A type III complement factor D deficiency: Structural insights for inhibition of the alternative pathway

To the Editor:

We investigated an alternative complement pathway (AP) deficiency in a patient with absent alternative pathway hemolytic activity but normal classical pathway hemolytic activity recovering from invasive meningococcal infection (for patient and sibling details, see Patient details in this article's Online Repository at www.jacionline.org). Serum reconstitution with proximal AP components suggested a factor D (FD) deficiency (Fig 1, *A*). Sanger sequencing of *CFD* identified a rare homozygous missense mutation (c.602G>C) in exon 4 in the patient (II-1) and sibling (II-2), resulting in an arginine to proline substitution (p. R176P) (see Fig E1, *A*, and reference E9 in this article's Online Repository at www.jacionline.org). This genotype cosegregated with an alternative pathway hemolytic activity–null phenotype, as the parents, both heterozygotes, had normal alternative pathway hemolytic activity (Fig 1, *B*). In contrast to previously confirmed FD deficiencies,[1-3] all members of the pedigree had normal levels of circulating FD, as corroborated by Western blot (see Fig E1, *B*). Meanwhile, identical circular dichroism spectra and melting curves of recombinant wild-type (WT) and R176P FD precluded gross changes in FD structure or stability, suggesting a functional deficiency (Fig 1, *C*, and see Fig E1, *C*). We assessed the cleavage of C3b-bound factor B (FB) by recombinant WT and mutant FD (R176P, R176A, R176Q). WT FD could cleave C3b-bound FB to produce fragments Bb and Ba. Conversely, R176P FD demonstrated diminished *in vitro* catalytic activity at all concentrations and had negligible activity at physiological concentration (0.04 μmol/L) (Fig 1, *D*, and see Fig E1, *D*). Reconstitution of FD-depleted serum with R176P FD also demonstrated impaired AP-mediated hemolysis (see Fig E1, *E*).

FD's serine protease activity depends on obligatory binding to the C3bB complex via 4 exosite loops (residues 132-135, 155-159, 173-176, 203-209). This leads to rearrangement of the self-inhibitory loop (199-202), allowing realignment of His41 and Asp89 with Ser183 to form the active catalytic triad (see Fig E2, *A* and *B*, in this article's Online Repository at www.jacionline.org).[4,5] Mutation R176P lies outside the active site, within one of the FB-binding exosite loops. We used molecular dynamics (MD) stimulations to study how the R176P mutation affects the FD protein fold (see Fig E2, *C*). In mutant FD, we observed a rearrangement of the exosite loop 155-161 within 50 nanoseconds of simulation (Fig 2, *A*). This was unexpected because loop 155-161 was not in direct contact with residue 176. Average structures generated from the final 50 nanoseconds of simulation
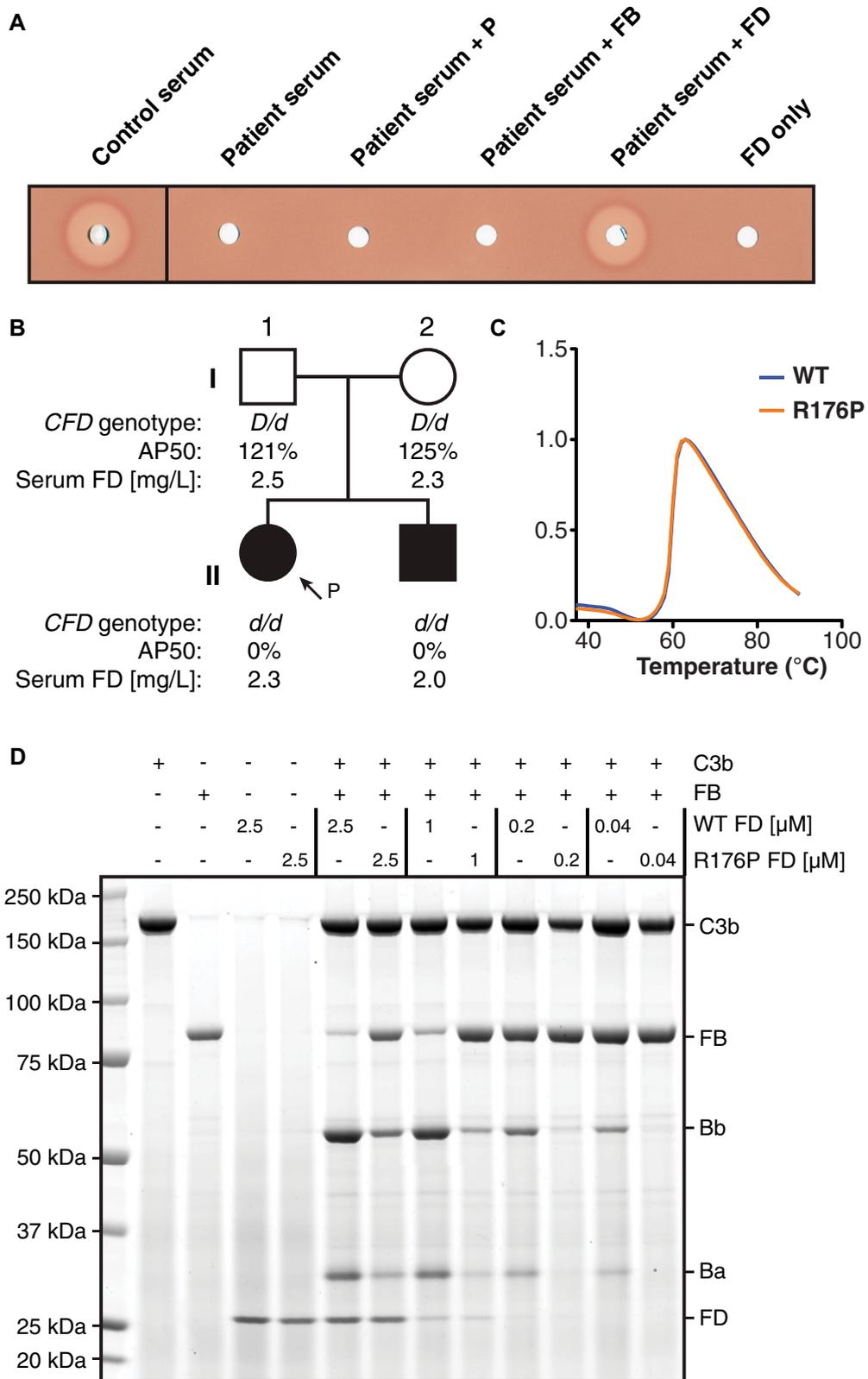
FIG 1. Assessing the contribution of mutation R176P to AP dysfunction. **A,** Alternative pathway hemolytic activity (AP50) assay assessing patient serum supplemented with properdin (P), FB, or FD. **B,** The immediate family pedigree of the patient with the *CFD* genotype, serum AP50, and serum FD concentrations displayed. *D* represents the WT allele and *d*, the mutant allele (c.602G>C). **C,** Thermal shift assay of WT and R176P FD. **D,** Serial dilutions of recombinant WT or R176P FD were incubated with C3b and FB. The SDS-PAGE gel, stained with AcquaStain, shows the individual proteins and resultant products.
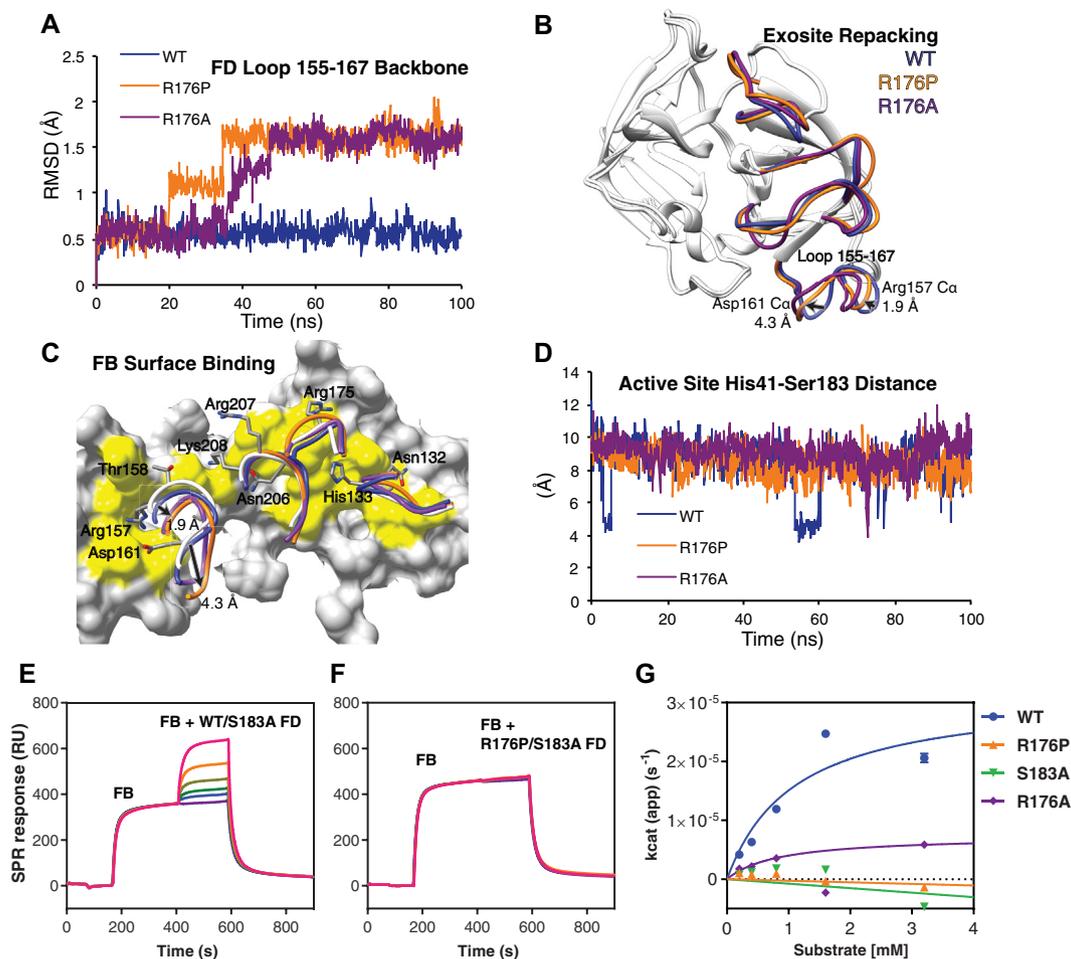
FIG 2. Defining the effects of the R176P mutation on FD function. **A** and **B,** FB-binding exosite loop 155-167 assumes a new conformation in mutant FD simulation. *Arrows* highlight average Cα position shifts of 2 residues that bind C3bB in the R176P FD simulation. **C,** Loss of shape complementarity at the FD-C3bB interface. FD exosite loops from published cocrystal structures (*white*, Protein Data Bank ID: 2XWB) overlaid with the simulated loops of WT and mutant FD. **D,** Distance sampled between the active site Nε2 nitrogen of His41 and Oγ of Ser183 during each simulation. The shorter distance is necessary for catalytic activity. **E** and **F,** Surface plasmon resonance binding measurement of enzymatically inactive recombinant FD (R176P/S183A or WT/S183A) to C3bB complex. **G,** Steady state kinetics for Z-Lys-SBzl cleavage by WT, R176P, R176A, and catalytically inactive control S183A FD. *RMSD,* Root-mean-square deviation.

for WT and mutant FD (R176P and R176A) demonstrated that key FB-binding residues Asp161 and Arg157 were shifted by 4.3 Å and 1.9 Å, respectively (Cα average position) (Fig 2, *B*). Superimposing these MD average structures onto the crystal structure of the C3bB-D complex revealed that Asp161 and Arg157 assumed a conformation that no longer supported binding due to loss of shape and charge complementarity to the FB surface (Fig 2, *C*). The other 3 exosite loops retained their binding-competent conformations. After assuming the new conformation, exosite loop 155-161 demonstrated higher conformational mobility (root mean square fluctuation) relative to WT (Fig E2, *D* and *E*). In contrast, the mobility of loops containing catalytic residues His41 and Asp89 decreased in the mutants. Using the distance between His41 and Ser183 during MD simulations as a proxy for the active site conformation, we observed that WT could sample the short distance necessary for a catalytically active conformation (Fig 2, *D*). Conversely, in both mutant simulations, the distance remained larger, consistent with His41 pointing away from the active site. Therefore, in

addition to disruption of key FB-binding residues, mutations R176P and R176A appear to stabilize the self-inhibited conformation of free FD.

To assess the binding of FD to C3bB, we used surface plasmon resonance. Coinjection of catalytically inactive FD (WT/S183A) with FB demonstrated a dose-dependent increase in binding to C3b and complex formation (Fig 2, *E*). In contrast, R176P/S183A FD lacked any detectable binding (Fig 2, *F*). Consistent with the stochastic transitions of free WT FD to the active conformation observed in the MD simulation, FD has a low level of esterolytic activity toward a small synthetic substrate, Z-Lys-SBzl (Fig 2, *D*). Surprisingly, R176P FD demonstrated a loss of esterolytic activity similar to the active site mutant, S183A (see Fig 2, *G*).

Deficiency of properdin, the most common AP deficiency, can result from absent (type I), low (type II), or normal but nonfunctioning (type III) protein levels (for reference, see reference E10 in this article's Online Repository at www.jacionline.org). Meanwhile, previously confirmed deficiencies of activating complement serine proteases have all

resulted in low or absent gene product. We have identified a unique deficiency: R176P FD is fully expressed and stable, but enzymatically inert, constituting a functional or type III deficiency. Recent preclinical evidence[6] that FD-deficient mice are susceptible to diabetes prompted metabolic assessment in the FD-deficient patients. No abnormality was detected (for details, see Functional FD deficiency does not result in impaired oral glucose tolerance section, Fig E3, and Table E1 in this article's Online Repository at www.jacionline.org).

Overactivation of AP is implicated in numerous inflammatory disorders, including age-related macular degeneration. Therefore, blockade of the AP by targeting the rate-limiting enzyme, FD, is an attractive approach to controlling disease progression. An anti-FD Fab fragment targeting the 2 distal exosite loops has shown some benefit in phase II clinical trials for treatment of dry age-related macular degeneration.[7] In vitro studies indicate that it inhibits binding to the C3bB complex but increases esterolytic activity toward small-molecule substrates.[8] This may result in unwanted clinical effects due to nonspecific activity or limit its efficacy in vivo. In the case of R176P FD, both FB-binding and esterolytic activity are abrogated through exosite hindrance and stabilization of the self-inhibited state. Loop 173-176 is thus a promising target for allosteric inhibitors of FD that stabilize the inhibitory loop in addition to binding-blockade. A structure-based design approach to targeting FD has recently succeeded in identifying candidate FD inhibitors where high-throughput screens had failed,[9] highlighting the benefits of integrating structural information into candidate drug screens. Comprehensive definition of the structural and molecular determinants of in vivo FD activity is critical for this. This study of the R176P mutation demonstrates how in-depth mechanistic analysis of rare complement deficiencies can deliver such insight validated clinically by in vivo human evidence of AP blockade.

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