the alternative pathway is triggered by a unique autoactivation mechanism involving the highly reactive thioester bond in C3. This phenomenon referred to as the "tick-over" mechanism, occurs spontaneously at a rate of ~ 1% of the total circulating C3 pool per hour, and it keeps the complement system alert and allows constant probing for danger signals. In its native form, the complement protein C3 has very few ligands and is inert, but upon hydrolysis of the thioester (by tickover) C3 converts into $C3_{H2O}$, exposing new binding sites. This $C3_{H2O}$ then binds factor B and makes it amenable to cleavage by factor D, allowing for the formation of the C3 convertase (C3bBb), which cleaves C3 to C3a and C3b (6,7). This reaction is normally kept under check by regulatory proteins, but upon exposure to an external stimulus like a microbial surface, this regulation is overcome and there is rapid activation of the alternative pathway. The cleavage of C3 exposes an extremely reactive, very short-lived thioester, which covalently binds to amine and hydroxyl residues on target surfaces. The C3 cleavage product C3b (generated from classical, lectin and alternative pathways) then activates the alternative pathway, by feeding into the loop **(Figure 3)**.

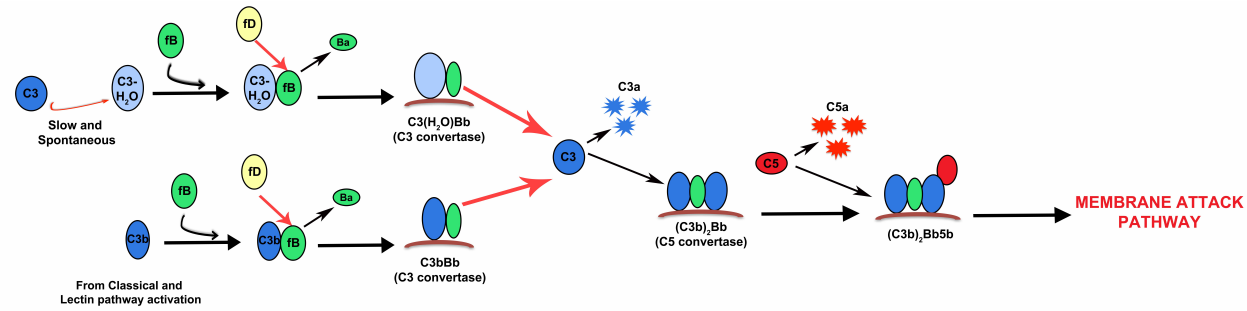


Figure 3: <u>Alternative pathway.</u>

Schematic figure showing the events occurring during alternative pathway activation. Red arrows indicate proteolytic cleavage reactions.

1.2.d. Membrane Attack Pathway

The C3b formed upon complement activation by any of the pathways binds the C3 convertase to form a C5 convertase (C4b2a3b/ C3bBbC3b) that cleaves C5 to C5a and C5b. The C5b fragment bound to the active convertase initiates the membrane attack pathway, also known as the terminal pathway. While still bound to the C3b in the convertase, C5b binds C6, which stabilizes the membrane-binding site of C5b and exposes a binding site for C7. Attachment of C7 causes release of the C5b67 complex from the convertase to the fluid phase, which then allows the complex to bind to the membrane by its hydrophobic membrane-binding site. C8 then binds this complex causing slight leakiness, but upon C9 binding C8 undergoes major conformational change to an elongated amphipathic form, which traverses the membrane. Additional C9 molecules are then recruited forming the multimeric membrane attack complex/ MAC (C5b-9) causing lysis of the target cell (8) **(Figure 4).**

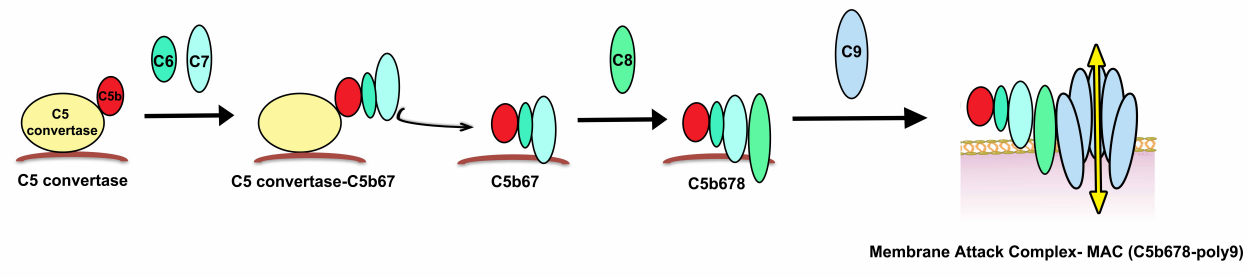


Figure 4: <u>Membrane attack pathway.</u>

Schematic figure showing the events occurring during the formation of the membrane attack complex (MAC).

1.3. C3 - THE CENTRAL MOLECULE OF COMPLEMENT

Complement C3 is the central component of the human complement system. Activation of C3 is central to all three complement pathways and results in inflammation and elimination of self and non-self targets. C3 is a 185 kDa protein consisting of an α-chain (~110 kDa) and a β-chain (~75 kDa) that are connected by disulfide bonds **(Figure 5)**. The complement serum proteins C3 and C4 and the protease inhibitor α_2 macroglobulin are all members of the C3/α_2M thioester protein family, an evolutionarily ancient and conserved family of molecules that contain an intrachain thioester bond. Being an evolutionarily old defense system, the complement system relies on a simple but extremely effective defense strategy based on an activated thioester - referred to as the "molecular warhead" - due to its extremely reactive nature. This highly reactive thioester (formed by the side chains of Cys 988 and Gln 991) is intact in the structure of C3 and is shielded from reacting with water ($t_{½} > 6$ days) or other small nucleophiles by a hydrophobic/aromatic pocket. These residues are conserved in the α_2M family, except for the related C5, which lacks the thioester moiety. The thioester is contained in a thioester domain (TED), allowing it to be tightly regulated by the arrangement of the various other domains of C3 and thus permitting C3 to patrol the bloodstream in an inactive conformation. The proteolytic cleavage of C3 to C3b and the conversion of C3 to $C3_{H2O}$ induces major conformational changes, allowing for the exposure of various cryptic binding sites, and thus C3b binds both many soluble plasma proteins and cell-surface receptors, while native C3 has very few binding partners. The exposure and activation of the thioester moiety are required for the covalent surface attachment of C3, to allow for continued complement activation, opsonization and other downstream effector functions.

In native C3 the TED domain is embraced by the CUB (Complement protein subcomponents C1r/C1s, Urchin embryonic growth factor and Bone morphogenetic protein 1) domain and held against the MG8 (Macroglobulin domain 8) domain and is thus protected from reacting with nucleophiles. But upon proteolytic cleavage and release of C3a, there is massive conformational rearrangement, where the CUB domain extends downwards and the TED domain is dropped, allowing for the formation of the highly reactive and extremely short-lived ($t_{1/2}$< 100 μs) acyl-imidazole intermediate, which can react covalently with the target surface (9). However, the covalent attachment of C3b to targets is rather inefficient, and only about 10% of the activated C3b molecules attach to the intended targets (10,11). Generally, the majority of thioesters react with water molecules in the fluid-phase in nascently generated C3b, and this serves to neutralize the reactive thioester to limit deposition on host cells (10). Covalent binding of C3b to certain plasma proteins such as C4, properdin and IgG upon C-activation has also been reported previously (12-14) in the context of immune complex clearance or complement pathway convertase formation. Cleavage of C3 into C3a (~9 kDa) and C3b (~177 kDa) causes conformational changes that expose several binding sites for complement proteins including factor B, properdin, factor H, CR1, CR2 and CR3. Additional cleavages in the α-chain of C3b (generating iC3b) mediated by factor I in association with soluble or membrane-bound co-factors prevent further convertase formation and profoundly alter the function of the protein. The first two cleavages release C3f (2 kDa), and the third cleavage in the remaining iC3b liberates C3c (135 kDa) from the target-bound C3dg (40 kDa) fragment. Trypsin, plasmin and elastase treatments are known to cause further cleavage of C3dg to C3d and C3g (15-20). Trypsin treatment can also produce further cleavage fragments of C3c, namely C3e (12 kDa) (21).

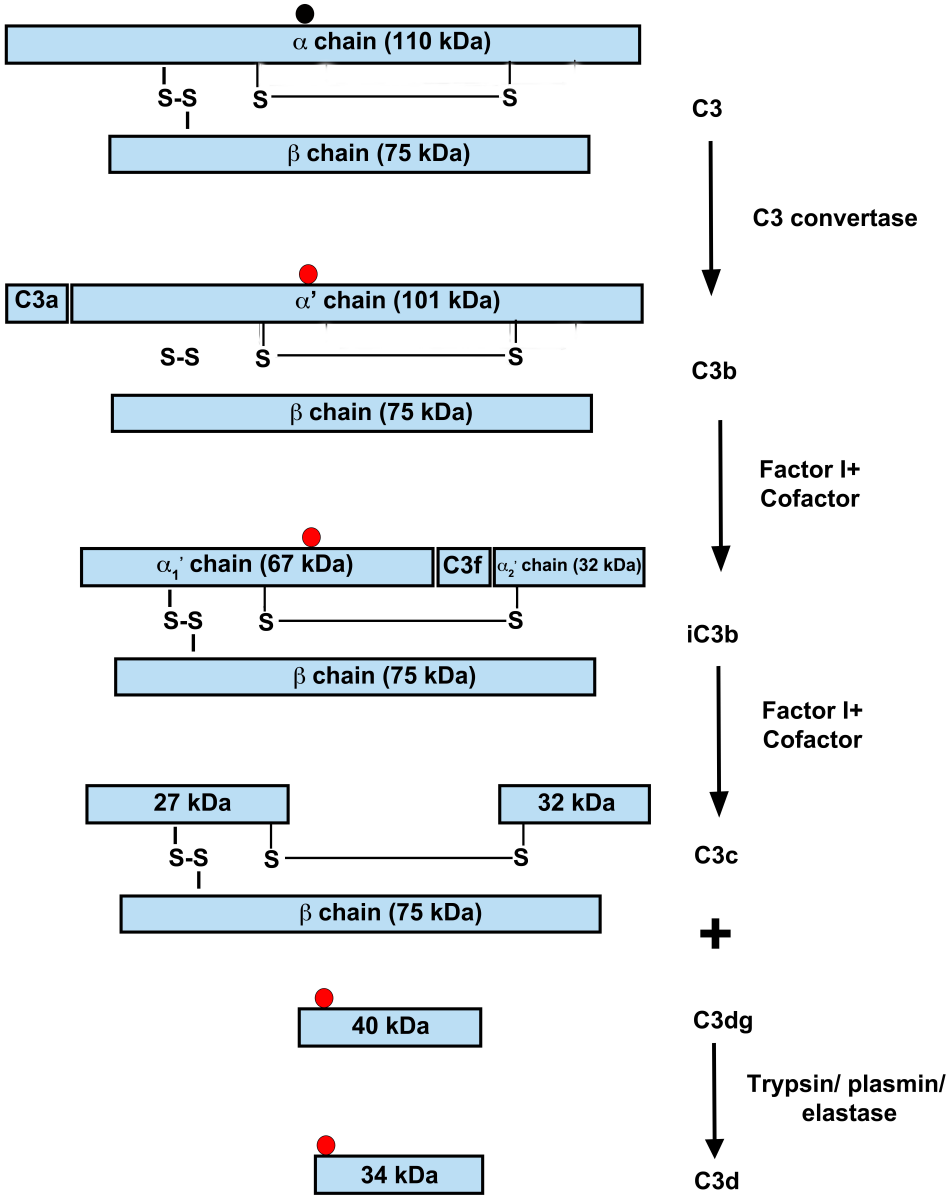


Figure 5: Complement C3 cleavage.

Schematic figure showing products of C3 cleavage and the proteases and cofactors that mediate each step. The black circle represents the C3 thioester and red circle represents the activated thioester.

1.4. EFFECTOR FUNCTIONS OF COMPLEMENT

1.4.a. Proinflammatory Signaling

The various activation products generated during C-activation carry out the multitude of functions of the complement system. C3a and C5a generated during this process are potent anaphylatoxins, which trigger pro-inflammatory signaling through their corresponding G-protein coupled receptors, C3aR and C5aR (CD88). C5a also acts through another receptor, C5L2, potentially a decoy receptor (22). C5a is a potent chemoattractant that recruits neutrophils, monocytes and macrophages to sites of complement activation, thus allowing them to participate in phagocytosis. C3a is chemotactic for eosinophils and mast cells.

1.4.b. Phagocytosis

The activated complement products that deposit on the target surface engage their corresponding receptors on the surface of phagocytes to allow uptake. Receptors for C3b and its cleavage products play a primary role in phagocytosis. CR1 is a receptor for C3b, C4b and the C3b cleavage product, iC3b (23). CR1 and CRIg promote phagocytosis by binding C3b and iC3b, while CR3 and CR4 mediate phagocytosis through iC3b (24-26). These phagocytic receptors are differentially expressed in blood cells and in tissues. CR1 is widely expressed on erythrocytes, monocytes/ macrophages, neutrophils, eosinophils, basophils, natural killer (NK) cells, B cells and some T cells, follicular dendritic cells, glomerular podocytes, Kupffer cells, while CRIg is expressed on macrophages. CR3 is present on neutrophils, eosinophils, basophils, monocytes/ macrophages, NK cells, microglial cells, platelets and Kupffer cells and follicular

dendritic cells, while CR4 is present on monocytes and macrophages. gC1qR, a receptor for the globular head regions of C1q, has several functions but also mediates phagocytosis (27).

1.4.c. Cell Lysis

Formation of the multimeric membrane attack complex (C5b–C9) during complement activation creates a pore in the target cell membrane causing lysis of the target cell (8).

1.4.d. Clearance of Immune Complexes and Cell Debris

The role of complement is not limited to just clearance of foreign organisms by phagocytes; it also contributes to safe clearance of modified self-cells undergoing apoptosis or necrosis. The modified cells rapidly shed the regulators CD46 and CD59 and allow for complement activation, followed by opsonization by C3b and C4b leading to phagocytic uptake. C1q is able to directly bind to apoptotic cells via its globular head domains, which leads to complement activation via the classical pathway, resulting in the opsonization of apoptotic cells with C4b, C3b or their degradation fragments. This opsonization further facilitates uptake by professional phagocytes via complement receptors (28). Thus C1q deficiency has been associated with defective clearance of apoptotic cells and increased susceptibility to autoimmune diseases in mice and humans. The complement receptor CR1 also plays a critical role in the clearance of immune complexes from circulation. The C3b bearing immune complexes are bound up to CR1 on erythrocytes delivering them to the reticuloendothelial system to be transported to the liver for disposal by phagocytic Kupffer cells (29).

1.5. COMPLEMENT ACTIVATING AGENTS

In the laboratory, various agents are used to activate the different complement pathways, such as Cobra Venom Factor (CVF) for the alternative pathway, aggregated human IgG (HAGG) for the classical pathway and zymosan (yeast cell wall) for the lectin pathway. CVF is the snake complement protein C3 and it functionally resembles the mammalian protein C3b and binds factor B, allowing it to be cleaved by factor D to form the C3 convertase (30). The C3 convertase CVFBb is physico-chemically far more stable than C3bBb, and it is also resistant to inactivation by the complement regulatory proteins factors H and I, thus making it a very potent activator of the alternative pathway.

Normally a circulating monomeric immunoglobulin molecule will not activate complement. When antibodies bind to antigens forming immune complexes they cluster, allowing two or more of the six arms of C1q to bind to the Fc domains of antibodies such as IgG or IgM. The binding of multiple arms to immune complexes is required to cause conformational changes that then allow the two C1r proteins in the complex (protease zymogens) to auto-activate, producing two C1r proteases that cleave and activate the two C1s protease zymogens in the complex. Heat-aggregation of IgG allows immune complexes to form, thus triggering the activation of the classical pathway.

Zymosan is prepared from actively growing yeast (*Saccharomyces cerevisiae*) by isolating their cell wall. Zymosan is primarily composed of a polymer of mannose resulting in activation of the lectin pathway via MBL; the alternative pathway of complement activates spontaneously on the zymosan. All pathways converge at the point of C3 cleavage, generating

C3b, the initial trigger of the alternative pathway. Thus, 80-90% of total complement activation is accounted for by the alternative pathway, even when triggered by the classical or the lectin pathway (31).

1.6. COMPLEMENT REGULATORS

The regulatory mechanisms of complement are finely balanced, such that activation is focused on invading microorganisms, while protecting host cells from complement-mediated damage. When mechanisms that regulate this fine balance go awry, that causes a tip in the balance between activation and regulation leading to self-attack. There are three major classes of complement regulators: fluid phase, cell-surface and complement clearance receptors **(Figure 6)**.

1.6.a. Fluid Phase Regulators

The fluid-phase regulators include properdin, carboxypeptidase N, complement factor H (fH), complement factor I (fI), C1 esterase inhibitor (C1-inh), C4 binding protein (C4BP), clusterin, and vitronectin. Properdin is the only positive regulator of complement that binds and stabilizes the alternative pathway C3 convertase (C3bBb). Recent studies have shown that properdin can directly bind to and initiate complement activation on a microbial surface and trigger local assembly and action of the alternative pathway C3 convertases (32,33). Carboxypeptidase N cleaves and partially inactivates the anaphylactic peptides C3a and C5a to their des Arg forms (34). Although this cleavage impairs signaling through the primary receptors C3aR and C5aR, it shifts the signaling pattern, as C3a desArg and C5a desArg themselves can trigger other important functions, for example, during hematopoietic stem-progenitor cells

(HSPC) mobilization or lipid metabolism. C1 esterase inhibitor (C1-inh) is a soluble protease inhibitor belonging to the serpin family that is a suicide inhibitor for C1r, C1s and MASP2, thus inhibiting classical and lectin pathway activation (35). C4BP acts as a cofactor for the factor I mediated inactivation of C4b, regulating the formation of the classical and lectin pathway convertases (36). Clusterin and vitronectin are soluble inhibitors of the terminal pathway of complement that bind C5b-7 to prevent the assembly of the membrane attack complex (MAC).

1.6.b. Factor H Family of Proteins

The Factor H family consists of seven different proteins that are organized in repetitive elements termed short consensus repeats (SCRs). Complement factor H is a 155-kDa plasma protein, which circulates at an average plasma concentration of 500 μg/ml. It contains 20 SCR domains and is the central soluble inhibitor of alternative pathway activation. Factor H regulates complement in both fluid-phase and on cell surfaces. There are three different regions in the fH molecule that express high affinity for C3b: both terminal ends of the molecule at SCRs 1-4 and 19-20 as well as in the center at SCRs 12-14. The polyanion binding sites are localized in SCR 7, SCR 12-14 and SCR 19-20. The C3b binding site in SCR1-4 is the only site essential for the cofactor activity of factor H in factor I mediated cleavage of C3b to iC3b, which can no longer form the convertase (37). Factor H also prevents the binding of factor B to C3b and dissociates the C3bBb complex (acting as a decay accelerating factor) (38). The C terminus of the protein (SCRs 18–20) mediates surface binding and target recognition. This C-terminal region includes binding sites for several ligands, such as C3b, C3d, heparin, cell surface glycosaminoglycans and microbial virulence factors, and hence is the most important site for preventing alternative pathway activation on host cells. While factor H binds C3b promptly in the fluid-phase, cell

surface inactivation of C3b depends on the composition of the surface to which C3b is bound. The presence of polyanions such as sialic acids, typically *N*-acetyl neuraminic acid which is found as the terminal sugar residue of oligosaccharide chains on the surface of cells, and the ability of factor H to bind these polyanions are what regulates spontaneous activation of complement on host surfaces (39). On host cells, the presence of sialic acid is responsible for creating a high affinity interaction between fH and C3b and thus the formation of AP C3 convertase (C3bBb) is restricted. Foreign cell membranes and surfaces generally lack sialic acid residues, resulting in a low affinity interaction between fH and C3b, favoring C3 convertase formation leading to AP amplification.

FHL1 is a 42 kDa protein that is a product of alternative splicing of the factor H gene and circulates at a plasma concentration of 10-50 μg/ml. It contains the seven N terminal SCRs of factor H and, consequently, FHL1 shares ligand binding and complement regulatory activity with the N terminus of fH, such as factor I cofactor activity and decay acceleration activity (40). There are 5 other factor H family members called complement factor H related proteins (CFHR) 1-5, which are encoded in the Regulators of Complement Activation (RCA) locus and are products of gene duplication. CFHR1 is made up of five SCRs, and the three C-terminal SCRs have homology with SCRs 18-20 of fH. CFHR 1 lacks decay acceleration or cofactor activity, but inhibits C5 converstase activity and terminal complex formation (41). The function of CFHR 2 remains unclear. CFHR 3 has five SCRs and binds heparin and C3b. CFHR3 blocks C5a generation and C5a-mediated chemoattraction of neutrophils and is thought to enhance cofactor activity of factor H (42). CFHR4 was also found to enhance fH cofactor activity, and when bound to CRP, can activate complement. Both CFHR3 and CFHR4 can ‘prime’ C3b to

inactivation by factor I in the presence of a cofactor (e.g. factor H/FHL1), although neither can act efficiently as a cofactor on its own and both proteins lack decay accelerating activity for the C3 convertase (43). CFHR5 is a 65-kDa protein made up of nine SCRs. It exhibits cofactor activity and possible fluid-phase decay accelerating activity, despite the fact that its SCRs do not contain homology with the N-terminus of fH, where these functions have been documented. CFHR5 also can bind heparin, C-reactive protein (CRP) and C3b due to its homology to SCRs 12-14 (heparin and CRP binding) and SCRs 19-20 (C3b binding) (44).

1.6.c. Factor I

One of the most important regulators of complement activation is complement factor I (fI), a 88 kDa serine protease that circulates in a zymogen-like state at a concentration of ~35 μg/ml and increases up to 100 μg/ml during inflammation, since it is an acute phase protein. It is primarily synthesized in the liver but is also produced in monocytes, endothelial cells and fibroblasts. Unlike most other plasma serine proteases that need to be proteolytically cleaved at the N terminus of the serine protease domain to be activated, factor I circulates in an active state and needs to be regulated. FI inactivates C3b by cleaving its α chain into iC3b, C3c and C3dg and, in an analogous way, C4b into C4c and C4d. To perform its functions, fI requires the aid of co-factors like factor H family proteins, membrane cofactor protein (MCP), CR1 and C4 binding protein (C4BP). It regulates the complement cascade by inactivating C3b or C4b and thus preventing the formation of C3/C5 convertases (45). Absence of factor I in the system leads to uncontrolled activation and amplification of the alternative complement pathway. The clinical manifestations of fI deficiency are similar to those of hereditary C3 deficiency, the most common feature being recurrent pyogenic infections which are secondary to the depletion of C3

and factor B caused due to uncontrolled alternative pathway activation. (46,47). There is also an increased susceptibility to *Neisseria meningitidis* infection. Patients with fI deficiency may also have "immune complex" type illness (48,49). Though factor I deficiency causes alternative pathway dysregulation in the same way as factor H deficiency, it has rarely been associated with a renal disease (50).

1.6.d. Membrane-bound Regulators and Receptors

The membrane-bound complement regulators include CD46/ MCP, CR1, CR2, CR3, CR4, complement receptor of the immunoglobulin family (CRIg), CD55/ decay accelerating factor (DAF) and CD59/ Membrane inhibitor of reactive lysis (MIRL). Many of these regulators are located on the same arm of human chromosome one, in the regulators of complement activation (RCA) locus. MCP regulates complement activation by functioning as a cofactor for factor I-mediated cleavage of C3b. CR1 is a multifunctional polymorphic glycoprotein which is variably expressed on the plasma membrane of erythrocytes, eosinophils, monocytes, macrophages, B lymphocytes, a subpopulation of CD4+ T cells, dendritic cells, Langerhans cells in the skin and glomerular podocytes. A non-membrane bound soluble form of CR1 (sCR1) found in plasma is released from leukocytes especially polymorphonuclear leukocytes into the circulation by cleavage of the surface form of CR1 (51). sCR1 shows complement inhibitory and anti-inflammatory activities, but the physiological plasma levels of sCR1 are very low (52). The CR1 molecule acts as a receptor for C3b and C4b thereby destabilizing and enhancing the decay of classical pathway C3 (C4b2a) and C5 (C4b2a3b) convertases and alternative pathway C3 (C3bCBb) and C5 (C3bBb3b) covertases. It also acts as a cofactor for factor I mediated inactivation of C3b and C4b (53). CR1 is also an immune adherence receptor that plays a key

role in immune complex clearance. Erythrocyte CR1 binds C3b/C4b opsonized immune complexes and transports them to the macrophages in the liver and spleen. Kupffer cells and other phagocytes in the liver and spleen metabolize these immune complexes (54). CR1 is also a major phagocytic receptor for complement-opsonized particles (23).

CR2 (also known as CD21) binds iC3b, C3c and C3dg. The CR2-C3dg interaction is believed to provide an important link between the innate and adaptive immune responses. On the membrane of B cells, CR2 acts as a co-receptor for surface-bound immunoglobulin, regulates B cell differentiation and maturation and instructs B cells to respond to C3d-coupled foreign antigens (55). CR3 (CD11b/CD18, MAC-1) and CR4 (CD11c/CD18) are very similar in structure, being the only heterodimeric complement receptors (integrins), and can recognize pathogen surface molecules as well as iC3b and thus contribute to immune clearance of pathogens through opsonization and phagocytosis (26). CR3 also functions in adhesion by binding to ligands found on many bacterial strains, including LPS. A recently characterized AP regulator, CRIg, aids in phagocytosis via iC3b and inhibition of AP activation through C3b binding, which blocks the formation of the C3 convertase (24). DAF is another regulator that acts on the central molecule of the complement pathways; it accelerates the decay of the C3 convertase via C2 and factor B dissociation (56). CD59 (MIRL), also known as protectin, prevents the MAC complex from inappropriately activating by blocking the binding of C9 to the C5b-C8 complex (57). This interferes with the completion of the MAC complex that creates a pore in the lipid bilayer of the target.

Membrane receptors for complement proteins also include gC1qR, C5aR, C3aR, and

C5L2. C5aR and C5L2 are receptors for the anaphylotoxin C5a, while C3aR binds C3a. Globular heads of the C1q receptor (gC1qR) recognizes the globular heads of C1q and plays a role in phagocytosis and signaling. It also modulates IL-12 on antigen-presenting cells and may play a role in regulating dendritic cell differentiation (58).

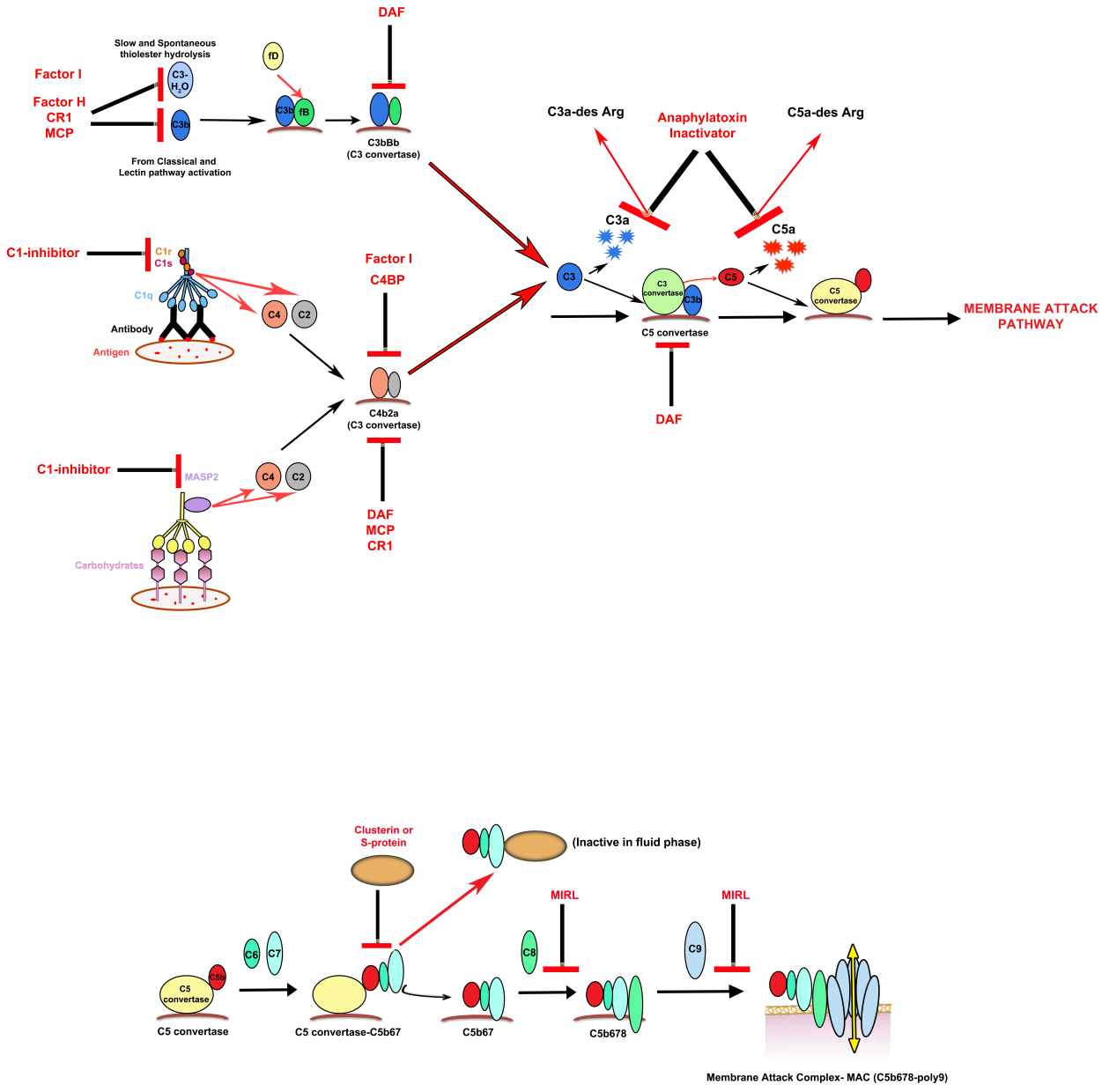


Figure 6: <u>Regulators of complement pathways.</u>

Schematic figure showing the regulation of the complement cascade at multiple different points.

1.7. COMPLEMENT DYSREGULATION AND DISEASES

Uncontrolled activation of the complement system due to various factors leads to a vicious cycle of inflammation causing tissue damage. Because of its spontaneous activating abilities, the alternative pathway requires active control, and hence a disturbance to this balance is associated with a wide range of diseases. This control mechanism can be overwhelmed by factors that favor activation, impaired function of regulatory proteins or increased activation due to high concentrations of alternative pathway components. Accordingly, various diseases like atypical hemolytic uremic syndrome (aHUS), age-related macular degeneration (AMD) and C3 glomerulopathies have been associated with AP dysregulation.

1.7.a. Atypical Hemolytic Uremic Syndrome

Hemolytic uremic syndrome (HUS) is a thrombotic microangiopathy that is characterized by a clinical triad of acute renal failure, microangiopathic anemia and thrombocytopenia. The typical form of HUS is associated with diarrhea caused by infection with *Escherichia coli* and is predominant in the younger age group. The atypical form (aHUS) is associated with genetic mutations of complement regulatory genes and affects both adult and juvenile populations. Low C3 levels as well as increased levels of fB and C3 breakdown products were reported in the plasma of a few patients with aHUS (11, 12). Granular C3 deposits were also observed in the glomeruli and arterioles from kidney biopsies in the acute phase of disease (13, 14). fH mutations account for up to 30% of reported cases, and the majority of fH mutations (60%–70%) are heterozygous missense mutations that cluster in the C-terminal region of fH. Functional studies of fH mutants in aHUS have demonstrated, for the most part, decreased binding to glycosaminoglycans and cultured endothelial cells. They have also shown a decreased

binding capacity for the fH ligand, C3b.

aHUS also has been associated with mutations of fluid phase alternative pathway inhibitors factor H, the membrane-bound inhibitor membrane cofactor protein (CD46), factor I and autoantibodies to factor H that block the C-terminal domain and inhibit cell surface regulation (59-61). Gain of function mutations in factor B and C3 have been associated with aHUS as well (61). Patients with aHUS have low levels of C3 and factor B in circulation, while C4 levels remain unaffected. C3 is also deposited in the glomeruli and arterioles of patients with aHUS. The main causes of kidney disease in aHUS are microthrombi formation in kidney blood vessels and endothelial cell damage due to dysregulation of complement activity on the surface of host cells. Deletion of CFHR 1 and 3 is associated with an increased risk of aHUS, and serum from these patients shows an impaired ability to protect erythrocytes from complement-mediated lysis (62). A mouse model that expresses a truncated fH molecule, which lacks the surface recognition region (SCRs 16–20), develops aHUS spontaneously (63). This demonstrates that defective control of complement activation on cell surface (renal endothelium) leads to aHUS.

1.7.b. Age-Related Macular Degeneration

Age-related macular degeneration is the most common cause of blindness in older populations in developed countries. The disease is characterized by damage to the macula (located at the center of the retina in the eye) and formation of drusen, which is the accumulation of debris in the retinal pigment epithelial cells and Bruch's membrane. The retinal drusen has been shown to contain complement components C3, C5, C6, C7, C8 and C9, complement regulators clusterin and vitronectin, as well as activation products C3a and C5a. These lesions

are structurally similar to those seen in the kidney in dense deposit disease (DDD) patients (64). A tyrosine-histidine polymorphism at amino acid 402 of factor H, a region that binds heparin and C-reactive protein, is associated with the development of AMD (65). Other complement genes with mutations and allele variants associated with AMD include factor B, C2, C3, and C4 (66,67).

1.7.c. C3 Glomerulopathy

C3 glomerulopathy is a recent disease classification that comprises several different glomerulonephritides (GN), including dense deposit disease (DDD), C3 glomerulonephritis (C3GN) and CFHR5 nephropathy. These disorders share the common histological feature of C3 deposition in the glomerulus with little or no immunoglobulin deposition and their etiology is associated with alternative pathway dysregulation.

CFHR5 nephropathy is a C3 glomerulopathy characterized by mesangioproliferative or membranoproliferative pattern. Electron microscopy reveals subendothelial and mesangial deposits with occasional subepithelial deposits. Serum C3 levels are normal, indicating that complement activation occurs locally in the glomerulus (68). The disease progresses to end stage renal failure during adulthood. Successful treatment with transplantation has been reported in ten patients (69), re-iterating the fact that complement dysregulation occurs locally in the kidney.

C3GN is a subtype of C3 glomerulopathy where subendothelial or subepithelial deposits are found in the mesangium or the capillary wall. Discontinuous intramembranous deposits are also sometimes seen by electron microscopy, but without the osmiophilic, ribbon-like

appearance characteristic of DDD. As in DDD, subepithelial 'hump'-like deposits classically associated with post-infectious GN may be present. Mass spectrometry has revealed C3 and MAC components in laser-dissected glomeruli, similar to DDD (70). Progression to end stage kidney disease is less common than in DDD, but does occur, with histological recurrence post-transplantation also reported (71).

DDD is a complex genetic disease of the glomeruli in the kidneys. The prevalence of DDD is about 2-3 cases per million. DDD is usually diagnosed in children although adult cases do occur; patients present with hematuria, proteinuria and hypertension. About half of DDD patients who have disease for more than 10 years develop end-stage renal disease. Some DDD patients also develop ocular drusen, which are whitish-yellow deposits in the Bruch's membrane beneath the retinal pigment epithelium of the eye. similar to the pathologic eye phenotype found in AMD (72). The disease takes its name from the transformation of the glomerular basement membrane (GBM) by extremely dark, ribbon-like electron-dense deposits located within the lamina densa (seen also within the mesangium, tubular basement membrane and Bowman's capsule) (73). The deposits contain complement components C3, C4, C5, C6, C8 and C9 and complement regulators CFHR1, CFHR5, clusterin and vitronectin (74). The absence of factor H in plasma has been observed to be a cause of DDD in humans, pigs and mice (75-77). Most DDD patients have an autoantibody against C3 convertase in the serum called C3 Nephritic Factor (C3NeF), which stabilizes the C3 convertase, making the enzyme less prone to inactivation by complement regulators (77-79). Though C3NeF is common in DDD, its prevalence is less so in C3GN and is absent in CFHR5 nephropathy. Autoantibodies to factor B that stabilize the C3 convertase and combined C3b and factor B autoantibodies have also been

reported in DDD patients (80,81). Autoantibodies to factor H that bind and inhibit the fluid phase AP regulatory role of factor H have also been implicated in the pathophysiology of DDD (82). In addition to these, common genetic variants including single nucleotide polymorphisms in the fH, C3 and CFHR5 genes have also been recognized as modifying risk of DDD (83) Complement haplotypes (or 'complotypes') combining polymorphisms in fH, CFHR1 and MCP conferring either increased risk or protection have also been delineated (84). Such polymorphic variations might be a factor in phenotypic differences between the histology of DDD and C3GN due to fH mutations.

Current therapies for DDD focus on decreasing progression of renal damage by improving renal hemodynamics and controlling infiltration of leukocytes in the kidneys (73). Some DDD patients undergo renal transplantation; however, recurrence of DDD occurs in most grafts and is the most common cause of renal failure in 15-50% of recipients (85). This further indicates that the dysregulation occurs in the fluid phase and is not localized to the kidney. Plasmapheresis has been useful in removing C3NeF from the circulatory system of DDD patients (86). Eculizumab, a monoclonal antibody that prevents C5 activation, has been approved for therapy in paroxysmal nocturnal haemoglobinuria (PNH) and aHUS patients and has shown promise in DDD patients as well (87). A possible treatment for patients with fH deficiency or protein defect is replacement of fH or other fluid-phase complement regulators (e.g., soluble CR1). Though administering exogenous functional factor H to patients with defective factor H has been shown to be efficacious, this approach will not be helpful in cases where genetic factors result in fH-resistant C3 convertases.

The function of fH has not only been studied in humans but also in pigs and mice by generating fH deficient animal models. Both the fH-deficient pig and mouse models develop a renal phenotype similar to human DDD (75-77). Mice lacking both fH and factor B (fB) proteins or fH and factor D (fD) do not have DDD, implying that activation of the AP is necessary in the development of DDD (77,88). Also, mice deficient in factor H and factor I proteins do not develop DDD, but upon addition of factor I, they develop the characteristic DDD renal pathology (89). Recent studies using mice lacking both fH and properdin have shown that properdin deficiency exacerbates renal injury in mice lacking fH (89). Conclusions from these studies using animal models of DDD have shown that alternative pathway dysregulation in the fluid phase and factor I mediated cleavage of C3b lead to the renal phenotype seen in DDD models.

1.8. EXTRACELLULAR CHAPERONES

Extracellular protein misfolding underlies most of the serious amyloidosis, including Alzheimer's disease, diabetes and spongiform encephalitis. Both the intracellular and extracellular compartments impose stress on protein structure due to fluctuation in pH, temperature and oxidative stress. Thus, it is necessary to maintain protein levels, structure and function in living systems, and this process is collectively known as protein homeostasis (proteostasis). The extracellular space is more oxidizing than the intracellular space, and there is an additional challenge posed to protein stability in the form of shear stress due to blood being pumped around the body. Chaperones play a key role in protecting against such stresses either by binding and preventing aggregation of the unfolded/misfolded protein, by facilitating refolding of such proteins or by clearance from circulation. Excessive misfolding/unfolding can

cause pathology due to disruption of function, gain of toxic function or inappropriate accumulation and disruption of tissues. Amyloidoses develop due to inappropriate accumulation of one or more proteins. Though the process of protein homeostasis has been studied extensively in the intracellular context, extracellular chaperones are understudied and characterization of these proteins and their functions has gained attention only in recent times. The number of extracellular chaperones continues to grow, and the list includes at least seven proteins now. The list includes clusterin, α_2-macroglobulin, haptoglobin, apolipoprotein E, Serum Amyloid P (SAP), caseins and fibrinogen.

Clusterin (apolipoprotein J) is a heavily glycosylated 60 kDa plasma protein that circulates in blood at a concentration of 35-105 μg/ml and plays roles in complement regulation, apoptosis, sperm maturation, membrane recycling and lipid transport. Clusterin is known to inhibit stress-induced protein aggregation by binding to exposed hydrophobic regions of non-native proteins and sequestering them to form high-molecular weight complexes, but it has no independent refolding activity. It also inhibits the fibrillar aggregation of a large number of amyloid forming clients including amyloid β (Aβ) peptide (90). The LDL receptor megalin (LRP 2/ Low density lipoprotein-related protein 2) is known to bind and internalize clusterin and clusterin-Aβ complexes (91). Supporting its role as an extracellular chaperone, clusterin is up-regulated in experimental models of oxidative stress, shear stress, heat stress and proteotoxic stress and is associated with extracellular deposits in age related macular degeneration, Creutzfeldt–Jakob disease, atherosclerosis and Alzheimer's disease.

α_2-macroglobulin is a major blood glycoprotein found at a concentration of 1.5-2 mg/ml.

α_2M is a broad-spectrum protease inhibitor which traps the protease covalently by its intramolecular thioester bond. α_2M forms stable complexes with misfolded proteins to inhibit their stress-induced aggregation and precipitation but is unable to promote independently their refolding (92). α_2M retains the ability to trap proteases after binding to misfolded proteins, and the complex is recognized by lipoprotein receptor-related protein (LRP), probably suggesting a role in clearance. α_2M-Aβ complexes are internalized via LRP and subsequently degraded (93). α_2M has been seen to be associated with extracellular deposits in Alzheimer's disease, dialysis related amyloidosis and Creutzfeldt–Jakob disease.

Haptoglobin is a secreted glycoprotein that binds hemoglobin and is found in plasma at a concentration of 0.3-2 mg/ml. Haptoglobin is known to inhibit stress-induced aggregation and precipitation of a wide variety of proteins in vitro induced by heat or oxidative stress. It binds and forms high molecular weight complexes with partially unfolded clients, but lacks independent refolding activity. Haptoglobin binds to a range of amyloid forming peptides and inhibits amyloid formation by binding to transient prefibrillar species. It is known to co-deposit with amyloid in senile plaques, drusen with age-related macular degeneration and in protein deposits associated with chronic glomerulonephritis. The acute-phase macrophage protein CD163 has been identified as a scavenger receptor for hemoglobin–haptoglobin complexes. It is possible that haptoglobin may facilitate the clearance of misfolded proteins via a similar mechanism to the clearance of hemoglobin–haptoglobin complexes (94).

ApoE is a 35 kDa secreted amphipathic glycoprotein that is known for its ability to mediate transport and clearance of cholesterol, triglycerides, and other lipids. ApoE is known for

its association with Alzheimer's disease and binds to aggregation prone polypeptides, such as tau and Aβ. It promotes and inhibits Aβ aggregation depending on the conditions and specific variant of Aβ peptide used. ApoE also co-localizes with Alzheimer's and Creutzfeldt-Jakob plaques. Just as observed for other extracellular chaperones such as clusterin and α_2M, complexes formed between ApoE and Aβ are efficiently taken up by receptor mediated endocytosis and promote subsequent degradation of Aβ. ApoE-Aβ complexes bind megalin and also bind Low density lipoprotein receptor-related protein 1 (LRP1) followed by internalization and subsequent degradation in the lysosomes (95,96).

Serum Amyloid P component (SAP) is a 25 kDa protein that circulates in blood at a concentration of 40 μg/ml. SAP is a refolding chaperone in the extracellular milieu that has ATP-independent refolding activity (97). SAP also binds highly specifically to amyloid and is universally found in amyloid deposits.

Casein is the main constituent of milk, and the family consists of four products: α_{S1}-, α_{S2}-, β-, and κ-casein. α_S-casein and β-casein inhibit the amorphous aggregation of a range of target proteins induced by heating, reduction and UV light (98). They bind to the target proteins to form high molecular weight complexes and thus prevent their aggregation and subsequent precipitation. They lack an independent refolding activity.

Fibrinogen is a 340 kDa plasma protein that circulates in plasma at a concentration of 2-4.5 mg/ml. Its plasma levels are increased during stress, and it is known to stabilize proteins in solution.

In summary, the extracellular chaperones mediate clearance of non-native proteins by binding to them and mediating their uptake via receptor-mediated endocytosis followed by lysosomal degradation. The primary sites of action of this process are likely to be the liver and the reticuloendothelial system.

1.9. COMPLEMENT C3 IN AMYLOID DISEASES

Consistent with what has been seen with thus far identified extracellular chaperones, complement C3 is deposited in amyloid plaques in Alzheimer's disease and age-related macular degeneration. Amyloid beta is known to activate the complement system through the classical pathway by binding to C1q (99,100). This activation causes covalent binding of C3 to Aβ42, and the circulating Aβ42 is subject to complement C3b-dependent adherence to complement receptor 1 (CR1) on erythrocytes, a classical set of mechanisms by which pathogens and proteins recognized as foreign are cleared from the bloodstream (101). The opsonized material can be transported to the liver, stripped from the erythrocytes by specialized reticuloendothelial cells (sessile macrophages), and degraded as a clearance mechanism. Studies using overexpression of soluble complement receptor-1 related gene/protein Y (sCrry) to inhibit C3 in an Alzheimer's disease mouse model, showed reduced microgliosis, increased Aβ plaque burden and neurodegeneration (102). Using a C3-deficient amyloid precursor protein (APP) transgenic Alzheimer's disease mouse model (APP;C3-/-), it was shown that C3 deficiency in APP mice resulted in increased cerebral deposition of Aβ and neuronal loss, thereby indicating a beneficial neuroprotective role for C3 in the brain (103).

2. MATERIALS AND METHODS

2.1. Reagents

Purified cobra venom factor (CVF), pre-activated Zymosan A and sheep erythrocytes were obtained from Complement Technology, Inc. (Tyler, TX). Human serum depleted of individual complement components (C3, C4, C5, factor B, factor D, properdin, factor H and factor I), and purified human complement proteins (C3, C3b, factor B, factor D, factor H and factor I) were all purchased from Complement Technology, Inc. The following purified human proteins were all obtained from Athens Research and Technology (Athens, GA): alpha-1 proteinase inhibitor (α_1PI), alpha-1 acid glycoprotein (α_1AG), immunoglobulin G (IgG), vitamin D binding protein (DBP) and neutrophil elastase. The following reagents for enzyme assays were purchased from Sigma-Aldrich: 2-p-toluidinylnaphthalene-6-sulfonate (TNS), N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (MeOSuc-AAPV-pNA) and MeOSuc-Ala-Ala-Pro-Val Chloromethyl Ketone (MeOSuc-AAPV-CMK). Bio-Gel P30 Gel with medium polyacrylamide beads (90–180 μm wet bead size) for size exclusion chromatography and Lowry protein assay based *DC* Protein Assay Kit were purchased from Bio-Rad. Pre-diluted bovine serum albumin (BSA) protein standards were purchased from Thermo Scientific. Purified actin was purchased from Cytoskeleton, Inc.

The IgG fraction of goat polyclonal anti-human DBP was purchased from DiaSorin (Stillwater, MN) and then affinity-purified in our laboratory using immobilized DBP. Chicken polyclonal anti-α_1PI antibody was obtained from ProSci Inc. (Poway, CA). Biotinylated rabbit polyclonal anti-α_1AG, goat polyclonal anti-human albumin, and rabbit polyclonal anti-kininogen and rabbit polyclonal anti- HK antibodies were all obtained from Abcam (Cambridge, MA).

Mouse monoclonal anti-human factor B (clone D33/3) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-C1 inhibitor and rabbit polyclonal anti-albumin antibodies were a generous gift from Dr. Berhane Ghebrehiwet, Stony Brook University. Chicken polyclonal anti-human C3 was obtained from Gallus Immunotech (Cary, NC). Mouse monoclonal anti-human factor I neutralization antibody (#A247) was obtained from Quidel (San Diego, CA). William's plasma (deficient in high and low molecular weight kininogen) was a generous gift from Dr. Alwin Schmaier, Case Western Reserve University.

2.2. Collection of human blood and in vitro activation of complement

Blood was collected from healthy, medication-free human subjects who gave informed consent using a protocol approved by the Stony Brook University Institutional Review Board (IRB). Vacutainer tubes (BD, Franklin Lakes, NJ) containing either 3.2% sodium citrate (for plasma) or a silica clot activator (for serum) were utilized. Individual serum and plasma samples from at least 5 subjects were pooled, aliquoted and frozen at -80°C. Pooled serum or citrated plasma (0.3 ml) was activated either with CVF (416 U/ml), zymosan A (10 mg/ml) or 50 µl heat-aggregated (heated at 63°C for 20 minutes) human IgG (10 mg/ml) and incubated at 37°C for the time indicated in each experiment. For activation of citrated plasma samples, 2 mM Mg^{2+} was added to overcome the chelation effect of sodium citrate. Sera depleted of the complement regulatory factors H and I spontaneously activate the alternative pathway and hence were stored in 0.1 mM EDTA. These samples were activated by adding 0.5 mM Mg^{2+} and incubating at 37°C. In certain experiments, depleted serum samples were reconstituted by adding back the purified protein to achieve its mean plasma concentration (factor B: 210 µg/ml; factor D: 1 µg/ml; C3: 1.3 mg/ml) along with 0.4 mM Mg^{2+} and then incubating at 37°C for 15 minutes.

2.3. Human Subjects with C3G

Twelve patients with biopsy-proven C3G (6 DDD and 6 C3GN) were selected from C3G registry from the University of Iowa for inclusion in this study based on histopathological data (light microscopy, immunofluorescence, electron microscopy) and the availability of sufficient plasma samples to complete all assays multiple times. The human research institutional review board at the University of Iowa approved all procedures, and all patients gave informed consent.

2.4. Gel electrophoresis and immunoblotting

For denaturing and reducing gels, all samples were separated using 8% polyacrylamide gels with sodium dodecyl sulfate (SDS) at 80 V for the stacking gel and 100 V for the resolving gel. Resolved gels then were transferred onto an Immobilon polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) at 100 V for 75 minutes. The PVDF membrane was blocked with 5% non-fat dry milk (NFDM) in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 30 minutes, followed by primary and horseradish peroxidase (HRP)-labeled secondary antibody incubations in 5% NFDM. Finally, blots were developed using HyGLO Quick Spray chemiluminescent detection reagent (Denville Scientific, Denville, NJ) and X-ray film. For native gels, the same procedure was carried out using 8% native polyacrylamide gels (without SDS).

2.5. In vitro complex formation and breakdown

To evaluate the role of activated C3 in complex formation, the alternative pathway was assembled in vitro using the purified proteins C3 (1.3 mg/ml), factor B (200 μg/ml) factor D (1 μg/ml) and DBP (400 μg/ml) or α_1AG (1 mg/ml) with 0.5 mM Mg^{2+} and incubated at 37°C for the specified amount of time. In control experiments, factor B was eliminated from the mixture to prevent activation. The molar ratios of the various components were maintained at physiological levels even when the exact concentrations could not be maintained due to dilution effects. The roles of regulatory proteins factors H and I in the breakdown of C3 complexes were determined by adding purified factor H alone (340 μg/ml), factor I alone (54 μg/ml) or both together with C3 (1 mg/ml), factor B (177 μg/ml), factor D (0.76 μg/ml), DBP (307 μg/ml) and 0.5 mM Mg^{2+} at 37°C along with CVF (416 U/ml) to activate the protein mixture. Complexes were evaluated by SDS-PAGE and immunoblotting.

2.6. C5a Sandwich ELISA

A sandwich enzyme-linked immunosorbent assay (ELISA) to detect human C5a and C5a des Arg was developed as previously described in detail (104). Briefly, Maxi-sorb 96 well plates were coated with 500 ng of mouse monoclonal anti-human C5a (clone 295003; R&D Systems, Minneapolis, MN) capture antibody at 4°C overnight. The coating solution was removed, and the wells were blocked with 300 μl of blocking solution [3% NFDM in PBS-T (phosphate buffered saline with 0.05% Tween-20)] for 1 hour at room temperature. After 3 washes, 100 μl of standards (78 pg/ml to 5 ng/ml of purified natural human C5a, Complement Technology, Inc.) or experimental samples were added and incubated at room temperature with shaking for 90 minutes. This was followed by 4 washes and incubation with 100 ng of the detection antibody,

biotinylated mouse monoclonal anti-human C5a (clone 295009, R&D Systems) for 60 minutes at room temperature with shaking. Finally, 40 ng of HRP-conjugated streptavidin (KPL, Gaithersburg, MD) was added to each well and incubated at room temperature for 30 minutes. After 5 washes, 100 µl of HRP substrate solution (KPL) was added until color development, followed by addition of 100 µl stop solution (KPL) and measurement of the absorbance at 450 nm using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA).

2.7. Hydrolysis of the C3 thioester

The thioester bond in C3 was inactivated by treating 250 µg C3 with 0.5 M hydroxylamine (NH_2OH) pH 7.5 for 2 hours at 21°C followed by exhaustive dialysis against Dulbecco's phosphate-buffered saline (DPBS) (1.8 L, 3x) at 4°C for a total of 18 hours (105). Inactivation of thioester was confirmed by hemolytic assays using rabbit erythrocytes for the alternative pathway, and antibody-coated sheep erythrocytes for the classical pathway as well as a C5a ELISA of the supernatant from the hemolysis assay with antibody-coated sheep erythrocytes (EAs). C3- depleted serum alone or C3-depleted serum reconstituted with either native C3 or C3-NH_2OH (at 0.5 mg/ml) was taken in a final dilution of 1:100 in GVB^{++} buffer along with 50 µl EAs (5 x 10^7/ml) and incubated at 37°C for 1 hour, after which the cells were centrifuged, supernatant collected and C5a levels quantified.

2.8. Antibody neutralization of factor I

Mouse monoclonal anti-human factor I, which inhibits the serine protease domain, was used to determine if enzyme activity of factor I is required to cleave these complexes. Anti-factor I (100

μg/ml) was added to factor H depleted serum and incubated on ice for 15 minutes to allow antibody binding, followed by incubation at 37°C for the specified amount of time.

2.9. Denaturation of proteins

The protein of interest was denatured using 5 M guanidine hydrochloride (GuHCl), 30 mM dithiothreitol (DTT), 10 mM EDTA, 100 mM Tris-HCl (pH 8.3) at room temperature for the time indicated in each experiment on a rotary shaker. Upon denaturation, the denaturing reagents were removed by size-exclusion chromatography using a Bio-Gel P-30 Gel column. Upon purification, protein levels were quantified in the eluates using a Lowry assay, and the proteins were tested for denaturation using functional assays.

2.10. TNS Assay

Native or denatured DBP (500 nM) or native or denatured HSA (500 nM) were incubated together with 30 nM TNS at room temperature for 20 minutes to allow binding, and the fluorescence was measured at an excitation wavelength of 322 nm and an emission wavelength of 465 nm.

2.11. Elastase Assay

Elastase (60 nM) was incubated at 37°C for 30 minutes with either 60 nM native α_1PI, 60 nM denatured α_1PI or 100 μM MeOSuc-AAPV-CMK (positive control) in assay buffer (100 mM HEPES, pH 7.25, with 500 mM NaCl, 0.05% Tween 20) to allow binding. MeOSuc-AAPV-pNA substrate (100 μM) was added, and the mixture was incubated at 37°C for 30 minutes. Absorbance was read at 405nm to assess elastase activity.

2.12. DBP:Actin complex formation

In vitro complex formed with either native DBP or denatured DBP (C3b:DBP) was incubated with actin at a 1:5 molar ratio of DBP:Actin for 30 minutes at room temperature. Complex formation was assessed using an 8% native polyacrylamide gel and immunoblotting for DBP to look for a shift in molecular weight upon binding to actin.

2.13. Human erythrocyte purification

Blood from normal healthy individuals was drawn into EDTA tubes and mixed well. The blood was transferred into a conical tube containing an equal volume of 3% dextran, mixed thoroughly but gently and allowed to sediment in upright position for 30 minutes at room temperature until a clear demarcation between the sedimented red cells and the pink supernatant was seen. The supernatant was aspirated, and the erythrocyte fraction was washed 3 times with DPBS by spinning at 1000 x g for 10 minutes at 15°C. The erythrocytes were suspended again in DPBS to be used for further experiments.

2.14. Complex binding to erythrocyte

Purified erythrocytes were treated with either normal human serum or CVF activated normal human serum (10^8 erythrocytes + 100 μl serum) for 2 hours at 37°C, following which the cells were centrifuged at 1000 x g for 3 minutes and washed twice with DPBS at 1000 x g at 4°C. The cell pellet was lysed using H_2O containing 1X protease inhibitor cocktail by incubating on ice for 30 minutes. The samples were centrifuged at 3300 x g for 10 minutes at 4°C. The supernatant containing the cell lysate was collected for analysis, following which the membrane fraction was

solubilized in 100 µl 0.5 M Tris HCl (pH 8.3) + 0.05% Triton X-100. The sample was centrifuged at 3300x g for 10 minutes at room temperature, and the soluble fraction was collected.

2.15. Complement activation using thermally denatured serum

Normal human serum (NHS) was heated to 60°C for 1 hour (Δ Serum), added to an equal volume of either NHS, factor B depleted serum, factor D depleted serum or C3 depleted serum and incubated at 37°C for the time indicated in the experiment. The NHS + Δ Serum sample was then activated using CVF, zymosan or aggregated IgG for 15 minutes. The samples were run on an 8% SDS polyacrylamide gel to look for factor B cleavage and iC3b generation. C5a levels in the samples were quantified using C5a ELISA.

3. CHAPTER 1: Complement activation induces the formation of C3b:plasma protein complexes

3.1. Complement activation induces generation of high molecular weight SDS-resistant forms of several plasma proteins

DBP has been shown to function as a chemotactic cofactor for C5a in vitro (106), and the initial goal of this study was to determine if complement activation converts DBP into an active chemotactic cofactor. Thus, possible structural changes in DBP were investigated by treating normal human serum (NHS) with activators of all three pathways and then analyzing samples by SDS-PAGE and immunoblotting. There was no apparent diminution in the 56 kDa native DBP band, and no lower molecular weight DBP bands were detected (**Figure 7A** and not shown). However, Figure 7 shows that activation of any complement pathway generates an SDS-resistant DBP band at approximately 200 kDa that is not observed in untreated serum. Moreover, simultaneous addition of EDTA with CVF to serum, inhibited complement activation (as shown by absence of factor B cleavage in **Figure 7D**) and prevented formation of this high molecular weight band.

To determine if formation of this high molecular weight band is specific to DBP, two other plasma proteins, α1-proteinase inhibitor (α_1PI) and α1-acid glycoprotein (α_1AG), both of similar size but greater abundance than DBP, were also examined in the C-activated serum samples. **Figure 7B and 7C** show that activation of any complement pathway also induces formation of high molecular weight SDS-resistant bands of α_1PI and α_1AG, and these are not

observed in untreated NHS or CVF-activated serum incubated with EDTA. Factor B cleavage shown in **Figure 7D** is used as a marker to verify complement activation. Thus, activation of serum complement in vitro correlates with the appearance of high molecular weight SDS-resistant bands of DBP, α_1PI and α_1AG.

3.2. Formation of higher molecular weight SDS-resistant bands parallels the time course of complement activation

The temporal correlation between complement activation and generation of these high molecular weight bands was investigated in both serum and citrated plasma to determine if the clotting process alters band formation. Samples were blotted for several proteins with different abundance, molecular weights and isoelectric points. All the proteins examined formed similar SDS-resistant bands upon complement activation in both serum and plasma **(Figure 8A)**, suggesting that the formation of SDS-resistant bands is generally not affected by blood clotting and not restricted to a certain class of proteins with a common structural motif. The temporal appearance of these SDS-resistant bands correlated with complement activation in both CVF-activated serum and plasma (**Figure 8A**). In serum, high molecular weight bands begin appearing at the 5-minute time point (**Figure 8A**), which is the precise time that complement activation products of factor B cleavage (**Figure 8A**) and C5a generation **(Figure 8B)** appear in the CVF-treated serum. Peak formation occurs at approximately 15 minutes and then gradually decreases in intensity (**Figure 8A**). In CVF-treated citrated plasma (**Figure 8A**), complement activation is delayed due to chelation of divalent cations by the sodium citrate, but the bands consistently appear at the 30-minute time point (**Figure 8A**), which correlates with factor B

cleavage (**Figure 8A**) and C5a generation **(Figure 8C)**. Interestingly, complement activation generates a single kininogen band in serum but a doublet in plasma. We surmise that this is due to the two forms of kininogen in blood, high molecular weight kininogen (HK) and low molecular weight kininogen (LK). HK is cleaved during the clotting process in serum so probably only the lower LK band appears, whereas both forms are found in plasma (**Figure 8A**). These results show that formation of high molecular weight SDS-resistant bands of several plasma proteins correlates temporally with complement activation in vitro and is not unique to specific blood proteins.

Complement dependence of band formation was further investigated using factor H depleted serum. The lack of this key complement regulator causes spontaneous activation of the alternative pathway. DBP, α_1PI and kininogen formed SDS-resistant bands spontaneously in factor H depleted serum (without the need for a complement activating agent) upon addition of 0.5 mM Mg^{2+} and incubation at 37°C **(Figure 9)**. Complement activation was assesed by factor B cleavage. Reconstitution to physiological levels (500 μg/ml) of factor H before 37°C incubation prevented band formation. When factor H depleted serum alone or fH depleted serum reconstituted with increasing concentrations of factor H (from 0.25X to 2.5X; X = physiological concentration) was activated with CVF in the presence of 0.5 mM Mg^{2+}, the intensity of the bands decreased with increasing concentration of reconstituted factor H. This closely correlated with the amount of factor B cleavage, further confirming the complement activation dependence of this process **(Figure 9).**

3.3. The doublet in the SDS-resistant higher molecular weight complex formed by kininogen contains both HK and LK

It is interesting to note that all plasma proteins tested form a single complex in both serum and plasma except kininogen, which forms a doublet in plasma but a single band in serum. We surmised that this difference was due to kinin activation during the clotting process to generate serum. To further explore the effect of C-activation on the formation of the kininogen bands, purified HK was added to serum, and it restored the higher band of the doublet upon C-activation (data not shown). Next, the kininogen composition (high versus low molecular weight forms) of these bands was investigated using two different antibodies, the first antibody (used in the blots shown below) detects both HK and low molecular weight kininogen (LK), the second antibody reacts with the light chain region of HK and does not detect LK (both HK and LK have identical heavy chains but LK has a very small light chain). In addition, we also utilized HK-depleted plasma (no HK but has LK) and the very rare William's trait plasma (no HK or LK, a generous gift from Dr. Alvin Schmaier, Case Western Reserve University). Using these antibodies and plasma samples two different approaches were employed: 1) activate HK-depleted and William's trait plasma with CVF for different times to determine if HK, LK or both are capable of forming these complexes; 2) use the antibody specific for the light chain of HK (that does not detect LK) to determine their position (upper or lower band) in the kininogen complexes.

Figure 10A shows that the lower band of the doublet contains LK while HK forms two different complexes, one that migrates at a similar molecular weight as the LK complex and another at the higher molecular weight. To further confirm this observation, the same experiment was repeated using William's trait citrated plasma. William's trait plasma contains no HK or LK

(Figure 10B) but when purified HK was added, a doublet band appeared only after C-activation. Likewise, addition of purified LK produced the single band of lower molecular weight, again only after C-activation **(Figure 10C).** Since both HK and LK form these high molecular weight complexes, it seems reasonable to assume that the heavy chain region (identical in HK and LK) is required for complex formation. To determine if the bradykinin sequence in the heavy chain is required for complex formation, the kallikrein cleaved form of HK, known as HKa, was utilized. Plasma kallikrein cleaves HK in a two- tep pattern to release bradykinin and yield a 64 kDa heavy chain linked by a single disulfide bond to a 45 kDa light chain, and this two-chain molecule has been termed HKa. The question was asked if the HKa form could restore the doublet when added to HK depleted plasma activated with CVF. **Figure 10D** clearly demonstrates that HKa is incapable of forming the high molecular weight complex upon C-activation, indicating that the bradykinin containing region may be essential for this process.

3.4. Complexes form even in the presence of cell surface regulators in blood

The affect of blood cells on the generation of these high molecular weights bands was investigated using citrated whole blood. **Figure 11** demonstrates that SDS-resistant bands of α_1PI and α_1AG form even in whole blood during complement activation, indicating that surface expression of complement receptors and regulatory proteins on blood cells does not prevent formation of these high molecular weight bands.

3.5. Complexes formed by different plasma proteins are independent of each other

The question of whether the high molecular weight SDS-resistant bands of the different plasma proteins can form independently of each other was addressed using William's plasma from a patient deficient in both HK and LK. **Figure 12** shows that no kininogen bands formed in William's plasma upon complement activation, but this did not alter generation of SDS-resistant bands of DBP, α_1PI and α_1AG, suggesting that the generation of each high molecular weight plasma protein band occurs independently.

3.6. Activation of the C3 thioester during C-activation causes formation of covalent complexes of C3b with plasma proteins

The evidence presented above indicates a strong correlation between complement activation and formation of high molecular weight SDS-resistant bands of several plasma proteins. To determine if these high molecular weight forms are generated as a result of complement activation, sera depleted of different complement components were utilized. **Figure 13A** shows the formation of high molecular weight SDS-resistant bands of DBP, α_1PI and α_1AG (as representative proteins) in various depleted serum samples activated with CVF; reconstitution of depleted serum with the purified missing component was performed in samples where depletion abolished band formation. Serum deficient in either C3, factor B, or factor D was unable to generate high molecular weight bands upon addition of CVF (**Figure 13A**), but formation was restored with addition of the deficient proteins. In contrast, generation of the bands was not affected in serum deficient in either properdin, C4, or C5. Since these results

clearly demonstrate that all steps leading to C3 cleavage are essential, the role of the C3 cleavage product C3b was examined next (**Figure 13B**). Addition of purified C3b to factor B-depleted serum did not lead to generation of high molecular weight forms of DBP, α_1PI and α_1AG, even though purified C3b was present in the system. Similarly, addition of C3b to C3-depleted serum did not result in high molecular weight forms of DBP, α_1PI and α_1AG, even though there was robust factor B cleavage in the C3-depleted serum (**Figure 13B**), indicating that a C3 convertase was formed but there was no native C3 to cleave. These results (**Figures 13A and 13B**) indicate that both native C3 and components needed to form a C3 convertase (factors B and D) are required to generate the high molecular weight forms of DBP, α_1PI and α_1AG.

Since native C3 is essential for this process, the role of the thioester bond in C3 was investigated next. C3 was treated with hydroxylamine (NH_2OH) to inactivate the thioester bond, and this inactivated C3 was run on a gel and silver-stained to detect possible structural changes. **Figure 14A** shows that the treatment did not cause a change in the apparent molecular weight of inactivated C3. Hydroxylamine treated C3 was then added to C3 depleted serum and tested for its ability to cause hemolysis using both rabbit erythrocytes (data not shown) and antibody-coated sheep erythrocytes. The supernatant from the antibody-coated sheep erythrocytes was tested for C5a generation. **Figure 14B** shows that hydroxylamine-treated C3, like C3b, could not attach to an activating cell surface and generate C5a indicating that hydroxylamine treatment effectively inactivated the thioester bond.

Once the loss of thioester activity was tested, the C3-NH_2OH was used to reconstitute C3 depleted serum; and this serum was then treated with CVF and formation of high molecular

weight DBP, α_1PI and α_1AG bands was determined. **Figure 14C** demonstrates that hydroxylamine inactivated C3 could not form complexes with DBP, α_1PI or α_1AG upon C-activation, indicating that an intact thioester bond is essential for this process. The C3 blot of the same samples shows that high molecular weight SDS-resistant bands are only observed in the CVFAS control, or when native C3 is added back to C3-depleted serum and activated with CVF (**Figure 14C**). Thus, the SDS-resistant high molecular weight bands of plasma proteins formed during complement activation are covalent complexes with nascently generated C3b.

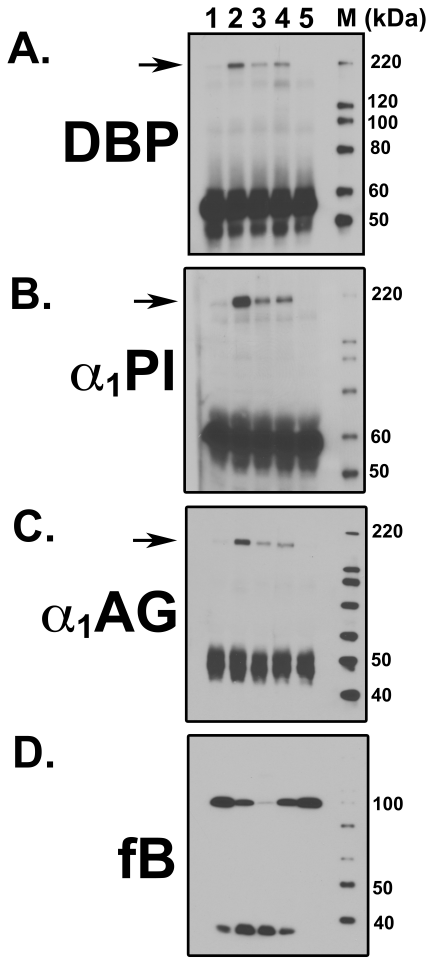


Figure 7: <u>Complement activation induces formation of high molecular weight SDS-resistant complexes of plasma proteins.</u>

Pooled normal human serum was sham-treated with PBS (lane 1) or complement was activated by incubating serum at 37°C with either 416 U/ml CVF (lane 2), 10 mg/ml zymosan A (lane 3), or 0.5 mg/300 µl heat-aggregated human IgG (lane 4). As a control to inhibit complement activation, 10 mM EDTA was added to serum prior to addition of CVF (lane 5). Serum aliquots were separated on an 8% SDS-PAGE and then immunoblotted for DBP, α_1PI or α_1AG. Arrows indicate the position of high molecular weight SDS-resistant bands formed during complement activation. The same samples were also blotted for factor B (fB) to verify cleavage as an indicator of complement activation (bottom panel).

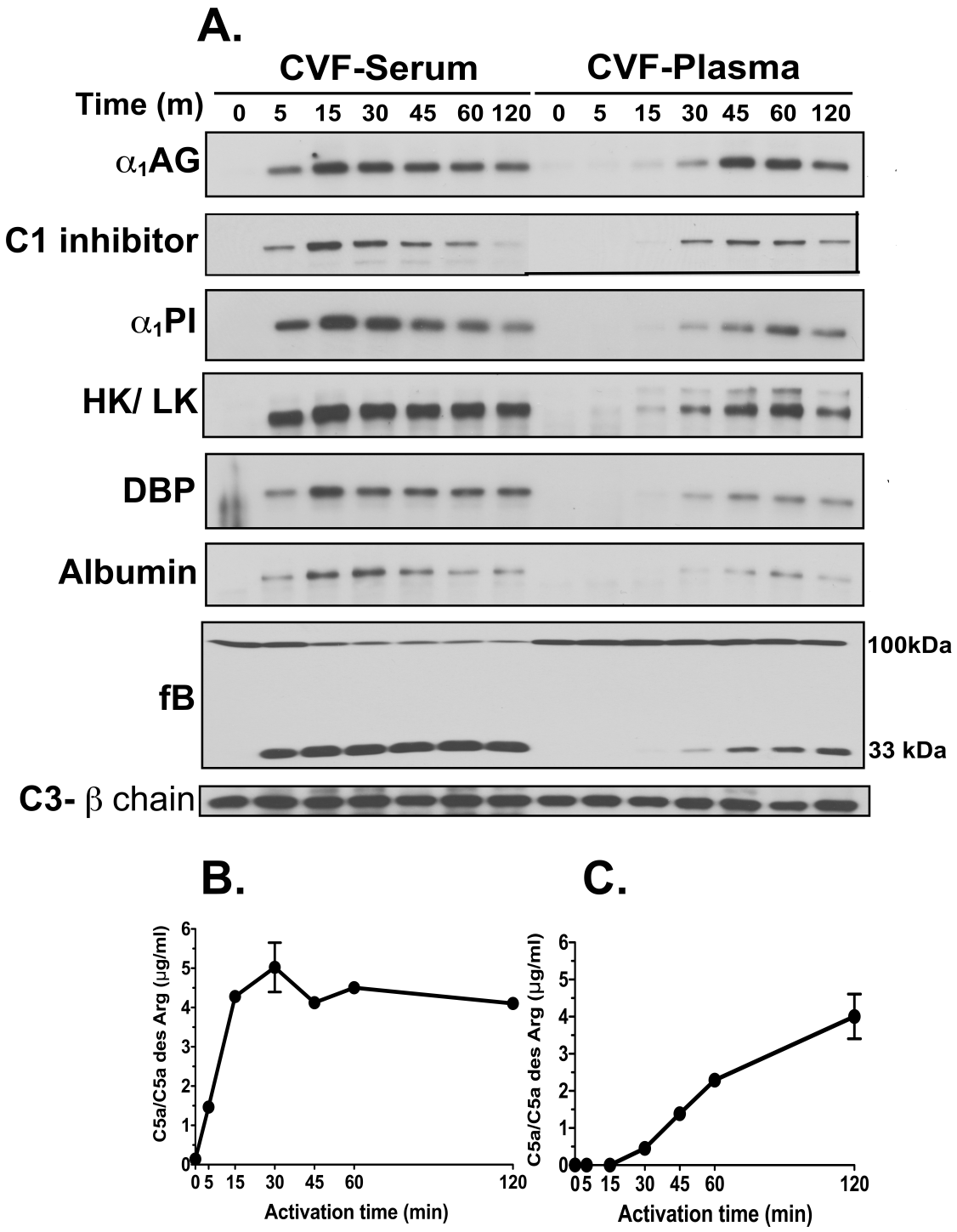

A.
CVF-Serum
CVF-Plasma
Time (m)
0 5 15 30 45 60 120 0 5 15 30 45 60 120
α1AG
C1 inhibitor
α1PI
HK/ LK
DBP
Albumin
fB
100kDa
33 kDa
C3- β chain
B.
C5a/C5a des Arg (µg/ml)
0 5 15 30 45 60 120
Activation time (min)
C.
C5a/C5a des Arg (µg/ml)
0 5 15 30 45 60 120
Activation time (min)

Figure 8: Formation of SDS-resistant bands correlates temporally with complement activation in vitro.

Pooled normal human serum or citrated plasma was activated at 37°C with 416 U/ml CVF for the indicated times. In addition to CVF, 2 mM Mg^{2+} was added to plasma overcome the chelation effects of sodium citrate. At each time-point, activation was stopped by placing the sample on ice. Panel A: Aliquots were separated on an 8% SDS-PAGE and then immunoblotted for the indicated proteins. Gels were cropped to focus only on the high molecular weight SDS-resistant bands. Samples also were blotted for factor B to verify cleavage as an indicator of complement activation. The 75 kDa β-chain of C3 was utilized as a loading control. Panel B: C5a ELISA of complement activation in serum. Panel C: C5a ELISA of complement activation in citrated plasma.

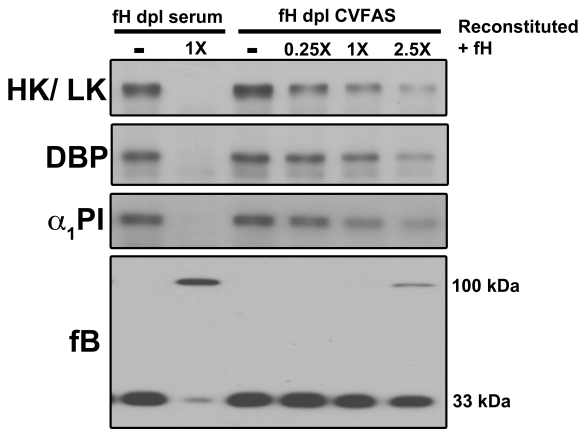
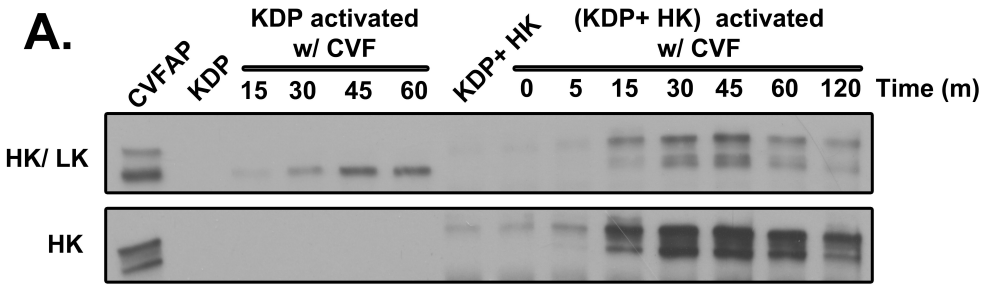
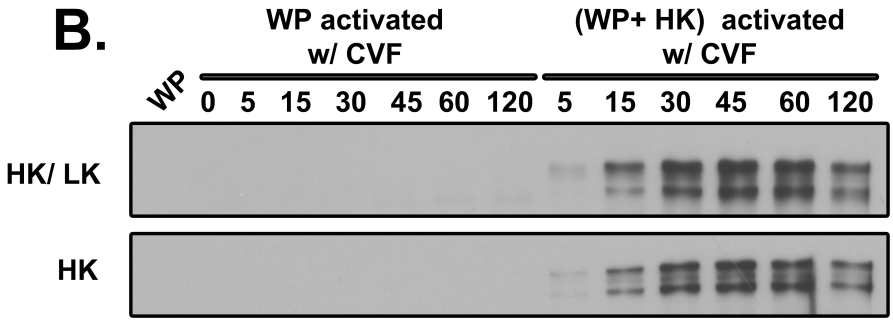
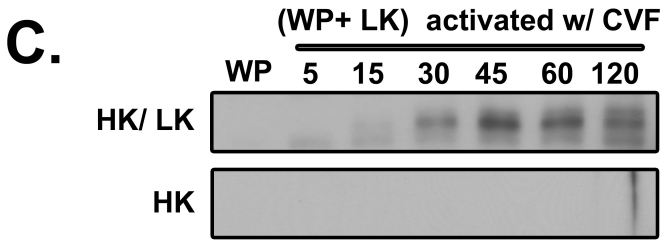


Figure 9: <u>The intensity of complexes formed correlates with the extent of complement activation.</u>

Factor H depleted serum (that was stored in 0.1 mM EDTA to prevent depletion of complement) alone or factor H depleted serum reconstituted with 500 μg/ml purified factor H was incubated at 37° C for 15 minutes with 0.5 mM Mg^{2+} to allow for complement activation. Lanes 3, 4, 5 and 6 contain factor H depleted serum reconstituted with either no factor H, 125 μg/ml factor H (0.25X), 500 μg/ml (1X) factor H or 1.25 mg/ml factor H (2.5X) followed by activation with CVF in the presence of 0.5 mM Mg^{2+} for 15 minutes. Aliquots were separated on an 8% SDS-PAGE and then immunoblotted for the indicated proteins. Gels were cropped to focus only on the high molecular weight SDS-resistant bands. Samples were also blotted for factor B to verify cleavage as an indicator of complement activation.


A.
KDP activated w/ CVF
(KDP+ HK) activated w/ CVF
CVFAP
KDP
KDP+ HK
15 30 45 60
0 5 15 30 45 60 120
Time (m)
HK/ LK
HK


B.
WP activated w/ CVF
(WP+ HK) activated w/ CVF
WP
0 5 15 30 45 60 120
5 15 30 45 60 120
HK/ LK
HK


C.
(WP+ LK) activated w/ CVF
WP
5 15 30 45 60 120
HK/ LK
HK

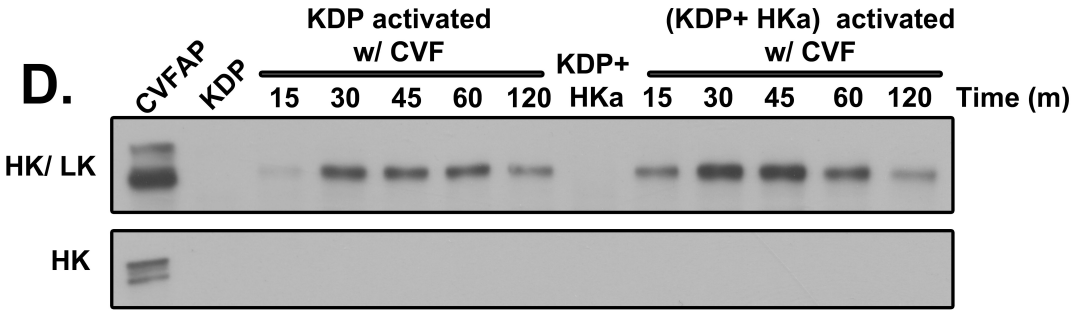

D.
KDP activated w/ CVF
(KDP+ HKa) activated w/ CVF
CVFAP
KDP
KDP+ HKa
15 30 45 60 120
15 30 45 60 120
Time (m)
HK/ LK
HK

Figure 10: The kininogen doublet seen in plasma consists of both HK and LK

Panel A: Kininogen depleted plasma (KDP) that was immuno-depleted of HK was activated with CVF for various time intervals between 0 and 120 minutes either by itself or after reconstitution of HK. Aliquots were separated on an 8% SDS-PAGE and then immunoblotted for kininogen using two different antibodies. The top panel was immunoblotted with an antibody that recognizes both HK and LK, and the bottom panel was blotted with an antibody that detects HK alone. Panel B: William's plasma from a patient deficient in both HK and LK was activated with CVF for various time intervals between 0 and 120 minutes either directly or after reconstitution with HK. Aliquots were separated on an 8% SDS-PAGE and then immunoblotted for kininogen using two different antibodies. The top panel was immunoblotted with an antibody that recognizes both HK and LK, and the bottom panel was blotted with an antibody that detects HK alone. Panel C: William's plasma from a patient deficient in both HK and LK was activated with CVF for various time intervals between 0 and 120 minutes either directly or after reconstitution with LK. Aliquots were separated on an 8% SDS-PAGE and then immunoblotted for kininogen using two different antibodies. The top panel was immunoblotted with an antibody that recognizes both HK and LK and the bottom panel was blotted with an antibody that detects HK alone. Panel D: Kininogen depleted plasma that was immuno-depleted of HK was activated with CVF for various time intervals between 0 and 120 minutes either directly or after reconstitution of HKa. Aliquots were separated on an 8% SDS-PAGE and then immunoblotted for kininogen using two different antibodies. The top panel was immunoblotted with an antibody that recognizes both HK and LK and the bottom panel was blotted with an antibody that detects HK alone. Gels in all panels were cropped to focus only on the high molecular weight SDS-resistant bands.

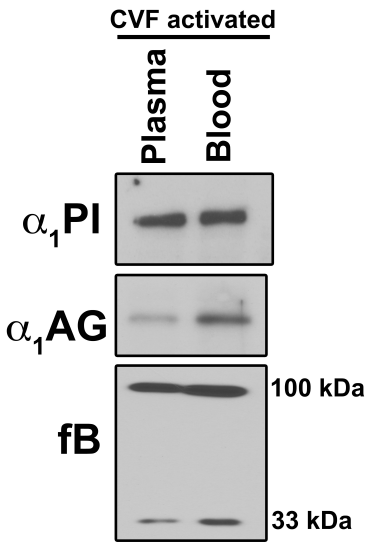


Figure 11: <u>High molecular weight SDS-resistant bands form in whole blood upon complement activation.</u>

Citrated whole blood or citrated plasma from the same blood donor was treated with 2 mM Mg^{2+} and CVF (416 U/ml) for 30 minutes at 37°C. PBS was added to plasma to compensate for blood cell volume and equalize the protein concentrations between the plasma and whole blood samples. After CVF-activation, whole blood was centrifuged to pellet the cells, and aliquots of both plasma samples were separated using an 8% SDS-PAGE and immunoblotted for α_1PI, α_1AG and factor B.

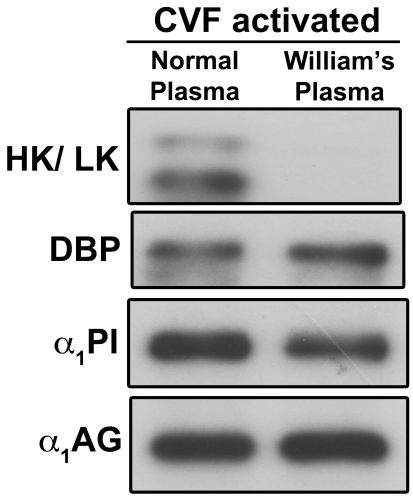
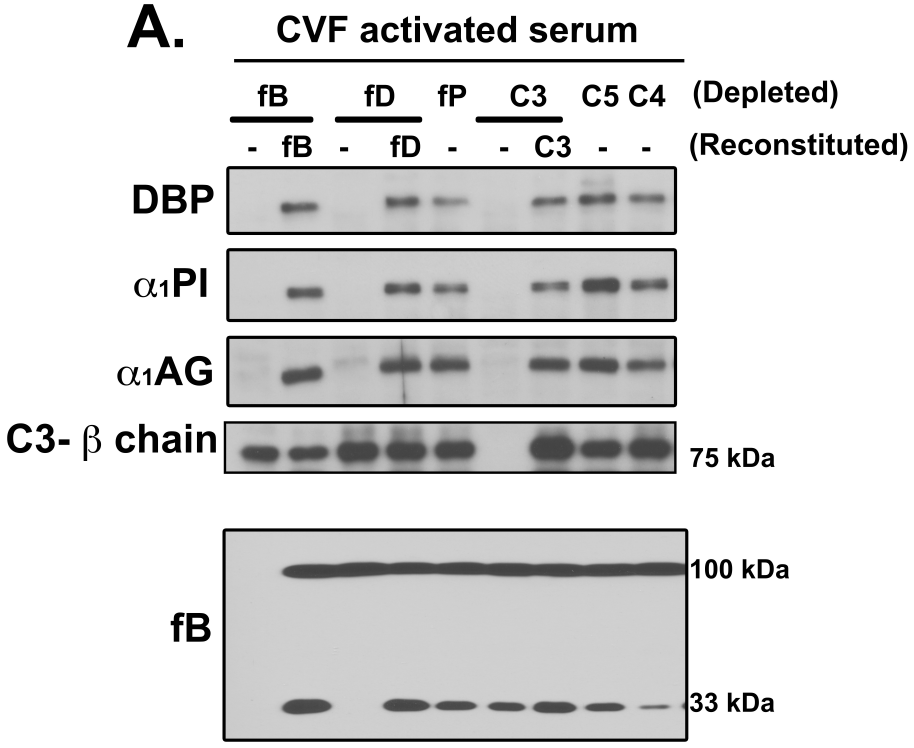


Figure 12: <u>High molecular weight SDS-resistant bands of the different plasma proteins can form independently of each other.</u>

Normal human citrated plasma or citrated William's plasma (deficient in both HK and LK) were activated with CVF (416 U/ml) and 2 mM Mg^{2+} for 60 minutes at 37°C. Aliquots were separated using 8% SDS-PAGE and immunoblotted for kininogen, DBP, α_1PI, and α_1AG.


A.
CVF activated serum
fB
fD
fP
C3
C5 C4
(Depleted)
- fB - fD - - C3 - -
(Reconstituted)
DBP
α1PI
α1AG
C3- β chain
75 kDa
fB
100 kDa
33 kDa

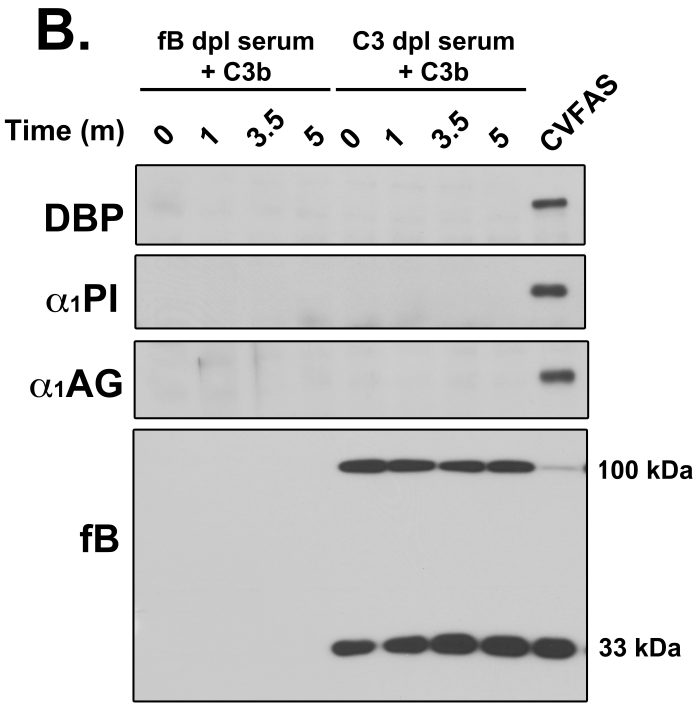

B.
fB dpl serum + C3b
C3 dpl serum + C3b
Time (m)
0 1 3.5 5 0 1 3.5 5 CVFAS
DBP
α1PI
α1AG
fB
100 kDa
33 kDa

Figure 13: C3 convertase and native C3 are both essential for the formation of high molecular weight SDS-resistant bands.

Panel A: Serum samples depleted of individual complement components (fB, fD, fP, C3, C5 and C4) were activated with CVF (416 U/ml) for 15 minutes at 37°C. Samples where complex formation was abolished were reconstituted to physiological levels with missing purified protein (fB, fD or C3) followed by activation with CVF. Aliquots were separated using 8% SDS-PAGE and immunoblotted for DBP, α_1PI and α_1AG. Samples also were immunoblotted for C3 β-chain band as a loading control and factor B to show complement activation. Panel B: Factor B-depleted and C3-depleted sera were reconstituted with purified C3b (1.3 mg/ml) and incubated at 37°C for 1, 3.5 or 5 minutes, separated using 8% SDS-PAGE and immunoblotted for DBP, α_1PI and α_1AG. Samples also were blotted for factor B to show complement activation in C3-depleted serum reconstituted with C3b. CVF activated normal human serum was included as a positive control.

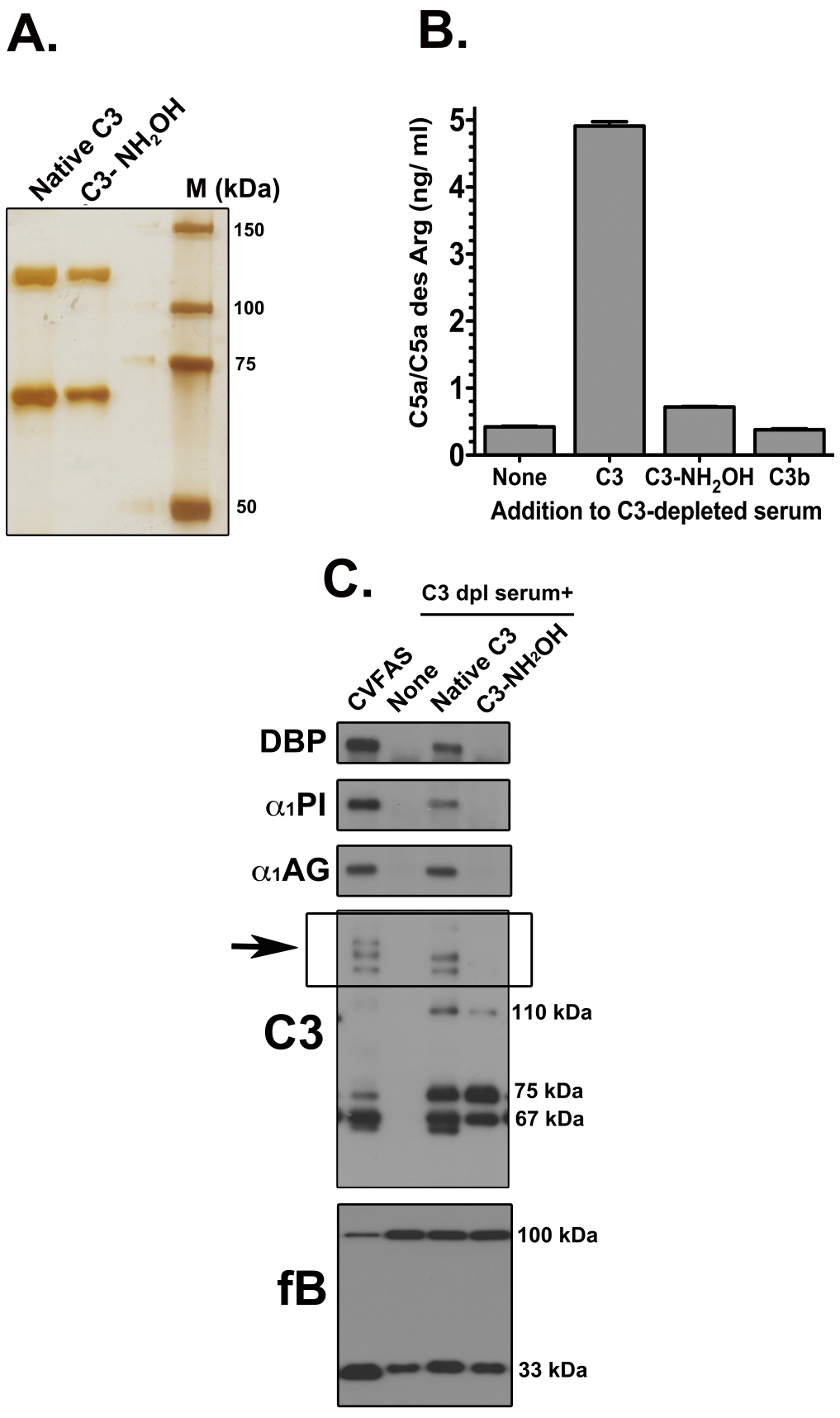
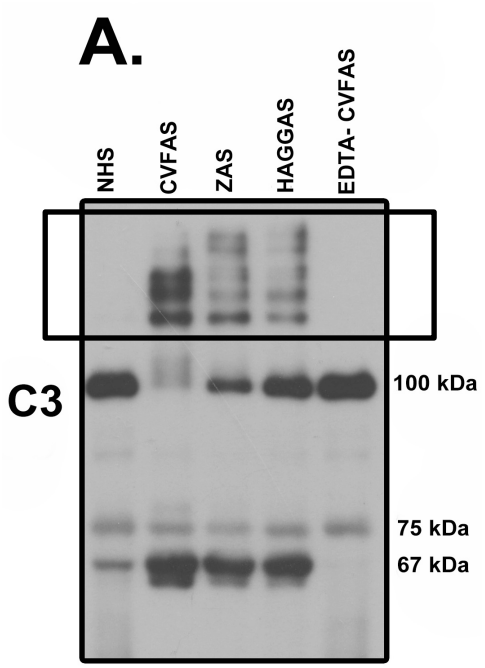

A.
Native C3
C3- NH2OH
M (kDa)
150
100
75
50
B.
C5a/C5a des Arg (ng/ ml)
0
1
2
3
4
5
None
C3
C3-NH2OH
C3b
Addition to C3-depleted serum
C.
C3 dpl serum+
CVFAS
None
Native C3
C3-NH2OH
DBP
α1PI
α1AG
C3
110 kDa
75 kDa
67 kDa
fB
100 kDa
33 kDa

Figure 14: Reaction with the thioester bond in C3 during complement activation causes formation of covalent complexes with plasma proteins.

Panel A: Hydroxylamine-treated C3 was run on an 8% SDS gel and silver stained to look for any gross structural changes during thioester inactivation. Native C3 was run along side as a control. Panel B: The amount of C5a generated in C3-depleted serum samples measured by ELISA. Antibody coated sheep erythrocytes were treated with either C3-depleted serum alone or C3-depleted serum reconstituted with 1.3 mg/ml native C3, hydroxylamine treated C3 (C3-NH_2OH) or C3b and incubated at 37°C for 1 hour. The supernatant was collected and the C5a levels were quantified by ELISA. Panel C: C3-depleted serum alone or C3-depleted serum reconstituted with native C3 or hydroxylamine treated C3 (C3-NH_2OH), both at 1.3 mg/ml, were activated with CVF (416 U/ml) in the presence of 0.4 mM Mg^{2+} for 15 minutes at 37°C. CVF activated normal human serum was included as a positive control. Aliquots were separated using 8% SDS-PAGE and immunoblotted for DBP, α_1PI, α_1AG, factor B and C3. The C3b:plasma protein complexes are highlighted by a box and an arrow.

The results presented thus far indicate that C3 forms covalent complexes with multiple plasma proteins. If our premise is correct then we would expect to see multiple higher molecular weight bands in a C3 blot upon C-activation. To examine this the samples from Figures 7 and 8 were run on an SDS gel and blotted for C3. **Figure 15A** shows that C3 forms multiple covalent complexes upon C-activation either using CVF, zymosan or aggregated IgG (HAGG), and the formation of these complexes is prevented in the presence of EDTA to inhibit complement activation. **Figure 15B** shows that the formation of these multiple higher molecular weight bands on the C3 blot, correlates temporally with complement activation as shown by factor B cleavage in Figure 8.


A.
NHS
CVFAS
ZAS
HAGGAS
EDTA- CVFAS
C3
100 kDa
75 kDa
67 kDa

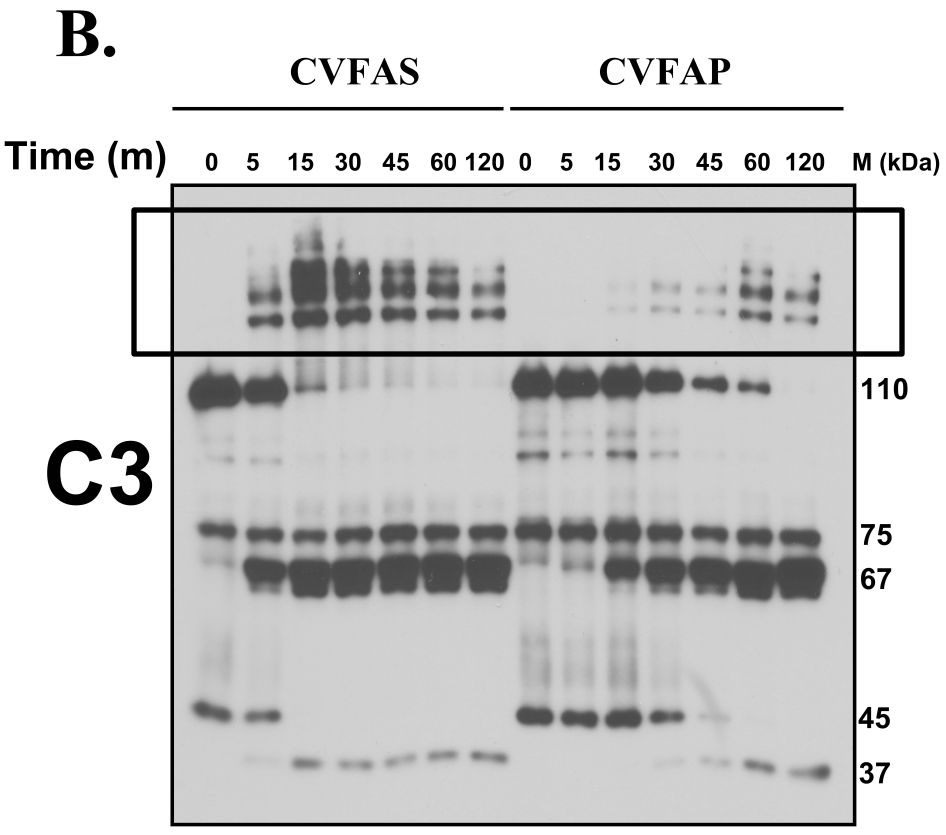

B.
CVFAS
CVFAP
Time (m)
0 5 15 30 45 60 120 0 5 15 30 45 60 120 M (kDa)
C3
110
75
67
45
37

Figure 15: Complement activation induces the formation of covalent complexes of C3 with multiple plasma proteins.

Panel A: Pooled normal human serum was sham-treated with PBS (lane 1) or complement was activated by incubating serum at 37°C using either 416 U/ml CVF (lane 2), 10 mg/ml zymosan A (lane 3), or 0.5 mg heat-aggregated human IgG (lane 4). As a control to inhibit complement activation, 10 mM EDTA was added to serum prior to addition of CVF (lane 5). Serum aliquots were separated on an 8% SDS-PAGE and then immunoblotted for C3. Panel B: Pooled normal human serum or citrated plasma was activated at 37°C with 416 U/ml CVF for the indicated times. In addition to CVF, 2 mM Mg^{2+} was added to plasma overcome the chelation effects of sodium citrate. At each time-point, activation was stopped by placing the sample on ice. Aliquots were separated on an 8% SDS-PAGE and then immunoblotted for C3.

4. CHAPTER 2: Breakdown of C3b:plasma protein complexes

4.1: The C3b:plasma protein complexes are degraded by complement regulators factor H and factor I

The time course of complex formation (**Figure 8A**) shows that the band intensity decreases over time, suggesting breakdown of these complexes. Since the complex is composed of C3b covalently linked to a plasma protein (**Figure 14C**), the role of the primary fluid-phase C3b regulators, factors H and I, was examined. Factor H depleted serum and factor I depleted serum were incubated at 37°C for 15 minutes to allow for complex formation, and then breakdown was studied by incubating at 37°C for 16 hours with or without adding back purified factor H or factor I (**Figure 16A**). There was no apparent breakdown of complexes in factor I depleted serum after 16 hours, whereas addition of purified factor I induced complete degradation of each complex. In contrast, factor H depleted serum showed significant degradation of complexes after 16 hours, and addition of purified factor H only marginally increased this breakdown. Interestingly, the complexes have a higher molecular weight in factor I depleted serum as compared to factor H depleted serum, and this size difference may reflect the factor I-mediated conversion of C3b to iC3b. These results indicate that factor I is essential for the process of complex breakdown, but factor H facilitates the process and perhaps factor H-like and/or factor H-related proteins can substitute in factor H depleted serum.

Factor H and similar proteins in serum (factor H-like, factor H-related) are essential cofactors for factor I to cleave C3b to iC3b. Since this conversion is very rapid, factor H depleted serum was examined for complex formation at very early time points to determine if higher

molecular weight complexes could be detected (**Figure 16B**). DBP, α_1PI and α_1AG complexes spontaneously formed in factor H depleted serum (without a complement activator), and the complexes appeared as doublets within one minute but were all cleaved to the lower molecular weight form by 5 minutes. No doublets were observed in normal serum even one minute after CVF was added (data not shown), indicating that complex cleavage is delayed in the absence of factor H, further confirming that although factor H facilitates complex breakdown, other cofactors can assist factor I in the absence of factor H. To confirm the role of factor I in the initial rapid cleavage reaction, purified factor I was added to factor I depleted serum. As expected, reconstitution of factor I to the depleted serum restored the initial cleavage (**Figure 16C**). Furthermore, the essential role of factor I also was examined using factor H depleted serum and an antibody that specifically blocks the serine protease domain of factor I (**Figure 16D**). Results show that this approach confirms that factor I mediates the initial cleavage of the complexes. These results demonstrate that the complexes are initially generated as higher molecular weight forms, and factor I along with factor H (or other cofactors in factor H depleted serum) mediate the initial cleavage (presumably C3b to iC3b) that is needed for further processing and clearance of these complexes. When normal human serum was treated with an antibody that specifically blocks the serine protease domain of factor I, the cleavage could not be blocked even when twice the amount of antibody used for factor H depleted serum was added **(Figure 16E).** This indicates that in the presence of a cofactor such as factor H, even trace amounts of factor I that is unblocked by the antibody will suffice to cause cleavage.

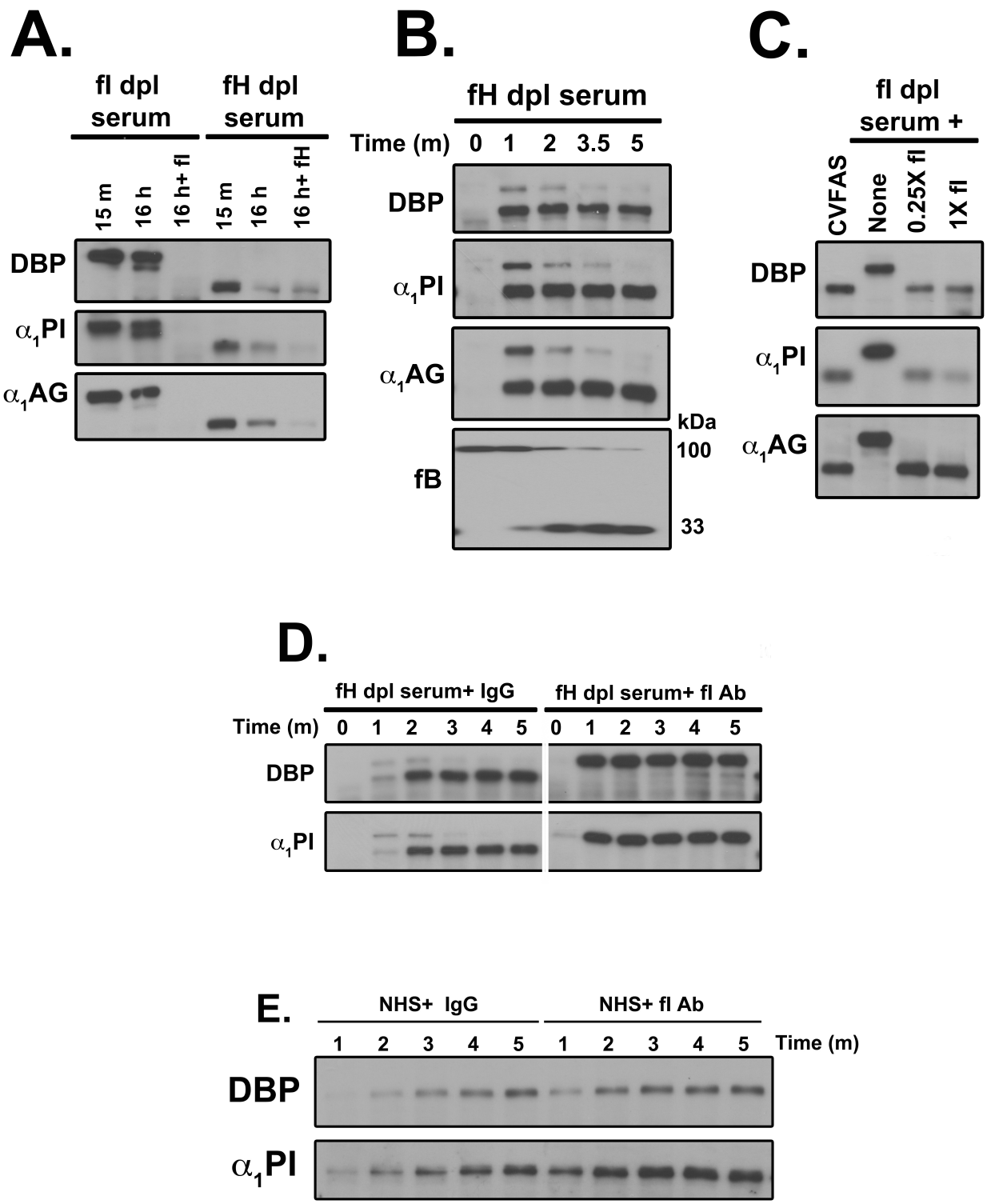

A.
fl dpl serum
fH dpl serum
15 m
16 h
16 h+ fl
15 m
16 h
16 h+ fH
DBP
α1PI
α1AG
B.
fH dpl serum
Time (m) 0 1 2 3.5 5
DBP
α1PI
α1AG
fB
kDa
100
33
C.
fl dpl serum +
CVFAS
None
0.25X fl
1X fl
DBP
α1PI
α1AG
D.
fH dpl serum+ IgG
fH dpl serum+ fl Ab
Time (m) 0 1 2 3 4 5 0 1 2 3 4 5
DBP
α1PI
E.
NHS+ IgG
NHS+ fl Ab
1 2 3 4 5 1 2 3 4 5 Time (m)
DBP
α1PI

Figure 16: C3b:plasma protein complexes are cleaved in serum by factor I with the help of a cofactor.

Panel A: Factor I-depleted serum and factor H-depleted serum were reconstituted with 0.5 mM Mg^{2+} and incubated at 37°C for 15 minutes, to allow for complex formation, followed by addition of 10 mM EDTA to stop the reaction. The samples were then reconstituted with either DPBS or the purified depleted component (factor I or factor H) and incubated for 16 hours at 37°C. Samples were separated using 8% SDS-PAGE and immunoblotted for DBP, α_1PI and α_1AG. Panel B: Factor H-depleted serum was reconstituted with 0.5 mM Mg^{2+} and incubated at 37°C for the indicated times. Serum aliquots were separated using 8% SDS-PAGE and immunoblotted for DBP, α_1PI, α_1AG and factor B. Panel C: Factor I-depleted serum alone or reconstituted with 25% (0.25X) or 100% (1X) of the plasma concentration of factor I (54 μg/ml) was activated with CVF (416 U/ml) for 15 minutes at 37°C. CVF activated normal human serum was included as a positive control for complex formation. Serum aliquots were separated using 8% SDS-PAGE and immunoblotted for DBP, α_1PI and α_1AG. Panel D: Factor H-depleted serum was treated with 100 μg/ml of either an irrelevant mouse IgG or an antibody that neutralizes the serine protease domain of factor I for 15 minutes on ice. Serum samples then were incubated with 0.5 mM Mg^{2+} at 37°C for the indicated times. Immunoblots for DBP and α_1PI complex formation are shown. Panel E: Normal human serum was treated with 200 μg/ml of either an irrelevant mouse IgG or an antibody that neutralizes the serine protease domain of factor I for 15 minutes on ice. Serum samples then were incubated with 0.5 mM Mg^{2+} at 37°C for the indicated times. Immunoblots for DBP and α_1PI complex formation are shown.

4.2. A significant proportion of C3b forms complexes with plasma proteins upon complement activation

Complexes form despite the fact that 91.5% of plasma is comprised of water (55 Molar), while only 7.5% is comprised of protein. Given that water reacts with the thioester bond and is in great molar excess as compared to plasma proteins, it is noteworthy that C3b:protein complexes form and can be detected in serum or plasma after complement activation. The amount of C3b:plasma protein complexes generated in complement activated normal serum, and particularly in factor I depleted serum, is considerable, accounting for a large percentage of the total immunoreactive C3 bands **(Figure 17).**

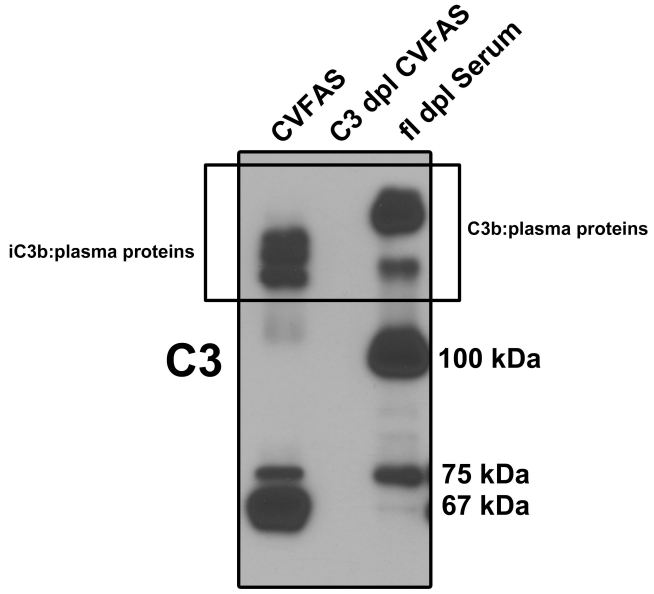


Figure 17: <u>A significant proportion of C3b forms complexes with plasma proteins.</u>

Normal human serum or C3-depleted serum was activated with CVF for 15 minutes at 37°C and factor I depleted serum with 0.5 mM Mg^{2+} added was incubated at 37° C for 15 minutes. Samples were run on an 8% SDS gel and immunoblotted for C3.

4.3. In vitro generation of C3b:DBP complexes using purified proteins

The preceding data demonstrated that formation of C3b:plasma protein complexes requires native C3, a C3 convertase and a target plasma protein. Therefore, to examine whether this system can be assembled in vitro, purified C3, factor B, factor D and a target protein of interest (DBP) were mixed at physiological ratios in the presence of Mg^{2+}. **Figure 18A** shows that a C3b:DBP complex was formed upon incubation at 37°C for either 15 or 30 minutes. This complex was the same size as the uncleaved complex in factor I depleted serum (**Figure 18A**). The absence of any one of these four components prevented complex formation; shown is the reaction at 15 and 30 minutes without factor B (**Figure 18A**). This confirms that just four components are necessary and sufficient for complex formation. Moreover, hydroxylamine-treated C3 was capable of activating the alternative pathway as evidenced by factor B cleavage but, in contrast to native C3, it could not form a covalent complex with DBP (**Figure 18B**), further indicating that an intact thioester in C3 is essential for complex formation.

Since the use of purified proteins provides a well-defined in vitro model, the breakdown of C3b:protein complexes was investigated using purified factors H and I. **Figure 19A** shows C3b:DBP complexes after 15 minutes, 2 hours or 16 hours with no regulators, factor H alone, factor I alone, or both factors H and I. Only the combination of both factors H and I mediated complex dissolution. These results using purified proteins confirm that C3b:DBP (or other plasma proteins) are initially generated as higher molecular weight complexes that are rapidly cleaved by factor I with the help of a cofactor to a lower molecular weight form, and that this complex represents the relatively stable SDS-resistant complex observed in serum and plasma following complement activation **(Figures 7-14).** Only the combination of both factor H and

factor I could initiate cleavage and breakdown of the C3b:DBP complex. Although the iC3b:DBP band completely disappeared after a 16 hour incubation, a intermediate cleavage product, which could potentially be C3dg:DBP, appeared at 15 minutes and was not further degraded even after 16 hours (**Figure 19B**). These results were confirmed using purified α_1AG as the target protein, where a prominent band at an approximate molecular weight corresponding to C3dg:α_1AG also appeared at 15 minutes and was present at 16 hours (**Figure 19C**). Similar results were also observed using purified α_1PI (**Figure 19D**). This putative C3dg:protein band does not appear in factor I depleted CVFAS reconstituted with purified factor I (**Figure 19C**), indicating that in serum there is a factor I independent mechanism that further cleaves the C3dg portion. This factor I-independent process is consistent with the known cleavage of C3dg by trypsin and trypsin-like proteases. Because no other intermediate cleavage products could be detected in serum samples, even when utilizing a broad range gradient gel (data not shown), it is reasonable to surmise that following the initial rapid factor I-mediated cleavage of C3b (to iC3b), a second slower factor I-dependent cleavage removes most of the remaining C3 (C3c fragment) leaving C3dg attached to the plasma protein. This is then followed by a rapid factor I independent cleavage removing the remaining C3d and perhaps leaving a small C3 peptide tag attached to the plasma protein, which would be indistinguishable from the native protein by immunoblotting.

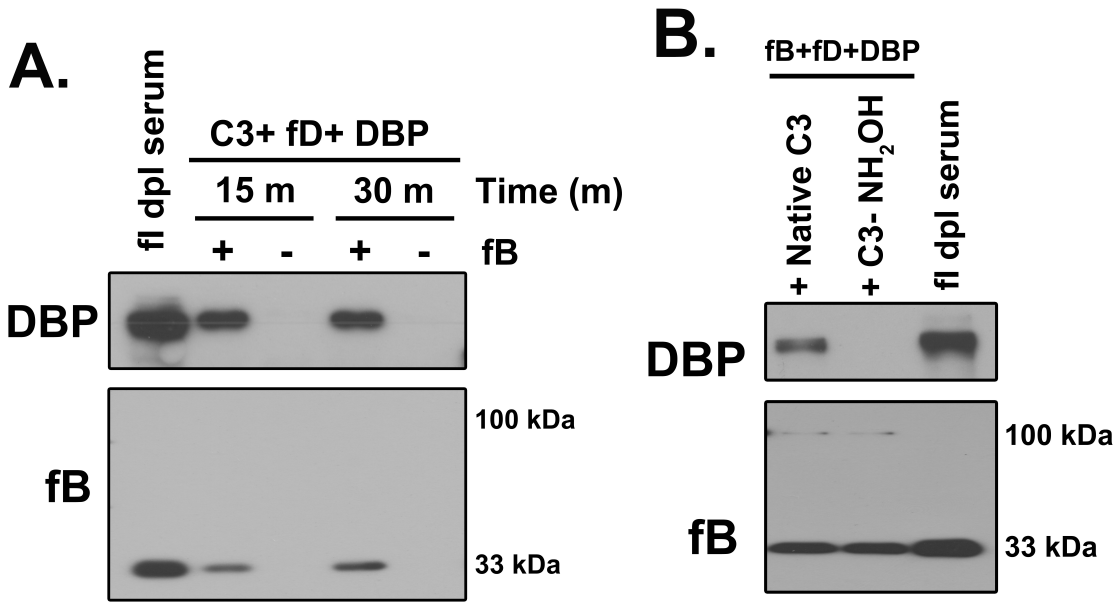
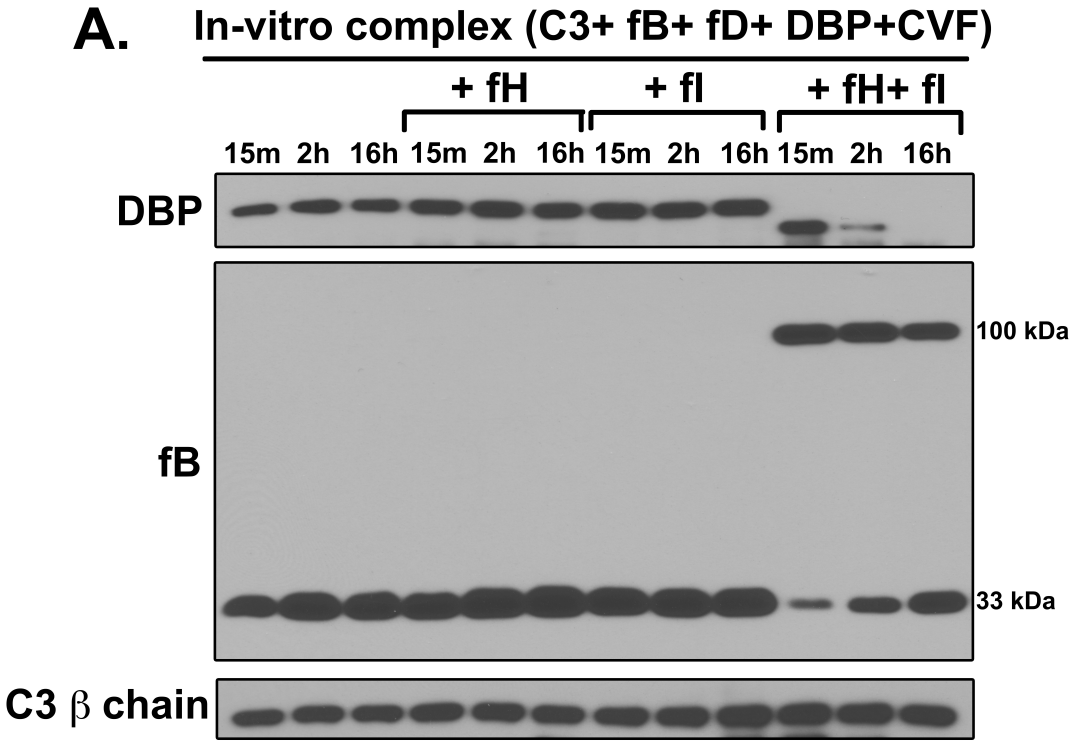
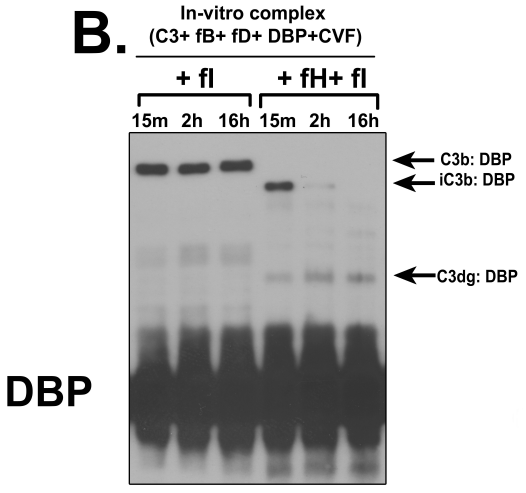
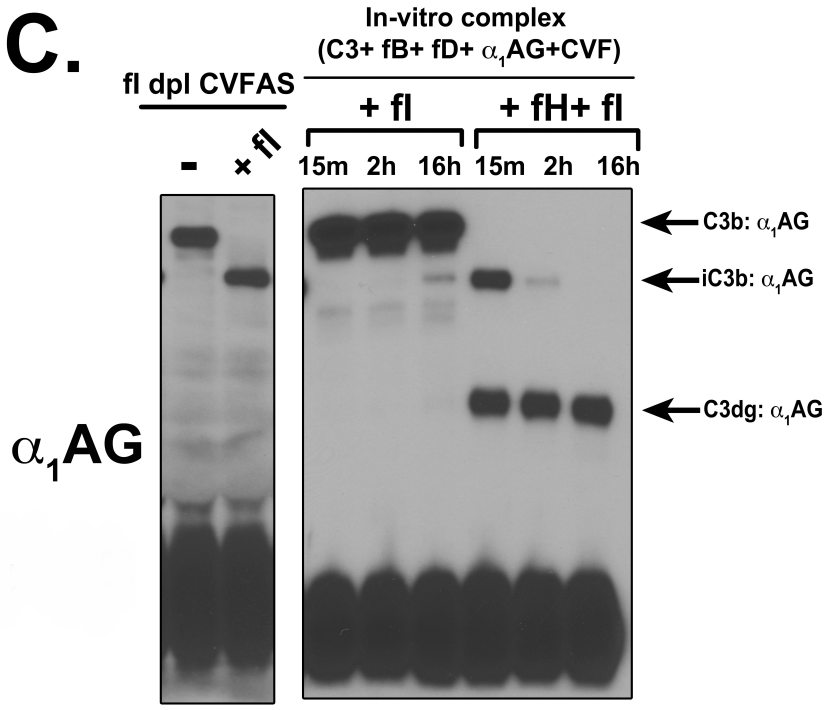


Figure 18: C3b:protein complexes can be formed in vitro using four purified proteins.

Panel A: Purified C3, fD, DBP and 0.5 mM Mg^{2+} were mixed in the presence or absence of fB at physiological concentrations and incubated at 37°C for either 15 or 30 minutes. Factor I-depleted serum incubated at 37°C for 15 minutes in the presence of Mg^{2+} was included as a positive control. Mixtures were immunoblotted for DBP (for complex formation) and factor B (for complement activation). Panel B: Purified fB, fD, DBP and 0.5 mM Mg^{2+} were mixed with either native C3 or hydroxylamine treated C3 (C3-NH_2OH), at physiological ratios and incubated at 37°C for 15 minutes. Factor I depleted serum incubated at 37°C for 15 minutes in the presence of Mg^{2+} was used as a positive control. Samples were immunoblotted for DBP to assess complex formation and factor B to verify complement activation.


A.
In-vitro complex (C3+ fB+ fD+ DBP+CVF)
+ fH
+ fI
+ fH+ fI
15m 2h 16h 15m 2h 16h 15m 2h 16h 15m 2h 16h
DBP
fB
100 kDa
33 kDa
C3 β chain


B.
In-vitro complex
(C3+ fB+ fD+ DBP+CVF)
+ fI
+ fH+ fI
15m 2h 16h 15m 2h 16h
C3b: DBP
iC3b: DBP
C3dg: DBP
DBP


C.
In-vitro complex
(C3+ fB+ fD+ α1AG+CVF)
fI dpI CVFAS
+ fI
+ fH+ fI
-
+ fI
15m 2h 16h 15m 2h 16h
C3b: α1AG
iC3b: α1AG
C3dg: α1AG
α1AG

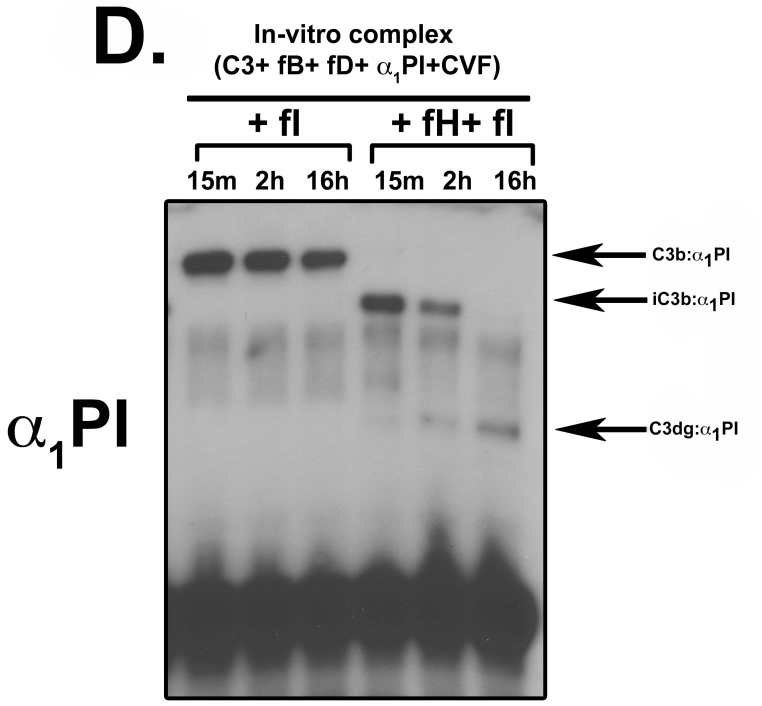

D.
In-vitro complex
(C3+ fB+ fD+ α1PI+CVF)
+ fI
+ fH+ fI
15m 2h 16h 15m 2h 16h
C3b:α1PI
iC3b:α1PI
C3dg:α1PI
α1PI

Figure 19: The breakdown of C3b:plasma protein complexes requires cleavage by factor I in the presence of factor H.

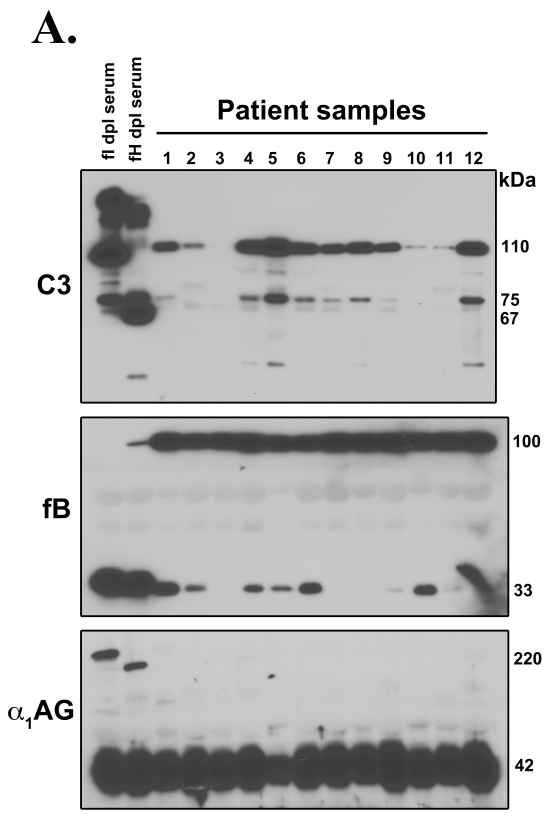
Panel A: Purified C3, fB, fD and DBP were mixed with either factor H alone, factor I alone or a combination of factor H and factor I at physiological ratios and then activated with CVF in the presence of 0.5 mM Mg^{2+} for the indicated times. Samples were immunoblotted for DBP to assess complex formation and factor B to verify complement activation; C3 β-chain was used as a loading control. Panel B: The complete DBP immunoblot of the last six lanes of panel A to show intermediate cleavage products. Panel C: Purified C3, fB, fD and α_1AG were mixed with either factor I alone or a combination of factor H and factor I at physiological ratios and then activated with CVF in the presence of 0.5 mM Mg^{2+} for the indicated times. Samples were immunoblotted for α_1AG to assess complex formation and breakdown. Factor I depleted serum alone or factor I depleted serum reconstituted with factor I was activated with CVF for 15 minutes, and the samples were run on a gel and immunoblotted for α_1AG to trace breakdown in serum in comparison to the in vitro purified system. Panel D: Purified C3, fB, fD and α_1PI were mixed with either factor I alone or a combination of factor H and factor I at physiological ratios and then activated with CVF in the presence of 0.5 mM Mg^{2+} for the indicated times. Samples were run on an 8% SDS gel and immunoblotted for α_1PI to assess complex formation and breakdown.

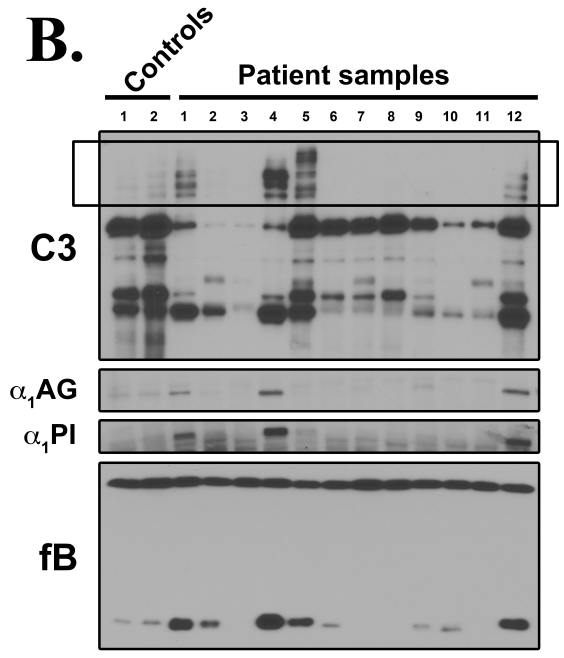
5. CHAPTER 3: C3b:plasma protein complexes in C3 glomerulopathies

The data presented above show that C3b:protein complexes are generated by complement activation and then degraded by the actions of factors H and I. Defective regulation may allow C3b:plasma protein complexes to accumulate in the circulation. Since alternative pathway dysregulation is associated with the etiology of C3 glomerulopathies, we wanted to test the presence of these complexes in circulation in these patients. CFHR5 nephropathy is caused due to local dysregulation of the alternative pathway in the kidney; this is in contrast to the fluid phase dysregulation observed in C3GN and DDD patients. Aliquots of EDTA plasma from six C3GN and six DDD patients were analyzed by SDS-PAGE and immunoblotting for C3b:protein complexes, but no sample showed high molecular weight SDS-resistant complexes when blotted for C3 and α_1AG **(Figure 20A)**. To determine whether complexes could form in these patient plasmas, samples were incubated at 37°C for 60 minutes in the presence of 7.5 mM Mg^{2+} to overcome the EDTA chelation, and in three samples (DDD patient samples 1, 4 and 12) complexes were then observed. No complex formation was observed in normal controls and C3GN samples, although multiple high molecular weight bands that did not correspond to complexes on the α_1PI and α_1AG blots were present on the C3 blot in C3GN sample 5 **(Figure 20B).**

Since many of the patient samples were significantly depleted of C3, purified C3 (1.3 mg/ml) and 7.5 mM Mg^{2+} were added to all patient samples followed by 37°C incubation for 30 minutes or 60 minutes. Five of six DDD samples (1, 4, 6, 11 and 12) formed complexes (**Figure 20C**). Sample 8, the only DDD sample that showed no complex formation, was also the only DDD sample negative for C3Nefs **(Table 1)**. The C3GN sample 7 clearly formed a complex with

α_1AG whereas samples 2 and 9 formed weak complexes and samples 3, 5 and 10 were essentially negative. Interestingly, the bands formed in the patient plasma samples corresponded to the approximate size of bands in factor H-depleted serum (**Figure 20C**). The presence of C3b:protein complexes in the blood of patients with DDD and to a lesser extent in C3GN patients, but not in plasma samples from healthy controls, indicates that there is dysregulation of the C3 convertase.


A.
fI dpl serum
fH dpl serum
Patient samples
1 2 3 4 5 6 7 8 9 10 11 12
kDa
C3
110
75
67
fB
100
33
α1AG
220
42


B.
Controls
Patient samples
1 2 1 2 3 4 5 6 7 8 9 10 11 12
C3
α1AG
α1PI
fB

C.

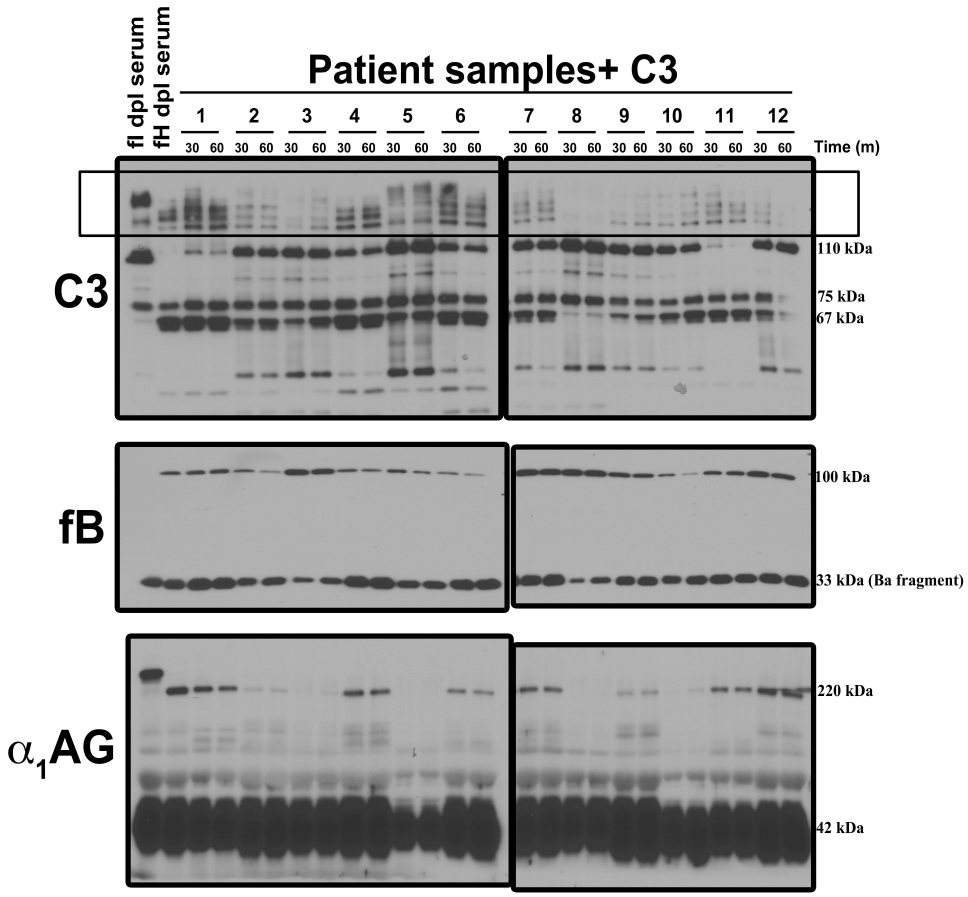

fI dpl serum
fH dpl serum
Patient samples+ C3
1
2
3
4
5
6
7
8
9
10
11
12
30 60 30 60 30 60 30 60 30 60 30 60 30 60 30 60 30 60 30 60 30 60 30 60
Time (m)
C3
110 kDa
75 kDa
67 kDa
fB
100 kDa
33 kDa (Ba fragment)
α1AG
220 kDa
42 kDa

Figure 20: C3b:plasma protein complexes form spontaneously in DDD patient samples.

Panel A: EDTA-plasma samples from DDD patients (n=6) and C3GN patients (n=6) were run on an 8% SDS gel and immunoblotted for C3, factor B and α_1AG. Factor H depleted serum and factor I depleted serum were used as positive controls. Panel B: EDTA-plasma from DDD patients (n=6) and C3GN patients (n=6) or healthy controls (n=2) were reconstituted with 7.5 mM Mg^{2+} and incubated at 37°C for 60 minutes. The samples were then separated using 8% SDS-PAGE and immunoblotted for C3, factor B, α_1PI and α_1AG. Panel C: 1.3 mg/ml of C3 and 7.5 mM Mg^{2+} were added to the EDTA-plasma from DDD patients (n=6) and C3GN patients (n=6) and incubated at 37°C for either 30 minutes or 60 minutes. As a reference for complex size, factor H and factor I depleted serum samples were included. The samples were separated using 8% SDS-PAGE and immunoblotted for C3, factor B and α_1AG. Samples 1, 4, 6, 8, 11 and 12 were from patients diagnosed with DDD, while samples 2, 3, 5, 7 and 9 were from C3GN patients. The iC3b:plasma protein complexes are highlighted by a box drawn around them.

Patient	Diagnosis	Birth year	Gender	C3Nef	Factor H mutations
1	DDD	1996	Female	Positive	Normal alleles
2	C3GN	1991	Male	Positive	CFH c.1699A>G, p.Arg567Gly; normal allele
3	C3GN	1996	Male	Positive	CFH c.2509G>A, p.Val837Ile; c.3435G>C, p.Glu1145Asp
4	DDD	1978	Male	Positive	Normal alleles
5	C3GN	2006	Male	Negative	CFH c. 497G>A, p.Arg166Gln; normal allele
6	DDD	2000	Male	Positive	Normal alleles
7	C3GN	1980	Female	Positive	Not done (no DNA)
8	DDD	1999	Male	Negative	CFH c.2651C>A,p.Ser884Tyr; normal allele
9	C3GN	1989	Male	Positive	Normal alleles
10	C3GN	1988	Male	Negative	CFH c.2867 C>T p. Thr956Met; normal allele
11	DDD	1996	Male	Positive	Normal alleles
12	DDD	1982	Male	Positive	Normal alleles

Table 1: Patient sample diagnosis with C3Nef existence and factor H mutations.

6. CHAPTER 4: Functional significance of C3b:plasma protein complexes

C3b binds more readily to proteins in their non-native state and could possibly play a role in their clearance.

6.1. C3b binds more readily to non-native DBP

The binding of C3b to plasma proteins during complement activation does not appear to be a completely random event. If that were the case, then albumin, the most abundant protein in plasma (~40 mg/ml concentration), would form the most intense complexes with C3b. But the intensity of C3b:albumin complexes was rather weak compared to other less abundant proteins **(Figure 8A).** Hence, there seems to be some specificity in this process. Furthermore, only a small proportion of the total plasma protein forms complexes with C3b. Since multiple plasma proteins are capable of forming these complexes, it seems unlikely that the common feature among the small population is a post-translational modification. It is known that the thioester binds hydroxyl or amine groups, and the availability of those groups could determine the binding efficiency of that particular plasma protein to C3. C3's role in the clearance of foreign or abnormal host molecules led us to hypothesize that C3 might bind to proteins in their non-native state. To test this, purified DBP was denatured using 6 M guanidine hydrochloride (GuHCl) and dithiotreitol (DTT), and this denatured DBP was used in the in vitro purified system to form C3b complexes. Loss of native structure of DBP was tested using the TNS assay, where DBP's ability to bind TNS dye was measured by fluorescence emission **(Figure 21A)**. Denatured DBP showed a greatly reduced ability to bind TNS, and hence reduced fluorescence. In the in vitro purified system, denatured DBP formed more complexes with C3b as compared to native DBP. The

intensity of the complex decreased when 80% less C3 was used and no complexes were observed when C3 was excluded, indicating that the complexes noted were indeed covalent complexes of C3b:DBP and not DBP aggregates formed due to denaturation **(Figure 21B).** In the C3 blot, two higher molecular weight bands were observed: the C3b:fB band that remains unchanged in intensity, and the C3b:DBP band that increased when denatured DBP was used.

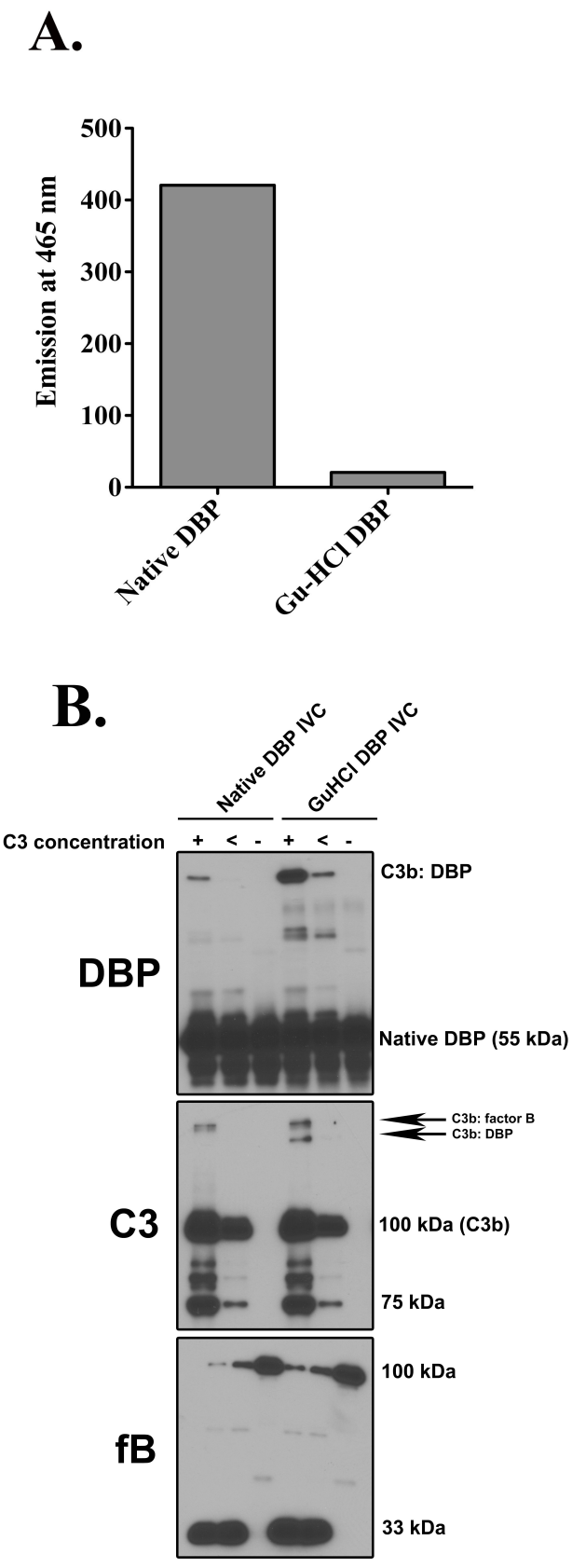

A.
Emission at 465 nm
500
400
300
200
100
0
Native DBP
Gu-HCl DBP
B.
Native DBP IVC
GuHCl DBP IVC
C3 concentration
+ < - + < -
DBP
C3b: DBP
Native DBP (55 kDa)
C3
C3b: factor B
C3b: DBP
100 kDa (C3b)
75 kDa
fB
100 kDa
33 kDa

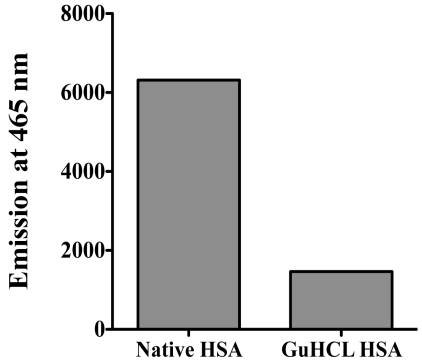
Figure 21: C3b binds more readily to non-native DBP as compared to native DBP.

Panel A: Native DBP and DBP denatured with guanidine hydrochloride (GuHCl) for 6 hours were tested for their ability to bind TNS. The fluorescence emitted by TNS upon binding to DBP was read at an excitation wavelength of 322 nm and emission wavelength of 465 nm. Panel B: Native DBP or GuHCl DBP were allowed to form complexes with either 1X (physiological concentration) of C3 (+) or 0.2X C3 (<), in the presence of fB, fD and 0.5 mM Mg^{2+} mixed at physiological ratios. The samples were incubated at 37°C for 15 minutes. Aliquots were run on an 8% SDS gel and immunoblotted for DBP and C3 (for complex formation) and factor B (for complement activation).

6.2. C3b binds more readily to non-native HSA

To further validate C3b's avidity for non-native protein structures, the system was tested using another plasma protein, human serum albumin (HSA). To test this, HSA was denatured using 6 M guanidine hydrochloride (GuHCl) and dithiotreitol (DTT) for 24 hours, and this denatured HSA was used in the in vitro purified system to form complexes. Loss of native structure of HSA was tested using the TNS assay, where HSA's ability to bind TNS was measured by fluorescence emission **(Figure 22A).** Denatured HSA showed reduced ability to bind TNS. Denatured HSA formed more intense complexes with C3 as compared to native HSA. The intensity of the complex decreased when a reduced level of C3 was used, and no complexes formed when C3 was omitted from the mixture, indicating that the complexes we observed were indeed C3b:HSA covalent complexes and not HSA aggregates formed due to denaturation **(Figure 22B).**

A.


8000
6000
4000
2000
0
Emission at 465 nm
Native HSA
GuHCL HSA

B.

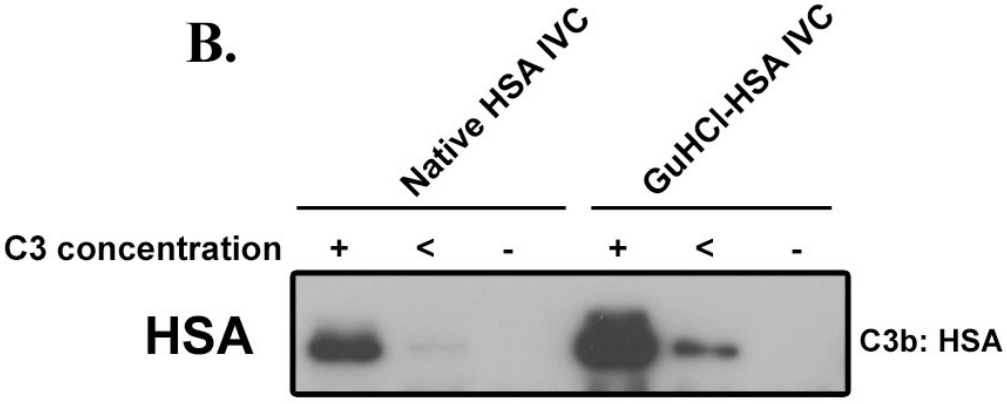
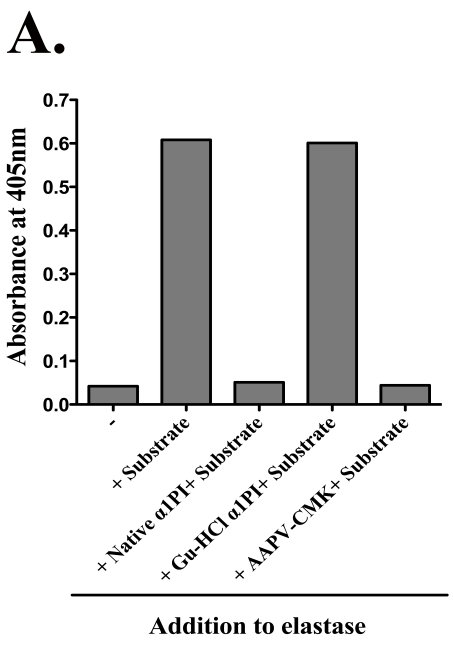

Native HSA IVC
GuHCl-HSA IVC
C3 concentration
+
<
-
+
<
-
HSA
C3b: HSA

Figure 22: C3b binds more readily to non-native HSA as compared to native HSA.

Panel A: Native HSA and HSA denatured with guanidine hydrochloride (GuHCl) for 6 hours were tested for their ability to bind TNS. The fluorescence emitted by TNS upon binding was read at an excitation wavelength of 322 nm and emission wavelength of 465 nm. Panel B: Native HSA or GuHCl HSA was allowed to form complexes with either 1X (physiological concentration) C3 (+) or 0.2X C3 (<), in the presence of fB, fD and 0.5 mM Mg^{2+} mixed at physiological ratios. The samples were incubated at 37°C for 15 minutes. Mixtures were run on an 8% SDS gel and immunoblotted for HSA.

6.3. C3b binds more readily to non-native α_1PI

This system was further tested using a third representative plasma protein, α_1PI. α_1PI was denatured and then tested in the purified in vitro system to form C3b complexes. To test if the denaturation procedure altered the structure of α_1PI, the ability of the protease inhibitor to bind elastase was determined. α_1PI covalently binds to its substrate elastase and inhibits its activity. A chromogenic substrate assay was used, where AAPV-pNA, a peptide substrate for elastase, turns blue upon elastase activity and emits absorbance at 405 nm. It was seen that the GuHCl-α_1PI lost elastase inhibiting ability, thus confirming the loss of the native state of this protein **(Figure 23A).** In the in vitro purified system, denatured α_1PI formed more intense complexes with C3b as compared to native α_1PI. The intensity of the denatured complex with C3b was decreased dramatically when 80% less C3 was added, and no complexes were seen when no C3 was used, indicating the complexes observed were indeed C3b:α_1PI covalent complexes. In the C3 blot, two higher molecular weight bands are noted. The C3b:fB band remained unchanged in intensity, while the C3b:α_1PI band showed an increase when denatured α_1PI was used **(Figure 23B).**


A.
Absorbance at 405nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-
+ Substrate
+ Native α1PI+ Substrate
+ Gu-HCl α1PI+ Substrate
+ AAPV-CMK+ Substrate
Addition to elastase

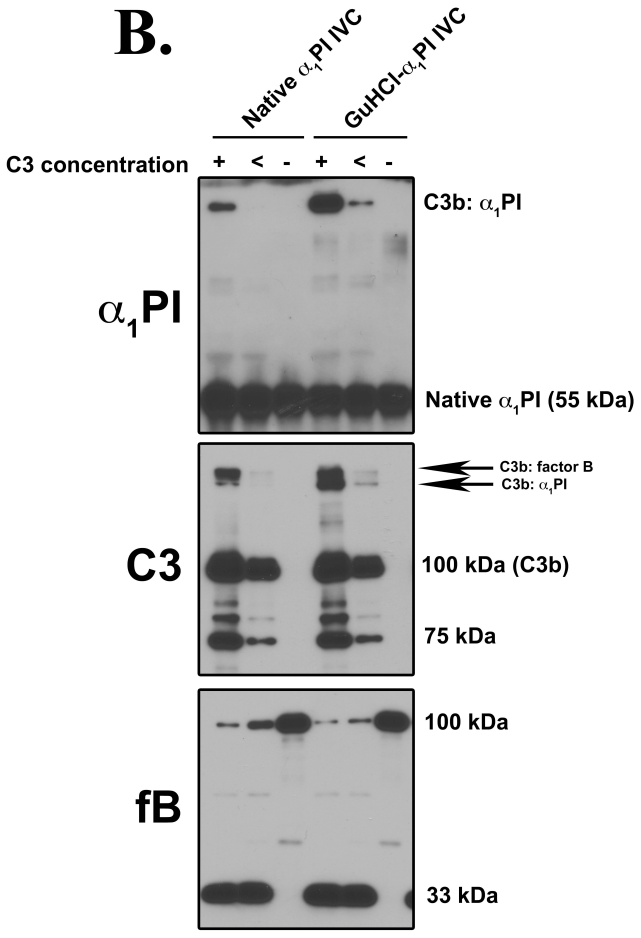

B.
Native α1PI IVC
GuHCl-α1PI IVC
C3 concentration
+ < - + < -
α1PI
C3b: α1PI
Native α1PI (55 kDa)
C3
C3b: factor B
C3b: α1PI
100 kDa (C3b)
75 kDa
fB
100 kDa
33 kDa

Figure 23: C3b binds more readily to non-native α_1PI as compared to native α_1PI.

Panel A: Native α_1PI and α_1PI denatured with guanidine hydrochloride (GuHCl) for 24 hours were tested for their ability to bind and inhibit elastase. Elastase activity was measured by a chromogenic reaction using a substrate, AAPV-pNA and by measuring absorbance at 405 nm. AAPV-CMK was used as a positive control for elastase inhibition. Panel B: Native α_1PI or GuHCl α_1PI was allowed to form complexes with either 1X (physiological concentration) C3 (+) or 0.2X (<) C3, in the presence of fB, fD and 0.5 mM Mg^{2+} mixed at physiological ratios. The samples were incubated at 37°C for 15 minutes. Mixtures were run on an 8% SDS gel and immunoblotted for α_1PI, C3 and factor B.

6.4. C3b binds plasma proteins in their native state, though it binds more readily to plasma proteins in their non-native form

In the in vitro system using purified proteins, a small fraction of the total target protein actually forms complexes with C3b, even when using the native plasma protein. This could either be due to the presence of a small proportion of proteins in their non-native state or due to C3b's ability to bind native proteins at a basal level. To test if the DBP in the in-vitro C3b:DBP complexes formed using non-denatured DBP is in its native state, we tested its ability to bind actin. The C3b:DBP in vitro complexes were allowed to bind actin, and the samples were run on an 8% native (non-denaturing) gel to evaluate actin binding **(Figure 24).** The results indicate that the DBP in the C3b:DBP complex still retains the ability to bind actin, as shown by the shift in molecular weight in samples where actin was added. This indicates that C3b probably binds proteins in their native state, but binds more readily to misfolded/unfolded proteins due to increase in accessibility of binding sites. The GuHCl denatured DBP did not run into the gel as expected, since non-native proteins form aggregates upon denaturation to protect their hydrophobic domains from the surrounding solvent. This further explains the inability to bind up all of the non-native protein in the complex. Though the functional binding assay indicates that GuHCl treatment completely denatured DBP, a large proportion of the protein runs at the native molecular weight on a denaturing gel even after being allowed to form complexes with C3b. This is probably due to aggregation allowing access to only a small proportion of the denatured protein, while much of it is masked within the aggregates.

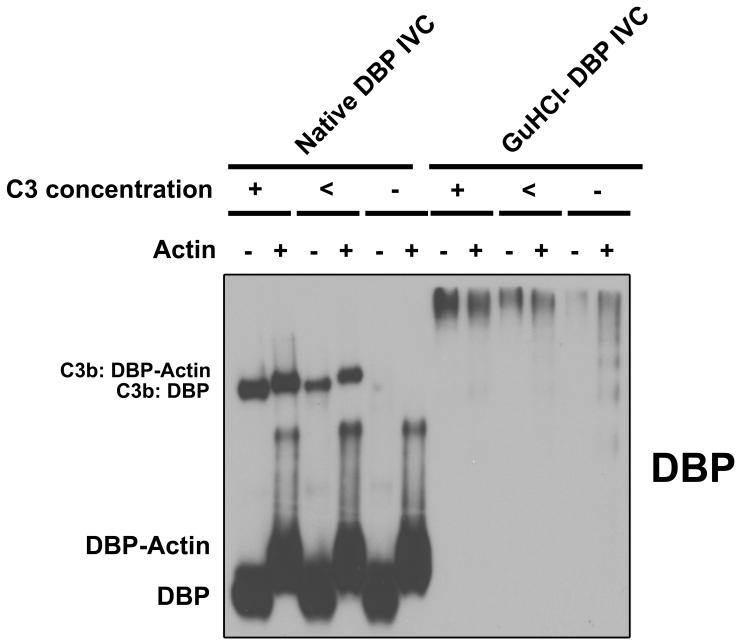


Figure 24: <u>DBP in C3b:native DBP complex retains actin binding ability.</u>

Native DBP or GuHCl DBP were allowed to form complexes with either 1X (physiological concentration) of C3 (+) or 0.2X (<) C3, in the presence of fB, fD and 0.5 mM Mg^{2+} mixed at physiological ratios. The samples were incubated at 37°C for 15 minutes. The in vitro complexes once formed were treated with actin in a 1:5 M ratio of DBP:actin and allowed to bind at room temperature. Mixtures were then run on an 8% native gel and immunoblotted for DBP.

6.5. Complement could play a role in the clearance of non-native proteins in vivo

Since C3b binds to plasma proteins, and more specifically to proteins in their non-native state, we wanted to test if these complexes, once formed, can bind erythrocytes to be cleared from circulation via the reticulo-endothelial system. To test this, erythrocytes purified from the blood of healthy individuals were treated with either normal human serum or CVF activated serum, to test if iC3b:plasma protein complexes bind to erythrocyte membrane receptors **(Figure 25).** It is well known that the erythrocyte CR1 receptor binds both C3b and iC3b and also plays a role in the clearance of C3b:Aβ42 complexes. It was observed that the erythrocyte membrane fraction contained iC3b:plasma protein complexes upon treatment with CVF activated serum, indicating binding to a membrane receptor and a potential mechanism for clearance.

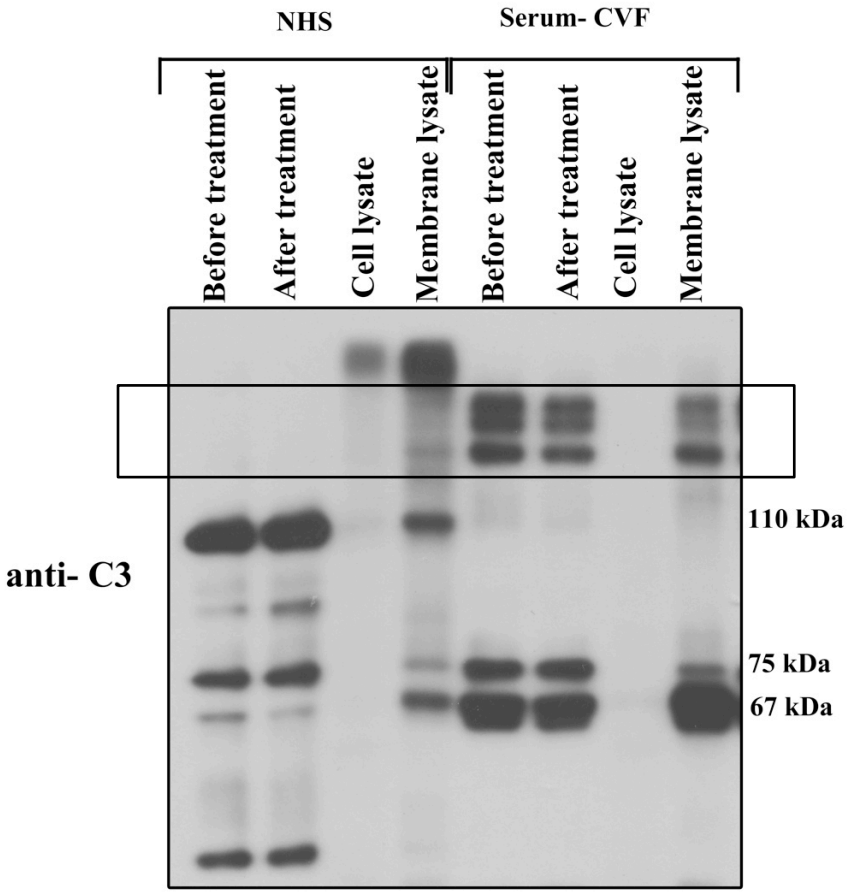


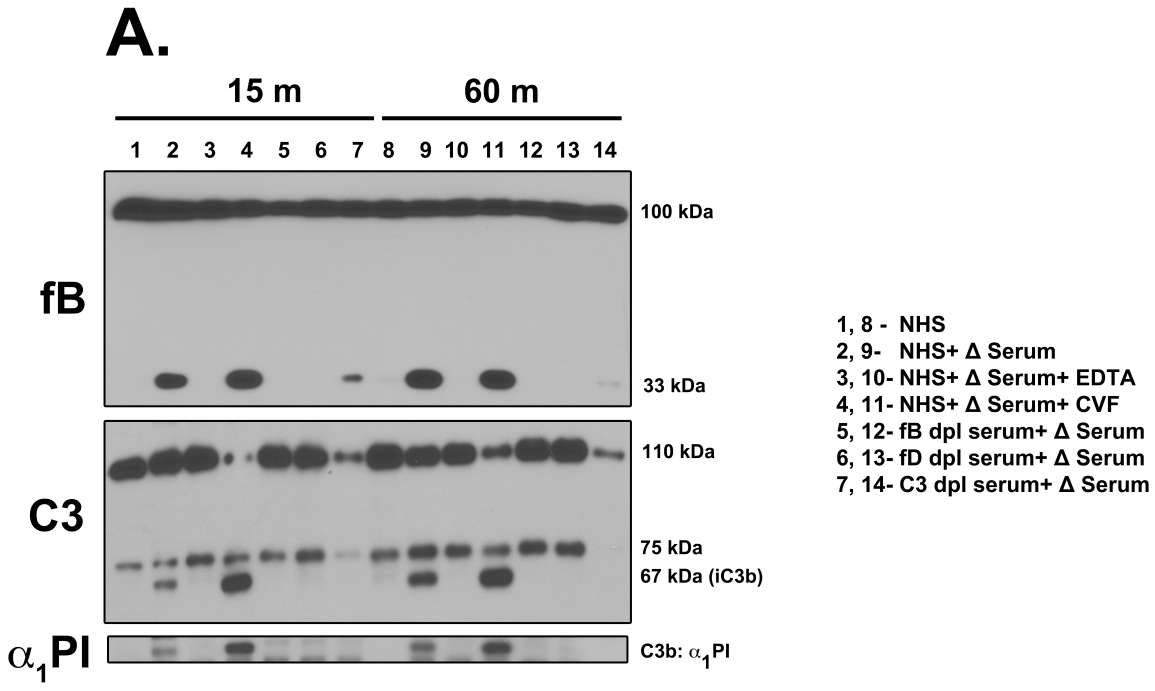
Figure 25: <u>iC3b:plasma protein complexes bind erythrocyte cell membrane.</u>

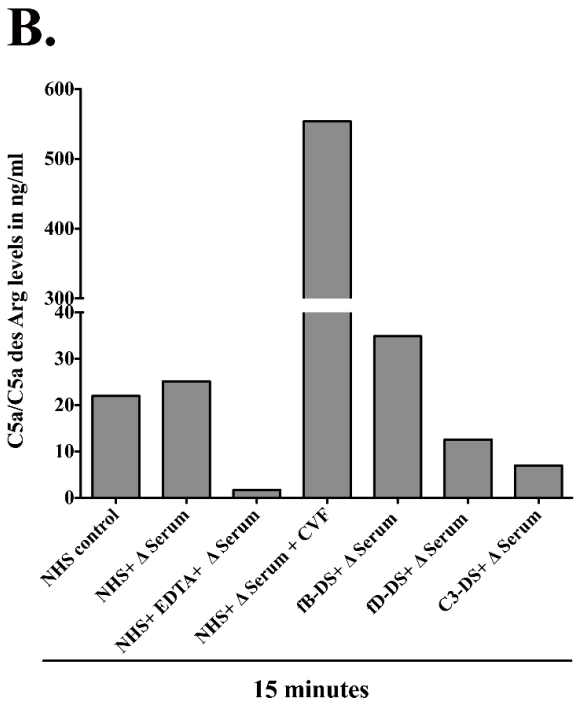
Erythrocytes purified from blood drawn from healthy individuals were treated either with normal human serum or CVF activated serum for 30 minutes at 37° C. The serum samples before and after treatment were run on the gel to look for a decrease in total intensity of complexes due to binding to erythrocytes. After treatment, RBCs were lysed with H_2O and the membrane fraction was purified and solubilized. The samples were run on an 8% SDS gel and immunoblotted for C3. The iC3b:plasma protein complexes are highlighted by a box drawn around them.

6.6. Thermally denatured proteins activate the complement cascade

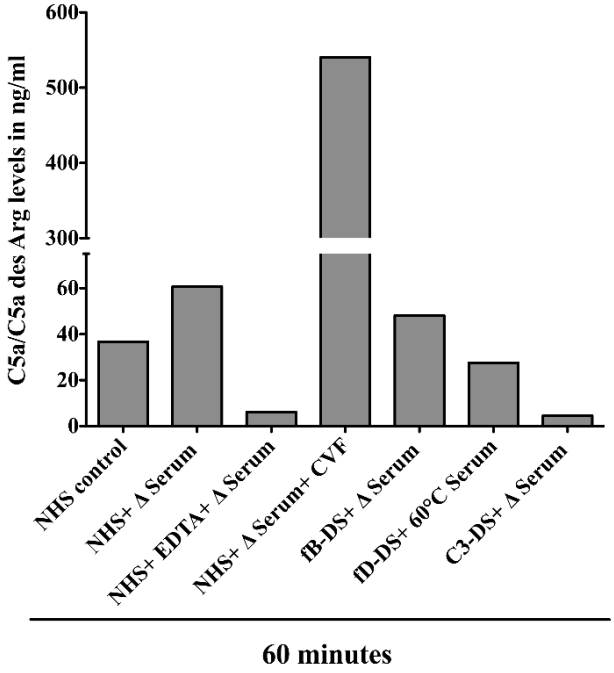
C3b's increased ability to attach to non-native proteins, as well as the activation of the complement by amyloid Aβ42, led us to hypothesize that thermally denatured proteins could also activate the complement system. To test this hypothesis, serum heated to 60°C for 1 hour was added to NHS, fB depleted serum, fD depleted serum or C3 depleted serum followed by incubation at 37°C for 15 minutes or 60 minutes. Complement activation was then assessed by factor B cleavage and iC3b generation. Controls included EDTA-treated and CVF-treated 60°C-heated serum. It was observed that serum heated at 60°C was able to activate the complement system in fresh serum at 15 minutes and 60 minutes, as shown by factor B cleavage and iC3b generation **(Figure 26A).** Treatment of normal human serum with 60°C denatured serum also caused the generation of C3b:plasma protein complexes as seen from the α_1PI blot **(Figure 26A)**. Interestingly, even though significant levels of factor B cleavage and iC3b generation were seen in these samples, there was no C5a generation **(Figure 26B and 26C).** Addition of CVF to the sample allowed C5a generation, indicating that the addition of thermally denatured proteins did not alter the ability of the sample to generate C5a. Since CVF can act independently of C3 and generate C5a, the NHS + heated serum was treated with zymosan and aggregated IgG (HAGG) to test if C5a can be generated. Zymosan, HAGG and CVF treatments could all induce C5a generation **(Figure 26D).** The levels of C5a generated were comparable in the NHS alone and NHS + heated serum upon both CVF and zymosan activation. Upon HAGG activation, 50% less C5a was seen in the NHS + heated serum sample, but that is due to half the level of active C2 present in the sample (C2 is heat sensitive and will be inactivated in the heated serum sample). This data indicates that upon addition of thermally denatured proteins, complement

activation occurs up until the stage of C3 cleavage, without the generation of C5a. This is consistent with the observation that complement is involved in the non-inflammatory clearance of apoptotic cells by preventing downstream effects of complement activation. No iC3b generation was seen in factor B or factor D depleted sera, indicating that complement activation by thermally denatured proteins requires the presence of active alternative pathway components.


A.
15 m
60 m
1 2 3 4 5 6 7 8 9 10 11 12 13 14
fB
100 kDa
33 kDa
C3
110 kDa
75 kDa
67 kDa (iC3b)
α1PI
C3b: α1PI
1, 8 - NHS
2, 9- NHS+ Δ Serum
3, 10- NHS+ Δ Serum+ EDTA
4, 11- NHS+ Δ Serum+ CVF
5, 12- fB dpl serum+ Δ Serum
6, 13- fD dpl serum+ Δ Serum
7, 14- C3 dpl serum+ Δ Serum


B.
C5a/C5a des Arg levels in ng/ml
600
500
400
300
40
30
20
10
0
NHS control
NHS+ Δ Serum
NHS+ EDTA+ Δ Serum
NHS+ Δ Serum + CVF
fB-DS+ Δ Serum
fD-DS+ Δ Serum
C3-DS+ Δ Serum
15 minutes

C.


C5a/C5a des Arg levels in ng/ml
600
500
400
300
60
40
20
0
NHS control
NHS+ Δ Serum
NHS+ EDTA+ Δ Serum
NHS+ Δ Serum+ CVF
fB-DS+ Δ Serum
fD-DS+ 60°C Serum
C3-DS+ Δ Serum
60 minutes

D.

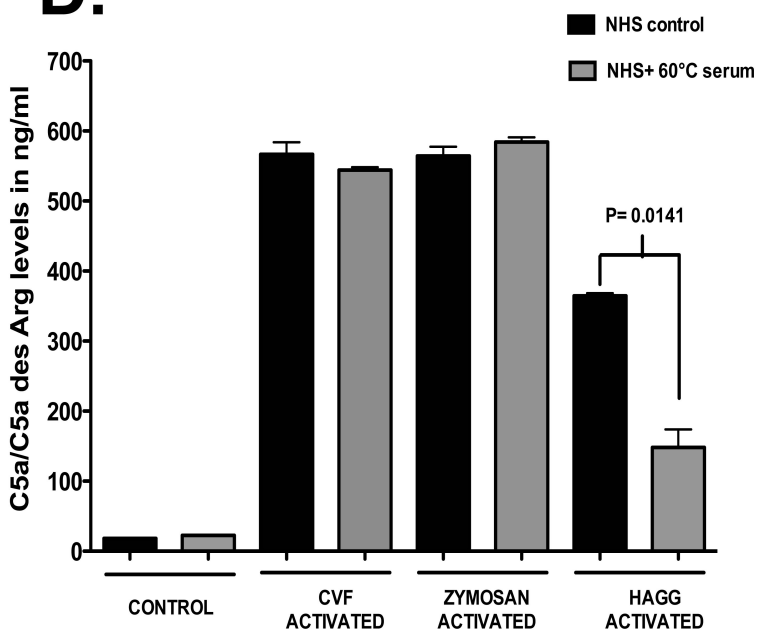

NHS control
NHS+ 60°C serum
C5a/C5a des Arg levels in ng/ml
700
600
500
400
300
200
100
0
P= 0.0141
CONTROL
CVF ACTIVATED
ZYMOSAN ACTIVATED
HAGG ACTIVATED

Figure 26: Thermally denatured proteins activate the complement system via the alternative pathway.

Panel A: Normal human serum or serum depleted of either factor B, factor D or C3 was treated with heated (Δ) serum (heated to 60° C for 1 hour) for either 15 minutes or 60 minutes at 37°C. EDTA was added to a sample to test complement dependence, and CVF was added to a sample as a positive control. The samples were separated on an 8% SDS gel and immunoblotted for factor B, C3 and α_1PI. Panel B: C5a levels were quantified by ELISA in normal human serum, or serum depleted of either factor B, factor D or C3 that was treated with Δ serum (heated to 60° C for 1 hour) for 15 minutes at 37°C. EDTA was added to sample to test complement dependence, and CVF was added to a sample as a positive control. Panel C: C5a levels were quantified in normal human serum, factor B, factor D or C3 depleted sera that were treated with Δ Serum (heated to 60°C for 1 hour) for 60 minutes at 37° C. EDTA was added to sample to test complement dependence and CVF was added to a sample as positive control. Panel D: C5a levels were quantified in normal human serum treated with Δ serum (heated to 60°C for 1 hour) for 60 minutes at 37°C. This sample was later activated with either CVF, zymosan or HAGG, and C5a levels were quantified by C5a ELISA.

7. DISCUSSION

7.1. C3b binds surrounding plasma proteins to neutralize the thioester and thus prevents unwarranted damage to host cells

As the major humoral component of innate immunity, the complement system is one of the first responders to pathogenic stimuli (1). The conversion of C3 to C3b is the central step during complement activation, and the C3b generated binds to target surfaces and tags them for clearance by phagocytes. Binding of C3b to the target surface is also necessary for continued complement activation on that surface. However, this reaction is rather inefficient, and only 10% of the C3b generated binds to the intended target, while 90% remains in the fluid phase bound to water molecules (10,11). The conversion of C3 to C3b induces a major structural rearrangement and formation of an acyl-imidazole intermediate, which is highly reactive towards hydroxyl nucleophiles. Proper orientation of the TED domain close to the target surface might be important for the highly reactive and short-lived ($t_{1/2}$ < 100 ms) acyl-imidazole intermediate to react covalently with the target surface instead of the surrounding solvent (9). In this study, we started by examining modifications to DBP upon C-activation and observed that activating the complement cascade resulted in the formation of high molecular weight SDS-resistant complexes of DBP **(Figure 7)**. This effect was not limited to DBP, and at least 10 different plasma proteins tested (DBP, α_1PI, α_1AG, HK, LK, albumin, C1-inh, C4, fB and fH) formed SDS-resistant high molecular weight complexes upon C-activation that correlated closely with the timing and extent of C-activation **(Figure 7-9)**. Using sera depleted of individual complement components, it was observed that C3 cleavage and all components necessary to form the convertase (fB, fD and C3) were required to form these complexes **(Figure 13).** Since

both native C3 and a C3 convertase were required for this process, the role of the thioester in C3 was examined. It was determined that upon activation, the exposure of the C3b thioester causes it to bind covalently to the surrounding plasma proteins **(Figure 14).**

It is well known that the neutralization of the C3 thioester by the surrounding solvent (water) helps prevent unwarranted damage caused by the extremely reactive thioester (armed molecular warhead) by limiting it to the site of activation. There have been very few reports in the literature concerning the covalent binding of C3 to plasma proteins, and these focused on complement proteins (C4, properdin) or activators (IgG) **(13,14,107)**. Here, we present evidence that upon activation, C3 can bind to various plasma proteins by its reactive thioester. This study demonstrates that at least ten different plasma proteins tested were capable of binding C3b. The amount of C3b:plasma protein complexes generated in complement activated normal serum was considerable, accounting for a large percentage of the total immunoreactive C3 bands. Examining factor I depleted serum, where formation occurs in the absence of cleavage and breakdown, is a good model to roughly quantify the amount of C3b:plasma protein complexes formed as compared to C3b(H_2O). It was noted that the amount of C3b neutralized by plasma proteins was comparable to the amount of C3b neutralized by H_2O (even given the differences in western blotting transfer capacity, owing to their increased size) **(Figure 17).** These complexes form despite the fact that 91.5% of plasma is water (55 Molar), and only 7.5% is protein. Given that water readily reacts with the thioester bond and is in great molar excess as compared to plasma proteins, it is noteworthy that C3b:protein complexes form and can be detected in serum or plasma after complement activation. Furthermore, the C3b:protein complex attachment must have some level of selectivity, because if the reaction was entirely nonspecific then C3b:albumin

would be the most prevalent, since albumin is the most abundant plasma protein. However, formation of a high molecular weight albumin band was considerably weaker than other much less abundant plasma proteins. Thus, binding of C3b to plasma proteins does not seem to be based just on abundance, but probably on the availability of nucleophile acceptor groups. This disparity in binding efficiency was also noted in another study, where activation of C3 by factors B and D (in the presence of Mg^{2+} ions) along with C4b led to 35% incorporation of nascent C3b into C3b:C4b complexes in the fluid phase. When human IgG was tested as an acceptor under similar conditions, only 12% of generated C3b was incorporated into C3b:IgG complexes **(108)**. Studies have also shown that the covalent C3b attachment site is Ser-1217 of human C4, and that the threonine corresponding to Thr144 in the CH1 domain of IgG is the major acceptor site for C3b (107,109). Thus the specificity seems to vary from protein to protein, though the C3b thioester seems to target hydroxyl group containing amino acids to form an ester linkage. Studies using model peptides have also shown that tyrosine reacts with the thioester of metastable C3b and that this reactivity is 11-fold better than that of threonine, 47-fold better than serine and 50-fold better than the reactivity of carbohydrates (110). The specificity to certain acceptor groups versus others within the protein structure requires further study using purified complexes and mass spectrometric analysis. Given that C3b:plasma protein complexes form in significant quantities under physiological conditions, this collateral by-product of complement activation could serve a passive regulatory role by limiting unintended deposition of C3b on host cells. In addition, since C3 has a major role in clearance of debris, this C3b binding efficiency could also determine the circulating half-life of various plasma proteins.

7.2. Breakdown of C3b:plasma protein complexes

The data presented in this work shows that C3b:plasma protein complexes once formed are broken down by the fluid-phase RCA proteins factors H and I. The C3b:protein complexes we have described are first produced as higher molecular weight forms at a 1:1 molar ratio of C3b to plasma protein. Consistent with what is known about C3b's sequential cleavage following complement activation, the C3b:protein complexes are then cleaved very rapidly by factor I and its cofactors to iC3b and then more slowly to C3dg, which is followed by a very rapid factor I independent cleavage, possibly leaving a very small peptide tag attached to the plasma protein **(15,16)**. Studies using factor I depleted serum show that in the absence of factor I, the complexes stay as higher molecular weight forms and do not break down even after 16 hours, indicating that factor I is absolutely essential to the process of complex degradation **(Figure 16A)**. This process of complex cleavage and breakdown occurs even in a sample depleted of factor I's major serum cofactor (factor H), indicating that there are other cofactors in serum that can substitute for factor H **(Figure 16A).** The series of events occurring during the sequential complex breakdown are as follows:

1. The initial cleavage of C3b:plasma protein to iC3b:plasma protein is extremely rapid, and hence the C3b:plasma protein complex could not be detected in normal serum upon C-activation even at the 1 minute time point. The C3b:plasma protein band could be detected only under two conditions: when factor I is depleted, hence causing no cleavage and stalling of the complex in the C3b:plasma protein form **(Figure 16A and 16C),** or when the primary cofactor (factor H) is depleted, resulting in the detection of uncleaved complex only for the first 5 minutes **(Figure 16B)**. Even though factor H appears to be

expediting the process, other cofactors allow the cleavage to occur but at a slightly delayed pace **(Figure 16B)**. This was also seen when a neutralizing antibody to factor I was used to stall the complexes as C3b:plasma proteins. Neutralization in factor H depleted serum prevented cleavage of these complexes **(Figure 16D)**, while neutralization in normal human serum followed by CVF activation did not **(Factor 16E).** This could be because neutralization with an antibody is not a 100% efficient process and will allow some factor I to escape inhibtion and cause cleavage. In the absence of factor H that amount of factor I was not sufficient to cause cleavage, while in the normal human serum, the presence of factor H allows extremely efficient cleavage by trace amounts of factor I.

2. The second cleavage of iC3b:protein to C3dg:protein complex is a slower reaction, and complexes that are detected in serum/plasma largely are iC3b:plasma protein complexes. This process requires the presence of factor I and a cofactor as evidenced in the purified in vitro system **(Figure 19B, C and D)**. But even in the presence of factor I and a cofactor (factor H), the processing and breakdown seems to stop at the stage of C3dg when using only purified proteins, indicating that further processing requires a factor I independent mechanism that is present in serum.

3. Further cleavage of C3dg:plasma protein complexes occurs only in serum or plasma and requires other proteases. This occurs very rapidly since C3dg:plasma protein intermediates are not detected in serum. This factor I independent process could possibly be mediated by elastase, plasmin or trypsin (15-20). This further processing could leave a

small C3 peptide tag attached to the plasma protein, making it indistinguishable from the native protein on SDS-PAGE.

To further understand the process of breakdown, purification of the complexes followed by mass spectrometric analysis of the cleavage products needs to be performed. During the course of this research an attempt was made to purify these complexes. Since we have seen that non-native denatured proteins form more complexes with C3b in vitro, this system was considered the ideal one to isolate the complex of C3b:plasma protein **(Figure 21B).** GuHCl denatured DBP was used in the in vitro system to form complexes, and purification was attempted from this mixture using fast protein liquid chromatography (FPLC). When the eluates were tested for complexes on SDS-PAGE, the complex eluted in all the fractions and there was a native protein band that showed up in all of them. This, along with the native gel result where none of the C3b:GuHCl DBP complexes or GuHCl-DBP ran into the gel, indicates that the denatured protein aggregates, and C3b will bind only to the most accessible monomers in those aggregates. This explains why using purified denatured protein in in vitro functional assays showed complete loss of protein function upon GuHCl treatment, but none of the proteins could be completely shifted up into the complex. However, when examined by denaturing gels (SDS-PAGE), the uncomplexed DBP monomers in the aggregates (that C3b had no access to) ran at the molecular weight of the native protein. Dispersing the aggregates after complex formation with C3b was attempted with low pH (pH 6), high salt (0.3 M, 0.45 M), low pH + high salt (pH 6 + 0.3 M, pH 6 + 0.45 M), 1% detergent, 0.1 M urea and 0.1 M GuHCl. None of these treatments dispersed the aggregates, making it difficult to purify the C3b:DBP complex. The other method to go about this would be to use large amounts of native DBP in the in vitro system to form

enough complexes to be purified. Once purified, the breakdown can be studied in more detail, using factor I alone or factor I and factor H together and with a combination of purified proteases to determine if the final breakdown leaves a small C3 peptide tag attached to the plasma proteins. This will also allow for mass spectrometric analysis of the complexes to identify the intermediate cleavage products to confirm that they are indeed iC3b:plasma protein and C3dg:plasma protein complexes.

7.3. C3b:plasma protein complexes in C3 glomerulopathies

Uncontrolled alternative pathway activation has been associated with several diseases including atypical hemolytic uremic syndrome (aHUS), C3 glomerulopathies (C3GN and DDD) and age-related macular degeneration (AMD) (111,112). AMD and aHUS are surface-phase diseases, with loss of tight complement regulation at the level of either the Bruch's membrane or endothelial cell, respectively. The C3 glomerulopathies, in contrast, are fluid-phase diseases in which complement dysregulation occurs in the plasma. The consequence of fluid phase dysregulation is massive conversion of C3 to C3b, which would lead to the formation of multiple different C3b:protein complexes. Since excessive amounts of C3b:plasma protein complexes would form due to alternative pathway dysregulation, as seen in factor H and factor I depleted sera, we wanted to test their presence and significance in C3Gs. Twelve patients with biopsy-proven C3G (6 DDD and 6 C3GN) were selected based on histopathological data (light microscopy, IF, EM) and were tested for circulating complexes. However, no complexes were detected when these plasma samples were directly tested by immunoblotting. We surmise that this could be because of rapid cleavage of these complexes to a plasma protein with a small C3 peptide tag, indistinguishable from the native plasma protein. As we have seen, the absence of

even a single factor either delays (factor H depletion) or pauses (factor I depletion and absence of serum factors) the breakdown. Thus the presence of additional cofactors such as CR1 and MCP in vivo on circulating blood cells and on the endothelial cells lining the bloodstream will help expedite this process even further, making it difficult to detect the intermediate forms of these complexes in vivo (113). Consistent with this observation, serum from factor H deficient mice, which develop DDD spontaneously, showed no circulating higher molecular weight C3 complexes (114). Complexes were detected in patient samples when plasma was either incubated at 37°C or spiked with native C3 in vitro, indicating that although dysregulation allows complexes to spontaneously form, they are rapidly degraded and/or cleared from the circulation. Furthermore, these results are consistent with the clinical finding of extremely low plasma C3 levels in these patients. It is intriguing to speculate whether these C3b:protein complexes have a direct role in the pathogenesis of the C3Gs.

These disorders share the key histological feature of C3 deposits in the glomerulus, with little or no immunoglobulin, the defining criterion for the new disease classification, 'C3 glomerulopathy'. Though C3GN and DDD are both associated with deposition of C3 in the glomerulus, DDD has been studied more extensively using animal models. Studies using mouse models have identified that activation of the alternative pathway is key to the pathogenesis of these diseases as seen in human patients (83,115). **Table 2** shows the correlation between the renal phenotype in the mouse models of DDD and DDD human patients with complex formation in our system. Factor H deficiency has been the observed cause of DDD in humans, pigs and mice (75-77). Though mice with factor H deficiency develop DDD spontaneously, factor H and factor B double knockout mice are protected from the disease, as are factor H and factor D

double knockout mice, due to the absence of alternative pathway activation (77). Interestingly, factor I knockout mice, as well as factor H and I double knockout mice, are protected and do not develop the disease even though they show dysregulation of alternative pathway in the fluid phase. This observation is consistent with the clinical phenotype associated with human factor I deficiency (46-48,112,116). This very rare primary immunodeficiency is inherited as an autosomal recessive trait and has been reported in only about 30 families. In the absence of factor I, C3 is depleted, and any C3b:protein complexes that form cannot be broken down **(Figure 16).** Because iC3b is not generated, neither are its smaller cleavage products like C3d, which through recognition by receptors on lymphoid and phagocytic cells stimulates phagocytosis and links the innate and adaptive immune systems. Defective opsonization makes factor I-deficient patients susceptible to recurrent pyogenic infections and aseptic meningitis (46-48,116). But what is notable is that although renal disease has been described in these patients, it is very uncommon and is associated with deposition of immune complexes, suggesting activation of the classic pathway.

In the factor I and factor H and I double knockout mice, the C3 deposition is mesangial as opposed to the glomerular basement membrane pattern seen in factor H knockout mice. We see that in the absence of factor I, C3b:plasma protein complexes that we observe stay as higher molecular weight uncleaved forms. It has been concluded that in order to develop DDD, factor I is required to generate C3 fragments in the circulation of factor H deficient mice (114). DDD and C3GN are associated with deposition of C3 and various complement proteins in the kidney (74,117). The exact composition of these deposits is yet to be determined. We propose that iC3b covalently coupled to plasma proteins is what gets deposited in the kidney. Thus, the protective

phenotype of the factor I knockout mice could be due to bigger sized complexes that influence the deposition pattern. The kidney GBM is extremely size and charge selective and its integrity is prime for the proper functioning of the kidney. Hence, we speculate that it is the cleaved C3b:plasma protein complexes (iC3b and C3dg) that are deposited in the kidney GBM. Moreover, we surmise that in factor I knockout mice, the lack of cleavage causes these C3b:protein complexes to remain in higher molecular weight form, which then deposit in the mesangium (due to their bigger size and different charge) thus providing a protective phenotype. This study also showed that plasma samples from factor I deficient as well as fH and fI double knockout mice, but not factor H deficient mice, showed multiple high molecular weight SDS-resistant forms of C3 that corresponded to the uncleaved C3b:plasma protein complexes reported herein. This study demonstrates that circulating C3b:protein complexes can be readily detected in vivo in the absence of factor I mediated degradation. But in the factor H deficient mice, in a similar fashion to our DDD patient samples, no circulating complexes were seen in the blood. We propose that this is due to rapid cleavage of the complex once formed to a small C3 peptide tag attached to the native protein, and hence it cannot be discerned from the native protein itself on SDS-PAGE.

In another study by the same group, the authors performed laser capture microdissection on the kidney glomeruli of factor I deficient mice and detected almost no 110 kDa C3b (alpha chain) band, though there was a distinct 75 kDa β-chain band (indicating C3 deposition) (118). Interestingly, there was a higher molecular weight C3 band in the factor I knockout mouse sample, perhaps indicating covalent attachment of the alpha chain of C3b to other proteins (the samples were run on a reducing, denaturing gel). This supports our hypothesis that the

C3b:plasma protein complexes are what are deposited in the kidney, constituting the electron dense deposits seen in the disease.

This hypothesis can be proved unequivocally by extracting protein from DDD patient kidney samples and testing them by western blot for deposition of C3 complexes. An attempt was made to do so by using kidney sections from DDD patients obtained from Stony Brook University Hospital. Unfortunately, these samples are stored as paraffin-embedded blocks after fixation of the specimens with paraformaldehyde, which makes it impossible to detect covalent complexes. Paraformaldehyde is a cross-linking agent and thus cross-links everything in the samples. Hence, frozen sections are required to test this, but storing paraffin blocks is the norm as of now. In the future, fresh frozen sections can be obtained from patients and tested for this. Our hypothesis can also be tested by obtaining frozen kidney sections from factor I and factor H deficient mice, extracting proteins and immunoblotting them for C3 and various other plasma proteins.

Shown below is a table drawing the correlation between complex formation in our system and renal phenotype as seen in humans or mouse models of DDD.

Complement profile	**Complex formation associated with the genotype in vitro**	**Renal phenotype**
fH -/- fB -/- mice	No	Normal
fH -/- fD -/- mice	No	Normal
C3 gain of function mutations, C3 nephritic factors (humans)	Yes	Renal disease
fH -/-, factor H autoantibody (mice and human)	Yes	Renal disease
fI -/-; fH -/- fI -/- mice	Yes	Deposits in the mesangium. No renal failure.

Table 2: Human and mouse genotype and renal phenotype correlated with complex formation in our system.

Currently, there are minimal disease-specific treatment options for the C3Gs (119). Based on our current understanding of pathogenesis, therapies that warrant consideration include drugs that can prevent ongoing dysregulation of the C3 convertase or drugs that can potentially 'mop up' the C3 breakdown products. Our demonstration that C3b binds to multiple different proteins to form C3b:protein complexes raises the intriguing possibility of identifying a protein to which C3b could bind and, once bound, become inaccessible to factor I and its cofactors, thus preventing subsequent cleavage. In addition, a major question for future studies will be to determine if different C3b:protein complexes have different clearance rates and associations with specific diseases. Furthermore, the fate and function of plasma proteins with an attached C3 peptide tag remain to be investigated. We believe that further work on characterizing C3b:plasma protein complexes is warranted, and these complexes possibly could be useful as a marker of dysregulated or excessive fluid-phase complement activation.

7.4. C3 as an extracellular chaperone

C3 has a well-documented role in the clearance of microbes and cell debris. However, C3 can also bind abnormal host proteins such as beta amyloid (Aβ42); this molecule is known to activate the complement system via the classical pathway by binding and activating C1q (99,100). As a consequence of complement activation, C3b covalently binds to Aβ42. Circulating Aβ42 is subject to complement C3b-dependent adherence to complement receptor 1

(CR1) on erythrocytes, a mechanism by which pathogens and proteins recognized as foreign are cleared from the bloodstream (101).

Inhibition of C3 activation in transgenic mice containing the human amyloid precursor protein (hAPP) can be achieved by expressing soluble complement receptor-related protein y (sCrry, a murine complement inhibitor) in the brain. Aβ deposition is 2- to 3-fold higher and is accompanied by a prominent accumulation of degenerating neurons when complement is inhibited. These results indicate that complement activation products can protect against Aβ-induced neurotoxicity and may reduce the accumulation or promote the clearance of amyloid and degenerating neurons (102). Similar results were also obtained using a hAPP plus C3-/- mouse, where accelerated amyloid β plaque deposition and neurodegeneration was observed (103). These results suggest a beneficial role for complement C3 in plaque clearance and neuronal health when covalently linked to Aβ42. This evidence led us to hypothesize that C3 might bind to plasma proteins in their non-native state in circulation, preventing their aggregation and providing a mechanism to clear them from circulation. This was tested using chemically denatured plasma proteins (guanidine hydrochloride), and it was observed that C3b binds more readily to proteins in their non-native state **(Figures 21-23).** This led us to test if the small proportion of proteins that bind C3b upon activation in plasma/serum, or in the in vitro system, is due to the presence of small levels of non-native protein. To test this, the in vitro complex formed with DBP was treated with actin to determine functional binding capacity. DBP in its native state is known to bind actin and is involved in actin clearance from circulation, preventing the formation of actin filaments in the blood. The denaturation of DBP was measured using the TNS assay. The binding of TNS to the DBP is associated with a strong increase of fluorescence

intensity and a concomitant shift in the wavelength of maximum emission (120). Interaction with actin results in alteration of certain physicochemical properties of DBP, and the fluorescence of the hydrophobic TNS probe is abolished when DBP complexes with actin (120), indicating that loss of native state (as assessed by the TNS assay) would abolish actin binding as well. But the in vitro complex formed with purified DBP could still bind actin, indicating that the DBP in that complex retains actin-binding ability **(Figure 24).** This suggests that C3b is capable of binding plasma proteins in their native state due to the presence of certain yet to be identified acceptor groups, but it binds more readily to non-native proteins probably due to the exposure of more binding groups. The major acceptor for the covalent attachment of C3b to IgG is known to be Thr144 in the CH1 domain of IgG, but a second more reactive residue Thr148 was also found. The crystal structure of IgG shows that the –Ser-Thr144-Ser- cluster is exposed to the solvent, while the Thr148 residue is buried and will not be available without conformational changes in the Fab structure (109). This could explain the enhanced binding seen upon loss of native structure. It is not known if C3 possesses holdase activity (capable of refolding these proteins), but given C3's conventional role in clearance, it is probable that C3 functions by binding and preventing non-native proteins from forming aggregates and helping to clear them from circulation. Erythrocytes are the most abundant blood cells and the major CR1 expressing cells in the bloodstream. Since CR1 is the receptor that binds and clears circulating C3 opsonized particles, we wanted to test if that could be a possible mechanism for the clearance of these complexes. Since cleavage of C3b:plasma protein to iC3b:plasma protein is extremely rapid, and iC3b:plasma protein is the form that persists longer, binding of the iC3b:plasma protein complex to erythrocyte membranes was tested. It was observed that the iC3b:plasma protein complexes

bind to erythrocyte membranes, probably to CR1 since that is the most abundant receptor on the surface **(Figure 25).**

Since Aβ42 can activate the complement system (99,100), we were also interested in testing if thermally denatured proteins can activate complement. In order to test this, serum heated to 60°C for an hour was added to fresh serum. Complement activation was tested by examining factor B cleavage and iC3b generation. It was observed that addition of 60°C serum could activate complement in 15 minutes, as seen by factor B cleavage and iC3b generation, and the level of activation increased at the 1 hour time point. Experiments using serum depleted of either factor B, factor D or C3 showed no factor B cleavage and iC3b generation, indicating that complement activation occurs through the alternative pathway **(Figure 26A)**. Interestingly, no C5a was generated in these samples even though C3 cleavage occurred **(Figure 26B and 26C)**. To test if adding heated serum affects the C5a generation pathway, the same samples were treated with CVF, and robust C5a production was detected. Since CVF can generate C5a independent of C3, C5a levels following addition of zymosan and aggregated IgG to these samples was tested, and C5a generation also was unaffected **(Figure 26D)**. This indicates that the innate ability of these samples to generate C5a was not altered and that thermally denatured proteins activate complement without triggering an inflammatory reaction, perhaps in much the same manner as apoptotic cells activate complement and are cleared without triggering inflammation.

This study provides evidence for an extracellular chaperoning role of C3 under physiological conditions. Further evidence would require testing serum/plasma from patients with amyloid diseases for C3b tagging and also examining plasma samples from burn injury cases for C3b:plasma protein complexes.

8. SUMMARY AND CONCLUSION

To summarize, activation of complement and subsequent conversion of C3 to C3b exposes the reactive C3 thioester that covalently attaches to adjacent plasma proteins in the fluid-phase. This could be a passive regulatory mechanism to intercept C3b from binding to host cells and thus limit collateral damage at the site of activation. Once formed, the C3b:plasma protein complexes are broken down by factor I and its cofactors (primarily factor H), generating several cleaved intermediate complexes. The final cleavage step appears to be mediated by a serum protease independent of factor I. These complexes form spontaneously in DDD patient samples that were C3Nef positive and to a lesser extent in C3GN patient samples. This spontaneous C3b complex formation confirms that these renal diseases exhibit dysregulation of the alternative pathway. Moreover, it is intriguing to speculate that these circulating complexes may have a role in disease pathogenesis. Excessive generation and/or poor regulation of C3b:plasma protein complexes may allow them to be deposited in the kidney and contribute to the formation of renal dense deposits. Although C3b can bind covalently to native proteins, its binding is enhanced considerably when the proteins are in their non-native state, suggesting that C3 could act as an extracellular chaperone by binding anomalous proteins and preventing their aggregation **(Figure 27)**. The iC3b:plasma protein complexes also are capable of binding erythrocyte membranes, possibly indicating a mechanism to clear complexes from the circulation **(Figure 28)**. Finally, thermally denatured serum is capable of activating the alternative pathway without C5a generation, providing further evidence that C3 can function as an extracellular chaperone. This dissertation demonstrates a predicted but heretofore not described role of the complement system and C3 in maintaining homeostasis. The C3b:plasma protein complexes described herein may be

diagnostic and/or pathogenic in several conditions where complement has been implicated in disease pathogenesis.

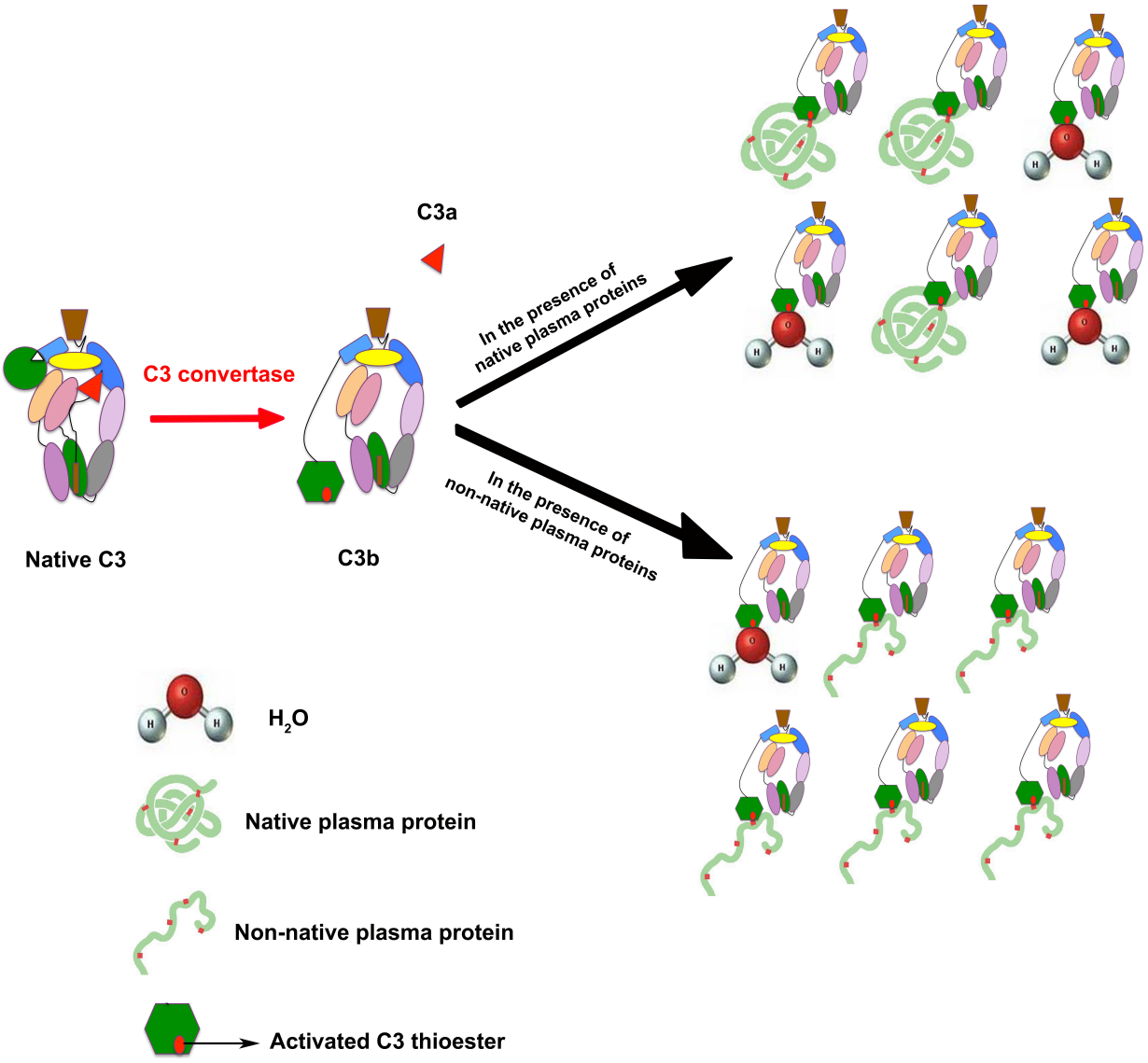

C3a
C3 convertase
In the presence of native plasma proteins
In the presence of non-native plasma proteins
Native C3
C3b
H_2O
Native plasma protein
Non-native plasma protein
Activated C3 thioester

Figure 27: Summary figure showing formation of C3b:plasma protein complexes.

A schematic representation of the events occurring during formation of the C3b:plasma protein complexes. Cleavage of C3 to C3b by the C3 convertase exposes a very short-lived active thioester, which binds to surrounding water molecules and plasma proteins covalently. C3b binds more readily to proteins in their non-native state, and hence the amount of C3b:plasma protein complexes formed in the presence of non-native proteins is increased significantly. Red arrows indicate proteolytic cleavage reactions.

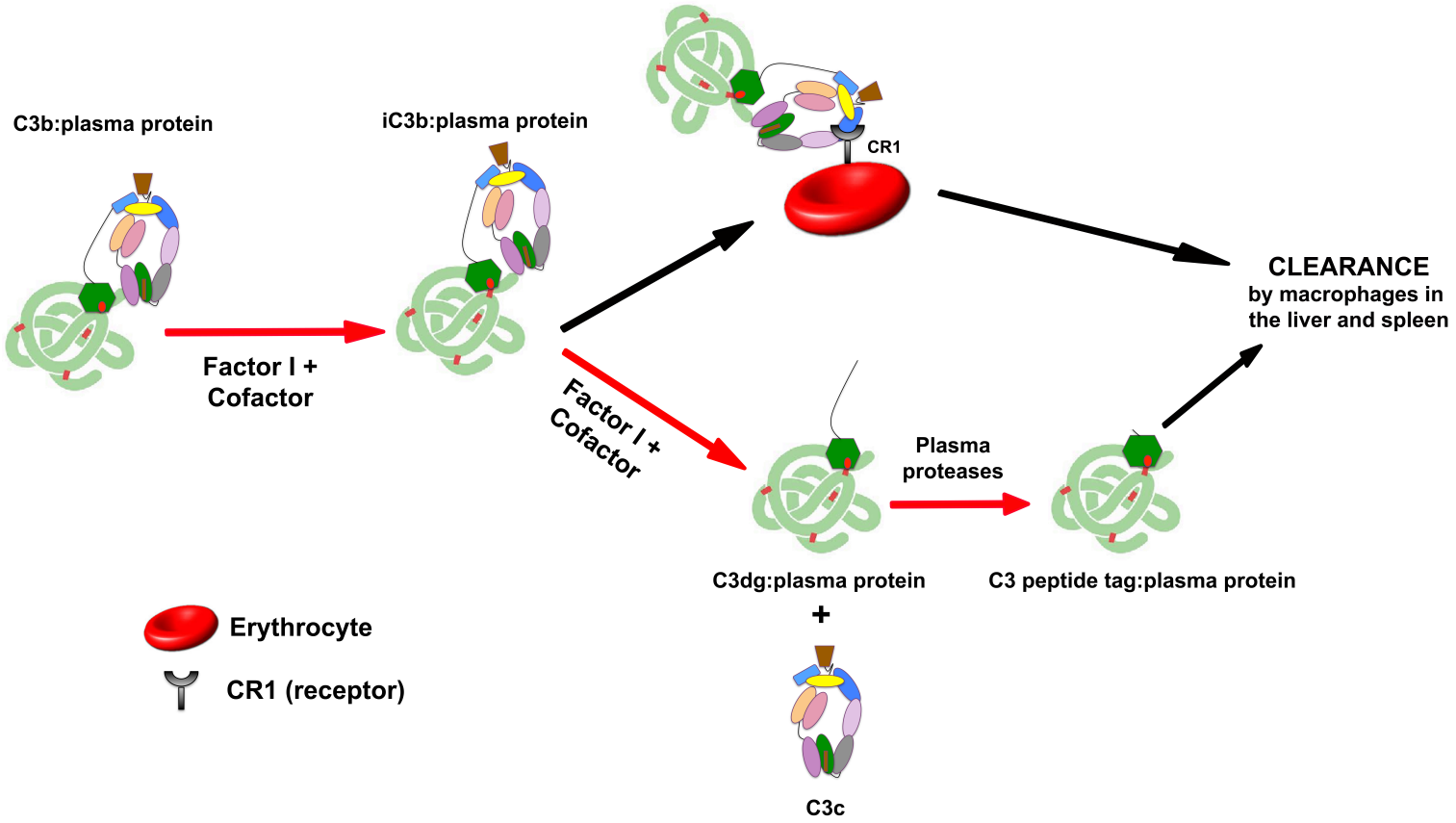

C3b:plasma protein
iC3b:plasma protein
CR1
CLEARANCE
by macrophages in
the liver and spleen
Factor I +
Cofactor
Factor I +
Cofactor
Plasma
proteases
C3dg:plasma protein
C3 peptide tag:plasma protein
+
C3c
Erythrocyte
CR1 (receptor)

Figure 28: Summary figure showing the breakdown and clearance of C3b:plasma protein complexes.

Schematic figure showing the breakdown and clearance of C3b:plasma protein complexes upon formation. The initial cleavage by factor I with the help of a cofactor generates iC3b:plasma protein complexes that are capable of binding to the CR1 receptor on the erythrocyte cell membrane and being cleared in the liver and spleen. The iC3b:plasma protein complexes can also be further broken down to C3dg:plasma protein complexes by factor I in the presence of a cofactor. The final cleavage by a factor I independent process mediated by other plasma proteases generates a complex of the plasma protein covalently linked to a small C3 peptide tag, making it indistinguishable from the native plasma protein based on molecular weight. These subsequent breakdown products can also be cleared in the liver and spleen by macrophages.

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