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Arranged Sevenfold: Structural Insights into the C-Terminal Oligomerization Domain of Human C4b-Binding Protein

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Abbreviations used: C2a, complement component 2a; C3, complement component 3; C3b, complement component 3b; C4b, complement component 4b; C4BP, complement component 4b binding protein; CCP, complement control domain protein

Highlights

Core crystal structure of a major modulator of complement system

Human C4BP core complex reveals heptameric ring structure

7 disulfide bonds and 3 layers of electrostatic interactions provide high stability

Molecular modeling provides insights into the structure of heterooligomeric isoforms

Running title

Crystal structure of human C4BP core complex

Abstract

The complement system as major part of innate immunity is the first line of defense against invading microorganisms. Orchestrated by more than 60 proteins, its major task is to discriminate between host cells and pathogens and to initiate immune response. Additional recognition of necrotic or apoptotic cells demand a fine-tune regulation of this powerful system. C4b-binding protein (C4BP) is the major inhibitor of the classical complement and lectin pathway. The crystal structure of the human C4BP oligomerization domain in its 7 α isoform and molecular simulations provide first structural insights of C4BP oligomerization. The heptameric core structure is stabilized by intermolecular disulfide bonds. In addition, thermal shift assays indicate that layers of electrostatic interactions mainly contribute to the extraordinary thermodynamic stability of the complex. These findings make C4BP a promising scaffold for multivalent ligand display with applications in immunology and biological chemistry.

Introduction

The complement system as part of the innate immune response is one of the oldest sections of the multicellular organisms defense repertoire. It is the first line of protection against invading microorganisms that paves the way for the adaptive immunity to react via initiation of antibody production or T cell response. In addition, complement activity on apoptotic and necrotic cells was reported.¹ The highly conserved complement system is found in all kinds of invertebrates and vertebrates² and is orchestrated by approximately 60 membrane bound and soluble plasma proteins.³ Once activated, a membrane attack complex is formed by components of the complement via a proteolytic cascade that eventually mediates lysis of pathogens by osmotic shock. The relevance of the complement system is underlined by the fact that congenital defects in activation and regulation increase the susceptibility to inflammatory diseases.⁴

Human C4BP (hC4BP) is a plasma glycoprotein complex of 570 kDa, which is mainly produced in the liver. C4BP is the major inhibitor of complement activation (Fig. 1). For human hC4BP four different isoforms are known. The major isoform consists of 7 α and one β -chain, while less abundant isoforms exist as 6 α 1 β , 7 α or 6 α , respectively. Each α -chain comprises eight complement control domain

proteins (CCPs) and a C-terminal oligomerization domain with a molecular mass of 75 kDa. In contrast, the smaller β -chain (40 kDa) of hC4BP has only three CCPs and a C-terminal oligomerization domain. The assembly results in a characteristic spider or octopus like structure of the C4BP protein complex.⁵ Both the α -chains and the β -chain of the core domain consist of approximately 60 amino acids and are linked together in most organisms by intermolecular disulfide bonds. Interestingly, the β -chain shares no sequence homology with the α -chain and it remains largely unclear how the assembly of this heterooligomeric complex occurs (Fig. 1c).⁶

C4BP modulates the lectin pathway that proceeds through lectin-mediated binding of complement factors to sugars residing on the target surface as well as the classical complement pathway by binding to C4b via a cluster of positively charged residues that are located in the first three CCP domains of the α -chains.⁷ C4b protein interacts with other components of the complement system as C2a and C3b. Binding of C4BP results in increased accessibility for proteolytic cleavage of C4b by factor I and inhibition of assembly of C3/C5 convertase (C4b, C2a and C3bC4b, C2a).⁸ Each C4BP α -chain alone is fully capable of binding C4b molecules, but due to steric hindrances up to four C4b can be bound simultaneously.⁹ The β -chain of hC4BP does not bind to C4b, but has high affinity to protein S, which is part of the coagulation system. It also is a cofactor for activated protein C that is important for degradation of coagulation factors Va and VIIIa. C4BP bound protein S is not able to act as cofactor in coagulation. Interestingly about 70% of protein S in human serum circulates in complex with C4BP, directly linking the complement to the coagulation system.⁵ While the CCPs of C4BP interact with a variety of proteins, for the C-terminal core domain and parts of CCP8, only few specific interaction partners have been identified so far. These are serum amyloid P¹⁰, A β ₁₋₄₂¹¹, islet amyloid polypeptide¹² and C-reactive protein¹³.

Moreover, C4BP is also an acute phase protein and its plasma levels are positively correlated to the progress of infection and inflammation. Cytokines (e.g. IL-6, IL-1 β and INF- γ) seem to up-regulate secretion of C4BP.¹⁴ Serum levels of C4BP isoforms may correlate with the progress of disease or inflammation.¹⁵ For instance, in patients with critical limb ischemia necrotic and apoptotic cells were found to bind to C4BP while the level of the 7 α isoform is up-regulated.¹⁶ Interestingly, numerous

pathogens evade the immune response by decorating themselves with C4BP via interaction of different surface proteins.¹⁷ Research is still in progress to identify other conserved bacterial membrane proteins that also hijack C4BP.^{18; 19} In addition to the intriguing physiological relevance of C4BP the oligomerization domain of the 7 α isoform has also been found to be beneficial for vaccination to act as an adjuvant leading towards prolonged plasma half-life or increased immune response.^{20; 21; 22; 23} So far the only published structural information of C4BP is an NMR structure of outermost CCPs 1 and 2 of the α -chain (PDB ID: 2A55).²⁴ Very recently, the X-ray structure of the 7 α core isoform of *Gallus gallus* (gC4BP) was deposited at the Protein Data Bank (PDB ID: 2YF2) by Lea, S.M. *et al.* However, sequence identity of the α -chain from *Gallus gallus* and *Homo sapiens* is only ~26%. For the human C4BP oligomerization domain only computational prediction of the secondary structure was available.²⁵ Here we present the first crystal structure of the heptameric C-terminal oligomerization domain of human C4b-binding protein in its 7 α isoform and provide data on the contribution of particular structural features to monomer and oligomer stability. Based on the crystal structure we utilized molecular simulations to generate other known isoforms, showing that the β -chain of hC4BP is likely to partially disrupt the structural stability of the core complex.

Results

Structure determination of the 7 α isoform of human C4BP

Since the C-terminal oligomerization domain of human C4BP (residues 540-597), hereafter referred to as hC4BP, contains 14 cysteines that form 7 intermolecular disulfide bridges, protein production was carried out in *E. coli* strain Origami (DE3) to provide an oxidative milieu.^{26; 27} In order to increase the yield of correctly folded protein, hC4BP was furthermore expressed as thioredoxin fusion protein.²⁸ Production of SeMet labeled hC4BP using *E. coli* B834 (DE3) yielded up to 10 mg per liter bacterial culture. This indicates that ambient oxygen during cell disruption is sufficient for formation of intermolecular disulfide bonds. The heptameric thioredoxin-hC4BP complex was purified as described in the experimental procedures. Due to the lack of a suitable homologue model of the hC4BP core domain, experimental phases had to be obtained. First attempts to solve the structure either by single

or multiple wavelength anomalous dispersion (SAD or MAD) using a SeMet hC4BP derivative failed, probably caused by weak diffracting crystals and/or abundant Se atoms (28) in the asymmetric unit. Replacement of the first two methionine residues with alanine residues (M2A M6A) in the artificial linker region resulted in crystals with the same tetragonal symmetry but with better diffraction and anomalous signal. The anomalous data at the selenium edge was used up to 3.4 Å for SAD. Fourteen Se sites (2 in each of the seven monomers) per asymmetric were found and used for initial model building with AutoBuild. This model was used as scaffold for the final manual build model. In most chains electron density was observed for residue range 543 to 595. In the longest chain (mono A) residues 541 to 597 and for the shortest chain (mono C) residues 543 to 592 were modeled. The final model was refined to $R_{\text{work}}/R_{\text{free}}$ of 21.2/24.7 with 2.8 Å resolution. Data and refinement statistics are summarized in Table S1.

Structure of the heptameric core complex of 7α hC4BP

The structure of the heptameric C-terminal core complex of hC4BP (residues 540-597) has a donut like shape in top view and a champagne cork like shape in side view (Fig. 2). The inner and outer diameter of the ring is 13 and 53 Å, respectively. This correlates with the EM data of Dahlbäck and coworkers from the 80s, who measured 13 Å and 60 Å for the inner and the outer diameter of the C4BP core domain, respectively.²⁹ Each monomer of the heptameric core complex of hC4BP comprises a short helix H1 (residues 545-557) and a second ring forming helix H2 (residues 562-597). A five residue long flexible linker with the sequence CLPNP links the two helices and contains two prolines in trans conformation leading to a special arrangement of the two helices in the ring described by three angles (Fig. 2c, Fig. S1). The distortion angles α and β provide the relative orientation of the two helices to each other, while the tilting angle γ shows the tilt of the long C-terminal helix in reference to the symmetry axis of the hC4BP core complex. For the 7α isoform of hC4BP the distortion angles α and β have an average of $20.3 \pm 0.7^\circ$ and $86.1 \pm 1.6^\circ$, respectively (Table S2). The average tilting angle of helix H2 in respect to the symmetry axis is $37.1 \pm 1.1^\circ$. Two cysteine residues, Cys546 and Cys558, are located at the beginning and the end of helix H1. Each monomer is linked via

disulfide bridges to the adjacent two monomers on either side of H1, making the bridged helices look like a continuous ring wrapped around the assembled ring of H2 helices, similar to a hoop of a wine barrel in top view (Fig. 2). The electrostatic surface potential in this orientation of the inner ring residues (Glu563, Glu570) is negative, while the “hoop” forming residues are neutral (Fig. 2b).

In addition to the above-mentioned disulfide connectivity, the fold sustaining forces can be depicted as three internal layers of non-covalent interactions along the vertical axis of the oligomer (Fig. 3). These are from top to bottom two layers of ionic bonds between Lys566 and Glu570 for the first and Lys573 and Glu577 for the second layer. The final internal layer in hC4BP is formed by hydrogen bonds between Gln580 and Gln584. Additionally, hydrogen bonds between Tyr572 and Glu582 (Fig. S2), which act as molecular clamps, form an external layer that further stabilizes the ring-forming helix H2 and the overall ring-like structure. On average 54 non-bonded contacts per monomer (e.g. hydrophobic interactions) are formed (Fig. S3, Table S3). Each monomer has a total surface area of 5200 Å² with an average interface area of 980 Å² to a neighboring chain. In the assembled ring 42 % (2170 Å²) of the total monomer surface is buried (Table S3). The sum of these interactions and bonds is essential for ring formation and the characteristic shape of hC4BP.

In the assembled ring three regions with high mobility were identified. Those are the N-terminal linker (aa 540-445), the linker between helix H1 and H2 (aa 558-562) and the C-terminus of H2 (aa 587-597). Here the B-values are almost twice as high as the average (Fig. 3e and S4). The flexibility of the N-terminus is consistent with its biological function as linker to the CCP domains. Not surprisingly, the main rigid part is found in a region of helix H2 where all above-mentioned stabilizing layers are located (aa 565-579). On the other hand, the C-terminal part of H2 has been shown, as described below, to be not essential for ring formation and hence the helices in this part are less well ordered.

Truncation of C-terminal helix correlates with structural features of the core complex

Previous studies have shown that oligomerization and ring formation of hC4BP subunits is initiated by C-terminal amphiphilic helices. ⁶ By successive C-terminal truncation of helix H2, it was possible to identify a critical length of 42-45 residues (aa 540-582, hC4BP^{ΔC15}) that is required for ring formation

(Fig. 4). In shorter C-terminal truncation variants (e.g. hC4BP^{ΔC20}, aa 540-577) ring formation was totally abolished (Fig. 4d and 4e). This indicates that the inner hydrogen bond forming layer (Gln580 and Gln584) contributes to C4BPs ring formation but is not of major relevance since the external molecular clamp (Tyr572 and Glu582) is still intact in the hC4BP^{ΔC15} variant.

Heptameric hC4BP⁵⁴⁰⁻⁵⁹⁷ shows strong synergistic effects upon melting

Circular dichroism (CD) measurements of recombinant hC4BP in a temperature range from 35°C to 95°C (Fig. S5) showed no significant change in secondary structure over the whole temperature gradient. This correlates with the data of Kask and co-workers,³⁰ who showed that human plasma purified full-length C4BP, as well as a C-terminal core domain truncation variant, have high thermal stability. To further investigate the nature of hC4BP core stability thermal shift assays were performed at reducing, denaturing as well as electrostatic destabilizing conditions in the presence of 1,4-dithio-D-threitol (DTT), guanidine hydrochloride, urea and/or NaCl (Fig. 5a). Addition of DTT (up to 50 mM) or NaCl (up to 3.9 M) did not disrupt the hC4BP core complex when heated up to 99 °C. In order to initiate melting of the complex, addition of 3.9 M NaCl with 1 mM DTT or 20 mM DTT with 1.5 M NaCl, was required resulting in a T_m of ~96°C or ~78°C, respectively. The lowest melting temperature (T_m = 75°C) was observed with 3.9 M NaCl and 50 mM DTT. This suggests strong synergistic effects of charged interaction and disulfide bridge formation in assembly of heptameric ring structure. The C-terminally truncated version hC4BP^{ΔC15} (aa 540-582) that lacks the internal H-bond layer (Gln480/Gln484), but not the external molecular clamp (Tyr572/Glu582), shows significant decrease in stability, reflected in a 10-15°C lower T_m -value compared to hC4BP under similar conditions (Fig. 5b). However, an increase of salt concentration showed a decrease of melting temperature for both variants, which indicates a weakening of the two layers of salt bridges between the C-terminal helices H2. Due to high background fluorescence of SYPRO Orange at high concentrations of guanidine hydrochloride (>3 M) and urea (>4 M) no T_m values at higher concentrations were obtained. However, incubation of hC4BP⁵⁴⁰⁻⁵⁹⁷ with 3 M guanidine hydrochloride or 4 M urea in combination with 2 M

NaCl and 10 mM DTT led to a further 7°C decrease of the T_m , confirming the extraordinary stability of the heptameric complex (Fig. 5c).

To further investigate the contribution of the cysteine residues to the C4BP core assembly and overall complex stability, the nature or position of the first cysteine (Cys546) has been altered by single site mutations. Neither loss of function (C546A) nor shortening of the relative distance of Cys546 to the second cysteine by a double mutation (C546A/V549C) impeded ring formation of C4BP (Fig. S6). Interestingly, even insertion of one additional helix-turn (aa VLT) after Cys546 still led to ring formation of hC4BP as verified on Blue-Native-PAGE (Fig. S6). These data suggest that disulfide formation is not a prerequisite for oligomer formation, but annealing of C-terminal helices might be the initial step of assembly. Formation of disulfides may occur later during assembly and is not crucial for the preservation of the heptameric structure. Nevertheless, once formed, disulfide bridges interlock neighboring monomers and covalently fix the oligomeric state of the hC4BP isoforms.

MD analysis of ring formation

In order to study the role of hydrogen and ionic bonds in the complex's stability, a 100 ns molecular dynamics simulation was performed using the 7 α crystal as a starting point (Fig. S12). Assessment of ionic interactions revealed that at every moment in simulation an average of three bonds were established in the Lys566 and Glu570 layer, as well as in the Lys573 and Glu577 one. On average Lys566-Glu570 and Lys573-Glu577 bonds were established 42.9 ± 3.7 % and 45.0 ± 7.2 % within the simulations time frame of 100 ns, respectively. These frequent strong interactions represent a significant contribution to the assembly's stability. A similar analysis was performed to assess the hydrogen bonds between Gln580 and Gln584, as well as between Tyr572 and Glu582. Remarkably, in the Gln580 and Gln584 layer hydrogen bonds were established only the 3.9 ± 1.8 % within 100 ns. While having a possible damping effect on the local oscillation of the H2 helices, these transient interactions cannot be considered as significant for the stabilization of the complex. This observation can explain why in C-terminal truncation experiments the Gln580-Gln584 layer has been found to have no major role in ring formation.⁶ Bonding between Tyr572 and Glu582 was slightly more frequent, taking place an average of 9.5 ± 4.8 % within 100 ns.

Human and chicken C4BP core complex is different

Although the overall fold of hC4BP with the recently deposited structure ggC4BP (**PDB ID: 2YF2**) is similar, their overall superposition with an RMSD of 25.4Å is relatively poor (Fig. S7). Alignment of separate chains resulted in an average RMSD of 1.5 ± 1.4 Å for 178 ± 29.3 aligned atoms. This is mainly caused by the one amino acid elongated, unrelated sequence of ggC4BP, which further separates the cysteines in H1 of ggC4BP (Fig. 1c). This causes a slightly larger ring of H1 helices and hence a changed geometry of it in respect to helix H2 in that the distortion angle α is 7° larger, while the other two angles stay constant within errors (Table S2 and S4). Furthermore, the inner diameters of the last stabilizing layer of ggC4BP (residues K440 and E444), which is formed by salt bridges and not hydrogen bonds, is 7 Å smaller compared to hC4BP (Fig. S8). Although the C-term of every second chain in the ggC4BP is truncated, the flexibility seen in the B-factor plot is comparable to regions of hC4BP (Fig. S7b).

***In silico*-modeling of naturally occurring C4BP isoforms**

In silico-modeling was performed to generate the core complexes of major known isoforms 6 α , 6 α 1 β , and 7 α 1 β . While in the 6 α 1 β ring only one chain had to be mutated to represent the β -chain, the 6 α as well as 7 α 1 β had to be generated *de novo* using one chain of the 7 α crystal structure and applying a 6-fold or 8-fold symmetry, respectively. For the 7 α 1 β isoform one monomer of the resulting 8 α ring had to be mutated to represent the β -chain. To minimize steric clashes all obtained *in silico* models have been energy minimized. Initial models, where residues 220-252 of the β -chain were aligned to H2 (aa 563-595) of the α -chain, showed that the internal stabilizing layers found in the crystal structure are not well maintained (Fig. 6a, c). Those important interactions are likely to be better maintained by shifting helix H2 of the β -chain upwards by seven amino acids upwards in respect to the α -chains. In this position E234 and K237/E241 of the β -chain are able to interact with the salt bridge forming layers K566/E570 and K573/E577 in the α -chain, respectively (Fig. 6b, d). However, in this position the β -chain projects ~10 nm out of the assembled ring (aa 217-221 modeled as coil, aa 222-225 as

helix) to allow H1 to form disulfide bridges with neighbouring α -chains. In both likely positions of the β -chain the hydrogen bond forming layer is not maintained, although Lys244 and Lys248 in the second models are more favourable than K237 and E241 in the first model (Fig. 6). The whole ensemble of all modelled isoforms and the 7 α crystal structure is displayed in Fig. 7. Interestingly, in contrast to the crystal structure the 6 α and the 7 α 1 β models, which were packed by symmetry *de novo* with a final energy minimization, seem to have an extended height when displayed in side view. The resulting distortion angle α is for both isoforms $\sim 3\text{--}7^\circ$ larger, while the distortion angle β is almost constant ($\pm 1^\circ$) for the 7 α 1 β isoform, but $\sim 4^\circ$ large in the case of the 6 α isoform (Table S2). A major difference is found in the tilting angle γ , which is $\sim 10^\circ$ and $\sim 14^\circ$ smaller for the 6 α and 7 α 1 β , respectively.

Discussion

The structure of the human C4bp α -chain heptameric core domain displays an assembly of two alpha helices in each subunit with interlocking disulfide bonds endowing it with extraordinary stability and rigidity. Interestingly, the sequence homology among different mammalian C4BP core domains is low and even the cysteine residues are not conserved as these are absent in murine C4BP (Fig. 1c). Recently, the structure of the chicken C4BP (*Gallus gallus*; ggC4BP) core domain was solved that displays a very similar fold despite significant differences in the amino acid sequences. Common to both sequences is the cysteine-bridged assembly of 7 alpha helices and the first two layers of oligomer stabilizing ionic and hydrogen bonding interaction. In contrast, the third internal layer in ggC4BP, which was demonstrated not to be important for ring formation in hC4BP, is stabilized by electrostatic interactions rather than hydrogen bonds (Fig. S8d). Furthermore, the external layer of hydrogen bonds (Tyr572 and Glu582) found in hC4BP are also absent.

hC4BP mainly is made up of alpha helices. The only sections of the hC4BP subunit, that are not part of the two helices, are the N-terminal linker region to the CCPs and the short five amino acid loop connecting them. The sequence CLPNP is located right next to the second cysteine of the first helix and the very first turn of the C-terminal helix. This short loop contains two prolines in trans

conformation and arranges the two helices almost in a 90° angles to each other. In addition, the N- and C-terminal ends of each subunit, as well as this loop represent a region of increased atomic mobility as seen in the B-factor plot (Fig. 3e and S4). It links the two most stable sections of the heptamer, in particular, the anterior helices containing the disulfides and the posterior ones providing four layers of intermolecular hydrogen bonds and salt bridges.

It was postulated that polymerization of C4BP subunits is initiated by C-terminal amphiphilic helices.⁶ By successive truncation of this helix, it was possible to identify a minimal length of hC4BP core complex (42-46 aa, residues 540-582/ 586) that is required for ring formation. This complements the data of Kask and coworkers, who showed that ring formation of full-length hC4BP (as a cysteine double mutant C546A/C558A) that lacks 12 aa at the C-terminus still occurred, while it was abolished upon removal of 27 aa.⁶

Intuitively, one would assume that intermolecular disulfide bond formation mainly contributes to oligomer stability. Our data show that even high concentrations of the relatively strong reducing agent DTT are insufficient to disassemble the oligomeric complex. The finding that complex stability depends on salt concentration corroborates the importance for heptamer stability of the salt bridges and hydrogen bonds formed by the C-terminal helix. A combination of high concentrations of reducing agent and high salt is required to lower the melting temperature of the complex, which even under these conditions still remains above the T_m for proteins of mesophilic organisms.³¹ In conclusion, it is the combination of disulfide bonds, hydrogen bonding, formation of salt bridges and the large number of hydrophobic interactions that contributes in a synergistic manner to the extraordinary stability of the complex.

The question why a protein of human complement system shows such an extraordinary stability that is based on the formation of 7 intermolecular hydrogen bonds, 14 intermolecular salt bridges and 7 disulfides needs to be addressed. Considering the spider-like overall structure of C4BP isoforms, occurrence of enormous shear forces that may act on the core domain is very likely. Moreover, being itself a huge polymer circulating in the blood stream it is interacting with a variety of other proteins, soluble or membrane bound. For example, the two isoforms of C4BP containing the β -chain appear to

be permanently complexed with protein S while each of the CCP domains interact with several C4b molecules.⁵ As a consequence, a strong scaffold stability may be required to hold the macromolecular assembly together.

At present, little is known about the *in vivo* assembly of the C4BP complex. The C4BP α -chain obviously spontaneously forms a heptamer, while the β -chain forms a mixture of monomers and disulfide-linked dimers in solution (Fig. S9). This is supported by the observation that some monomerized 7α -chains after treatment with heat and DTT reassembled spontaneously into rings when dialyzed overnight (Fig. S9). However using this simple heating/reducing procedure to insert purified β -chains into the core complex was not successful. Hence, it remains still unclear whether the hetero-assembly of α - and β -chains is a spontaneous/autonomous process or whether molecular helpers such as chaperones are involved in hetero-oligomer formation *in vivo*. Moreover, the underlying mechanism of the formation and coexistence of different isoforms in the blood is currently unknown as is the biochemical basis of the distribution shift of isoforms observed in various diseases.³² Certainly further systematic biochemical and cell biological studies are required to address these points.

The major isoform of hC4BP contains an additional β -chain. Notably, in each of the two likely positions of the β -chain in the *in silico* models the crystal structure stabilizing layers are not fully maintained and break the symmetry of the core complex (Fig. 6 and 7). In the most favored model the β -chain projects out of the ring by ~ 10 Å. Whether this is of any biological relevance in terms of assembly stays unclear. However, the models show that in nature the β -chain is not likely to fit perfectly in the otherwise symmetric core complex and might weaken its stability. This might be an indication why there are no isoforms with more than one β -chain.

It is noteworthy that the symmetric heptameric structure of the human C-terminal C4BP oligomerization domain, its small size of only 57 residues per monomer unit and the extremely high oligomer stability renders it an interesting scaffold for future applications in the field of protein engineering and design.

Materials and methods

Cloning of expression vector pET32aTrxTEVhC4BP

The vector pEF-IRES containing the gene of the C-terminal oligomerization domain of hC4BP α -chain (residues 540-597; **GenBank ID: 416733**) served as template for the initial construct. The oligonucleotides NcoI_C4BP_up (5'-GCGCGCCCATGGGCGCGCACATGGGGTGGGAGACC CCGAAGGC-3') and C4BP_HindIII_lo (5'-GCGCGCAAGCTTGAATTCTTAGATT-AGTTCTTTATCCAA-3') with restriction sites *NcoI* and *HindIII* were used to amplify the C4BP α -chain by PCR and purified with the Promega PCR and Gel cleanup kit. After *NcoI* and *HindIII* restriction the purified fragment was ligated to similarly cleaved pET32a vector (Novagen) followed by transformation of electrocompetent *E.coli* Top10. Positive clones were checked by colony-PCR and sequencing using the primers T7_Promotor_up (5'-TAATACGACTCACTATAGGG-3') and T7-Terminator_lo (5'-TAGAGGCCCAAGGGGTTAT-3').

In order to decrease the overall number of selenomethionine residues, codons of two methionines of the N-terminal linker sequence MGAHMG of the initial construct were replaced by alanines via amplification of the hC4BP core domain coding sequence using the primer pairs BglII_TEV_C4BP_up (5'-CGCGCAGATCTGGAAAACCTGTACTTCCAGTCCGCCGGCGCGC-ACGCCGGGTGGGAGACCCCGAAGGC-3') and C4BP_XhoI_lo (5'-GCGCGCCTCGAG-TGCGGCCGCAAGCTTGAATTCTTAGATTAGTTCTTTATC-3') followed by cleavage with *BglII* and *XhoI* and ligation into similarly cleaved pET32a.

Expression of Trx-C4BP α -chain variants

Transformed *E.coli* cells were grown in 1 L LB-medium at 30 °C and 180 rpm to an OD₆₀₀ of 1. In case of Origami (DE3) administering of 0.5 mM IPTG induced expression. After 3 h cells were harvested by centrifugation, cell pellets were resuspended in 7.5 mL PBS per liter cell culture and stored at -80°C until further use.

Expression of SeMet C4BP variants was based on a previous published protocol ³⁴. *E. coli* B834 (DE3) cells were grown in LB media to an OD₆₀₀ of ~1.0. For each washing step cells were

pelletized by centrifugation at 6660 rpm, 13 min, 4 °C. Cells were gently washed twice by resuspending them in 50 mL and 100 mL ice-cold water. The washed and centrifuged cells were transferred to 50 mL M9 medium, which was used to inoculate 1 liter of M9 medium with supplement (10 mM glucose, 1 mM CaCl₂, 1 mM MgCl₂, 0.0005% (w/v) thiamine and 50 mg L-selenomethionine (Calbiochem)) added. Production of Trx-C4BP α -SeMet was instantly started by addition 0.5 mM IPTG. Growth conditions and expression time were the same as for the native protein.

Purification of the C-terminal oligomerization domain of hC4BP 7 α isoform

Frozen cells were thawed and cell lysis was performed four times using a French pressure cell (Aminco) with 1200 psig pressure. The lysate was centrifuged at 20,000 rpm, 20 min, 4 °C and the supernatant filtered with a 0.45 μ m sterilfilter (Sarstedt). The His-tagged Trx-C4BP variants were purified via two 1 ml His Trap IMAC columns (GE Healthcare) in a row attached to an Äkta FPLC purifier system (GE Healthcare). Bound protein was eluted in fractions with 200 mM and 500 mM imidazole. For TEV-cleavage 10fold TEV-buffer (1.5 M NaCl, 500 mM Tris/HCl pH 7.5) and recombinant TEV-protease (generated in house, ratio 1:25) were added and incubated over night at room temperature. The cleaved protein was dialyzed in 5 liter PBS over night (12-14 k MWCO dialysis tube). The cleaved Trx-tag was removed by reapplying the dialyzed protein to a second IMAC. Elution with 20 mM imidazole yielded the cleaved target protein, which was concentrated using a Vivaspin 15 with a MWCO of 10,000 (Sartorius Stedim biotech).

The final purification of hC4BP was performed using size exclusion chromatography on a Superdex 75 pg 16/60 (GE Healthcare) with 20 mM NaCl and 20 mM Tris/HCl pH 7.8. Purified protein was concentrated, flash-frozen in liquid nitrogen and stored at -80 °C.

C-terminal truncation of hC4BP core complex

Successive truncation of the C-terminal oligomerization domain was obtained using the following primers: Trx_AS_91-96_up (5'-AAAGTGGGTGCACTGTCT-3'), C4BP Δ C11_lo (5'-GCGC-GCCTCGAGTTAGTCTCTCTGTAGTTCCAG-3'), C4BP Δ C15_lo (5'-GCGCGCCTCGAGTTATT-

CCAGTTGTTCAATTTTC-3'), C4BPΔC20₁₀ (5'-GCGCGCCTCGAGTTATTCCAGAGACAGCT-TATA-3'), C4BPΔC27₁₀ (5'-GCGCGCCTCGAGTTACTCCAGGGCCATTTTCAC-3'). Resulting PCR products were digested using *MscI* and *XhoI* and ligated into pET32 expression vector in frame with the thioredoxin coding sequence. Truncated proteins were produced and purified according to the wild type variant. TEV-cleavage mixture was concentrated and applied to a Superdex 75 pg 16/60 column for size exclusion without further purification.

Thermal shift assay (TSA)

A thermal shift assay was used to test the stability of hC4BP and the truncated hC4BP^{ΔC15} variant. The environmentally sensitive fluorescence dye SyproOrange was used, which fluoresces when bound to hydrophobic patches of the protein upon melting. All reactions contained 50 mM Tris pH 7.8 and 10x SyproOrange (Sigma-Aldrich, stock: 5000x) and 0.2 mg/mL protein (dialyzed or stored in 50 mM Tris pH 7.8, 50 mM NaCl). Concentrations of up to 50 mM DTT and 3.9 M NaCl were used to test their influence on hC4BP heat stability. In the case of the denaturing agents guanidinium hydrochloride or urea concentrations up to 4 M were tested with constant concentration of 10 mM DTT and 2 M NaCl. Measurements were performed in triplicate in 96-well format using a BioRad 96CFX RT-PCR detection system with 0.5°C/10sec to 99 °C. T_m -values were obtained from melting curves using the detection systems analysis software and plotted with Qtiplot. Error bars were calculated using the standard deviation from three independent experiments.

Crystallization and structure determination

Initial crystallization of C4BP SeMet was probed by mixing equal volumes of 6.8 mg/mL protein and precipitant in commercial screens JCSG I-IV. Before crystallization set up the protein was centrifuged at 15.000 rpm for 5 min. Initial crystals were found in tube 32 of JSCG III (0.1M imidazole pH 8.0, 40% (v/v) PEG 400) with 6.8 mg/ml protein. Successive optimization of this condition led to crystals diffracting to ~3.0-3.5Å. Mutated C4BP SeMet (M2A M6A) did crystallize in similar conditions. Diffraction-quality SeMet (M2A M6A) crystals were obtained after several days at 19 °C by mixing equal volumes of concentrated protein solution with precipitant solution (46.5 % (v/v) PEG 400, 5 %

(v/v) MPD, 0.1 M Tris pH 7.3 and 13.7 mg/ml protein) by hanging-drop vapour-diffusion crystallization in 24-6 well plates (EasyXtal; QIAGEN). Crystals were flash-frozen in liquid nitrogen. High redundant SAD data was collected at 100 K at the selenium peak wavelength (0.980 nm) at BESSY MX-14.1 in Berlin.

Data were indexed, integrated and scaled with the XDS/XSCALE³⁵ package. Determination of selenium positions, density modification and initial model building were performed using AutoSol/Autobuild from the PHENIX suite³⁶. The initial model was manually rebuilt with COOT.³⁷ The C4BP structure was completed using alternate cycles of manual fitting in COOT and refinement in PHENIX suite with experimental phase and geometry restraints. For NCS refinement two independent groups (residue range 546-560 and 562-590) per monomer were chosen. The stereochemical quality of the final model was assessed using MolProbity.³⁸ Figures and structure alignments were prepared using PyMOL.³⁹ Data and refinement statistics are summarized in Table S1.

Molecular assembly

In order to produce the 6 α 1 β model, an α -chain from the 7 α crystal was mutated into a β -chain using VMD mutator tool.⁴⁰ α and β sequences were aligned so that the small helix H1 could establish two disulfide bridges with its neighbors, and the long helix H2 could contribute to stabilize the interaction layers (K566-E570 and K573-E577 of the α -chain) with its residues K237 and E241 facing inwards. The connection between H1 and H2 was designed *de novo* using Modeller.⁴¹ Since secondary structure prediction indicates that beta is mostly helical,⁴² residues 222-225 were designed as helical and residues 217-221 as coil. The resulting structure was minimized with 1000 conjugate gradient steps using the CHARMM27 force field⁴³ and NAMD molecular dynamics engine.⁴⁴ Minimization was performed in implicit solvent, using 80 as dielectric constant.

In order to model the 6 α and 7 α 1 β assemblies, new structures having a stoichiometry equal respectively to 6 and 8 had first to be generated. To this end, important interactions at monomers interface were first identified (K566[NZ]-E570[OE1], K573[NZ]-E577[OE2], Q580[NE2]-Q584[OE1] and C546[SG]-C558[SG]). Models having 6 and 8 units, minimal energy and presenting the same interface interactions were obtained using POW (further details in Supplementary Data). The

energy of the resulting 6 α and 8 α multimers were minimized using the same protocol detailed above. In order to produce the 7 α 1b model, an alpha chain of the 8 α model was mutated to beta using the same protocol adopted for the 6 α 1 β model.

Molecular Dynamics

The 7 α crystal was solvated in a rectangular box of pre-equilibrated TIP3 water, and the resulting system's total charge neutralized by the addition of Na⁺ and Cl⁻ ions. Molecular dynamics has been performed CHARMM27 force field on NAMD molecular dynamics engine, with SHAKE algorithm on all the bonds, and Particle-mesh Ewald treating the electrostatic interactions in periodic boundary. We used an integration step of 2 fs. The system had its energy initially minimized by mean of 1000 conjugate gradient steps, the water was then equilibrated for 50 ps at 1 atm and 300K, with harmonic restraints on the protein's backbone. The unrestrained system was subsequently heated from 0 to 300 K in 500 ps at 1 atm. 100 ns simulation was finally run in the nPT ensemble at 1 atm and 300 K. Temperature was controlled by mean of Langevin forces, using a damping constant of 1 ps⁻¹. Assessment of protein's RMSD showed that equilibration was reached at approximately 15 ns. From the resulting simulation, one frame every 100 ps was extracted (i.e. 1000 frames). For every frame, the presence of ionic bonds between all Lys566 and Glu570 couples and between Lys573 and Glu577 ones was assessed, using as criteria a distance smaller than 3.2 Å. The presence of hydrogen bonds between Gln580 and Gln584 and between Tyr572 and Glu582 was also assessed, using as a criteria a distance smaller than 3 Å and an angle smaller than 20°.

Accession codes

The atomic coordinates and structure factors have been deposited into the Protein Data Bank with accession code 4B0F.

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Supplementary Data

Supplementary data to this article can be found online at

References

1. Gaipf, U. S., Kuenkele, S., Voll, R. E., Beyer, T. D., Kolowos, W., Heyder, P., Kalden, J. R. & Herrmann, M. (2001). Complement binding is an early feature of necrotic and a rather late event during apoptotic cell death. *Cell Death Differ.* **8**, 327-34.
2. Fujita, T., Matsushita, M. & Endo, Y. (2004). The lectin-complement pathway--its role in innate immunity and evolution. *Immunol. Rev.* **198**, 185-202.
3. Zipfel, P. F. & Skerka, C. (2009). Complement regulators and inhibitory proteins. *Nat. Rev. Immunol.* **9**, 729-40.
4. Sjöholm, A. G., Jonsson, G., Braconier, J. H., Sturfelt, G. & Truedsson, L. (2006). Complement deficiency and disease: an update. *Mol. Immunol.* **43**, 78-85.
5. Dahlbäck, B., Smith, C. A. & Müller-Eberhard, H. J. (1983). Visualization of human C4b-binding protein and its complexes with vitamin K-dependent protein S and complement protein C4b. *Proc. Natl. Acad. Sci. U. S. A.* **80**, 3461-5.
6. Kask, L., Hillarp, A., Ramesh, B., Dahlbäck, B. & Blom, A. M. (2002). Structural requirements for the intracellular subunit polymerization of the complement inhibitor C4b-binding protein. *Biochemistry* **41**, 9349-57.
7. Blom, A. M., Webb, J., Villoutreix, B. O. & Dahlbäck, B. (1999). A cluster of positively charged amino acids in the C4BP alpha-chain is crucial for C4b binding and factor I cofactor function. *J. Biol. Chem.* **274**, 19237-45.
8. Rawal, N., Rajagopalan, R. & Salvi, V. P. (2009). Stringent regulation of complement lectin pathway C3/C5 convertase by C4b-binding protein (C4BP). *Mol. Immunol.* **46**, 2902-10.
9. Ziccardi, R. J., Dahlbäck, B. & Müller-Eberhard, H. J. (1984). Characterization of the interaction of human C4b-binding protein with physiological ligands. *J. Biol. Chem.* **259**, 13674-9.
10. Villoutreix, B. O., Hardig, Y., Wallqvist, A., Covell, D. G., Garcia de Frutos, P. & Dahlbäck, B. (1998). Structural investigation of C4b-binding protein by molecular modeling: localization of putative binding sites. *Proteins* **31**, 391-405.

11. Trouw, L. A., Nielsen, H. M., Minthon, L., Londos, E., Landberg, G., Veerhuis, R., Janciauskiene, S. & Blom, A. M. (2008). C4b-binding protein in Alzheimer's disease: binding to Abeta1-42 and to dead cells. *Mol. Immunol.* **45**, 3649-60.
12. Sjölander, J., Westermark, G. T., Renstrom, E. & Blom, A. M. (2012). Islet amyloid polypeptide triggers limited complement activation and binds complement inhibitor C4b-binding protein, which enhances fibril formation. *J. Biol. Chem.* **287**, 10824-33.
13. Sjöberg, A. P., Trouw, L. A., McGrath, F. D., Hack, C. E. & Blom, A. M. (2006). Regulation of complement activation by C-reactive protein: targeting of the inhibitory activity of C4b-binding protein. *J. Immunol.* **176**, 7612-20.
14. Criado Garcia, O., Sanchez-Corral, P. & Rodriguez de Cordoba, S. (1995). Isoforms of human C4b-binding protein. II. Differential modulation of the C4BPA and C4BPB genes by acute phase cytokines. *J. Immunol.* **155**, 4037-43.
15. Esparza-Gordillo, J., Soria, J. M., Buil, A., Souto, J. C., Almasy, L., Blangero, J., Fontcuberta, J. & de Cordoba, S. R. (2003). Genetic determinants of variation in the plasma levels of the C4b-binding protein (C4BP) in Spanish families. *Immunogenetics* **54**, 862-6.
16. Martin, M., Gottsater, A., Nilsson, P. M., Mollnes, T. E., Lindblad, B. & Blom, A. M. (2009). Complement activation and plasma levels of C4b-binding protein in critical limb ischemia patients. *J. Vasc. Surg.* **50**, 100-6.
17. Blom, A. M., Villoutreix, B. O. & Dahlbäck, B. (2004). Complement inhibitor C4b-binding protein-friend or foe in the innate immune system? *Mol. Immunol.* **40**, 1333-46.
18. Ho, D. K., Riva, R., Kirjavainen, V., Jarva, H., Ginstrom, E., Blom, A. M., Skurnik, M. & Meri, S. (2012). Functional Recruitment of the Human Complement Inhibitor C4BP to Yersinia pseudotuberculosis Outer Membrane Protein Ail. *J. Immunol.* **188**, 4450-9.
19. Ho, D. K., Tissari, J., Jarvinen, H. M., Blom, A. M., Meri, S. & Jarva, H. (2011). Functional recruitment of human complement inhibitor C4B-binding protein to outer membrane protein Rck of Salmonella. *PLoS One* **6**, e27546.

20. Ogun, S. A., Dumon-Seignovert, L., Marchand, J. B., Holder, A. A. & Hill, F. (2008). The oligomerization domain of C4-binding protein (C4bp) acts as an adjuvant, and the fusion protein comprised of the 19-kilodalton merozoite surface protein 1 fused with the murine C4bp domain protects mice against malaria. *Infect. Immun.* **76**, 3817-23.
21. Dervillez, X., Huther, A., Schuhmacher, J., Griesinger, C., Cohen, J. H., von Laer, D. & Dietrich, U. (2006). Stable expression of soluble therapeutic peptides in eukaryotic cells by multimerisation: application to the HIV-1 fusion inhibitory peptide C46. *ChemMedChem* **1**, 330-9.
22. Kessel, C., Kreuz, W., Klich, K., Becker-Peters, K., Vorpahl, F., Dietrich, U., Klingebiel, T. & Konigs, C. (2009). Multimerization of peptide mimotopes for blocking of factor VIII neutralizing antibodies. *ChemMedChem* **4**, 1364-70.
23. Draper, S. J., Moore, A. C., Goodman, A. L., Long, C. A., Holder, A. A., Gilbert, S. C., Hill, F. & Hill, A. V. (2008). Effective induction of high-titer antibodies by viral vector vaccines. *Nat. Med.* **14**, 819-21.
24. Jenkins, H. T., Mark, L., Ball, G., Persson, J., Lindahl, G., Uhrin, D., Blom, A. M. & Barlow, P. N. (2006). Human C4b-binding protein, structural basis for interaction with streptococcal M protein, a major bacterial virulence factor. *J. Biol. Chem.* **281**, 3690-7.
25. Gjelstrup, L. C., Andersen, S. H., Petersen, S. V., Enghild, J. J., Blom, A. M., Vorup-Jensen, T. & Thiel, S. (2011). The role of higher-order protein structure in supporting binding by heteroclitic monoclonal antibodies: the monoclonal antibody KIM185 to CD18 also binds C4-binding protein. *Mol. Immunol.* **49**, 38-47.
26. Bessette, P. H., Aslund, F., Beckwith, J. & Georgiou, G. (1999). Efficient folding of proteins with multiple disulfide bonds in the Escherichia coli cytoplasm. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 13703-8.
27. Prinz, W. A., Aslund, F., Holmgren, A. & Beckwith, J. (1997). The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the Escherichia coli cytoplasm. *J. Biol. Chem.* **272**, 15661-7.

28. LaVallie, E. R., DiBlasio, E. A., Kovacic, S., Grant, K. L., Schendel, P. F. & McCoy, J. M. (1993). A thioredoxin gene fusion expression system that circumvents inclusion body formation in the *E. coli* cytoplasm. *Biotechnology (N Y)* **11**, 187-93.
29. Dahlbäck, B. & Müller-Eberhard, H. J. (1984). Ultrastructure of C4b-binding protein fragments formed by limited proteolysis using chymotrypsin. *J. Biol. Chem.* **259**, 11631-4.
30. Kask, L., Villoutreix, B. O., Steen, M., Ramesh, B., Dahlbäck, B. & Blom, A. M. (2004). Structural stability and heat-induced conformational change of two complement inhibitors: C4b-binding protein and factor H. *Protein Sci.* **13**, 1356-64.
31. Kumar, S., Tsai, C. J. & Nussinov, R. (2000). Factors enhancing protein thermostability. *Protein Eng.* **13**, 179-91.
32. Kishiwada, M., Hayashi, T., Yuasa, H., Fujii, K., Nishioka, J., Akita, N., Tanaka, H., Ido, M., Okamoto, T., Gabazza, E. C., Isaji, S. & Suzuki, K. (2008). Regulatory mechanisms of C4b-binding protein (C4BP)alpha and beta expression in rat hepatocytes by lipopolysaccharide and interleukin-6. *J. Thromb. Haemost.* **6**, 1858-67.
33. Khalil, A. S. & Collins, J. J. (2010). Synthetic biology: applications come of age. *Nat. Rev. Genet.* **11**, 367-79.
34. Guerrero, S. A., Hecht, H. J., Hofmann, B., Biebl, H. & Singh, M. (2001). Production of selenomethionine-labelled proteins using simplified culture conditions and generally applicable host/vector systems. *Appl. Microbiol. Biotechnol.* **56**, 718-23.
35. Kabsch, W. (2010). Xds. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **66**, 125-32.
36. Adams, P. D., Afonine, P. V., Bunkoczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C. & Zwart, P. H. (2010). PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **66**, 213-21.
37. Emsley, P. & Cowtan, K. (2004). Coot: model-building tools for molecular graphics. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **60**, 2126-32.

38. Chen, V. B., Arendall, W. B., 3rd, Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S. & Richardson, D. C. (2010). MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **66**, 12-21.
39. Schrodinger, LLC. (2010). The PyMOL Molecular Graphics System, Version 1.5.0.
40. Humphrey, W., Dalke, A. & Schulten, K. (1996). VMD: visual molecular dynamics. *J. Mol. Graph.* **14**, 33-8, 27-8.
41. Eswar, N., Webb, B., Marti-Renom, M. A., Madhusudhan, M. S., Eramian, D., Shen, M. Y., Pieper, U. & Sali, A. (2006). Comparative protein structure modeling using Modeller. *Curr. Protoc. Bioinformatics* **Chapter 5**, Unit 5 6.
42. Cole, C., Barber, J. D. & Barton, G. J. (2008). The Jpred 3 secondary structure prediction server. *Nucleic Acids Res.* **36**, W197-201.
43. MacKerell, A. D., Bashford, D., Bellott, M., Dunbrack, R. L., Evanseck, J. D., Field, M. J., Fischer, S., Gao, J., Guo, H., Ha, S., Joseph-McCarthy, D., Kuchnir, L., Kuczera, K., Lau, F. T. K., Mattos, C., Michnick, S., Ngo, T., Nguyen, D. T., Prodhom, B., Reiher, W. E., Roux, B., Schlenkrich, M., Smith, J. C., Stote, R., Straub, J., Watanabe, M., Wiorkiewicz-Kuczera, J., Yin, D. & Karplus, M. (1998). All-atom empirical potential for molecular modeling and dynamics studies of proteins. . *J. Phys. Chem. B* **102**, 3586-3616.
44. Phillips, J. C., Braun, R., Wang, W., Gumbart, J., Tajkhorshid, E., Villa, E., Chipot, C., Skeel, R. D., Kale, L. & Schulten, K. (2005). Scalable molecular dynamics with NAMD. *J. Comput. Chem.* **26**, 1781-802.
45. Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.* **16**, 10881-90.
46. Gouet, P., Courcelle, E., Stuart, D. I. & Metoz, F. (1999). ESPript: analysis of multiple sequence alignments in PostScript. *Bioinformatics* **15**, 305-8.

Figure legends

Fig. 1. (a) Schematic drawing of the major 7 α 1 β isoform of human C4BP. Seven α -chains and one β -chain containing 8 and 3 CCPs, respectively, are arranged around the C-terminal oligomerization domain. (b) Overview of all naturally occurring oligomerization domains of C4BP. The subunit compositions are from top to bottom: 6 α , 6 α 1 β , 7 α and 7 α 1 β , respectively. (c) Alignment of C4BP α -chains and β -chain from *Homo sapiens*, *Mus musculus* and *Rattus norvegicus*. Multiple sequence alignment was performed with MultAlin⁴⁵ and rendered using ESPript 2.2.⁴⁶ Secondary structure information was obtained from hC4BP⁵⁴⁰⁻⁵⁹⁷ coordinates (this study). Amino acids marked in red are identical to hC4BP sequence; amino acids highlighted in red are conserved for all aligned sequences. Sequence labels on top of the alignment refer to human C4BP α -chain. The blue frames show the consensus of at least two amino acids. The consensus sequence (bottom line) was calculated with a threshold of 0.5. Consensus sequence: uppercase is identity, lowercase is consensus level > 0.5, \$ is anyone of LM, # is anyone of NDQEBZ. Green numbers indicate the position of disulfide bridges in respect of the template.

Fig. 2. Overview on hC4BP core complex structure assembled by C-terminal residues of the α -chain. Monomer A is colored in blue and comprises an N-terminal and a C-terminal helix H1 and H2, respectively. Cysteine residues (C547 and C558) of intermolecular disulfide bonds are labeled in blue. (a) Top view of the heptameric ring in cartoon (left hand site) and surface electro potential (right hand site) representation. One monomer in the cartoon representation is colored blue. Carbons of cysteine's are colored red and sulfur atoms yellow. A blue sphere indicates N- terminus of one monomer. Inner diameter of the ring is ~13 Å. (b) Side view representation of hC4BP core structure in cartoon (left hand site) and surface electro potential (right hand site) representation. Coloring as in (a). The C-terminus is marked with a red sphere. The outer diameter of the ring is ~53 Å. The electro potential was displayed from -125 to +125 mV. (c) Distortion angles α and β in top, side and in profile view

(left hand site). Tilting angle γ is displayed in respect to the vertical symmetry axis of hC4BP core complex (right hand site). (See also Fig. S1)

Fig. 3. hC4BP core complex stabilizing ring regions and thermal stability. (a) Overview on the three rings in surface mode that stabilize the hC4BP core structure. hC4BP helices are shown as white cartoon. Ring 1, 2 and 3 are colored blue, orange and red and are labeled A, B and C, respectively. (b) Top view of the first stabilizing ring that is formed by K566 and E570 of hC4BP. The clipped surface is shown in blue, while the residues are shown in stick mode. Yellow dotted lines indicate salt bridges. Inner diameter of this ring is ~ 13.7 Å. (c) Top view of the 2nd stabilizing ring that is formed by K573 and E577. The clipped surface is shown in orange, while the residues are shown in stick mode. Yellow dotted lines indicate salt bridges. Inner diameter of this ring is ~ 13 Å. (d) Top view of the 3rd stabilizing ring that is formed by Q580 and Q584. The clipped surface is shown in red, while the residues are shown in stick mode. Yellow dotted lines indicate H-bonds between Q584 and Q580. Inner diameter of this ring is ~ 20 Å. (e) Thermal mobility of the hC4BP core complex. B-factors for hC4BP main chain residues are indicated by rainbow colors, whereas blue, thin tube indicate a low B-factor and red, thick tubes a high B-factor (see scale bar). In the top view representation (left hand site) for all monomers the B-factor putty is displayed, but in the side view only one monomer is colored for the sake of clarity. The B-factors indicate that the N- as well C-terminal ends have high atomic mobility (see also Fig. S4).

Fig. 4. Comparison of SEC-chromatograms of hC4BP wild type oligomerization domain and C-terminal truncated variants. TEV-cleavage mixture of fusion proteins was separated on a Superdex 75 pg 16/60 column. (a) Calibration proteins: Ovalbumin: 44 kDa (Ov), Carbonic anhydrase: 29 kDa (CA), Ribonuclease A: 13.7 kDa (RA) and Aprotinin: 6.5 kDa (Ap). (b)-(f) Elution peaks on the left correspond to heptamers (7α) of wt (b) and various C-terminal truncated variants of hC4BP core complex (c)-(f). Elution peaks at 78 mL represent cleaved thioredoxin (THX) (17.1 kDa). Monomers (α) are observed when more than 15 amino acids have been removed from the C-terminus (d)-(f). (b)

hC4BP: 52.5 kDa, (d) hC4BP^{ΔC11}: 43.1 kDa, (d) hC4BP^{ΔC15}: 39.5 kDa, (e) C4BP^{ΔC20}: 35.4 kDa, (f) hC4BP^{ΔC27}: 29.4 kDa.

Fig. 5. Thermal shift assay with hC4BP and hC4BP^{ΔC15}. Melting temperature of hC4BP (a) and hC4BP^{ΔC15} (b) as a function of DTT and NaCl concentration. (c) Melting temperature of hC4BP as a function of urea (squares) and guanidinium hydrochloride (circles) concentration in presence of 2 M NaCl and 10 mM DTT. Error bars from three experiments.

Fig. 6. Possible interactions of α and β -chain of hC4BP. (a) Initial model of β -chain (green) position in reference to its two neighbouring α -chains. The β -chain was modelled so that H2 is completely inserted in the resulting ring. This results in disruption of the three inner stabilizing layers (highlighted in blue, orange and red). The two neighbouring α -chains are coloured white and blue. Red full circles indicate missing interacting residues of the β -chain. Residues of the α -chain that assemble stabilizing layers are coloured in the respective layer colours as used before. (b) Optimized model of β -chain position to maximize the interaction of β -chain residues with ring stabilizing layers of the α -chain as described in experimental procedures. Though the β -chain projects out of the ring by ~ 10 Å the second stabilizing layer can be maintained by K237 and E241 of the β -chain. Colouring and labelling as in (a). (c) Sequence alignment of α - and β -chain (C-terminal core domain) of the initial model displayed in A. (d) Sequence alignment of the optimized positioning of β - with α -chains as displayed in (b). Residues that form (α -chain) or maintain (β -chain) the stabilizing layers are coloured red. Stabilizing layers are coloured and highlighted as in (a) and (b).

Fig. 7. Models of naturally occurring isoforms of C4BP. The coordinates of the 7 α C4BP C-terminal oligomerization domain were used to model (see Supporting information/Material and methods) the C-terminal core complex of the three other known isoforms of C4BP. Coloring of helices and stabilizing rings 1-3 as in Fig. 2. Each isoform is shown in side (left), top (middle) and bottom view (right) as indicated by labels and arrows. (a) 6 α C4BP. (b) 6 α 1 β C4BP. (c) 7 α C4BP (crystal). (d) 7 α 1 β C4BP.

β -chain in (b) and (d) is highlighted in green. Residues of β -chain that interact with stabilizing rings 1-3 of the α -chain are colored light blue, sand and yellow, respectively. Note that stabilizing rings 1 (blue) and 3 (red) are disrupted by the β -chain in the $6\alpha 1\beta$ as well as $7\alpha 1\beta$ models (b) and (d).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7