Regulators of complement activity mediate inhibitory mechanisms through a common C3b-binding mode

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Abstract

Regulators of complement activation (RCA) inhibit complement-induced immune responses on healthy host tissues. We present crystal structures of human RCA (MCP, DAF, and CR1) and a small-pox virus homolog (SPICE) bound to complement component C3b. Our structural data reveal that up to four consecutive homologous CCP domains (i–iv), responsible for inhibition, bind in the same orientation and extended arrangement at a shared binding platform on C3b. Large sequence variations in CCP domains explain the diverse C3b-binding patterns, with limited or no contribution of some individual domains, while all regulators show extensive contacts with C3b for the domains at the third site. A variation of ~100° rotation around the longitudinal axis is observed for domains binding at the fourth site on C3b, without affecting the overall binding mode. The data suggest a common evolutionary origin for both inhibitory mechanisms, called decay acceleration and cofactor activity, with variable C3b binding through domains at sites ii, iii, and iv, and provide a framework for understanding RCA disease-related mutations and immune evasion.

Keywords complement; regulators of complement activity; cofactor activity; decay-accelerating activity; immune evasion

Introduction

The complement system is an important arm of the humoral immune system in mammals (Sjoberg et al., 2009; Ricklin et al., 2010; Merle et al., 2015a,b). Activation of the complement leads to C3b opsonization of targeted cells and particles inducing killing and clearance of the target. To protect host cells and tissues from inadvertent complement activation, mammals express complement regulators, such as factor H (FH), complement receptor 1 (CR1, CD35), membrane-cofactor protein (MCP, CD46), and decay-accelerating factor (DAF, CD55; Zipfel & Skerka, 2009; Merle et al., 2015a). These regulator proteins stop C3b opsonization by breaking down the C3 convertases. Lack of protection, due to familial mutations in the complement genes (Liszewski & Atkinson, 2015a; Martinez-Barricarte et al., 2015) or the presence of autoantibodies against regulators (Luzzatto & Gianfaldoni, 2006; Dragon-Durey et al., 2010), has been linked to, for example, atypical hemolytic uremic syndrome (aHUS) and C3 glomerulopathies (C3G) in kidneys and age-related macular degeneration (AMD) in eyes. Moreover, conditions of chronic and acute inflammations, as in rheumatoid arthritis, strokes, and heart attacks, become aggravated by complement activation against the disturbed tissue (Sjoberg et al., 2009; Merle et al., 2015b). These pathological conditions underscore the importance of balancing complement activation and regulation (Ricklin & Lambris, 2013a).

Proteins of the “regulators of complement activation” (RCA) gene cluster consist of strings of consecutive complement control protein (CCP) domains of ~60–70 amino-acid residues (Kirkkitadze & Barlow, 2001). Complement regulators CR1, MCP, and DAF are expressed on cell membranes, where they provide immediate protection to host cells. The soluble regulator FH stops complement activation in the fluid phase and discriminates “self” from “non-self” cells and matrix material by binding host molecular patterns (Clark et al., 2013). The protective function can be attributed to 3–4 consecutive CCP domains (Kirkkitadze & Barlow, 2001; Zipfel & Skerka, 2009; Merle et al., 2015a), which may act through two inhibitory mechanisms. DAF, CR1, and FH have so-called decay-accelerating activity. Decay acceleration enhances the irreversible
dissociation of protease fragments from C3 convertases, either C4b2a or C3bBb (Nicholson-Weller & Wang, 1994; Hourcade et al., 1999). Linked to this first activity, the regulators also block the binding of proenzymes and prevent the formation of new convertases. MCP, CR1, and FH exert cofactor activity. By binding to C3b or C4b, these regulators provide a binding platform for the protease factor I (FI). Bound FI cleaves C3b and C4b to yield fragments that cannot form convertases. Viruses and bacteria utilize these host protection mechanisms to avoid complement response. While certain bacteria express surface-bound proteins that bind host FH to provide protection (Zipfel et al., 2013; Merle et al., 2015b; Schmidt et al., 2015), orthopox and herpes viruses encode their own protection proteins, which structurally and functionally mimic host regulators (Ahmad et al., 2007; Ojha et al., 2014). Variola virus “smallpox inhibitor of complement enzymes” (SPICE) consists of four CCP domains and exhibits both decay-accelerating and cofactor activities (Rosengard et al., 2002) and is 40% identical in amino-acid sequence to human MCP.

A structural basis of regulator binding to C3b was provided by crystal structures of C3b in complex with FH domains CCP1–4 (Wu et al., 2009) and C3d in complex with FH CCP19–20 (Kajander et al., 2011; Morgan et al., 2011). The structure of C3b-FH CCP1–4 shows C3b in its characteristic arrangement of a key-ring-shaped core consisting of 6 macroglobulin domains (MG1–6) connected by a linker domain, two domains (MG7–8) on top of the key ring that connect to an arm formed by a “complement C1r/C1s, UEGF, BMP1” (CUB) domain and thioester-containing domain (TED); and a C-terminal C345c (CTC) domain at the top of C3b (Janssen et al., 2006; Wiesmann et al., 2006; see Fig 1). The FH CCP1–4 binding site spans C3b from the N-terminal (α′ NT) region bound to MG7, through MG6, MG2, and CUB, down to MG1 and TED. The C-terminal CCP19–20 domains of FH bind at a distinct binding site provided by the TED domain. Taken together, “mini-FH” chimeras consisting of the CCP1–4 and 19–20 showed strong decay-accelerating and cofactor activities, similar to those of full-length FH (Hebecker et al., 2013; Schmidt et al., 2013). Structure comparison of C3b-FH CCP1–4 and the C3 convertase (C3bb) in complex with SCIN (Roosijnakers et al., 2009) suggested that FH displaces Bb by steric hindrance upon binding to C3b, thereby exerting its decay-accelerating activity. Furthermore, crystal structures of FB in complex with C3b or the homologous cobra-venom factor (CVF; Janssen et al., 2009; Forneris et al., 2010) revealed overlapping binding sites for the CCP1–2 domains of FB and the CCP1–2 domains of FH, which explains why binding of FH to C3b blocks convertase formation. In the case of cofactor activity, regulator binding to C3b provides a binding platform for the protease FI. Roversi et al. presented a crystal structure of FI (Roversi et al., 2011) and suggested that FI possibly binds at a niche formed by the regulator domains CCP1–3 of FH and the CTC domain of C3b, providing a potential explanation for the regulator role in cofactor activity of FH.

Regulators vary in activity and C3b- and C4b-binding behavior, even though all regulators consist of strings of homologous CCP domains (Kirkitatde & Barlow, 2001; Ahmad et al., 2007; Harris et al., 2007; Zipfel & Skerka, 2009; Merle et al., 2015a). Both MCP and DAF require their CCP3–4 for binding to C3b (Liszewski et al., 2000; Harris et al., 2007) and CCP2–4 for cofactor activity (Liszewski et al., 2000; Riley et al., 2002) and decay-accelerating activity, respectively (Harris et al., 2007). However, their CCP2–4 domains map to CCP2–4 and CCP1–3 of FH, respectively (McLure et al., 2004). CR1 bears three clusters of CCP domains displaying differential C3b/C4b binding and associated regulatory activities. The CCP1–3 domain binds weakly to C3b and strongly to C4b (Krych et al., 1991) and predominantly shows decay acceleration in both classical and alternative pathways (Krych-Goldberg et al., 1999), whereas the two domain clusters CCP8–10 and CCP15–17, identical in sequence with the exception of four amino acids, bind strongly to both C3b and C4b (Krych et al., 1994) and display mostly cofactor activity (Krych et al., 1994, 1998). Furthermore, FH and CR1 binding to C3b depends on interactions with the α′ NT region, whereas MCP does not (Lambris et al., 1996; Krych et al., 1998; Omar & Isenman, 1999; Makou et al., 2013). Extensive mutagenesis data for MCP (Liszewski et al., 2000) show that C3b binding of domain CCP4 of MCP is incompatible with the observed arrangement of the equivalent domain in C3b-FH CCP1–4 complex (Wu et al., 2009). Various mutations of regulators linked to aHUS, C3G, and AMD have been reported (Weisman et al., 1990; Zipfel & Skerka, 2009; Rodriguez et al., 2014; Liszewski & Atkinson, 2015b; Schramm et al., 2015). These mutations identify multiple sites on the surface of C3b, FH, MCP, and CR1 responsible for effective complement regulation. The available structural data from the C3b-FH (CCP1–4; Wu et al., 2009) and C3d-FH (CCP19–20; Kajander et al., 2011; Morgan et al., 2011) complexes and the structures of isolated regulators (Smith et al., 2002; Uhrinova et al., 2003; Williams et al., 2003; Lukacik et al., 2004; Persson et al., 2010) provided initial guidance for structural mapping of these mutations. However, the differences in the sequences and structural orientations of the various CCP domains of the regulators did not provide an unambiguous molecular explanation for several disease-related mutations (Dragon-Durey & Fremeaux-Bacchi, 2005; Rodriguez et al., 2014; Liszewski & Atkinson, 2015b; Martinez-Barricate et al., 2015; Schramm et al., 2015). In addition, Gropp et al. (2011) indicated β2-glycoprotein 1, which consists of five CCP domains, to act as a complement regulator (Gropp et al., 2011). To provide a structural basis for binding of the regulators and their activities, we determined the crystal structures of human DAF domains CCP2–4, MCP CCP1–4, CR1 CCP15–17, and the variola virus SPICE (full-length CCP1–4) in complex with human C3b. In addition, we report the structure of “free” C3b at 2.8 Å resolution, thereby improving the resolution of an earlier report (Janssen et al., 2006). These data indicate a common binding site on C3b for the regulators and suggest a common structural origin for the complement-inhibitory activities by the “regulators of complement activation (RCA)”.

Results and Discussion

Structural data

We determined the crystal structures of C3b-MCP (CCP1–4), C3b-SPICE (CCP1–4), C3b-CR1 (CCP15–17), and C3b-DAF (CCP2–4) to 2.4, 2.5, 3.3, and 4.2 Å resolution, respectively (Table 1 and Fig 1A). Previously, we reported the crystal structure of C3b-FH (CCP1–4) at 2.7 Å resolution (Wu et al., 2009). For these fragments, we observed Kd’s of 0.35 μM for MCP (CCP1–4), 1.3 μM for SPICE (CCP1–4), 1.9 μM for CR1 (CCP15–17), 6.1 μM for DAF (CCP1–4),...
and 7.2 μM for FH (CCP1-4); see Fig EV1. Furthermore, initial crystallization trials of C3b-CR1 (CCP15-17) and C3b-b2GPI yielded crystals containing only C3b. One of these crystals was used to collect a C3b data set up to 2.8-Å resolution, superseding the C3b structure at low resolution currently available in the PDB (Janssen et al, 2006).
Table 1. Diffraction, data collection, and refinement statistics.

<table>
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<tr>
<th></th>
<th>C3b-DAF (CCP2–4)</th>
<th>C3b-CR1 (CCP15–17)</th>
<th>C3b-MCP (CCP1–4)§</th>
<th>C3b-SPICE (CCP1–4)</th>
<th>C3b</th>
</tr>
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<tr>
<td>Space group</td>
<td>P2₁,2₁,₂</td>
<td>P₁</td>
<td>P2₁,2₁,₂</td>
<td>P₁</td>
<td>P₂₁</td>
</tr>
<tr>
<td>Cell parameters</td>
<td>a = 117.4 Å  (\alpha = 90.0^\circ)</td>
<td>a = 104.3 Å  (\alpha = 90.0^\circ)</td>
<td>a = 82.8 Å  (\alpha = 90.0^\circ)</td>
<td>a = 68.9 Å  (\alpha = 75.0^\circ)</td>
<td>a = 57.6 Å  (\alpha = 90.0^\circ)</td>
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<td>X-ray source</td>
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<td>ESRF ID23 EH1</td>
<td>ESRF ID29</td>
<td>ESRF ID14 EH4</td>
<td>ESRF ID14 EH4</td>
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<tr>
<td>Wavelength (Å)</td>
<td>0.975</td>
<td>0.933</td>
<td>0.999</td>
<td>0.999</td>
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<tr>
<td>Resolution (Å)</td>
<td>117.83–4.20 (4.43–4.20)</td>
<td>60.15–3.30 (3.48–3.30)</td>
<td>69.95–2.40 (2.44–2.40)</td>
<td>47.80–2.50 (2.55–2.50)</td>
<td>70.07–2.80 (2.89–2.80)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>96.5 (92.6)</td>
<td>98.4 (98.2)</td>
<td>99.0 (96.2)</td>
<td>97.2 (96.3)</td>
<td>96.8 (98.3)</td>
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<tr>
<td>Rmerge (%)</td>
<td>0.175 (0.673)</td>
<td>0.225 (0.878)</td>
<td>0.116 (0.583)</td>
<td>0.128 (0.774)</td>
<td>0.122 (1.098)</td>
</tr>
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</table>

Refinement

<table>
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<tr>
<th></th>
<th>Rmerge/Rfree (Å)²</th>
<th>Average B-factors (Å²)³</th>
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<tr>
<td>Overall</td>
<td>0.258/0.296</td>
<td>0.250/0.291</td>
</tr>
<tr>
<td>C3b only</td>
<td>0.188/0.219</td>
<td>0.198/0.229</td>
</tr>
<tr>
<td>Regulator only</td>
<td>0.229/0.268</td>
<td>1.07</td>
</tr>
<tr>
<td>Number of atoms</td>
<td>27,275</td>
<td>14,478</td>
</tr>
<tr>
<td>Protein</td>
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<td>14,036</td>
</tr>
<tr>
<td>Ligands</td>
<td>0</td>
<td>28</td>
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<tr>
<td>Waters</td>
<td>492</td>
<td>207</td>
</tr>
<tr>
<td>R.M.S. deviations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.005</td>
<td>0.003</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.04</td>
<td>0.83</td>
</tr>
</tbody>
</table>

Ramachandran statistics

| Favored (%) | 93                | 96                       |
| Allowed (%) | 6                 | 4                        |
| Disallowed (%) | 1                | 0                        |

4In the crystal structure of C3bMCP (CCP1–4), only CCP domains 3 and 4 could be modeled.
5Values in parentheses are for reflections in the highest resolution shell.
6Rmerge = \( \Sigma_l (I - \langle I \rangle) / \Sigma_l \langle I \rangle \), where \( I \) is the observed intensity for a reflection and \( \langle I \rangle \) is the average intensity of symmetry-related observations of a unique reflection.

C3b-regulator arrangements

The regulators bound to C3b show extended arrangements of their CCP domains, while C3b displays its characteristic organization of MG1–8, linker, CUB, TED, and CTC domains (Jansen et al, 2006, 2005; Wiesmann et al, 2006; Fig 2A). The extended CCP arrangements are in agreement with the structures of unbound regulators and regulator fragments (Smith et al, 2002; Uhrinova et al, 2003; Williams et al, 2003; Lukacik et al, 2004; Persson et al, 2010; Appendix Fig S3). Overall, the regulators bind C3b at the same site, formed by the \( \alpha' \)NT region and domains MG7, MG6, CUB, MG2, MG1, and TED (top to bottom as shown in Fig 2A), similar to that observed in the complex of C3b with FH CCP1–4 (Wu et al, 2009). The C3b-regulator contact interfaces extend over a length of up to 130 Å burying surface areas up to 2,300 Å² (see Fig 2 for details).
Figure 2. Domain organization in the C3b-regulator complexes and molecular interactions.

A "Open-book" surface representation of C3b and regulator molecules, highlighting their domain organizations using different colors as shown in the neighboring schemes. Domains in complement regulators are labeled using domain numbering from CCPi to CCPiv based on their contact sites on C3b. The corresponding CCP domains in each regulator described in this work are shown in the bottom-left scheme. Domains CCP1-2 of MCP are shown in shaded gray because they are not involved in C3b binding. The table reports the extent of the regulator contact surface on C3b and the associated buried surface area.

<table>
<thead>
<tr>
<th>regulator complex</th>
<th>interface extent (Å)</th>
<th>buried surface area (Å²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3b-MCP</td>
<td>70</td>
<td>1215</td>
</tr>
<tr>
<td>C3b-SPICE</td>
<td>125</td>
<td>1730</td>
</tr>
<tr>
<td>C3b-FH</td>
<td>130</td>
<td>2296</td>
</tr>
<tr>
<td>C3b-CR1(15-17)</td>
<td>100</td>
<td>1908</td>
</tr>
<tr>
<td>C3b-DAF(2-4)</td>
<td>65</td>
<td>1575</td>
</tr>
</tbody>
</table>

B Opened view of the molecular footprint of the regulator-C3b interaction (as shown in Fig 2A), highlighting the C3b domains on the regulator surface (top) and the regulator domains on C3b (bottom).
C3b-MCP differs markedly from C3b-FH CCP1–4 in two aspects. First, in C3b-MCP, only CCP3–4 makes contact with C3b. No density and non-interpretable density were observed for CCP1 and CCP2, respectively (Fig 1D), whereas the density was well resolved for the CCP3 and CCP4 domains of MCP (Appendix Fig S1). These observations are in agreement with biochemical data that CCP1 and CCP2 of MCP do not contribute to C3b binding (Adams et al., 1991). Second, the orientation of MCP CCP4 differs markedly from FH CCP4. Though MCP CCP4 domain binds at the same site to C3b, it is rotated by ~100° around the long axis of the domain compared to CCP4 of FH in C3b-FH (Fig 3A and B, Appendix Fig S4A). Figure 3C shows that mutagenesis data on CCP4 of MCP (Liszewski et al., 2000) is in full accordance with the observed domain orientation in the crystal structure. The competitive binding of the GB24 antibody to MCP, identified by mutagenesis around residues Phe230 and Phe242, further supports the observed MCP interaction site (Liszewski et al., 2000). The CCP4 domain of SPICE binds C3b in the same orientation as MCP CCP4, although the electron density for SPICE CCP4 was weak, indicating disorder and suggesting weak local interactions (Fig 1D). Limited contribution by SPICE CCP4 to C3b binding is supported by improved binding of vaccinia virus complement control protein (VCP) CCP1–3 fused to MCP CCP4 (Ahmad et al., 2010). Putatively, FH may be considered deviant from the other presented structures (Fig 1C, Appendix Fig S4D). Variations in CUB-TED and CTC positions and between the β-ring top and bottom parts were previously observed in C3bB, C3BBD (Forneris et al., 2010) and the SCIN-stabilized C3bB convertase (Rooijakkers et al., 2009), in its bovine ortholog (Fredslund et al., 2006), and in paralogs such as alpha-2-macroglobulin (Marrero et al., 2012), C5 (Fredslund et al., 2008), and the C5b-C6 complex (Aleshin et al., 2012; Hadders et al., 2012).

The CTC domain of C3b adopts different orientations with the “neck” that links CTC to the MC core found in two distinct conformations. This neck region (residues 1,496–1,517) connects CTC to MG8 through the main chain and participates in three disulfide bonds; one internal (Cys1506–Cys1511) and one each connecting to MG7 (Cys873–Cys1513) and to the CTC domain (Cys1518–Cys1590; Appendix Fig S5). During conversion from C3 to C3b, this region undergoes a complete change of its secondary structure. In C3, residues 1,507–1,517 fold into a 3-helix (Janssen et al., 2005; Fredslund et al., 2006). In unbound C3b, the internal disulfide bond connects two short β-strands (respectively, 1,503–1,508 and 1,509–1,515) that fold into a short β-turn-β configuration (Janssen et al., 2006; Wiesmann et al., 2006). This conformational change positions CTC in a more upward orientation, while the MG7-MG8-CUB arrangement does not change (Appendix Fig S5). In the structures of C3b-SPICE, C3b-CR1, and C3b-DAF, the neck region adopts the β-turn-β configuration (Appendix Fig S5), as for all reported C3b structures except C3b-FH CCP1–4 (Janssen et al., 2006; Wiesmann et al., 2006; Katschke et al., 2009; Rooijakkers et al., 2009; Wu et al., 2009; Forneris et al., 2010; Garcia et al., 2010). The same conformational change is observed for conversion from C4 to C4b (Kidmose et al., 2012; Mortensen et al., 2015) and from C5 to C5b (Fredslund et al., 2008; Aleshin et al., 2012; Hadders et al., 2012). In the C3b-MCP and C3b-FH structures, the neck region adopts the α-helical configuration and is almost identical to that observed in C3. In these structures, the CTC shows a pronounced downward orientation (Fig EV3A). In this down position, a protruding loop of CTC (residues 1,585–1,600) contacts CUB, with aromatic residues Phe1558 and Trp1612 of CTC surrounding CUB residue Pro972 (Appendix Fig S6B). This arrangement is similar to the CTC-CUB contact observed in native C3. In C3b, the CTC loop docks on the β-hairpin conformation of the neck region through π–π stacking of the aromatic residues with Arg1507 and Arg1512 (Appendix Fig S6B). Since MCP, CR1 (CCP15–17), and FH have strong cofactor activity, the observed arrangements of CTC in C3bFH (CCP1–4) and C3bMCP (CCP1–4) may possibly reflect a preferred conformational arrangement for binding of FI; however, this arrangement is not observed for C3bCR1 (CCP15–17).

Besides the variations in C3b CTC positions (Fig EV3A), we observe differences in the placement of CUB and TED domains (Fig EV3B–D). The series of structures can be separated into two sets that differ by ~6–9 Å in CUB position as shown for residues 1,311–1,317 (Fig EV3C and D). Structures of C3b-DAF (CCP2–4) and C3b-CR1 (CCP15–17) show CUB-TED, with high B-factors, positioned in a downward orientation, while C3b-FH, C3b-MCP, and C3b-SPICE have CUB-TED in an upward position (Fig EV3B). The largest distinction between these two sets of structures is the

C3b structural variations

The C3b structures in all regulator complexes display the typical domain organization of C3b (Appendix Fig S4B). Notably, CUB-TED and CTC show variable positioning with respect to the main body of C3b. The characteristic β-ring of C3b (Fig 2A) can be divided into a bottom (MG1, 4–5) and top part (MG2–3, 6) with flexibility for the bottom part that is higher as observed in C3b-SPICE and the unbound C3b at 2.8-Å resolution compared to that observed in the other presented structures (Fig 1C, Appendix Fig S4D). Variations in CUB-TED and CTC positions and between the β-ring top and bottom parts were previously observed in C3bB, C3BBD (Forneris et al., 2010) and the SCIN-stabilized C3bB convertase (Rooijakkers et al., 2009), in its bovine ortholog (Fredslund et al., 2006), and in paralogs such as alpha-2-macroglobulin (Marrero et al., 2012), C5 (Fredslund et al., 2008), and the C5b-C6 complex (Aleshin et al., 2012; Hadders et al., 2012).

The CTC domain of C3b adopts different orientations with the “neck” that links CTC to the MC core found in two distinct conformations. This neck region (residues 1,496–1,517) connects CTC to MG8 through the main chain and participates in three disulfide bonds; one internal (Cys1506–Cys1511) and one each connecting to MG7 (Cys873–Cys1513) and to the CTC domain (Cys1518–Cys1590; Appendix Fig S5). During conversion from C3 to C3b, this region undergoes a complete change of its secondary structure. In C3, residues 1,507- to 1,517-fold into a 3-helix (Janssen et al., 2005; Fredslund et al., 2006). In unbound C3b, the internal disulfide bond connects two short β-strands (respectively, 1,503–1,508 and 1,509–1,515) that fold into a short β-turn-β configuration (Janssen et al., 2006; Wiesmann et al., 2006). This conformational change positions CTC in a more upward orientation, while the MG7-MG8-CUB arrangement does not change (Appendix Fig S5). In the structures of C3b-SPICE, C3b-CR1, and C3b-DAF, the neck region adopts the β-turn-β configuration (Appendix Fig S5), as for all reported C3b structures except C3b-FH CCP1–4 (Janssen et al., 2006; Wiesmann et al., 2006; Katschke et al., 2009; Rooijakkers et al., 2009; Wu et al., 2009; Forneris et al., 2010; Garcia et al., 2010). The same conformational change is observed for conversion from C4 to C4b (Kidmose et al., 2012; Mortensen et al., 2015) and from C5 to C5b (Fredslund et al., 2008; Aleshin et al., 2012; Hadders et al., 2012). In the C3b-MCP and C3b-FH structures, the neck region adopts the α-helical configuration and is almost identical to that observed in C3. In these structures, the CTC shows a pronounced downward orientation (Fig EV3A). In this down position, a protruding loop of CTC (residues 1,585–1,600) contacts CUB, with aromatic residues Phe1558 and Trp1612 of CTC surrounding CUB residue Pro972 (Appendix Fig S6B). This arrangement is similar to the CTC-CUB contact observed in native C3. In C3b, the CTC loop docks on the β-hairpin conformation of the neck region through π–π stacking of the aromatic residues with Arg1507 and Arg1512 (Appendix Fig S6B). Since MCP, CR1 (CCP15–17), and FH have strong cofactor activity, the observed arrangements of CTC in C3bFH (CCP1–4) and C3bMCP (CCP1–4) may possibly reflect a preferred conformational arrangement for binding of FI; however, this arrangement is not observed for C3bCR1 (CCP15–17).

Besides the variations in C3b CTC positions (Fig EV3A), we observe differences in the placement of CUB and TED domains (Fig EV3B–D). The series of structures can be separated into two sets that differ by ~6–9 Å in CUB position as shown for residues 1,311–1,317 (Fig EV3C and D). Structures of C3b-DAF (CCP2–4) and C3b-CR1 (CCP15–17) show CUB-TED, with high B-factors, positioned in a downward orientation, while C3b-FH, C3b-MCP, and C3b-SPICE have CUB-TED in an upward position (Fig EV3B). The largest distinction between these two sets of structures is the
presence of a CCP domain binding to the MG1-TED site on C3b. With CUB-TED in the up position, more interactions between CUB and MG2 domains are observed forming a more tightly packed concave interface for interactions with the regulators (Fig EV3B–D). Additional hydrogen-bonding and salt-bridge interactions between these two domains involve the side chains of Arg979 with main chain carbonyl of Leu198, Arg937 with Glu226, Lys930 with Glu197, a proximate interaction (not forming hydrogen bonds in the structures) between Ser1315 and Gln177, and the main chain nitrogen of Leu1319 with the carbonyl of Pro174 of CUB and MG2, respectively (Appendix Fig S6A). Moreover, the upward orientation of CUB-TED facilitates the possible interactions with loop 1,607–1,622 of CTC. However, both up and down positions are observed in the two structures of (unbound) C3b and that of other C3b complexes (Janssen et al, 2006; Wiesmann et al, 2006; Katschke et al, 2009; Rooijakkers et al, 2009; Wu et al, 2009; Forneris et al, 2010; Garcia et al, 2010). This indicates that the energy difference between the two forms is small. The CUB-TED domains in the structure obtained at 2.8Å resolution (reported here) are better packed (as judged by the B-factors and crystal contacts) and are placed in the up position. In the previously reported structure at 4Å resolution (Janssen et al, 2006), these domains are in the down position with higher B-factors. Overall, the ~6–9Å upward movement of CUB-TED observed for FH, MCP, and SPICE is likely induced by concerted bridging contacts between MG2 and CUB with the C-terminal part of CCP3 and contacts between the N-terminal part of CCP4 with TED. Together, these contacts result in a rearrangement of C3b domains MG2, CUB, and TED that has recently been indicated as critical for the strong cofactor activity of these regulators (Gautam et al, 2015).
Interactions between C3b and CCP domains: sites i–ii

The N-terminal CCP domains interact with C3b only through residues from their C-terminal “bottom” and linker to the next, second CCP domain (generic CCP domains and their binding sites are referred to as CCP-i–CCPiv; see Fig 2A). This CCPi–ii site constitutes a contiguous surface that interacts with a small hydrophobic patch surrounded by negatively charged residues from the \( \alpha' \)NT region and MG7 domain on C3b (Fig EV4). The domains binding to this \( \alpha' \)NT-MG7 site (SPICE CCP1, FH CCP1, CR1 CCP15, and DAF CCP2) differ by rigid-body rotations of 2–12° in the various complexes (Appendix Fig S4C). The diversity in domain positions yields large variations among the specific contacts observed in the C3b-regulator structures (as summarized in Fig EV2). DAF almost completely lacks hydrophobic interactions at this site, and the differences between the two C3b-DAF copies observed in crystal structure indicate weak binding of the regulator to this site (Appendix Fig S2). Previous mutagenesis data (Appendix Table S1) identified a group of amino-acid residues in DAF (located at its CCP2–CCP3 linker) as critical for decay-acceleration activity (Kuttner-Kondo et al., 2001). Among these, a group of positively charged residues R134–R135–K160 interacts with the \( \alpha' \)NT of C3b in one of the two copies observed in the C3b-DAF (CCP2–4) crystal structure. In the other copy, the displacement of DAF CCP2 does not allow any contacts of this domain with C3b (Appendix Fig S2). In agreement with our data and interpretation, DAF CCP2 is not required for binding DAF to C3b (Harris et al., 2007), replacement of VCP CCP1 with DAF CCP2 resulted in loss of C3b binding for VCP (Ahmad et al., 2010), and the DAF CCP2–3 domain orientation is flexible, as shown by NMR (Uhrinova et al., 2003; Appendix Fig S3E). In contrast, rigidity in the domain–domain arrangement was indicated based on NMR structures of CR1 CCP15–16 and CCP16–17 (Smith et al., 2002), structures of related C3b-binding domains CR1 CCP1–2 and CCP2–3 (Park et al., 2014), and solution studies of CR1 (Furtado et al., 2008; Appendix Fig S3D). CR1 CCP15 shows limited but very specific interactions at this site, which are mostly localized around the loop proximate to the \( \alpha' \)NT and the C-terminus of the CCP15 domain. These contacts constitute the specific anchoring point for CR1 CCP15–16 to the C3b platform and are consistent with previous reports (Lambris et al., 1996; Oran & Isenman, 1999). CR1 contacts with C3b at this site include the hydrophobic interactions surrounding CCP15 Tyr978 with \( \alpha' \)NT Ile756 and MG7 Phe920, as well as the hydrogen bond between CCP15 Arg 980 and \( \alpha' \)NT Asp754 (although observed only in one of the two independent complexes in the crystal). The multiple contacts observed for C3b-FH and C3b-SPICE involve few conserved and several non-conserved interactions at this site, among which electrostatic interactions and hydrophobic contacts located between the CCP1–2 interface and \( \alpha' \)NT as well as CCP2 and MG6. These contacts include the conserved charged interaction between FH Arg83 (Arg84 in SPICE) with \( \alpha' \)NT Glu759, the

### Table 2: Summary of interactions details between C3b and regulators, divided by CCP domain*

<table>
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<tr>
<th>Buried surface area (Å²)</th>
<th>CCP1: 0</th>
<th>CCP1: 15</th>
<th>CCP1: 17</th>
<th>CCP2: 0</th>
<th>CCP2: 1</th>
<th>CCP2: 15</th>
<th>CCP2: 17</th>
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<tr>
<td>C3b-MCP (CCP1–4)</td>
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<td>CCP1: 419</td>
<td>CCP15: 284</td>
<td>CCP2: 313</td>
<td>CCP1: 303</td>
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<td></td>
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<tr>
<td>C3b-SPICE (CCP1–4)</td>
<td>CCP1: 537</td>
<td>CCP16: 802</td>
<td>CCP17: 865</td>
<td>CCP15: 34</td>
<td>CCP16: 83</td>
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<td>CCP1: 419</td>
<td>CCP15: 284</td>
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<td>CCP17: 49</td>
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<tr>
<td>C3b-MCP (CCP1–4)</td>
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<td>CCP1: 419</td>
<td>CCP15: 284</td>
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<tr>
<td>C3b-SPICE (CCP1–4)</td>
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<td>C3b-MCP (CCP1–4)</td>
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<td>CCP1: 419</td>
<td>CCP15: 284</td>
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<tr>
<td>C3b-SPICE (CCP1–4)</td>
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<td>CCP16: 83</td>
<td>CCP17: 49</td>
<td>CCP15: 36</td>
</tr>
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**a**Values reported are obtained from computational analyses using the PISA software (Krissinel & Henrick, 2007).

**b**For the C3b-CR1 (CCP15–17) and C3b-DAF (CCP2–4) structures, the values reported refer to averages between the two copies in the asymmetric unit.

**c**Values for buried surface area are defined as sum of areas from C3b and regulator.

**d**Electrostatic contacts defined as sum of residues involved in hydrogen bonds and salt bridges.

**e**Number of strong hydrophobic contacts defined as the sum of residues showing buried area percentages higher than 40% according to PISA interface analysis.

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hydrophobic region defined by FH Pro84-Gly86 (Arg85-Pro87 in SPICE) proximate to Val762, the conserved CCP2 residue Asp90 (Asp91 in SPICE) forming a hydrogen bond with MG6 Lys796, and the hydrophobic groove defined by FH Phe96, Leu98, and Phe104 (Leu97, Ile99, and Val102 in SPICE, respectively) with MG6 Phe794 (Fig EV2).

**Figure 4. Structural mapping of disease-related mutations on the C3b-regulator surfaces.**

A Mapping of known disease-related mutations on C3b (Appendix Table S4). The high-resolution C3b three-dimensional structure is used as structural template, shown as white surface. The regulator contact surfaces (based on C3b-FH (CCP1–4; Wu et al, 2009) and C3d-FH (CCP19–20; Kajander et al, 2011; Morgan et al, 2011)) are shown as black contour. All mutations reported in Appendix Table S4 are shown in blue on the C3b surface. Disease-related mutants reported to affect regulator binding are shown in red and labeled in the right magnified panel.

B Mapping of disease-related mutations on the FH (CCP1–4) surface using data from Appendix Table S5. Colors as in (A).

C Mapping of disease-related mutations on the MCP (CCP3–4) surface using data from Appendix Table S5. Colors as in (A).

**Interactions between C3b and CCP domains: sites ii and iii**

Next, the regulator domains superposing on to FH CCP2 and CCP3 interact with domains MG2, MG6, and CUB of C3b (Fig EV2). These interactions involve polar and charged amino-acid side chains and main chain carbonyl and amino moieties of C3b MG2 forming...
hydrogen bonds with the regulator domains. In particular, the β-strand formed by residues 175–186 of MG2 exposes several negative charges that form an elongated binding site, which is involved in interactions as observed in all C3b-regulator structures (Fig EV2). A fully conserved interaction is observed for main chain NH of residue Ser181 in C3b with the main chain CO of the regulator’s residue preceding a sharp turn, that is, Gly171 in FH, Gly169 in SPICE, Leu184 in MCP, Gly1088 in CR1, and Arg246 in DAF. On the regulator side facing the CUB domain, amino-acid residues interacting with CUB surround residue Arg1310 of C3b, whose side chain points toward negatively charged residues (Glu177 in MCP, Glu163 in FH, Glu1083 in CR1) or aromatic side chains (Tyr162 in SPICE). In addition, highly variable interaction sub-sites are observed and are summarized in Fig EV2. Few disease-related mutations of C3 have been reported that map on the regulator binding sites formed by MG2 and MG6. The known mutations, R592W/Q, R161W, and T162R, are located on the periphery of the CCPii-iii binding regions in C3b. This CCPii-iii binding site is, however, also involved in interactions with FB at two steps in the convertase formation (Janssen et al., 2009; Rooijakkers et al., 2009; Forneris et al., 2010, 2012). First, in the “loading” state, FB binds C3b with its CCP2 in a position that overlaps with FH CCP2 (Janssen et al., 2009; Rooijakkers et al., 2009). Second, in the “activation” state of C3bB, the helical extension of the FB-serine protease domain covers the FH CCP3 binding site (Fig EV5; Forneris et al., 2010). Mutations in the CCPii-iii binding regions may thus affect both FB and FH binding to C3b. Disturbing FB binding would likely reduce or stop complement activation and, if binding of FB to C3b is still intact, disturbing FH binding would lead to uncontrolled, that is, more, complement activation. Possibly, the central role of FB binding to C3b in complement activation limits the number of disease-related mutations observed in the overlapping FB and regulator binding sites (CCPii-CCPiii) on MG2/6 of C3b.

Mutagenesis experiments on CR1 CCP clusters provided details for the putative C3b/C4b binding sites and for the residues implicated in decay-accelerating and cofactor activities (Krych et al., 1991, 1994, 1998; Krych-Goldberg et al., 1999, 2005). A summary of this comprehensive analysis is provided in Appendix Table S2 and Appendix Fig S7A–C. Consistent with our structural data, all mutations significantly affecting C3b binding in CCP8–10 and CCP15–17 map onto the C3b-CR1 (CCP15–17) interface observed in the crystal structure and are localized at C3b binding sites. Also the mutations affecting C3b/C4b binding on the CCP1–3 domains of CR1 (Appendix Table S2A) entirely map to this region. The most prominent differences between the CCP1–3 and CCP8–10, and CCP15–17 clusters are all located at the C3b-CR1 (CCP15–17) interface (Appendix Fig S7A–C). Furthermore, the epitope for the 3D9 antibody, responsible for abolishing the CR1-C3b binding (Krych et al., 1998), overlaps with the contact site between C3b and CR1 at the bottom side of CCP17 (Appendix Fig S7B). In DAF, mutagenesis data (summarized in Appendix Table S2 and Appendix Fig S8A) show that mutations affecting decay-acceleration activity are distributed all over the C3b-binding side of DAF, mostly through charged residues in CCP3–4 (residues R240 and R246). Domain swapping of CCP2 in the orthopox regulator Kaposica with DAF CCP3 results in loss of cofactor activity of this viral inhibitor (Gautam et al., 2015). The DAF side not involved in interactions with C3b shows that residues critical for decay acceleration are clustered on the CCP2–3 domains only, consistent with the expected binding data for the Bb fragment (Harris et al., 2007) and data from domain swapping with MCP (Gautam et al., 2015). Mutagenesis of SPICE and the homologous VCP (Yadav et al., 2008; Liszewski et al., 2009; Appendix Table S3) indicated that SPICE residue Asn163 (located on CCP3) is solely responsible for a four-fold increase in C3b binding, when replaced with Glu163 in VCP (Sfyroera et al., 2005; Yadav et al., 2008). In the C3b-SPICE (CCP1–4) structure, this residue lies at the interface between C3b and SPICE, in the vicinity of MG2 Asp178 side chain (although not forming hydrogen bonds with this residue; Fig 3E). Hence, the presence of a negatively charged residue in VCP in this position may be responsible for the reduced binding affinity due to charge repulsion, as initially suggested by previous computational electrostatic modeling of VCP and SPICE chimeras (Sfyroera et al., 2005).

Interactions between C3b and CCP domains: site iv

A C-terminal interaction site is observed for FH, MCP, and SPICE CCP4 with MG1 and TED domains of C3b. As described above, the most prominent feature of this interaction site is the marked structural difference in the orientation of the CCP4 domains of MCP and SPICE binding to C3b compared to FH. Moreover, the high B-factors for C3b-SPICE at this interaction site suggest flexibility and reduced interactions between SPICE CCP4 and C3b in comparison with the FH and MCP. SPICE CCP4 bears numerous negative charges at the bottom-edge of this domain in comparison with FH and MCP (Fig EV4). Remarkably, removal of one of the few positive charges located in the observed contact interface with C3b at the “bottom” of VCP CCP4 (residue Lys233, corresponding to SPICE Thr233, Fig 3E) did not affect C3b binding (Yadav et al., 2008). This observation supports the notion that SPICE CCP4 contributes less to complex formation. SPICE and the highly homologous VCP are evolutionarily more closely related to MCP than to other human regulators (Ciulla et al., 2005). This is in agreement with the structural arrangement observed in C3b-SPICE (CCP1–4), which shows strong similarity to MCP in the orientation of the CCP3 and CCP4 domains bound to C3b (Fig 3A and B). Whereas charged residues are broadly distributed over the whole interaction surface of FH CCP4 providing interactions with both MG1 and TED, MCP and SPICE are binding to C3b only through the upper half of their CCP4 domains and only with TED, that is, without specific interactions with the MG1 domain of C3b (Fig EV2B).

Mapping disease-related mutations and disease-predisposing polymorphisms

Over the years, a large number of mutations and disease-predisposing polymorphisms associated with pathological conditions such as aHUS, AMD, and C3G have been reported involving C3b and/or complement regulators FH and MCP (summarized in Appendix Tables S4 and S5). Our structures enable precise mapping and provide a structural interpretation of the effects for the majority of these mutations. Except for those clearly identified as involved in binding of FH CCP19–20 (Jokiranta et al., 2000; Kajander et al., 2011; Morgan et al., 2011), all pathogenic C3b mutations known to affect regulator-C3b binding map on residues
located on MG1, MG2, MG6, and MG7 at or near the regulator binding interface (Fig 4A). Our data now facilitate a structural basis to observed functional differences between regulators due to these mutations. C3G-related deletion mutation 923-ADG located on MG7 of C3b (Martinez-Barricarte et al., 2010) influences the conformation of the αNT region. This region provides critical contact sites for FH, whereas the structural variability observed in the two C3b-DAF (CCP2–4) monomers found in the crystal structure of the complex indicates that this region is dispensable for DAF binding (Appendix Fig S8B). Consistently, this mutation impairs decay acceleration and binding by FH, but not by DAF (Martinez-Barricarte et al., 2010). aHUS mutations Q185E/H on MG2 (Noris et al., 2010) and R592Q on MG6 (Fremeaux-Bacchi et al., 2008; Fig 4A) affect binding and cofactor activity of FH, but not those of MCP nor CR1 (Schramm et al., 2015; Appendix Table S4). In C3b-FH, these C3b mutations disrupt the hydrogen bonds with the side chain of residue Glu116 on CCP2 (Wu et al., 2009). Such hydrogen-bonding network is not present in C3b-MCP or C3b-CR1, as shown by the very weak and flexible interactions of MCP CCP2, and the presence of a threonine residue (CCP16 residue 1,033) in CR1 replacing FH Glu116 (Fig EV2B). Mutations on regulators can be divided into two major groups in general. One group maps onto the C3b-regulator interfaces and likely affects interactions with C3b, whereas the second group is located on the opposite side of the regulator and cannot be explained by current structural data. The latter mutations likely affect the interactions of the regulator with factor I in cofactor activity or with the convertase fragment Bb in decay-acceleration activity. Despite extensive studies on pathogenic mutations of FH located on its CCP1-4 domains (summarized in Appendix Table S5), their effects on C3b binding were only studied for very few mutations. The V62I mutation showed increased binding affinity for C3b and enhanced cofactor activity (Tortajada et al., 2009). Pechtl et al. (2011) found that aHUS mutation R78G has the opposite effect, reducing FH binding to C3b, as well as cofactor and decay-accelerating activity. As we already reported for the C3b-FH (CCP1–4) crystal structure (Wu et al., 2009), this mutation maps in the middle of the C3b-FH contact interface at the CCP1 site (Fig 4B). In MCP, numerous pathogenic mutations leading to aHUS and C3G phenotypes affect domains CCP3 and CCP4 (Appendix Table S5). For five of these mutations, all associated with aHUS, binding studies report reduced C3b binding and decreased cofactor activity (Richards et al., 2003; Caprioli et al., 2006; Fremeaux-Bacchi et al., 2006). Consistent with our structural data, these five mutations are located at the C3b-MCP contact sites, as shown in Fig 4C. In CR1, the only documented mutation in the CCP15–17 region is Q1022H, which may be linked to adaptive evolutionary events to protect individuals from cerebral malaria in endemic regions of Asia (Birmingham et al., 2003; Thomas et al., 2005). This mutation is located on CCP16, in proximity to the CCP15–16 linker, and is conserved in all three CR1 CCP clusters that bind C3b (Appendix Fig S7A). Presence of this mutation increases CR1 binding affinity to C4b but not to C3b (Birmingham et al., 2003). In the C3b-CR1 (CCP15–17) structure, this residue is in close proximity (approximately 6 Å) to C3b, but does not make direct contacts with C3b (Appendix Fig S7D). Superposition with the recently published three-dimensional structure of C4b (Mortensen et al., 2015), however, does not suggest a straightforward explanation for the alteration in CR1-C4b binding affinity due to this mutation.

Conclusions

The four presented and one previously published crystal structures of C3b-regulator complexes show that the consecutive CCP domains harboring the complement-inhibitory activities of human MCP, DAF, FH, CR1, and of vaccinia virus-derived SPICE bind in the same orientation to the same binding platform on C3b. Nevertheless, marked deviations from the common binding mode are apparent with differences in participation of individual domains to the binding (consistent with biochemical data) and in virtually all details of the interacting residues at the C3b-regulator interface.

The largest known binding platform on C3b for regulator fragments stretches from the αNT-MG7 site, through sites on MG6, MG2, and CUB to MG1 and TED, as previously observed for C3b-FH (CCP1-4; Wu et al., 2009), with an additional binding site for FH (CCP19–20) at TED (Jokiranta et al., 2000; Kajander et al., 2011; Morgan et al., 2011). The CCP1–CCP4 domains of FH and SPICE bind with the N-terminal domains (CCP1) to the αNT-MG7 site, CCP2 to MG6-MG7, CCP3 to MG2 and CUB, and the C-terminal CCP4 to MG1 and TED, defining the generic CCPi-CCPiv domains and binding sites. MCP only binds through its CCP3–4 domains at the CCPii-CCPiv binding sites on C3b. The orientation of MCP and SPICE CCP4 differs by 10° from that of FH however without changing the overall global binding of the extended CCP configuration and, thus, adhering to the common binding mode. The domains CCP2–4 of DAF and CCP15–17 of CR1 bind at the sites CCP1–iii, in agreement with biochemical binding data (Krych et al., 1994; Harris et al., 2007; Gautam et al., 2015). The similarity in C3b binding of the decay-accelerating regulator DAF, cofactor-regulator MCP and CR1 (CCP15–17), and dual-activity regulators FH and SPICE suggests that the two distinct complement-inhibition mechanisms (decay-acceleration and cofactor activities) may have evolved from a common C3b-regulator binding origin. Furthermore, the strong decay accelerator DAF binds both C3b and Bb and both binding sites appear important for decay activity (Harris et al., 2007). However, what the roles of the two binding sites are in the molecular mechanism of decay acceleration remains unclear. Based on sequence and structural homologies, the regulators of the classical pathway of complement activation (that is thought to have evolved from the alternative pathway (Nonaka & Yoshizaki, 2004)) likely bind C4b using the same binding mode as in C3b-regulator binding. The extensive variations during evolution may have served to adapt precise regulator functionality. Concatenation of the three C3b/C4b binding sites in CR1 may provide synergistic effects (Krych-Goldberg et al., 2005). The fluid regulator FH may have adapted its CCP4 orientation from a putative canonical orientation as observed for MCP CCP4 and SPICE CCP4, to account for subsequent CCP domains involved in host interactions of the soluble FH. Similarly, the surface-bound regulators MCP and DAF may have evolved to enhance their cofactor- and decay-acceleration activity, respectively, at the expense of losing the potential of dual function. Thus, we conclude that the two functional activities, decay-acceleration and cofactor activities, are based on one common C3b-regulator binding mode.
Regulators with decay-acceleration and/or cofactor activity vary in CCP domains bound to the CCP-i-iv binding sites on C3b. Biochemical data (Krych et al., 1991, 1994; Gordon et al., 1995; Kuttner-Kondo et al., 2001, 2007; Harris et al., 2007; Wu et al., 2009; Gautam et al., 2015) show that domains CCPi-CCPiii suffice for decay-accelerating activity. The structural data support the observation that predominantly CCP iii (as for DAF CCP3–4) account for the C3b binding interactions (Harris et al., 2007). Domains CCPi-ii (CCP2–3) of DAF are important for binding the Bb protease fragment (Kuttner-Kondo et al., 2003; Harris et al., 2007); thus, CCP iii form a minimal functional fragment in decay acceleration (Kuttner-Kondo et al., 2001, 2007; Harris et al., 2007). Strong cofactor activity of MCP requires binding of only CCP3–4 to sites CCPii–iv (Liszewski et al., 2000; Gautam et al., 2015). Nevertheless, an associated CCPi domain is linked to cofactor activity (Krych et al., 1994, 1998; Yadav et al., 2008; Gautam et al., 2015); presumably, it is required for binding of Fl. Regulators with both decay acceleration and cofactor activity bind C3b through CCPi up to CCPiv. This combines C3b binding interaction at CCPi–ii for decay acceleration and those for cofactor activity at CCPii–iv for regulatory activity.

Structures of C3b with MCP, FH, or SPICE reveal many interactions with C3b at the CCPii binding site. At the CCPiv site, the CCPi and CCPii domains of FH, MCP, and SPICE differ in interactions with C3b. MCP and SPICE CCPi binds in the same orientation and interact with TED. FH CCPi differs by ~100° (around the longitudinal axis) and interacts with both MG1 and TED. The density and B-factor for CCPi of SPICE suggest weaker binding for SPICE at this site. In the structures of C3b–FH (CCP1–4) and C3b–MCP, the interactions of CCPi with TED appear correlated with a 6–9 Å tilt of CUB-TED. In addition, this lifting of CUB-TED facilitates interactions between MG2, CUB, and CTC. The CUB–CTC arrangement in the latter two C3b complexes is remarkably similar to the CUB–CTC contact in native C3. Moreover, interactions bridging MG2–CUB induced by regulators with cofactor activity may be critical for Fl binding and cofactor activity (Gautam et al., 2015). However, whether and how these conformational changes affect the cofactor activity requires structural data of the ternary complex of C3b-regulator with a bound Fl.

Our data are in good agreement with mutagenesis and functional characterizations of regulator-C3b binding (Krych et al., 1991, 1994, 1998; Krych-Goldberg et al., 1999, 2005; Liszewski et al., 2000; Kuttner-Kondo et al., 2001, 2007; Sfyroera et al., 2005; Harris et al., 2007; Yadav et al., 2008; Ahmad et al., 2010; Gautam et al., 2015). Moreover, the presented structural data explain the differential effect of C3b mutations on regulators. Depending on the location of the mutant in the extended binding site, some but not all regulators may be affected by that mutation (Martinek-Barricarte et al., 2010; Schramm et al., 2015). Disease-related mutations affecting C3b-regulator interactions are located at or near the CCP interaction sites. Most mutations are located near the CCPiv site, while CCPii and CCPiii have less disease-related mutations known to date. The CCPii and CCPiii sites, however, also provide a contact interface for FB in convertase formation, both in the “loading” and “activated” conformation of FB bound to C3b (Fig EV5). Perhaps, the C3b interactions with FB (preceding the interactions with regulators in the complement pathways) restrict the number of mutations affecting regulator binding at the CCPii and CCPiii sites. Mutations in FH, MCP, and CR1 affecting Fl binding (Krych et al., 1991; Krych-Goldberg et al., 1999; Liszewski et al., 2000; Rodriguez et al., 2014) or in DAF affecting Bb binding (Kuttner-Kondo et al., 2003; Harris et al., 2007) are distant from the observed C3b-regulator interfaces; thus, the structural data support the interpretation that these mutation affect either Fl or Bb interactions. We could, however, not confirm previously reported C3b interactions or regulator activity for β2GPI (Gropp et al., 2011; Fig EV1). Even though CCP1–4 of β2GPI resembles complement regulators in domain composition and overall shape, its amino-acid composition differs markedly from complement regulators of the RCA family. In particular, the putative C3b-binding site on CCP3 displays numerous hydrophobic residues, where regulators contain hydrophilic residues critical for C3b binding (Appendix Fig S9A). Furthermore, β2GPI Asn162 in CCP3 is glycosylated (Bouma et al., 1999), whereas residues at the equivalent site of the regulators are interacting with C3b (Appendix Fig S9B). Thus, the Asn162 linked glycan of β2GPI likely precludes binding to C3b.

In summary, we have shown that C3b-regulator binding can be classified as extensive variations on a common binding mode. We hypothesize that one evolutionary origin may underlie this C3b-regulator binding mode that facilitates both decay-acceleration and cofactor activities in complement inhibition for host protection or immune evasion. The data highlight how molecular mimicry may impact infection and immunity, that is, how the evolution of structural and functional similarities of SPICE from variola virus, and homologs from other orthopox viruses, with human RCA assists its evasion of complement response (Ahmad et al., 2007; Ojha et al., 2014). Finally, the presented structural data will be instrumental to characterize the functional consequences of genetic polymorphisms (Rodriguez et al., 2014) that have been associated with pathologies and may guide the further development of regulator-based therapeutics for the treatment of complement-mediated diseases (Ricklin & Lambris, 2013b).

Materials and Methods

Protein production and C3b affinity measurements

Complement component C3b was obtained by isolating C3 from fresh human plasma, followed by specific cleavage of C3 into C3b by recombinant human factor B and factor D as described before (Forneris et al., 2010). Iodoacetamide was used to block the nascent thioester to prevent dimerization of C3b. The regulator fragments were produced in E. coli, P. pastoris, and human embryonic kidney (HEK) 293 cells. CR1 CCP1–15 (residues 942–1,136) was produced in P. pastoris using established methods (Kirkitazde et al., 1999). MCP CCP1–4 (residues 35–285), and full-length SPICE CCP1–4 (residues 19–263) were expressed in inclusion bodies in E. coli, refolded and purified (Sfyroera et al., 2005; Fremeaux-Bacchi et al., 2008). N-terminal His-tagged DAF CCP2–4 (residues 97–285) and MCP CCP1–4 (residues 35–285), as glycosylated alternative to the E. coli product, were purified from medium after expression in HEK293.

Purified MCP (CCP1–4) from both E. coli and HEK293 showed comparable activity (Fig EV1), confirming previous observations that glycosylation at the three potential glycosylation sites does not affect cofactor activity (Liszewski et al., 1998). Full-length β2-glycoprotein I (β2GPI) and its fragment CCP1–4 were recombinantly
expressed in HEK293 cells. For crystallization purposes, we used N-acetylgalactosaminyltransferase I-negative HEK293 cells (HEK293-ES, U-Protein Express B.V. Utrecht, the Netherlands; Reeves et al., 2002), which lack the ability to synthesize complex Asn-linked glycans and hence produce short-chain, and more homogeneous, glycosylated proteins that can be crystallized more readily. All proteins were purified to homogeneity by either metal-affinity chromatography or ion-exchange chromatography and gel-filtration prior to crystallization according to previously established protocols (Wu et al., 2009; Forneris et al., 2010).

C3b binding affinity of the purified regulator proteins and protein fragments was determined by surface plasmon resonance (SPR), using a Biacore 3000 instrument (GE Healthcare) at 25°C. Comparative affinity was assessed on a CM5 sensor chip surface with covalently immobilized C3b. For this purpose, standard amine coupling chemistry and amplifying C3b deposition via multiple injection cycles of factors B and D (200 mM each) and C3 (1 μM) in HBST buffer (10 mM HEPES, 150 mM NaCl, 0.005% Tween-20, pH 7.4) containing 1 mM NiCl₂. Binding affinities to C3b-SPICE (CCP1–4), C3b-MCP (CCP1–4), and C3b-DAF (CCP2–4) and flash-frozen in liquid nitrogen for data collection. Diffraction data were collected at beamlines of the European Synchrotron Radiation Facility (ESRF) and the Swiss Light Source (SLS; details in Table 1) and processed using MOSFLM, XDS, and AIMLESS (Collaborative Computational Project, Number 4, 1994; Kabsch, 2010; Battye et al., 2011). The observed resolution limits of the diffraction data, selected by evaluating the correlations between half data sets (CC1/2; Karplus & Diederichs, 2012), were 2.8 Å for C3b, 2.4 Å for C3b-MCP (CCP1–4), 2.5 Å for C3b-SPICE (CCP1–4), 3.3 Å for C3b-CR1 (CCP15–17), and 4.2 Å for C3b-DAF (CCP2–4). All structures were solved by molecular replacement by Phaser (McCoy et al., 2007) using the coordinates from the crystal structures C3c minus its CTC domain (PDB ID 2A74; Janssen et al., 2005), followed by sequential positioning of the TED, CTC, and CUB domains obtained from the three-dimensional structure of C3b (PDB ID 2I07; Janssen et al., 2006). Search models for regulators were generated from the crystal structures of MCP (CCP1–4; PDB ID 3OBE; Persson et al., 2010), DAF (CCP1–4; PDB ID 1OK3), and the NMR structures of CR1 (CCP15–17; PDB IDs 1GKN and 1GKG, resp.; Smith et al., 2002). Alternating cycles of automated refinement using phenix.refine (Adams et al., 2010) and manual model building using COOT (Emsley et al., 2010) were applied to generate the structural models. Validation of the final structural models was carried out using MOLPROBITY (Chen et al., 2010) and PDB-CARE (Lutteke & von der Lieth, 2004). Complete data collection details and final model statistics are available in Table 1. In all structures, all domains of C3b could be modeled (Appendix Fig S2). For C3b-MCP (CCP1–4), only domains CCP3–4 of MCP could be modeled due to lack of density for CCP1 and lack of interpretable density for CCP2 (Fig 1D). All four CCP domains of SPICE were modeled; however, CCP4 showed notably weak density (Fig 1D). Two C3b-CR1 (CCP15–17) complexes were present in the asymmetric unit. The two C3b-CR1 (CCP15–17) complexes displayed minor differences in the orientation of domain CCP15, while neither of the two copies of CCP15 was directly involved in crystal contacts. Non-crystallographic symmetry was applied to each CCP domain of CR1 separately (excluding the linker peptides), thus allowing adjustment of the binding orientations and positions of the CCP domains with respect to C3b in each copy. Also for C3b-DAF (CCP2–4), two copies of the complex were present in the asymmetric unit with significant differences in orientation of the CCP2 and CCP3 domains. Extensive crystal contacts between CCP2 of one copy with CCP4 of another resulted in 17º rotation of the regulator (compared to its bound C3b)
and a displacement of over 20 Å (as measured at the N-terminal edge of CCP2; Appendix Fig S2A). For modeling and refinement against the 4.2-Å resolution C3b-DAF (CCP2–4) data set, external structural restraints were applied based on the 2.8 Å-resolution C3b structure (this work) and the high-resolution structures of free DAF (Lukacik et al, 2004).

Coordinates and structure factors for C3b and the complexes of C3b-MCP (CCP1–4), C3b-CR1 (CCP15–17), C3b-DAF (CCP2–4), and C3b-SPICE (CCP1–4) have been deposited at the Protein Data Bank (PDB) with accession numbers 5FO7, 5FO8, 5FO9, 5FOA, and 5FOB, respectively. For consistency, UniProt residue numberings were used throughout, including in the deposited structure coordinates. We refer to the sequences of UniProt database entries P01024 (C3/C3b), P15529 (MCP), Q89859 (SPICE), P08603 (FH), P17927 (CR1), P08174 (DAF), and P02749 (β2GPI). The reference sequence for the vaccinia virus complement control protein (VCP) used for comparison with SPICE was UniProt entry P68638.

Expanded View for this article is available online.

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Author contributions

JW, FF, and XX purified C3b. XX and JCM purified DAF(2–4). RH, PB, and MKL produced and purified CR1. GS produced and purified MCP and SPICE. FF produced and purified β2GPI. JW crystallized and solved the structures of C3b-CR1, C3b-MCP, and C3b-SPICE. XX crystallized and solved the structure of C3b-DAF. FF refined and analyzed all structural data. DR, ZL, and AT performed surface plasmon resonance experiments and analysis. EV and JPA assisted in interpretation of genetic data. FF prepared figures. JD.L and PG supervised the project. FF, DR, XX, and PG performed data analysis and interpretation and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References


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