



Human C3 mutation reveals a mechanism of dense deposit disease pathogenesis and provides insights into complement activation and regulation

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Dense deposit disease (DDD) is a severe renal disease characterized by accumulation of electron-dense material in the mesangium and glomerular basement membrane. Previously, DDD has been associated with deficiency of factor H (fH), a plasma regulator of the alternative pathway (AP) of complement activation, and studies in animal models have linked pathogenesis to the massive complement factor 3 (C3) activation caused by this deficiency. Here, we identified a unique DDD pedigree that associates disease with a mutation in the C3 gene. Mutant C3_{923ADG}, which lacks 2 amino acids, could not be cleaved to C3b by the AP C3-convertase and was therefore the predominant circulating C3 protein in the patients. However, upon activation to C3b by proteases, or to C3(H₂O) by spontaneous thioester hydrolysis, C3_{923ADG} generated an active AP C3-convertase that was regulated normally by decay accelerating factor (DAF) but was resistant to decay by fH. Moreover, activated C3b_{923ADG} and C3(H₂O)_{923ADG} were resistant to proteolysis by factor I (fI) in the presence of fH, but were efficiently inactivated in the presence of membrane cofactor protein (MCP). These characteristics cause a fluid phase-restricted AP dysregulation in the patients that continuously activated and consumed C3 produced by the normal C3 allele. These findings expose structural requirements in C3 that are critical for recognition of the substrate C3 by the AP C3-convertase and for the regulatory activities of fH, DAF, and MCP, all of which have implications for therapeutic developments.

Introduction

Complement is a major component of innate immunity, with crucial roles in microbial killing, apoptotic cell clearance, immune complex handling, and modulation of adaptive immune responses. Complement is activated by 3 independent activation pathways: the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP). The critical steps in complement activation are the formation of unstable protease complexes, named complement factor 3-convertases (C3-convertases; specifically, C3bBb for AP and C4b2a for CP and LP), and the cleavage of C3 by the convertases to generate C3b. Convertase-generated C3b can form more AP C3-convertase, providing exponential amplification to the initial activation. Binding of C3b to the C3-convertases generates the C5-convertases with the capacity to bind and cleave C5, initiating formation of the lytic membrane attack complex (MAC). In contrast to the CP and the LP, whose activation is triggered by immune complexes and bacterial mannose groups, respectively, the AP is intrinsically activated. Spontaneous activation of C3 in plasma occurs through the tick-over mechanism,

which is initiated by hydrolysis of the internal C3 thioester to generate a C3b-like molecule, called C3i or C3(H₂O). Activation of C3 also occurs by the continuous low rate cleavage of C3 to C3b by plasma proteases (1). Progression of complement activation results from the balance between the rate at which the initial activation is amplified and the rate at which C3b and the AP C3-convertases are inactivated. Foreign substances on microbial pathogens (AP), antibodies (CP), or mannan (LP) disturb the balance in favor of amplification, causing target opsonization, leukocyte recruitment, inflammation, and cell lysis. In health, activation of C3 in plasma is kept at a very low level, and deposition of C3b and further activation of complement is limited to the surface of pathogens by multiple regulatory proteins, including factor H (fH), C4b-binding protein (C4BP), membrane cofactor protein (MCP), decay accelerating factor (DAF), complement receptor 1 (CR1), and CD59. These control complement activation and avoid wasteful consumption of components by inactivating C3b or C4b, by dissociating the C3/C5-convertases, or by inhibiting membrane attack complex (MAC) formation (2–4).

Dense deposit disease (DDD) is a rare form of glomerulonephritis that affects children and young adults and frequently develops into end-stage renal disease (ESRD; ref. 5). It is characterized by proliferation of mesangial and endothelial cells and by thickening of the peripheral capillary walls in the glomeruli (due to subendo-

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**Table 2**

Complement data in DDD patients and relatives

Patient	Relation	C3	C4	fH	fI	fB antigenic	fB hemolytic
GN28	Index case	94.2	26	21	100%	6.3	30%
I-1	Father	179	36	36	100%	25	100%
III-1	Son	94.4	22	22	100%	10	18%
III-2	Son	80.5	18	32	>100%	5.5	27%
II-3	Sister	114	26	43	>100%	34.6	100%
II-4	Brother	111	16	28	>100%	27	100%

Values are shown as mg/dl with the exception of fI and fB hemolytic, which are shown as percent of control. Reference ranges are as follows: C3, 80–177 mg/dl; C4, 14–47 mg/dl; fH, 10–35 mg/dl; fB antigenic, 7.5–28 mg/dl. C3 and fB values for the 3 individuals carrying the C3 mutation c.2767_2774delACGGTG (p.923ΔDG) are shown in bold; sequencing of the *CFH*, *CFB*, *CFI*, and *MCP* genes did not identify additional mutations in these individuals.

(13) and that the lytic pathway is not involved in the pathogenesis (14). Because fH regulates complement both in circulation and on cell surfaces, it is still debatable whether cell surface dysregulation plays a relevant role in DDD pathogenesis.

Here we report the first case to our knowledge of DDD caused by a mutation in the C3 gene, the functional characterization of which provided unique insights into DDD pathogenesis. Our findings provided conclusive evidence in humans that fluid phase-restricted AP dysregulation, which caused continuous generation of C3b in plasma, plays a major role in DDD pathogenesis. Furthermore, analysis of this C3 mutation advanced our understanding of the activation and regulation of the AP C3-convertase, providing useful information to unravel the structural requirements underlying the substrate recognition and regulatory activities of fH, DAF, and MCP.

Results

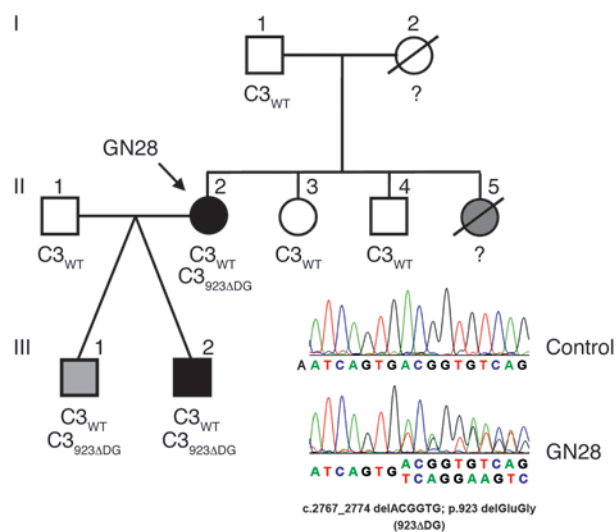
Familial case of DDD. We present the case of a 53-year-old woman (patient GN28; II-2) and her 26-year-old identical twin sons (referred to herein as III-1 and III-2). Their clinical presentation and development of disease are described in detail in Methods and summarized in Table 1. Biopsies taken from GN28, III-1, and III-2 illustrated similar findings by light and immunofluorescent microscopy that were consistent with a diagnosis of membranoproliferative glomerulonephritis (Figure 1). GN28 and III-2 reached ESRD after a prolonged period of progressive deterioration. III-1 showed persistent microhematuria and limited proteinuria, but still preserved renal function. Nevertheless, the profound pathological alterations found in the kidney biopsy from III-1 suggests that the patient will follow the same course to ESRD as his affected relatives. GN28 has been transplanted 3 times, with the disease recurring in all 3 allografts following the same course of disease as the original kidney.

Definitive diagnosis of DDD in GN28, III-2, and III-1 was established on the basis of EM analyses in renal biopsies performed at early stages of the disease. These analyses illustrated an electron-dense ribbon-like accumulation along the GBM and local electron-dense deposits in the mesangium. The latter was the predominant lesion found in the kidney biopsy of III-1. This finding may explain why III-1 still preserves renal function and suggest that, as disease progresses, increasing electron-dense deposits will be found in the GBM in this patient. The findings by EM matched the histology and immunofluorescence results (Figure 1) and were also con-

tent with the observations in biopsies from 2 allografts received by GN28 in which disease recurred.

Complement analysis in the 3 DDD-affected patients illustrated decreased levels of both C3 and fB (low-normal range) compared with their healthy relatives (Table 2), suggestive of activation through the AP. No activated C3 fragments were detected in plasma by Western blot, but terminal complement complex (TCC) levels were slightly elevated in GN28 and III-1 compared with controls (2.4 and 1.8 μg/ml, respectively; upper limit of normal, 1.24 μg/ml), indicating persistent low-grade C3 activation. Notably, although decreased, C3 levels in these patients were substantially higher than those usually found in DDD patients. Assays to identify C3Nef or anti-fH autoantibodies were negative. Levels of MCP on the surface of peripheral blood lymphocytes were normal in all 3 DDD patients.

Genetic analyses identify a C3 mutation associated with DDD. GN28, III-1, and III-2 were found to carry a mutation in heterozygosis in the C3 gene (Figure 2). The mutation, c.2767_2774delACGGTG (C3_{923ΔDG}) in exon 21, results in a mutated protein (C3_{923ΔDG}) lacking 2 amino acids (Asp923 and Gly924) in the MG7 domain of C3. No mutations were found in any other screened gene, including *CFH*, *MCP*, *CFI*, and *CFB*, and the C3_{923ΔDG} mutation was not detected in more than 300 unrelated individuals. In our pedigree, C3_{923ΔDG} was exclusively present in all 3 DDD-affected members (Figure 2). It was asso-

**Figure 2**

Mutation in the C3 gene in a multiaffected DDD pedigree. Pedigree of index case GN28 is illustrated. Individuals are identified by numbers within each generation. Affected individuals are indicated by black symbols. The twin brother III-1 (gray symbol) has not developed ESRD, but shows early signs of disease. II-5 is a sister of GN28 whose death at 13 years of age was attributed to glomerulonephritis. C3 alleles carried by the individuals are shown. The chromatogram corresponding to the DNA sequence surrounding the mutated nucleotides in C3 is shown for GN28 and a control sample. The corresponding amino acid sequences for the WT and mutated alleles are indicated. Amino acid numbering refers to the translation start site (Met +1), and the nucleotide nomenclature refers to nucleotide A in the ATG translation initiation codon, according to Human Genome Variation Society recommendations for description of sequence variants.



ciated with decreased C3 levels, probably secondary to C3 consumption, as the mutation also associated with low fB levels (Table 2). This evidence of complement activation and the observation that C3_{923ΔDG} was expressed normally in cells transfected with an expres-

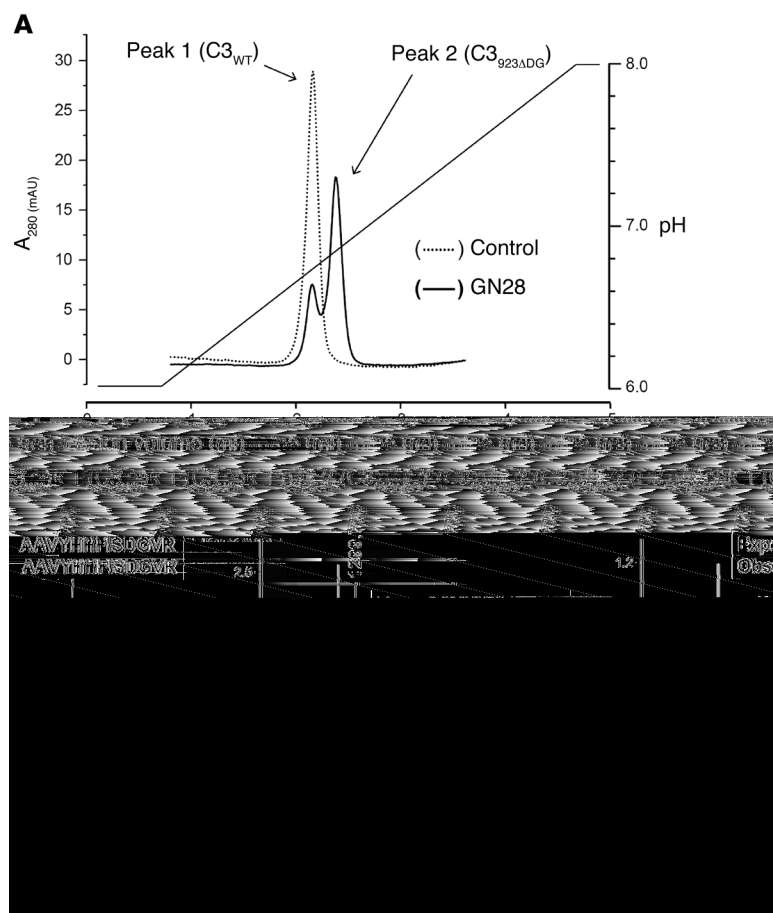


Figure 4
Purification of the mutant allele C3_{923ADG}. (A) C3_{923ADG} and C3_{WT} were separated as described in Methods. The elution profiles of this chromatographic separation of C3 prepared from control plasma (dotted line) and from GN28 plasma (solid line) are indicated. (B) The identity of the C3 variants was determined by mass spectrometry, as described in Methods. The minor C3_{WT} peak showed some contamination of the major C3_{923ADG} mutant protein (asterisk). Exp, expected; Obs, observed.

used SPR to monitor C3bBb formation and dissociation in real time (15). Hydrolyzed C3_{WT} (1,224 RU) or C3_{923ADG} (1,067 RU) was thiol-coupled to a CM5 chip, and convertase formation was analyzed by flowing fB (270 to 17 nM) over the surface in the presence of fD (43 nM). Kinetics were analyzed according to the Langmuir 1:1 binding model, and convertase formation by C3(H₂O)_{923ADG} and C3(H₂O)_{WT} was found to be comparable (Figure 6A). We tested next whether the AP C3-convertase generated from C3(H₂O)_{923ADG} was capable of activating C3_{WT}. We generated a C3-convertase immobilized onto a Biacore chip using either C3(H₂O)_{923ADG} or C3(H₂O)_{WT} and flowed C3_{WT} over the surface. The mutant C3-convertase was able to activate C3_{WT}, although it showed approximately 50% of the activity of the WT AP C3-convertase (Figure 6B).

C3(H₂O)_{923ADG} generates a C3-convertase resistant to fH inactivation. We have shown here that C3_{923ADG} activated spontaneously and that the activated C3(H₂O)_{923ADG} interacted normally with fB to generate an active C3-convertase. To determine whether the mutant C3-convertase is regulated efficiently by fH, we immobilized C3(H₂O)_{WT} or C3(H₂O)_{923ADG} as described above and flowed fH (1 μM to 8 nM) over the surface. The affinity of C3(H₂O)_{923ADG} for fH was reduced

compared with C3(H₂O)_{WT} (Figure 7A). In complementary experiments, we found that this decreased binding impaired the capacity of fH to both decay the convertase generated from C3(H₂O)_{923ADG} (Figure 7B) and act as a cofactor in the fI-mediated inactivation of C3(H₂O)_{923ADG} (Figure 7D). In contrast to the results obtained with fH, the C3-convertase generated from C3(H₂O)_{923ADG} was efficiently decayed by DAF (Figure 7C).

MCP, but not fH, catalyzes fI cleavage of C3b_{923ADG} and C3(H₂O)_{923ADG}. We generated large amounts of C3b_{923ADG} using trypsin and C3(H₂O)_{923ADG} using 0.33 M potassium isothiocyanate, and used them to test the cofactor activities of fH and MCP for fI-mediated proteolysis. In agreement with our findings described above, C3(H₂O)_{923ADG} interacted with and consumed fB in the presence of fD and Mg²⁺; trypsin-generated C3b_{923ADG} behaved similarly (Supplemental Figure 4). The trypsin-generated C3b_{923ADG} also formed an active AP C3-convertase on a Biacore chip, although it showed less than 5% the activity of the AP C3-convertase generated with C3b_{WT} (data not shown). Addition of C3b_{923ADG} to normal human sera activated complement and consumed C3 (Figure 8), which indicated that, despite its low activity, the mutant convertase was able to dysregulate the AP, most likely because C3b_{923ADG} is resistant to inactivation by fI and fH. As expected, C3b_{WT} had some capacity to activate C3 in normal serum, but this was much reduced compared with C3b_{923ADG}.

In order to confirm the differences in cofactor activity of fH and MCP in the fI-mediated proteolysis of C3b_{923ADG} and C3(H₂O)_{923ADG}, we performed a fluid phase assay. Identical amounts of C3b_{923ADG} and C3b_{WT} were added to purified fH (or MCP) in the presence of fI and incubated for 1, 2, 5, 10, 15, and 30 minutes at 37°C. C3b_{923ADG} was resistant to inactivation by fI in the presence of fH, but was inactivated by fI with soluble MCP (sMCP) at the same rate as C3b_{WT} (Figure 9). The same cofactor selectivity was found for C3(H₂O)_{923ADG} (Figure 10).

Discussion

Here we report the identification and functional characterization of C3_{923ADG}, which we believe to be the first C3 mutation associated with DDD. The functional consequences of this mutation are remarkable, providing fundamental insights into both DDD pathogenesis and structural aspects of AP C3-convertase control

Table 3
C3 proteins in the plasma of C3_{923ADG} mutation carriers

Patient	Allele	C3 (mg/dl)	Percent total C3
GN28	C3 _{WT}	26	27%
GN28	C3 _{923ADG}	68	73%
III-1	C3 _{WT}	31	32%
III-1	C3 _{923ADG}	64	68%
III-2	C3 _{WT}	28	35%
III-2	C3 _{923ADG}	52	65%

Total C3 levels were 94 mg/dl in GN28, 95 mg/dl in III-1, and 80 mg/dl in III-2.

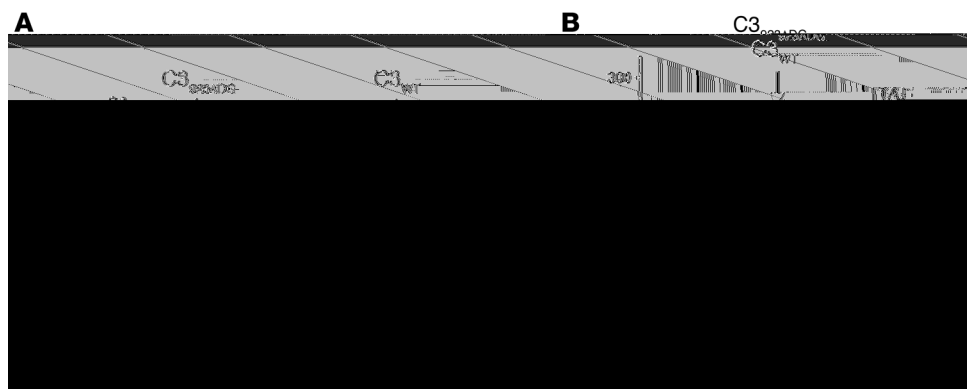


Figure 5

Resistance of purified C3_{923ADG} to cleavage by the AP C3-convertase. (A) C3_{923ADG} and C3_{WT} were purified to homogeneity and tested for their capacity to be cleaved to C3b in the presence of fB and fD; only the α chain of C3_{WT} was cleaved. Of note, C3_{923ADG} consumed fB, illustrating formation of a AP C3-convertase. This experiment was repeated twice. Lanes were run on the same gel but were noncontiguous (white lines). (B) C3_{WT} (1,000 RU) was immobilized via amine coupling to a CM5 Biacore chip. Convertase was formed by flowing fB (2.6 μ M) and fD (43 nM) in the presence of Mg²⁺. At the indicated time (arrows), C3_{WT} (gray line) or C3_{923ADG} (black line) was flowed over the surface. Remaining convertase was decayed using sDAF, and deposition of nascent C3b was measured. Resp. diff., response difference.

by the complement regulatory proteins fH, DAF, and MCP. The C3_{923ADG} mutation was found in heterozygosis in 2 DDD patients and in a relative in the early stages of the disease who present decreased levels of C3 and constitutive activation of the complement AP. C3_{923ADG} causes the deletion of 2 amino acids within MG7, but this does not affect C3 expression or its overall structure. The mutant C3_{923ADG} protein was the predominant C3 protein in the plasma of C3_{923ADG} carriers, where it circulated in the form of native, nonactivated, C3. As a consequence of the mutation, the mutant C3_{923ADG} could not be activated to C3b by the AP

C3-convertase. This explains why levels of C3 in the DDD patients carrying C3_{923ADG} were reduced only by 50%, in contrast to the complete C3 consumption found in most DDD patients. Crucially, we demonstrate that the C3_{923ADG} mutant could be activated to C3b_{923ADG} by proteases, or to C3(H₂O)_{923ADG} by the spontaneous hydrolysis of the thioester, and both generated an active AP C3-convertase that cleaved WT C3 to generate C3b. Moreover, these mutant C3-convertases were resistant to inactivation by fH, and neither C3b_{923ADG} nor C3(H₂O)_{923ADG} could be proteolyzed by fI in the presence of fH.

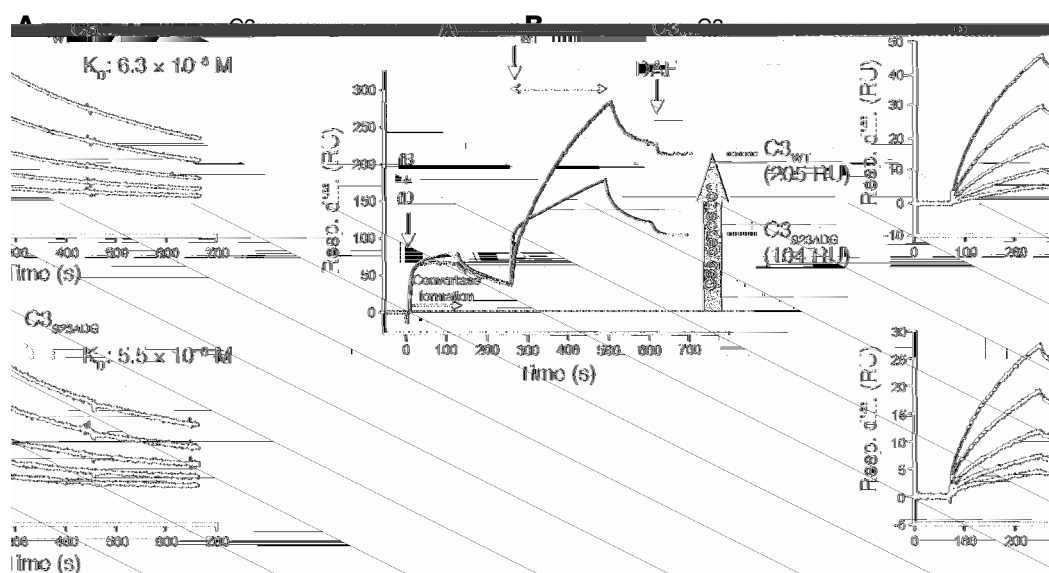


Figure 6

AP C3-convertase formation by C3_{923ADG} and C3_{WT}. (A) Hydrolyzed C3_{WT} (1,224 RU) or C3_{923ADG} (1,067 RU) was thiol-coupled to a CM5 Biacore chip. Convertase formation was analyzed by flowing fB (270 to 17 nM) over the surface in the presence of fD (43 nM). Kinetics were analyzed according to the Langmuir 1:1 binding model. Convertase formation by mutant C3_{923ADG} and C3(H₂O)_{WT}, measured as K_D, was comparable. (B) Hydrolyzed C3_{WT} (1,640 RU) or C3_{923ADG} (2,000 RU) was thiol-coupled to a CM5 Biacore chip. fB and fD were flowed over the surfaces to form either C3(H₂O)_{WT}Bb (black line) or C3(H₂O)_{923ADG}Bb (gray line). C3_{WT} was injected over the surface, where it was cleaved to nascent C3b and deposited on the surface via the thioester group. Remaining convertase after deposition was decayed using sDAF, and bound C3b was measured as change from baseline.



These altered functions provide a pathogenic mechanism that explains the development of DDD in our patients. C3_{923ADG} circulates in the plasma of the patients at stable and high levels and constantly produces activated C3 molecules by the tick-over mechanism (or through non-complement-mediated proteolysis), which cannot be inactivated by fH in plasma. In turn, the activated mutant C3 molecules generate active AP C3-convertases that cannot be regulated by fH, resulting in complement dysregulation in the fluid phase and substantial consumption of the WT C3 protein and fB in these heterozygote DDD patients.

In contrast to the situation in plasma, in which AP complement regulation depends almost exclusively on fH, on cell surfaces, complement regulators like MCP and DAF efficiently regulate the generation and stability of the AP C3-convertase. Our demonstration that the AP C3-convertases generated from the mutant C3_{923ADG} were decayed normally by DAF, and that the activated molecules C3b_{923ADG} and C3(H₂O)_{923ADG} were efficiently inactivated by fI in the presence of MCP, is most relevant to DDD pathogenesis. Thus, not only is C3_{923ADG} resistant to cleavage to C3b by the AP C3-convertases, limiting the deposition of C3_{923ADG} on the cell surface to the very few molecules that may spontaneously activate in its vicinity, but also these few mutant molecules will be efficiently controlled by DAF and MCP on the cell membranes. These data provide conclusive evidence that DDD in our patients results exclusively from a fluid phase-restricted AP dysregulation.

C3 is the most abundant protein of the complement system (~1.3 mg/ml). The structure of native C3 has been resolved at atomic resolution using X-ray crystallography (16) and reveals an intri-



fB. Cleavage of C3 into C3b, or generation of C3(H₂O), induces a huge conformational displacement of the TED domain, exposing the reactive group. In addition, this conformational change generates binding sites for a number of molecules, including the AP convertase component fB and the complement regulators fH, DAF, and MCP (reviewed in ref. 17). Because the 2-amino acid deletion caused by the C3_{923ADG}85 0 01_1 le h373794570226744.069 m44.891 743.01 44.941 741.95396 1 1 1 1 1 TBd/Td71.s9()-10(H)9 1 1 c



that fH and MCP have distinct structural requirements of C3b for their function. In agreement with early work (21, 22), our data indicate that the interaction between fH SCR1–SCR2 and C3b is critical for its cofactor activity, whereas for MCP, the interaction with this site in C3b does not substantially contribute to its cofactor activity (23, 24).

C3_{923ADG} could not be cleaved by the AP C3-convertase. Since the mutation locates distant to the AP C3-convertase cleavage site, one possibility is that it alters a region in C3 recognized by the substrate binding site in the C3b component of the AP C3-convertase. It was recently proposed that this interaction between C3 and C3b involves a large area on the same face of C3 (or C3b) that includes the domains MG3, MG4–5, and MG6–8 (25). Interestingly, this area of C3 overlaps with binding sites for the inhibitors compstatin (26), CRIg (27), and antibody S77 (28), which block substrate binding to the C3-convertase. In fact, the C3_{923ADG} mutation coincides with the interaction site of S77, which involves the His897 amino acid residue in C3 (28). It is therefore very likely that the mutant C3_{923ADG} could not be cleaved by the C3bBb convertase because the sequence on C3 recognized by the highly specific substrate binding site located on the C3b component of the C3bBb convertase has been lost (Supplemental Figure 6). These data may also explain why the AP C3-convertase generated by the mutant C3b_{923ADG} showed minimal activity compared with that generated by C3_{WT}. In this context, however, it is remarkable that C3(H₂O)_{923ADG} built a relatively normal AP C3-convertase that efficiently cleaved C3_{WT} (Figure 6). This suggests that the structural requirements for the substrate (C3) recognition in C3b are influenced by the presence of the C3a domain in C3(H₂O). This hypothesis, together with earlier data by Bexborn et al. (29) showing that the C3(H₂O)Bb convertase is more resistant to inactivation by fH and fI, warrant further research – out of the scope of the present study – into the role and specific properties of the C3-convertase generated through the tick-over mechanism.

In conclusion, our identification and functional characterization of the C3_{923ADG} mutation provided fundamental insights into both the pathogenic mechanisms underlying DDD and the structural aspects of substrate recognition and regulation of the C3bBb convertase. Previous studies have shown that the control of complement activation is impaired in DDD patients and that dysregulation of the complement system may result from decreased activity of the complement regulatory protein fH. We showed here that fluid phase complement dysregulation may also be a consequence of mutations in the AP C3-convertase components making the convertase resistant to inactivation by fH. This has important therapeutic implications. Although replacement therapies providing fH may be successful in the former case, they will be ineffective if C3 is mutated. In this latter case, however, patients may benefit from therapies involving soluble forms of membrane-associated regulators like MCP. Recently, several mutations and polymorphisms in the genes encoding fH, MCP, and DAF have been associated with a number of disorders involving complement dysregulation (30). Interestingly, the functional characterization of these genetic variations and laboratory mutants reveals that, despite their common evolutionary origin and overlapping functions, fH, MCP, and DAF have distinct structural requirements in their regulatory activities. The structural characterization of the C3_{923ADG} mutant may help to delineate the interaction sites for the different complement regulators in C3b, which, again, may have important implications in the design of therapeutic agents.

Methods

Patients. GN28 (II-2), a 53-year-old woman, presented with hypertension, microscopic hematuria and proteinuria at age 25 years, during the third trimester of her first and only identical twin pregnancy. She had an episode of nephritis at age 7. After 2 years of persistent microhematuria, proteinuria rising to 1.5 g/day and plasma creatinine (Cr) of 0.9 mg/dl, a renal biopsy was performed that illustrated segmental mesangial hypercellularity with thickened, brightly eosinophilic segments of basement membrane (Figure 1, A–D). There were prominent and diffuse C3 deposits, granular and nodular in some glomerular areas (Figure 1G). Transmission EM demonstrated the presence of a ribbon-like, osmophilic deposit in the GBM (Figure 1, E and F). Mild deposits of C1q, IgA, and IgM were also present. Anti-nuclear (ANA) and anti-DNA antibodies were negative. Levels of C4 were normal, whereas C3 and fB were in the low-normal range. During the following 6 years, renal function progressively declined with proteinuria, reaching a nephrotic range of 7 g/d. In 1991, at age 35, the patient started dialysis. In 1992, she received a cadaver kidney allograft that lasted until 1997. Interestingly, beginning the second month after transplant, the patient presented with hypertension, microhematuria, and progressive proteinuria that reached nephrotic range in the fourth posttransplant year. The patient went back to dialysis the following year. In 1998, she received a second cadaver kidney allograft that lasted until 2003, following a similar period of progressive renal insufficiency and proteinuria, this time beginning 3 years after transplantation. Biopsies taken from this and the previous kidney allograft, illustrated microscopic findings similar to those found in the original kidney. In 2006, the patient received a third cadaver kidney allograft that is still functioning. However, the patient presents microhematuria, proteinuria, and progressive renal insufficiency that is accelerating in the third posttransplant year.

III-2, 26 years old, is one of the identical twin sons of GN28. At age 2, coincident with an episode of fever, he presented with microhematuria. In 1999 (age 16 years), he was admitted to hospital because of hyperuricemia (9.1 mg/dl), showing proteinuria of 1.5 g/d, microhematuria (10–25 erythrocytes/field), and Cr of 1.4 mg/dl, corresponding to a creatinine clearance (CCr) of 73 ml/min. Like GN28, levels of C4 were normal, but C3 and fB were in the low-normal range. 5 years later (age 21 years), renal function started to decline with a Cr of 2 mg/dl, CCr of 46 ml/min, proteinuria of 1.1 mg/dl, and persistent microhematuria. Another 6 months later, Cr and CCr rose to 3 mg/dl and 33 ml/min, respectively. Renal biopsy at this time showed membranoproliferative glomerulonephritis with intense C3 deposits similar to those observed in the kidney biopsies of GN28. 9 years later, his renal function deteriorated to ESRD. The patient is currently on peritoneal dialysis.

III-1, 26 years old, is one of the identical twin sons of GN28. Despite microhematuria being evident in occasional follow-up visits, the patient was not available for assessment until 2006 (age 23 years). In his first visit to the nephrologist, he showed hyperuricemia of 8.5 mg/dl, Cr of 1.4 mg/dl with a CCr of 105 ml/min, proteinuria of 0.4 mg/dl, and microhematuria (12 erythrocytes/field). These values were slightly increased in 2010 (Cr, 1.6 mg/dl; CCr, 96 ml/min; proteinuria of 0.5 mg/dl), suggestive of progressive deterioration. As with the affected relatives, levels of C4 were normal, whereas C3 and fB were in the low-normal range. Renal biopsy showed membranoproliferative glomerulonephritis with intense mesangial C3 deposits and very limited C3 deposition within the GBM. This finding is in contrast with the kidney biopsies from GN28 and III-2, which may explain why the patient still preserves renal function.

The studies described herein received IRB approval (Comision de Bioetica, Consejo Superior de Investigaciones Cientificas, Madrid, Spain). Patients and their relatives gave their informed consent.



Complement analysis. Plasma or serum C3 and C4 levels were measured using standard nephelometric assays (Siemens). fH, fI, and fB levels were measured by sandwich ELISA as previously described (31–33). Anti-fH and C3Nef autoantibodies were detected as described previously (34, 35). Their concentrations in plasma were calculated by reference to the appropriate calibration curve prepared from purified proteins and expressed as mg/dl plasma, or percent of control for fI concentration. fB hemolytic activity was tested according to Lesavre et al. (36).

Mutation screening and genotyping. Genomic DNA was obtained from peripheral blood mononuclear cells using Puregene Blood Core kit B (QIAGEN) according to the manufacturer's instructions. Each exon of the *CFH*, *MCP*, *CFI*, and *CFB* genes was amplified from genomic DNA using specific primers derived from the 5' and 3' intronic sequences, as described previously (37–39). Exons of the *C3* gene were amplified from genomic DNA using the primers described in Supplemental Table 1. Automatic sequencing was performed in an ABI 3730 sequencer using a dye terminator cycle sequencing kit (Applied Biosystems).

Biosensor analysis. To measure affinity of fB or fH for C3, hydrolyzed C3_{WT} (1,224 RU) or C3_{923ADG} (1,067 RU) was thiol-coupled to a CM5 Biacore chip according to the manufacturer's instructions (ligand thiol coupling method; GE Healthcare). Convertase formation was analyzed by flowing fB (270 nM to 17 nM) over the surface in the presence of fD (1 µg/ml; Comptech) in HBS/Mg/P (10 mM HEPES, pH 7.4; 150 mM NaCl; 1 mM MgCl₂; and 0.005% surfactant-P20). The affinity for native fH was similarly analyzed by flowing fH (1 µM to 8 nM) over the surface. All samples were injected using the Kinject command, flowed at 30 µl/min, and analyzed at 25°C. Kinetic experiments were carried out on a Biacore T100 (GE Healthcare); fB affinity was calculated according to the Langmuir 1:1 binding model, and fH affinity was determined by steady-state analysis (Biaevaluation v1.1).

In order to assess fH-mediated decay of the convertases, hydrolyzed C3_{WT} or C3_{923ADG} was thiol-coupled to the chip, convertase was formed on each surface by flowing fB and fD, and, after 60 seconds of natural decay, either native fH or sDAF was injected for 60 seconds. The same injection of fH was performed in the absence of convertase formation, and the binding curve was subtracted to control for fH binding to the surface; the resultant curve was therefore representative of Bb decay only. Accelerated decay was evident by the sharp increase in the dissociation rate.

Cofactor activity was assessed by flowing fH (0.33 µM) and fI (0.11 µM) across the surface for 5 minutes at 5 µl/min. The capacity of C3(H₂O) on the surface to form a convertase was assessed before and after fH and fI injection by flowing fB and fD; decrease in convertase formation indicated cleavage of C3(H₂O) to iC3(H₂O).

To determine whether convertase formed by C3(H₂O)_{923ADG} was active and able to cleave C3_{WT} to C3b, hydrolyzed C3 was thiol-coupled to a CM5 Biacore chip on a Biacore 3000 (GE Healthcare), and convertase was formed by flowing fB and fD in HBS/Mg/P for 120 seconds. After a short dissociation period (approximately 120 seconds), C3_{WT} was flowed over the surface (22 µM) for 240 seconds, Bb was decayed using sDAF, and C3b bound to the surface was assessed by the change in baseline as indicated. To determine whether C3_{923ADG} was a substrate for the C3_{WT} convertase, C3b_{WT} (1,000 RU) was coupled to the surface via the thioester as previously

described (15). Convertase was formed in HBS/Mg/P by flowing fB (2.7 µM) and fD (43 nM) for 120 seconds. Following a period of dissociation, C3_{923ADG} was injected at a concentration of 0.4 µM at 10 µl/min for 120 seconds. Remaining convertase was decayed using 0.4 µM sDAF. The convertase was formed again as described above, and 0.4 µM C3_{WT} was similarly flowed over the surface. Cleavage and deposition of C3b was assessed by change in the baseline after regeneration.

Activation of C3 in fluid phase. Purified C3 (2.7 µM), fB (0.5 µM), and fD (0.17 µM) in 20 mM sodium phosphate buffer (pH 7), 40 mM NaCl, and 2 mM MgCl₂ were incubated in a water bath at 37°C. Aliquots of 5 µl were extracted from the mix at 0, 0.5, 1, 2, 4, 8, and 16 minutes; mixed with SDS-PAGE sample buffer (2% SDS, 62.5 mM Tris, 10% glycerol, and 0.75% bromophenol blue) to stop the reaction; and loaded into a 10% reducing SDS-PAGE gel. The gels were stained using Coomassie brilliant blue R-250 (BioRad).

fH and sMCP cofactor activity for fI-mediated proteolysis of fluid phase C3b. The fluid-phase cofactor activities of fH and sMCP were determined in a C3b proteolytic assay using purified proteins. In brief, C3b, fH or sMCP, and fI were mixed in 10 mM HEPES (pH 7.5), 150 mM NaCl, and 0.02% Tween 20. Final concentrations in one set were 1.9 µM C3b, 0.2 µM fI, and 0.46 µM sMCP, and in the other set 0.42 µM C3b, 43 nM fI, and 47 nM fH. Molarities were calculated using the following masses: fI, 88 kDa; C3, 185 kDa; fH, 155 kDa; fD, 23 kDa; fB, 93 kDa; sDAF, 28 kDa; sMCP, 28 kDa. Mixtures were incubated at 37°C in a water bath, and 6-µl aliquots were collected at 0, 1, 2, 5, 10, 15, 30, and 60 minutes. The reaction was stopped by the addition of 5 µl SDS sample buffer. Samples were analyzed in 10% SDS-PAGE under reducing conditions. Gels were stained with Coomassie brilliant blue R-250 (BioRad), and proteolysis of C3b was determined by analyzing the cleavage of the α' chain.

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- Amara U, et al. Interaction between the coagulation and complement system. *Adv Exp Med Biol.* 2008;632:71–79.
- Lachmann PJ. The amplification loop of the complement pathways. *Adv Immunol.* 2009;104:115–149.
- Walport MJ. Complement- second of two parts. *N Engl J Med.* 2001;344(15):1140–1144.
- Walport MJ. Complement- first of two parts. *N Engl J Med.* 2001;344(14):1058–1066.

- Smith RJH, et al. New approaches to the treatment of dense deposit disease. *J Am Soc Nephrol.* 2007;18(9):2447–2456.
- Walker PD. Dense deposit disease: new insights. *Curr Opin Nephrol Hypertens.* 2007;16(3):204–212.
- Ault BH, et al. Human factor H deficiency. *J Biol Chem.* 1997;272(40):25168–25175.
- Dragon-Durey MA, et al. Heterozygous and homozygous factor h deficiencies associated with hemo-

- lytic uremic syndrome or membranoproliferative glomerulonephritis: report and genetic analysis of 16 cases. *J Am Soc Nephrol.* 2004;15(3):787–795.
- Zipfel PF, Heinen S, Józsi M, Skerka C. Complement and diseases: Defective alternative pathway control results in kidney and eye diseases. *Mol Immunol.* 2006;43(1–2):97–106.
- Licht C, et al. Deletion of Lys224 in regulatory domain 4 of Factor H reveals a novel pathomechanism



- for dense deposit disease (MPGN II). *Kidney Int.* 2006;70(1):42–50.
11. HögÅsen K, Jansen JH, Mollnes TE, Hovdenes J, Harboe M. Hereditary porcine membranoproliferative glomerulonephritis type II is caused by factor H deficiency. *J Clin Invest.* 1995;95(3):1054–1061.
 12. Pickering MC, et al. Uncontrolled C3 activation causes membranoproliferative glomerulonephritis in mice deficient in complement factor H. *Nat Genet.* 2002;31(4):424–428.
 13. Rose KL. Factor I is required for the development of membranoproliferative glomerulonephritis in factor H-deficient mice. *J Clin Invest.* 2008;118(2):608–618.
 14. Pickering MC, et al. Prevention of C5 activation ameliorates spontaneous and experimental glomerulonephritis in factor H-deficient mice. *Proc Natl Acad Sci U S A.* 2006;103(25):9649–9654.
 15. Harris CL, Abbott RJM, Smith RA, Morgan BP, Lea SM. Molecular dissection of interactions between components of the alternative pathway of complement and decay accelerating factor (CD55). *J Biol Chem.* 2005;280(4):2569–2578.
 16. Janssen BJC, et al. Structures of complement component C3 provide insights into the function and evolution of immunity. *Nature.* 2005;437(7058):505–511.
 17. Gros P, Milder FJ, Janssen BJC. Complement driven by conformational changes. *Nat Rev Immunol.* 2008;8(1):48–58.
 18. Torreira E, Tortajada A, Montes T, Rodríguez de Córdoba S, Llorca O. 3D structure of the C3bB complex provides insights into the activation and regulation of the complement alternative pathway convertase. *Proc Natl Acad Sci U S A.* 2009;106(3):882–887.
 19. Torreira E, Tortajada A, Montes T, Rodríguez de Córdoba S, Llorca O. Coexistence of closed and open conformations of complement factor b in the alternative pathway C3bB(Mg2+) proconvertase. *J Immunol.* 2009;183(11):7347–7351.
 20. Wu J, Wu YQ, Ricklin D, Janssen BJC, Lambris JD, Gros P. Structure of complement fragment C3b-factor H and implications for host protection by complement regulators. *Nat Immunol.* 2009;10(7):728–733.
 21. Gordon DL, Kaufman RM, Blackmore TK, Kwong J, Lublin DM. Identification of complement regulatory domains in human factor H. *J Immunol.* 1995;155(1):348–356.
 22. Kuhn S, Skerka C, Zipfel PF. Mapping of the complement regulatory domains in the human factor H-like protein 1 and in factor H1. *J Immunol.* 1995;155(12):5663–5670.
 23. Liszewski MK, Leung MK, Schraml B, Goodship THJ, Atkinson JP. Modeling how CD46 deficiency predisposes to atypical hemolytic uremic syndrome. *Mol Immunol.* 2007;44(7):1559–1568.
 24. Richards A, et al. Implications of the initial mutations in membrane cofactor protein (MCP; CD46) leading to atypical hemolytic uremic syndrome. *Mol Immunol.* 2007;44(1–3):111–122.
 25. Rooijakkers SHM, et al. Structural and functional implications of the alternative complement pathway C3 convertase stabilized by a staphylococcal inhibitor. *Nat Immunol.* 2009;10(7):721–727.
 26. Janssen BJC, Halff EF, Lambris JD, Gros P. Structure of compstatin in complex with complement component C3c reveals a new mechanism of complement inhibition. *J Biol Chem.* 2007;282(40):29241–29247.
 27. Wiesmann C, et al. Structure of C3b in complex with CR1 gives insights into regulation of complement activation. *Nature.* 2006;444(7116):217–220.
 28. Katschke KJ, et al. Structural and functional analysis of a C3b-specific antibody that selectively inhibits the alternative pathway of complement. *J Biol Chem.* 2009;284(16):10473–10479.
 29. Bexborn F, Andersson PO, Chen H, Nilsson B, Ekdahl KN. The tick-over theory revisited: Formation and regulation of the soluble alternative complement C3 convertase (C3(H₂O)Bb). *Mol Immunol.* 2008;45(8):2370–2379.
 30. Rodríguez de Córdoba S, Goicoechea de Jorge E. Translational mini-review series on complement factor H: genetics and disease associations of human complement factor H. *Clin Exp Immunol.* 2008;151(1):1–13.
 31. Goicoechea de Jorge E, et al. Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome. *Proc Natl Acad Sci U S A.* 2007;104(1):240–245.
 32. Gonzalez-Rubio C, Ferreira-Cerdan A, Ponce IM, Arpa J, Fontan G, Lopez-Trascasa M. Complement Factor I deficiency associated with recurrent meningitis coinciding with menstruation. *Arch Neurol.* 2001;58(11):1923–1928.
 33. Hakobyan S, et al. Measurement of factor H variants in plasma using variant-specific monoclonal antibodies: application to assessing risk of age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2008;49(5):1983–1990.
 34. Abarrategui-Garrido C, Martínez-Barricarte R, Lopez-Trascasa M, Rodríguez de Córdoba S, Sánchez-Corral P. Characterization of complement factor H-related (CFHR) proteins in plasma reveals novel genetic variations of CFHR1 associated with atypical hemolytic uremic syndrome. *Blood.* 2009;114(19):4261–4271.
 35. Rother U. A new screening test for C3 nephritis factor based on a stable cell bound convertase on sheep erythrocytes. *J Immunol Methods.* 1982;51(1):101–107.
 36. Lesavre PH, Hugli TE, Esser AF, Müller-Eberhard HJ. The alternative pathway C3/C5 convertase: chemical basis of factor B activation. *J Immunol.* 1979;123(2):529–534.
 37. Fremeaux-Bacchi V, et al. Complement factor I: a susceptibility gene for atypical haemolytic uraemic syndrome. *J Med Genet.* 2004;41(6):e84.
 38. Perez-Caballero D, et al. Clustering of missense mutations in the C-terminal region of factor H in atypical hemolytic uremic syndrome. *Am J Hum Genet.* 2001;68(2):478–484.
 39. Richards A, et al. Mutations in human complement regulator, membrane cofactor protein (CD46), predispose to development of familial hemolytic uremic syndrome. *Proc Natl Acad Sci U S A.* 2003;100(22):12966–12971.