Structural Requirements for the Intracellular Subunit Polymerization of the Complement Inhibitor C4b-Binding Protein†

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Received April 17, 2002; Revised Manuscript Received June 1, 2002

ABSTRACT: C4b-binding protein (C4BP), an important inhibitor of complement activation, has a unique spider-like shape. It is composed of six to seven identical α-chains with or without a single *β*-chain, the chains being linked by disulfide bridges in their C-terminal parts. To elucidate the structural requirements for the assembly of the α-chains, recombinant C4BP was expressed in HEK 293 cells. The expressed C4BP was found to contain six disulfide-linked α-chains. Pulse−chase analysis demonstrated that the recombinant C4BP was rapidly synthesized in the cells and the polymerized C4BP appeared in the medium after 40 min. The α-chains were polymerized in the endoplasmic reticulum (ER) already after 5 min chase. The polymerization process was unaffected by blockage of the transport from the ER to the Golgi mediated by brefeldin A or low temperature (10 °C). The C-terminal part of the α-chain (57 amino acids), containing 2 cysteine residues and an amphiphatic α-helix region, was required for the polymerization. We constructed and expressed several mutants of C4BP that lacked the cysteine residues and/or were truncated at various positions in the C-terminal region. Gel filtration analysis of these variants demonstrated the whole α-helix region to be required for the formation of stable polymers of C4BP, which were further stabilized by the formation of disulfide bonds.

C4b-binding protein (C4BP)[1] is an important regulator of the classical pathway of complement activation. It interferes directly with the formation of C3 convertase (complex of C4bC2a) and enhances the natural decay of this enzymatic complex (*1*). Furthermore, upon binding to C4BP, C4b becomes susceptible to degradation by the plasma protease factor I (*2*). C4BP is a high molecular mass (570 kDa) plasma glycoprotein, its major isoform being composed of seven α-chains and one *β*-chain (α7*β*1) that are linked together by disulfide bonds (*2*−*4*). In the electron microscope, C4BP demonstrated a spider- or octopus-like shape, with the α-chains forming extended tentacles (*5*). In addition to the major isoform of C4BP (α7*β*1), two additional variants have been found in plasma. One contains only seven α-chains (α7*β*0), whereas the other consists of six α-chains and one *β*-chain (α6*β*1) (*6*). The α- and *β*-chains contain eight and three complement control protein (CCP) domains, respectively. CCP domains, which are found in many complement regulatory proteins, contain 50−70 amino acids organized in *β*-strands (*7*). In both types of subunits in C4BP, the CCP modules are followed by C-terminal extensions of about 60 amino acids, which are required for the polymerization of the subunits. Both types of subunits of C4BP contain binding sites for other proteins. CCP 1−3 of the α-chains comprise the C4b-interacting site (*8*), whereas CCP1−2 of the *β*-chain binds the vitamin K-dependent protein S with high affinity (*9*).

C4BP is an acute phase protein, and its concentration in blood increases 7-fold upon inflammation (*10*, *11*). Mainly the α-chain synthesis increases during acute phase, and consequently the molecules formed in this situation lack the *β*-chain and cannot bind protein S. As a result, the level of free protein S does not decrease during inflammation (*10*). The differential regulation of α- and *β*-chain synthesis is important since decreased levels of free protein S increase the risk of venous thrombosis (*12*, *13*). Although the major isoform of C4BP in plasma contains one *β*-chain, C4BP molecules consisting exclusively of α-chains can be detected in plasma from healthy individuals. Moreover, cells transfected with cDNA encoding the α-chain express C4BP that contains six α-chains (*14*). Thus, the *β*-chain is not necessary for the polymerization process. This is in analogy with IgM, in which the J-subunit is not required for the polymerization of hexameric IgM during synthesis (*15*).

The ability of C4BP α-chains to polymerize can be used as a biotechnological tool. Polymeric molecules consisting

† This work was supported by grants from the Swedish Research Council, the Foundation for Strategic Research, Tore Nilson's Trust, Greta and Johan Kock's Trust, Alfred Österlunds Trust, the Crafoord Trust, the Louis Jeantet Foundation, the Royal Physiographic Society in Lund, and research grants from the University Hospital in Malmö and the medical faculty of Lund University.

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[1] Abbreviations: C4BP, C4b-binding protein; CCP, complement control protein; CD, circular dichroism; CR1, complement receptor type 1; ER, endoplasmic reticulum; FTIR, Fourier transform infrared.

10.1021/bi025980+ CCC: $22.00
Published on Web 07/04/2002

of subunits with Fab fragments or complement receptor 1 (CR1) domains linked to the C-terminal extension of C4BP have been created (*16*, *17*). Such molecules were efficiently expressed as polymers in eukaryotic cells. This demonstrates that the C-terminal extension of the C4BP α-chain contains the information needed for the polymerization process (*16*). The C-terminal region of human C4BP contains two cysteine residues that form the two disulfide bridges that link the α-chains. Mouse C4BP lacks these cysteine residues, and the mouse α-chain polymer is formed by noncovalent bonds (*18*, *19*). The aim of this study was to investigate the structural requirements for the polymerization process of C4BP. Using a panel of recombinant C4BP variants, the C-terminal extensions of the α-chains, which are predicted to form amphipathic helices, were shown to be crucial for the polymerization process. The role of the two cysteines was to further stabilize the polymer by forming disulfide bridges.

EXPERIMENTAL PROCEDURES

1. cDNA Clones for Recombinant Proteins. Full-length cDNA encoding the human C4BP α-chain (*3*) was cloned into the eukaryotic expression vector pcDNA3 (Invitrogen). Mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene) and the following primers: C498A (5′ACC CCC GAA GGC GCC GAA CAA GTG CTC ACA 3′), C510A (5′ AGA CTC ATG CAG GCC CTC CCA AAC CCA GAG 3′), Δ514−549 (5′ CTC CCA AAC TAG GAG GAT GTG AAA ATG 3′), Δ525−549 (5′ GCC CTG GAG GTA TAT TAG CTG TCT CTG G 3′), Δ537−549 (5′ CTG GAA CTA CAG TAG GAC AGC GCA AGA 3′), and Δ493−549 (5′ AAG TGT GAG TGG TAG ACC CCC GAA GGC 3′). The mutant codons are underlined. The cysteine mutations (C498A and C510A) were introduced in wild-type (wt) as well as truncated α-chains variants. All mutations were confirmed by automated DNA sequencing using Big dye terminator kit (Applied Biosystems).

2. Purification of Recombinant Proteins. Human embryonic kidney (HEK) 293 cells (ATCC no. 1573-CRL) were transfected with the various C4BP constructs using lipofectin (Gibco), according to the manufacturer's instructions. The neomycin analogue, G418, at a concentration of 400 μg/mL, was used for selection of the transfected cells. Colonies of cells showing the highest expression levels, as judged by immunoblotting, were expanded in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 3.4 mM glutamine, 100 units/mL each of penicillin and streptomycin, and 400 μg/mL G418. Medium from transfected cells was collected in 3-day periods during which the cells were cultured in Optimem Glutamax. It was then stored at −20 °C until about 4 L of medium was collected. The medium was applied on an affinity column with mAb 104, directed against CCP1 of the α-chain, coupled to Affi-Gel 10 (BioRad), and the recombinant protein was subsequently eluted with 3 M guanidinium chloride and dialyzed immediately against a large volume of TBS (*20*). All preparative work was done at 4 °C. The yield of each purified protein was about 2 mg/L of medium, and the exact concentrations were determined from the amino acid composition analysis after 24 h hydrolysis in 6 M HCl.

3. Gel Filtration Analysis. Superose 6 (Amersham Biosciences), attached to an Äkta Explorer liquid chromatography system (Amersham Biosciences), was equilibrated with TBS, and 0.2 mL of recombinant wt C4BP or mutant C4BP (0.5 mg/mL) was applied at a flow rate of 0.4 mL/min. The absorbance at 280 and 214 nm in the eluate was continuously monitored in order to detect the protein. A Stoke's radius calibration curve was obtained as described previously (*21*) using ferritin (61 Å), catalase (52.2 Å), aldolase (48.1 Å), BSA (35.5 Å), ovalbumin (30.5 Å), chymotrypsin A (20.9 Å), and ribonuclease A (16.4 Å) that were all obtained from Amersham Biosciences. The full-length C498A/C510A mutant was also analyzed by gel filtration in the presence of 1 M NaCl, 3 or 6 M guanidinium chloride (ICN Biomedicals), 5−40% ethylene glycol (Merck), 0.12 mM Tween 20 (Merck), or 1.8 mM Triton X-100 (Merck).

4. Partial Proteolytic Degradation with Chymotrypsin. The wt C4BP expressed by HEK 293 cells was partially cleaved by chymotrypsin, essentially as described before (*22*), to determine the number of α-chains in the recombinant molecule. Briefly, wt C4BP (1.8 mg/mL) was incubated with α-chymotrypsin (27 μg/mL, Sigma) in 0.1 M ammonium acetate, 0.05 M NH_4HCO_3, pH 7.35 at 37 °C. Aliquots were taken (12.5 μL) at several time points and mixed with 50 μL of sample buffer for electrophoresis. The proteins were separated on a 2.5−10% SDS−polyacrylamide gel and visualized by silver staining.

5. Pulse−Chase Analysis. Cells expressing the α-chain of C4BP in a stable manner were grown to 80% confluency in DMEM supplemented with 10% FCS, 3.4 mM glutamine, and 100 units/mL each of penicillin and streptomycin. The medium was then replaced for 30 min by serum-, methionine-, and cysteine-free DMEM supplemented with glutamine. The cells were labeled for 10 min with [^{35}S]-methionine (0.1 mCi/plate). After different times of chase in medium containing 1 mg/mL bovine serum albumin, 3.4 mM glutamine, 2 mM methionine, and 2 mM cysteine, the plates were placed on ice, and the medium was collected. In some experiments, 10 μg/mL brefeldin A was added to the chase medium. The cells were rinsed once with ice-cold PBS, and 1 mL of solubilization buffer was added (20 mM Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl, 1% Triton X-100, 5 mM EDTA, 1% Trasylol, 1 mM PMSF). The solubilized cells were centrifuged at 13 000 rpm for 5 min, and the supernatants were collected and used for immunoprecipitation. To 1 mL of the cell extracts and 1.5 mL of the cell media was added 2 μL of rabbit antiserum (24 mg/mL) against human apolipoprotein B (Dakopatts) to remove molecules that bind antibodies unspecifically. After 2 h of incubation at 4 °C, 50 μL of a suspension of Pansorbin (10% cell suspension w/v, Calbiochem) was added, and after 1 h incubation, the mixtures were centrifuged for 5 min at 13 000 rpm. The supernatants were collected, and 30 μg of purified rabbit anti-C4BP antibodies (PK 9008) was added. After 16 h of incubation, 50 μL of Pansorbin was added, and the immune complexes were collected by centrifugation as described above. The pellets were washed twice with 10 mM Tris-HCl, pH 7.5, containing 0.4 M NaCl, 1% Triton X-100, and 5 mM EDTA and twice with 10 mM Tris-HCl, pH 8.0. The immune complexes were separated by SDS−polyacrylamide gel electrophoresis (SDS−PAGE, 3−15% gradient gel), and the ^{35}S-labeled C4BP was visualized using a

phosphoimager (Molecular Dynamics). Immunoprecipitates from lysates of cells chased for 15 min and media from cells chased for 90 min were incubated with 25 milliunits/mL endoglycosidase H (Boehringer Mannheim) for 18 h at 37 °C in 50 mM sodium acetate, pH 5.6, and analyzed under reducing conditions on a 10% SDS−PAGE gel.

6. Circular Dichroism (CD) Analysis of the C-Terminal Part of the C4BP α*-Chain.* Recombinant wt C4BP, expressed in eukaryotic cells and purified by affinity chromatography, was cleaved by 2% (w/w) chymotrypsin (Sigma) for 2 h at 37 °C (*23*). The amino-terminal fragments of the α-chains were removed by affinity chromatography on the mAb 104 column, and the unbound material, containing the central core of C4BP, was further purified by gel filtration chromatography on a Superose 12 gel (Amersham Pharmacia Biotech). The peak corresponding to the central core was collected, concentrated (Centricon YM-10, Millipore), and extensively dialyzed against 10 mM sodium phosphate, pH 7.4, for 48 h. The concentration of the central core was determined by measurement of the absorbance at 280 nm, using an extinction coefficient of 17 340 $L \cdot mol^{-1} \cdot cm^{-1}$, as calculated by the LaserGene software (DNA STAR). CD spectra were recorded on a J-720 spectropolarimeter from Jasco. Quartz cuvettes (Hellma), with 0.5 mm path length, were used. All spectra were recorded at 25 °C from 250 to 185 nm with a scan-speed of 5 nm/min, a response time of 16 s, and a step resolution of 1 nm. To improve the signal-to-noise ratio, the spectrum was collected 8 times. At first, the presence of helical components in the central core was evaluated with the method of Scholtz et al. (*24*), which focuses on the ellipticity measured at 222 nm. We have also used the self-consistent (SelCon) method (*25*) in which the experimental spectra were compared with different sets of reference spectra obtained for proteins with known secondary structures.

7. Fourier Transform Infrared (FTIR) Analysis of the C-Terminal Part of the C4BP α*-Chain.* Infrared spectra of C4BP from plasma, purified as described before (*26*), and a recombinant mutant containing only CCP1−8 (Δ493−549) were recorded on a Perkin-Elmer 1750 Fourier transform infrared spectrometer equipped with a triglycine sulfate detector. The proteins were dialyzed against deionized H_2O and freeze-dried. Before the samples were analyzed, they were reconstituted in dueterated buffer (0.01 M PBS/D_2O, pD 7.4). Spectral data were acquired from a 10 μL volume gastight CaF_2 cell (path length 6 μm), and the temperature of recorded spectra was 30 °C. The spectrometer was continuously purged with dry air to eliminate water vapor absorptions from the spectral region of interest. A sample shuttle was employed to permit the sample to be signal-averaged with the background. For each sample, 200 scans were signal-averaged at a resolution of 4 cm^{-1}. Absorbance spectra of the samples were obtained by digital subtraction of a spectrum of PBS/D_2O buffer recorded under identical conditions to that of the sample spectrum, so that a straight baseline was observed in the 2100−1800 cm^{-1} region. Detailed analysis of the amide I band was carried out using a second-derivative procedure. Second-derivative spectra were calculated using a GRAMS Derivative function with a 13 data point Savitzky−Golay smoothing window (Grams Research tm for Paragon 1000 FTIR, version 3.01A Level II, Driver Version 1.03).

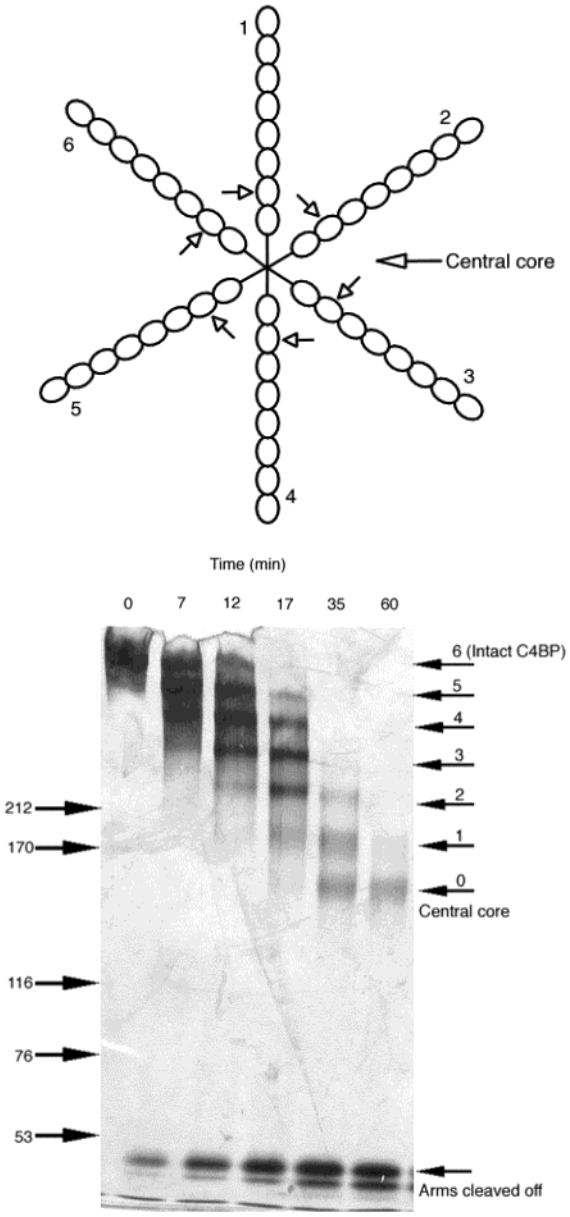

FIGURE 1: Chymotrypsin digestion of recombinant wt C4BP. C4BP was incubated at 37 °C with chymotrypsin, and aliquots were taken at given time points. The aliquots were mixed with sample buffer and applied to a 2.5−10% SDS−polyacrylamide gel. The bands were visualized by silver staining. The molecular markers are given in kDa. A schematic model of the C4BP molecule is shown at the top of the figure. The arrows point to the position of chymotrypsin cleavage in CCP7.

RESULTS

Chymotryptic Digestion of Recombinant C4BP. Recombinant C4BP was obtained from HEK 293 cells stably transfected with α-chain cDNA and therefore consisting exclusively of α-chains. To determine the number of α-chains in wt C4BP, the protein was subjected to cleavage with chymotrypsin. Chymotrypsin preferentially cleaves two peptide bonds in CCP7 of the α-chain, which liberates the α-chain fragment from the central core of C4BP (Figure 1, upper panel). The proteolysis was monitored by SDS−PAGE, which allowed calculation of the number of cleaved α-chains. This demonstrated the recombinant C4BP to consist of six α-chains (Figure 1, lower panel).

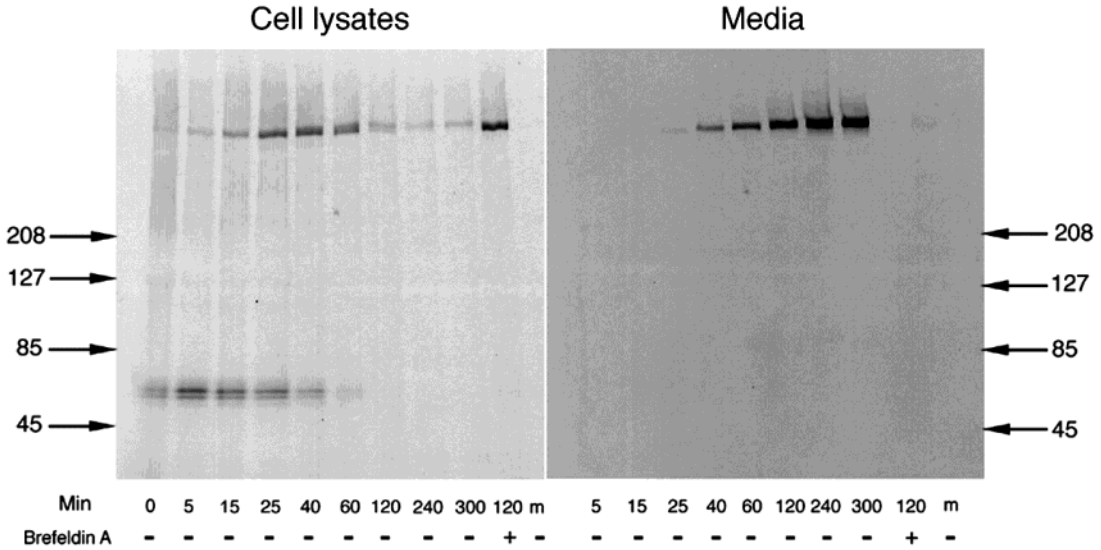

FIGURE 2: Pulse−chase analysis. HEK 293 cells stably expressing the α-chain of C4BP were labeled with [^{35}S]-methionine for 10 min and then chased. At intervals, the labeled C4BP was immunoprecipitated from the media and the solubilized cells. The immunoprecipitates were analyzed by SDS−PAGE followed by exposure on a Phosphoimager screen. Two of the samples were obtained from cells treated with brefeldin A, which inhibits intracellular transport between endoplasmic reticulum and Golgi apparatus. The molecular markers are given in kDa, and m stands for mock-transfected cells.

C4BP α-Chains Are Assembled in the Endoplasmic Reticulum (ER). As a first step in the elucidation of the assembly process of C4BP, we performed a pulse−chase analysis using stably transfected HEK 293 cells expressing the α-chain of human C4BP. Newly synthesized proteins were labeled with [^{35}S]-methionine for 10 min (pulse) and then chased with cold methionine. At intervals, the medium was collected, and the cells were lysed. C4BP was immunoprecipitated with a polyclonal antibody and analyzed by SDS−PAGE under nonreducing conditions. The primary translation product, appearing after 5 min of pulse, was a polypeptide with an apparent molecular mass of 70 kDa (Figure 2, left panel). Upon chase, the apparent molecular mass of the protein increased to >500 kDa, as expected for fully polymerized C4BP. We could not detect any intracellular assembly intermediates between the monomeric α-chain and the fully assembled form of C4BP. The polymeric C4BP was detectable already after 5 min of chase, indicating that the assembly process occurred early during the secretory pathway, most probably in the ER. This was further confirmed by the fact that brefeldin A, an inhibitor of intracellular transport between the ER and Golgi apparatus, did not inhibit the polymerization of C4BP but prevented secretion of the protein to the medium (Figure 2). Furthermore, we were able to detect polymerized C4BP in cells chased at 10 °C, a condition in which the transport between ER and Golgi is blocked (not shown). Fully assembled C4BP appeared in cell medium after 40 min of chase (Figure 2, right panel). After 2 h of chase, most of the labeled C4BP was found in the medium, illustrating the fast rate of biosynthesis of C4BP.

To confirm that the polymerization of C4BP occurred in the ER, we treated the synthesized C4BP with endoglycosidase H, which cleaves the high-mannose type of carbohydrate chains that are found on proteins during their presence in the ER. Immunoprecipitates obtained from cells chased for 15 min or from medium from cells chased for 90 min were treated for 18 h with endoglycosidase H, and the samples were separated by SDS−PAGE under reducing conditions.

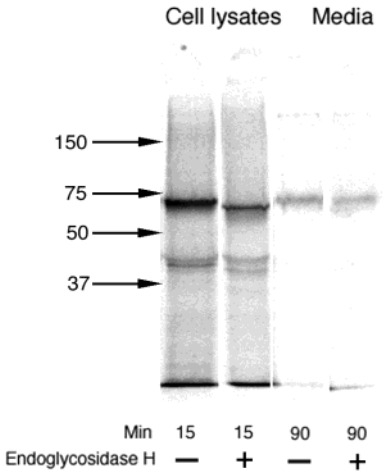

FIGURE 3: Endoglycosidase H treatment of C4BP. Immunoprecipitates from cells that had been chased for 15 min and from medium of cells that were chased for 90 min were treated with endoglycosidase H. The samples were separated by SDS−PAGE (10%) under reduced conditions, and the bands were visualized by radiography. The molecular markers are given in kDa.

The α-chain was fully sensitive to cleavage with endoglycosidase H (band shift) after 15 min of chase (Figure 3), whereas the mature protein in medium was resistant (Figure 3). This indicates that the C4BP, which is formed during the 15 min of chase, is still present in the ER. A significant proportion of the α-chains were already assembled to polymerized C4BP at this time point. Collectively, our results demonstrate C4BP to be assembled into a polymer in the ER.

The Role of Cysteine Residues in the Polymerization of C4BP. The mechanisms involved in the assembly of polymeric C4BP and the structural requirements for the process are unknown. There are two cysteine residues in the C-terminal extension of the α-chain, which covalently link the subunits. To evaluate the importance of these two cysteine residues for the polymerization process, we exchanged them into alanines (Figure 4). Four C4BP variants

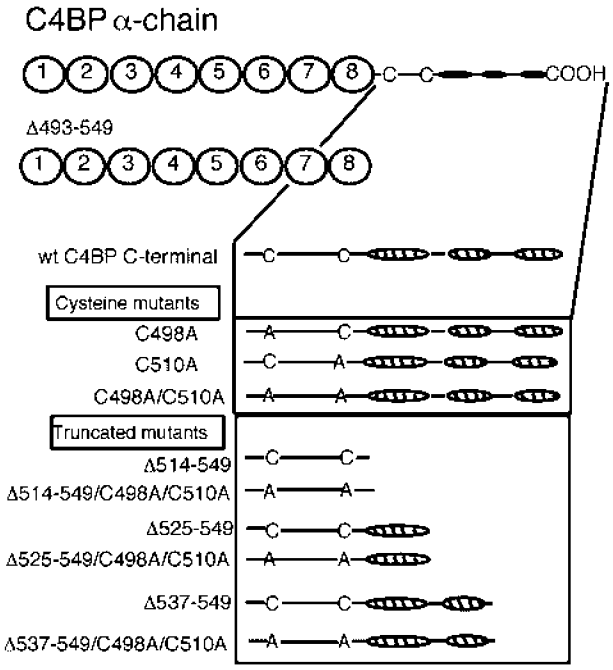

FIGURE 4: Schematic representation of recombinant wt C4BP and the mutants used in the present study. Three full-length variants lacking one or both cysteines were constructed. Six truncated mutants were prepared, lacking various lengths of the C-terminal region predicted to form an amphipathic helix. Three of the truncated mutants contained the two cysteine residues, whereas the other three had both cysteine residues being replaced by alanines. Schematic representations of wt C4BP α-chain and the mutant without the C-terminal extension (Δ493−549) are also shown.

(C498A, C510A, C498A/C510A, and Δ493−549, a monomeric α-chain lacking the entire C-terminal extension) and wt C4BP were expressed and purified by affinity chromatography. Purified proteins were separated by SDS−PAGE on 10% gels after reduction (Figure 5, upper panel) or on 3−10% gradient gels for nonreduced proteins (Figure 5, lower panel) and stained with Coomassie Brilliant Blue. Upon reduction, wt C4BP and C498A, C510A, and C498A/C510A mutants migrated as 76 kDa bands, whereas the Δ493−549 variant was slightly smaller (71 kDa). On unreduced gels, wt C4BP migrated as a 500 kDa polymer, whereas the Δ493−549 variant migrated as a 64 kDa monomer. The C498A/C510A variant behaved as a monomer in the presence of SDS, whereas mutants with single cysteine replacements displayed a small fraction of 135 kDa dimers (15% for C498A and 7.4% for C510A), with the bulk of the protein being in the monomeric form. We further analyzed the two single cysteine mutants, C498A and C510A, by pulse−chase and found only a small fraction of the newly synthesized protein to be dimeric, which was consistent with the results obtained with the purified proteins (not shown). Since SDS disrupts most noncovalent interactions, we further analyzed our mutants by gel filtration in order to assess whether they were polymerized into high molecular weight complexes (Figure 6). Purified proteins were applied on a Superose 6 column, and the Stoke's radius for each mutant was calculated (Table 1) based on the calibration curve constructed from analysis of proteins with known Stoke's radii. In agreement with previously published results of analytical centrifugation, wt C4BP eluted as a large protein with a Stoke's radius of 110.5 nm. The truncated mutant,

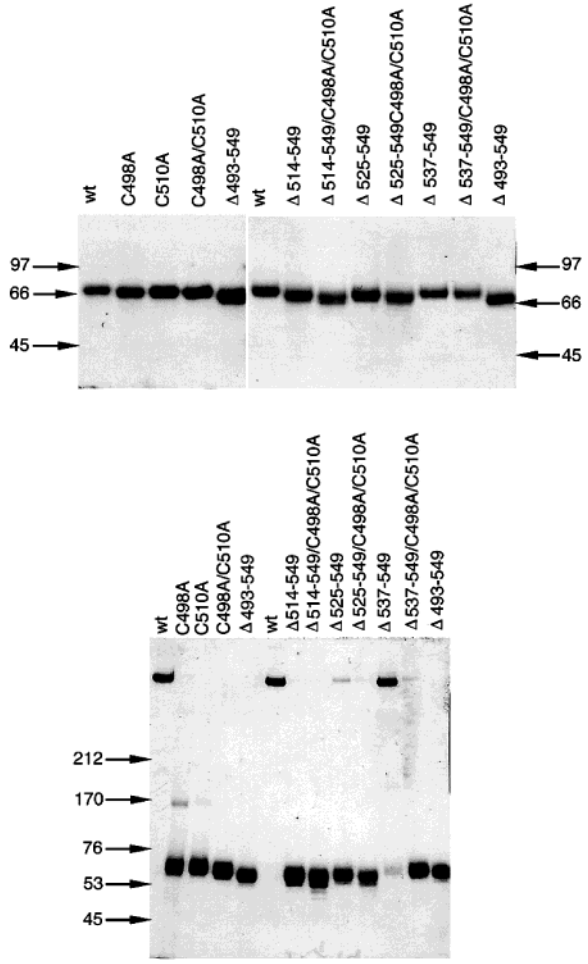

FIGURE 5: SDS−PAGE analysis of purified C4BP, mutants C498A, C510A, and C498/C510, and truncated forms of C4BP. The recombinant proteins (approximately 2 μg/well) were separated by SDS−PAGE electrophoresis and then subjected to Coomassie staining. The polyacrylamide concentrations were 10% and 3−10% for reduced (upper panel) and unreduced (lower panel) samples, respectively. The molecular markers are given in kDa.

Δ493−549, lacking the C-terminal extension, had a Stoke's radius of 55.9 nm (analyzed also on a Superose 12 column for better accuracy in this range of molecular mass). As this corresponds to a globular protein with an apparent molecular mass of 119 kDa, these results strongly suggest the monomeric α-chain to be elongated, which is in good agreement with other proteins containing CCP domains. When the protein was analyzed by gel filtration in the presence of 6 M guanidinium chloride, it eluted at a similar position excluding the possibility that dimers of the Δ493−549 mutant were formed in TBS. The three mutants lacking one or both cysteine residues in the C-terminal extensions were able to polymerize, to a high degree, into forms that were indistinguishable on gel filtration from wt C4BP (Figure 6B). Interestingly, 97% of the mutant lacking both cysteines was present in polymeric form (the peak area was calculated by Unicorn software, Amersham Biosciences). This implies that the disulfide bridges are not required for the polymerization and that other noncovalent forces are involved in the process. These results that were obtained with proteins purified from one selected cell clone were confirmed by gel filtration analysis of media from cells transiently expressing the mutants. In this case, the presence of C4BP in the gel

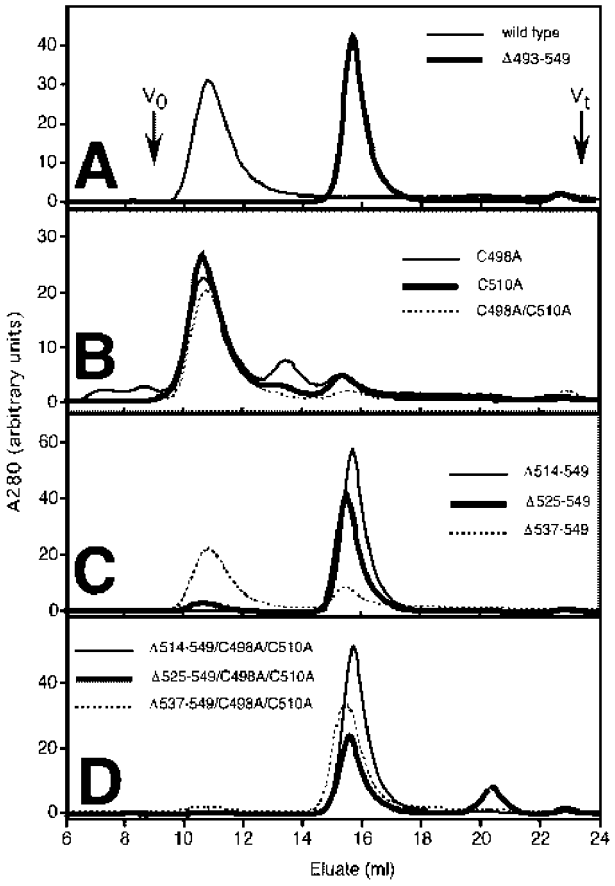

FIGURE 6: Gel filtration analysis. Recombinant wt C4BP and the mutants were applied on a Superose 6 column equilibrated with TBS and their elution volumes determined. Thymidine was present in the samples, and its elution position yielded V_t. V_0 was determined with blue dextran in a separate experiment, and the Stoke's radius was calculated for the proteins. Each mutant was analyzed twice, and one representative chromatogram is shown.

Table 1: Gel Filtration Analysis of C4BP Variants[a]

	Stoke's radius (Å)
wt C4BP	110.5
C498A	113.5 (72), 80.7 (10), 63.7 (17)
C510A	113.2 (84), 79.8 (8.5), 64.4 (7.5)
C498A/C510A	111.9 (97), 62.4 (3)
Δ514−549	63.1
Δ514−549/C498A/C510A	62.8
Δ525−549	113.1 (8), 62.6 (92)
Δ525−549/C498A/C510A	80.3 (10), 62.2 (90)
Δ537−549	110.2 (79), 63.7 (21)
Δ537−549/C498A/C510A	114.9 (2) 64.7 (98)
Δ493−549	60.9

[a] The proteins were applied on Superose 6 and their elution volumes estimated (Figure 6). The Stoke's radii were then calculated based on the calibration curve constructed from several standard proteins. When the protein eluted as several peaks, the percentage of each form is provided in parentheses. The numbers are the mean values from two chromatograms.

filtration fractions was measured by ELISA (results not shown). The results obtained with transiently expressed C4BP variants were identical to those obtained with the corresponding purified protein, showing the expression/polymerization patterns not to be cell clone specific but a general property of the proteins.

C-Terminal Truncations Affecting the Polymerization of C4BP. The C-terminal 57 amino acid residue long extension of the α-chain is sufficient for the polymerization process to occur (*11*, *12*). Secondary structure prediction suggested the formation of an amphipathic helix in the C-terminal region (*24*). To elucidate whether this helix was involved in the polymerization process, we constructed six C4BP variants in which this region was truncated to three different lengths (Figure 4). In three of these variants, the two cysteine residues were left intact, whereas in the other three, these residues were replaced with alanines. When analyzed by SDS−PAGE after reduction, the six C4BP variants had similar electrophoretic mobility (Figure 5, upper panel). On nonreducing gels, the amount of polymerized C4BP was found to depend on the length of the putative helix and the presence or absence of the two cysteine residues (Figure 5, lower panel). Thus, the variant Δ514−549, which lacked most of the putative helix region, demonstrated no polymerized form. Trace amounts of polymers were detected in the Δ525−549 variant, whereas most of the Δ537−549 variant appeared as polymeric C4BP.

Similar results were obtained in gel filtration analysis of the mutants (Figure 6C,D). When most of the C-terminal extension was removed (Δ514−549 variants), the protein was unable to polymerize irrespective of the presence or absence of the two cysteines (Table 1). The Δ525−549 variant, which contained half of the C-terminal extension, contained only a small fraction (8−10%) of polymers, whereas the corresponding variant lacking the two cysteines formed no polymers. When the last 13 amino acids were deleted, but the cysteines were replaced with alanines (Δ537−549/C498A/C510A), only about 2% of the molecules were polymeric. In contrast, the corresponding variant that contained the cysteines (Δ537−549) was mainly polymeric (approximately 80%). These results imply that both the disulfide bonds and the interactions between the amphipathic helices in the C-terminal extensions are important for the formation of stable polymers of C4BP.

Gel Filtration in Various Conditions. In an attempt to characterize the nature of the interaction between the amphipathic helices of C4BP, the C498A/C510A mutant was subjected to gel filtration under various conditions (Figure 7). The presence of 1 M NaCl or 3 M guanidinium chloride did not affect the polymerization level, whereas 6 M guanidinium chloride was found to disrupt the interchain bonds. To probe the importance of hydrophobic interactions, 5, 10, 20, and 40% ethylene glycol (Figure 7D) were tested as well as 0.12 mM Tween 20 or 1.8 mM Triton X-100. The mutant eluted as a monomer under all conditions except in 5 and 10% ethylene glycol, implying that hydrophobic surfaces of the amphipathic helices are involved in formation of the polymer, while charged surfaces are facing the solvent.

To investigate whether the protein that had been depolymerized by guanidinium chloride or ethylene glycol could repolymerize, the C498A/C510A mutant was incubated for 1 h in 6 M guanidinium chloride or 40% ethylene glycol and then dialyzed against TBS overnight. Gel filtration analysis indicated that the protein disrupted by ethylene glycol repolymerized whereas guanidinium chloride caused irreversible disruption of the polymer.

Analysis of the Secondary Structure of the C-Terminal Extension. Secondary structure prediction suggested at least a part of the C-terminal extension of the C4BP α-chain formed an amphipathic helix (*24*). We used CD and FTIR

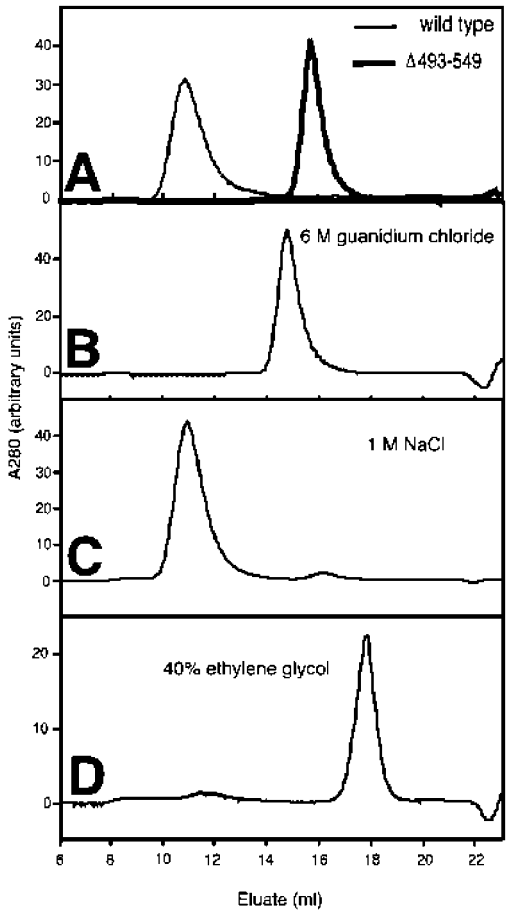

FIGURE 7: Gel filtration analysis of the mutant C498A/C510A under various conditions. The C498A/C510A mutant lacking both cysteines in the C-terminal extension was analyzed by gel filtration in 6 M guanidinium chloride (B), 1 M NaCl (C), and 40% ethylene glycol (D).

analysis to experimentally confirm this suggestion. The central core of recombinant wt C4BP, that was used for the CD analysis, was obtained after chymotrypsin digestion. It consisted of the disulfide-linked C-terminal parts of the α-chains, which was composed of the last 39 amino acids of CCP7 followed by CCP8 (57 amino acids) and the C-terminal extension (57 amino acids) (*23*). CD spectra obtained for the central core (Figure 8) were analyzed both by the method of Sholtz and by the self-consistent (SelCon) method. When using the method of Sholtz, which focuses only on the helical content of the analyzed protein, the analysis showed that the central core contained 20% α-helix, which implied that around 31 amino acids (of the total 153) of the protein fold into helical structure. Using the SelCon method, we have compared the whole spectrum with 2 sets of reference spectra including 37 and 29 proteins (*25*). The method yielded a helical content of 26−28%, which equals 40−41 amino acids.

In the FTIR analysis, plasma purified C4BP was compared with the monomeric C4BP variant lacking the C-terminal extension (Δ493−549, results not shown). Information regarding the secondary structure of proteins and polypeptides can be obtained from the frequency of amide I bands in the range of 1650−1695 cm^{-1} (*26*, *27*). Detailed analysis of the amide I bands was made using a second-derivative procedure. Second-derivative spectra were calculated using GRAMS Derivative function with a 13 data point Savitzky−

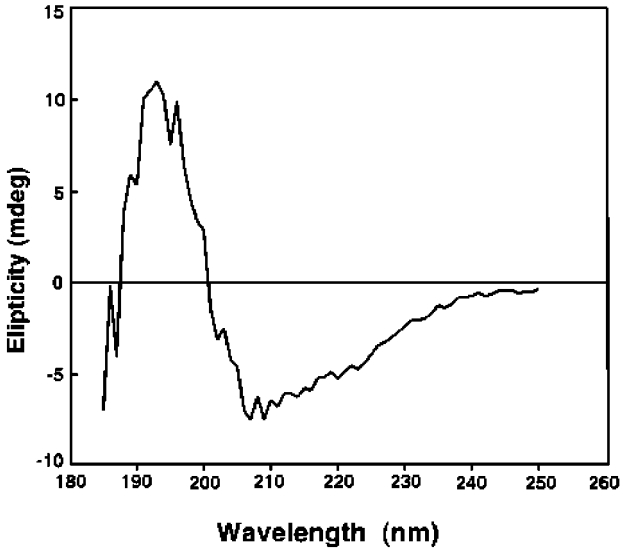

FIGURE 8: Circular dichroism spectra. Shown is the spectrum obtained for the central core of wt C4BP that was isolated after partial digestion with chymotrypsin. The spectrum was collected 8 times, and the mean values for each wavelength were calculated. Approximately 20−28% of the signal is according to the calculation derived from the α-helix.

Golay smoothing window. All band assignments made were taken from (*28*, *29*). A comparison of the FTIR second-derivative spectra of Δ493−549 and wt C4BP in PBS and D_2O (pD 7.4) was made. Δ493−549 and wt C4BP adopt a predominantly β-sheet structure, which is in agreement with the fact that both proteins are composed mainly of CCP domains. A minor component observed at 1658 cm^{-1} in the wt C4BP spectrum indicates the presence of an α-helical structure of about 5% compared to Δ493−549, again corresponding to ∼30 amino acids. Taken together, molecular modeling, CD, and FTIR imply that the C-terminal extension of the C4BP α-chain adopts an α-helical structure.

DISCUSSION

It has been demonstrated that the 57 amino acid residue long C-terminal extension of the C4BP α-chain is required and sufficient for the polymerization of C4BP and that recombinant chimeric proteins having the CCP units replaced by other protein domains can be efficiently expressed. Thus, a molecule containing both CR1 and single-chain Fv anti-Rh(D) components was shown to assemble into a covalent polymer by the C-terminal part of the C4BP α-chain (*17*). This heterofunctional molecule was able to bind to erythrocytes, thus localizing CR1 to the surface of the erythrocytes. Therefore, it is a potential therapeutic agent for diseases manifesting impaired immune complex clearance due to low levels of CR1 on erythrocytes (systemic lupus erythematosus, hemolytic anemia, AIDS). In addition, a polymeric CD46 (membrane cofactor protein, MCP) produced by a similar approach was an efficient inhibitor of measles infection (*27*). MCP is an epithelial receptor for pili present on the surface of measles virus, and the polymeric MCP inhibited the binding of the virus to the epithelial cells (*28*).

For many proteins, oligomerization occurs in the ER (*29*, *30*). For others, properly folded subunits are transported to the Golgi apparatus where polymerization is finalized (*31*,

32). The mechanisms that account for these differences are not understood. Our present results suggest that C4BP belongs to the group of proteins that are assembled in ER. Thus, in pulse−chase analysis, the polymeric form of C4BP was detectable in the cell lysates already after 5 min chase, and inhibition of the transport from ER to the Golgi apparatus by brefeldin A did not affect the polymerization of C4BP α-chains. Further support for the hypothesis that C4BP is polymerized in the ER was gained by the observation that endoglycosidase H cleaved off high-mannose-type sugar residues from polymerized C4BP present in the cell lysate early during the pulse−chase. Moreover, the polymerization occurred also when the chase was performed at 10 °C, a condition known to inhibit transport from ER to Golgi.

The two cysteines in the C-terminal extension following CCP8 of the α-chain were found to be important for the stabilization of the C4BP polymers, but they were not required for the polymerization process. The observation that secretion was unaffected by single cysteine mutations suggested that free thiol groups were buried inside the polymeric central core of C4BP since intracellular transport is often hindered by exposed thiols as they form reversible disulfide bonds with the protein matrix of ER.

Using a series of truncation mutants, we found that most of the C-terminal region was required for the polymerization process. In variants having both cysteines, less of the C-terminus was required for the formation of stable polymers. Thus, it can be concluded that successful polymerization of C4BP depends on protein−protein interactions between the C-terminal extensions and that the polymers are further stabilized by formation of disulfide bridges. The mutant lacking both cysteine residues (C498A/C510A) allowed further elucidation of the nature of the protein−protein interactions. As the polymers were disrupted by high concentrations of ethylene glycol or detergent, but not by high levels of salt, it is probable that the interactions are of hydrophobic nature and occurring between amphipathic helices. Such helices were predicted by secondary structure prediction, and their presence in the C-terminal extensions of the α-chain was supported by results of CD and FTIR analysis. The CD spectra of the central core of C4BP containing the C-terminal extension together with CCP8 and half of CCP7 suggested 20−28% α-helix structure (31−41 amino acids). This is in a good agreement with the theoretical prediction, which suggested 53% (30 amino acids) of the C-terminal extension (57 amino acids) to form α-helix. Recently, the 3-D structures of several CCP domains from various proteins were determined by X-ray crystallography and NMR (*33*, *34*). A typical CCP domain has a compact hydrophobic core wrapped in β-sheets whose strands lie approximately parallel to the long axis of the domain (*35*). In the FTIR experiment, both wt C4BP and the monomeric α-chain lacking the C-terminal extension (Δ493−549) yielded mostly β-strand signal. However, in the FTIR spectrum recorded for wt C4BP, there was a clear signal for α-helix content comprising approximately 30 amino acids. Since the CCP domains mainly contain β-strands, the α-helix signal recorded for wt C4BP must come from the C-terminal extension.

The Stoke's radius obtained for the monomeric α-chain was 55 Å, which suggests the α-chain to be elongated. The frictional ratio (f/f_0) calculated from the Stoke's radius for the α-chain was 2.03 (f/f_0 for sphere = 1.0). The protein can be modeled as a prolate ellipsoid (rodlike shape) with a long semiaxis "*a*" and two shorter semiaxes "*b*" which are of identical length. Using the hydrated volume of the protein, we calculated that the total length of the long axis for the α-chain is 320 Å and the two shorter axes are 27 Å each. These calculations were performed as previously described in detail (*36*). The calculated dimensions of the α-chain of C4BP correspond well with our 3-D model (*37*) and with electron microscopy images (*5*) and suggest that the monomeric protein is fully extended in solution. Our results are also in agreement with the observation that CCP domains in several complement regulatory proteins are not in extensive contact with one another (*38*, *39*).

The major form of C4BP in blood consists of seven α-chains and one β-chain (α7β1) and is synthesized by hepatocytes. Other forms of C4BP in plasma have been described, one having seven α-chains but lacking the β-chain (α7β0) and the other having six α-chains and one β-chain (α6β1) (*6*). It is therefore not surprising that cells transfected with the cDNA coding for α-chain synthesize polymeric C4BP molecules of the α6β0 type. Following transfection of eukaryotic cells with β-chain cDNA, the β-chain is synthesized, but the protein is retained intracellularly irrespective of the cell type tested (unpublished observation). Thus, we conclude that the β-chain is not required for the polymerization of the α-chains, and it is not able to form polymers by itself. The latter is noteworthy since the C-terminal extension of β-chain resembles that of the α-chain in that it also contains two cysteine residues and is predicted to form an amphipathic α-helix. The role of the β-chain is possibly similar to that of the J chain in the biosynthesis of IgM molecules (*15*).

In summary, we show the C4BP α-chains to be assembled in the ER and that almost all of its C-terminal extension is required in the polymerization process. The formation of disulfide bridges stabilizes the polymer that is formed by hydrophobic protein−protein interactions involving the α-helical parts of the C-terminal α-chain extension.

ACKNOWLEDGMENT

We gratefully thank Dr. Sara Linse, Lund Institute of Technology, for assistance with circular dichroism analysis.

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BI025980+