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Characterization of Complement C3 Dysregulation Predisposing to Two Human Disease States

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Characterization of Complement C3 Dysregulation Predisposing to Two Human Disease States

By

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Abstract of the Dissertation

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Professor John P. Atkinson, Chairperson

The complement system is an essential branch of the innate immune system and acts as a bridge to the adaptive immune system. It serves as the first line of defense against pathogens, as well as in the clearance of immune complexes and apoptotic cells. Deficiencies in many of the complement components lead to an increase in bacterial infections while others predispose to autoimmune conditions, especially systemic lupus erythematosus (SLE). Regulation of the complement system is important for protection of the host tissues against inappropriate activation. In the absence of appropriate regulation, commonly due to mutations in regulatory proteins, complement can attack self-cells. This thesis work will examine two situations in which the central component of complement, C3, is involved in a human disease state. In the first case, there is a loss of appropriate regulation of C3 and in the second an autoantibody to the C3 convertase leads to a secondary C3 deficiency.

Atypical hemolytic uremic syndrome (aHUS) is a thrombotic microangiopathy that primarily affects the kidneys. This disease is characterized by

microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. Heterozygous mutations in the regulatory proteins Factor H, CD46 and Factor I have been shown to predispose to the disease. This is due to a lack of proper control of complement activation on endothelial cells in the renal microvasculature. The first goal of this thesis work was to characterize the functional consequences of heterozygous mutations in C3 that have been identified in patients with aHUS. These mutant C3 proteins were expressed recombinantly in mammalian cells and characterized for functional changes. In ELISA binding studies, fluid phase cofactor assays and surface plasmon resonance, the majority of these proteins (11/17) had a defect in their interaction with CD46 or Factor H, both critical C3 regulatory proteins. Additionally, one mutation, R139W, led to increased binding of Factor B, resulting in formation of an inappropriately stabilized alternative pathway C3 convertase and, secondarily, to an undesirable increase in C3 activation.

Complete C3 deficiency predisposes to multiple pyogenic bacterial pathogens and was lethal in early childhood prior to the advent of antibiotics. Additionally, C3 deficiency impairs the antibody response to vaccinations and causes a defect in dendritic cell maturation. In the second part of this thesis, I will describe a case of C3 deficiency in an 18 year-old patient who presented with a life-threatening *Neisseria meningitides* infection. He had a zero whole complement titer (CH50) and C3 antigen was not detected in the standard hospital laboratory tests. Sequencing of his C3 DNA did not reveal any mutations or truncations. Further analysis of his serum demonstrated quite low levels of C3 degradation products. Mixing the patient's serum with normal human serum led to an accelerated C3 turnover

followed by the expected degradation of the C3 activation products. In hemolytic assays performed to assess the decay of the classical pathway C3 convertase, his serum stabilized the convertase, preventing its decay. Finally, it was determined that the factor stabilizing the C3 convertase was in the IgG fraction of the patient's serum. This factor, presumably an acquired autoantibody to the convertase, appears analogous to a previously reported C4 nephritic factor observed in a few patients with glomerulonephritis. However, this patient's presentation is unique and two years later he does not have renal disease.

The studies presented here establish that mutations in C3 predispose to the development of aHUS due to inadequately regulated activation of the complement cascade. These data are the first described example of C3 mutations leading to this disease. The second part of this work focuses on a novel case in which stabilization of the classical pathway C3 convertase by an autoantibody led to complete loss of functionally intact C3. Together, this work emphasizes the requirement for precise regulation of complement activation at the key C3 step.

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List of Abbreviations

Ab	Antibody
aHUS	Atypical hemolytic uremic syndrome
AMD	Age-related macular degeneration
ANA	Anaphylatoxin domain
AP	Alternative pathway
AP-C3 con	Alternative pathway C3 convertase
C3-Neph	C3 nephritic factor
C4BP	C4 binding protein
C4-Neph	C4 nephritic factor
CA	Cofactor activity
CCP	Complement control protein
CHO	Chinese hamster ovary
C/L C3-con	Classical/Lectin pathways C3 convertase
Cos-7	Green monkey kidney epithelial cell
CR1	Complement receptor 1
CUB	C1r/C1s, Uegf, Bmp1 domain
DAA	Decay accelerating activity
DAF	Decay accelerating factor
DDD	Dense deposit disease
DGVB	Dextrose gelatin veronal buffer
DMEM	Dulbelcco's modified eagle medium
ELISA	Enzyme linked immunosorbant assay

ESRF	End stage renal failure
FB	Factor B
FD	Factor D
FH	Factor H
FI	Factor I
h	Hour
HRP	Horseradish peroxidase
HEK293T	Human embryonic kidney 293T cell
kDa	Kilodalton
MA	Methylamine
MAC	Membrane attack complex
MASP1	MBL-associated serine protease 1
MASP 2	MBL-associated serine protease 2
MBL	Mannose binding lectin
MCP	Membrane cofactor protein
MG	Macroglobulin domain
MPGN II	Membranoproliferative glomerulonephritis
MW	Molecular weight
NHS	Normal human serum
P	Properdin
PNH	Paroxysmal nocturnal hemoglobinuria
PG	Protein G sepharose
Pt	Patient

RBC	Red blood cell
RCA	Regulators of complement activation
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SPR	Surface plasmon resonance
TBS-T	Tris buffered saline with Tween-20
TED	Thioester domain
TSR	Thrombospondin repeat
TTP	Thrombotic thrombocytopenia purpura
WT	Wild type

CHAPTER 1

Introduction

The complement system: historical perspective

The complement system is a critical component of the innate immune system. It is composed of multiple proteins, present abundantly in serum, whose early steps are activated in a step-wise fashion. Complement was first described over 100 years ago as the heat labile component that "complements" the humoral response by the work of Jules Bordet and Paul Ehrlich[1,2]. The initial observation was that a heat stabile element (antibody) plus a heat labile component (complement) would lead to pathogen lysis, whereas antibody without complement would not. It was subsequently elucidated that this "factor" was not a single protein, but rather an activation cascade[3]. The complement system is highly conserved evolutionarily, being present in species that have only primitive immune systems and lack adaptive immunity. The complement system arose as a part of the original innate immune system[4].

The complement system: function

The complement system serves as the first line of defense against invading pathogens and can be activated in seconds in the blood or hemolymph[5–8]. Through opsonization, it marks pathogens for phagocytosis. Initiation of the complement system also triggers activation of the terminal pathway, which leads to lysis of a target through formation of the membrane attack complex. Next, the complement system serves as a bridge ("nature's adjuvant") between the innate immune system and the adaptive immune system[9]. The production of anaphylatoxins recruit inflammatory cells such as neutrophils and eosinophils to a site of infection. Signaling through the anaphylatoxin receptors

leads to cell activation, production of reactive oxygen species and mast cell and neutrophil degranulation. Additionally, the C3d frament binds to CD19/CD21 on the surface of B cells, leading to their activation and stimulation of the adaptive immune response[10]. Deficiency in the central component, C3, has been associated with a poor response to vaccinations[11–13]. Finally, the complement system plays an important role in waste disposal as it marks immune complexes and apoptotic cells for elimination by phagocytic cells[14].

The complement system: pathways

The complement system is composed of three distinct activation pathways: the classical, lectin and alternative. The classical and lectin pathways are triggered via specific pattern recognition[15], whereas the alternative pathway is initiated by a spontaneous tickover mechanism[16]. Though a different trigger initiates each pathway, they all converge at the central step of cleavage of C3 (Figure 1.1). Through careful biochemical studies the individual components of each pathway have been identified and purified from serum.

The classical pathway is initiated when the C1 complex binds to antibody bound to antigen (i.e. an immune complex)[17]. The C1 complex is composed of C1q, C1r, and C1s. C1q is a protein composed of 6 globular heads each with a collagen-like stalk. C1r and C1s are serine proteases that associate with C1q. For C1 to be activated, the globular heads of C1q first bind to the Fc portion of IgG or IgM in the immune complex. Once in the bound conformation, the serine protease C1r autoactivates, and then in turn activates

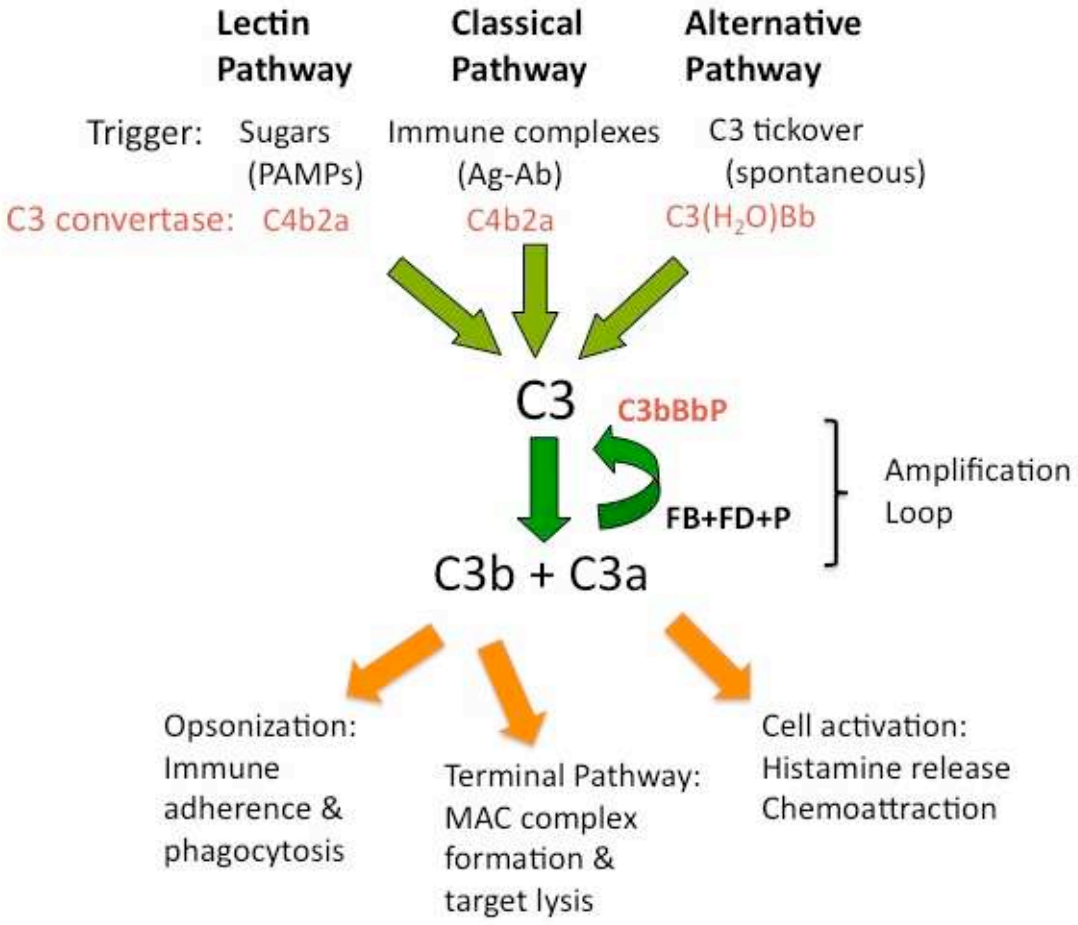

Figure 1.1: Diagram of the pathways of complement activation.

the serine protease C1s. C1s then cleaves C4 and C2 to form the classical pathway C3 convertase, C4b2a.

In a mechanism analogous to the classical pathway, the lectin pathway is initiated when mannose-binding lectin (MBL) recognizes specific carbohydrate moieties on pathogen surfaces[15]. MASP-1 and MASP-2 are the serine proteases that are associated with MBL and are akin to C1r and C1s[18]. Once activated, they also cleave C4 and C2 to form the C3 convertase.

The alternative pathway is initiated by a spontaneous turnover of C3. This "tickover" of C3 is secondary to hydrolysis of the thioester, which induces a conformational change in C3 to form $C3(H_2O)$[5]. It is now able to bind to Factor B (FB), an inactive serine protease (zymogen). Once FB has bound to $C3(H_2O)$, Factor D (FD) cleaves FB to generate Bb and releasing the smaller fragment, Ba. Bb remains bound to $C3(H_2O)$ and the complex forms the initiating alternative pathway C3 convertase, $C3(H_2O)Bb$. Once initiated, the alternative pathway acts as an efficient amplification loop, generating more and more C3b, which can bind to FB to form more C3 convertases.

The cleavage products formed by the action of the C3 convertases are C3a and C3b. These activation fragments participate in the two most important effector functions of the complement system. C3a is an anaphylatoxin and chemoattractant. C3b opsonizes target surfaces, which leads to immune adherence followed by phagocytosis of the pathogen by macrophages or neutrophils. Additionally, C3b can form a covalent attachment to the C3 convertase to generate the C5 convertases $C3b_2Bb$ and C4b2a3b.

The C5 convertases cleave C5 to C5a and C5b[19]. C5a is also a chemoattractant and anaphylatoxin. C5b initiates the terminal pathway activation and, through sequential binding of C6, C7, C8 and C9, forms the membrane attack complex (MAC). The MAC contains many molecules of C9 (up to 12), and forms a pore in the cell membrane that may lead to osmotic lysis of the target.

The complement system convertases

The C3 and C5 convertases are bi- or tri-molecular enzyme complexes, respectively (Figure 1.2). As described in the previous section, they are formed by proteins of the complement cascade that are activated by proteolytic cleavage. Activated fragments of C4 and C2 form the classical and lectin pathway C3 convertase (C/L C3-con): C4b2a. C4b2a has a higher affinity for C3 and much lower affinity for C5[19,20]. C4b2a cleaves C3 through the serine protease domain contained in C2a to C3a and C3b. The newly formed C3b can covalently attach to the C4b in the complex, which forms the high affinity C5 convertase, C4b2a3b.

The alternative pathway C3 convertase (AP-C3 con) is initially formed by $C3(H_2O)$ binding to Factor B. When bound to $C3(H_2O)$, FB is now susceptible to cleavage by FD, which cleaves FB to Ba and Bb[21]. Bb remains attached to $C3(H_2O)$ and is the catalytic serine protease containing domain of the complex. This convertase also cleaves C3 to form C3a and C3b. The newly formed C3b can either form a new AP C3 convertase by interacting with FB, or bind to the C3b in the original convertase, forming the AP C5 convertase, $C3b_2Bb$.

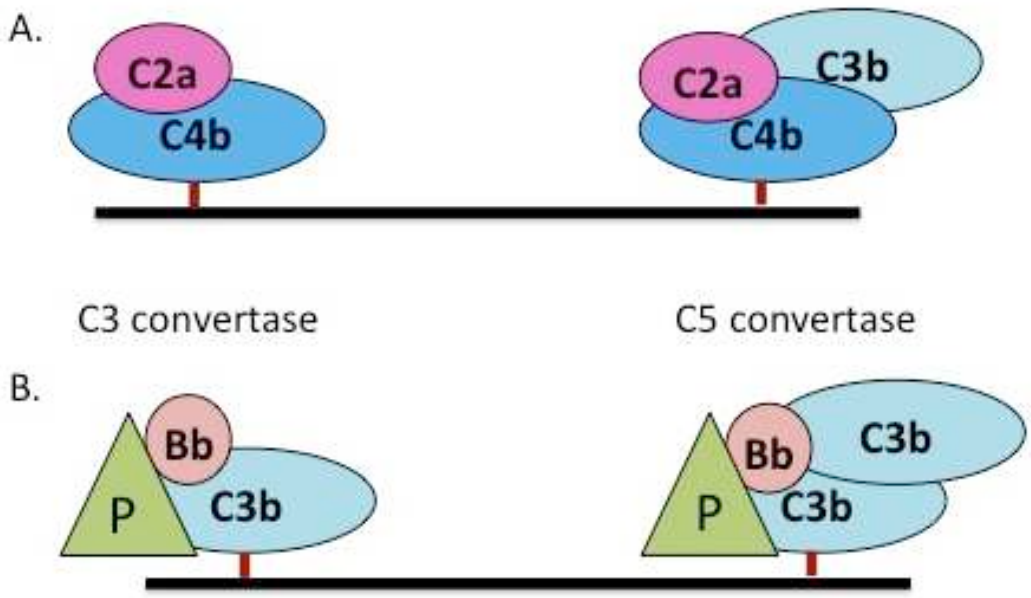

Figure 1.2. A. Classical/Lectin pathway convertases. B. Alternative pathway convertases.

The convertases of both pathways are labile complexes that are susceptible to spontaneous decay. The bonds between the two subunits are not covalent and the two will naturally, over time, dissociate. The half-life of the CP C3 convertase is less than five minutes. The half-life of the AP C3 convertase is even shorter (~90 sec). However, the AP C3 convertase can associate with properdin. Properdin is a positive regulator of the complement cascade. It increases the half-life of the AP C3 convertase from ~90 sec to five to ten min[22]. Properdin is a protein of 55 kDa, composed of thrombospondin repeats. Each monomer of properdin contains 6 TSR repeats. Properdin can be found as a monomer or as dimers, trimers and tetramers. In general, the higher order oligomers are thought to have more functional significance. Additionally, properdin has recently been shown to be able to function as a platform for assembly of the AP C3 con[23].

When C3b is initially generated it can either a) covalently deposit on an activating surface (with an ester linkage), such as a bacterial membrane or zymosan (yeast cell well particles); b) similarly deposit on a non-activating surface, such as an epithelial cell; or c) be hydrolyzed by H_2O. This covalent binding reaction takes place very quickly with a half-life of 60 μsec. If the C3b is deposited on a non-activating surface, it will be quickly cleaved by Factor I (FI) with the help of a cofactor Factor H (FH) or membrane cofactor protein (MCP) and thereby be permanently inactivated. However, if it is deposited on an activating surface, it will not be subject to immediate regulation and therefore complement activation will proceed and mark the target for phagocytosis or lysis.

Complement system regulation

Due to the feedback loop of the AP, it is necessary to have regulators to prevent complement attack on healthy self-cells. This regulation is provided by a combination of plasma and cell surface inhibitory proteins (Table 1). Many of these proteins are encoded by a cluster of genes named the Regulators of Complement Activation (RCA) on chromosome 1[24]. This gene locus includes membrane cofactor protein (MCP; CD46), Factor H (FH), Complement Receptor 1 (CR1; CD35), C4 binding protein (C4BP) and Decay Accelerating Factor (DAF; CD55). A common structural feature of these RCA proteins is the complement control protein repeat (CCP). These are about 60 amino acid units, usually encoded by a single exon, that are organized in a "beads on a string" arrangement.

MCP is a 60 kDa membrane protein expressed by most cells, except erythrocytes in man, and is composed of four contiguous CCP repeats. FH is a serum protein of 150 kDa composed solely of 20 contiguous CCPs. CR1 is an ~250 kDa membrane protein whose ectodomain is composed of 30 CCP repeats. C4BP is also a serum protein consisting of CCP domains. It forms oligomeric structure, most often composed of seven α chains, each containing 8 repeats, and one β chain with three CCPs. Finally, FI is a 100 kDa plasma serine protease that is expressed on chromosome 4. It is composed of a 55 kDa heavy and a 35 kDa light chain that contains the catalytic domain.

Regulatory Protein	Location	Function
Factor H	Blood/plasma	DAA, CA
Factor I	Blood/plasma	CA (protease)
Membrane Cofactor Protein (MCP; CD46)	Membranes of most human cells except RBCs	CA
Complement Receptor 1 (CR1; CD35)	Membranes of RBCs, B cells, PMNs, monocytes, FDCs, podocytes	DAA, CA
Decay Accelerating Factor (DAF; CD55)	Membranes of RBCs, T cells, B cells, monocytes, PMNs, platelets, endothelial cells, epithelial cells	DAA

Table 1.1: **Regulatory proteins for C3 of the complement system.**
Abbreviations: RBC: red blood cells; PMN: polymorphonuclear leukocytes; FDC: follicular dendritic cells; DAA: Decay accelerating activity; CA: Cofactor activity.

There are two main mechanisms for regulation of the complement system: decay accelerating activity and cofactor activity[25] (Figure 1.3). Decay acceleration protects cells from damage by inhibiting the assembly of the convertases on the cell surface, as well as disassembling those already present. DAF, C4BP, CR1, and FH can all participate in DAA by enhancing the dissociation of C2a from C4b or Bb from C3b. Cofactor activity describes the permanent inactivation of C3b (or C4b) by proteolytic cleavage carried out by the serine protease FI. For FI to bind C3b, C3b must first bind to a cofactor protein, which may be MCP, FH or CR1. Then, FI can cleave C3b in two locations to release a small fragment, C3f, leaving iC3b on the membrane surface. iC3b is unable to bind FB, thus blocking the amplification loop of the complement system.

C3

C3 is the central component of the complement cascade. It is the most abundant complement protein in blood and is primarily synthesized by the liver[26]. C3 has a MW of ~190 kDa, being composed of 1663 amino acids that are encoded by 41 exons on chromosome 19 in humans. It is a member of the α-2 macroglobulin family of proteins, which also includes two other complement components, C4 and C5, as well as the anti-protease, α-2-macroglobulin. C3 is translated as a single polypeptide chain (pro-C3), which is post-translationally cleaved to form the disulfide linked alpha and beta chains. Post-translational modifications of C3 include N-linked glycosylation at two sites and thioester bond formation (Figure 1.4).

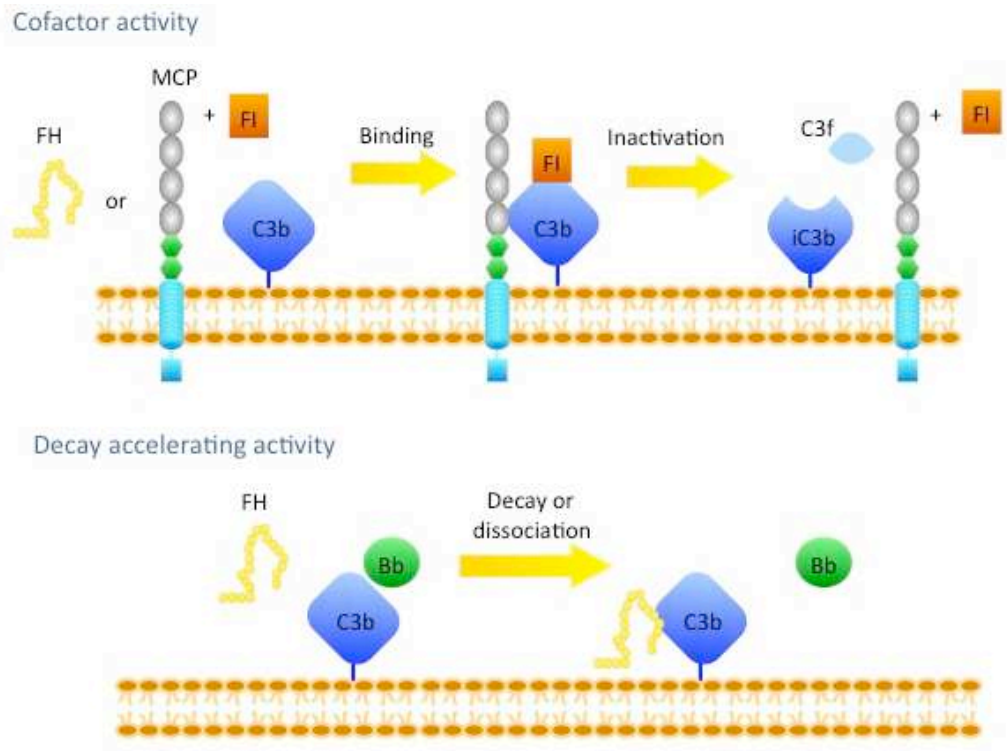

Figure 1.3 : **Two mechanisms of complement regulation.** A. Cofactor activity is carried out by FI in association with MCP, FH or CR1. B. Decay accelerating activity through FH in the fluid phase or DAF on the cell surface dissociates Bb from C3b (or C2a from C4b). Adapted from Fang CJ. *Br J Haematol 143:336-348, 2008*

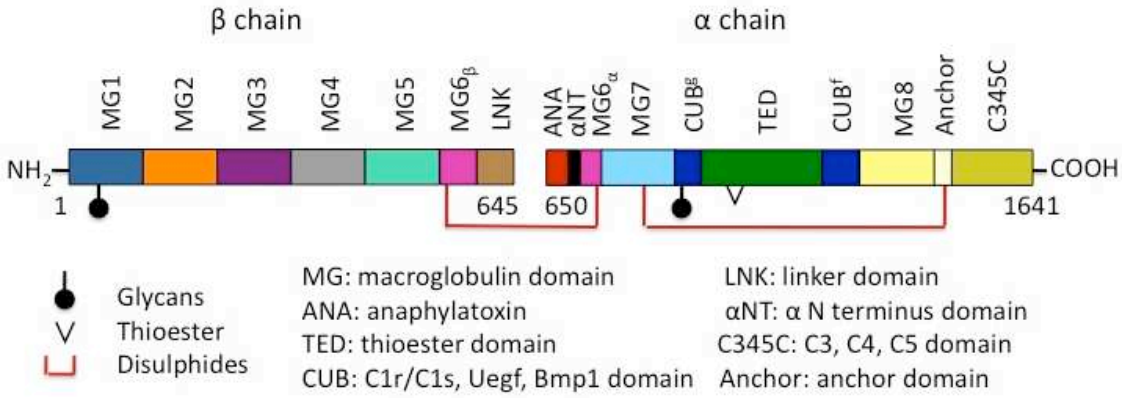

Figure 1.4: Diagram of the linear organization of C3 protein domains.

Thioester bond formation is a common feature of most members of the α-2 macroglobulin family. It is a highly labile bond that, in C3, is formed between the cysteine residue in position 988 and the glutamine residue at 991[27]. Upon activation of C3, the thioester bond is exposed and undergoes nucleophilic attack. The free acyl-imidazole group can now form an attachment to hydroxyl or amine groups on a target surface, with a preference for hydroxyls[28]. The presence of an intact thioester can be assessed in several ways: susceptibility to autolytic cleavage, susceptibility to cleavage by a C3 convertase, and hemolytic activity[29]. When proteins containing an intact thioester (C3, C4, α-2 macroglobulin) are subjected to heating under denaturing conditions, the thioester bond is susceptible to nucleophilic attack[30]. The amino acid upstream of the cysteine attacks the bond and causes a break in the polypeptide chain. The result is two α-chain fragments observed on a reducing SDS-PAGE Western blot. If the thioester has already been hydrolyzed, this cleavage cannot occur.

Requirements for formation of an intact thioester have been suggested to include specific residues in the immediate area that can contribute bulky side chains that can pack around the thioester to exclude water[29,31]. Specifically, two prolines (at positions 985 and 998) have been shown to be absolutely critical to forming the thioester bond, these prolines are conserved in all species studied, including cobra and lamprey C3. Substituting these residues results in production of inactive C3 with no hemolytic or autolytic cleavage activity, or susceptibility to a C3 convertase.

The mechanism of thioester bond formation has been investigated and debated, with no resolution to date. One attractive hypothesis is that there may be a chaperone

protein necessary for formation of this bond. One study investigating thioester formation used a reticulocyte cell free protein synthesis system to generate C3[32]. This system is unable to generate an intact thioester bond. However, when rabbit liver homogenates were added to this system, the C3 generated had an intact thioester. Another study utilized C3 deficient guinea pigs[33]. These animals were found to have normal C3 DNA and C3 specific mRNA. Cells from these animals were able to synthesize C3, but that C3 did not have an intact thioester and was more susceptible to trypsin digestion. This data suggested that formation of the thioester is critical for secretion of stable C3, and that there is an extrinsic factor that is needed for thioester formation. However, this factor, if it exists, has never been identified.

The crystal structures of C3, C3b, and C3c have recently been solved,[27,34,35] providing interesting insights into C3 activation (Figure 1.5). The structure and borders of the domains had not been previously predicted in part because they were found not to coincide with exon/intron borders (at all). The beta chain is mostly composed of five macroglobulin domains (MG) of ~100 amino acids each that have very similar folds, despite having no sequence homology. Interestingly, half of MG6 is encoded in the beta chain and half in the alpha chain. The two halves of this domain are separated by the linker domain (LNK) of the beta chain as well as the C3a (anaphylatoxin; ANA domain) and α-NT′ domains in the alpha chain, suggesting that these domains arose as insertions into the primitive C3 gene (Figure 1.4). The α-chain also contains the thioester-containing domain (TED) that is important for the attachment of C3 to targets.

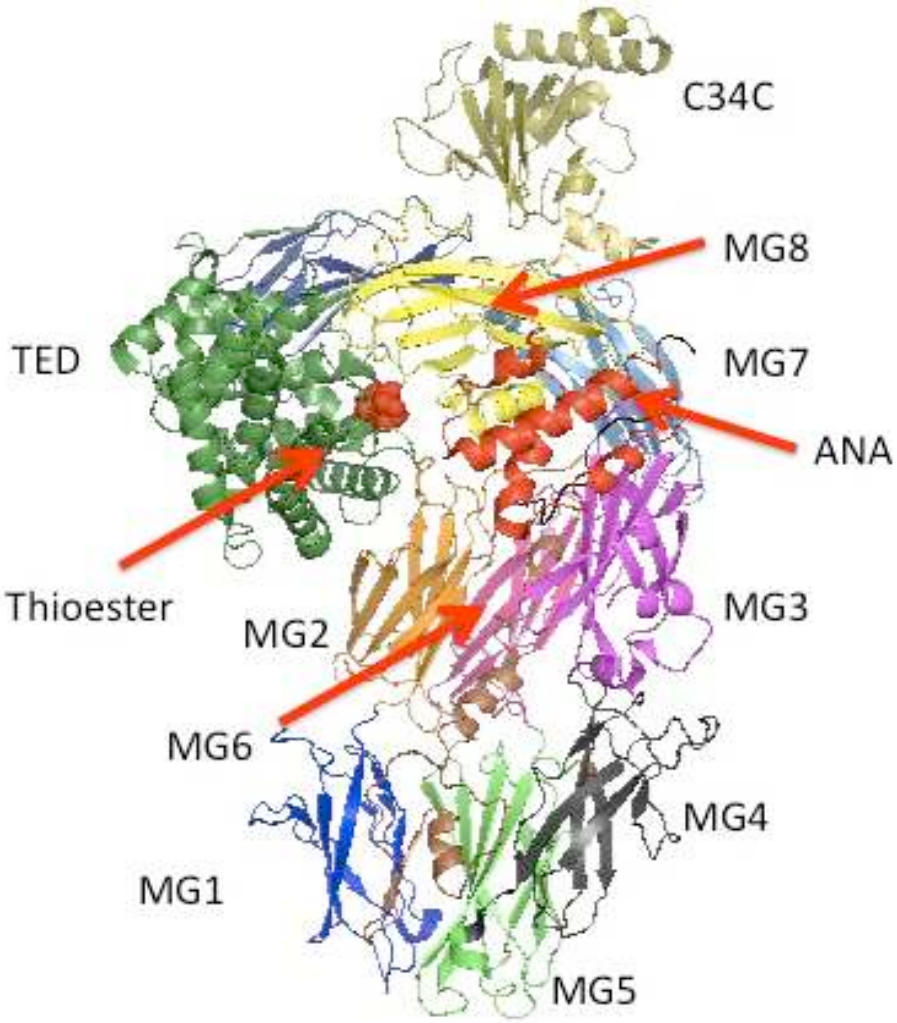

Figure 1.5: **Crystal structure of complement C3.** Ribbon representation with each domain represented by a different color (corresponding to the colors in Figure 4). The thioester residues are highlighted spheres (shown in red).

Structure coordinates published in: Janssen, B.J. et al, Nature 437, 505-511 (22 September 2005)

Additionally, the α-chain possesses two more MG domains (MG7 and MG8), the CUB domain, and the C345C domain. The CUB domain contains the cleavage sights for FI that are critical in the regulation of C3. The function of the C345C domain has not been well defined. Interestingly, all family members (C3, C4, and C5) contain this domain that appears to be sitting as the "head" of the protein. In C3b, it appears important for interaction with Bb, while in C5, it has been shown that the C345C domain is important for binding to C6/7[36].

The proteolytic cleavage of C3 to C3b releases the ANA domain (C3a) and induces a substantial conformational rearrangement[34,35] (Figure 1.6). This change results in many new ligand binding epitopes being exposed. C3b has multiple binding partners that include other complement components (C4b, FB, properdin, C2a, and C5), regulatory proteins (FH, MCP, FI, and DAF), and receptors (C3aR, CR1, CR2, CR3, CR4, and CRIg)[37,38]. The binding sites on C3 for several of these binding partners have been described through mutagenesis, peptide blocking, and structural studies. However, several interactions remain undefined.

The activation product of C3 formed during the spontaneous tickover of the protein, C3(H_2O), has long been thought to have a C3b-like structure. This assumption was based on the fact that it can bind to the same ligands as C3b, as well as participate in the AP C3/C5 convertase. The structure of C3(H_2O) has been studied recently[39]. The

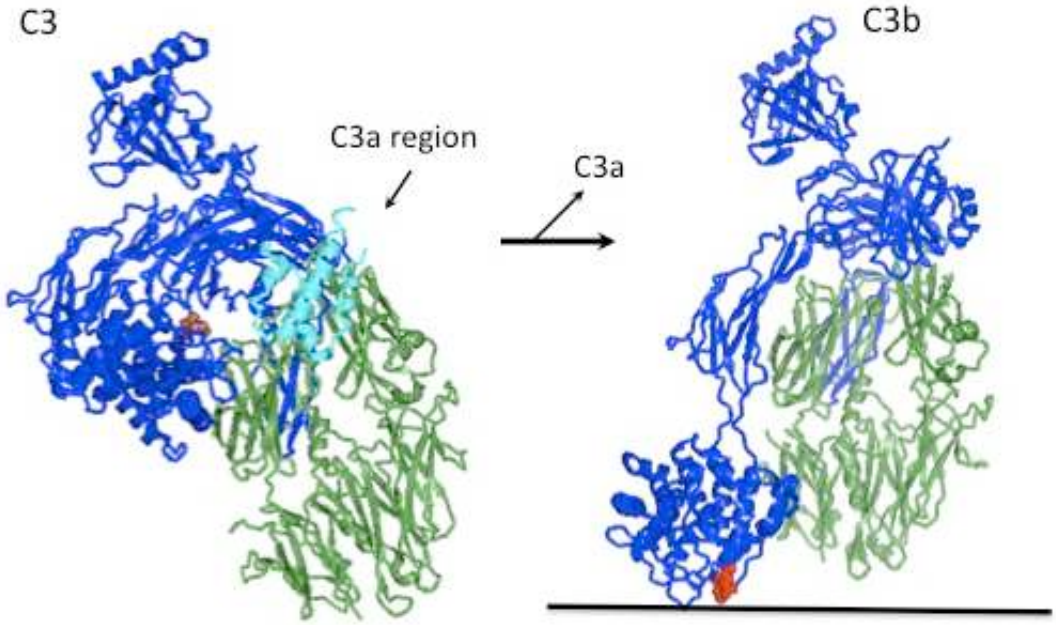

Figure 1.6: **Schematic diagram of the conformational change during the C3 to C3b conversion**. The α-chain is highlighted in blue, the β-chain in green and C3a is in cyan. The thioester residues are in red.

data presented in this paper show that while C3(H_2O) does undergo the dramatic conformational shift that is seen in C3b, the resulting structure is subtly different. In C3(H_2O), the TED and CUB domains are further extended from the body of the protein. However, the newly exposed sights are otherwise similar to those exposed in C3b, suggesting that the activity and ligand binding properties of these two activation products will be similar, which is in agreement with the previous literature. In an EM study of C3 and the activation products of C3 (C3b, C3(H_2O) and iC3b), it was shown that there are multiple conformations that can be seen for each C3 state[40]. This data suggests that there is quite a bit of flexibility in the conformational arrangement of these C3 activation products.

C3 deficiency

Complete C3 deficiency is a rare disorder. In a 2000 review, 19 families were described worldwide with a genetic defect in *C3*[41,42]. Of these families, those characterized are caused by point mutations or truncations. Patients with a homozygous C3 deficiency present with pyogenic infections. Specifically, infections with organisms such as *Streptococcus pneumonia*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Neisseria meningitides* are typically seen. C3 deficiency may also lead to reduced efficacy of vaccination, with particularly poor antibody responses to encapsulated pathogens[11,12]. Lack of C3 has also been associated with impairment in dendritic cell differentiation and maturation[13,43].

A secondary C3 deficiency may be induced when activation of complement is dysregulated. Patients deficient in either of the regulatory proteins FI or FH consume their C3[41]. One function of Factors H and I is to control the spontaneous turnover of the AP in blood by inactivating C3b to iC3b by the protease FI. If either of these regulatory proteins is missing, the AP cannot be held in check. Another cause of secondary C3 deficiency is C3 nephritic factor (C3-Neph). It is an autoantibody to the AP C3-con that stabilizes the enzyme complex so that it does not decay normally. In this manner, the convertase is over-activated and can turnover all of the C3. Rarely, C4 nephritic factor (C4-Neph) has been described[44–46]. It is an autoantibody that stabilizes the classical/lectin pathway C3 convertase (C4b2a) and also leads to consumption of C3. Secondary C3 deficiencies typically present with a glomerular renal disease termed membranoproliferative glomerulonephritis type II (MPGN II) or dense deposit disease (DDD).

Atypical hemolytic uremic syndrome

Atypical hemolytic uremic syndrome (aHUS) is a thrombotic microangiopathy that primarily affects the kidneys[47]. While the typical form of HUS (diarrheal positive) is primarily caused by infection with the Shiga toxin producing strains of *E. coli*[48], the atypical form has been described as sporadic or familial, is non-enteropathic, and thought to be triggered by injury and other types of endothelial stress or damage[49,50]. Patients with this disease generally present with the triad of microangiopathic hemolytic anemia (RBCs are sheared by fibrin strands), thrombocytopenia (platelets are consumed) and

acute renal failure (due to clots in small blood vessels). In aHUS, the underlying hypothesis for blood clot formation is complement activation that is excessive for the degree of endothelial injury.

In the past decade, mutations in complement regulators have been shown to predispose to the development of aHUS. Through genetic screening, mutations in the genes for MCP, FH, and FI have been identified in about 50% of aHUS patients[51]. These mutations lead to insufficient regulation of the complement system. The amplification loop of the AP is not held in check, resulting in an inappropriate acute inflammatory response that predisposes to microthrombi formation. Autoantibodies to FH have also been identified in aHUS patients[52]. Many of the patients with these autoantibodies were found to have a deletion of one or more of the FH-related genes[53].

Additionally, mutations in FB have been recently described[54,55]. These mutations allow a higher affinity interaction between C3b and FB to occur. They form a more stable C3 convertase and thus lead to the undesirable deposition of C3b on cells. In contrast to the loss of function mutations in the regulatory proteins, these FB mutations are primary gain of function mutations. In both situations, there is too much alternative pathway activation. Finally, work to be presented here will describe mutations in the central component, C3, that have been recently identified in patients with aHUS.

Treatment options for aHUS have been limited, with variable success[50]. The use of plasma infusion or plasma exchange is temporarily helpful in some cases. Kidney transplantion is usually unsuccessful due to recurrent aHUS. It is now understood that the success or failure of the graft is largely dependent on the type of mutation the patient

carries[53,56]. If the patient carries a mutation in a plasma protein, the new kidney will still be susceptible to complement attack due to the dysregulation in blood. However, if a patient has a mutation in the membrane protein MCP, the new kidney is more likely to survive because it now has the expected regulatory activity. Recently, combined liver/kidney transplants in young infants with refractory aHUS have been reported that may have a greater chance of success[56].

Another treatment that has been used recently is Eculizumab (Alexion)[57–59]. This drug is a function blocking monoclonal antibody against C5 that had been previously approved in use for treatment of paroxysmal nocturnal hemoglobinuria (PNH). In several recent case reports, it was effective in aHUS patients and led to recovery of kidney function. It is approved by the FDA to treat aHUS. Additionally, in the recent outbreak of *E. coli* induced HUS in Europe, the drug may have been effective in some cases.

The focus of this thesis work is on two human disease states, aHUS and secondary C3 deficiency, which can be attributed to defective complement regulation. In the first set of studies, I will describe mutations in the central component, C3, and the studies that have been accomplished to elucidate the effect these mutations on the activation state of the complement system. The second section of this thesis describes a patient with a secondary form of C3 deficiency and the efforts to discern the molecular mechanism of this deficiency.

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CHAPTER 2

Characterization of the functional consequences of C3 mutations in aHUS

Some of the data presented in this chapter has been published in two papers:

Frémeaux-Bacchi, V. *et al*. Mutations in complement C3 predispose to development of atypical hemolytic uremic syndrome. *Blood* **112**, 4948-4952 (2008) © by the American Society of Hematology.

Roumenina, L.T. *et al*. A prevalent C3 mutation in aHUS patients causes a direct C3 convertase gain-of-function. *Blood* (2012). doi:10.1182/blood-2011-10-383281 © by the American Society of Hematology.

These reports are reproduced in their entirety in Appendix 1 and 2.

INTRODUCTION

Hemolytic uremic syndrome (HUS) is a thrombomicroangiopathy that primarily affects the kidneys[1]. It is characterized by microangiopathic hemolytic anemia (shearing of red blood cells), thrombocytopenia (consumption of platelets) and acute renal failure (caused by microthrombi in the renal vasculature). There are two forms, typical or enteropathic and atypical. Typical HUS is the more common form and is associated with diarrheal (D+) *E. coli* infection in which the bacterium secretes a shiga toxin. Atypical HUS (aHUS) is not preceded by a diarrheal illness and is familial or sporadic. It is a rare disease, affecting 1 to 2 per 1,000,0000[2]. Compared to Shiga toxin based typical HUS, the rate of renal failure and severity of disease is much worse in aHUS, with 25% of patients dying and 50% of patients requiring dialysis[3].

Changes in complement levels have been observed in rare patients with HUS for many years[4]. In the 1970's it was noted that occassional patients with HUS had low serum C3 levels during the acute phase of the disease, which tended to normalize when the patient recovered. Later in the same decade, low C3 levels as well as raised Factor B (FB) and C3 activation fragments were reported in a few patients with aHUS[4]. Granular C3 deposits in the glomerulus and arterioles in aHUS patients have also been described[5,6]. Subsequently, two cases of siblings with a FH deficiency who developed aHUS were reported[7,8]. Taken together, these data suggested a role for activation of the alternative pathway of complement in the pathogenesis of aHUS.

In 1998, a genetic association study employing satellite markers linked this disease to chromosome 1q32[9], which encodes the genes for the regulators of complement

activation[10]. Following this observation, candidate gene sequencing was performed on complement genes. Heterozygous mutations were identified in Factor H (FH) first[11–13], followed by Factor I (FI)[14–16] and membrane cofactor protein (MCP)[17–20]. These regulatory genes control the level of C3 activation by permanently inactivating C3b such that it cannot participate in the amplification loop of the complement system's alternative pathway (AP). A relatively large number of mutations have now been identified in patients, and functional studies of the mutations have shown convincingly that, if the levels of regulatory proteins of the complement system are haploinsufficient, a predisposition to aHUS occurs.

Proper regulation of complement C3 is critical to maintain homeostasis; that is, to prevent inappropriate deposition on host tissues, but yet allow rapid deposition of C3 on a target. C3 is the central component of the complement system and its activation is essential for complement function. However, because of the efficient amplification loop of the alternative pathway, activation of C3 must be tightly controlled[21]. Loss of regulation is detrimental to the host, leading to complement attacking self-tissues. The importance of C3 as a susceptibility factor for human disease has been emphasized recently by studies documenting association of the polymorphism C3 R80G (C3 slow and fast) with both age-related macular degeneration[22] and long-term renal allograft survival[23]. This polymorphism had previously been tentatively associated with childhood cirrhosis, IgA nephropathy and partial lipodystrophy[24].

C3 is a complex protein. It undergoes multiple conformational changes following activation and then degradation by regulatory proteins[25] (Table 2.1). C3 in its native

Table 2.1. **Comparison of C3 and its activation products.**

	Thioester	C3a	Participates in a convertase	Interacts with regulatory proteins
C3	Intact	Present	No	No
C3i, C3(MA), $C3(H_2O)$	Hydrolyzed	Present	Yes	Yes
C3b	Hydrolyzed	Removed	Yes	Yes
iC3b	Hydrolyzed	Removed	No	Yes

form is a substrate for the C3 convertases, bimolecular enzyme complexes that cleave C3 to its active form, C3b[26]. However, once activated to C3(H_2O) by spontaneous tickover or C3b by convertases, a large number of protein-protein interactions become possible. This includes associations with other complement components as well as regulators and receptors. C3b is degraded by limited proteolysis to iC3b and then C3c and C3dg through the action of the regulatory proteins. These newly generated fragments also have distinct protein-protein interactions. The binding sites for many of these proteins on C3b are just beginning to be defined[27–29]. The naturally occurring mutations in C3 may give clues as to the location and quality of these protein-protein interactions.

In this chapter I will describe the functional characterization of 16 C3 mutants. Our approach to study these proteins was to produce the proteins recombinantly and analyze their ability to interact with other complement proteins. The initial hypothesis was that the mutations would lead to a loss of regulation, hence a secondary "over-activation" state, leading to tissue destruction. Another possibility was that the mutations would strengthen C3 binding to Factor B or properdin, thereby increasing convertase function.

MATERIALS AND METHODS

Materials

Purified C3, C3b, iC3b, Factor B (FB), Factor H (FH), Factor I (FI) and properdin were purchased from Complement Technologies (Tyler, TX). Polyclonal antibodies to C3, Factor I and Factor H were also purchased from Complement Technologies. The chicken anti-human C3 polyclonal antibody was from Biodesign International (Saco, ME). A monoclonal anti-C3d antibody was from Quidel (San Diego, CA). CM5 BIAcore sensor chips were obtained from GE Healthcare. Methylamine-hydrochloride was from Sigma (St. Louis, MO).

Construct preparation

C3 cDNA was provided by David Isenman, Toronto in the pSV vector[30] and was sequenced and compared to the published one. Two single base pair changes were found in the cDNA and altered (QuikChange Multi Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA) to match the published sequence of the C3 used in the structural analysis[28]. Mutant C3 constructs were generated using the QuickChange XL Site-Directed Mutagenesis Kit from Stratagene. Clones were sequenced to screen for the desired nucleotide substitution and then the selected clones were sequenced in their entirety to ensure that there were no other mutations. Plasmid DNA was purified from *E. coli* cultures using the Promega Midi/Maxi Prep system.

Transient transfections

Transient transfections were performed in mammalian cell lines in T-75 flasks. Chinese hamster ovary (CHO), Green monkey kidney epithelial (Cos-7) and Human embryonic kidney 293T (HEK 293T) cells were all transfected with 10 μg DNA per flask. CHO cells were transfected with Fugene (Promega), COS cells with Lipofectamine (Invitrogen) and HEK 293T cells with TransIt-293 (Mirus). Cells were incubated for 72 hrs in serum free DMEM, and supernatants were then collected and routinely concentrated approximately 40-fold.

Western blotting

C3 proteins produced by transient transfection were analyzed by Western blotting. Samples were electrophoresed on 10% Tris-glycine gels (Invitrogen). Protein was transferred to nitrocellulose membrane at 25V for 90 min and membranes were blocked in 5% non-fat dry milk in PBS-T overnight. Two antibodies, which recognize distinct epitopes, were used to assess C3. The goat anti-human C3 polyclonal Ab was diluted 1:5,000 and the chicken anti-human C3 polyclonal Ab was diluted 1:10,000 in 5% non-fat dry milk. The goat Ab recognizes both the α-chain and the β-chain, whereas the chicken Ab recognizes primarily the α-chain. Membranes were incubated in the Ab for 1 h at 37° C, followed by three 10 min washes in PBS-T. Secondary Abs were a rabbit anti-goat IgG 1:3,000 or a donkey anti-chicken 1:10,000. Membranes were incubated with the appropriate secondary Ab for 1 hr at RT, followed by washing in PBS-T. Blots were developed using West Pico SuperSignal (Thermoscientific).

Western blots for the regulatory proteins FI, FH, MCP and CR1 were also performed. Samples were electrophoresed as above and probed with the following Abs at the indicated dilutions: goat anti-human Factor I, (1:3,000), goat anti-human FH, (1:5,000), rabbit anti-human MCP, (1:5,000), and rabbit anti-CR1 (TP10), (1:10,000).

C3 ELISA

To determine the concentration of C3 in the transfection supernatants, a C3 ELISA was performed[31]. The anti-C3d monoclonal antibody was coated on ELISA wells at a concentration of 2 μg/ml in PBS (100 μl/well) and wells then incubated overnight at 4° C. Wells were next blocked with 10 mM Tris pH 7.4, 150 mM NaCl containing 1% BSA and incubated at 37° C for 90 min. Standard curve and sample dilutions were prepared in 10 mM Tris pH 7.4, 150 mM NaCl containing 4% BSA. Standard curves were prepared with purified serum C3 with a starting concentration of 10 ng/ml followed by serial 1:2 dilutions. Dilutions of transfection supernatants were prepared in ELISA dilution buffer (minimum of 4). Standard curve or sample (100 μl) was plated in each well and samples were incubated at 37° C for 90 min, followed by washing with Tris buffered saline with Tween (TBS-T). The polyclonal chicken anti-human C3 (diluted 1:10,000 in ELISA dilution buffer) was added (100 μl/well) and wells were incubated for 1 hr at 37°C followed by washing with TBS-T. The secondary antibody, donkey anti-chicken IgG was next added (1:10,000 dilution) 100 μl/well. Wells were incubated for 1 hr at 37° C followed by washing with TBS-T. Wells were developed using TMB Substrate (Pierce) and absorbance at 630 nm was determined.

Methylamine treatment of C3 proteins

To inactivate C3, the proteins were treated with methylamine[32]. A methylamine solution was prepared in 0.1 M Tris, pH 8.0 containing 0.4 M methylamine hydrochloride (Sigma, St. Louis). The C3 proteins were mixed 1:1 with an equal volume of methylamine solution (final methylamine concentration of 0.2 M) and incubated at 37° C for 1 hr.

Autolytic cleavage assay

To assess the state of the thioester bond in the C3 preparations, the supernatants were subjected to the autolytic cleavage assay[33]. C3 (20 ng) in 10 µl of PBS was mixed with 10 µl of 0.1 M Tris-acetate, pH 8.3 and 2 µL of 10% SDS. After 1 h incubation at 80°C, 11 μl of 3X reducing electrophoresis buffer was added. The sample was heated for 5 min at 95°C and then electrophoresed on a 10% polyacrylamide gel. Western blot analysis using the polyclonal chicken anti-C3 antibody was employed to monitor the ⟨–chain fragments.

C3 purification: affinity column preparation

For purification of recombinant C3 from the transfection supernatants, a CR1 affinity column was prepared. CR1 was coupled to CnBr-activated sepharose (GE Healthcare) following the manufacturer's instructions. Briefly, sepharose was activated by resuspending in 1 mM HCl, followed by washing four times with the HCl solution. After centrifugation, the sepharose was resuspended in 5 ml of 2 mg/ml solution of sCR1

in coupling buffer ($NaHCO_3$, pH 8.3). The sepharose and ligand were incubated overnight at 4° C, followed by washing in the coupling buffer. Remaining active groups were blocked by addition of 0.1M Tris-HCl, pH 8.0. Sepharose was washed with alternating cycles of 0.1 M acetic acid/sodium acetate pH 4.0 and 0.1 M Tris pH 8.0. Column was then washed with 5 mM KPO_4, 25 mM NaCl buffer (pH 6.5) and stored at 4° C.

C3 purification

C3(MA) preparations were applied to the CR1 column in KPO4 buffer, pH 6.5, at low salt (25 mM NaCl). Following extensive washing, proteins were eluted using a stepwise salt gradient in 5 mM KPO_4 buffer pH 6.5 (50-300 mM NaCl). Intact C3(MA) eluted in the 150-250 mM NaCl fractions. The fractions containing C3 were pooled together and assessed by silver staining for purity.

ELISA based ligand binding assays

Proteins were coated on wells using 2 μg/ml of sMCP, FH, sCR1 (gift of H. Marsh, Avant Immunotherapeutics, Inc, Needham, MA) or FB in PBS overnight at 4°C. This was followed by a blocking step at 37°C for 60 min (10 mM Tris, pH 7.4, with 25 mM NaCl, 1% BSA and 0.05% Tween-20). Dilutions of wild-type and mutant C3 proteins were prepared in a low salt ELISA buffer for the assessment of binding to sMCP, FH and sCR1 (10 mM Tris pH 7.4, 25 mM sodium chloride, 0.05% Tween 20, 4% BSA) or, for assessing binding to FB, in low salt buffer containing 10 mM $MgCl_2$. Following an

incubation at 37°C for 1 h, the sample was washed (10 mM Tris, 25 mM NaCl, 0.05% Tween) and affinity purified chicken anti-human C3 (1:10,000) was applied for 1 h at 37°C. After washing, an HRP-linked donkey anti-chicken IgY (1:10,000) was applied for 1 h at 37°C. Following washing, TMB substrate (Pierce) was added and absorbance was assessed at 630 nm. Values are given as % of binding to wild-type C3. These binding assays are typically carried out under low ionic strength conditions because of the low affinity of receptors and regulators to monomeric C3(MA) or C3b at physiologic ionic strength[31].

Cofactor assays

Cofactor assays were modified from a previously reported protocol[34]. C3 preparations were incubated for 0 to 30 min at 37°C with factor I (5 ng in MCP assays and 20 ng in factor H assays) and a cofactor protein sMCP (50 ng, recombinant) or FH (200 ng) in 15 μl of buffer (10 mM Tris, pH 7.4, 150 mM NaCl). To stop the reaction, 7 μl of 3X reducing Laemmli sample buffer was added. Samples were boiled at 95°C for 5 min, electrophoresed on 10% Tris-glycine polyacrylamide gels, transferred to nitrocellulose and blocked overnight with 5% non-fat dry milk in PBS. These blots were probed with either a 1:10,000 dilution of chicken anti-human C3 (Biodesign International) followed by HRP-conjugated donkey anti-chicken IgG or, after stripping (see below), a 1:5000 dilution of goat anti-human C3 followed by HRP-conjugated donkey anti-goat IgG. The blots were developed with SuperSignal substrate (Pierce). Membranes were stripped by washing with double-distilled water for 5 min, followed by

washing in 0.2 M sodium hydroxide for 5 min and a final water wash of 5 min.

Surface plasmon resonance

SPR analysis was performed using the BIAcore 2000. sMCP, FH, sCR1 and anti-C3d mAb were coupled to individual flow paths using standard amine coupling technology from GE. One flow path in each chip, lacking a coupled protein was activated as above to serve as a reference flow path. The running buffer was composed of 10 mM Hepes, pH 7.4, 0.005% Tween-20 and NaCl (25 mM, 75 mM or 150 mM). Binding of serum C3, C3b and C3(MA) were assessed at multiple concentrations at the three different salt concentrations. WT and mutant recombinant C3 proteins binding was assessed in low salt buffer only. C3 proteins were injected for 90 sec at 30 μl/min. Following the injection, dissociation was monitored for 300 sec. The chip was regenerated by injection of 0.5 M NaCl after each C3 injection. Each protein was analyzed at four concentrations, with at least two injections performed per each concentration. The experiments were performed with three independently produced and quantitated protein preparations. Data was analyzed using the BIAeval software available from BIAcore.

RESULTS

Identification of mutations

Sequencing of aHUS patients was performed by groups in France, England and the US. The inclusion criteria for these studies were the presence of thrombocytopenia, microangiopathic hemolytic anemia, acute renal failure and normal functional activity of ADAMTS-13 (to rule out cases of TTP). The initial sequencing for C3 mutations was performed on 26 patients, nine in England and 17 in France, who had consistently low C3 serum levels and no mutations in FH, MCP, FI or FB. Of this group, 11 patients had a C3 mutation. Further analysis of patient's families identified three affected individuals who had a C3 mutation and also suffered from aHUS. Three of the mutations were in more than one patient, giving a total of nine distinct mutations (Table 2.2). One mutation, R713W, was identified in both France and England. Of these 14 patients, seven recovered renal function after the first episode of aHUS, and four of those went on to have recurrent episodes. Among the 14 patients there have been twelve renal transplants, and five of these were affected by recurrent disease.

The family of one patient with the R570Q C3 mutation was analyzed separately in a follow up study[35]. In this report, nine family members harbored this mutation. All carriers showed reduced or borderline low C3 levels. Chronic kidney disease was identified in two carriers, and five carriers had microhematuria. Hypertension was present in six of these carriers, but in only one of the non-carrier family members. This suggests that C3 mutations may be associated with not only aHUS but also may predispose to other disorders.

Table 2.2. List of *C3* mutations identified in three aHUS cohorts.

Cohort	Nucleotide change	Amino acid change	Number of patients
England	1774 C>T	R570W	3
France	1775 G>T	R570Q	3*
England/France	2203 C>T	R713W	2
France	2562 C>G	Y832X	1
France	3281 C>T	A1072V	1
England	3343 G>A	D1093N	1
France	3474 C>G	C1136W	1
England	3481 C>A	Q1139K	1
France	4390 C>G	H1442D	1
France	---	R139W	14
France	---	K43N	2
France	---	K133Q**	3
France	---	I1073S	1
France	---	P1092L	1
US	1807 T>G	F581V	1
US	3125 G>T	R1020L	1

Blue, patients with low C3 levels; purple, follow up screening on French cohort; green, mutations from US cohort.

*Following initial studies, a familial study was done with one of these patients in which more carriers were identified who suffered from chronic kidney disease.

** K133Q was seen in 5 controls as well, and is a rare polymorphism.

After obtaining these results, the remainder of the French cohort was screened for mutations, regardless of their C3 serum levels. Five additional mutations were identified (Table 1), K43N*, K133Q, R139W, I1073S and P1092L. K43N was observed in two patients. K133Q probably represents a rare polymorphism, being in four controls as well. I1073S and P1092L were in one patient each. There was one C3 mutation, I734T identified in a patient with dense deposit disease (DDD), another complement related kidney disease.

R139W was seen in fourteen patients, representing about 50% of the patients with C3 mutations in the French cohort. This mutation was not found in 550 normal controls. Most of the patients and 150 of the normal controls came from the north of France. Screening of healthy family members was also performed in some of the cases and 11 healthy carriers were identified. Patients carrying this mutation rapidly progressed to end stage renal failure (ESRF) and also showed an increased frequency of cardiac and neurologic complications.

An American group reported two additional C3 mutations in aHUS patients, F581V and R1020L[2]. This group also identified a C3 mutation in a DDD patient, K1181R.

* Per recent correspondence with our collaborators, we are unsure of the substitution at this location, it may be K43Q, not K43N, that is carried by the patient. The sequencing will be repeated if necessary.

C3 protein production and purification

Mammalian cell lines

To produce the recombinant C3 proteins, transient transfections of mammalian cell lines three cell lines were initially tested: CHO (Chinese hamster ovary), COS (Green monkey epithelial), and 293T (Human embryonic kidney) cells. The use of COS cells for recombinant C3 production had been previously reported in the literature[36], while CHO and 293T cells are routinely used in our laboratory for protein production. 293T cells consistently produced the most C3. However, the protein generated in these mammalian cell lines was not hemolytically active, as assessed by autolytic cleavage assays and hemolytic assays.

To determine if the thioester bond was formed but then hydrolyzed, add-back experiments were performed. In these experiments, purified serum C3 was added to the cell cultures, and the cultures were incubated for three days (to mimic our standard culture conditions). The serum derived C3 ~50% of it's hemolytic activity. This result suggested that the secreted C3 is probably hemolytically inactive.

Baculovirus protein production

To increase our protein yield as well as to generate hemolytically active protein, the baculovirus system of protein production was used to produce recombinant C3 in insect cells[37]. The C3 cDNA was cloned into the pVL1393 baculovirus transfer plasmid and this vector was sent to Allele Technologies for virus production. Protein production

was tested on a small scale in three cell lines: Sf9, Sf21 and T.ni. C3 was produced in all cell lines, with Sf9 providing the highest yield. However, upon scaling up in Sf9 cells, the C3 protein α-chain was degraded. The virus was sent to Allele Technologies for protein production but they also obtained only degraded protein. Due to the proteolytic cleavage of C3 in this system, this approach was not further pursued.

Background cleavage in mammalian C3 production

Based on the above results, I decided to primarily produce the C3 proteins in 293T cells. Efforts were therefore made to increase the yield. However, upon scaling up, a significant amount of background C3 cleavage was now present in the supernatants (Figure 2.1). PMSF and a commercially available protease inhibitor cocktail (Roche) were added to the cultures but neither blocked the protein degradation.

Two pieces of evidence led to the hypothesis that this cleavage was mediated by FI. The first was that the degradation fragments observed by Western blotting of the cell supernatants had similar M_r to the fragments produced by FI cleavage of C3 α-chain. The second supporting piece of information was that the standard protease inhibitors could not block this cleavage. It is known that the serine protease FI is not susceptible to blockade by most protease inhibitors[38].

To assess if FI was present in the cell supernatants, Western blot analysis was performed. FI was seen in all the supernatants (Figure 2.1B). FI requires the presence of a cofactor protein (MCP, FH or CR1) to cleave C3. Therefore, western blot analysis was also utilized to detect these cofactor proteins. Bands corresponding to MCP but not FH

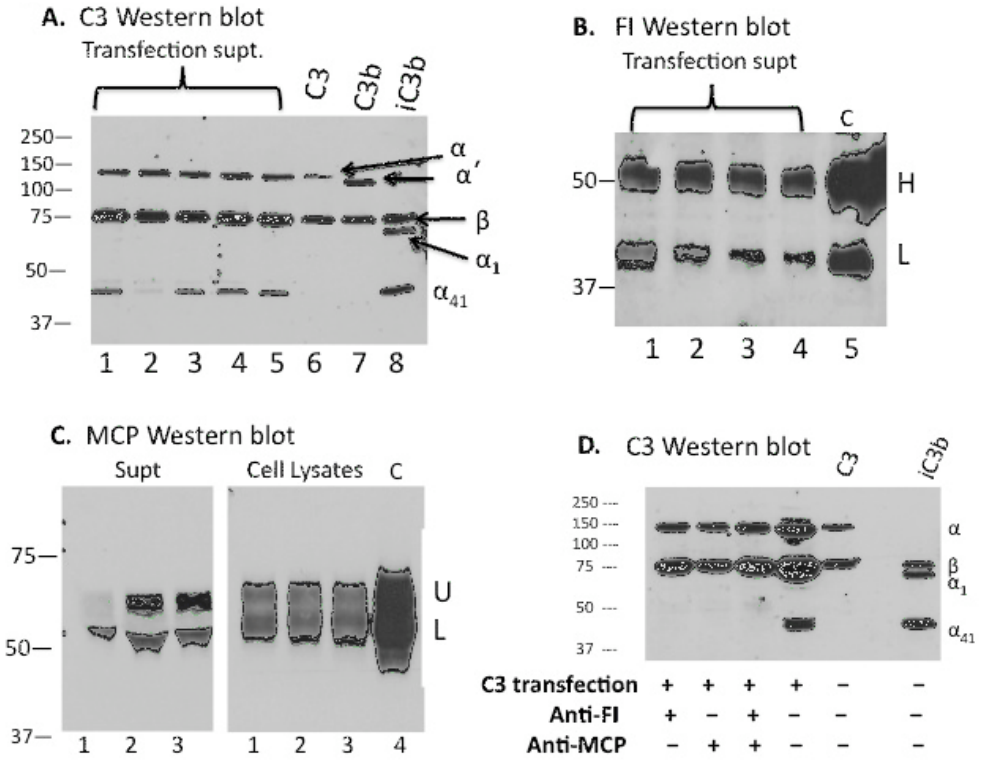

Figure 2.1. The presence of FI and MCP in 293T transfection supernatants lead to background cleavage of C3. Supernatants from transfected cells were electrophoresed on 10% Tris-glycine gels. The proteins were then transferred to nitrocellulose membranes and probed with (A) goat anti-human C3 1:5,000 (B) goat anti-human FI 1:3,000; the control lane is purified FI, H and L refer to heavy and light chains (C) rabbit anti-human MCP 1:5,000. The positive control (lane 4) is a HepG2 lysate, U and L, upper and lower bands of MCP (D) Transient transfections were performed in the presence of function blocking FI and MCP Abs, followed by Western blot analysis using the goat anti-human C3 antibody. C3 and iC3b are purified protein controls (10 ng/lane). All samples in Figure 1 were prepared under reducing conditions.

or CR1 were observed in the supernatants (Figure 2.1C). Further, if blocking antibodies to either FI or MCP were added to the cultures at the time of transfection, this blocked the cleavage of C3 (Figure 2.1D).

C3 purification

Because there is a mixture of proteins in the transfections supernatants as well as C3 degradation products, purification of the C3 from the transfection supernatant was undertaken for SPR (surface plasmon resonance). To accomplish this, an affinity column in which sCR1 is coupled to sepharose beads was prepared. To test this column, purified C3b was mixed with conditioned media from 293T cells (to mimic transfection conditions). After adjusting the ionic strength to 25 mM, the C3b was applied to the column. The column was then washed extensively before the protein was eluted with a stepwise salt gradient of 25 to 300 mM NaCl in a phosphate buffer at pH 6.5. Through this approach, I recovered C3b as expected and also separated it from the contaminating iC3b.

To purify recombinant protein from the transfection supernatants, 293T supernatants were pooled, concentrated to a small volume, ionic strength was adjusted to 25 mM NaCl, and then applied to the CR1 column. Purified C3 was recovered with this method (Figure 2.2). However, routinely a greater than 50% loss was observed. Therefore, most experiments discussed below were performed using transfection supernatants, and the purified protein was used in selected experiments.

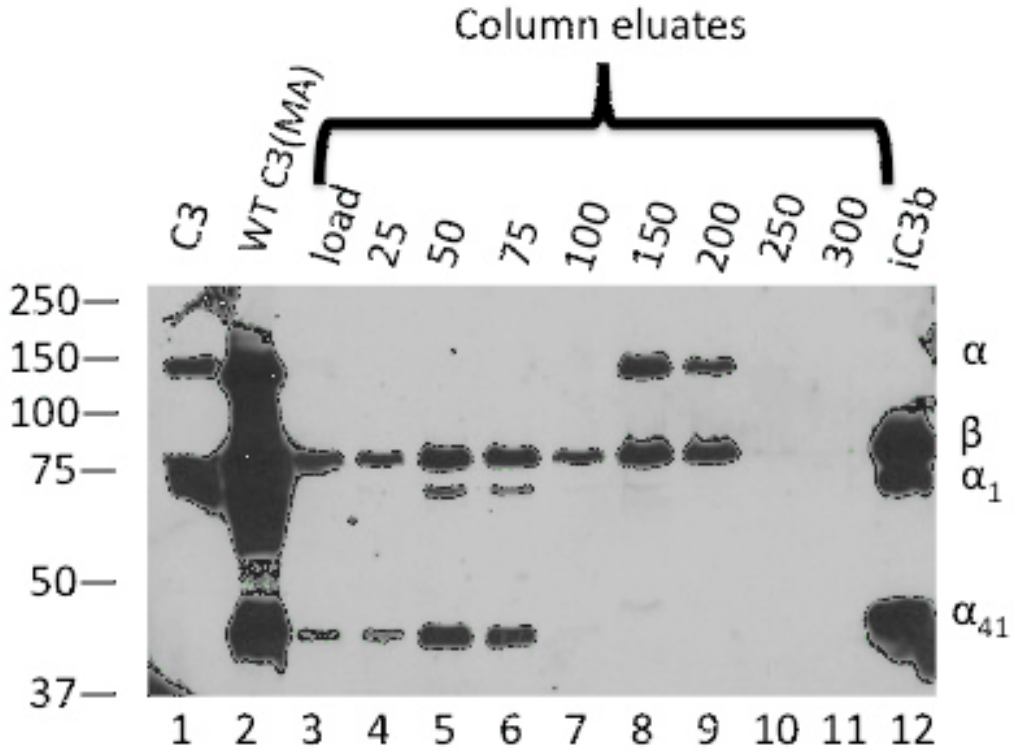

Figure 2.2. Purification of C3 from cell supernatants with a soluble complement receptor 1 (sCR1) affinity column. C3 containing transfection supernatants were applied to the sCR1 affinity column and then eluted with a stepwise (25 to 300 mM) NaCl gradient at pH 6.5. Fractions were electrophoresed on a 10% Tris-glycine gel (10 µl/lane), followed by transfer to a nitrocellulose membrane. Western blot was probed with the goat anti-human C3. Lanes 1 and 12 are purified protein controls (10 ng/lane). Lane 2 represents the transfection sample before it was applied to the column.

C3 mutants in patients with low C3

The initial sequencing for C3 mutations was performed in patients that presented with low C3. There were nine mutations (in 14 patients) initially identified in these groups: R570W, R570Q, R713W, Y832X, A1072V, D1093N, C1136W, Q1139K and H1442D. R570W and R570Q are in the β-chain, while the others are in the α-chain. The location of the mutants was mapped on the structure of C3 and C3b (Figure 2.3).

C3 mutant expression

Constructs of each of these mutants were prepared (with the exception of Y832X) and transient transfections of 293T cells performed. The first observation was that C1136W was not secreted (Figure 2.4), being undetectable in the cell supernatants by Western blot or ELISA. Upon preparing cell lysates, C1136W protein was observed, but at a reduced level compared to WT protein. The intracellular C1136W protein also had a different M_r, suggesting that the protein was misfolded or degraded. Other mutants were all expressed at levels similar to WT.

C3 ligand binding assays

Based on the data showing the critical involvement of regulatory proteins in the pathogenesis of aHUS, binding of the C3 mutants to the regulatory proteins MCP, FH and CR1 was assessed (Figure 2.5). These assays revealed a deficiency in binding of C3 to MCP by five mutants: R570W, R570Q, A1072V, D1093N, and Q1139K. These

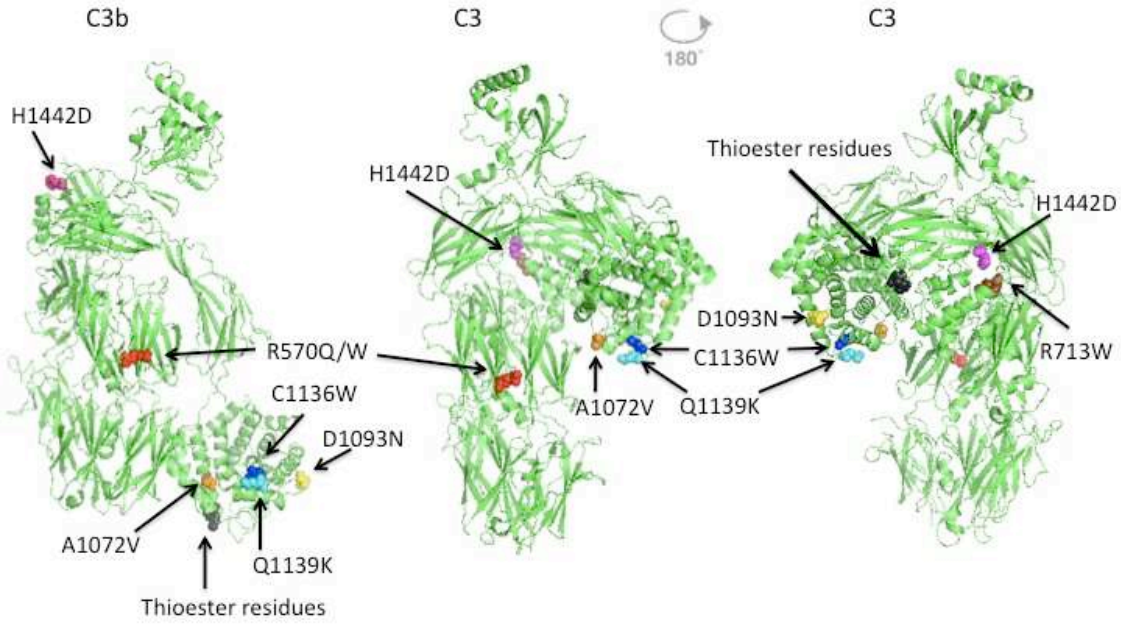

Figure 2.3 Location of C3 mutations. The crystal structures of C3 and C3b are shown. The location of the mutations is highlighted. The structural coordinates were obtained from the Protein Data Bank; PDB ID numbers 2A73 (C3) and 2I07 (C3b).

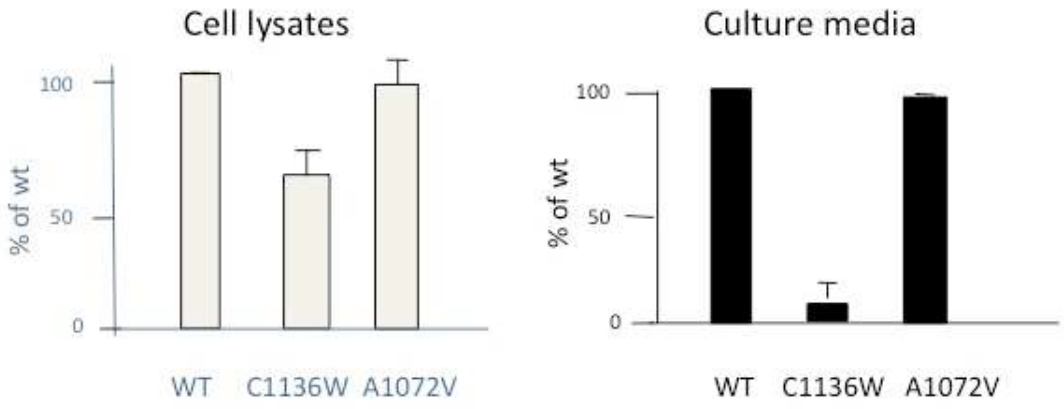

Figure 2.4. The mutant C1136W C3 protein is not secreted. Transfection supernatants and cell lysates from 293T cells were analyzed by ELISA for C3 protein. WT, wild type; and A1072V, a mutant that is expressed normally.

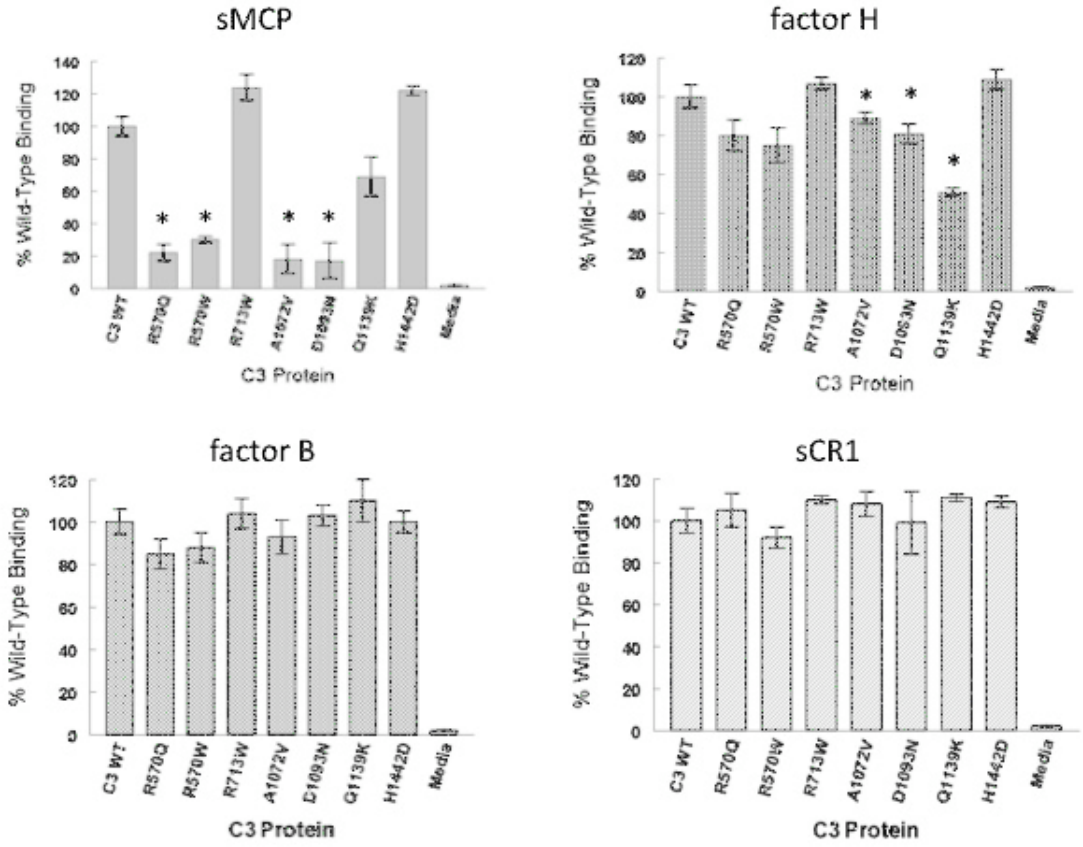

Figure 2.5. Ligand binding studies of mutant C3 proteins. WT and mutant C3 proteins derived from transient transfections were concentrated and then quantitated by ELISA. These proteins were next assessed for their binding capacity to MCP, FH, FB and sCR1 by ELISA. Concentration curves were performed in all cases. The data shown are at one concentration: 15 ng/ml for MCP, FH and CR1, and 200 ng/ml for FB. Asterisks, $p<0.05$.

mutants also had a corresponding defect in binding to Factor H, although the magnitude of the defect was less. None of the mutants had a change in binding to CR1.

FB binds to C3b to form the AP C3 pro-convertase, C3bB. FB is then converted by the serine protease Factor D to Bb, resulting in the functional AP-C3 convertase (C3bBb). If C3b had enhanced binding to FB, this would lead to a gain of function convertase. Therefore, the ability of the mutant C3 proteins to bind to Factor B was tested by ELISA. None of the mutants had an altered level of Factor B binding, suggesting that the problem in these mutants is not a "super" AP C3 convertase.

C3 Cofactor assays

Fluid phase cofactor assays were performed to assess the time course of C3(MA) inactivation by the serine protease FI and the cofactor proteins MCP or FH (Figure 2.6). By monitoring the level of intact α-chain, as well as appearance of the $\alpha_{41\text{-}43}$ degradation products, modulation of C3 inactivation by FI can be assessed. The MCP cofactor assays paralleled the results of the binding assays, with R570W, R570Q, A1072V, D1093N and Q1139K exhibiting reduced cofactor mediated cleavage. In the FH cofactor assays, defective cleavage of the Q1139K protein was seen, while all other proteins exhibited normal levels of cleavage.

These data represent the initial description of C3 mutations in aHUS. The decrease in binding to the regulatory proteins leads to a secondary gain of function in these mutants. Importantly, the location of these mutations is consistent with structural and mutagenic analysis of the binding site for FH on C3b. In fact, in the year following

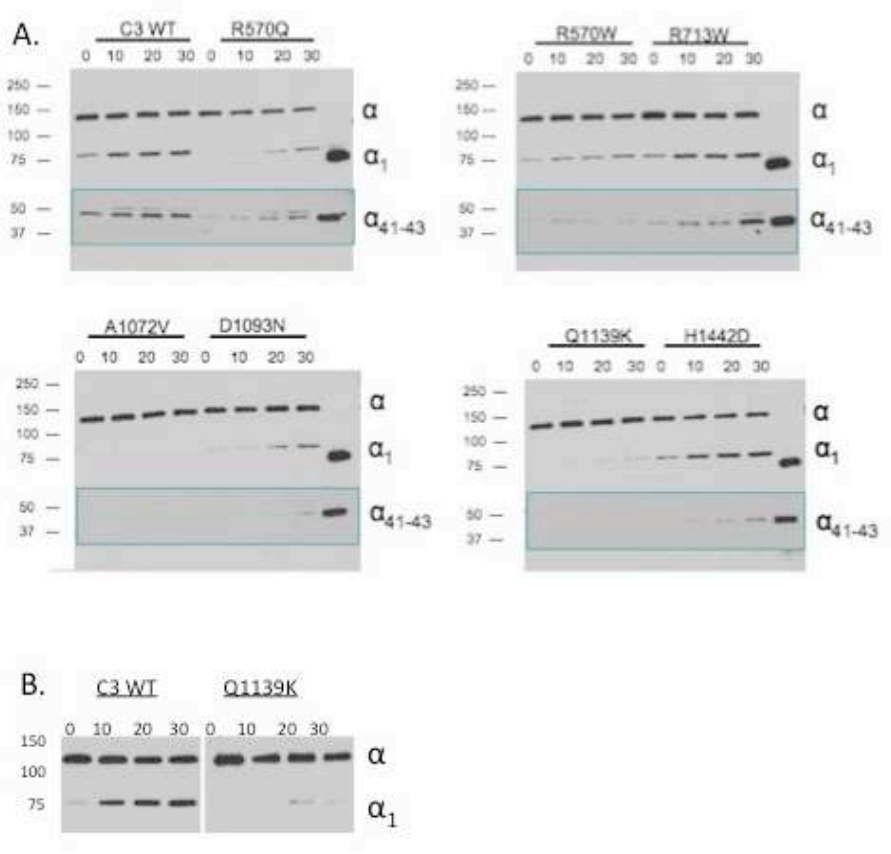

Figure 2.6. Cofactor assays utilizing the mutant C3 proteins. C3 preparations were incubated for 0 to 30 min at 37°C with FI and a cofactor protein (MCP in panel A, FH in B). The time zero control is prior to the addition of FI. The last lane is a purified iC3b control (10 ng/lane). Samples were reduced and analyzed by Western blotting using either chicken anti– human C3 or goat anti–human C3 (insets) followed by an HRP-linked secondary Ab. Cofactor activity is assessed by a reduction in the quantity of the α-chain and appearance of the α_1 and $\alpha_{43\text{-}41}$ kDa cleavage fragments. Data are representative of 5 experiments. The chicken Ab recognizes poorly the β-chain, while the goat Ab stains more efficiently the α_{41} and α_{43} fragments.

publication of this report, the structure of C3b-FH (1-4) was published[39]. In that report, the C3 mutations described here served as a basis for verification of the structure.

C3 mutants in patients with normal C3 levels

Following the initial characterization of the first nine C3 mutations in aHUS, identified based on low C3 levels, all aHUS patients in several cohorts were screened for C3 mutations. Five novel mutations were identified in the French cohort: K43N, K133Q, R139W, I1073S, and P1092L. Additionally, an American group recently reported that they screened for mutations in all complement genes known to be predisposing to aHUS in their patient cohort. The novel C3 mutations they identified were F581V and R1020L. Two C3 mutations were also identified in patients with a glomerulopathy known as dense deposit disease (DDD), I734T and K1181R. In this section, I will describe my studies on eight of these mutations. R139W is a special case and will be discussed in the next section.

The locations of the mutations are indicated on the crystal structure of C3b in Figure 2.7. They localize to several domains on both chains. K43N is in MG1, K133Q in MG2, F581V in LNK domain and I734T in the α′NT domain while R1020L, I1073S, P1092L and K1181R are in the TED domain. As in my first study, many of the mutations are clustered in the TED domain. Interestingly, there are also several mutations in domains not previously reported to house mutations (MG1, MG2, LNK and α′NT.

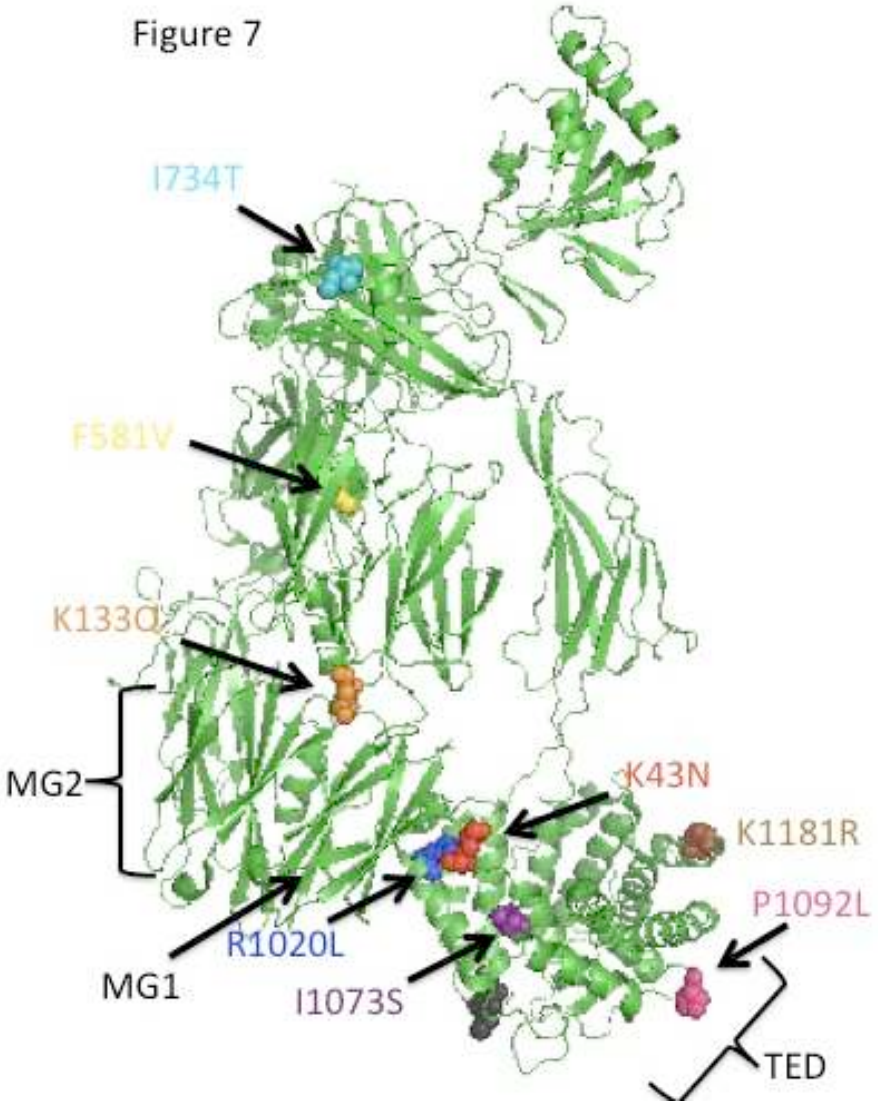

Figure 2.7. Location of C3 mutants from aHUS patients with normal C3 levels. The crystal structure of C3b is shown with the location of mutants highlighted. The structural coordinates were obtained from the Protein Data Bank (PDB ID 2I07).

C3 ligand binding studies

As in our previous study, we began by performing ELISA binding assays (Figure 2.8). The C3 mutants K43N, I1073S and R1020L had decreased binding (48, 34, and 40% of WT binding, respectively) to MCP. K133Q, F581V, P1092L and K1181R had unaffected MCP binding. Interestingly, I734T had increased MCP binding, being 185% of WT. Factor H binding assays indicated that K43N, K133Q, I734T, R1020L, I1073S and P1092L all had defective binding to this regulator, with levels of 65, 83, 71, 80, 48 and 31% of WT. The binding of F581V and K1181R to FH was only slightly reduced (not statistically significant). All of the mutants had normal binding to CR1 and Factor B. Thus, most of these new mutants exhibited defective binding to MCP, FH or both of the key C3b regulators.

Cofactor assays

Fluid phase cofactor assays were next performed employing MCP and FH as the cofactor protein to enable FI to cleave C3(MA). Densitometric scanning of the α-chain was used to monitor cleavage of the α-chain chain over time. I1073S exhibited a complete block in cleavage in MCP cofactor assays (summarized in Figure 2.9, Western blots are shown in Appendix 3, Figure A3.1 and A3.2). R1020L had a 50% reduction in α-chain cleavage in the MCP cofactor assays. The other mutants had normal levels of MCP mediated cleavage. In FH cofactor assays, K133Q, R1020L, I1073S and P1092L had substantially reduced α chain cleavage.

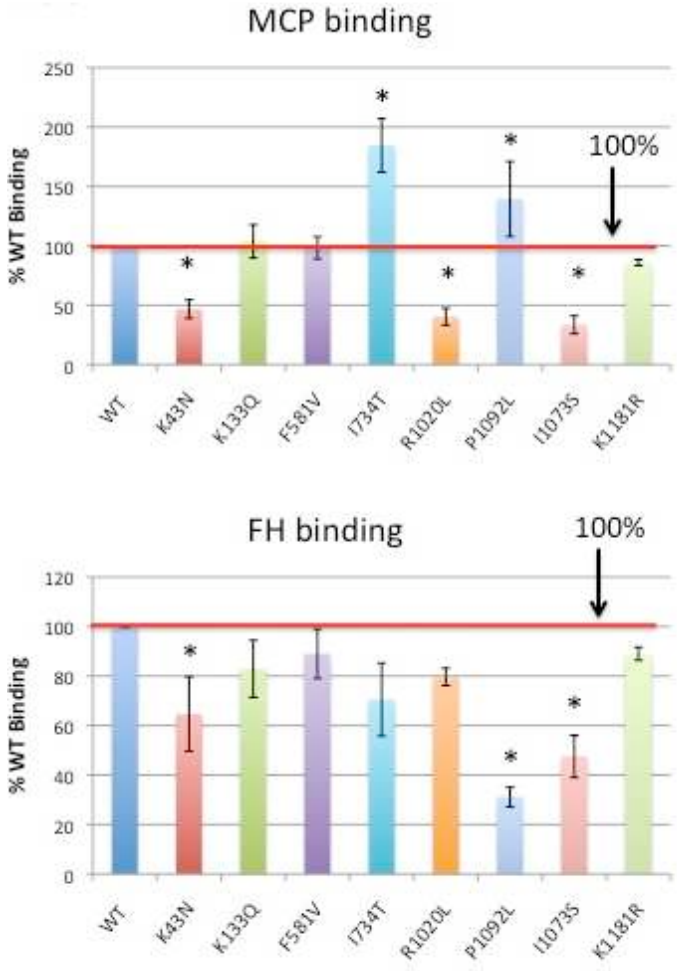

Figure 2.8. ELISA ligand binding studies of mutant C3 proteins. Ligands (MCP and FH) were coated on ELISA wells at 2 μg/ml. WT and mutant C3 proteins were applied to the wells in Tris buffer containing 25 mM NaCl. The binding of C3 was assessed using a chicken anti-human C3 antibody (1:10,000) followed by a donkey anti-chicken HRP IgG (1:10,000). Assays were developed using TMB substrate and absorbance was read at 630 nm. WT binding was set to 100% and values are represented as percent of WT binding. Shown is the mean +/- SEM of four experiments. Asterisks, $p<0.02$. Note the different y-axes.

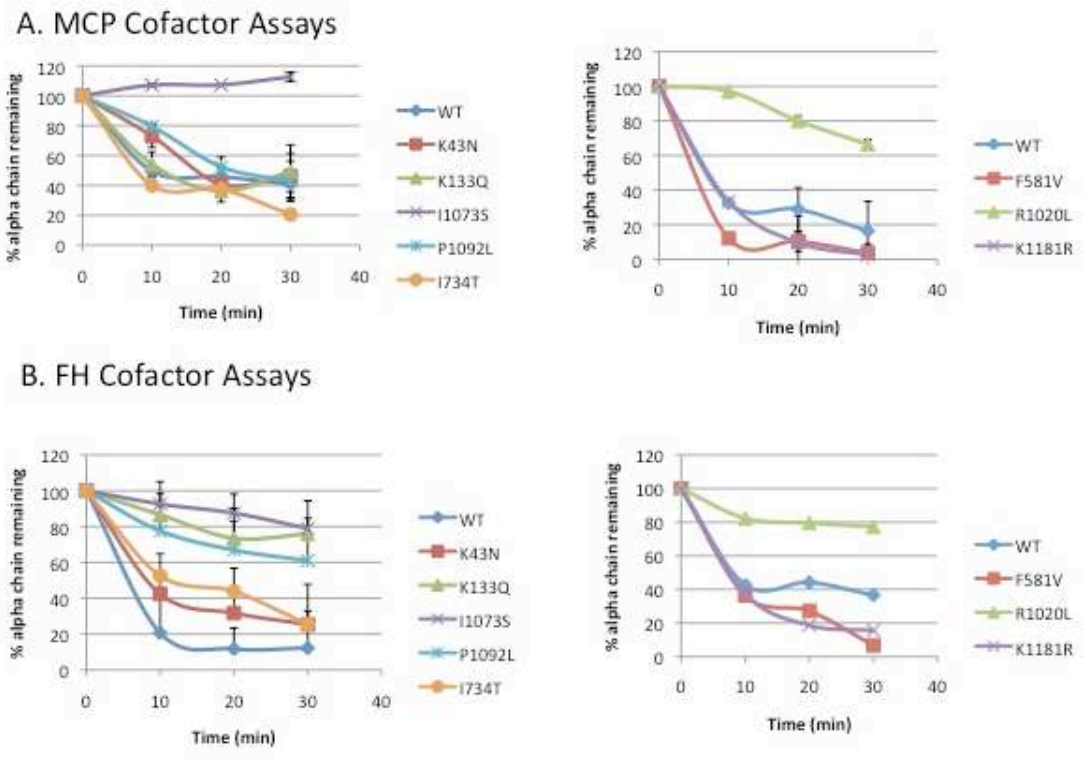

Figure 2.9. Cofactor assays of mutant C3 proteins. C3 preparations were incubated for 0 to 30 min at 37°C with FI and a cofactor protein (MCP or FH). Samples were reduced and analyzed by Western blotting using goat anti-human C3 followed by an HRP-linked secondary Ab. As in Figure 6, cofactor activity was assessed by the loss of the α-chain and appearance of α_1 and $\alpha_{43\text{-}41}$. Bands were quantitated by densitometry and the % of the α-chain remaining was determined. Graphs present the mean % α-chain remaining +/− SEM from four experiments (MCP, panel A) or three experiments (FH, panel B).

Surface plasmon resonance (SPR) analysis

To further define the interactions of the mutant C3 proteins with complement regulatory proteins, SPR experiments were performed. The initial strategy was to couple purified WT and mutant C3 proteins to a CM5 Biacore sensor chip, and then flow over the ligands (MCP, FH, CR1). However, all attempts to couple the C3 to the chip failed. The most likely explanation for this is that the C3 concentrations were too low to effectively couple to the chip. In fact, if purified serum C3(MA) was diluted to concentrations that were similar to those of the recombinant protein, coupling to the chip was also impaired; however, C3(MA) was able to couple to the chip when it was applied at higher concentrations.

Next, I tried antibody capture. Antibody to C3 was coupled to the chip and then the C3 proteins were applied. The ligand of interest could then be applied to the flow paths. Four different monoclonal antibodies were tested, and only one of them was able to form a complex with C3(MA). However, this complex dissociated quickly and also raised the concern that a mAb might mask important epitopes. This method was not further pursued.

Coupling of the ligands to the chip by amine coupling was then examined. With the ligands coupled to the chip, the C3(MA) proteins became the analyte. Initially, serum derived purified C3(MA) protein was used to define the parameters of the system. The binding of C3(MA) and C3b (positive controls) was tested at multiple concentrations and at two ionic strengths (Figures 2.10-2.11). Dose response curves were generated in 150 mM NaCl containing buffer and analyzed using the 1:1 Langmuir binding model in the

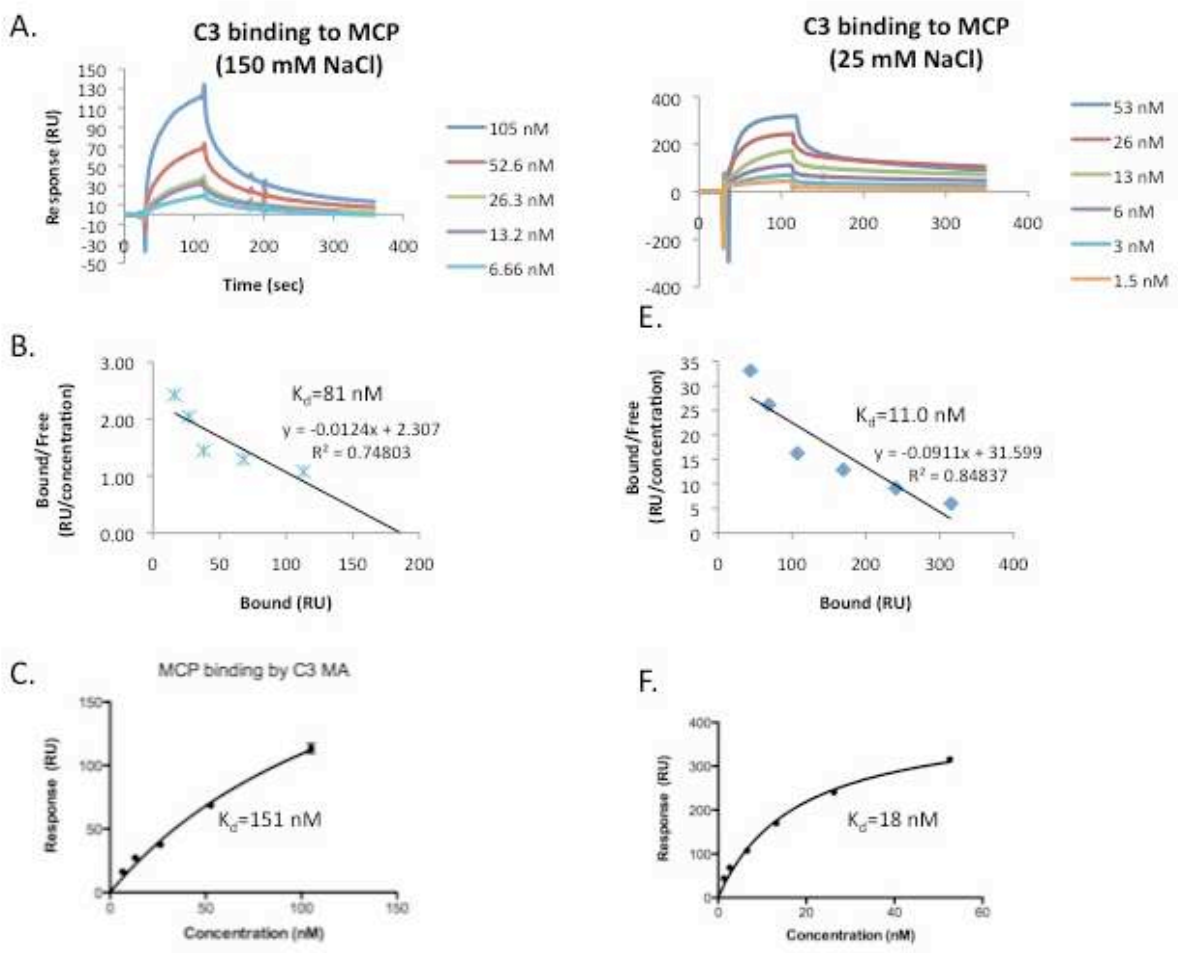

Figure 2.10. Ionic strength dependence of C3 binding to soluble MCP (sMCP) in surface plasmon resonance (SPR). sMCP was coupled to a CM5 sensor chip using standard amine coupling. C3 was applied to chip in buffer containing 150 mM NaCl (A) or 25 mM NaCl (D). Scatchard (B and E) and non-linear regression (C and F) analyses were performed on each data set.

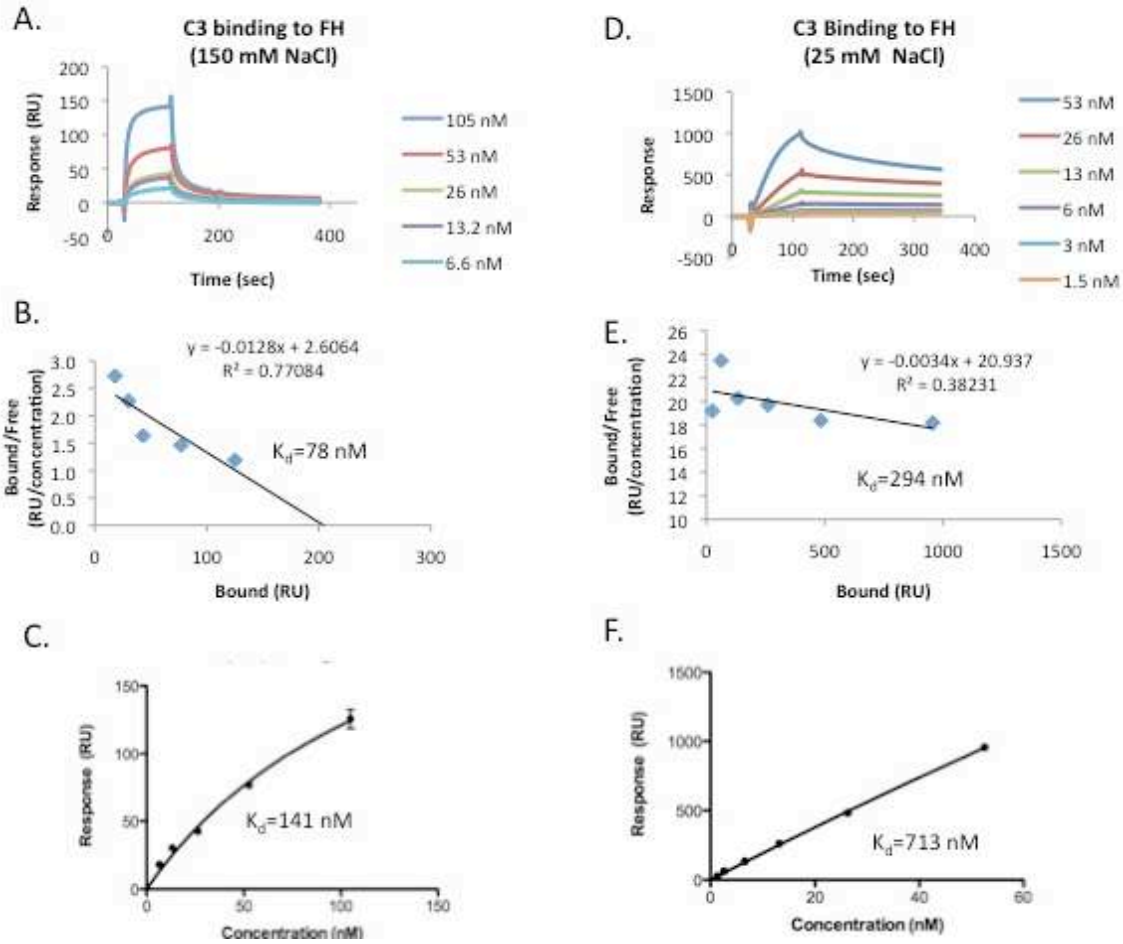

Figure 2.11. Ionic strength dependence of C3 binding to FH in surface Plasmon resonance (SPR). FH was coupled to a CM5 sensor chip using standard amine coupling. C3 was applied to chip in buffer containing 150 mM NaCl (A) or 25 mM NaCl (D). Scatchard (B and E) and non-linear regression (C and F) analyses were performed on each data set.

BIAeval software to determine kinetic parameters. At 25 mM NaCl, a dose response was again seen, but in this case it did not fit well to the models. This is not entirely surprising as these curves were generated in a low salt buffer, whereas the models assume a physiologic ionic strength. Nevertheless, in both cases reproducible, dose-dependant binding curves of the C3 proteins interacting with MCP and FH were generated.

To further analyze the system, Scatchard and nonlinear regression analyses were performed (Figures 2.10-2.11). This analysis demonstrated that equilibrium was not reached, making a strict quantitative analysis problematic. Qualitative differences can still be appreciated.

Having established the parameters of this system, the C3 mutant proteins were analyzed. It quickly became apparent that these assessments would need to be done in low salt buffer (25 mM NaCl). The low concentration of the recombinant proteins prohibited their use at 150 mM NaCl. The C3 mutants, treated with methylamine to assure conformational homogeneity, were flowed over the chip at various concentrations (0.1 nM to 5 nM) for 90 sec, followed by a dissociation period of 300 sec. Between each injection, 0.5 M NaCl was injected for 10 sec as a regeneration step followed by washing of the flow cells.

In a comparison of WT to the mutant C3 proteins, six mutants (K43N, K133Q, F581V, R1020L, P1092L, and K1181R) demonstrated reduced binding to MCP (Figure 2.12). In one mutant, I1073S, the binding levels were similar to those of WT. In the FH binding sensorgrams, all mutants demonstrated reduced binding to FH (Figure 2.13). To confirm that the recombinant proteins injected were at the same concentration, the fourth

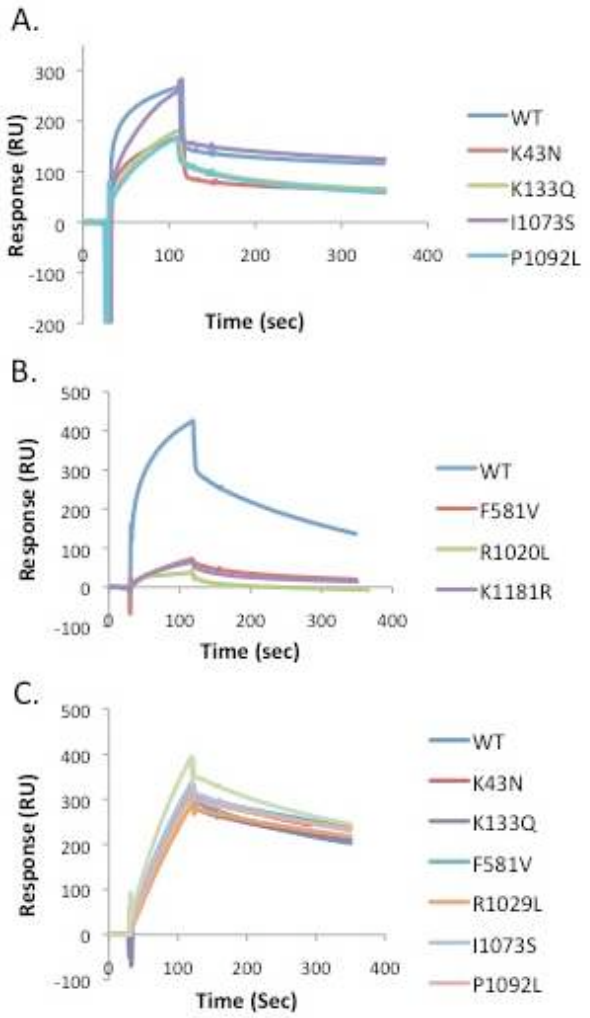

Figure 2.12. Surface plasmon resonance analysis of WT and mutant C3 proteins binding to MCP. MCP was coupled to a CM5 sensor chip. WT and mutant C3 proteins were applied for 90 sec, followed by a 300 sec dissociation period. Each protein was analyzed at four concentrations with three injections each. Representative curves at 2.5 nM of C3 protein added are shown (A and B). C. Representative curves of recombinant C3 proteins binding to anti-C3d Ab that was coupled in a parallel flow path.

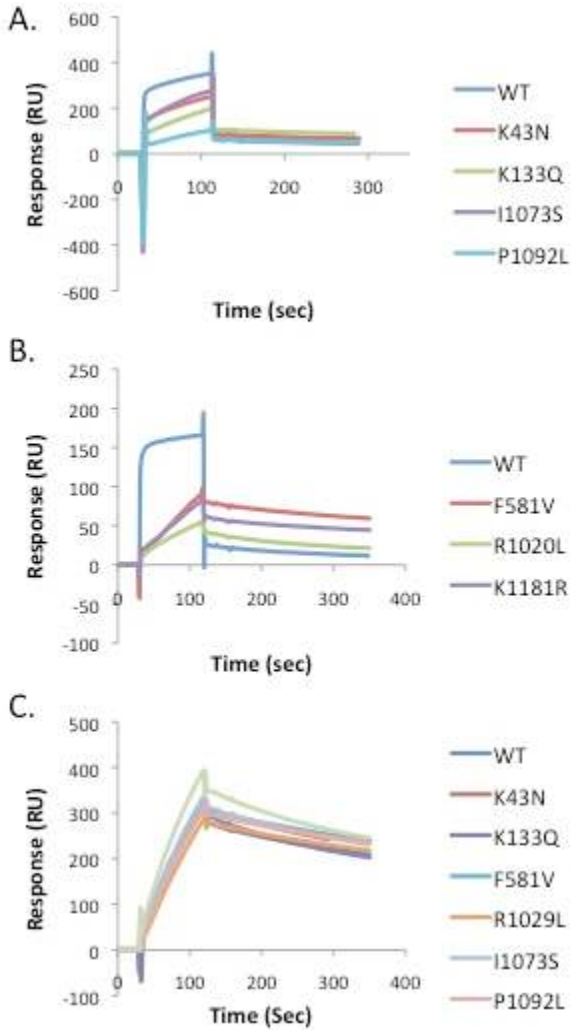

Figure 2.13. Surface plasmon resonance analysis of WT and mutant C3 proteins binding to FH. FH was coupled to a CM5 sensor chip by. WT and mutant C3 proteins were applied to the chip for 90 sec, followed by a 300 sec dissociation period. Each protein was analyzed at four concentrations with three injections each. Representative curves at 2.5 nM of C3 protein added are shown (A and B). C. Representative curves of recombinant C3 proteins binding to anti-C3d Ab that was coupled in a parallel flow path.

flow path was coupled with an anti-C3d monoclonal antibody and levels of C3 binding were monitored. The overlays show that the proteins were injected at equivalent levels (Figure 2.12 and 2.13C). Titration overlays for all of the mutants tested are shown in Appendix 3 (Figures A3.3 and A3.4).

The results of these experiments confirm that these mutations lead to a resistance to inhibition by regulatory proteins. Reduced binding capacity to one or both regulators was observed for all mutants. Additionally, this result translates to a reduction in FI mediated cofactor cleavage of the protein.

The comparison of ELISA and the SPR data demonstrate that more than one method should be used to assess mutation defects. In the ELISA, some changes in regulator binding by the mutants did not appear to be significant; however, when analyzed by SPR, a defect was apparent. This may simply be that the SPR experiments are more sensitive. It may also be due to a difference in the orientation of the proteins.

Studies on the C3 mutant R139W

R139W is a prevalent mutation that has been identified in 14 patients in the north of France. It was not identified in 150 normal controls taken from the same region.

R139W binding to regulators

Binding to the proteins MCP, FH, CR1 and Factor B was assessed for this mutant. Binding of R139W C3 to MCP was reduced to about 35% of WT binding in the ELISA

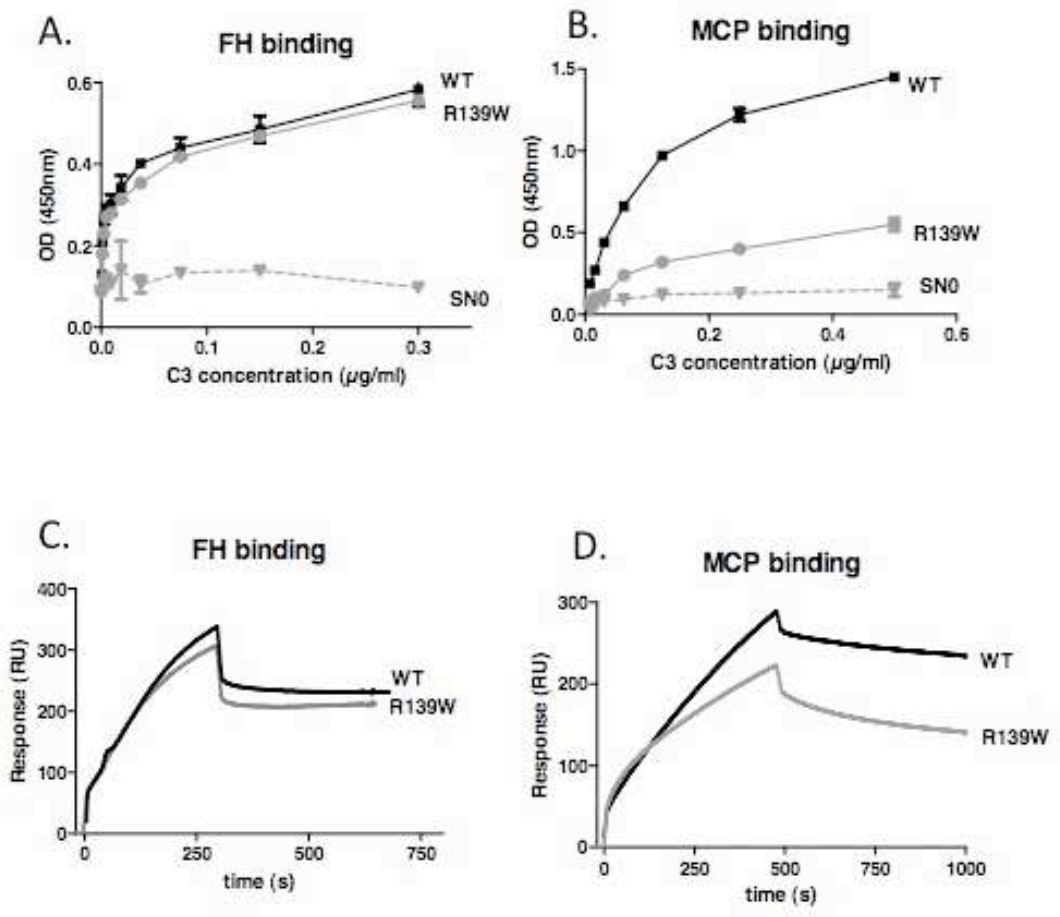

Figure 2.14. C3 mutant protein R139W demonstrates normal FH but reduced sMCP binding. (A) and (B) Recombinant WT and R139W C3 proteins were applied to ELISA wells coated with FH or sMCP. SNO is a negative control transfection supernatant. Binding of C3 to the regulatory proteins was detected with a chicken anti-human C3 Ab, followed by an HRP conjugated donkey anti-chicken secondary Ab. Absorbance at 450 nm was monitored. (C) and (D) SPR analysis of WT and R139W binding to FH and MCP was performed by coupling FH and MCP to a CM5 sensor chip and monitoring the binding of the C3 proteins.

binding assays (Figure 2.14). The binding of R139W to FH, however, was similar to WT levels. Interestingly, binding of R139W to CR1 was modestly reduced (70% of WT binding). R139W is the only C3 mutant seen to have a reduction in binding to CR1. To confirm the ELISA binding data, SPR analysis was next performed with MCP or FH coupled to the chip (Figure 16). In these experiments, R139W was again seen to have a decrease in binding to MCP, but not FH.

C3 cofactor assays

Fluid phase cofactor assays employing MCP, FH or CR1 as the cofactor protein demonstrated a delay in MCP mediated cleavage of R139W as compared to WT as well as an alteration in the cleavage fragments generated (Figure 2.15). With the WT protein, there was transient generation of the α_{43} fragment that precedes the α_{41} fragment generation. With the R139W protein, there was a change in this pattern. The α_{43} was more prominent than α_{41} at all time points, suggesting that the mutation is affecting the second FI cleavage site resulting in more of the larger sized fragment.

In cofactor assays utilizing FH and CR1, there was no delay in cleavage in the α chain, suggesting that there was no significant alteration in binding to FH and CR1 by R139W, and supporting the data from the binding assays. However, a distinct cleavage pattern was again seen. The α_{43} fragment was a darker band than α_{41} in both assays. This result further suggests that R139W mutation has an effect on the ability of FI to cleave at the second location (perhaps due to a steric effect).

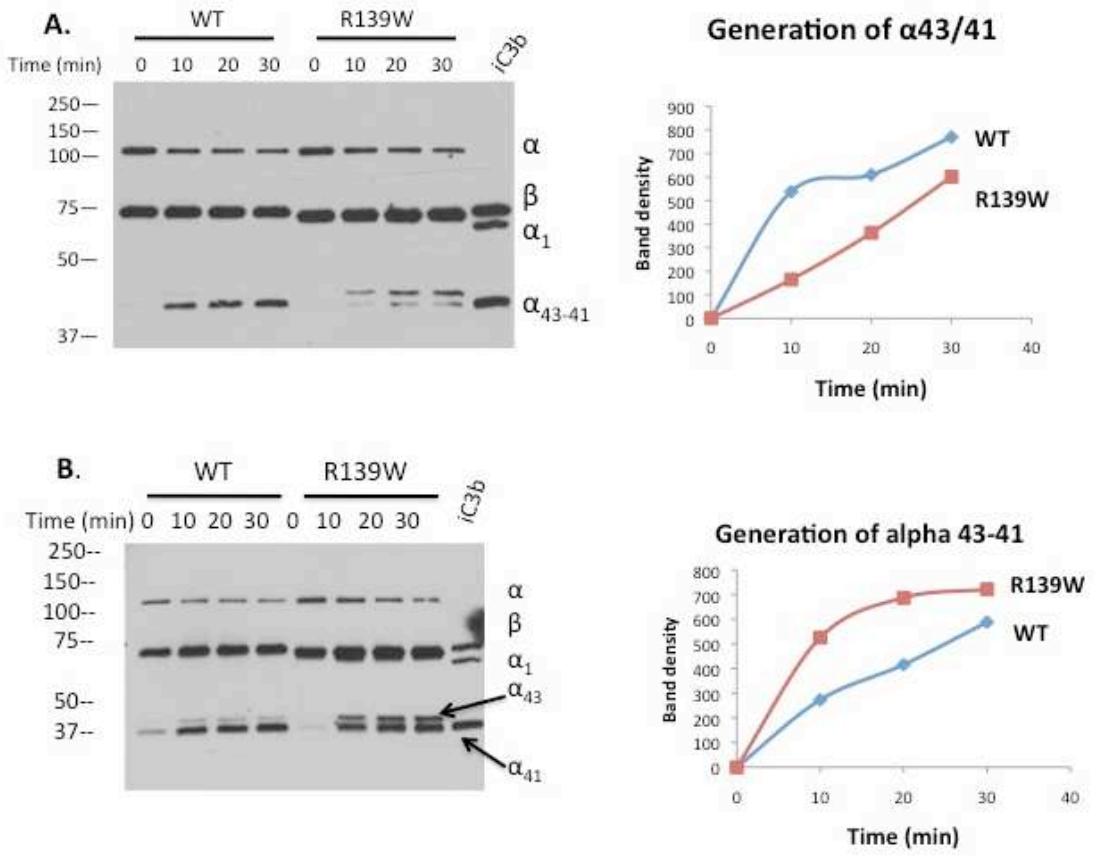

Figure 2.15. R139W exhibits reduced MCP mediated cofactor activity. C3 preparations were incubated for 0 to 30 min at 37°C with FI and a cofactor protein- (A) MCP or (B) FH. The zero control is prior to the addition of FI. The last lane is an iC3b control (10 ng/lane). Samples were reduced and analyzed by Western blotting using the goat anti– human C3 followed by an HRP-linked secondary Ab. Cofactor activity is assessed by the loss of the α chain and appearance of the α_1 and $\alpha_{43\text{-}41}$ major cleavage fragments. Data are representative of 3 experiments. Generation of

the $\alpha_{43\text{-}41}$ fragment was monitored by densitometry.

Factor B binding by R139W

To analyze the interaction of R139W with Factor B, ELISA binding studies were performed. In these assays, R139W had a higher level of binding to FB than WT C3. To determine whether this increase in binding translated to a hyperfunctional convertase, the AP C3 convertase was assembled on the BIAcore chip. C3 (WT or R139W) was injected along with Factor B and Factor D. Following convertase assembly, C3 by itself was injected. The convertase formed with R139W was more efficient, leading to more C3b being deposited on the chip.

The R139W mutation is the first description of a C3 mutation with a double-hit. This mutation has lost susceptibility to regulatory proteins but also experienced a gain of function relative to its FB interactions.

DISCUSSION

The characterization of 16 mutations in human C3 identified in aHUS patients and two mutations in patients with DDD is described here. The majority had a decrease in their susceptibility to regulation by FH and/or MCP (summarized in Table 3.3). Additionally, two mutants (one in this study and one report from another group[40]) have an increased binding activity with Factor B, leading to a more stable C3 convertase. In either situation, though, the end result is the same- increased complement activation. In aHUS, this presumably leads to endothelial cells in the microvasculature of the kidney becoming having a more procoagulant phenotype.

The studies presented here represent the first description of C3 mutations associated with aHUS. Prior studies provided evidence that mutations in the negative regulators of complement predispose to aHUS[12–15,17,41,42]. The work presented here, together with the recent studies of FB[43,44], provides clear evidence that a primary gain of function in activating proteins of the AP also predispose to aHUS. Altogether, ~70% of aHUS cases can be explained by a mutation in one of the regulatory proteins, C3 or FB, or by the presence of autoantibodies to FH[45,46]. Additionally, common haplotype risk factors are now being identified that may be a second hit along with a heterozygous mutation to lead to disease[3,47].

The majority of the aHUS patients are heterozygous and the patients have about half normal levels of the WT protein. There are three major negative regulators involved in control of the C3 activation. A single point mutation in any one of these regulatory

proteins predisposes to aHUS. Haploinsufficiency is thus not adequate to maintain homeostasis. These proteins do not have overlapping functions. They are not redundant, as was proposed initially by multiple groups. A 50% decrease in any one predisposes to aHUS.

In these studies, there were two cases of C3 haploinsufficiency, Y832X and C1136W. They do not fit the paradigm of a gain of function in an activating protein predisposing to aHUS. A case of haploinsufficiency of an activating protein should protect an individual from aHUS secondary to a lesser amount of C3 available for the AP. These mutations may be merely coincidental to the disease pathogenesis and development of aHUS.

The mutations described here also lend insight into the domains of C3 important for interactions with regulatory proteins. There have been reports (prior to the crystal structure of C3) of peptide sequences important for FH, CR1, and MCP interactions with C3. The structure of C3b in complex with FH 1-4 has been recently solved[39]. In this structural report, the authors determined that repeats 1-4 of FH contact C3b in multiple locations including in the α′NT, MG1, MG2, MG6, MG7, CUB and TED domains. Additionally, they mapped the mutations described in our first group of patients and found that most of these mutants with a defect in binding to FH mapped to the binding interface. These findings are therefore a conformation of our functional studies.

Recent reports of the interactions of C3b with FH domains 19-20 indicate that FH19-20 interacts with an area of the TED domain that is separated from the FH 1-4 interface. FH 1-4 is responsible for the cofactor activity, while FH 19-20 binds to C3b

and to anionic surfaces. Interestingly, one of the C3 mutants studied here (P1092L) is in the FH 19-20 binding region and had decreased FH binding. However, in my cofactor assays there was reduced cleavage of P1092L C3 by FH, suggesting that this binding interface may also influence FH 1-4 mediated cofactor activity.

The relative severity of aHUS associated with C3 mutations has yet to be well defined due to the small number of cases reported. Mutations in FH and FI lead to the worst outcome. However, aHUS in patients with C3 and FB mutations may also be as severe. Patients carrying MCP mutations have the best prognosis, probably because a donor kidney will replace the mutated MCP with normal MCP. With the recent identification of additional C3 mutations[48], we will be able to compile more data on the disease progression of C3 associated aHUS.

Table 3.3. Functional consequence of C3 mutations in aHUS patients.

MUTANT	ELISA BINDING		COFACTOR ASSAY		BIACORE	
	FH	MCP	FH	MCP	FH	MCP
K43N	++	+	+++	++/+++	↓	↓
K133Q	++	+++	+	+++	↓	↓
R139W	+++	+	+++	++	N	↓
R570Q	++	+	+++	+	NT	NT
R570W	++	+	+++	+	NT	NT
F581V	+++	+++	+++	+++	↓	↓
R713W	+++	++++	+++	+++	NT	NT
I734T	++	++++	+++	+++	NT	NT
R1020L	++	+	+	+	↓	↓
A1072V	++	–	+++	–	NT	NT
I1073S	+	+	+	–	↓	N
P1092L	+	++++	+++	++/+++	↓	↓
D1093N	++	–	+++	+	NT	NT
Q1139K	++	++	+	–	NT	NT
K1181R	++	+++	+++	+++	↓	↓
H1442D	+++	++++	+++	+++	NT	NT

Binding assay activity was graded as follows: ++++, >110%; +++, 90-110%; ++, 50-89%; +, 20-49%; –, < 20%.

Cofactor activity was defined as: +++, normal; ++, 50-90% of WT; +, 20-50% of WT; –, < 20% of WT.

For Biacore analysis ↓= less than WT binding levels, N= normal levels of binding, ↑=increased binding compared to WT C3.

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CHAPTER 3

Autoimmunity to the classical pathway C3 convertase leading to C3 deficiency and *Neisseria meningitidis* septicemia and meningitis

Introduction

Primary C3 deficiency is a rare disorder, in a 2000 review it was described in 19 families worldwide[1]. Patients with a C3 deficiency typically present with recurrent pyogenic infections cause by primarily encapsulated organisms such as *Staphylococcus aureus, Haemophilus influenzae, Streptococcus pneumoniae,* and *Neisseria meningitides.* These infections can lead to sepsis and become life-threatening[1,2]. C3 deficiency may also reduced the efficacy of vaccination, with particularly poor antibody responses to encapsulated pathogens[3,4]. The genetic cause of a primary C3 deficiency is commonly either an amino acid substitution that may lead to protein misfolding, or a premature stop codon[1,5,6].

Secondary C3 deficiency is seen in patients that synthesize C3 protein normally, but its consumption is accelerated. These patients may have a deficiency in a C3 regulatory protein such as Factor H (FH) or Factor I (FI)[1]. Deficiency in either of these inhibitors causes accelerated alternative pathway (AP) turnover and excessive turnover of C3. Another cause of secondary C3 deficiency is an autoantibody to the AP C3 convertase, C3bBb[7]. These antibodies are known as C3 nephritic factors (C3-Neph) due to their association with glomerular renal disease. When patients carry a C3-Neph, the C3 convertase is stabilized indefinitely and thus converts (by proteolysis) C3 to C3b. This C3b is then fragmented by FI in conjunction with a cofactor protein and the resulting proteolytic pieces are cleared from the circulation. More rarely, C4-nephritic factor (C4-Neph) has been reported[8–10]. C4-Neph is an autoantibody that stabilizes the

classical pathway convertase, C4b2a, and has been reported in a few patients with glomerular disease and two patients with lupus.

The C3 convertases are bimolecular enzyme complexes that cleave C3 to C3a and C3b[11]. The C3 convertase shared by the classical and lectin pathways (CP/LP) is composed of two subunits, C4b, which attaches covalently to the target, and the catalytic subunit C2a. The AP C3 convertase consists of C3b and the serine protease Bb, the large catalytic subunit of the zymogen Factor B. The convertase subunits are attached to one another non-covalently. The complexes are relatively unstable, each having a half-life of less than five min. Regulators in the blood and on cell surfaces can accelerate decay. These proteins include FH, Decay Accelerating Factor (DAF), Complement Receptor 1 (CR1) and C4 binding protein (C4BP)[12]. C4BP specifically accelerates decay of the CP/LP convertase, FH is specific for the AP convertase, and CR1 and DAF are able to regulate the both the CP/LP and AP convertases.

In this chapter, we studied an 18-year-old patient with a first-time *Neisseria meningitides* infection as the initial manifestation of his C3 deficiency. We show here that the C3 deficiency was caused by an autoantibody that stabilized the classical pathway C3 convertase.

Materials and Methods

Materials

Purified human complement proteins (C1, C2, C3, C3b, iC3b, C3c, C3d, C4, FB, C4 binding protein, and properdin), polyclonal antibodies against C3, FB and properdin and antibody sensitized sheep erythrocytes (EA cells) were purchased from Complement Technologies (Tyler, TX). A polyclonal antibody against C4 binding protein was purchased from AbCam (Cambridge, MA). Protein G agarose was obtained from Thermo Scientific (Rockford, IL). $DGVB^{++}$ buffer (veronal buffered saline: 0.015% sodium 5′, 5′′-diethylbarbiturate (pH 7.35) and 71 mM NaCl supplemented with 2.5% dextrose, 0.1% gelatin, 1 mM $MgCl_2$ and 0.15 mM $CaCl_2$) was prepared as described previously[13]. EDTA-GVB buffer contained 0.1% gelatin, 0.015% sodium 5′, 5′′-diethylbarbiturate (pH 7.35), 71 mM NaCl and 40 mM EDTA[13].

Sample collection

Informed consent was obtained under a protocol at Children's Hospital of Wisconsin approved by their internal review board. Blood samples were procured from the patient in EDTA vacutainer tubes for DNA analysis. For serum samples, blood was allowed to clot for 30 min at RT and, after centrifugation, the serum was removed and frozen in aliquots until analysis.

Genomic sequencing

DNA was isolated from the peripheral blood of the patient using a QIAmp Blood Maxi Kit (Qiagen, Valencia, CA). Sequencing primers for genomic C3 were designed using the Primer 3 software. Twenty-eight pairs of primers were used to cover the 41 exons and intron/exon borders. A minimum of 50 base pairs into each intron was sequenced. Primer sequences are listed in Table 1.

Western blotting

For C3 analysis, sera and purified complement proteins were electrophoresed on 10% Tris-Glycine polyacrylamide gels followed by transfer to a nitrocellulose membrane[14]. Membranes were blocked overnight with 5% non-fat dry milk in Phosphate Buffered Saline-Tween (PBS-T). Blots were probed with a 1:5,000 dilution of a goat anti-human C3 antibody (Complement Technologies, Tyler, Texas), followed by a rabbit anti-goat IgG HRP secondary antibody (Sigma, St Louis, MO). The blots were developed with Super Signal West Pico Substrate (Thermo Scientific, Rockford, IL).

C3 ELISA

Native C3, C3b and iC3b were coated on ELISA wells at 2 µg/ml in PBS. After overnight incubation at 4°C, wells were blocked with TBS (10 mM Tris, pH 7.4, 150 mM NaCl) containing 1% BSA. Serum samples were diluted in TBS containing 4% BSA and applied to the ELISA wells. Following an incubation at 37 °C, samples were removed by washing with TBS-Tween. Secondary antibodies to IgG or IgM were applied to the wells

Table 3.1. Genomic DNA sequencing primers for the C3 gene

Exon	Sense	Antisense
1	GGAAAGGCAGGAGCCAG	AAATGTCTGCTTCCACCCC
2	CACATCCGTGGAATGACAAG	GAGGGGCTCAGGAGGAG
3-4	GGCCTTGGAACAGACCC	TTGCCTCTCCTAAGCCTGTG
5-7	TCCGAAGAGCCACTTTATCC	GTCTTCACCTGGTCCCTCAC
8-9	ATTCTCCAGGAGGGATGGAC	CTTCTGACCTGGTCTCCCC
10-11	GGAGGTCTAATCCTGAGGGG	CCTGTACCGTCTTCCCAGTG
12	TCCCAGGTCTCAGGGATTC	GAAACAAGGAGGAGGCGG
13	AGCCTGATGCCAGCCTGT	GAGCCCAGGGCACACTTAC
14	CTCTCCCAGGGCTGACTTC	GTGCCTCCGCCTCTTCTC
15-16	ACACAGGTGCATATGTGGGG	TCCCCTCCTCCCTCTCTG
17	GAAGTCCTCCCTGGGGTC	TCCCTCCTCAGACAGGAGTC
18	GTGACCGCTGAGGAAGTAGG	GCAGGTGCATGCAAATTAAA
19	CCCCTCTACCACCCTGCTA	TGCCAAGAAACATAGGGTTG
20-21	CACTCCCCGACCTTGACAC	CCTGAGTCAATAGTACGAAGACC
22-23	TGCTGACCATCTGTGTGTCTG	CCATGCCCTCCTGGGAC
24	GTCACCCACCTGGCTTGAG	ATGAGGTGGGATCTTAGGGG
25	CTGTCCCCTCTCTGCACC	CCTAAGGGACCACCCCTG
26	GTTGACATGGCAGTCTCTGG	ACTCAGGAGCCCCTCTCTTC
27	GATGACTGCCATGTGTGGAC	GTGCTCTGCATCGGGTAAG
28	ACAGCAATGCCCACATGAC	AACTTCAGCCATGCATCTCC
29	AGGCTGGACAGAGCAGTGAG	CTAGGAGGCCAGTGGGAAG
30-31	CTAGCCACTTTCCCAGGCTC	AGAGGAGATGGTCCCTCTGG
32-33	TCCTAGTCCTGTGGGCTGAC	GAGATAGAGGGATGGCCAAG
34-35	CTGACCCCTCCTTGGGAC	CCAGATAGAGGTCAGGGTGC
36	CTCCCAAGACAATGCTGGAC	ACTCTCAGGCCCCTGGAAC
37-38	AGGGAGGCCCTTATCCTCTC	CACACACAGCTAAACATGCG
39-40	CCTCATGGTCAACCTAGCCC	ACAATGGTGTGGGCGTG
41	CACCATTGTCACGCCCC	GGCAAAGAACTCCAGACACG

(goat anti-human IgM (Sigma) or donkey anti-human IgG (Jackson Immunoresearch) using a 1:3000 dilution and plates were incubated for 1 h at 37 °C. Following a washing step, plates were developed using TMB substrate (Thermo Scientific) and the absorbance at 630 nm was determined[15].

Preparation of EAC14b2a cellular intermediates

The classical pathway C3 convertase was assembled on antibody-sensitized sheep erythrocytes (EA) as previously described[16,17]. Briefly, EA cells (5×10^8 cells/ml) were washed three times in $DGVB^{++}$. Purified human C1 was added to the cells and incubated at 30° C for 15 min, followed by washing in $DGVB^{++}$. Next, human C4 was added and cells were incubated for 15 min at 30° C, followed by washing in $DGVB^{++}$. Finally, purified human C2 was added in a limiting fashion (0.3 μg C2 to 5×10^8 cells), and cells were incubated at RT for 4 min, followed by a washing step. EAC14b2a cells were resuspended in $DGVB^{++}$ and utilized immediately.

Convertase stabilization hemolysis assay

NHS and the patient's serum were heat inactivated at 56° C for 30 min and then diluted 1:5 in $DGVB^{++}$. EAC14b2a cells (50 μl) were mixed with 50 μl of $DGVB^{++}$, NHS or patient's serum. Samples were incubated at 30° C to allow for decay of the convertase. At the indicated time points, guinea pig serum, diluted 1:20 in 40 mM EDTA-GVB buffer, was added as a source of complement components. Samples were

incubated for 1 h at 37° C. After centrifugation, hemolysis was assessed by measuring the absorbance of the supernatants at 414 nm.

In other experiments the sera were first pre-treated with Protein G agarose to remove immunoglobulin (per the manufacturer's instructions). An equal volume of Protein G agarose slurry was added to the heat inactivated sera. Samples were incubated on a rotator for 2 hrs at RT. Following centrifugation at 1,000 x g for 5 min, the supernatants were collected. Western blots were performed on the Protein G treated samples to visualize the efficiency of IgG removal. The blots were probed with a donkey anti-human IgG HRP labeled antibody (Jackson Immunoresearch).

C5 convertase stabilization assays

Two approaches were used for C5 convertase assessment. In the first approach, the C3 convertase was assembled as in the previous assays. Following C3 convertase assembly, the patient's IgG, a normal IgG control, patient's serum, NHS, or buffer alone was added at 4°C for 5 min. Next, cells were washed with $DGVB^{++}$, centrifuged, and the pellets were resuspended in $DGVB^{++}$. Purified C3 was then added to each sample and incubated for 5 min at RT. The cells were washed with $DGVB^{++}$, resuspended in EDTA-GVB and allowed to decay. C3-depleted serum was diluted 1:20 in EDTA-GVB and 400 µL was added to each tube as a source of terminal pathway components. After a 1 hr incubation at 37° C, samples were centrifuged and absorbance of the supernatants determined at 414 nm.

The second strategy first required assembly of the C5 convertase on the EA cells. EAC14b cells were prepared first as described above. C2 and C3 were then added simultaneously and incubated for 4 min at RT. Following washing and centrifugation, the cells were resuspended in $DGVB^{++}$ and treated with buffer alone, patient's IgG, normal IgG, patient's serum or NHS. Samples were allowed to decay for various times. The degree of hemolysis was ascertained by addition of C3 depleted serum (see above).

IgG purification

The IgG fraction was purified from the patient's serum and a control NHS using a Protein A column (Bio-Rad, Hercules, CA). The column was equilibrated in 50 mM borate (pH 8.5) containing 150 mM NaCl. The serum was applied to the column followed by extensive washing in the equilibration buffer. Immunoglobulins were eluted with 200 mM glycine (pH 2.5) containing 500 mM NaCl. The eluate fractions were neutralized by the addition of 1 M Tris (pH 9.0). The IgG containing fractions were pooled and dialyzed against PBS overnight. The IgG concentration was determined at an OD 280 nm using an absorbance coefficient of 1.35. Purity of the purified IgG was assessed by SDS-PAGE under reducing and non-reducing conditions followed by Coomassie Blue staining.

RESULTS

Patient description

The patient presented with *Neisseria meningitides* at 18 years of age. He had no prior history of pyogenic infections or major illnesses. At the time of hospitilization, a CH50 (complete complement titer) was performed and was below the limit of detection, and C3 antigen levels were also undetectable (Table 1). Further testing determined that his FB, C2 and C4 levels were normal, while his C5 level was somewhat reduced (~50% of normal). After a two-year follow-up the patient has remained healthy, while his C3 levels are still undetectable. There was no family history of complement deficiency.

Sequencing of patient's C3 gene revealed no mutations

Genomic DNA was sequenced using twenty-seven pairs of primers that encompassed all 41 exons and intron/exon borders of the *C3* gene. The sequences were aligned to the reference sequence available in GenBank with the accession number NG 009557. The sequence of the patient's *C3* gene was normal, with no truncations, splice site defects or amino acid substitutions identified. The patient was homozygous for the slow form of C3 (Arg80) and carried the more common Pro at position 292.

The sequencing results suggested that the C3 deficiency was not primary, but more likely related to accelerated turnover. One possibility was that, while the *C3* DNA was intact, the synthesis of the thioester bond, which is critical to C3 stability and function, was defective. However, this hypothesis was less likely in view of the fact that the patient has normal levels of C4, a second thioester containing protein of the

Table 3.2. Patient's complement levels in serum*

	Patient	Reference range
CP-CH50	<10	31-66 (units/ml)
AP-CH50	0	77-159 (units/ml)
C2	2.0	1.6-3.5 (mg/dL)
C3 (antigen)**	<11	82-185 (mg/dL)
C3 (function)	0	11249-42887 (units/ml)
C4	35	15-53 (mg/dL)
C5	3.4	5.5-11.3 (mg/dL)
Factor B	24	13-28 (mg/dL)

*Levels of Factor H, C4 binding protein and Factor I were comparable to those in normal serum by western blotting.
** C3 antigen was also measured in the patient's family father: 89; mother: 95; siblings: 85, 120, 101, and 93 (mg/dL); all within the normal range.

complement system that is closely related to C3. Another possibility was that the patient synthesized C3 normally, but it was prematurely cleared by, for example, an autoantibody to C3.

To screen for the presence of an autoantibody to C3, ELISA was performed. In comparison to NHS, there was no evidence of autoantibodies specific for C3 in the patient's serum (not shown). Therefore, we considered alternative mechanisms by which accelerated consumption of C3 could be occurring.

Patient's serum demonstrates C3 degradation products

On multiple occasions, the standard clinical laboratory test for C3 antigen (nephelometry) detected no protein in this patient's serum. Thus, if C3 were present in the patient's serum, it would be at very low levels. Therefore, his serum was analyzed in a western blot at a 1:5 or 1:10 dilution in contrast to NHS which is routinely diluted 1:500 or 1:1000 for western blots. In the NHS sample, as expected, the intact α chain (110 kDa) and intact β chain (75 kDa) were observed. In the patient's serum, an intact C3 α chain was not present; however, there was evidence of C3 α chain degradation fragments (α_{41} and α_{27}; 41 kDa and 27 kDa, respectively), as well as the intact 75 kDa β chain (Figure 3.1). These bands correspond to those observed in the C3c control.

C3c is normally generated from C3b through cofactor activity which is carried out by the serine protease FI in conjunction with a cofactor protein[18]. In the serum, the major cofactor protein is FH, and on the cell surface the most widely expressed cofactor protein

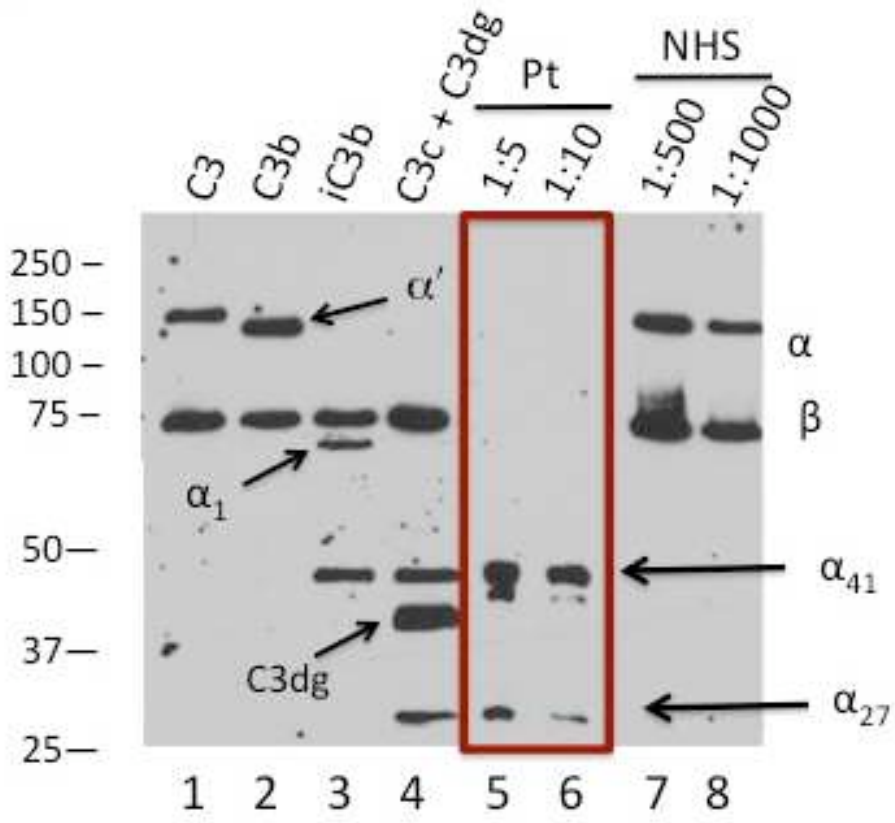

Figure 3.1. Western blot of patient's serum demonstrates C3 degradation fragments. Patient's serum (1:5 and a 1:10 dilution) and normal human serum (NHS) (1:500 and a 1:1000 dilution) were separated by electrophoresis on a 10% Tris-glycine gel under reducing conditions followed by transfer to a nitrocellulose membrane. The blot was developed with a goat anti-human C3 polyclonal (1:5000) Ab followed by a rabbit anti-goat IgG-HRP (1:3000) Ab. Purified C3, C3b, iC3b and C3c (10 ng each) were loaded as controls. Representative of 3 experiments.

is Membrane Cofactor Protein (MCP; CD46) whereas Complement Receptor 1 (CR1; CD35) has a more limited cellular distribution. Upon binding to C3b, FI cleaves it in two locations to first generate iC3b, releasing C3f, and then at a third site to generate C3c and C3dg (Figure 3.2). These results suggest that the patient's liver synthesizes C3, but that it is undergoing accelerated activation to C3b, followed by the expected pathway to inactivate C3b, which generates C3c. The patient therefore has a small amount of C3c in his serum which is derived from cofactor activity. This protein is not observed in normal human serum as it is only generated in small amounts in normal individuals and is rapidly cleared.

Normal C3 is degraded by the patient's serum

To assess if there was a factor in the patient's serum capable of activating C3, serum-mixing experiments were performed (Figure 3.3). At a 1:50 dilution of the patient's serum, no C3 fragments are visualized (Figure 3.3, lane 8). When NHS was mixed with the patient's serum, C3 degradation fragments are visible (lanes 2, 3 and 4) consistent with iC3b. This effect was dose dependent, as the evidence of consumption diminished as the amount of the patient's serum added was decreased (compare lane 2 to lanes 3-7). These results indicate that there is a factor in the patient's serum capable of activating normal C3 to C3b.

Additionally, the cleavage pattern is consistent with FH and FI mediated cleavage of newly generated C3b, based on the presence of the β chain and these two α chain fragments. If the cleavage events were mediated by a non-specific protease, one would

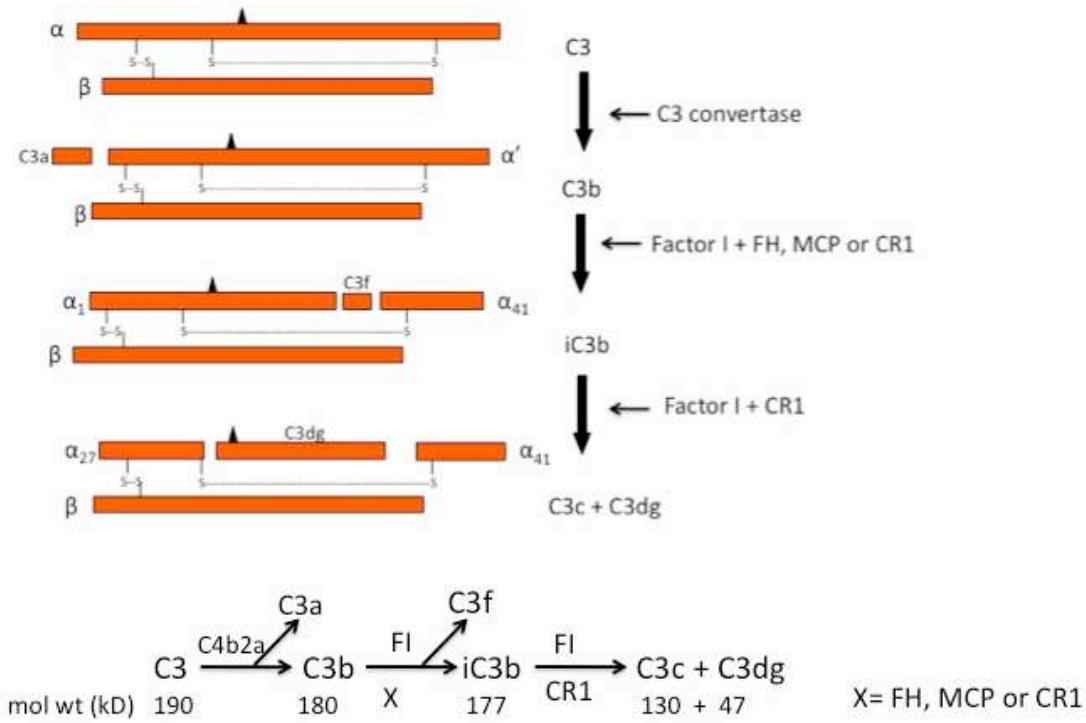

Figure 3.2. Schematic diagram of C3 activation and degradation.

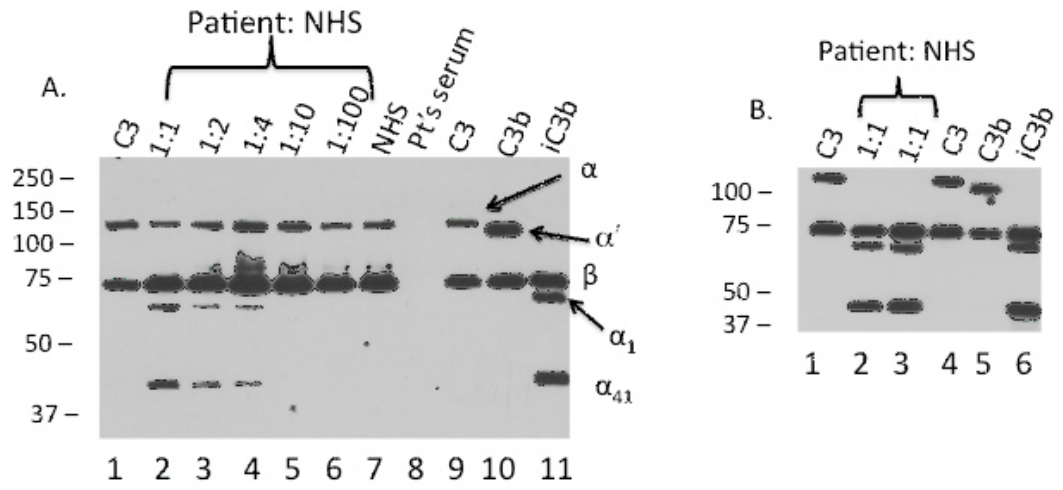

Figure 3.3. Patient's serum activates C3 in normal human serum (NHS). A. Pt's serum and NHS were diluted 1:50 in PBS and mixed together at the indicated ratios. At the 1:50 dilution, no C3 fragments are detectable in the patient's serum (lane 8). Therefore, all C3 bands seen by western blot are from the NHS. Samples were incubated for one h at 37°C. Reactions were electrophoresed on a 10% Tris-glycine gel under reducing conditions and then transferred to a nitrocellulose membrane. Western blots were developed as per Fig 1. Lanes 1, 9, 10 and 11 are controls (10 ng each). Data shown are representative of 8 independent experiments. B. Pt's serum and NHS were mixed together undiluted at a 1:1 ratio, and then incubated overnight at 37°C. Reactions were electrophoresed and western blot was developed as in panel A. Lanes 1, 4, 5, and 6 are controls (10 ng). Lanes 2 and 3 are two patient serum samples which were obtained ~6 months apart.

expect to see bands of other molecular weights on the western blot; instead, the bands correspond to those generated by FI (cofactor activity). The lack of cleavage from iC3b to C3c in these mixing experiments can be explained by the fact that there is no CR1 present in serum. CR1 is the predominant cofactor for FI in the further cleavage of iC3b to C3c. In the patient, C3c may be seen because CR1 is expressed on his cells that mediate the final cleavage event by FI.

Because native C3 is not susceptible to cofactor activity, this suggests that in this patient his C3 is being activated to C3b and then degraded by FI. In the complement cascade, the activation of C3 to C3b is mediated by either the classical/lectin pathway (C4b2a) and alternative pathway (C3bBb) C3 convertases. If either of these were to be stabilized, this could lead to consumption of C3. For example, C3 nephritic factor (C3-Neph) is an autoantibody to the alternative pathway C3 convertase that stabilizes this enzyme complex[19,20]. Patients with C3-Neph present with low C3 and FB levels secondary to consumption by the stabilized AP C3 convertase. However, no C3 nephritic factor was detected in this patient's serum and the FB level was normal (Table 3.2). Therefore, we hypothesized that the classical pathway convertase was stabilized in this patient.

The patient's serum stabilizes the classical pathway C3 convertase

To determine if the patient's serum stabilizes the classical pathway C3 convertase, decay accelerating assays were performed using hemolysis of antibody sensitized sheep red blood cells (EA) as the readout. In these assays, the convertase is assembled on EA

and then allowed to decay for various times before addition of the terminal pathway complement components to produce hemolysis. The convertase spontaneously decays (natural decay), resulting in less hemolysis at later time points. If the convertase is stabilized, more hemolysis will be observed.

The samples to which buffer alone was added demonstrate the expected natural decay of the convertase (Figure 3.4). If the patient's serum was added to the EA cells, there was no spontaneous decay of the C3 convertase. The accelerated decay of the convertase (as compared to the buffer alone control) with exposure to NHS is due to C4BP in the NHS.

Taken together, these results indicate that a factor in the patient's serum is both capable of stabilizing the CP C3 convertase and of inhibiting decay by C4BP (normal in his serum- see Table 3.2). If the assay time was extended up to eight hours, there was still no decay in the presence of the patient's serum (not shown). Additionally, if exogenous C4BP was added to the samples containing patient's serum or normal serum, convertase decay was still not observed. This confirms that the factor in the patient's serum is inhibiting convertase decay by C4BP.

IgG is the factor in the patient's serum that is stabilizing the classical pathway C3 convertase

To assess if the convertase stabilizer/decay inhibitor was in the IgG fraction, we depleted the patient's serum and NHS of IgG using Protein G beads (Figure 3.5). Untreated, the patient's serum prevented decay of the convertase (as previously

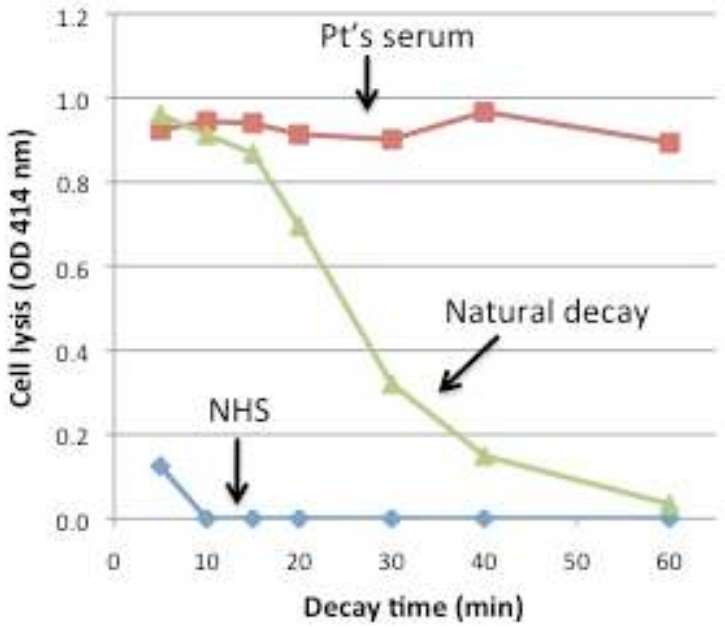

Figure 3.4. The classical pathway C3 convertase is stabilized in the presence of the patient's serum. The classical/lectin pathway C3 convertase (C4b2a) was built up on antibody sensitized sheep erythrocytes (EA). The patient's serum, NHS or buffer (natural decay) was added and then the convertase was allowed to decay at 30° C. At the indicated time points, EDTA-treated guinea pig serum was added and samples were incubated for an additional h at 37° C. Following centrifugation, hemolysis was assessed by reading the absorbance at 414 nm. Results are representative of 3 independent experiments.

demonstrated). Following a single adsorption of the serum with protein G beads, the level of hemolysis was reduced by 50% (Figure 3.5A). A second exposure to protein G beads reduced the hemolysis to 16%, comparable to that in the samples that received NHS. This stepwise loss of stabilization paralleled the removal of IgG from the serum as determined by western blotting (Figure 3.5B).

To further confirm this result, the IgG fractions purified from the patient's serum and NHS were assessed for purity by SDS-PAGE followed by Coomassie blue staining (Figure 3.6). The IgG samples were also analyzed in hemolysis assays (as described in Figure 3.3). The IgG from the patient's serum stabilized the classical pathway C3 convertase while the IgG from NHS did not (Figure 3.7). Thus, the factor leading to consumption of the C3 in this patient is likely an autoantibody to the classical pathway C3 convertase.

C5 convertase stabilization assays

To determine whether the patient's IgG was also capable of stabilizing the C5 convertase, C5 convertase stabilization assays were performed. These experiments were performed in two ways. In the first approach, the C5 convertase was assembled on EA cells (EAC14b2a3b). Following washing, the cells were treated with the patient's serum or IgG, NHS or normal IgG, or buffer alone and then incubated for various times to allow for decay. The results of these experiments (Figure 3.8) indicated that the IgG in the patient's serum could not stabilize the preformed C5 convertase.

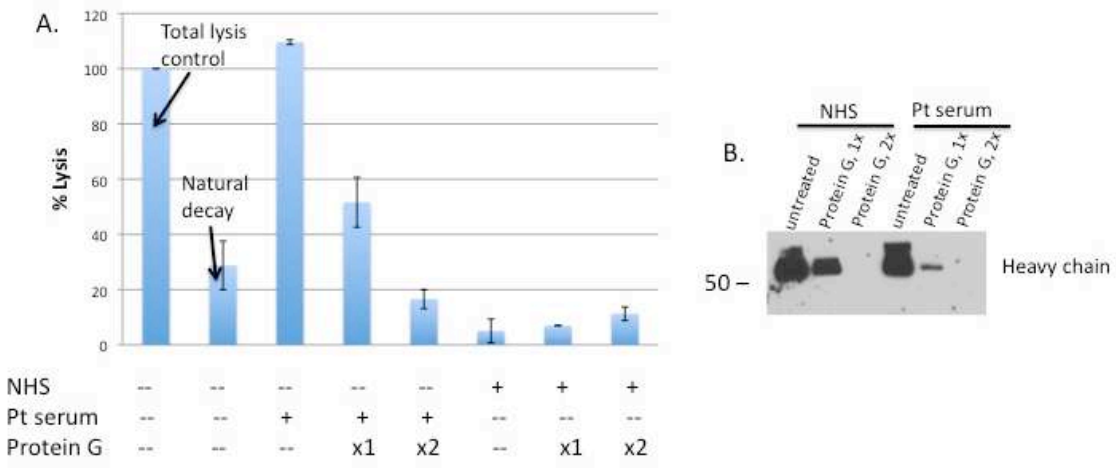

Figure 3.5. Stabilization of the classical/lectin pathway C3 convertase by the patient's serum is IgG dependent. Serum samples were treated with Protein G (PG) Sepharose to remove IgG. (A) Following centrifugation, the supernatants were employed in a classical pathway hemolytic assay to assess decay of the C4b2a enzyme complex (as per Fig 3). Following addition of the supernatants, EA cells were incubated at 30° C for 30 min to allow for convertase decay. Shown is the mean +/-SEM of 3 experiments. (B) PG treated serum samples were electrophoresed on a 10% Tris-glycine gel and transferred to a nitrocellulose membrane for western blotting. The blot was developed with a donkey anti-human IgG (1:5,000 dilution) Ab.

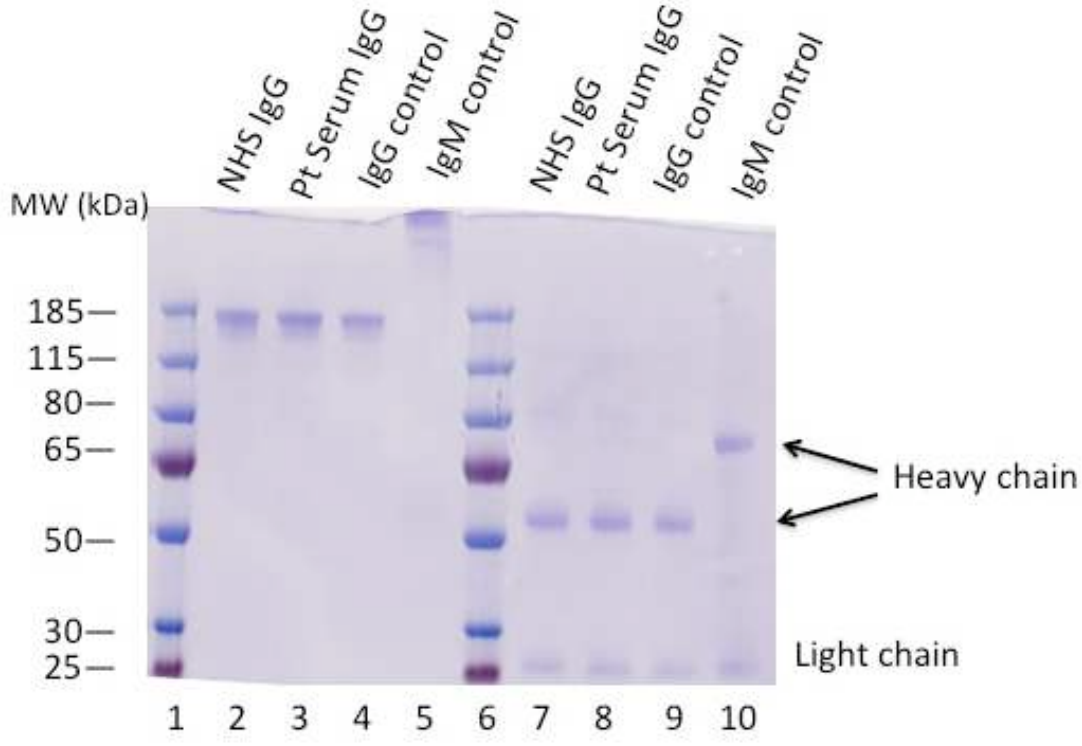

Figure 3.6 Purified IgG from patient's serum and NHS exhibit the expected electrophoretic mobility. Samples from the pooled fractions were electrophoresed on a 4-12% Bis-Tris gel. Commercially available IgG and IgM were used as control proteins (2 μg per lane). The gel was stained with 0.05% Coomassie blue. Lanes 1-5, non-reducing conditions; lanes 6-10, reducing conditions.

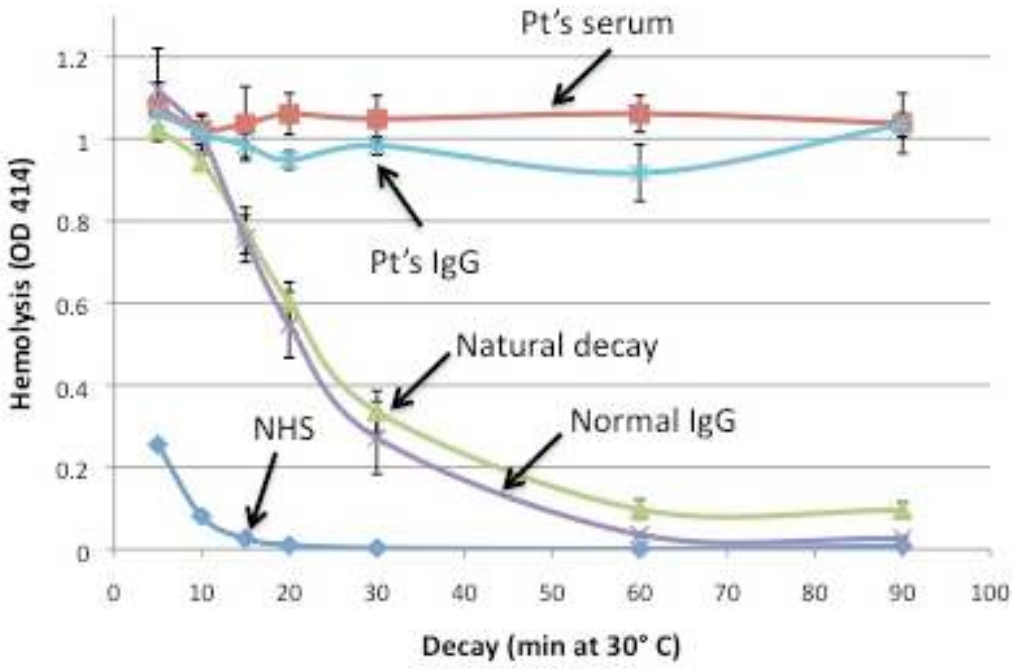

Figure 3.7. Patient's purified IgG stabilizes the classical pathway C3 convertase. IgG was isolated from NHS and the patient's serum by a Protein A column. The convertase containing cells were incubated with 25 μg of IgG at 30° C for the indicated times. At each time point, an aliquot was removed and EDTA-treated guinea pig serum added. The samples were then incubated for 1 h at 37° C. Following centrifugation, absorbance at 414 nm was measured. Shown is the average of two independent experiments, +/- SEM.

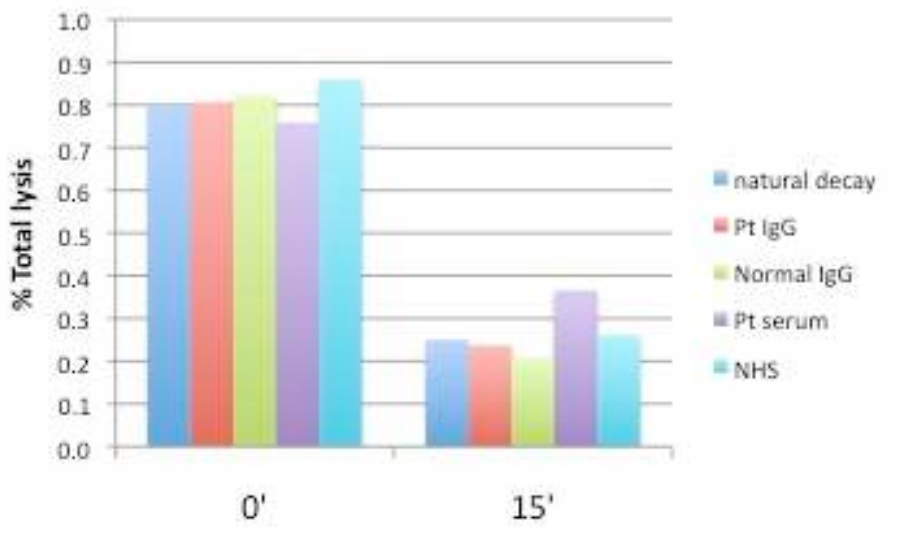

Figure 3.8. Patient's purified IgG does not stabilize the classical pathway C5 convertase. IgG was isolated from NHS and the patient's serum as described. The C5 convertase containing cells were incubated with 25 µg of IgG at 30° C for the 15'. At each time point, an aliquot was removed and EDTA-treated C3-depleted serum added. The samples were then incubated for 1 h at 37° C. Following centrifugation, absorbance at 414 nm was measured. The data shown is representative of three independent experiments.

The second approach was a two-step assembly. The C3 convertase was assembled, followed by addition of the patient's or the normal samples, and then, C3 was added to form the C5 convertase. This experiment design more closely mimics the *in vivo* formation of a C5 convertase. The results of these experiments demonstrated that there was stabilization of the C5-convertase when the cells were treated with the patient's serum, but not with the patient's IgG. In further experiments, purified IgG from the patient's serum was added back to NHS that had been depleted of IgG by protein G. (Figure 3.9). In these samples, there was no stabilization of the convertase.

From this set of experiments, we conclude that the patient's IgG cannot stabilize the preformed C5-convertase. However, the results from the two-step C5-convertase assembly assays are unclear. The patient's whole serum stabilized the C5-convertase, but the patient's purified IgG did not. One possibility is that the C5 stabilizing activity did not co-purify with the C3-convertase stabilizer and was lost during purification. The IgG was purified on a Protein A column, which does not bind human IgG3. If the C5-convertase stabilizer was a different isotype than the C3-convertase stabilizer, perhaps it was lost during the purification.

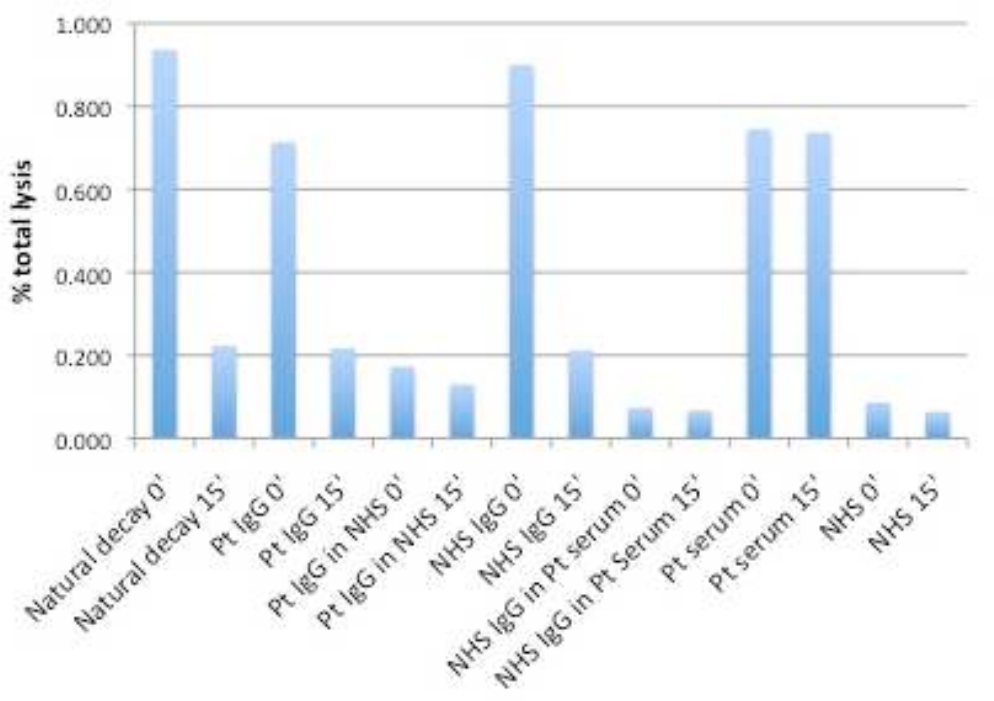

Figure 3.9. The patient's IgG and serum give different results in a two-step C5 convertase stabilization assay. The C3 convertase was assembled on EA cells by the subsequent addition of C1, C4, and then C2. Next, NHS, patient's serum, purified patient's IgG, purified normal IgG, purified patient's IgG added back to NHS, purified normal IgG added back to pt's serum, or buffer alone were added to the cells. Following a 5' incubation on ice, native C3 was added. Cells were incubated at RT for 4' and then washed. Following resuspension of the cells, the convertase was allowed to decay at 30°C for 0' or 15'. C3 depleted serum in EDTA-GVB was added and cells were allowed to lyse for 1 hr at RT. Following centrifugation, the absorbance of the supernatants was read at 414 nm.

DISCUSSION

Here we describe a patient who presented with a life-threatening *Neisseria meningitides* sepsis and meningitis. Clinical laboratory tests revealed an undetectable whole complement titer secondary to an absence of C3. Genomic sequencing of the patient's C3 gene did not identify a deletion, mutation or splice site defect. Western blot analysis established that there was a very low level of degraded C3 antigenic fragments in the patient's serum, suggesting that C3 was being synthesized. If the patient's serum was mixed with NHS, activation of normal C3 was observed. The factor cleaving C3 was identified as the classical/lectin pathway C3 convertase. This observation was accounted for by identification of an autoantibody that stabilized the CP/LP C3 convertase.

The development of an autoantibody to the AP C3 convertase (C3-Neph) was described in the 1970's[20,21]. It was usually found in association with a membranous glomerulonephritis. In particular, it was described with Type II MPGN (Dense Deposit Disease), partial lipodystrophy and SLE. There have only been a few reports over the past 30 years of this phenomenon[19].

There have also been a rare reports of a C4 nephritic factor (C4-Neph), an autoantibody that stabilizes the classical/lectin pathway C3 convertase[8,10,16]. In the largest series in the literature, about 100 patients with membranous glomerulonephritis and C3 levels less than 40% of normal were screened for C3 and C4 nephritic factors[16]. Of the 37 patients who met these criteria, about one-third each had C3-Neph, C4-Neph or both. In another study of lupus patients, two of 16 screened were shown to have C4-

Neph[10]. The complement levels reported in the MPGN patients with C4-Neph and the patient described here are similar in that C3 was low and C4 and FB levels were normal. However, our patient had no renal abnormalities at presentation or during a two-year follow up.

Interestingly, the patient described here had been previously healthy with no history of unusual infections until he developed the *Neisseria* infection at the age of 18, suggesting that the stabilizing antibody was recently acquired. One possibility to explain the newly acquired antibody is that, during the course of an infection, the adaptive immune system mistakenly recognized a neoepitope on the convertase. However, it is known that, like the alternative pathway, the classical pathway also undergoes a continuous low-grade tick-over, secondary to the low level, spontaneous activation of C1[22]. This classical pathway tick-over indicates that a C3 convertase is continuously being formed in small amounts in blood. Why this patient generated a B cell response to this autoantigen is unknown.

This patient's presentation and clinical course to date seems to be unique. We could find no case in the literature in which a patient with C4-Neph came to medical attention because of a *Neisserial* infection. As noted, C4-Neph has been described with MPGN in three reports[8,9,16] and with two SLE patients in another[10]. We could find no reports on such patients since 1994. This patient does not have glomerulonephritis or SLE as the reported patients with C4-Neph did. It will be of interest to monitor this patient's clinical case to see if it results in glomerular disease in the future.

The patient's C5 level was about 50% of normal and of course, he could not generate a C5 convertase. It is still unclear whether his low C5 is induced by a stabilized C5 convertase or an increase in C5 cleavage by the C3 convertase. The absence of properdin or any component of the membrane attack complex (C5-9) predisposes to *Neisserial* infections[7]. In our patient, the lack of C3 was presumably why he developed this infection.

This antibody could be a useful tool. The structural analysis of the classical pathway C3 convertase has been hampered by the highly transient nature of the complex. Having an antibody that stabilizes this complex could facilitate such structural studies and provide further insight into the assembly and function of the classical/lectin pathway C3 and C5 convertases. Also, this type of a reagent could be utilized to selectively deplete C3 *in vivo*.

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CHAPTER 4

Concluding remarks and future directions

Introduction

The complement system is an essential branch of the innate immune system[1]. It serves as a first line of defense against invading pathogens as well as having an important role in debris clearance. Through three protein cascades, the classical, lectin and alternative ptathways, it recognizes danger associated molecular patterns, and leads to the deposition of the central component, C3, on a target[2,3]. A properly functioning complement system requires tight regulation[4]. Because the complement system can be quickly amplified through the alternative pathway's amplification loop, mechanisms are in place to prevent inappropriate complement deposition on host cells and tissues. When this homeostatic regulation is disrupted, pathogenic states usually develop[5]. Some examples of these include paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS), dense deposit disease (DDD) and age-related macular degeneration (AMD)[6]. The focus of this dissertation has been to examine complement dysregulation at the step of C3 activation in two human disease states.

Summary and conclusions: aHUS

The first aim was to study the role of C3 mutations in aHUS. In this disease, patients present with microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure. Previous studies have shown that mutations in the complement regulatory genes FH, FI and MCP predispose to development of the disease[7–9]. Here, I described mutations in the central component, C3, that were identified in aHUS patients.

Sequencing of the C3 gene was carried out in three cohorts of aHUS patients. The mutations were heterozygous in all cases. To study the mutant C3 proteins, I produced them recombinantly in mammalian cells and then characterized their interaction with the regulatory proteins MCP, FH and CR1, as well as the activating component FB. Utilizing ELISA, a fluid phase cofactor assay and SPR analysis, in the majority of the cases (13/16) the mutation caused defective interactions with one or more regulatory protein(s). This decreased binding to an inhibitor leads to a secondary gain of function. One mutation, R139W, also had an increased ability to bind to Factor B, resulting in a longer-lived C3 convertase. In endothelial cell assays, an increase in C3 deposition was observed with serum containing R139W C3 compared to WT C3.

These experiments represent the first examples of mutations in C3 that predispose to aHUS. Together with the studies of the mutations in the complement regulatory genes, abnormalities in the complement system have now been strongly linked to development of aHUS. The identification and characterization of these mutations has pointed to inhibitors of the complement system as a therapeutic target for aHUS patients[7,10]. It has also focused attention on the critical nature of complement regulation on the endothelial cells of the kidney microvasculature.

Model: aHUS

A model aHUS development is shown in Figure 4.1. There is low-level activation of the AP at all times, through spontaneous hydrolysis of C3 (~1-2% per hour)[5]. In a normal kidney, this activation in the microvasculature is held in check through the action

of regulatory proteins. Each endothelial cell has approximately 1.5 million copies of CD59 (MAC inhibitor), 400,000 copies of MCP and 200,000 copies of DAF (personal communication, Anna Richards). If C3b is deposited on a cell surface, it is inactivated to iC3b by MCP or FH and FI[9]. However, in the glomerulus of a patient with a mutation in a complement gene (Figure 1, bottom panel), the inhibition is less efficient[9,11]. Upon a triggering event causing endothelial cell stress or damage, the complement system is further activated. If the amplification loop is not regulated normally, excessive complement activation leads to undesirable deposition and damage to endothelial cells. Adhesion molecules are upregulated on the endothelial cells, probably by C3a and C5a. This receptor engagement also recruits inflammatory leukocytes. Endothelial cell swelling or detachment may lead to exposure of the subendothelial matrix, further inducing a prothrombotic state. The result is formation of a fibrin and platelet rich clot in the microvasculature.

Future directions: aHUS

Genetic studies are ongoing in aHUS patients. Approximately 70% of patients have a mutation in a complement gene or autoantibodies to FH (<10%)[10,12]. However, 20 to 30% of patients still have no genetic defect identified. It will be of interest to determine if these patients have mutations in other complement- or coagulation-related genes. Also, while a defect in the complement system is a predisposing factor to aHUS development, the mechanisms linking this to clot formation have not been defined.

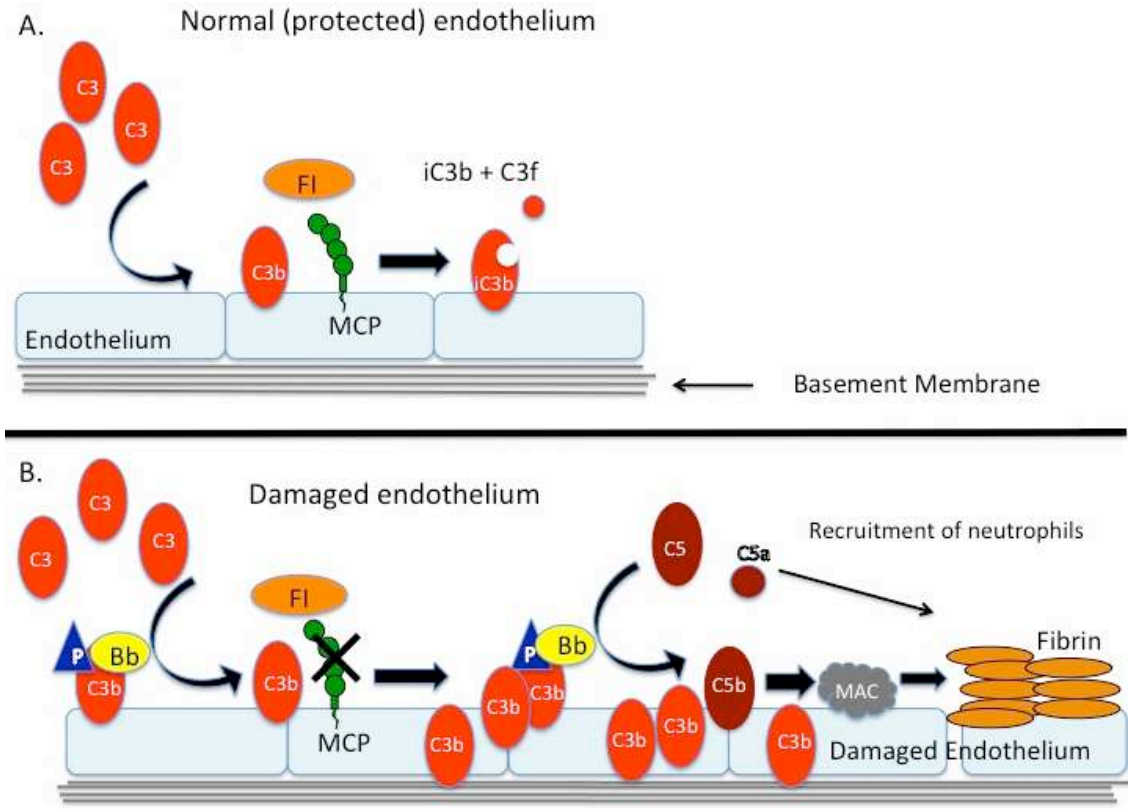

Figure 4.1. Model of damage to endothelium in aHUS. In a normal kidney (A) regulators of C3b quickly inactivate it to prevent convertase formation and further activation. When complement is dysregulated (B) deposition of C3b leads to amplification of the AP, thus more C3b deposition, C5 convertase formation and MAC formation. This results in damage to the endothelial cells, neutrophil recruitment and fibrin clot formation.

It is clear that there is cross talk between complement and coagulation. Studies have shown that C5a can be generated in the absence of C3, and have implicated the role of thrombin in this event[13]. Another report revealed that C5a is able to induce tissue factor expression on endothelial cells[14]. Tissue factor leads to the generation of thrombin, which is the key factor converting fibrinogen to fibrin[15]. The formation of a fibrin clot causes ischemic events in the renal glomerulus in aHUS. Finally, mutations in thrombomodulin have also been described in aHUS[16]. Thrombomodulin is a membrane bound anticoagulant protein that down-regulates thrombin generation and has recently been shown to inhibit the AP by enhancing FI mediated inactivation of C3b.

Further studies of the interaction between the complement system and the coagulation cascade will help to elucidate mechanisms of disease development, as well as provide insights into the cross-talk between these two systems that are critical components of immunity and homeostasis.

Summary and conclusions: Secondary C3 deficiency

The second aim of this work focused on a case of C3 deficiency. This patient presented at the age of 18 with a life-threatening infection with *Neisseria meningitides*. His history was notable in that he had no prior history of serious pyogenic infection or other major illnesses. Clinical laboratory testing revealed an undetectable whole complement titer and absence of the C3 antigen. I sequenced his DNA and identified no abnormalities in the C3 gene. Western blot analysis of his serum revealed trace C3 fragments. Importantly, in mixing experiments, his serum activated the C3 in normal

human serum. Hemolytic assays indicated that this was due to stabilization of the classical pathway C3 convertase and that the lytic activity was in the IgG fraction. Thus, we concluded that this patient had an autoantibody to the classical pathway C3 convertase. This stabilized convertase led to complete consumption of his C3, inducing a secondary C3 deficiency. A few cases of secondary C3 deficiency such as in our patient were previously described as C4 nephritic factors[17–19]. However, there have been no reports of this phenomenon over the past nearly twenty years. This autoantibody was so-named due to the presence of glomerulonephritis in all of these patients. However, this patient did not have renal disease at presentation or after a two year of follow-up.

The generation of C3b by the classical pathway C3 convertase is usually regulated by three mechanisms. The first is the natural decay of the convertase, with a $t_{1/2}$ of two to three minutes. The second mechanism is decay accelerating activity (facilitated decay), which is provided by DAF on cells and C4BP in the fluid phase. The autoantibody in this patient prevents natural decay and blocks decay accelerating activity by the two regulatory proteins. The third mechanism is cofactor activity. The inability of MCP or C4BP to bind to the CP C3 convertase bound by the autoantibody prevents them from serving as a cofactor protein for cleavage of C4b by FI. The outcome therefore of this pathologic situation is massive consumption of C3 and inappropriate deposition of C3 fragments. Typically, in the limited literature, this leads to a glomerular disease[20]. This patient does not have glomerulonephritis but rather presented with a *Neisseria* infection which is more commonly associated with a complete deficiency in properdin or one of the terminal pathway components (C5-C9)[21].

Finally, the study of this C3 deficient patient supports the concept of continuous classical pathway turnover. A recent study demonstrated that C1 undergoes spontaneous activation leading to cleavage of C4 and C2[22]. In this patient, the spontaneous turnover would provide a constant supply of antigen (convertase) for the autoantibody to bind and stabilize.

Future directions: C3 deficiency

Further studies of the binding interaction of the antibody with the classical pathway C3 convertase are ongoing. It is most likely that the antibody is directed towards a neoepitope formed when the two subunits (C4b and C2a) are in a C3 convertase. The patient also has half normal levels of C5, so perhaps he is also turning over this component. Formation of a C5 convertase is inefficient in the fluid phase, but the C3 convertase can also cleave C5, although with less efficiency than it cleaves C3[23]. Perhaps the stabilized convertase is now able to cleave more C5, in the setting of its longer half-life and low levels of its preferred substrate, C3. Purification of this antibody may prove to be useful for studies of the classical pathway convertase, which has not been crystallized due to its short half-life. Finally, the ability to selectively deplete C3 *in vivo* in order to prevent undesired complement deposition could also be possible with this autoantibody.

Final thoughts

The role of the complement system in health and disease is still being elucidated. Several disorders have been linked to poorly controlled complement activation, including aHUS, DDD, AMD, PNH and forms of glomerulonephritis. It is clear that a fine balance must be maintained between activation and regulation. The two cases presented here provide an interesting contrast relative to complement regulation. In the first case, there is a relative defect in regulation (haploinsufficiency) of the mutated C3b once it is deposited on self-cells. This lack of appropriate regulation leads to endothelial cell damage and clot formation. In the second case, regulation of the convertase is blocked by the autoantibody. Therefore, all of the C3 is turned over, resulting in C3 deficiency. Interestingly, in many cases of secondary C3 deficiency such as this one, the patient presents with renal disease. In both cases then, there is excessive complement deposition leading to tissue damage in the kidney. Additional analysis of the patient may uncover why this patient has not developed kidney disease. Defining the specific mechanisms that maintain homeostasis of the complement system will further our understanding of disease states and lead to improved therapy for these conditions.

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APPENDIX 1

Frémeaux-Bacchi, V. *et al*. Mutations in complement C3 predispose to development of atypical hemolytic uremic syndrome. *Blood* **112**, 4948-4952 (2008) © by the American Society of Hematology.

Brief report

Mutations in complement C3 predispose to development of atypical hemolytic uremic syndrome

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Atypical hemolytic uremic syndrome (aHUS) is a disease of complement dysregulation. In approximately 50% of patients, mutations have been described in the genes encoding the complement regulators factor H, MCP, and factor I or the activator factor B. We report here mutations in the central component of the complement cascade, C3, in association with aHUS. We describe 9 novel *C3* mutations in 14 aHUS patients with a persistently low serum C3 level. We have demonstrated that 5 of these mutations are gain-of-function and 2 are inactivating. This establishes C3 as a susceptibility factor for aHUS. (Blood. 2008;112: 4948-4952)

Introduction

Mutations in the genes encoding the complement regulators factor H,[1-6] factor I,[7,8] and membrane cofactor protein (MCP; CD46),[9,10] as well as in the activating component factor B,[11] have been detected in approximately 50% of patients with atypical hemolytic uremic syndrome (aHUS).[12] A proportion of the remaining patients have persistently low serum levels of C3. In this study we have examined the hypothesis that mutations in the gene encoding C3 could be associated with aHUS in these patients.

C3 is the pivotal component of the complement system.[13] Activation of the classical, lectin, and alternative pathways results in cleavage of C3 to generate C3b and the anaphylatoxin C3a. When C3b is produced, the thioester is cleaved, and then this highly reactive species may bind covalently to targets. Interaction of the zymogen factor B with C3b and subsequent cleavage of factor B by factor D results in formation of the alternative pathway C3 convertase C3bBb. This set of reactions represents an amplification loop.

A series of complement regulators including factor H and MCP prevent feedback via this loop by increasing the rate of dissociation of C3bBb and/or by serving as cofactors for the serine protease factor I to cleave C3b. Mutations in the gene encoding factor B were recently found to enhance formation of C3bBb or increase resistance to inactivation.[11]

The importance of *C3* as a susceptibility factor for human disease has been emphasized by recent studies documenting that a common nonsynonymous coding change in *C3* (rs2230199, Arg80Gly, corresponding to C3S and C3F) is both a susceptibility factor for age-related macular degeneration[14] and associated with long-term renal allograft survival.[15]

Methods

Subjects

In 2 independent cohorts of aHUS patients (Paris, France and Newcastle upon Tyne, United Kingdom), 26 patients (17 Paris, 9 Newcastle) with a serum C3 level persistently below the lower end of the normal range of 680 to 1380 mg/L were identified. In these patients functionally significant mutations in *CFH*, *MCP*, *CFI*, and *CFB* had not previously been detected. Mutation screening of *C3* was undertaken in these patients.

Approval for this study was obtained from the Departement de la Rechereche Clinique et du Developement, DRRC Ile de France, France and the Northern and

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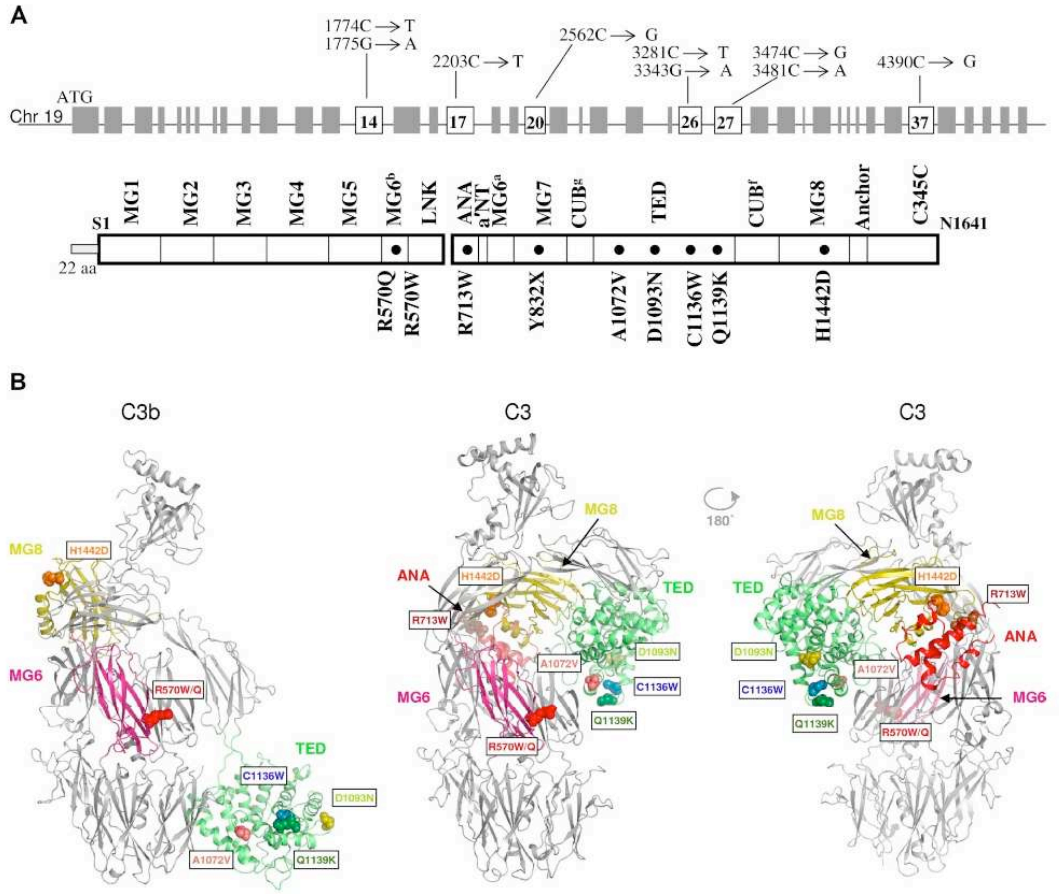

Figure 1. Location of *C3* mutations associated with aHUS. (A) Gene structure of *C3*, domains of the encoded protein, and position of the mutations. Note that the genomic structure numbering begins with the start site ATG, while the protein structures begin with the first amino acid of the mature protein. (B) Structures of complement component C3 and C3b showing the locations of mutations identified in atypical hemolytic uremic syndrome (aHUS) patients. Ribbon representation of C3b and C3 (2 views) with the domains containing mutations (labeled spheres).

Yorkshire Multi-Center Research Ethics Committee, United Kingdom. Informed consent was obtained in accordance with the Declaration of Helsinki.

Mutation screening

The coding sequence of *C3* was amplified with flanking primers (Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Direct sequencing was undertaken using a 96-capillary Sequencer 3700 (Applied Biosystems, Courtaboeuf, France) using the dye terminator method. For the genomic DNA sequence the first nucleotide A of the initiator ATG codon is denoted as nucleotide +1. The amino acid numbering does not include the 22 residues of the signal peptide.

Functional studies

C3 cDNA (gift of David Isenman, University of Toronto, Toronto, ON)[16] was sequenced and compared with the sequence published for the C3 crystal structure.[17] Two single basepair changes were found in the cDNA and altered (QuikChange Multi Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA) to match the published sequence of the C3 used in the structural analysis.[17] Mutant clones were produced (QuikChange XL Site-Directed Mutagenesis Kit; Stratagene) and sequenced in both directions.

The mutant and WT C3 DNAs were transiently transfected into either 293T or COS-1 cells using Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA). Two to 3 days after transfection, the supernatants were collected and concentrated. C3 was quantitated by ELISA and examined by Western blotting (see Document S1). Conversion of C3 to iC3 was accomplished by storage at 4°C or repetitive freeze thawing and monitored by autolytic cleavage[18] (see Document S1). C3b ligand-binding assays were undertaken using recombinant MCP (see Document S1 for protocol), factor H (Complement Technologies, Tyler, TX), soluble CR1 (gift of H. Marsh, Avant Immunotherapeutics, Needham, MA) and factor B (Complement Technologies). Cofactor assays were undertaken using wild-type and the mutant C3 proteins, human factor I (Complement Technologies) and the above noted cofactor proteins.

See Document S1 for methodology relating to assays for cofactor protein binding to C3 and cofactor activity.

Results and discussion

In 11 patients, we identified a heterozygous *C3* mutation. Three additional family members were also affected by HUS, 2 from a

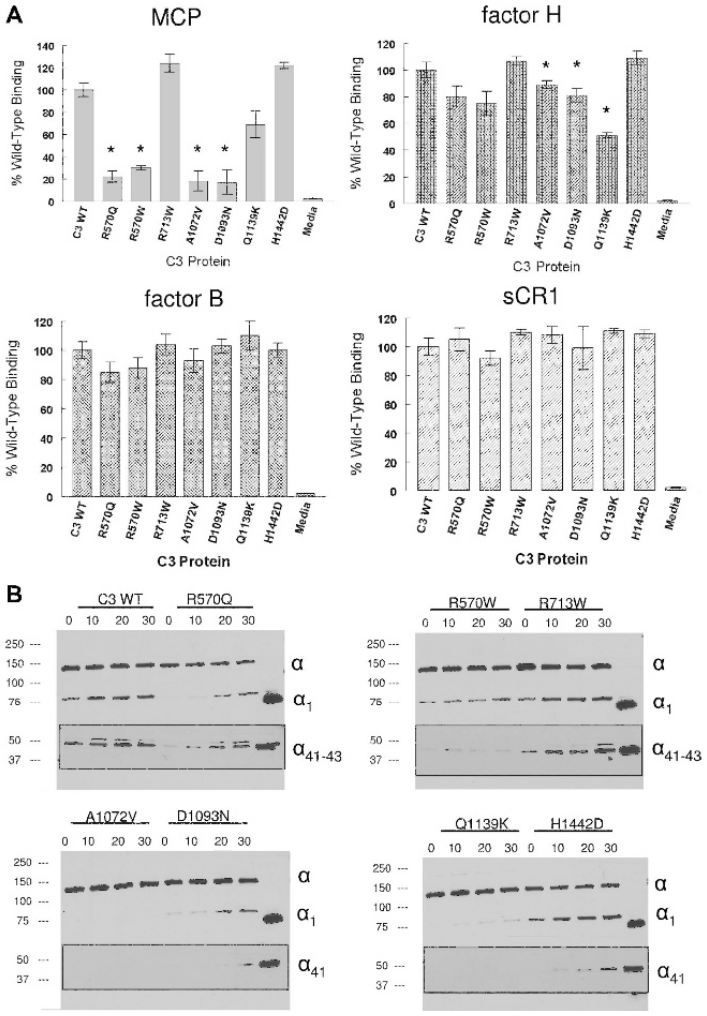

Figure 2. Ligand binding and cofactor activity of the C3 proteins. Proteins were transiently expressed in 293T cells, concentrated and quantified before analysis (see Document S1). (A) Binding to MCP, factor H, factor B, and soluble CR1 (sCR1) in ELISA. For MCP and factor H, C3 protein was incubated at 15 ng/mL and for factor B, 200 ng/mL. Detection was made with chicken anti–human C3 and HRP-linked donkey anti–chicken IgY. * indicates a significant reduction in binding ($P < .05$). (B) Kinetic analysis of cofactor activity. C3 preparations were incubated for 0 to 30 minutes at 37°C with factor I and a cofactor protein (MCP, factor H, or sCR1). The zero control is before the addition of factor I. The last lane is an iC3b control. Samples were reduced and analyzed by Western blotting using either chicken anti–human C3 or goat anti–human C3 (insets) followed by an HRP-linked antiglobulin (see Document S1). Cofactor activity is assessed by the loss of the α chain and appearance of the α_1 and α_{41} kDa major cleavage fragments. The minor α_{43} kDa cleavage fragment is more variable but no pattern was observed that was specific for a mutant. Data are representative of 5 similar experiments.

pedigree (Figure S1) in which an unaffected individual also carried the same change, and another from an affected sibling pair. Therefore, 14 affected persons were found to harbor a mutation. There were a total of 9 distinct mutations identified in the initial 11 patients, the same mutation was carried by 2 unrelated persons. There were 8 heterozygous missense mutations (R570W, R570Q, R713W, A1072V, D1093N, C1136W, Q1139K, and H1142D) and one heterozygous nonsense mutation (Y832X). The positions of these are shown in Figure 1. None of these variants was present in 200 chromosomes from healthy persons.

Clinical and laboratory data are presented in Table S2. Median age at presentation was 6.5 years (range, 8 months to 40 years). Seven of the 14 recovered renal function after their initial presentation, and 4 of these had recurrences. Among the 14 patients there have been 12 renal transplantations (9 cadaveric and 3 live-related), 5 of which have been affected by recurrent disease.

Next, 8 of 9 C3 mutant constructs (except for the missense mutation) were prepared and transfected into mammalian cells. The C1163W mutant was either minimally or not detectably expressed. Of the remaining 7, each was expressed in approximately comparable amounts, and protein bands in gels migrated identically to wild-type C3 (Figure S2).

Assays were performed to assess the interaction of the 7 secreted C3 mutants with MCP.[19] Binding of R570Q, R570W, A1072V, D1093N, and Q1139K was 22%, 30%, 18%, 17%, and 69% of wild-type, respectively (Figure 2A). Binding of R713W and H1442D was not statistically different from wild-type. The results of the cofactor assays paralleled the ligand binding data (Figure 2B). The 2 mutants with normal MCP binding also had normal cofactor activity, whereas the 5 mutants with decreased MCP binding had decreased cofactor activity. Specifically, there were no detectable cleavage fragments in 4 and modest cleavage by mutant R570Q. Based on the loss of the α-chain and appearance of major α_1 and α_{41} kDa cleavage fragments, there

was more than 90% reduction in cofactor activity for these 5 mutants. Thus, the reduced interaction of 5 of the 7 secreted C3 mutant proteins with MCP (the major membrane cofactor regulator of C3b) is likely to lead to a gain of function relative to complement activation.

We next evaluated the interaction of the 7 secreted mutants with complement receptor 1 (soluble CR1, sCR1) and the plasma regulatory protein factor H. Both of these inhibitors also bind C3b and are cofactors for its cleavage by factor I. There were no statistically significant differences between binding of sCR1 to wild-type C3 versus that of the 7 mutants. For factor H, however, the binding profile generally resembled that of MCP except that the magnitude of the decrease in binding was less. However, there was a statistically significant decrease in iC3 binding of A1072V, D1093N, and Q1139K, while R570Q and R570W were modestly (10%-20%) but not statistically significantly decreased. Cofactor activity for Q1139K was also decreased compared with native C3 (Figure S3).

We also assessed binding to factor B. Factor B interacts with C3b or iC3 to begin the formation of the alternative pathway C3 convertase. There were no statistically significant alterations in binding of these mutants to factor B. Taken together, these results further suggest that it is a modification in interactions with regulators that leads to a secondary gain of function in these C3 mutants.

Homozygous C3 deficiency in association with recurrent bacterial infections has been recognized for many years.[20] Following the description of the genomic structure of *C3* in 1990,[21] a molecular basis for C3 deficiency was first described.[22] Subsequently, inherited C3 deficiency has been identified in 19 families (reviewed in Reis et al[23]) and the underlying molecular mechanism established in 8 of these. In this study we have identified 9 heterozygous mutations in *C3*. Five (R570Q, R570W, A1072V, D1093N, and Q1139K) lead to impaired binding to the regulator MCP and, thus, resistance to cleavage by factor I.

Recent structural studies of human C3 have provided unique insights into the functional domains of the molecule.[17,24] Putative binding sites for factor H, complement receptors 1 and 2 (CR2; CD21), properdin, and factor B have been mapped to the structure of C3.[13] The 5 mutations associated with impaired binding to MCP also provide additional information on potential interactive sites for this regulator as well as possibly for factor I.

The functional significance of R713W and H1442D mutations remains to be elucidated as they had normal binding to MCP and subsequent cleavage by factor I. While the C3 mutants with a resistance to regulator binding fit with current concepts of aHUS pathogenesis, the mutations Y832X and C1136W lead to impaired secretion of C3 and haploinsufficiency. The latter is more difficult to understand relative to the concept of increased complement activation for a given degree of cellular injury as the fundamental predisposing event for aHUS.

In summary, this study establishes a new association between heterozygous mutations in C3 and aHUS.

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Authorship

Contribution: V.F.-B., T.H.J.G., and J.P.A. designed the research, analyzed the data, and wrote the paper. V.F.-B., E.C.M., M.K.L., M.-A.D.-D., L.S., R.G., and R.H. performed the research. J.B., A.L.B., N.M., B.S.K., R.A.W., K.L., G.K., T.M., H.N., W.W., S.G., B.H. de L., M.F., V.M., C.L., and W.H.F. contributed clinical information. B.J.C.J. undertook the structural analysis. E.C.M., M.K.L., R.G., and R.H. prepared the mutant proteins and performed the ligand binding and cofactor assays.

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APPENDIX 2

Roumenina, L.T. *et al.* A prevalent C3 mutation in aHUS patients causes a direct C3 convertase gain-of-function. *Blood* (2012). doi:10.1182/blood-2011-10-383281

A prevalent C3 mutation in aHUS patients causes a direct C3 convertase gain-of-function

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Abstract

Atypical hemolytic uremic syndrome (aHUS) is a rare renal thrombotic microangiopathy commonly associated with rare genetic variants in complement system genes, unique to each patient/family. Here we report 14 sporadic aHUS patients carrying the same mutation, R139W, in the complement C3 gene. The clinical presentation was with a rapid progression to end stage renal disease (6/14) and an unusually high frequency of cardiac (8/14) and/or neurologic (5/14) events. Although resting glomerular endothelial cells (GEnC) remained unaffected by R139W-C3 sera, the incubation of those sera with GEnC pre-activated with pro-inflammatory stimuli led to increased C3 deposition, C5a release and pro-coagulant tissue factor expression. This functional consequence of R139W-C3 resulted from the formation of a hyperactive C3 convertase. Mutant C3 showed an increased affinity for Factor B and a reduced binding to MCP (CD46), but a normal regulation by Factor H (FH). Also, the frequency of at-risk FH and MCP haplotypes was significantly higher in the R139W-aHUS patients, as compared to normal donors or to healthy carriers. These genetic background differences could explain the R139W-aHUS incomplete penetrace. These results demonstrate that this C3 mutation, especially when associated with an at-risk FH and/or MCP haplotypes, becomes pathogenic following an inflammatory endothelium damaging event.

Introduction

The atypical hemolytic uremic syndrome (aHUS) is a rare kidney predominant thrombotic microangiopathy, associated with genetic abnormalities in the regulators and activators of the alternative complement pathway [1]. A complement genetic abnormality has been identified in more than 60% of patients [2]. Most commonly, they correspond to rare distinct point mutation variants unique for each patient/family [3]. The small number of patients sharing a particular mutation often precludes investigations on the consequence of each particular mutation and on the influence of additional factors for the disease manifestation. Therefore it is still unclear why aHUS has incomplete penetrance among the mutation carriers, despite the clear functional consequences of the mutations. Complement dysregulation is linked, by as yet not well understood mechanisms, to the induction of a pro-coagulant phenotype on glomerular endothelial cells (GEnC). Thrombi are formed in the kidney microvasculature, resulting in end stage renal disease (ESRD) one year after the first flare in about 50% of the cases [1].

Complement is an innate immune surveillance system, designed to fight infections and to handle damaged or apoptotic cells and debris [4]. It may be activated by three pathways, all leading to the cleavage of the central component C3 by an enzymatic complex called a C3 convertase. The C3 convertase of the alternative pathway, C3bBb, is composed of the active fragments of C3 and Factor B (FB).

To avoid accidental host tissue injury, the complement system is tightly controlled on self surfaces by regulators such as Factor H (FH), Factor I (FI) and membrane cofactor protein (MCP, CD46). In aHUS, these regulators are frequently mutated and unable to efficiently protect the endothelium from complement attack [1]. C3 mutations were recently discovered in aHUS and only few reports describe the clinical outcome of such patients [5-8]. Functional analysis of 9 of these C3 mutations revealed that most of them result in impaired regulation by MCP and hence in indirect, or secondary, gain-of-function of the C3 convertase [5]. It has also become evident that aHUS could be associated with an intrinsically hyperactive C3 convertase. Three such mutations have been reported in FB [9, 10]. They resulted in the formation of a more potent C3 convertase, resistant to decay by complement regulators and leading to enhanced C3 deposition on resting GEnC. A particular hyperactive C3 convertase, formed with mutated C3, was described in patients with Dense Deposit Disease due to in frame deletion of two aminoacid residues [11]. To date, no C3 mutations in aHUS have been described that are able to directly enhance the function of the C3 convertase.

Here we report the first large series of aHUS patients carrying the same genetic abnormality in C3, R139W. This is the first description of a direct gain-of-function mutation in C3 that forms a hyperactive C3 convertase as well as being resistant to regulation by MCP, but not by FH. This C3 mutation, especially if associated with at-

risk FH and MCP haplotypes, becomes pathogenic following an endothelium damaging event.

Patients and Methods

Patients

Patients were recruited from the French aHUS cohort of patients (n=343). Diagnosis of HUS was defined by the simultaneous and acute occurrence of at least three of the following four criteria: acute renal failure, microangiopathic hemolytic anemia, thrombocytopenia and/or histological thrombotic microangiopathy. Further definitions and the case reports are given in the Supplementary section for Patients and Methods. The clinical history of patients P7 and P9 has been reported previously [12].

Structure analysis

The crystal structures of C3 [13], C3b [14], C3b-FH1-4 [15], C3bBb [16], C3bB [17] and MCP [18] are available in Protein Data Bank. Molecular graphic imaging and analysis were produced using the Pymol and UCSF Chimera package [19] (http://www.cgl.ucsf.edu/chimera/). Protein numbering throughout this study is according to the sequence of the mature protein, lacking the signal peptide. Wherever appropriate, the numbering will be according to the gene sequence (starting with c.) and to the protein with the 22-amino-acid-long leader peptide (starting with p.).

Endothelial cell assays

Conditionally immortalized GEnC [10, 20] (passage 29-35) and third passage primary HUVEC were used for this study. Detailed of culturing conditions are given in the Supplement (Patients and Methods). Briefly, GEnC and HUVEC, either resting or stimulated with TNFα/IFNγ, were exposed to normal human sera (NHS) from 50 donors or R139W sera or FH-depleted (FH-dpl) serum. Alternatively, blocking anti-FH (Ox24 [21]) monoclonal antibody was added to these sera or anti-MCP (GB24 [22]). Also R139W sera were supplemented with different concentrations of purified FH (Comptech, Tylor TX). Released C3a, C5a and sC5b9 were measured in the supernatant using specific ELISAs (Quidel, San Diego, CA). Cells were incubated with a mouse anti-human C3c mAb (Quidel, San Diego, CA), followed by a secondary anti-mouse IgG-PE or an anti-Tissue Factor-PE antibody (BD Pharmingen) and analyzed by flow cytometry as described in the Supplement (Patients and Methods).

Recombinant C3 production

The R139W mutant C3 was produced from the wild type (WT) C3 plasmid as described [5]. The expression level of the plasmids was compared after multiple transient transfections using Lipofectamine. The plasmid was introduced into CHO cells via stable transfection using selection with G418. The constructs were sequenced in their entirety to confirm that no additional mutations had been introduced. The synthesis of C3 was assessed by a sandwich ELISA, using immobilized anti-human C3 antibody for capture and biotinylated anti-human C3, followed by streptavidin-Horseradish Peroxydase (HRP) (Amersham, Buckinghamshire, GB) for detection. The expression levels were similar between WT and mutant plasmids. Supernatants derived from stable transfected cells containing recombinant WT and R139W-C3 as well as supernatants of the mock-

transformed cells (SN0), were used to purify C3 by ion exchange chromatography using DEAE Sepharose (GE Healthcare) and eluted with 0.2M NaCl.

Surface plasmon resonance

The interaction of wild type and mutant C3 with FH, MCP and FB was analyzed using surface plasmon resonance (SPR) technology with Biacore2000 equipment. In the first approach, FH and MCP were coupled to the CM5 biosensor chip (GE Healthcare) using standard amide-coupling technology, according to the manufacturer's instructions. Purified recombinant wild type and mutant C3 were used as an analyte at concentrations of 1, 0.5, 0.25, 0.125, and 0.06nM. The flow rate was 10 μl/min in a HEPES buffer (10 mM HEPES, 25 mM NaCl, pH 7.4). An empty activated/deactivated flowcell served as a control.

Alternatively, the anti-C3d antibody (Quidel, San Diego, CA) was coupled to the four flowcells of the chip. Purified WT and R139W C3 and an equivalent purification fraction from the SN0 were loaded on three of the flowcells. No binding was detected in the case of SN0. WT and R139W C3 were always loaded to equivalent resonance units. FB was applied as an analyte at 0.06, 0.125, 0.25, 0.5 and 1 nM in 1 mM $MgCl_2$-containing HEPES buffer (without regeneration). The complex was allowed to decay spontaneously before the next concentration was injected.

Data were analyzed using BIAevaluation software and the RU from the blank flowcell was subtracted. Kinetic parameters were calculated by fitting the obtained

sensorgrams into 1:1 interaction with a drifting baseline algorithm to give the lowest chi square.

C3 convertase formation

Alternative pathway C3 convertase was assembled on a CM5 chip by pre-programmed injections of native and recombinant C3, FB and FD. Native and recombinant C3 were mixed to mimic the heterozygous situation found in patient sera. In each case three different samples were prepared: 1) purified recombinant WT (10 ng) or R139W C3 (10 ng) or equivalent volume of SN0 plus human C3 purified from plasma (Calbiochem, San Diego, California; USA, 10 ng), 3 μg of FB (Calbiochem, San Diego, California, USA) and 0.2 μg of FD (Calbiochem, San Diego, California, USA) were added in 10 mM HEPES, 25 mM NaCl, 1 mM $MgCl_2$ running buffer to a final volume of 100 μl. 2) 7μg FB and 0.5μg FD in 200 μl of running buffer, 3) purified recombinant WT (10 ng) or R139W C3 (10 ng) or equivalent volume of SN0 with human C3 purified from plasma (10 ng) in a final volume of 100 μl. Samples were injected as follows: sample 1, followed by sample 2, then sample 3 and, at the end, sample 2 again, followed by 100s of running buffer only. Alternatively, 1 mM $NiCl_2$ was used instead of $MgCl_2$ to stabilize the C3 convertase.

Results

Genetic analysis

Mutation screening

Direct sequencing of the C3 gene allowed identification of 14 patients (4% of the French aHUS cohort and ~ 50% of patients with C3 mutations) carrying the same heterozygous missense mutation: c.481C>T (Figure 1A), encoding for p.R161W substitution if the first Met was counted as 1 (i.e. with a signal peptide of 22 residues, Figure 1B) or R139W of the mature protein (without the signal peptide). The affected R139 residue is partially exposed on the surface of C3 MG2 domain (Figure 1C). This mutation was absent in 550 healthy controls. Most of the patients and 150 of the normal controls came from the North of France. Two of the patients carried a second genetic abnormality in the FH gene (R341H for P9 and F960S for P11, Table 1). No missense or splice site mutations were present in the genes of FH, FI, MCP, C3, FB and thrombomodulin. No complete deletion of CFHR1/3 was found. Eleven healthy R139W carriers were identified among 19 healthy individuals from the six families available for screening.

FH and MCP haplotypes analysis

The frequency of FH and MCP SNPs and haplotypes [23] was evaluated in the R139W-aHUS patients and their relatives and compared to healthy donors (Table 2). FH (FH_{TGTGT}) and MCP (MCP_{GGAAC}) at-risk haplotypes were significantly higher in R139W-aHUS compared to 190 normal donors. These two haplotypes were also

more frequent in R139W-aHUS, as compared to healthy R139W carriers. The presence of FH_{TGTGT} together with R139W gave a 4-fold higher chance for disease development compared to R139W alone. Similarly, the presence of MCP_{GGAAC} with R139W increased 3-fold the risk for development of R139W-aHUS. Also, 69% of R139W-aHUS were homozygous for the minor TT genotype of the rs3753394 SNP in the promoter region of FH, while it occurred only in 6% of normal donors and was not observed in healthy R139W carriers.

Four of the patients carried homozygous FH risk haplotype TGTGT and five patients homozygous MCP risk haplotype GGAAC (Table 1). Two of the patients (P2 and P9) carried both risk haplotypes. If the presence of the FH or MCP at-risk haplotype is considered as 1 risk copy when in a heterozygous form and as 2 risk copies when in a homozygous form, the sum of at-risk haplotype copies in FH and MCP could be estimated in R139W-aHUS and R139W healthy carriers. We were able to assign the individual haplotype alleles for 11 patients and 10 healthy carriers. Eighty-two % of patients had 2 or more at-risk copies in FH and/or MCP versus 30 % of the healthy carriers.

aHUS presentation

The onset of the R139W-aHUS was in the pediatric population (under 18 years) in 5 cases and in adults in 9 cases. A triggering event was suspected in 10 out of 14 patients including an infection in 3 of 5 pediatric cases, while the adults < 50 years

of age developed the disease post-partum (3/7) or in relation to drugs or toxins (4/7). No triggering event was identified in the patients > 50 years of age (2/2). The pediatric cases had nearly equal distribution of male (2/5) and female (3/5) patients while in adults 90% of the patients (8/9) were female. Shiga toxin-producing *Escherichia coli* were not present in any patient.

Initial clinical symptoms and laboratory test results are detailed in Table 1. Treatment consisted in plasma therapy in 10 patients: plasma exchange (30 to 80 ml/kg per session) or exchange transfusions in one case (P1). Cardiac events were reported in 8/14 patients. Echocardiography demonstrated a dilated cardiomyopathy with a reduction in left ventricular function in 7 patients. One patient (P13) died in relation to a cardiovascular event but the myocardial process was not defined. Heart failure occurred mostly at the R139W-aHUS onset but two patients (P10 and P1) experienced a delayed cardiomyopathy 2 and 6 months respectively after the hematologic remission. Cardiac function slowly improved with medical treatment but a left ventricular dysfunction persisted. Neurological events occurred in 5/14 patients, always during the acute phase of the illness. Four patients had seizures and one had brachioplegia. Cranial computed tomographic scans were abnormal in 2 patients, showing diffuse multiple small infarcts. Neurologic manifestations resolved except for one (patient P2), who developed recurrent seizures requiring chronic therapy. Altogether, 9 out of 14 patients developed an ESRD, 6 before one year of follow-up. Seven kidney transplantations

were performed in 4 patients. Four transplanted kidneys were lost after 1 to 23 months due to an aHUS relapse, one of them under preventive plasma therapy.

Complement component assessment

Low C3 at the acute phase of the disease was detected in 6 out of 9 patients, for whom samples were available from the acute phase. In contrast, all screened healthy carriers (samples available for 8 individuals) had normal C3 levels. No patient had anti-factor H autoantibodies. FH and FI antigenic levels and MCP expression (assessed by flow cytometry) were normal in all tested patients.

Functional characterization of the R139W mutation

Using R139W-C3 containing sera

The R139W mutation led to complement activation on stimulated but not resting endothelial cells

Resting, adherent HUVEC or GEnC were stimulated with proinflammatory cytokines (TNFα+IFNγ) and then incubated with sera from three patients with R139W-aHUS (P2, 5 and 14), three healthy carriers of the mutation from patient P5's family, two mutation-free members from the same family and compared to 50 different healthy donors (normal controls) and FH-depleted serum (FH-dpl) (positive control) (Figure 2). Minimal C3 deposition was observed on resting cells incubated with normal sera, aHUS patients' sera and all but one healthy carrier's sera (the second sister of patient P5 – P5.S2) (Figure 2A). FH-dpl led to increased deposition of C3 on

the resting cell surface. The high C3 deposition observed P5.S2 serum, with GEnCs as well as with HUVECs from 5 different donors, is mainly alternative pathway dependent (persistent in presence of EGTA-Mg). The screening of FB and FH polymorphisms and CFHR1 copy numbers showed no peculiarity in this subject and, unfortunately, new serum samples were not available for further analysis. On cytokine-stimulated cells, C3 deposition from all tested R139W-HUS patients and healthy R139W carriers was increased, as compared to healthy donors and was similar to the FH-dpl (Figure 2A). In the large family tested, the increase of C3 deposition perfectly correlated with the presence of the mutation (as could be seen from the genealogic tree in Supplemental Figure 1). The augmented C3 deposition was accompanied by increased C3a and C5a release, sC5b-9 formation and tissue factor expression. Doubling the amount of FH present in R139W sera (corresponding to 1/3 dilution of serum in our assay) led to C3 deposition comparable to that of normal donors. These effects were observed both using HUVEC (Supplemental Figure 1) and GEnC (Figure 2A).

The C3 deposition from R139W serum on stimulated EC was controlled by FH but not by MCP.

To determine if the increase in C3 deposition from R139W serum was caused by defective regulation of the R139W C3 by FH or by MCP, function blocking antibodies were utilized (Figure 3A). FH-dpl was used as a control for defective regulation by FH. If the blocking anti-FH OX24 antibody was applied to NHS, C3 deposition on

resting cells was increased 2-fold as compared to NHS and was equivalent to that of the FH-dpl. The same antibody caused a more than 4-fold increase in C3 deposition when applied to a serum containing R139W-C3. Similar experiments were performed using blocking anti-MCP GB24 antibody. Addition of this antibody to NHS caused a 2.5-fold increase in C3 deposition, similar to FH-dpl. When the anti-MCP antibody was applied to FH-dpl or to R139W serum, the C3 deposition exceeded control levels by 5-fold and 3 fold respectively. Addition of purified FH to sera of R139W-aHUS patient or healthy relatives but carrying the same mutation resulted in a decreased C3 deposition on activated GEnC (Figure 3B). The C3 deposition reached levels obtained with NHS when the FH concentration was doubled.

Using recombinant proteins

The R139W mutation affected C3 interaction with MCP, not with FH

The affected residue R139 is in close proximity to the FH CCP3 binding site [15] and to the suggested MCP CCP3 binding site [18] (Figure 4A and 4B). ELISA (Figure 4C and 4D) and SPR (Figure 4E and 4F) revealed a normal interaction with FH, but the binding to MCP was decreased. Kinetic analysis indicated a statistically significant 2-fold decrease in the association rate (7.11×10^4 Ms^{-1} for the WT versus 2.97×10^4 Ms^{-1} for R139W, $p = 0.016$, $n = 4$, t-test) and no difference in the dissociation rate, resulting in a 3-fold reduction in the apparent binding affinity (1.57×10^8 M^{-1} for the WT versus 5.15×10^7 Ms^{-1} for R139W, $p = 0.045$, $n = 4$, t-test). No difference was observed for the kinetic parameters of the interaction with FH. The decay

acceleration activity of FH was identical for the WT and R139W-C3 (Supplementary Figure 2A). Both C3 forms were cleaved by Factor I in presence of FH as a cofactor, but the cleavage of R139W was slightly delayed compared to the WT (Supplementary Figure 2B).

R139W is a gain-of-function mutation leading to a hyperactive C3 convertase

Structural aspects of the formation of the alternative complement pathway C3 convertase C3bBb have been recently described [16, 17]. R139W mapped far from FB in the closed structure of C3bB and C3bBb. However, it was in close proximity to the SP-domain of FB in the so-called open form of C3bB, which is capable of binding FD and allows generation of the active convertase C3bBb (Figure 5A). Therefore the influence of R139W on the interaction with FB was analyzed. Both ELISA (Figure 5B) and SPR (Figure 5C) demonstrated an increased binding of FB to R139W, specifically a nearly two-fold increase in the binding affinity secondary to enhanced association rate. No difference was observed in dissociation parameters. In order to determine if this increased binding could lead to hyperfunctional C3 convertase, the formation of the C3 convertase was assessed by SPR. In the presence of R139W the convertase was more efficient, leading to increased C3b deposition on the chip (Figure 5D). This phenomenon was observed both in the presence of Mg (Figure 5D) and of Ni ions (not shown).

Discussion

Atypical HUS is a predominant renal thrombotic microangiopathy strongly associated with complement genetic abnormalities. Nevertheless, the contributions of the complement system mutations to the expression of a pro-coagulant phenotype of the glomerular endothelium and to the disease manifestation remain elusive. Here we report a large series of aHUS patients who carry the same genetic abnormality, R139W, in the central complement component C3. This mutation, found in 14 unrelated patients, accounted for half of the C3 mutations associated with aHUS in France. Most of the patients came from the north of France, but did not share a high level of polymorphisms in the vicinity of the C3 gene (data not shown). This suggests that, if there was a founder effect, it was ancient. R139W was not identified in normal controls, confirming it as a mutation and not a rare polymorphism. Given the high frequency of this genetic abnormality, it could be considered as a prototypical aHUS C3 mutation, similar to R1210C in FH [24].

The functional consequences of R139W were analyzed using sera containing R139W-C3 and recombinant proteins. Incubation of resting endothelial cells (both HUVEC and GEnC) with R139W positive sera did not result in increased C3 deposition, except in one case of a healthy carrier. These results are in contrast to what we observed previously for two mutations in FB, which caused increased C3 deposition even on resting cells [10]. No significant increase in TF expression was

observed if resting GEnC were cultured with R139W serum, compared to healthy controls. The fact that the presence of R139W-C3 is tolerated by healthy endothelium could explain why the disease associated with this mutation occurs at variable ages, in the majority of the cases after a triggering event, and has an incomplete penetrance. Atypical HUS flares are frequently preceded by a triggering event, as in 10 out of the 14 patients reported here. Herein we observed increased complement deposition on glomerular endothelial cells exposed to R139W sera, if cells had been stimulated by pro-inflammatory cytokines.

All tested R139W containing sera caused increased C3-deposition compared to normal donors and a perfect correlation was observed between this C3 deposition and the presence of the mutation in patient P5's family. This result was similar to our model conditions of complement dysregulation, using FH-depleted serum or a blocking FH with an inhibitory antibody. The enhanced C3 deposition was accompanied by increased release of C3a, C5a and formation of the soluble terminal complement complex C5b9 (sC5b9), indicating that complement activation on the cell surface proceeded to its final stages. C5a and sC5b9 are known to induce TF expression on HUVEC [25, 26]. Stimulated GEnC upon culture with R139W sera also expressed higher TF levels, as compared to the same cells cultured with normal human sera. Of note, R139W sera from patients and healthy carriers resulted in similar levels of deposited or released complement active fragments and of TF expression. Thus, we provide experimental evidence that complement

hyperactivation in aHUS is indeed linked to the expression of a pro-coagulant phenotype of the GEnC and therefore participates in the thrombotic process.

The functional consequences of this mutation were studied at the molecular level. Positioning of R139 on the structure of C3b in complex with three of its most important ligands revealed that this residue is in proximity to the binding site for CCP3 of FH [15], the putative binding site for CCP3 of MCP [18] and the binding site of the FB's serine protease (SP) domain in the open conformations as part of the C3 pro-convertase C3bB. Structural aspects of the C3 convertase formation have been extensively studied [16, 17, 27-29]. Binding of FB to C3b induces a dynamic equilibrium between a closed (loading) and open (activation) form. This conformational change is accompanied by a reorientation of FB catalytic SP domain, which comes into a contact with the MG2 and the CUB domains of C3b in the open form. The activation form allows Factor D binding and formation of the active convertase C3bBb. The R139 residue is far from the FB binding site in the loading form, but the SP domain comes close to this residue in the active form. Experimental results here demonstrated a two-fold increase in the binding of R139W to FB, due to a faster association rate. Furthermore, mutant C3 formed a hyperactive C3 convertase. These results are in agreement with the structural data and confirm the importance of the MG2 domain for binding of FB.

This is the first rigorously analyzed case of a direct gain-of-function of a C3 mutation. All mutations previously described [5] are indirect gain-of-function, resulting from ineffective regulation by MCP. Interestingly, the direct binding of

R139W C3 to FH and its decay acceleration activity were undistinguishable from wild type C3, both by ELISA and SPR, notwithstanding the close proximity of the mutated residue to FH binding site. The cofactor activity of FH towards R139W cleavage by Factor I was only moderately decreased, compared to the wild type. The interaction with MCP was affected by the mutation, causing a strong decrease in binding (measured by ELISA) and a three-fold decrease in binding affinity (studied by SPR), due to a slower association rate. Once formed, the complex had the same dissociation rate as the WT. These results demonstrate that the binding sites for FH and MCP are not identical and do not involve the same contact residues, since R139W influences differentially the interaction with these two complement regulators.

The notion that R139W could be regulated by FH but not by MCP was confirmed by a different approach, using function blocking anti-FH and anti-MCP antibodies in R139W containing serum. When anti-MCP blocking antibody was applied to R139W sera, C3 deposition was slightly increased, suggesting that in this experimental set-up MCP does not contribute much to the regulation of C3 deposition. In contrast, anti-FH blocking antibody in R139W sera raised C3 deposition to the levels observed in the absence of both MCP and FH. Also, addition of purified FH was able to control the C3 deposition in R139W positive sera in the normal range of healthy donors. These results confirm the capacity of FH to regulate R139W C3 and emphasize FH essential role in complement regulation in sera harboring this mutation

Although no family history of aHUS was found in all 14 patients, 11 R139W healthy carriers were identified in 6 families in which genetic analysis was performed. Two patients carried additional mutations in FH outside the hotspot region CCP19-20. To find out whether healthy carriers differed in their genetic background from the R139W-aHUS patients, the frequency of the aHUS at-risk haplotypes in FH (FH_{TGTGT}) and in MCP (MCP_{GGAAC}) were compared. The R139W-aHUS patients had significantly higher frequency of these at-risk haplotypes compared to healthy controls, as previously reported in patients with aHUS [30]. In contrast, these frequencies were similar in healthy R139W carriers and in normal donors. The presence of R139W together with FH_{TGTGT} conferred 4-fold higher risk and with $MCP_{GGAAC,}$ 3-fold higher risk for development of aHUS respectively, as compared to R139W alone. Among the R139W-aHUS patients, 82% had two or more at-risk alleles in FH and/or MCP genes, compared to 30% of the healthy carriers. This clear distinction between healthy carriers and patients demonstrates the critical role of the FH and MCP genetic background for the development of aHUS in the presence of a mutant C3.

The R139W-aHUS had variable disease manifestations and both severe and milder renal phenotypes were found. Nevertheless, the R139W-aHUS was marked by a high frequency of extra-renal complications (more than 60% of the patients). Particularly, 60% of the patients had cardiac events and 35% had neurological events either during the acute phase or at distance from the aHUS episodes. Cardiac symptoms in HUS and are rarely mentioned, mostly in isolated case reports. A dilated cardiomyopathy (as in our R139W-aHUS patients) has been the most

frequent observation [31-34]. In a series of 64 autopsied HUS cases, heart involvement was detected in 19 patients [35]. Only one study of aHUS pediatric cases reported a high frequency of cardiomyopathy, affecting 10 children (43% of the cohort). Nevertheless, no mutation screening was performed in these two patients' series [36]. Cardiac complications have been reported in patients with a FH mutations leading to haploinsufficiency [34] in 1 out of 7 children with autoimmune aHUS [37] and in 3 out of 45 cases of the French autoimmune aHUS [38]. Further studies are needed to determine if there exists a specific association of cardiac complications with R139W-aHUS. Nevertheless, results suggest that echocardiographic screening of patients with aHUS should be performed at admission and during the follow-up, since cardiac complications could be more frequent than reported or associated with particular forms of aHUS, as is the case here.

Starting from the observation that R139W causes a hyperactivity of C3 and reduced binding to MCP, a rational therapeutic strategy could be suggested for these patients. An optimal situation would be an agent capable of controlling the mutant convertase or its consequences. We found that the addition of purified FH to the R139W-C3 harboring sera was capable to reduce C3 deposition on activated GEnC in a dose dependent manner. Therefore, purified FH, or FH regulatory domains containing constructs (as the FH-CR2 hybrid inhibitor TT30[38]), could be considered as a therapeutic option for R139W-aHUS. Experimental assessment for the susceptibility of a mutant convertase to control by FH should be considered as a mean to find potential putative responders to purified FH treatment. Some

mutations such as the FB gain-of-function [10] appear to be resistant to regulation by FH. Independently of the localization of the mutation, the generation of adverse products of complement hyperactivation, such as C5a and C5b9, can be efficiency blocked by Eculizumab, a monoclonal antibody blocking the cleavage of the complement protein C5. This therapeutic agent has reduced the magnitude of the thrombotic microangiopathy, restored kidney function and improved quality of life in a small number of aHUS patients [39, 40].

In summary, we describe the genotype-phenotype and structure-function relationships for a frequent direct gain-of-function mutation in C3. The number of patients and healthy carriers with the R139W form of C3 on different genetic backgrounds indicates that this mutation, particularly if associated with an at-risk FH and/or MCP haplotypes, becomes pathogenic in association with an endothelium damaging event.

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Authorship Contributions

L.T.R., M.F., L.H.M, J.P.A., E.C.M., A.L. and V.F.B, designed the research. L.T.R., M.F., and E.M. performed the research. P.B., S.B. and C.H. provided technical assistance. F.P., C.M., C.N. and A.L. provided clinical information and are in charge for the patients. S.C.S. and P.W.M. provided the GEnC cell line. L.T.R., M.F, E.C.M., M.A.D.D., C.S.F., L.H.M., J.P.A., A.L. and V.F.B, analyzed and discussed the data. L.T.R., M.F., E.C.M.., J.P.A., L.H.M. and V.F.B. wrote the manuscript.

The authors have no conflicting interests.

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Figure legends

Figure 1. Localization of R139W C3 mutation. A) Position within the C3 gene and within the protein primary structure. B) Representative histogram for the sequencing of a patient, carrier of R139W. C) Mapping of R139W on the surface of C3 using Pymol software.

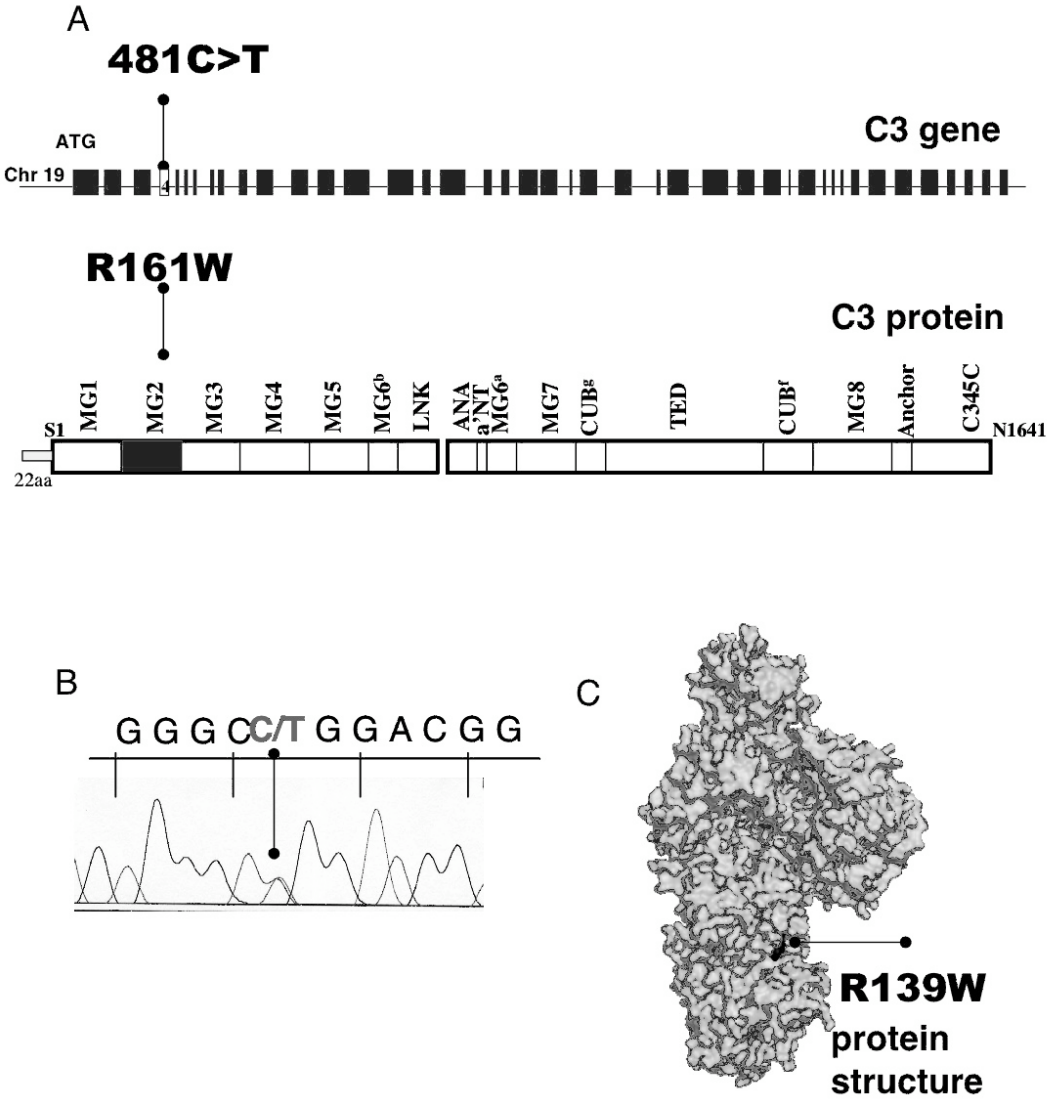
Figure 2. Complement activation on glomerular endothelial cells, incubated with sera from R139W-aHUS patients and their healthy relatives. A) C3 deposition on resting or TNFα/IFNγ activated GEnC in the presence of sera from 50 individual normal donors, FHdpl (four different lots), R139W-aHUS patients (P2, P5 and P14), healthy relatives of patient P5 bearing the mutation (5.F – father, 5.S1 and 5.S2 – sisters) or mutation-free relatives of patient P5, indicated as family (5.M – mother and 5.B – brother). C3 depositions (RFI) obtained with each patient or healthy donor were normalized by the C3 deposition from one normal human serum on resting cells, considered as a standard and run in each experiment in order to obtain the fold increase. Each point is a mean of 3-5 independent experiments, statistical significance (***$p<0.001$) was calculated by ANOVA. B) Levels of C3a, C5a and soluble C5b-9, released after incubation of the TNFα/IFNγ activated GEnC with serum from normal donors (n=6) or R139W sera (n=3), were measured by ELISA. The level of C3a, C5a or sC5b-9 in the supernatant (1/3 diluted serum) from resting cells was subtracted from the corresponding levels on activated cells, in order to obtain the specific amount of C5a and sC5b-9 released due to complement activation. Results are expressed as fold increase, when compared to a standard normal serum as in A). The statistical analysis was a Mann-Whitney test. C) Tissue factor expression on TNFα/IFNγ activated or resting GEnC after overnight incubation with sera from normal donors (n=6) or R139W positive sera (n=4). The percentage of TF-positive cells was measured by flow cytometry. The statistical analysis was Mann-Whitney test.

Figure 3. Effects of blocking FH and MCP on the regulation of C3 deposition from WT or R139W sera. A) GEnC pre-activated with TNFα/IFNγ were incubated for 30 min with the standard normal human serum, FHdpl or R139W sera, in the presence or absence of blocking mAbs against FH (Ox24) or MCP (GB24). The C3 deposition was evaluated by flow cytometry as in Fig. 2A. *** $p<0,0001$, unpaired t-test. Unless specifically mentioned, the comparison was made with the NHS (the first bar with a black and white pattern) B) GEnC activated with TNFα/IFNγ were incubated for 30 min with normal human sera, FHdpl or R139W sera in the presence of increasing doses of purified FH. C3 deposition in the absence of FH for the FHdpl or R139W positive sera (from aHUS patient and healthy carriers) was

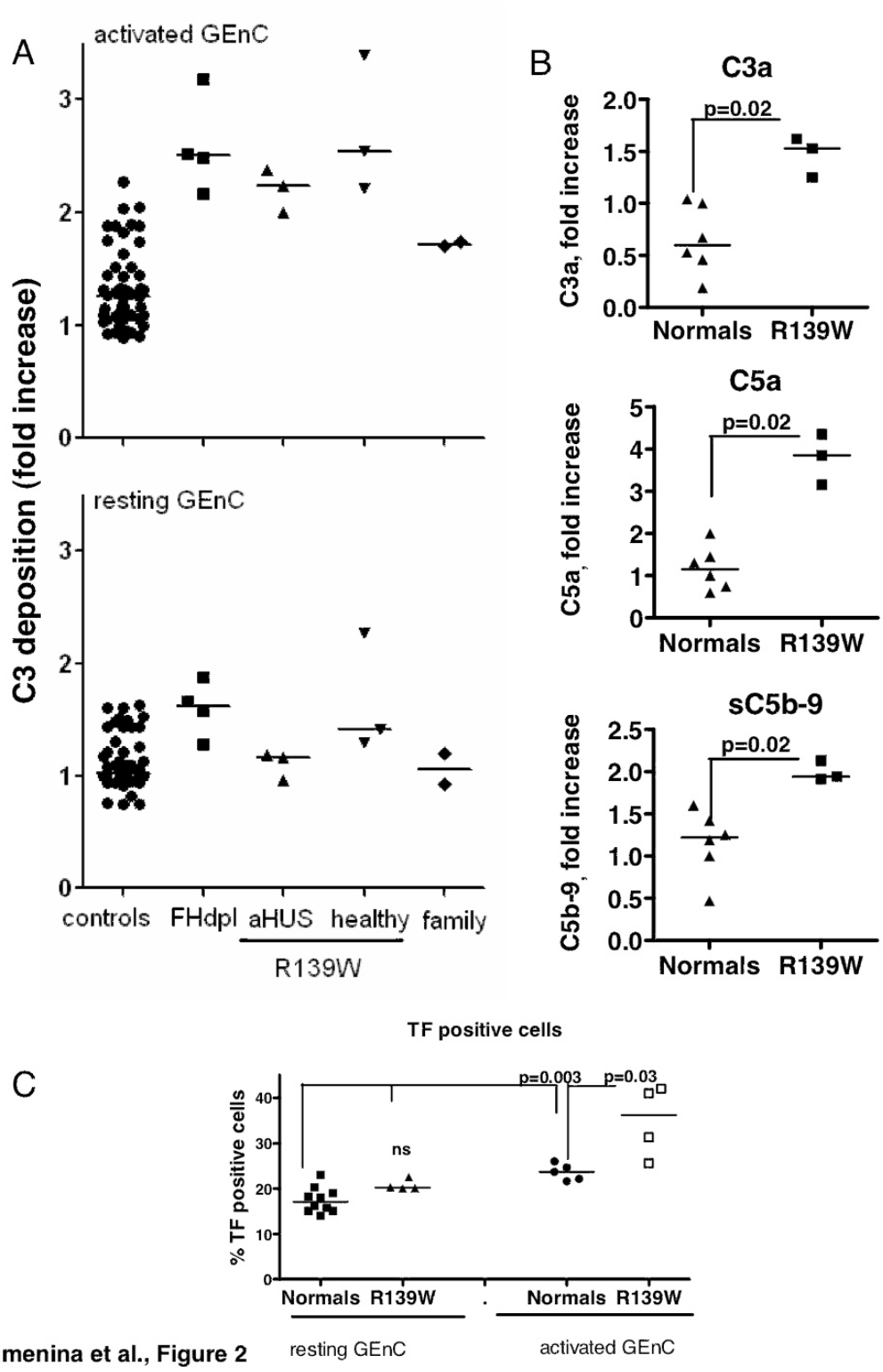
taken as 100% and each C3 level percentage, in the presence of different doses of FH, was calculated. The starting FH concentration was in the normal range for P5, 5.F and 5.S1. The level of C3 deposition from a normal serum is given as a straight line.

Figure 4. Interaction of R139W with MCP and FH. R139 position on the structure of C3b in a complex with FH CCP1-4 (A) or in a model complex with MCP (B). R139 is close to CCP3 binding site of both FH and MCP. Direct binding of recombinant WT or mutant C3 to FH (C) or MCP (D), studied by ELISA. Fetal calf serum free supernatant, containing recombinant WT or mutant C3 produced by stably transfected CHO cells, was used as a source of C3. SN0 is the supernatant of cells transfected with a plasmid not containing the C3 gene. The real time binding of recombinant WT or mutant C3 to FH (E) or MCP (F) was studied by surface plasmon resonance, using recombinant C3 proteins purified from serum-free culture supernatants by DEAE-Sepharose column.

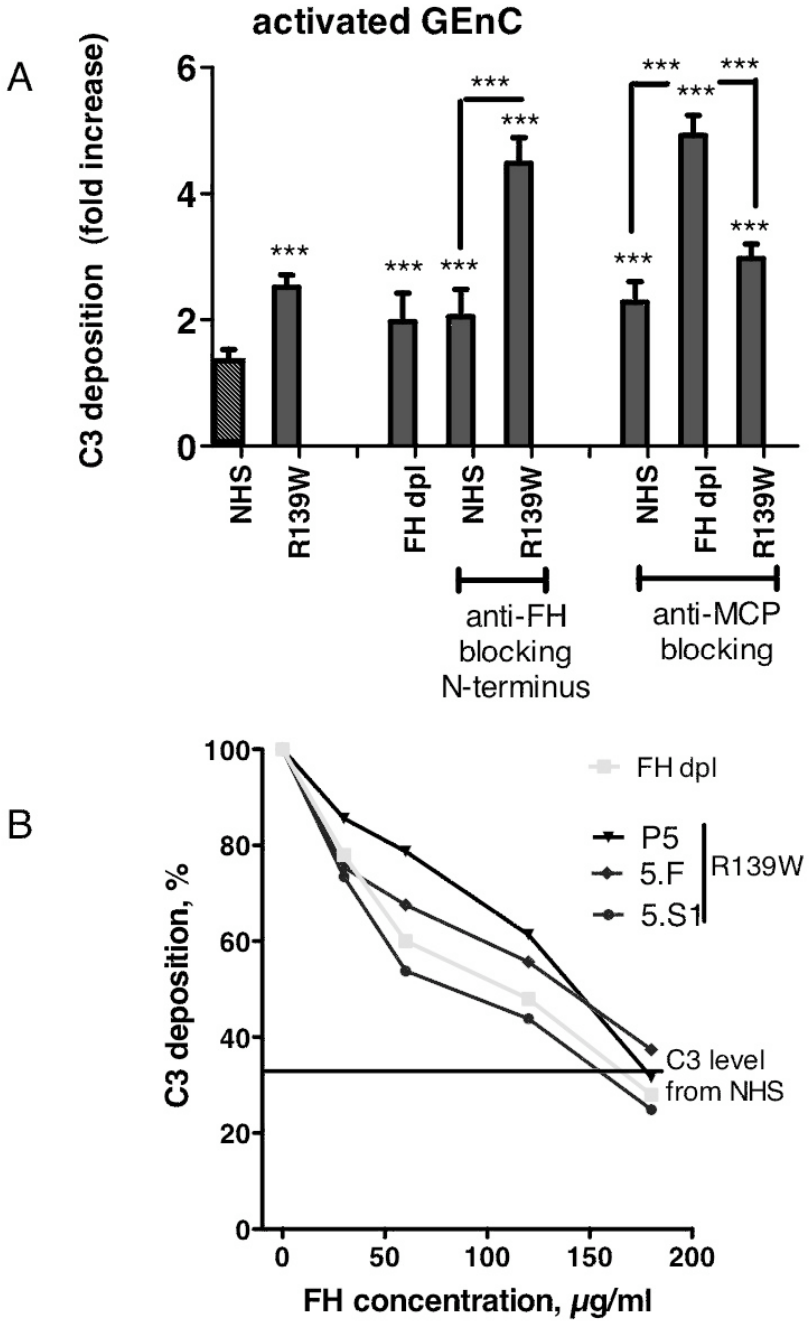
Figure 5. Interaction of R139W with Factor B. A) R139 position on the structures of C3b with FB in closed (refractory to cleavage by FD) and open (prone to cleavage by FD) conformations and on the structure of the C3bBb complex. The α and β chains of C3b are depicted in blue and green and FB is colored in magenta. B) Binding of FB to WT or mutant recombinant C3, bound to an anti-C3d monoclonal antibody, coated to the ELISA plate. Serum-free supernatant was used as a source of recombinant C3 molecules. C) The binding of FB to recombinant WT or R139W C3, bound to an anti-C3d monoclonal antibody on the CM5 biosensor chip, was studied by surface plasmon resonance using Biacore. Recombinant C3 proteins, purified from serum-free culture supernatants by DEAE-Sepharose column, were used. D) Formation of a C3 convertase on the biosensor chip by subsequent injection of C3+FB+FD, followed by an injection of FB+FD. Purified WT or R139W recombinant C3 were mixed with native C3, purified from plasma. Proteins deposition on the chip was followed over time. For panels B), C) and D) one representative experiment out of 3, performed with 3 independent productions of C3, is presented.



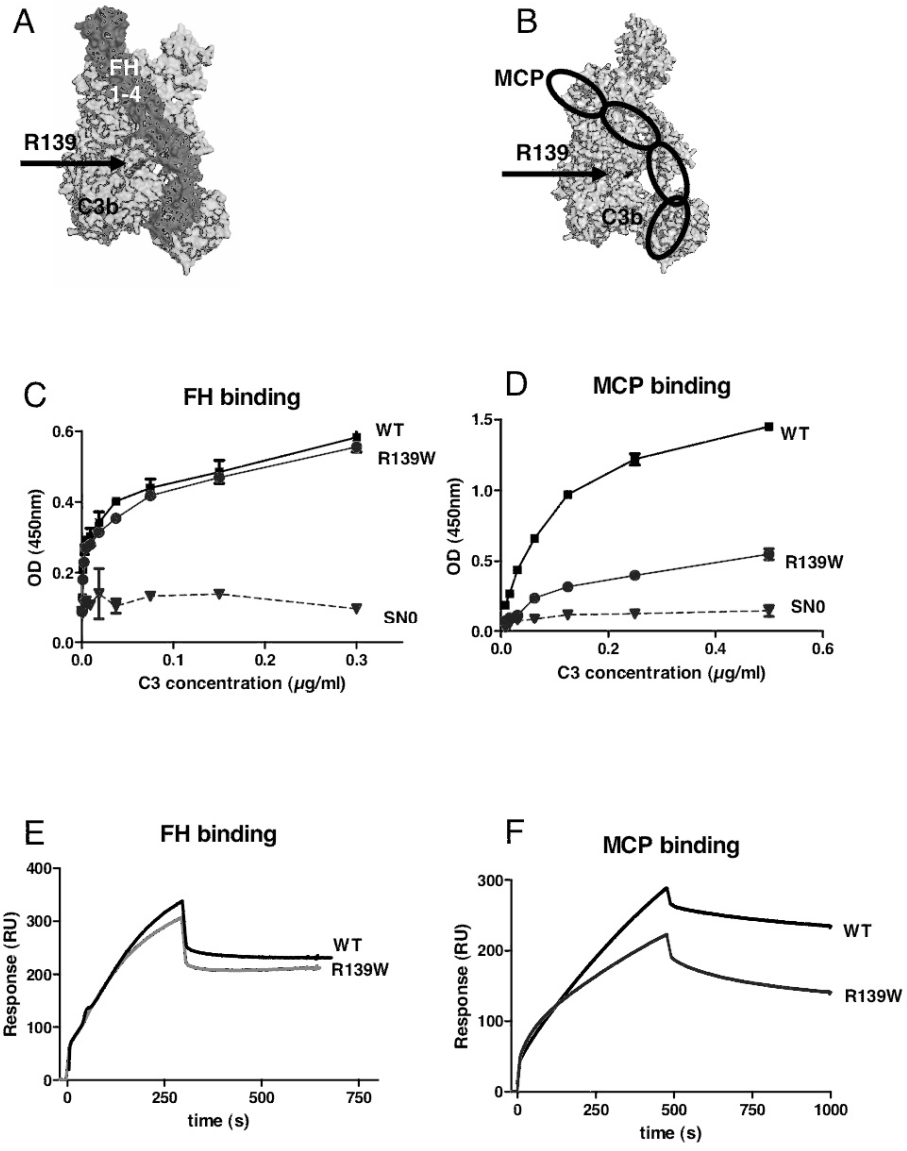
Roumenina et al., Figure 1



Roumenina et al., Figure 2



Roumenina et al., Figure 3



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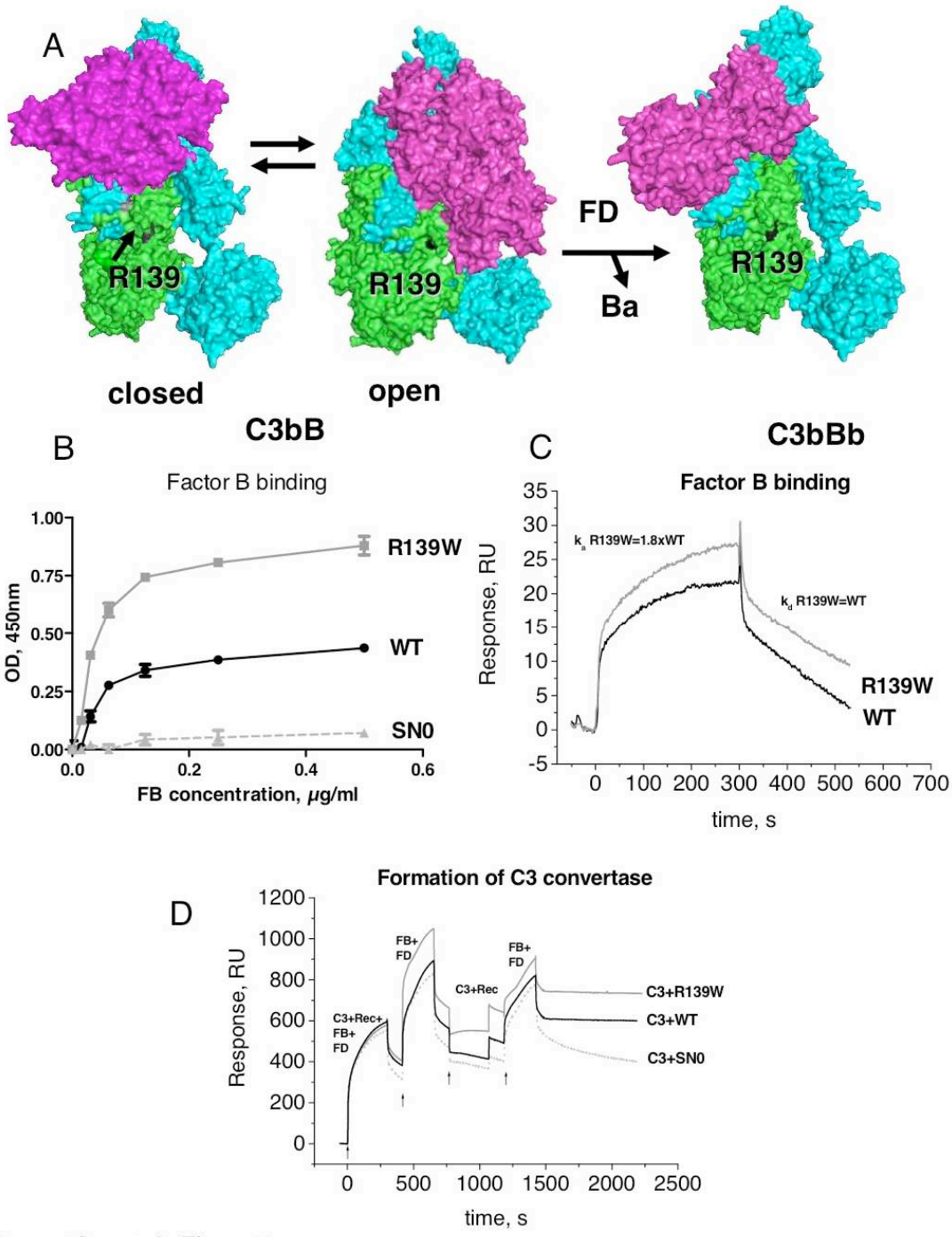

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Appendix 3

Supplementary data for Chapter 2

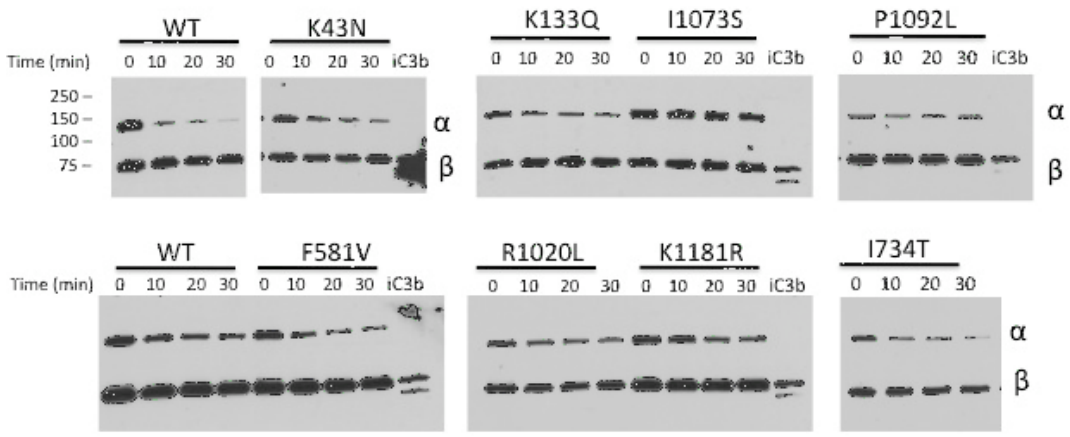

Figure A3.2. **C3 cleavage in FH cofactor assays**. WT and mutant C3 proteins were incubated with 20 ng FI and 200 ng FH at 37°C for the indicated times. The reactions were stopped with addition of 3X reducing sample buffer and then electrophoresed on 10% Tris-glycine gels. Proteins were transfered to a nitrocellulose membrane, and Western blots were probed with a goat anti-human C3 (1:5,000) followed by a rabbit anti-goat IgG HRP (1:3,000). Blots were developed with SuperSignal. Representative of four similar experiments.

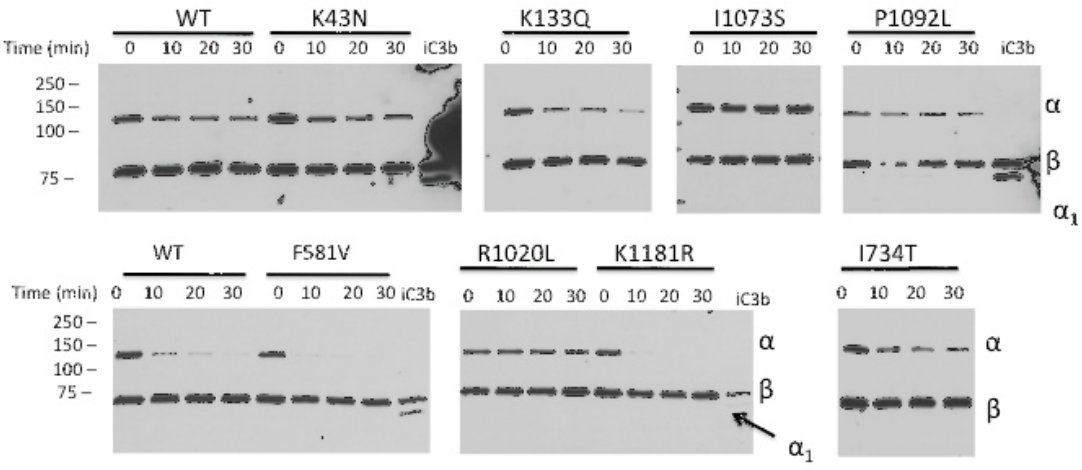

Figure A3.2. **C3 cleavage in MCP cofactor assays.** WT and mutant C3 proteins were incubated with 20 ng FI and 200 ng FH at 37°C for the indicated times. The reactions were stopped with addition of 3X reducing sample buffer and then electrophoresed on 10% Tris-glycine gels. Proteins were transfered to a nitrocellulose membrane, and Western blots were probed with a goat anti-human C3 (1:5,000) followed by a rabbit anti-goat IgG HRP (1:3,000). Blots were developed with SuperSignal. Representative of four similar experiments.