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 C3923ADG, 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, C3923ADG generated an active AP C3-convertase that was regulated normally by decay accelerating factor (DAF) but was resistant to decay by fH. Moreover, activated C3b923ADG 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 mannann (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-

Authorship note: Rubén Martínez-Barricarte and Meike Heurich, as well as Claire L. Harris and Santiago Rodríguez de Córdoba, contributed equally to this work.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest doi:10.1172/JCI43343.
thelial and intramembranous dense deposits) that present a double-contour appearance upon light microscopy. The morphological hallmark of DDD is the presence of dense deposits within the glomerular basement membrane (GBM), as resolved by EM (6). The chemical composition of the dense deposits is largely unknown. Notably, IgG is absent from them and from other regions of the glomerulus, which excludes a role for immune complexes in their formation. DDD is associated with complement abnormalities that lead to intense deposition of C3 activation products in GBM and persistent reduction of C3 serum levels. Among the different factors associated with these complement abnormalities are the deficiency of the plasma AP regulator fH, as a result of mutations in the CFH gene or the presence of autoantibodies against fH, or the presence of autoantibodies against the C3-convertase (C3 nephritic factors; C3Nef). Familial cases of DDD are exceptional. There are only approximately 6 patients described in the literature in which deficiency of fH, either heterozygous or homozygous, is associated with the development of DDD. In all but one of these cases, the fH deficiency is caused by mutations in CFH that result in truncations or amino acid substitutions that impair secretion of fH into circulation (7–9). The exception is the case of a CFH mutation in the complement regulatory region of fH that markedly reduced both the fH-mediated C3b cofactor activity and the AP C3-convertase decay-accelerating activity of fH (10).

The severe dysregulation of the AP observed in DDD patients is consistent with data from animals presenting this renal phenotype. In the pig, fH deficiency results in accelerating activity of fH (10). and the AP C3-convertase decay-accelerating activity of fH are also markedly reduced. The mouse model has also shown that the development of DDD requires fI, which suggests that the C3b, generated at high levels in the absence of fH, needs to be proteolyzed to iC3b, C3c, and C3dg to produce this pathology (13). Furthermore, transplantation studies in the fH-deficient animals illustrate that glomerular C3 deposition derives from circulation also resembles human DDD (12). These fH-deficient animals have been very useful in demonstrating that the uncontrolled activation of C3 in plasma resulting from the lack of fH is essential for DDD development (12). The mouse model has also shown that the development of DDD requires fI, which suggests that the C3b, generated at high levels in the absence of fH, needs to be proteolyzed to iC3b, C3c, and C3dg to produce this pathology (13). Furthermore, transplantation studies in the fH-deficient animals illustrate that glomerular C3 deposition derives from circulation.

**Table 1**

Progression of DDD patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>First clinic visit</th>
<th>Current kidney status</th>
<th>Time to ESRD</th>
<th>Biopsy</th>
<th>Renal transplant</th>
<th>Graft recurrence</th>
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<tr>
<td>GN28</td>
<td>53</td>
<td>Female</td>
<td>28 yr 0.9 mg/dl 1.5 g/24 h Yes</td>
<td>ESRD</td>
<td>7 yr</td>
<td>Yes</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>III-1</td>
<td>26</td>
<td>Male</td>
<td>23 yr 1.4 mg/dl 0.4 g/24 h Yes</td>
<td>Functioning&lt;sup&gt;2&lt;/sup&gt;</td>
<td>–</td>
<td>Yes</td>
<td>No</td>
<td>–</td>
</tr>
<tr>
<td>III-2</td>
<td>26</td>
<td>Male</td>
<td>16 yr 1.6 mg/dl 0.6 g/24 h Yes</td>
<td>ESRD&lt;sup&gt;2&lt;/sup&gt;</td>
<td>9 yr</td>
<td>Yes</td>
<td>No</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>1</sup>Two recurrences, and current transplant indicative of early stages of disease.
<sup>2</sup>Early stages of disease: persistent microscopic hematuria, 96 ml/min glomerular filtration rate.

![Figure 1](http://www.jci.org)

**Figure 1**

Histology, immunofluorescence, and EM findings. The first kidney biopsy in GN28 was performed in 1985. Although there was considerable variation in glomerular changes, there was remarkable similarity in the light, immunofluorescence, and ultrastructural findings in the original kidney biopsy and 2 allograft biopsies of GN28 and the kidney biopsies from III-1 and III-2. The characteristic histological lesion consisted of segmental mesangial hypercellularity with thickened, eosinophil-rich segments of basement membrane (A and B, arrows). The affected glomerular segments were PAS positive and reacted to trichrome stain (D, arrow). The affected tufts showed hypercellularity, leukocyte infiltration, and endothelial swelling. The mesangium showed variable expansion, matrix accumulation, and a lobular pattern (C and D, arrows). The main immunofluorescence findings were prominent and diffuse C3 deposits, granular and nodular in some glomerular areas (G and J). Mild deposits of C1q, IgA, and IgM were also associated with these deposits (not shown). All biopsies showed similar ultrastructural alterations consisting of a ribbon-like, osmiophilic deposit present in the GBM (E and I, red arrows). These deposits occasionally showed signs of dissolution with translucent areas (F, red arrows). The mesangial areas showed increased mesangial matrix with electron-dense deposits (H and I, yellow arrows). Original magnification: ×400 (A, B, G, and J); ×500 (C and D); ×2,200 (E and F); ×5,500 (H); ×7,800 (I).

Patient number is indicated within each panel.
that the patient will follow the same course to ESRD as his affective 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-1, and III-2 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 consistent 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 (p.923DG), was included in a panel of 300 unrelated individuals. In our pedigree, C3DG was exclusively present in all 3 DDD-affected members (Figure 2). It was associated with a C-terminal truncation, with the exception of fB 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.923DG) are shown in bold; sequencing of the CFH, CFB, CFI, and MCP genes did not identify additional mutations in these individuals.

Table 2

<table>
<thead>
<tr>
<th>Patient</th>
<th>Relation</th>
<th>C3</th>
<th>C4</th>
<th>fH</th>
<th>fI</th>
<th>fB antigenic</th>
<th>fB hemolytic</th>
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<tr>
<td>GN28</td>
<td>Index case</td>
<td>94.2</td>
<td>26</td>
<td>21</td>
<td>100%</td>
<td>6.3</td>
<td>30%</td>
</tr>
<tr>
<td>I-1</td>
<td>Father</td>
<td>179</td>
<td>36</td>
<td>36</td>
<td>100%</td>
<td>25</td>
<td>100%</td>
</tr>
<tr>
<td>III-1</td>
<td>Son</td>
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<td>22</td>
<td>100%</td>
<td>10</td>
<td>18%</td>
</tr>
<tr>
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<td>Son</td>
<td>80.5</td>
<td>18</td>
<td>32</td>
<td>&gt;100%</td>
<td>5.5</td>
<td>27%</td>
</tr>
<tr>
<td>II-3</td>
<td>Son</td>
<td>114</td>
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<td>43</td>
<td>&gt;100%</td>
<td>34.6</td>
<td>100%</td>
</tr>
<tr>
<td>II-4</td>
<td>Brother</td>
<td>111</td>
<td>16</td>
<td>28</td>
<td>&gt;100%</td>
<td>27</td>
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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 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.

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C3 purifycated from GN28, approximately half the C3 was cleaved, corresponding 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 expression plasmid in vitro (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI43343DS1) suggest that C3_923ΔDG is likely a gain-of-function mutation causing constitutive activation of complement. C3 mutation screening in all DDD patients in our cohort (n = 8) failed to identify additional patients carrying the C3_923ΔDG mutation or additional C3 mutations.

The C3_923ΔDG mutation deletes 2 amino acids within MG7 in the polypeptide linking the MG7 and CUB domains. The deletion shortens the distance between the MG7 and CUB domains, likely displacing the upstream and downstream amino acids from their original positions. It is likely that these structural changes alter the function of C3 in a way that results in DDD. To test this possibility, we purified the mutant C3_923ΔDG protein and assessed its function. C3_923ΔDG is the major circulating C3 protein in the DDD patients. C3 was purified to homogeneity from EDTA-plasma obtained from GN28, III-1, III-2, and normal controls (Supplemental Methods and Supplemental Figure 2). Interestingly, when mixed with fB and fD in the presence of Mg²⁺, the C3 purified from GN28, III-1, and III-2 was largely resistant to C3b generation (Figure 3). The presence of potential inhibitory contaminants in the C3 preparations from GN28, III-1, and III-2 was ruled out by showing that in a C3 pre-treatment containing equal amounts of normal control C3 and mutant C3 purified from GN28, approximately half the C3 was cleaved, corresponding to the amount of normal protein present (Figure 3).

Separation of C3_923ΔDG from C3_WT in the C3 preparation from GN28, III-1, and III-2 was achieved using a Mono S HR 1.6/5 cation exchange column (GE Healthcare) with a pH 6–8 gradient (20 mM Na/K phosphate, 40 mM NaCl). In this chromatographic setting, C3 from GN28 produced 2 well-separated peaks, the first comigrating with the single C3 peak obtained from a similar experiment with a C3 preparation from a control individual (Figure 4A). Proteomic analysis of the C3 protein contained in each peak confirmed that the second peak corresponded to pure C3_923ΔDG mutant protein (Figure 4B). EM analyses of the C3_WT and C3_923ΔDG proteins purified from the GN28 plasma resulted in 3D structures that were indistinguishable at the resolution level of this technique (approximately 25Å), which indicates that the deletion of the 2 amino acids in the C3_923ΔDG mutant did not cause gross changes in the structure of the C3 protein. The structures also demonstrated that C3_923ΔDG circulates in plasma mainly in the native (nonactivated) C3 conformation (Supplemental Figure 3). Similar proportions (approximately 1:2) of C3_WT and C3_923ΔDG were present in the plasma samples from the 3 C3_923ΔDG mutation carriers (Table 3).

When purified C3_923ΔDG was incubated with fB and fD in the presence of Mg²⁺, no generation of C3b was observed despite cleavage of fB and generation of the Bb fragment; conversely, C3_WT was completely converted to C3b under these conditions (Figure 5A). To confirm that C3_923ΔDG could not be activated to C3b by the AP C3-convertase, we performed surface plasmon resonance (SPR; Biacore) experiments with the C3_923ΔDG and C3_WT proteins purified from GN28 and healthy controls, respectively. We immobilized a small amount of C3_WT onto a Biacore chip and generated a nidus of convertase with fB and fD. Then, either C3_WT or C3_923ΔDG was flowed over the surface as substrate of this convertase; activation of C3_WT or C3_923ΔDG exposes the thioester and results in accumulation of protein onto the Biacore chip via a covalent link. In agreement with previous experiments, when C3_923ΔDG was flowed, no protein deposited on the surface, in contrast to the deposition evident when C3_WT was flowed (Figure 5B), which indicated that the mutant C3_923ΔDG was not converted into nascent C3b by the AP C3-convertase. As a whole, these experiments demonstrated that C3_923ΔDG is the major C3 protein circulating in the plasma of the DDD patients, because the constitutive AP activation present in these individuals exclusively consumed the C3_WT produced by the normal C3 allele.

C3_923ΔDG activates C3/H2O_923ΔDG and generates an active AP C3-convertase. We next sought to determine why C3_WT produced by the normal allele was consumed in C3_923ΔDG carriers. C3_923ΔDG was not converted to C3b when mixed with fB and fD in the presence of Mg²⁺ (Figure 5A). However, in these experiments, fB was completely consumed to Bb, which suggests that the preparations contain some hydrolyzed C3_923ΔDG that binds and activates fB and is therefore able to form an active AP C3-convertase. We have previously...
used SPR to monitor C3bBb formation and dissociation in real time (15). Hydrolyzed C3wt (1,224 RU) or C3ΔDG (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)ΔDG and C3(H2O)WT was found to be comparable (Figure 6A). We tested next whether the AP C3-convertase generated from C3(H2O)ΔDG was capable of activating C3wt. We generated a C3-convertase immobilized onto a Biacore chip using either C3(H2O)ΔDG and C3(H2O)WT and flowed C3wt over the surface. The mutant C3-convertase was able to activate C3wt, although it showed approximately 50% of the activity of the WT AP C3-convertase (Figure 6B).

C3(H2O)ΔDG generates a C3 convertase resistant to fH inactivation. We have shown here that C3ΔDG activated spontaneously and that the activated C3(H2O)ΔDG 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)ΔDG as described above and flowed fH (1 μM to 8 nM) over the surface. The affinity of C3(H2O)ΔDG 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)ΔDG (Figure 7B) and act as a cofactor in the fI-mediated inactivation of C3(H2O)ΔDG (Figure 7D). In contrast to the results obtained with fH, the C3-convertase generated from C3(H2O)ΔDG was efficiently decayed by DAF (Figure 7C).

MCP, but not fH, catalyzes fI cleavage of C3β2ΔDG and C3(H2O)ΔDG. We generated large amounts of C3β2ΔDG using trypsin and C3(H2O)ΔDG 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)ΔDG interacted with and consumed fI in the presence of fD and Mg2+; trypsin-generated C3β2ΔDG behaved similarly (Supplemental Figure 4). The trypsin-generated C3β2ΔDG 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 C3wt (data not shown). Addition of C3β2ΔDG 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 C3β2ΔDG is resistant to inactivation by fI and fH. As expected, C3β2ΔDG had some capacity to activate C3 in normal serum, but this was much reduced compared with C3β2ΔDG.

In order to confirm the differences in cofactor activity of fH and MCP in the fI-mediated proteolysis of C3β2ΔDG and C3(H2O)ΔDG, we performed a fluid phase assay. Identical amounts of C3β2ΔDG and C3wt 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. C3β2ΔDG 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 C3wt (Figure 9). The same cofactor selectivity was found for C3(H2O)ΔDG (Figure 10).

Discussion
Here we report the identification and functional characterization of C3β2ΔDG, 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 C3β2ΔDG mutation carriers

<table>
<thead>
<tr>
<th>Patient</th>
<th>Allele</th>
<th>C3 (mg/dl)</th>
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<td>GN28</td>
<td>C3wt</td>
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<td>27%</td>
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<td>GN28</td>
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<td>III-1</td>
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<tr>
<td>III-1</td>
<td>C3ΔDG</td>
<td>64</td>
<td>68%</td>
</tr>
<tr>
<td>III-2</td>
<td>C3wt</td>
<td>28</td>
<td>35%</td>
</tr>
<tr>
<td>III-2</td>
<td>C3ΔDG</td>
<td>52</td>
<td>65%</td>
</tr>
</tbody>
</table>

Total C3 levels were 94 mg/dl in GN28, 95 mg/dl in III-1, and 80 mg/dl in III-2.
The Journal of Clinical Investigation

http://www.jci.org

The C3$^{923\Delta DG}$ mutation was found in heterozygosis in 2 DDD patients and in a relative in the early stages of the disease who presented decreased levels of C3 and constitutive activation of the complement AP. C3$^{923\Delta DG}$ causes the deletion of 2 amino acids within MG7, but this does not affect C3 expression or its overall structure. The mutant C3$^{923\Delta DG}$ protein was the predominant C3 protein in the plasma of C3$^{923\Delta DG}$ carriers, where it circulated in the form of native, nonactivated, C3. As a consequence of the mutation, the mutant C3$^{923\Delta DG}$ could not be activated to C3b by the AP C3-convertase. This explains why levels of C3 in the DDD patients carrying C3$^{923\Delta DG}$ were reduced only by 50%, in contrast to the complete C3 consumption found in most DDD patients. Crucially, we demonstrate that the C3$^{923\Delta DG}$ mutant could be activated to C3b$^{923\Delta DG}$ by proteases, or to C3(H$_2$O)$^{923\Delta DG}$ 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$^{923\Delta DG}$ nor C3(H$_2$O)$^{923\Delta DG}$ could be proteolyzed by fI in the presence of fH.

Figure 5
Resistance of purified C3$^{923\Delta DG}$ to cleavage by the AP C3-convertase. (A) C3$^{923\Delta DG}$ 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$^{923\Delta DG}$ 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 μM) and fD (43 nM) in the presence of Mg$^{2+}$. At the indicated time (arrows), C3$^{WT}$ (gray line) or C3$^{923\Delta DG}$ (black line) was flowed over the surface. Remaining convertase was decayed using sDAF, and deposition of nascent C3b was measured. Resp. diff., response difference.

Figure 6
AP C3-convertase formation by C3$^{923\Delta DG}$ and C3$^{WT}$. (A) Hydrolyzed C3$^{WT}$ (1,224 RU) or C3$^{923\Delta DG}$ (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$^{923\Delta DG}$ and C3(H$_2$O)$^{WT}$, measured as K$_{SC}$, was comparable. (B) Hydrolyzed C3$^{WT}$ (1,640 RU) or C3$^{923\Delta DG}$ (2,000 RU) was thiol-coupled to a CM5 Biacore chip. fB and fD were flowed over the surfaces to form either C3(H$_2$O)$_{Bb}^{923\Delta DG}$ (black line) or C3(H$_2$O)$_{Bb}^{WT}$ (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. C3923ΔDG 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 C3923ΔDG were decayed normally by DAF, and that the activated molecules C3923ΔDG and C3(H2O)923ΔDG were efficiently inactivated by fH in the presence of MCP, is most relevant to DDD pathogenesis. Thus, not only is C3923ΔDG resistant to cleavage to C3b by the AP C3-convertases, limiting the deposition of C3923ΔDG 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 intricate arrangement of 13 domains, including a core of 8 homologous macroglobulin domains forming a ring; a TED domain, linked to this ring by a CUB domain, that contains the reactive thioester; and the C345C domain, which participates in the interaction with fB.

Figure 7
Reduced affinity of fH for hydrolyzed C3923ΔDG impairs both decay of the mutant C3-convertase and fI-mediated inactivation of hydrolyzed C3923ΔDG. (A) Hydrolyzed C3WT (1,224 RU) or C3923ΔDG (1,067 RU) were thiol-coupled to a CM5 Biacore chip. The affinity for native fH was analyzed by flowing fH (1 μM to 8 nM) over the surface and determined by steady-state analysis. The affinity of fH for C3(H2O)923ΔDG was reduced 2-fold compared with C3WT. Values are mean ± SD of 3 determinations. (B) Convertase was formed on each hydrolyzed C3 surface by flowing fB and fD. After a period of natural decay, fH (0.9 μM) was injected for 60 seconds. Convertase formed by C3(H2O)WT (gray line) was efficiently decayed by fH, whereas the C3(H2O)923ΔDG convertase (black line) was inefficiently decayed. Binding (RU) of fH to the surface in the absence of the convertase was subtracted; curves illustrate decay of Bb. In contrast, mutant convertase was efficiently decayed by DAF (0.4 μM). (D) Hydrolyzed C3WT or C3923ΔDG was coupled to a CM5 Biacore chip. Initial formation of convertase on each surface was assessed by flowing fB and fD (black line). After complete decay of the convertase, fH (0.33 μM) and fI (0.11 μM) were flowed across the surface for 5 minutes at 5 μl/min. Convertase was then formed again (gray lines) using identical conditions. Enzyme formation by C3(H2O)WT convertase was reduced 50% by fI/fH treatment, whereas enzyme formation from C3(H2O)923ΔDG convertase was hardly affected.

Figure 8
C3b923ΔDG activates C3 cleavage in normal human serum. The capacity of C3b923ΔDG and C3bWT to activate C3 in normal serum was tested by incubating 180 ng of each C3b with a 1:20 dilution of normal serum at 37°C in AP buffer (5 mM Veronal; 150 mM NaCl; 7 mM MgCl2; 10 mM EGTA, pH 7.4). Samples (5 μl) were taken at the indicated times and loaded into a 10% SDS-PAGE. C3 activation was measured by the appearance of the 43-kDa fragment of C3 α′ chain, as detected by Western blot. The α′ 43 band of iC3b is shown for comparison.
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 C3ΔDG mutation lies within, and likely alters, these interaction surfaces (Supplemental Figure 5), we analyzed the interaction of C3bΔDG and C3(H₂O)ΔDG with fB, fH, DAF, and MCP. Our data showed no differences in the assembly and stability of the AP C3-convertase formed by WT and mutant C3, which indicates that the mutation does not substantially modify the surfaces interacting with fB. In contrast, the C3ΔDG mutant showed a differential sensitivity to regulation by fH, DAF, and MCP, clearly illustrating that there are distinct structural requirements underlying the decay regulatory activities of fH and DAF, as well as the fI cofactor activities of fH and MCP.

The crystal structure of the complex between C3b and a truncated form of fH has shown that the first 4 short consensus repeat domains (SCRs) of fH, responsible for its decay-accelerating and fI cofactor activities, bind C3b in an extended configuration, which partially overlaps the site involved in the interaction with fB in the initial steps of the AP convertase assembly (Supplemental Figure 5 and refs. 18–20). These structural data explain previous mutagenesis experiments and functional analyses of disease-associated mutations indicating that fH competes with fB in the formation of the C3b proconvertase and interferes with the positioning of the Bb fragment, destabilizing C3bBb, the active AP C3-convertase. Our data support the hypothesis that the C3ΔDG mutation specifically alters the site of interaction of fH SCR1–SCR2 with C3b. Because the mutation does not affect the function of DAF, these data also support early mutagenesis results indicating that DAF and fH have distinct structural requirements for their decay activity and that whereas fH SCR1–SCR2 interacts with C3b, DAF SCR1–SCR2 interacts primarily with Bb (15, 21, 22).

fH and MCP are vital cofactors for fI-mediated inactivation of C3b in the CUB domain, which yields the inactive iC3b species. As with the differential decay acceleration of the mutant C3ΔDG convertase by DAF and fH, the C3ΔDG mutation affected cofactor activity of fH, but not of MCP, which indicates

**Figure 9**
C3ΔDG is inactivated by fI and MCP, but not by fH and fI. Activated C3bWT or C3bΔDG was incubated with fI and either fH (A) or sMCP (B). Cleavage of the α′ chain was indicated by generation of the α43 and α45 products. The experiment was repeated twice with identical results.

**Figure 10**
C3(H₂O)ΔDG is resistant to inactivation by fI in the presence of fH, but not in the presence of sMCP. Hydrolyzed C3ΔDG or C3WT were incubated with fI and either fH (A) or sMCP (B). Cleavage of the α′ chain was indicated by generation of the α74 and α43 products. The experiment was repeated twice.
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<sub>923ΔDG</sub> 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 C3bBb convertase. Previous studies have shown that the control of structural aspects of substrate recognition and regulation of the AP C3-convertase is more resistant to inactivation by fH (23, 24). In agreement with early work (21, 22), our previous studies 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<sub>923ΔDG</sub> 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, eosinophilic 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, CCCR of 46 ml/min, proteinuria of 1.1 g/dl, and persistent microhematuria. Another 6 months later, Cr and CCCR 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 deterioration 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 CCCR 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; CCCR, 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, fl, 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 fl 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. ligation 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 specific primers derived from the 5 CM5 Biacore chip on a Biacore 3000 (GE Healthcare), and 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) was active and able to cleave C3b to C3b, hydrolyzed C3 was thiol-coupled to a CMS 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), C3bw especially 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 C3bw was a substrate for the C3bw convertase, C3bw (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, C3bw 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 C3bw 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; with SDS-PAGE sample buffer (2% SDS, 62.5 mM Tris, 10% glycerol, and 0.05% 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 fl, and 0.46 μM sMCP, and in the other set 0.42 μM C3b, 43 nM fl, and 47 nM fH. Molarities were calculated using the following masses: fl, 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.

Acknowledgments
We are grateful to the patients and their relatives for their participation in this study. We thank Vivian de los Rios (Proteomics and Genomics Facility, CIB), the members of Segucten S.L., and the DNA sequencing laboratory at CIB for invaluable technical assistance with patient sequencing and genotyping. We thank Svetlana Hako byan (Cardiff University) for measuring TCC in plasma samples and Susan Lea (University of Oxford, Oxford, United Kingdom) for gifts of sDAF and sMCP. This work was funded by the Spanish Ministerio de Educación y Cultura (grants SAF2008-00226, SAF2008-00451, and SAF2006-02948), the Ciber de Enfermedades Raras (INTRA/08/738.2), the Red temática de investigación cooperativa en cáncer (RD06/0020/1001), the Fundación Renal Inigo Alvarez de Toledo, the Fundación Areces, the Human Frontiers Science Program (RGP39/2008), and MRC UK project grant 84908.

Received for publication April 14, 2010, and accepted in revised form July 21, 2010.

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