

Supporting Information

Torreira et al. 10.1073/pnas.0810860106

SI Materials and Methods

Preparation of the Complement C3b Fragment. C3 was prepared from pooled normal human plasma. Plasma was subjected to a 4 to 12% cut with PEG4000, the pellet was solubilized in 10-mM Na/K phosphate pH7.6, 5-mM NaCl, and applied to a DEAE-Sepharose anion exchange column attached to an Akta Prime system (GE Healthcare). Protein was fractionated using a gradient to 0.5M NaCl, C3-containing fractions were identified by SDS/PAGE, dialyzed against 20-mM Na/K phosphate pH6, 60-mM NaCl and applied to a Source S cation exchange column (GE Healthcare). Protein was eluted with a gradient to 0.5M NaCl. C3-containing fractions were pooled and concentrated. C3b was generated by limited digestion with trypsin as previously described (1) and repurified by gel filtration on a SuperoseTM 6 10/300 column (GE Healthcare). C3b was obtained without any detectable contaminants or aggregates [supporting information (SI) Fig. S1A].

Production and Purification of Recombinant fB. The D279G amino acid substitution was introduced in the fB cDNA by using QuikChange site-directed mutagenesis kit (Stratagene) and appropriate primers. Both cDNAs encoding full length fB-WT and fB-D279G were introduced in the eukaryote expression vector pCI-Neo (Promega) and the resulting clones entirely sequenced to confirm a correct DNA sequence. CHO cells were maintained in Ham-F12 medium (GIBCO-BRL) supplemented with 10% FCS, L-glutamine (2 mM final concentration), penicillin and streptomycin (10 U/ml and 100 μ g/ml). The neomycin analogue, G418 sulfate (Geneticin; GIBCO-BRL), at 500 μ g/ml, was used for selection of transfected cells. Factor B-cDNA transfections were performed using Lipofectine (Invitrogen), as recommended by the manufacturer. Cells were plated in p60 plates 1 day before transfection at 5×10^5 cells per well. Transfections were carried out with 10 μ g of the pCI-Neo constructs and 24 μ l of Lipofectine in a total volume of 1 ml of medium per well. Stable transfected CHO cells were cloned by limiting dilution and clones producing the highest levels of fB (fB-WT 20 μ g/ml; fB-D279G 2 μ g/ml) were expanded for production. Factor B concentration was quantified by ELISA as previously described (2). The recombinant fBs were purified from tissue culture supernatant by affinity chromatography using the JC1 monoclonal antibody (anti-human Bb, a gift from Prof. B.P. Morgan, Cardiff, U.K.) coupled to a HiTrap NHS-activated column according to the manufacturer's instructions (GE Healthcare). Bound fB was eluted with 0.1M Glycine/HCl pH 2.5, immediately neutralized with 2M Tris pH 8.6, and re-purified by gel filtration on a SuperoseTM 6 10/300 column (GE Healthcare). Both fB-WT and fB-D279G were obtained

without any detectable contaminants or aggregates (see Fig. S1A).

Generation and Purification of C3bB Complexes. The Ni²⁺ cation was used instead of Mg²⁺ to promote a stable C3bB(Ni²⁺) complex that is otherwise undistinguishable from the physiological C3bB(Mg²⁺) proconvertase. Purified C3b and fB were mixed in a (1:2) molar excess of fB in 20-mM Tris, pH = 7.6 buffer containing 50-mM NaCl and either 20-mM EDTA or in 5-mM NiCl₂, and allowed to interact for 15 at room temperature. Subsequently, both preparations were size-fractionated by gel-filtration chromatography on a calibrated SuperoseTM 6 10/300 column (GE Healthcare) (Fig. S1B). In the absence of divalent cations (EDTA-sample), fB does not interact with C3b and both proteins elute separately from the Superose column as illustrated by the SDS/PAGE analysis of the corresponding fractions (Fig. S1C). In the presence of Ni²⁺ and a 1:2 molar excess of fB, C3b readily interacts with fB to form C3bB(Ni²⁺) complexes. SDS/PAGE characterization of the elution fractions from the Superose column illustrates the presence of the C3bB(Ni²⁺) complex, which elutes from the column in a single peak that precedes and partially overlaps the peak containing the single C3b molecules (see Fig. S1 B and C). To avoid a potential contamination of single C3b molecules in the fractions corresponding to the C3bB(Ni²⁺) complex, samples for the EM analysis were collected exclusively from the initial elution fractions of the peak containing the C3bB(Ni²⁺) complex.

Generation and Purification of C3bBb Complexes. We have used the fB-D279G mutant instead of the fB-WT protein to promote a relatively stable active C3bBb convertase. For the generation of the C3bB complex, purified C3b and fB-D279G were mixed in a (1:2) molar excess of fB in 20-mM Tris, pH = 7.6 buffer containing 50-mM NaCl and 5-mM MgCl₂ and were incubated for 15 min at room temperature. Subsequently, we added fD (Comptech, Inc.) at a 1:500 molar ratio to fB, incubated for 1 min at 37 °C and size-fractionated the mix by gel-filtration chromatography on a calibrated SuperoseTM 6 10/300 column (GE Healthcare) (Fig. S4A). The presence of C3bBb complexes was demonstrated by SDS/PAGE analysis, illustrating that, as expected, most of the Bb fragment eluted in the fractions containing the C3b molecule (Fig. S4B). The C3bBb complex eluted from the column in a single peak that significantly overlapped the peak containing the single C3b molecules (see Fig. S4A and B). To minimize the contamination of single C3b molecules in the fractions corresponding to the C3bBb complex, samples for the EM analysis were collected exclusively from the initial elution fractions of the peak containing the C3bBb complex.

1. Sánchez-Corral P, et al. (1989) Separation of active and inactive forms of the third component of human complement, C3, by fast protein liquid chromatography (FPLC). *J Immunol Methods* 122 (1):105–113.

2. Goicoechea De Jorge E, et al. (2007) Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome. *Proc Natl Acad Sci USA* 104(1):240–245.

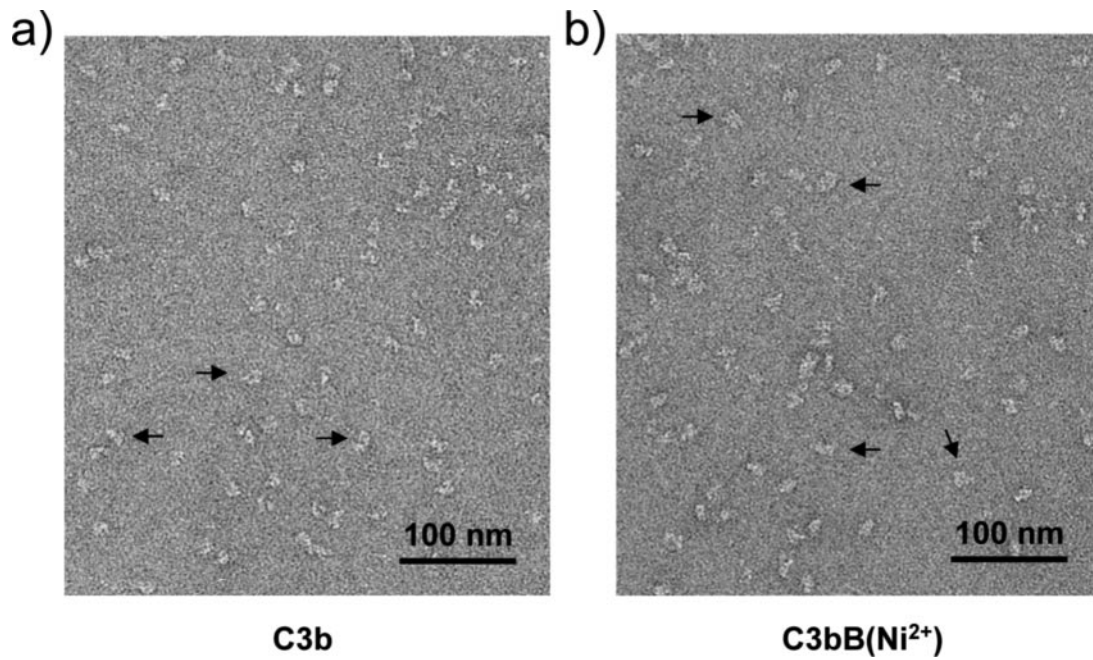


Fig. S2. Electron micrographs of C3b and C3bB(Ni²⁺). Selected field of an electron micrograph of C3b (a) and C3bB(Ni²⁺) complex (b). (Scale bar, 100 nm.) Some representative images of individual molecules are indicated using arrows.

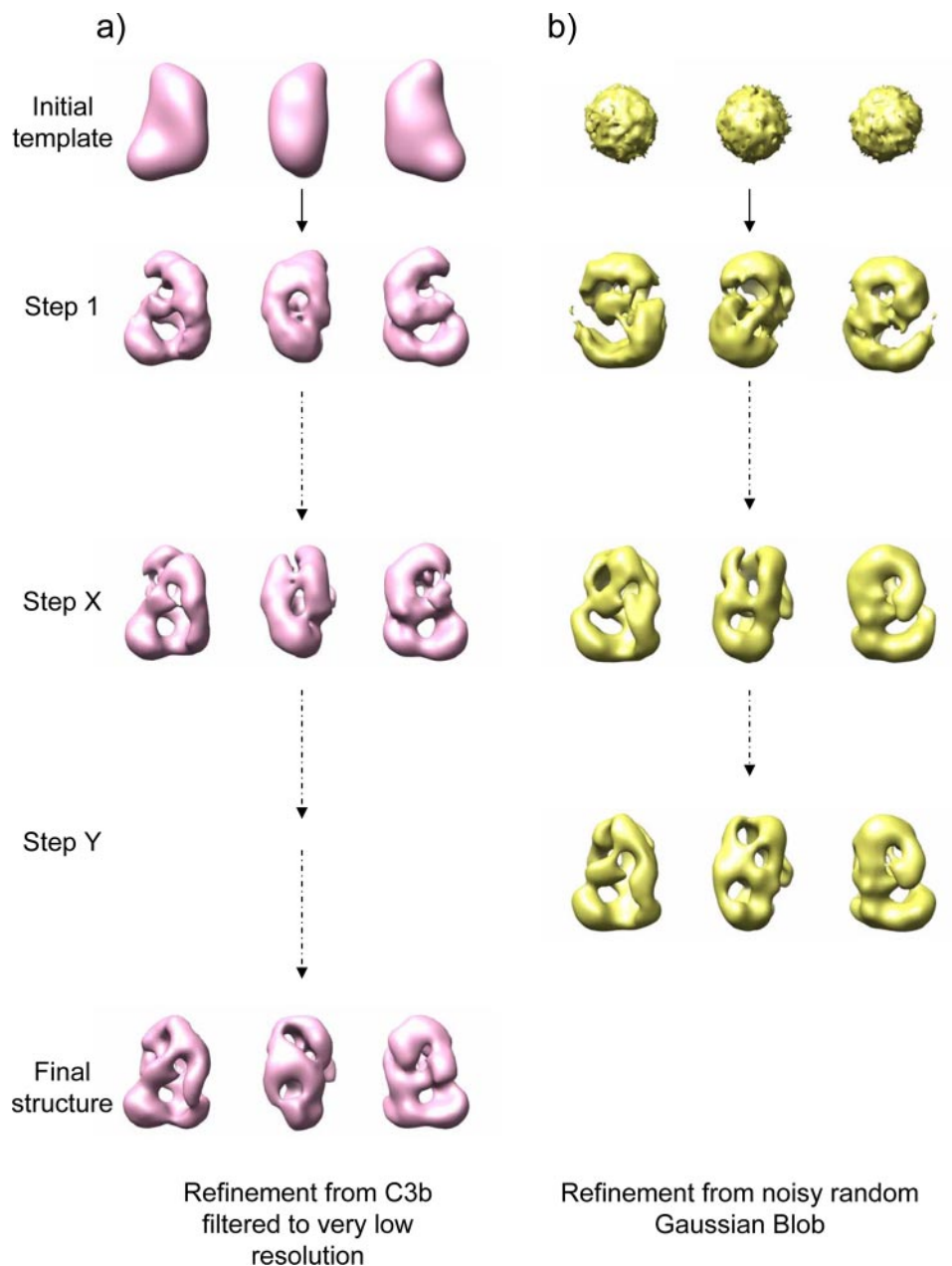


Fig. S3. Image processing of C3bB(Ni²⁺) using angular refinement methods. Angular refinement was performed in 2 independent experiments from 2 different volumes, either a low-pass filtered version of the atomic structure of C3b (a) or a random noisy Gaussian blob (b). Three views of the initial template in each case and of several output volumes along the progression of the refinement are shown. It can be noted that two very distantly related initial references converge into a similar 3D structure, indicating the absence of bias of the initial template during the refinement and the accuracy of the final reconstruction.

