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Rare Loss-of-Function Mutation in Complement Component C3 Provides Insight into Molecular and Pathophysiological Determinants of Complement Activity

Georgia Sfyroera,*1 Daniel Ricklin,*1 Edimara S. Reis,* Hui Chen,* Emilia L. Wu,† Yiannis N. Kaznessis, † Kristina N. Ekdahl, ‡,§ Bo Nilsson, ‡,§ and John D. Lambris*1

The plasma protein C3 is a central element in the activation and effector functions of the complement system. A hereditary dysfunction of C3 that prevents complement activation via the alternative pathway (AP) was described previously in a Swedish family, but its genetic cause and molecular consequences have remained elusive. In this study, we provide these missing links by pinpointing the dysfunction to a point mutation in the β-chain of C3 (c.1180T > C; p.Met373Thr). In the patient’s plasma, AP activity was completely abolished and could only be reconstituted with the addition of normal C3. The M373T mutation was localized to the macroglobulin domain 4 of C3, which contains a binding site for the complement inhibitor compstatin and is considered critical for the interaction of C3 with the AP C3 convertase. Structural analyses suggested that the mutation disturbs the integrity of macroglobulin domain 4 and induces conformational changes that propagate into adjacent regions. Indeed, C3 M373T showed an altered binding pattern for compstatin and surface-bound C3b, and the presence of Thr373 in either the C3 substrate or convertase-affiliated C3b impaired C3 activation and opsonization. In contrast to known gain-of-function mutations in C3, patients affected by this loss-of-function mutation did not develop familial disease, but rather showed diverse and mostly episodic symptoms. Our study therefore reveals the molecular mechanism of a relevant loss-of-function mutation in C3 and provides insight into the function of the C3 convertase, the differential involvement of C3 activity in clinical conditions, and some potential implications of therapeutic complement inhibition. The Journal of Immunology, 2015, 194: 000–000.

The versatile functions of the complement system in host defense, immune surveillance, and cell homeostasis are tightly linked to a proper function and interplay of its individual components. Whereas an encounter with microbial intruders induces a forceful response that involves rapid amplification of opsonization, proinflammatory signaling, and induction of downstream immune responses, the removal of cellular debris and apoptotic cells requires a much more gentle approach (1). It is not surprising, therefore, that mutations, deficiencies, and dys-
only a limited number of studies are available in which the molecular causes of C3 deficiency and dysfunction have been delineated (7, 13, 17).

In this study, we provide important molecular insight into a case of C3 dysfunction that had initially been reported two decades ago (18). We were able to ascribe the cause of this C3 dysfunction to a single point mutation (p.Met123Thr) on the β-chain of C3, and we describe the molecular mechanism that leads to impaired complement activation. In particular, we found that the mutation causes structural changes in a functionally important region of C3, which renders the protein unable to bind to the alternative pathway (AP) C3 convertase (i.e., C3bbBb) and limits its ability to amplify the response. Overall, our findings emphasize the importance of the structural integrity of C3 for the effective function of the complement system. Additionally, the absence of a well-defined clinical consequence of this major impairment in activating complement contributes to our understanding of complement inhibition at the level of C3.

Materials and Methods

Patient and family

In 1992, Nilsson et al. (18) reported the case of a then 42-y-old white woman with systemic lupus erythematosus (SLE)–like syndrome associated with a dysfunctional C3 protein and lack of AP-mediated complement activity. The affected patient presented several autoimmune manifestations, including fatigue, swollen fingers, arthralgia, SLE-like symptoms, and the presence of antinuclear, anti-dsDNA, anti-SSA, and anti-centromere Abs (18). Although the patient showed SLE-like symptoms, she did not classify as an SLE patient according to the criteria set by the American College of Rheumatology. The plasma concentration of C3 (0.4–0.7 mg/ml) was significantly below physiological range (0.7–1.5 mg/ml), and hemolytic activities for both the classical pathway (CP; <44% of normal activity) and AP (<15%) were largely impaired (18). By the time of the C3 dysfunction diagnosis, the patient had received plasmapheresis, which initially induced acute inflammatory reactions, including proteinuria, hematuria, proinflammatory cytokines, and a positive lupus band test but had subsequently led to a gradual improvement in the quality of life (19).

Pertinent to the family history, an older sister (sister I) with similar complement profile (C3 of 0.5 mg/ml; CP and AP activities at 43 and 1%, respectively) suffered from recurrent otitis media during adolescence and one severe episode of meningococcal meningitis at age 43 y (18). No clinical complications had been reported for the oldest of the patient's sisters (sister II) despite reduced complement activity (C3 of 0.6 mg/ml; CP and AP activities at 72 and 6%, respectively) (18). Whereas both parents had normal CP and AP function at the time of the study, the father’s serum showed low C3 levels (0.3 mg/ml); both parents were apparently healthy (18).

Sample collection

Serum and EDTA plasma samples from the patient and family members were previously collected at the University Hospital of Uppsala (Uppsala, Sweden). Serum and EDTA plasma samples from healthy donors were collected at the University of Pennsylvania (Philadelphia, PA). All samples were stored at −70°C until assayed. The study was approved by the regional Ethics Committees, and informed consent was obtained from all donors involved in the study.

DNA sequencing

Genomic DNA was isolated from blood leukocytes using the FlexiGene DNA kit (Qiagen). All 41 exons of the C3 gene were amplified by PCR using 500 ng genomic DNA, 50 ng specific primers (Supplemental Table I), 0.2 mM dNTPs, and 2.5 U Taq DNA polymerase (Life Technologies) as instructed by the manufacturer. The products were amplified using 30 cycles at 95°C for 5 min, 95°C for 10 s, 58–62°C for 10 s, 72°C for 30 s, and finally 72°C for 10 min. The PCR products were analyzed on a 2% agarose gel and further purified using the QIAquick PCR purification kit (Qiagen). PCR fragments were then sequenced using the DNA sequence Long Read IR 4200 (LI-COR Biosciences) according to the standard protocol using the forward or reverse oligonucleotides described in Supplemental Table I. All nucleotide sequences were determined at least twice for both strands and compared with the deposited human C3 cDNA sequence (20).

Proteins and peptides

C3 was isolated from EDTA plasma, collected from the patient or healthy donors, and converted into C3b using standard protocols (21, 22). C3b was site-specifically biotinylated at the thiostere moiety as previously described (23). Factor H (FH), FB, FD, and C5 were prepared using established protocols (24) or purchased from Complement Technologies (Tyler, TX). Complement convertase antibodies 41(Mec)W and 4W9A were prepared using solid-phase peptide synthesis (25, 26).

Complement activation assays

Classical pathway. The ability of the patient’s plasma to initiate the CP was evaluated using an established ELISA-based assay (25). In brief, plates were coated overnight with 1% chicken OVA at 4°C and blocked with 1% BSA. Ag/Ab complexes were formed on the plate by adding rabbit polyclonal anti-chicken OVA Ab (1:1000 dilution). Following a 1-h incubation, plasma samples were serially diluted in 5 mM barbital (pH 7.4) with 145 mM NaCl and 0.1% gelatin (gelatin veronal buffer [GVB]) containing 0.5 mM MgCl2 and 0.15 mM CaCl2 (i.e., GVB+). GVB containing 20 mM EDTA (GVB-EDTA), or GVB containing 0.1 M MgCl2 and 0.1 M EGTA (GVB-MgEGTA) and incubated at room temperature for 1 h. Deposed C3b was detected using an HRP-conjugated anti-human C3 Ab (MP Biochemicals; 1:1000 dilution), followed by addition of the ABTS substrate. The OD was measured at 405 nm and plotted against the plasma dilution using Origin 6.0 (OriginLab).

Alternative pathway. Plates were coated overnight with 40 µg/ml LPS from Salmonella typhosa (Sigma-Aldrich) at 4°C and then blocked with 1% BSA. Plasma samples were serially diluted in GVB-MgEGTA or GVB-EDTA, added to the plate, and incubated at room temperature for 1 h. Detection of deposited C3b and plotting of the data were performed as described above.

Where indicated, CP- and AP-dependent complement activity were determined in the patient’s or a C3-depleted plasma in the absence or presence of additional normal human C3 purified as described above.

AP C3 convertase formation in solution

To assess the ability to form initial AP C3 convertases, normal C3 and C3 M373T were treated with 50 mM methylamine (pH 8.0) for 4 h at 37°C to yield their thoister-activated forms, which are conformationally equivalent to C3 hydrolyzed via the tickover mechanism (27). For simplicity, these forms are referred to as C3(H2O). The formation of the initial AP C3 convertase in the fluid phase was assayed by incubating normal C3(H2O) or C3(H2O) M373T (250 µg/ml) with FB (250 µg/ml) and FD (4 µg/ml) in PBS (pH 7.4) containing 5 mM MgEGTA. The reaction mixture was incubated at 37°C for 30 min and analyzed by 9% SDS-PAGE under reducing conditions. The generation of FB cleavage fragments (i.e., Ba and Bb) was used as an indicator of the formation of AP C3 convertases. The assay was repeated with native forms of C3 under the same conditions, and generation of the C3 β-chain was monitored in addition to Ba and Bb to test the ability of initial AP C3 convertases to activate C3.

Surface-based convertase formation and C3b deposition

The ability of normal and mutant C3b to form surface-based AP C3 convertases and activate C3 was evaluated using surface plasmon resonance (SPR) on a Biacore 2000 instrument (GE Healthcare). Assays were performed at 25°C using 10 mM HEPES (pH 7.4) with 150 mM NaCl, 1 mM MgCl2, and 0.005% Tween 20 (HBS-Mg) as running buffer. A 1:1 mixture of FB and FD (100 nM each) was injected onto thoister-immobilized C3b (normal or M373T) on a streptavidin-coated sensor chip (GE Healthcare) for 2 min at 10 µl/min to form the C3bBb complex. The regular decay of the convertase was observed for 1 min before either normal C3, C3 M373T, or a buffer control was injected for 1 min. After monitoring potential C3b deposition on the chip for 1 min, FH (1 µM) was injected to accelerate convertase decay. Finally, a wash step (1 M NaCl injection for 1 min) was included to remove bound FH, and the postinjection baseline was compared with the initial baseline to measure the levels of covalently deposited C3b. Data were processed by subtracting the signals from an uncoated streptavidin flow cell and analyzed using Scrubber (v2.0; BioLogic).

Ligand binding studies

All ligand binding studies were performed using SPR analyses on a Biacore 3000 instrument (GE Healthcare) at 25°C. Thoister-specifically bio-

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tinted C3b and C3b M373T were captured on separate flow cells of a streptavidin-coated sensor chip (GE Healthcare) and equilibrated overnight. To evaluate the C3/C3b interaction that is considered an essential step for convertase-mediated C3 activation, C3 (1 μM) was injected in the presence or absence of compstatin (analog 4W9A; 1 μM) for 1 min, with a dissociation phase of 2 min in 10 mM sodium phosphate (pH 7.4) with 150 mM NaCl and 0.005% Tween 20 (PBS-T); compstatin alone was injected as a control. Additionally, FH (200 nM), FB, C3, and C5 (500 nM each) were injected in Mg-Mg buffer for 2 min, with 5 min dissociation, to compare the binding pattern of the relevant ligands to normal and mutated C3b. The C3b surface was regenerated by injecting 0.2 M sodium carbonate (pH 9.0) twice for 1 min each. Data processing and analysis were performed as described above.

The effect of the M373T mutation on the binding of compstatin was further investigated by performing detailed quantitative interaction analysis. Compstatin analog 4W9A was injected as a 2-fold linear dilution series (10 nM–5 μM) in PBS-T for 1 min over immobilized C3b and C3b M373T (see above). No regeneration was required between injections. SPR signals were processed by Scrubber; in addition to subtracting the reference surface signals, averaged responses from two buffer injections were subtracted from all compstatin responses (double referencing). Processed signals were plotted against the analyte concentration to determine the steady-state affinity (K_{a}) using a single binding site model. Moreover, kinetic association rate (k_{a}) and dissociation rate (k_{d}) constants were determined by globally fitting the responses to a Langmuir 1:1 binding model.

### Mass spectrometric characterization of C3

Changes in the protein sequence of the patient’s C3 were characterized by mass spectrometry (MS) after in-gel trypsin digestion. Normal C3 and C3 M373T (20 μg) were run on a 7.5% SDS-PAGE gel and stained with Coomassie blue. Bands of interest were excised and destained with 100 mM ammonium bicarbonate and acetonitrile. Gel pieces were swollen in 10 mM DTT with 100 mM ammonium bicarbonate and alkylated by incubation in 55 mM iodoacetamide. Each protein sample was digested with trypsin (20 ng/μl) at 37°C for 16 h. The tryptic peptides were mixed with a matrix of α-cyano-4-hydroxycinnamic acid in acetonitrile/0.1% trifluoroacetic acid (1:1). One microfiltration of this mixture was quickly spotted onto a MALDI-TOF mass spectrometer in reflector mode (MALDI micro MX; Waters, Milford, MA) at an acceleration voltage of 20 kV. Typically, 40 single-shot mass spectra were summed to give a composite spectrum. All data were processed using Waters Mass Lynx software. Postsource decay data were used for peptide sequencing by Waters MassLynx software.

### Hydrogen–deuterium exchange mass spectrometry

Hydrogen–deuterium exchange MS (HDX-MS) experiments were performed based on previously described protocols (28, 29). In brief, aliquots of C3 or C3 M373T were deuterated by the addition of 6 D2O (pH 7.4) to 10 mM sodium phosphate, 150 mM sodium chloride (pH 7.4) to 120 μM D2O (99.9%, Cambridge Isotope Laboratories, Andover, MA). After timed intervals at room temperature (10–10,000 s), 10–1 μl aliquots of the protein/D2O solution were removed, added to 10 μl 0.2% trifluoroacetic acid, and immediately frozen in liquid nitrogen to quench the amide HDX process (final pH 2.5).

All samples were passed through two immobilized protease columns (pepsin and fungal protease XIII, respectively), and peptic peptides were separated by reversed phase HPLC. A mass spectrometer (ThermoFinnigan LCQ, capillary temperature of 200°C) was directly coupled to HPLC to analyze the eluent. All analyses were performed on ice to avoid back exchange. Initial identification of proteolytic fragments was determined by tandem MS, followed by data analysis using the SEQUEST and DXMS software programs (ThermoFinnigan, San Jose, CA, and Sierra Analytics, Modesto, CA, respectively) (30). Peptide deuterium levels were extracted from the centroid of individual mass envelopes according to the method of Zhang and Smith (31) after accounting for end effects and proximal residues. The percentage deuterium incorporation at each time point was compared for the corresponding C3 and C3 M373T peptides to calculate the average difference in deuterium across all time points; values below ~10% and above +10% were considered significant.

### Molecular dynamics simulation

All molecular dynamics (MD) simulations were performed with the program NAMD using the CHARMM27 force field. The available crystal structure of the C3c fragment in complex with compstatin analog 4W9A (PDB 2QKD) (32) was used for all calculations. The systems were first minimized in three consecutive steps, during which the protein was initially held fixed and water molecules were allowed to move for 10,000 conjugate gradient steps; next, only the protein backbone was held fixed for 100,000 steps, and, finally, all atoms were allowed to move for an additional 10,000 steps. The particle mesh Ewald method (33) was used to treat long-range electrostatic interactions in a periodic boundary condition, with a grid of ~1 point/A. Bond lengths involving bonds to hydrogen atoms were constrained by using SHAKE (34). The time step for all MD simulations was 2 fs. A total of 100 ps MD simulations were carried out at constant volume, during which the systems were heated to 310 K in increments of 30 K, and a subsequent isothermal isobaric MD simulation was used for >25 ns to adjust the solvent density without any restraints on all the solute atoms for these three simulations. Finally, the last structures were extracted from the MD equilibrated trajectory files for the structure analysis and free energy calculation.

For the calculation of the difference in free energy changes between 1) the normal C3c and 2) the mutated form (C3c M373T), binding to compstatin was assessed according to free energy perturbation methodology, in conjunction with the dual-topology paradigm (35) and the program NAMD (36).

### Results

**Missense mutation in exon 10 leads to M373T substitution in patient’s C3**

In an attempt to identify the molecular basis for the C3 deficiency in the patient of interest, the 41 exons that encode the α- and β-chains of C3 were amplified and sequenced. The sequencing results revealed a novel missense mutation at position 1180 of the C3 cDNA. Although the open reading frame remained unchanged, nucleotide T was replaced by C, resulting in a double peak in the sequencing chromatogram (Fig. 1A; shown as an A/G mutation in the reverse strand). At the protein level, this mutation results in substitution of methionine 373 by threonine (i.e., C3 M373T). The double peak observed in the chromatogram indicates that the subject is heterozygous for this specific mutation.

Similar genetic analysis was applied to the C3 genes of the mother, father, and sister I of the patient (Fig. 1B). Additionally, DNA samples from healthy volunteers were used as a control. The sequencing results showed that the same missense mutation was present in heterozygosity in the C3 gene of the mother and sister I, whereas no alterations were detected in the DNA of the patient’s father or in control individuals. Therefore, the family tree reveals that the subject and sister I inherited the mutated C3 allele from their mother (Fig. 1B).

**The patient’s plasma shows an impaired ability to activate and amplify the AP**

To confirm and extend the previous evaluation of the complement activation profile of the patient (18), CP- and AP-dependent complement activities were determined in the patient’s plasma. Plasma from a donor with C3 levels in the physiological range was used as a control. Activation of the CP by immune complex formation was assessed in the presence of Mg²⁺/Ca²⁺, Mg-EGTA, or EDTA. Whereas Mg-EGTA served as a negative control that inhibits both pathways. Whereas a 50% CP activation level was achieved at a 500-fold dilution of the control plasma, the same levels of CP activation were obtained with a 100-fold dilution of the patient’s plasma, indicating that the patient’s plasma is able to activate the CP, although to a lower extent than the normal plasma control (Fig. 1C). The Mg-EGTA sample showed very low levels of complement activation, even in the control sample, thereby indicating that the contribution of the AP was <1% in this assay (data not shown).

Next, to determine the ability of the subject’s complement system to initiate the AP, plasma was incubated in the presence of...
LPS in Mg-EGTA buffer. The patient’s plasma was unable to activate the AP, whereas a 50% AP activation was achieved with control plasma diluted 65-fold (Fig. 1D). To confirm the association between impaired complement activation and the mutated C3 protein found in this patient (Fig. 1C, 1D), the patient’s plasma was reconstituted with purified normal C3. The CP activity in the patient’s plasma could be restored to levels close to the control by the addition of C3 at 1 mg/ml (Fig. 1C), thereby confirming that the observed activity differences were caused by C3. Evaluation of the AP-dependent activation also indicated increased complement activity in the patient’s plasma in response to the addition of exogenous C3 (Fig. 1D). Even though the activation levels in the patient’s plasma were still ~6-fold lower than those of control plasma, C3 reconstitution in patient’s plasma had an effect similar to that obtained with equivalent reconstitution of a C3-depleted plasma (Fig. 1D). Because both the C3-depleted and control plasmas showed similar levels of CP activation (Fig. 1C), the relatively lower AP activation in the C3-depleted plasma after C3 reconstitution cannot be attributed to a general impairment of this plasma. Of note, although the ability of the patient’s plasma to activate the lectin pathway was not specifically evaluated, the outcomes are expected to be highly similar to the CP activities, because both pathways employ the same convertase to activate C3.

To confirm the presence of mutated C3 M373T in the patient on the protein level, we isolated the protein from plasma using anion exchange chromatography. The elution profile of the C3 M373T was comparable to that of control C3, and SDS-PAGE analysis revealed the same mobility for both the α- and β-chains of the patient’s C3 when compared with C3 from control plasma (data not shown). The excised protein bands were subjected to in-gel tryptic digestion and analyzed by MS to characterize the protein at
the molecular level. MS analysis of control C3 and C3 M373T identified tryptic peptides that covered 64% of the α-chain and 78% of the β-chain. Although most of the peptides were identical between the samples, the analysis identified a unique peptide with a molecular mass of 2749.57 Da in the control sample. In contrast, a peptide of 2719.55 Da was found only in the patient’s C3 sample (Fig. 1E). The peptide in the control C3 corresponded to a 24-aa sequence that spanned the region between residues 363 and 386 of the molecule. The mass difference of 30.02 Da in the peptide from the patient’s C3 can only be accounted for by the change from methionine to threonine at position 373 (Fig. 1F), which corresponds to the mutation that was identified on the genetic level. Importantly, no normal form of peptide 363–386 was detected in the patient’s plasma, thereby essentially confirming that the patient expresses only the mutated form of C3.

**C3 M373T can form the initial fluid-phase AP C3 convertase**

Previous studies have indicated that C3b M373T, when artificially prepared by limited trypsinization, is able to form the final AP C3 convertase (C3bBb) (18). To better understand the mechanism of the patient’s C3 dysfunction and assess the residual complement convertase (C3bBb) (18). To better understand the mechanism of the tickover mechanism (1, 38). To determine whether the patient’s fluid-phase AP activation could be initiated properly, we converted C3 from the patient and from a normal donor to the respective C3(H2O) forms by methylamine treatment (see Materials and Methods) and incubated them with FB and FD. The proteins were then analyzed by SDS-PAGE for the occurrence of the FB cleavage products Ba and Bb, which are generated once FB binds to C3(H2O) and is activated by FD (Fig. 2A). A similar amount of Bb generation was observed in both samples, indicating that the M373T mutation does not significantly affect the formation of the initial C3(H2O)Bb convertase. We then extended our investigation to determine whether spontaneous hydrolysis of C3 M373T could induce convertase-mediated C3 activation. Again, generation of FB fragments was observed in the sample containing C3 M373T, although at a visibly lower rate than in the normal C3 sample (Fig. 2B). Whereas most normal C3 was converted to C3b, as shown by the occurrence of an α′-chain band, the α-chain of C3 M373T remained intact during the course of the experiment. In both assays, control samples lacking FD showed no degradation of FB or C3, thereby eliminating the possibility of protease contamination (Fig. 2A, 2B). These studies confirm that the patient can indeed form the C3(H2O)Bb convertase in the fluid phase, but the resulting convertase complex is unable to activate C3 M373T.

**C3 M373T cannot be activated by AP C3 convertases**

To unravel the mechanism behind the inability of this C3 M373T to participate in AP activation and amplification, we examined the underlying molecular processes using an established SPR-based convertase assay (13, 24, 39) (Fig. 2C). After immobilizing both C3b and C3b M373T in parallel on a sensor chip through their thioester moiety, we injected a mixture of FB and FD to allow the formation of the C3bBb convertase. Following a short period of regular decay, either normal C3 or C3 M373T was injected as a substrate for the convertase, and its activation was detected as covalent deposition of newly generated C3b on the chip surface. Whereas convertase formation was similar between the normal and mutated forms of C3b, the subsequent activation of C3 showed marked differences, with ∼6-fold less C3b being deposited by the mutated convertase (Fig. 2C, red lines). Subsequently, the assay was repeated using C3 M373T as a substrate (Fig. 2C, blue lines). Although there was a residual, yet very low, cleavage activity of C3 M373T by the normal convertase, the presence of the M373T mutation in both the convertase-associated C3b and the C3 substrate led to a complete abrogation of C3b deposition. Therefore, these findings clearly identify the impaired binding to and cleavage by the AP convertase as major causes of the dysfunction in the patient’s C3.

**C3 M373T prevents substrate binding to the convertase**

Mapping of the identified Met-to-Thr mutation at position 373 on the available crystal structure of C3 (37) revealed that the affected residue is located in macroglobulin domain (MG)4, in proximity to domain MG5 (Fig. 1F). Importantly, recent studies have indicated that a heterodimerization between the C3 substrate and convertase-bound C3b represents the initial step of C3 activation and have suggested that a large contact interface, including MG4/5 of both proteins, mediates that crucial interaction (40, 41). We therefore used an SPR-based assay to analyze whether the M373T mutation in this critical area might have disrupted the C3-to-C3b binding (Fig. 3A). Control C3b or C3b M373T was immobilized on a sensor chip, and normal C3 was injected onto both proteins. Strikingly, C3 showed a much stronger binding to control C3b than to C3b M373T (Fig. 3B, blue lines), thereby supporting the concept that the M373T mutation negatively affects this interaction.

We then used a saturating concentration of the well-described C3 inhibitor compstatin (25, 42, 43) to block the MG4/5 contact interface and found that the inhibitor markedly reduced the binding of C3 to normal C3b, whereas the already low interaction with C3b M373T showed a very modest further reduction (Fig. 3B, green lines). Of note, binding of C3 to both forms of C3b reached the same level in the presence of compstatin, indicating that the residual SPR signal may be caused by an additional binding interface or by an impurity in the plasma-purified C3. Therefore, this analysis indicates that, in functional terms, the C3 M373T mutant behaves similarly to compstatin-inhibited normal C3.

Based on the structural investigations of the AP C3 convertase and the complex between C5 and cobra venom factor (i.e., a structural homolog of C3 that forms inherently stable C3 and C5 convertases), it had recently been hypothesized that the MG4/MG5 interface not only mediates convertase binding of C3 but also of C5 (44). We therefore also compared the interaction of C5 with surface-immobilized normal and mutated C3b. In general, the signal intensity for the binding of C5 was much stronger when compared with that for C3 at equimolar concentrations. More importantly, and in contrast to the impairment in C3 binding, the M373T mutation caused a small but significant increase in C5 binding (Supplemental Fig. 1).

**Mutation at position 373 leads to structural perturbations in C3**

Although the binding studies above strongly indicated a disruption of the binding between C3 and C3b, Met373 is not exposed on the surface, but is rather situated within the domain, thereby rendering participation in any direct ligand interactions unlikely. We therefore investigated the possibility of mutation-induced structural perturbations in C3 by employing structural analysis, MD, HDX-MS, and ligand binding experiments.

Among the few structural methods capable of tracking conformational changes, HDX-MS is particularly powerful because it is able to analyze large protein complexes with high sensitivity (45). The method has previously been used to detect conformational rearrangements in C3 and its fragments (28, 29). When
applied to C3 M373T, HDX-MS analysis identified seven peptides with significant differences in exchange rate (Fig. 4A, Supplemental Fig. 2, Table I). Most of these peptides were located in MG4 or the adjacent MG3 and MG5 domains. Interestingly, two peptides covered areas distant from the site of the mutation (i.e., the anaphylatoxin and thioester-containing domains), indicating that the structural changes in MG4 may propagate across several domains and reach other functional areas of C3 (Fig. 4B). Note, however, that the limited availability of C3 isolated from the patient allowed for only comparatively low sequence coverage (∼55%) in the HDX-MS study (Supplemental Fig. 2) as compared with >90% in previous studies (28, 29), opening the possibility that not all areas of structural change may have been identified. Because some of the identified peptides are located in areas that are involved in ligand binding, we evaluated the differential binding pattern of the two major functional ligands, FB and FH, to normal C3b and its M373T mutant using SPR. In contrast to the reduced binding as seen for C3 (Fig. 3B), the signal intensity remained unchanged for FB, yet was visibly increased in the case of FH (Fig. 4C). Whereas the limited sequence coverage of the HDX experiment did not allow for a detailed binding site analysis, the reported C3b contact areas for FH are far distant from the M373 locus and do not involve MG4 (Fig. 4C, structural model); the observed effect on FH binding therefore supports our HDX-based finding that the M373T mutation results in more extended changes on the C3 structure.

The MG4/MG5 region also harbors the binding site of the clinically important compstatin family of complement inhibitors, which have been shown to prevent convertase-mediated activation of C3 (32, 42). Owing to the tight interaction of compstatin with C3 M373T can form the initial fluid-phase AP C3 convertase but cannot be activated by the AP C3 convertase. (A) Analysis of C3(H2O)Bb convertase formation. Purified C3(H2O) M373T and control C3(H2O) were mixed with FB and FD in fluid phase, and the mixture was analyzed by SDS-PAGE. Generated FB fragments are indicated on the right side of the figure; the appearance of Bb indicates convertase formation. (B) Analysis of convertase-mediated generation of C3b and FB activation fragments in solution. Purified C3 M373T and control C3 were mixed with FB and FD in fluid phase, and the mixture was analyzed by SDS-PAGE. Proteins added to each reaction mixture are indicated below the corresponding lane. Generated fragments of C3 and FB are indicated on the right side of the figure; the appearance of Bb indicates the formation of C3 convertases, whereas the disappearance of the C3 α-chain signals the activation of C3. (C) Monitoring of convertase formation and activation/deposition of C3b by SPR. On-chip formation of the C3 convertase was established by injecting a mixture of FB and FD (500 nM each) onto an SPR chip immobilized with C3b M373T (upper panel; 1700 RU) or C3b (lower panel; 1800 RU). After the end of the injection (180 s), a moderate decay of the convertase could be observed. As shown on the upper panel, the injection of 1 μM C3 (red line) led to limited binding of C3 and deposition of C3b on the chip. When the same amount of C3 M373T (blue line) was injected, no binding to the convertase or further cleavage to C3b was observed. Buffer alone (black line) had no effect on the preformed convertase. In the lower panel, injected C3 (red line) bound strongly to the C3bBb convertase, whereas injection of C3 M373T had only minimal effect. Decay of the convertases was accelerated upon injection of FH (1 μM). A final wash step was introduced at the end of each experiment to evaluate changes in baseline levels resulting from convertase-deposited C3b (double-headed arrows). RU, resonance units. The figures depict a representative experiment out of three experiments.
a shallow pocket formed by these two domains, we suspected that any perturbation at this domain interface might influence the binding of compstatin. Indeed, when we investigated the binding of a compstatin analog to normal C3b and to C3b M373T using SPR, we observed a significantly stronger binding affinity for the mutated form than for normal C3b (Fig. 5A). Kinetic analysis showed that these differences were caused solely by an increased stability of the compstatin complex (as determined by the lower dissociation rate constant; Fig. 5B), thereby indicating changes in the quality of the intermolecular contacts. Using the crystal structure of the same compstatin analog in complex with C3c as a template (32), we mutated Met in position 373 to Thr in silico and subjected the two complexes to MD simulation. As expected, superimposition of the mutated (red) and control complexes (blue) revealed no changes in the overall domain arrangement (Fig. 5C, left) but showed important deviations in the MG4 and MG5 areas (Fig. 5C, right). In particular, our findings showed that, when compared with Met373 in normal C3c, Thr373 in the complex structure of mutated C3c forms an extended hydrogen bond network with Thr358 and Thr360 (Fig. 5B, insert). This altered intradomain connection network is likely responsible for the structural changes that are observed in the compstatin binding area (Fig. 5D, Supplemental Fig. 3) and supports the hypothesis that the mutation induces structural perturbations to the MG4/MG5 domains.

**Discussion**

In this study, we describe the genetic cause and functional consequences of a clinically observed dysfunction in complement component C3 in a family of patients; this mutation leaves AP activation severely impaired, yet does not appear to lead to a uniform or permanent clinical manifestation. C3 polymorphisms have been frequently observed and associated with increased risk for diseases such as age-related macular degeneration, kidney pathologies, and systemic vasculitis, provided that they result in a gain of function of C3 (5, 7, 12, 46, 47). Alternatively, complete deficiencies and dysfunctions in C3 are rarely reported; they are mostly associated with increased susceptibility for pyogenic infections, especially during childhood, and a higher risk of immune complex–mediated diseases (2, 3, 8).

Genetic analysis has identified a point mutation on C3 (c.1180T>C) that results in a Met-to-Thr substitution in domain MG4 of the patient’s protein (p.Met373Thr). The same C3 mutation has been identified in her mother and sister I, suggesting maternal inheritance. Whereas the C3 gene of sister II could not be analyzed because of limitations in sample availability, the previously reported low AP activity profiles (18) indicate that she also inherited the mutation. Additionally, the low C3 levels observed in all three sisters as well as the father may be indicative of a paternal
null allele. This observation is further corroborated by a previous finding that the entire C3 in the patient and both her sisters carries another, although common, polymorphism (p.Arg^{162}Gly), whereas only the Arg^{162} form of C3 is detected in the father (18). Moreover, deposits previously found in the patient’s skin biopsies lacked the presence of C3 that are usually observed in SLE; only deposits previously found in the father (18). This result essentially excludes the low C3 levels in the patient as result of changes in the overall conformation of the C3 molecule, which impair its interaction with the AP C3 convertase (C3bBb). The plasma from the patient containing the C3 M373T mutation could not be activated in response to LPS (AP) and showed only limited activation in response to immune complexes (CP). Both AP- and CP-dependent activation could be reconstituted with addition of exogenous C3 to link the plasma impairment to the C3 molecule. The nonfunctional nature of C3 M373T was confirmed by its inability to generate C3b in the presence of FB and FD. Even though the mutated C3 can be processed to C3b by extrinsic proteases (18) and can be hydrolyzed to C3(H2O) through the tickover mechanism, thereby forming initial AP C3 convertases, it cannot be activated by those active convertase complexes. As revealed by the crystal structure of C3 (37), amino acid residue 373 is located within the MG4 domain of the β-chain, in an area that has not typically been associated with changes in disease risk. Another polymorphism in MG4 (p.Arg^{425}Cys) has recently been reported in a Japanese aHUS patient, but the functional consequences of this mutation have not yet been investigated (48). Moreover, because additional polymorphisms in the genes of FH, complement factor I, and thrombomodulin were gated (48). Moreover, because additional polymorphisms in the genes of FH, complement factor I, and thrombomodulin were detected in this patient (48), a direct disease correlation remains challenging. Although mutations in the MG4 domain of C3 appear to be rare, recent structural insight and data from studies on C3 inhibitors and mutations provide strong evidence for the functional importance of this region (41). Crystallization of the C3bBb complex in the presence of the bacterial inhibitor SCIN resulted in a stabilized dimeric complex (C3bBbSCIN2), with a dimerization site that includes the MG4 and MG5 domains of C3b. The high degree of conservation in the β-chains of C3 and C3b led to the hypothesis that these domains, alongside MG3 and MG6–MG8 on the same side of C3b, form the contact interface that allows the substrate C3 to bind to the C3bBb complex, thereby inducing activation (40). In our study, we now provide direct evidence that C3 can interact with surface-deposited C3b and that the MG4 M373T mutation impairs that interaction. Whereas recent structural studies have led to the hypothesis that C5 may bind to C5 convertases via the same interface (44), the binding of C5 to C3b was affected by M373T in a distinct way when compared

null allele. This observation is further corroborated by a previous finding that the entire C3 in the patient and both her sisters carries another, although common, polymorphism (p.Arg^{162}Gly), whereas only the Arg^{162} form of C3 is detected in the father (18). Moreover, deposits previously found in the patient’s skin biopsies lacked the presence of C3 that are usually observed in SLE; only after treatment of the patient with normal plasma could C3 be detected in the deposits (18). This result essentially excludes the low C3 levels in the patient being caused by consumption of C3 rather than a paternal null allele. Finally, our analysis of the patient’s C3 by MS did not reveal any normal C3. Although such a genetic constellation seems statistically unlikely, the combined data from this and previous studies indeed strongly suggest that all three sisters inherited the same C3 background. Therefore, both parents maintained normal complement activity because they had reduced but sufficient levels of functional C3, but the sisters, who all appear to have inherited one dysfunctional and one null C3 allele, showed a severe drop, particularly in their AP activation potential.

Our molecular analysis showed that the C3 dysfunction resulting from the M373T mutation resulted in defective amplification of the AP as a result of changes in the overall conformation of the C3 molecule, which impair its interaction with the AP C3 convertase (C3bBb). The plasma from the patient containing the C3 M373T could not be activated in response to LPS (AP) and showed only limited activation in response to immune complexes (CP). Both AP- and CP-dependent activation could be reconstituted with addition of exogenous C3 to link the plasma impairment to the C3 molecule. The nonfunctional nature of C3 M373T was confirmed by its inability to generate C3b in the presence of FB and FD. Even though the mutated C3 can be processed to C3b by extrinsic proteases (18) and can be hydrolyzed to C3(H2O) through the tickover mechanism, thereby forming initial AP C3 convertases, it cannot be activated by those active convertase complexes.

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with C3 binding. This difference may indicate that the MG4/MG5 interface is less, or differently, involved in the interaction of C5 with C3b and, by analogy, the C5 convertase. However, in the absence of an experimental model, additional structural and biochemical studies are needed to characterize this binding mode in the context of C5 convertase activity. At any rate, our study underscores the functional importance of the MG4/MG region for C3 convertase activity and validates the previously proposed model (40).

It is interesting that several natural and synthetic inhibitors, such as the complement receptor of the Ig superfamily (CRIg), the therapeutic Ab S77, and compstatin, all bind to the proposed C3/C3b contact region (32, 41, 49, 50). Notably, the binding site of compstatin is located directly at the interface between MG4 and MG5, in close proximity to the M373T mutation in C3. The observation that the addition of compstatin leads to an impairment in the C3/C3b interaction therefore provides strong support for a mode of action in which compstatin prevents the binding of the C3 substrate to the convertase. Moreover, our SPR data also suggest that the M373T mutation results in similar functional consequences, because it prevents the initial binding of C3 to the C3bBb complex via the dimerization interface. Whereas the presence of the M373T substitution in only the C3 substrate or the convertase-bound C3b resulted in low residual C3b deposition,
hence indicating a weakened but not totally attenuated dimerization, no activity was observed in the physiologically relevant case of the patient with M373T present in both convertase and substrate. The fact that the affected residue is not surface exposed but rather located deeper inside the MG4 domain suggests the involvement of conformational changes in the disruption of the binding interface. Indeed, our MD simulations of the compstatin complex with normal and mutated C3c, the altered binding activity of compstatin to C3 M373T, and the detection of differentially solvent-exposed peptides within the MG4/MG5 region all support the local structural perturbation of the binding/dimerization interface. Our HDX experiments even indicate that those local changes in C3 M373T may propagate into adjacent domains and lead to a more global change of the contact site for the convertase and, potentially, other ligands.

A comparison with gain-of-function mutations in C3 that affect C3 activation via the C3 convertase reveals important similarities and differences. The deletion mutant C3 p.923DG, which has been strongly associated with the development of a familial form of DDD, lacks two amino acids in the MG7 domain that is part of the proposed extended C3/C3b dimerization site (13). Despite being far distant from MG4 and residue Met373 (Fig. 6A), this mutation also showed an impaired ability to activate C3 via the C3bBb convertase. Additionally, the deletion in MG7 also affects the binding site for FH and leads to an impaired deactivation of initial convertases that are induced by the tickover or proteases, resulting in strong fluid-phase activation of normal C3 in heterozygous patients and likely driving the familial manifestation of DDD (13). Another C3 mutation affecting the functionality of C3 activation, that is, C3 p.Val1636Ala (reported as p.Val1636Ala based on sequence numbering of the mature protein), has recently been identified in a family with late-onset aHUS (17). This mutation in the C345C domain of C3 (Fig. 6A) is not part of the proposed C3/C3b contact interface and does not affect the susceptibility of C3 to convertase-mediated cleavage; rather, it increases the binding affinity for FB and thereby forms more stable convertases. As in the case of C3 923DG, the resulting higher turnover of C3 through the AP contributes to inflammatory kidney diseases. Therefore, the disrupted balance between FB-mediated activation and FH-mediated regulation attributed to these gain-of-function mutations appears to be responsible for the inflammatory consequences. In contrast, the C3 M373T loss-of-function mutation does not profoundly alter the binding pattern of FB or FH (the slight increase in FH binding is not likely to be of strong functional significance) and largely impairs turnover of C3. The resulting apparent absence of inflammatory kidney disorders (e.g., aHUS or DDD) is consistent with the observation that the risk for developing such clinical manifestations is low in the case of mutations or treatment options that strictly reduce AP activity (5, 43). Taken together, these studies offer valuable information with regard to the requirements for the proper functioning of the AP C3 convertase and its clinical consequences.

The impact of the M373T mutation on potential immune complex–mediated complications is more difficult to assess. C3 is considered an important contributor to the clearance of immune complexes (51), and an apparent reduction in immune complex solubilization has previously been reported for the patient (18). However, because SLE-type symptoms had only been observed in the patient and not in her two sisters, the loss of function in C3 activation itself cannot fully explain this clinical manifestation. Other than what is usually observed in SLE, the deposits found in the patient’s skin biopsies originally lacked C3, which, however, was detectable after infusions with normal plasma (18). Whereas this finding indicates an involvement of complement, factors other than C3 appear to have been driving the SLE-type syndrome. Indeed, in contrast to deficiencies in other CP components such as C1q or C4, C3 deficiency is not typically associated with SLE.

![Image](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org)
In previous analyses, the patient persistently showed low CP-mediated hemolytic activity (0–44%), which was only slightly affected by treatment with plasma (18, 19). Based on the available data, it is difficult to state whether and how much the CP status and/or other dysfunctions contributed to the clinical status of the patient. Also, it cannot be excluded that the C3 dysfunction has contributed to the SLE-type syndrome, because it has previously been associated with some cases of C3 deficiency (8). Nevertheless, the manifestation of SLE symptoms in only one out of three affected individuals suggests that this complication is part of a more complex constellation.

Finally, the close functional analogy between C3 M373T and comaptin-inhibited normal C3 in their inability to bind the C3 convertase may also prove interesting in the context of therapeutic C3 inhibition. Infections in individuals carrying the M373T mutation, such as recurrent otitis and one episode of meningitis in sister I, were episodic and manifested primarily during the early stages of life. Nevertheless, given the potential severity of these infections, antimicrobial control using vaccination against encapsulated bacteria and standby antibiotics need to be considered, as is already done in patients undergoing anti-C5 therapy. Also, patients may be tested for other complement-related parameters such as CP activity to reduce any interference with immune complex clearance. Importantly, and in contrast to the patient and her sisters who have an irreversible dysfunction of C3 activation, any therapeutic modulation of C3 activity via C3/convertase-targeted drugs may be interrupted in the case of complications, thereby restoring AP activity. Moreover, indications such as hemodialysis-related inflammation likely require only time-restricted intervention with C3 inhibitors (52) and may already include standard antimicrobial measures as in the case of prevention of transplant rejection. In any case, each complement-targeted treatment regimen needs to be carefully assessed and its risk weighed against the benefits of treatment. Although clinical experience still needs to be gained, the C3 mutation described in the present study alongside the use of complement inhibitors on the market and in clinical trials are not indicative of definite complications during well-controlled treatment of complement-related diseases.

In summary, our genetic and functional analysis of a unique loss-of-function mutation in the β-chain of C3 provides a molecular explanation for the impairment of AP activation in affected patients. It shows that the substitution of Met73 with Thr changes the intramolecular contact network, which affects functionally relevant regions of C3 and prevents C3 from binding to C3 convertases. Therefore, although this mutant is able to form initial AP C3 convertases and is susceptible to cleavage by extrinsic proteases, the activation of the mutated C3 and amplification via the AP are largely impaired (Fig. 6B). In contrast to known gain-of-function mutations in C3, this functional deficiency does not fuel inflammatory conditions such as DDD, but rather leads to less defined clinical manifestations such as potential exacerbation of immune complex disorders, or affects the risk for certain infections. Our genetic and molecular study of a rare mutant form of the major complement component C3 and its comparison with previously reported C3 polymorphisms and mutations therefore provides important insights into complement activation mechanisms, susceptibility to clinical manifestation, and the potential effects of C3-targeted therapeutic complement intervention.

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Disclosures
J.D.L. and D.R. are the inventors of patents and patent applications that describe the use of complement inhibitors for therapeutic purposes. J.D.L. is the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors for clinical applications. The other authors have no financial conflicts of interest.

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