Isolation and Characterization of a Novel Plasma Protein Which Binds to Activated C4 of the Classical Complement Pathway*

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We report here the isolation and partial characterization of a previously unrecognized protease-sensitive plasma protein identified during the development of a novel protocol for the purification of the second component of human complement (C2). This new protein is physicochemically similar to C2. It coprecipitates with C2 on polyethylene glycol fractionation and specifically binds, like C2, to Sepharose-bounded IC4/C4b. Binding occurs both in the presence and absence of C2. The purified protein has a chain structure similar to C2, as determined by sodium dodecyl sulfate-gel electrophoresis in the presence or absence of reducing agent and has a molecular mass of 120 kDa, only somewhat greater than C2 at 95 kDa. Both proteins radiiodinate under similar conditions to the same specific activities with each of two different methods that yield 10-fold disparate results. Quantitative Mancini analysis identifies 300 μg/ml of the 120-kDa protein in plasma and serum. The protein is present at normal concentrations in serum from individuals genetically deficient in C2, has no C2 functional activity, and is not cleaved as is C2 when serum complement is activated. Potent monospecific polyclonal anti-serum to each do not cross-immunoprecipitate using standard gel techniques. However, these anti-sera identify epitopes in common by Western blotting. The data presented indicate that the 120-kDa protein is a distinct plasma component and suggest that the protein is not an "immature" form of C2. Initial experiments to delineate a functional role for the 120-kDa protein have demonstrated a consistent inhibition of C1 site generation on EAC4b which is dose-dependent and reversible. Thus, this protein appears to be a new complement regulatory factor.

C'4 is a 200-kDa three-chain glycoprotein present in plasma at a concentration of about 350 μg/ml that functions as the second complement protein in the classical complement pathway activation sequence (1, 2). The binding of an appropriate antibody to a substrate leads to binding and activation of the complex complement protein C1. Activated C1 cleaves a 9-kDa fragment C4a, from the NH2-terminal end of the C4 α chain (3) exposing an internal thio ester linking amino acids at positions 991 and 994 within the C4d region of the C4 α subunit, as deduced from the cDNA of pro C4 (4). Upon exposure, this highly reactive group undergoes nucleophilic attack forming a covalent bond with the target substrate. The major fragment of C4 (C4b) covalently bound to a target following cleavage and release of C4a acts as a receptor for C2 of the classical pathway. C2 is bound to C4b and in turn is cleaved by active C1 to continue the complement cascade (5). Several proteins in addition to C2 have been identified that bind to C4b. The C4-binding protein binds to fluid-phase C4b as well as to C4b when precipitated at high density on a target surface to facilitate its cleavage and degradation (6, 7). Cell-bound C4b is cleaved following association with factor I as part of the degradation pathway. At least two cell membrane-bound proteins also bind to C4b. These include CR1 or the C3b/C4b complement receptor, and gp45-70 which presumably supports C3b and C4b degradation on cells which lack CR1 (8, 9). A third membrane-associated protein termed decay-accelerating factor which exists on a wide variety of cell types is a potent inhibitor of the C3 convertase of the classical and to a lesser extent the alternative complement pathway (10). It is not yet clear whether this single chain glycoprotein of M, 70,000 interacts with C4b/C3b, or the enzymatic units (C2a or Bb) of the C3 convertases (11, 12).

Recently in developing a new purification procedure for the complement protein C2,2 we identified a new plasma protein that binds to C4b. To our knowledge this protein, present in relatively high amounts in plasma, has not been identified before. Its isolation and partial characterization are included here.

MATERIALS AND METHODS

Analytical Procedures

Complement Hemolytic Assays

Buffers—The following buffers were used for functional assays: isotonic Veronal-buffered saline, pH 7.4 (VBS), was prepared as described (13); VBS containing 0.1% gelatin, 0.15 mM CaCl2, and 1.0 mM MgCl2 (GVBSM); VBS diluted to 40% with 5% dextrose solution and containing 0.1% gelatin, 0.15 mM CaCl2, 1.0 mM MgCl2, and 0.06 M NaCl (DGVBSM); isotonic VBS containing 0.02 M EDTA was prepared by mixing eight parts of GVBS and two parts of 0.1 M EDTA, pH 7.4 (EDTA-GVBS).

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‡ The abbreviations used are: C, human complement; the individual components are designated by number and their proteolytic cleavage fragments followed by lower case letters; EAC4b, sheep cell erythrocyte (E) sensitized with anti-Forssman antibody (A) and carrying the first and fourth components of complement; PEG, polyethylene glycol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WBS, whole human serum; VBS, Veronal-buffered saline; GVBS, VBS with gelatin and NaCl; GVBSM, GVBS with metal ion(s); IEP, immunoelectrophoresis; BCA, bicinchoninic acid; μ, conductivity at 0 °C in millisiemens (mS); NPGB, p-nitrophenyl-p'-guanidino benzoate; SBTI, soybean trypsin inhibitor; HSA, human serum albumin.

2 A procedure for rapid isolation of pure human C2 with full specific functional activity (Hammer, C. H., Brickman, C. M., Jacobs, R. M., and M. M. Frank, manuscript in preparation).
**Complement Components**—The human complement (C) components C2, C3, C4, C5, C6, and C7 used to prepare functional reagents for the titration of the individual proteins were prepared as described (14, 15). With the exception of C1, the individual proteins were titrated according to the procedures described for each component. Guinea pig C1 (16), C8 (17), and C9 (18) were also purified according to methods used in functional reagent preparations. Most general component assay sheep erythrocytes (E) were sensitized with anti-Forsman antibody (A) and concentrated to EAC1,4b (19) by incubation with 500 and 200 units/ml of guinea pig C1 and human C4, respectively. Titters for the individual C components C1 through C9 are expressed as the average of the product Z X reciprocal dilution obtained from three or more experiments. Results for the assay of Cr-51-labeled human C1 were expressed as the number of cpm/ml lysis. Human C2 was assayed according to Gaither and Frank (20) using EAC4b (1.5 × 10^6/ml). Human C2 was assayed in a titered in a system containing 0.1 ml of indicator cells and 0.1 ml of sample dilution at 30 °C for 4 h. Human C4 was assayed according to Gaither et al. (21) using EAC4b (1.5 × 10^6/ml). Human C2 was assayed in a system containing 0.1 ml of indicator cells and 0.1 ml of sample dilution at 30 °C for 4 h (generally four min). This was followed by the addition of 1 ml of C-EDTA, serving as a source of C3-9, on ice and incubation for 1 h at 37 °C. C-EDTA was prepared by dilution of guinea pig serum 20-5fold in EDTA-GVBS buffer and incubation at 37 °C for 15 min to chelate metals. Human C3, C5, C6, and C7 were titered using EAC1,4b generally as described for guinea pig C7 (21). One unit/ml corresponds to 1.5 × 10^5 effective molecules in a system containing 0.1 ml of indicator cells (1.5 × 10^6/ml), 0.2 ml of sample dilution, and 0.2 ml of converting reagent. Human C5 and C9 were titered using EAC7 and the appropriate guinea pig terminal component reagents as described (21). Human C4 was assayed by the method of Gaither et al. (22), and C1 inhibitor was assayed by the method of Gigi et al. (23). Kinetic and dose experiments were performed to assess the effect of the 120-kDa protein incubation with EAC4b at 30 °C on subsequent C1 site formation. Cells treated with 120-kDa protein for the times and concentrations indicated below as well as untreated cells as a control were incubated with C1 at a concentration of 1-2 units/ml to form 1-2 sites/cell. The assay was performed essentially as described above (20). EAC4b treated with 120-kDa protein that received no C4 showed the same low background lysis as untreated EAC4b.

**Gel Electrophoresis**

**SDS-PAGE**—Composition and purity of crude and purified protein preparations were examined by SDS-PAGE on discontinuous minigel slabs as described by Maizel (24). The monomer acrylamide concentration of the gel was 8.0% with an acrylamide/bisacrylamide ratio of 37:1:1. Samples were prepared and run concurrently under both reducing (2% mercaptoethanol) and nonreducing conditions with a protein concentration of 2% weight/volume. The gels were stained for protein using 0.25% Coomassie Blue R-250 for 30 min. In some experiments the indicated alkaline gels lacking SDS were run (24).

Such gels were also used to obtain detergent-free protein which was used as an immunogen in a goat. All reagents, standards and minigel unit for SDS-PAGE were obtained from Bio-Rad.

**Radioiodination of Proteins**—The electrophoretic transfer of proteins from SDS-PAGE slab gels to nitrocellulose was performed in a Bio-Rad unit by standard procedures (25) usually for 1 h on ice at 100 V (constant) and about 300-400 mA. Prestained molecular weight standards (Bethesda Research Laboratories, or Bio-Rad) were used to identify the molecular size of the blotted proteins.

**Radioautographic Analysis**—Slab gels were prepared for autoradiography by drying the gels at 80 °C for 2 h. Radioiodinated proteins in the gels were visualized by exposing the dried gels to Kodak X-Omat AR x-ray film with the use of Quanta III intensifying screens at -80 °C (Du Pont).

**Immunoelectrophoresis (IEP)**—IEP analysis (26) of proteins and proteins and antisera was performed on 3 × 5-inch plastic film sections of Gel-bond film (FMC Corporation) containing 1% low M, agarose (Bio-Rad). The gel was prepared in 60 mm sodium Veronol buffer, pH 8.6, containing 50 mM NaCl, 10 mM EDTA, and 5 mM NaF. Following a wash in hypertonic salt solution and air drying, the electrophoretic patterns were developed by staining with Cromwell stain (27).

**BCA Protein Assay**

Protein was assayed by the bicinchoninic acid (BCA) method (28) for micro volumes as described by the manufacturer (Pierce Chemical Co.). Bovine serum albumin was used as a reference standard.

**Single and Double Radial Immunodiffusion**

Antigenic levels of 120-kDa protein were estimated using polyclonal monospecific antibody prepared in a rabbit by the single radial diffusion method of Mancini et al. (29). Double radial immunodiffusion analyses of column fractions were performed on plates prepared as above with 1% agarose in 0.05 M phosphate-buffered saline containing 10 mM EDTA and 0.005% NaCl.

**Antisera to Purified Proteins**

For analytical purposes, small volumes of select monospecific antisera to human proteins were obtained from the following suppliers: goat anti-factor B, C1r, ceruloplasmin, and C-reactive protein (Atlantic Antibodies); rabbit anti-haptoglobin (Boehringer Mannheim); rabbit anti-C4b, a, lipoprotein, a, macroglobulin, a HS glycoprotein, antilipoprotein B, and hemopexin (Cappel); goat anti-IgA and rabbit anti-WHS (Cappel); goat anti-C6 and factor I (Genzyme); goat anti-IgG (Meloy); goat anti-HMW kininogen and sheep anti-C2 (Miles), and sheep anti-inter a, troutin inhibitor (Sera-Tec). Antisera to purified C2, C1 esterase inhibitor (C1EI), fibronectin, and factor H were prepared by immunization of goats and to C4 by immunization of a burro. Immunizations were performed with 50-100-μg amounts of purified protein emulsified in 50% complete Freund's adjuvant. Immunization of a goat with pure 120-kDa protein was unsuccessful with an accumulative dose of 250 μg of antigen. A total of 2 ml of extract was used for bacterial injections and given to the animal's back as well as intramuscular sites in the thigh. Booster injections were given at 2-3-week intervals. Successful immunization was accomplished in a rabbit. Rabbits were immunized in a similar fashion, also with pure C2 and factor B. Although the 120-kDa protein was assessed to be pure by IEP and SDS-PAGE, it was adsorbed with 1% case-bound IgG anti-C2, factor B, IgG, IgG, C3, factor H, and C1EI prior to use as an immunogen. Likewise, pure C2 was treated with the same adsorbing lacking IgG anti-C2.

**Antisera Fractionation, Immuno- and Affinity Adsorbent Preparation**

The procedure for using octanoic acid for the isolation of IgG from mammalian sera developed by Steinbuch and Audran (30) was employed with minor modifications to fractionate antisera and immune plasma. Highly purified IgG obtained was coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.) at 10 mg/ml, overnight at 4 °C, by the method of March et al. (31). Following coupling the unretracted groups were blocked with a solution of 0.1 M ethanolamine, pH 9, at room temperature for 2 h. The affinity adsorbents Ig/4/C4b-Sepharose and HSA-Sepharose were prepared as above for IgG-Sepharose. Here, highly purified C4 from large scale complement preparation (14, 15) not depleted of trace amounts of C3 and allowed to "age" at 4 °C for at least 1 month was coupled at 10 mg/ml and high purity HSA (Sigma) at 6 mg/ml, respectively.

**Radioiodination of Purified Proteins**

Homogeneous C2a and 120-kDa protein were each labeled with ^125I by using Bolton-Hunter reagent (Du Pont-New England Nuclear) as described by Lawley et al. (32) or with iodinebeads (Pierce Chemical Co.). Iodinated proteins were stored frozen at -80 °C in the presence of highly purified 5% bovine serum albumin (Sigma) previously treated with p-nitrophenyl-p'-guanidino benzote (NPG) at 25 μM.

**Purification Procedure**

The purification scheme for the isolation of 120-kDa protein and C2 is presented for isolation of both proteins (Fig. 1) since they coelute on PEG fractionation and on IgG/C4b-Sepharose affinity chromatography. Resolution of the two protein on DEAE-Sepacel is obtained only by careful selection of the elution conditions. Both C2 and 120-kDa protein losses due to adsorption and aggregation were reduced by including 0.005% gelatin in all buffers. All procedures were performed at 4 °C. PEG fractionation steps were equilibrated for 1 h and centrifugation of PEG-precipitated solutions was conducted out at 350,000 × g/min in a Sorvall RC2B centrifuge. All stock buffers were Millipore-filtered prior to dilution and use, and the conductivity of working buffers was measured at 0 °C. NPG at a final concentration of 25 μM was added to all working buffers prior to use.

**Buffers**—The following buffers were used for the purification of the proteins: a 10 times concentrated stock of sodium Veronol, pH 7.35, was prepared at 50 mM and contained 0.05% gelatin (GBV, 10 X); GBV, pH 7.35, diluted to 5 mM Veronol and 0.005% gelatin, μ =
Affinity Chromatography on iC4/C4b-Sepharose—To a 1.5-cm diameter column containing 16 ml of iC4/C4b-Sepharose equilibrated in GVBSM, \( \mu = 5.6 \) mS, was added the resolubilized PEG fraction containing C2 and 120-kDa protein. Following application of the sample at 30 ml/h, the column was washed at the same flow rate with equilibration buffer for about 10 column volumes. The column was eluted with about four column volumes of GVB, \( \mu = 15.2 \) mS, at 60 ml/h. The entire chromatographic profile was screened for protein by the BCA micro method and for C2 hemolytic activity. All protein and C2 functional activity eluted by the 15.2 mS buffer (15-25 ml) were pooled and prepared for anion exchange chromatography on DEAE-Sephalogel by dilution with GVB buffer, \( \mu = 0.5 \) mS, to yield 5.6 mS.

DEAE-Septachel Chromatography—The 120-kDa protein pool prepared above (35-65 ml) was applied to a 1 x 5-cm column containing 4 ml of DEAE-Sephalogel equilibrated with GVB, \( \mu = 5.6 \) mS, at 12 ml/h. Following application of the sample, the column was washed with two column volumes of equilibration buffer to complete elution of C2. The 120-kDa protein was recovered with a linear salt gradient of 40 ml to 15 mS also at 12 ml/h. The entire chromatographic profile was again assayed for C2 function and protein. Two pools were prepared: one contained all the C2 protein identified in the drop-through; the second (120-kDa protein) identified by a single uniform peak of BCA protein lacking C2 was recovered from the gradient at 6–7 mS. Both the C2 and 120-kDa protein pools were rechromatographed on the iC4/C4b-Sepharose column to allow removal of trace contaminants and effect final purification.

Final Purification of the 120-kDa Protein on iC4/C4b-Sepharose—The iC4/C4b-Sepharose column was regenerated by treatment with 45 ml of GVB, \( \mu = 48 \) mS, and equilibrated with GVBSM, \( \mu = 5.6 \) mS. To effect final purification and determine the requirement for C2 in the binding of 120-kDa protein to the C4 ligand, the 120-kDa containing pool was again chromatographed on a C2 affinity column. Following dilution to 5.6 mS by the addition of GVB, \( \mu = 0.5 \) mS, the 120-kDa protein was reapplied to the iC4/C4b-Sepharose column at 30 ml/h. The column was washed with 120 ml of 5.6 mS buffer prior to step elution with GVB at 15 mS. Protein was determined by the micro BCA protein assay.

Stability of 120-kDa Protein during Purification, in Plasma, and Enhancement by Protease Inhibitors—IEP was used to assess the physical stability of the 120-kDa protein during its purification. Kinetic experiments utilizing this technique were also performed in aliquots of plasma at 4 °C to study the cleavage of this protein. Other experiments which studied factors responsible for control of 120-kDa protein cleavage were performed using a series of protease inhibitors. \( \epsilon \)-amino caproic acid (EACA, 50 mM), Polybrene (100 \( \mu \)g/ml), isoamyl acetamide (20 mM), soybean trypsin inhibitor (SBTI, 50 \( \mu \)g/ml), NPGB (25 mM), EDTA (10 mM), and NaN3 (25 mM) were obtained from Sigma; aprotinin (1.5 \( \mu \)g/ml) leupeptin (1 \( \mu \)g/ml), and pepstatin A (1 \( \mu \)g/ml) were obtained from Boehringer Mannheim.

RESULTS

Isolation of Pure 120-kDa Protein—The results of preliminary experiments to determine optimal conditions for the isolation of C2, which resulted in the coisolation of the 120-kDa protein, led to establishment of the protocol shown in Fig. 1. The method utilizes affinity chromatography of a resolubilized PEG plasma fraction on iC4/C4b-Sepharose followed by DEAE-Sephalogel. One repetition of the affinity chromatography step for each component is required to obtain pure proteins. The data presented here derive from 22 repetitions of this protocol; a representative summary of the yield at each step is shown in Table I. C2 distribution and yield parallel that for the 120-kDa protein through the PEG fraction. Data for C2 are detailed elsewhere. Treatment of NPGB/EDTA plasma with 7.6% PEG allowed recovery of 8.2 g of the protein in the supernatant along with the majority of 120-kDa protein and all of the C2 functional activity. When the 7.6% PEG supernatant was brought to 21.8% PEG, 1.8 g or 22% of the protein was recovered. This step successfully concentrated the 120-kDa antigen. Qualitative analysis of the distribution, physical structure, and mobility of the 120-kDa protein during a PEG fractionation was assessed by IEP as shown in Fig. 2. This IEP was developed with a polyclonal rabbit antiserum prepared with pure 120-kDa protein and shown here to be monospecific. The majority of the 120-kDa protein was concentrated in the 7.6-21.4% PEG cut and shows a single and intense immunoprecipitate of slow mobility identical to that observed in fresh serum (wells 6 and 8). Only moderate reactivity was observed in the 7.6% PEG-resolubilized precipitate, and little reactivity was found in the 21.4% supernatant. A single precipitin arc was found on IEP of all fractions, suggesting that only a single immunoreactive component is isolated and concentrated when the separation is performed rapidly in the cold. Finally, as shown in wells 7 and 9, respectively, 120-kDa protein concentrated in the 7.6-21.4% PEG cut stored for 2 days at 4 °C or stored for 8 days at plasma concentration was cleaved into at least two fragments with distinct antigenic determinants. This conversion resulted in the generation of fragments with \( \alpha \) and \( \beta \) mobility that arose rapidly in the concentrated preparation and more
A New C4b-binding Control Protein of the Complement System

TABLE I

Summary of 120 kDa protein purification

| Step                                           | Volume (ml) | Conc. mg/ml | BCA protein Total mg | Yield % | Conc. mg/ml | RID antigen Total µg | Yield % |
|------------------------------------------------|-------------|-------------|----------------------|---------|-------------|----------------------|---------|
| EDTA/NPGB/STBI plasma                          | 134         | 65.1        | 8,720                | 100.0   | 284         | 38,100               | 100.0   |
| 7.6-21.4% PEG ppt                              | 34.5        | 54.5        | 1,880                | 21.6    | 1,310       | 45,100               | 118.0   |
| iC4/C4b-Sepharose 1                            | 39.5        | 0.110       | 4.35                 | 0.05%   | 57.3        | 2,260                | 5.9     |
| DEAE-Sephacel (C2 pool)                        | 9.5         | 0.176       | 1.670                | 0.019   | 0           | 0                    | 0       |
| DEAE-Sephacel (120-kDa pool)                   | 26.5        | 0.070       | 1.860                | 0.021   | 55.8        | 1,480                | 3.9     |
| iC4/C4b-Sepharose 2 (120 kDa pool)             | 9.5         | 0.088       | 0.840                | 0.010   | 72.2        | 686                  | 1.8     |

Fig. 2. IEP analysis of the 120-kDa protein during purification and storage. The majority of the 120-kDa protein was found in the 7.6-21.4% PEG fraction and showed a single, intense immunoprecipitate of slow α mobility (well 4). Storage of this cut for two days (well 7) or serum for 8 days (well 9) at 4 °C resulted in the conversion of the 120-kDa protein into at least two fragments with distinct antigenic determinants. Antigens in the other wells were: well 1, fresh plasma; well 2, 7.6% PEG precipitate; well 3, 7.6% PEG supernatant; well 5, 21.4% PEG supernatant; wells 6 and 8, serum (−80 °C). All troughs contained rabbit anti-120-kDa protein serum.

slowly in serum or plasma. The instability of the 120-kDa protein described above parallels that observed for C2 during separation with PEG and emphasized the need for a rapid purification scheme to obtain stable preparations of these components.

When the 7.6-21.4% PEG cut was solubilized in GVBSM, \( \mu = 5.6 \), and immediately applied to the iC4/C4b-Sepharose affinity column only 0.06% of the protein applied remained bound (Fig. 3). Elution with 15 mS of GVBS recovered less than 6 mg of protein. Interestingly, coisolating with the C2 and comprising more than half the recovered protein as assessed by SDS-PAGE was a single component of somewhat higher molecular weight. Relative to C2 at 95 kDa this new component stained intensely at 120 kDa.

The relatively “basic” C2 binds weakly to DEAE-Sephacel. The absence of the 120-kDa protein in preparations of C2 isolated in pilot experiments where DEAE-Sephacel was utilized first suggested that the 120-kDa protein was relatively acidic. Thus, we were able to separate the two proteins using DEAE-Sephacel; the results shown in Fig. 4 demonstrate the separation of 5.6 mS of all C2 function in the drop-through fractions prior to gradient elution of a single peak of coisolating protein at 6–8 mS. The yield of 120-kDa protein at this step which was about 1.9 mg or 4% of the antigen (Table I) was usually less than C2.

The last purification step for 120-kDa protein entailed a second adsorption at 5.6 mS to the iC4/C4b-Sepharose column which was diluted and applied at 5.6 mS to a column containing 4 ml of DEAE-Sephacel. Complete recovery of C2 was obtained in the drop-through step which was about 1.9 mg or 4% of the antigen (Table I) was usually less than C2.

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Fig. 4. DEAE-Sephacel chromatography to effect separation of C2 from the coisolating protein. The 120-kDa and C2 protein pool at 15 mS from the iC4/C4b-Sepharose column was diluted and applied at 5.6 mS to a column containing 4 ml of DEAE-Sephacel. Complete recovery of C2 was obtained in the drop-through step which was about 1.9 mg or 4% of the antigen (Table I) was usually less than C2.
to the affinity column, and eluted as in Fig. The pool from the DEAE-Sephacel step was diluted to 5.6 mS, applied binding of 120-kDa protein to the affinity matrix, presumably to "C4" was recovered in the fragments. 

The distribution, purity, and relative molecular size of the 120-kDa protein and C2 were assessed by SDS-PAGE (8% gel) under reducing conditions with Coomassie Blue staining. With nonreducing conditions identical patterns of electrophoresis were obtained for both proteins. Repeat affinity chromatography resulted in the final purification for both C2 (lane 6) and 120 kDa (lane 7). Western blot analysis not shown here demonstrated that the 120-kDa protein was isolated from plasma in an intact form as judged by antigen distribution.

Radioiodination—For binding studies and to further assess purity, each protein was labeled with $^{125}$I by both N-chlorobenzenesulfonamide (Iodobead) and Bolton-Hunter reagent. $^{125}$I incorporation was identically 10 times greater by the former method than by the Bolton-Hunter method in which both the 120-kDa protein and C2 were labeled to $3 \times 10^8$ cpm/µg. As expected, $^{125}$I-C2 labeled at tyrosine groups at $3 \times 10^6$ cpm/µg did not retain functional activity (33). Use of Bolton-Hunter reagent to label free amino groups preserved almost all lytic function. Both the 120-kDa protein and C2 radioiodinated at amino functions with Bolton-Hunter reagent are shown in the autoradiogram in Fig. 7. Their biochemical purity is evident from the lack of other well-defined bands. The trailing weak smear seen in the 120-kDa protein lane was probably due to aggregation of the parent molecule. With nonreducing conditions the smear was exaggerated, even more so in the higher specific activity preparation (not shown). Under more native conditions, the nonlabeled protein showed none of this effect (Fig. 6).

Functional Characterization—Functional complement assays sensitive to contamination levels well below that detectable by SDS-PAGE and autoradiographic analytic techniques were performed on the DEAE-Sephacel 120-kDa isolate. When assayed at 440 µg/ml some trace level of functional contamination is apparent for a few complement components at this stage of purification (Table II); these are most likely removed following the last step of purification in iC4/C4b-Sepharose.

Table I, 0.84 mg of protein representing 0.69 mg of antigen or 1.8% of the 120-kDa protein was recovered. As described below, significantly more 120-kDa protein is present in plasma which was not obtained by the method used here.

Physicochemical Properties of 120-kDa Protein—The distribution and purity of the 120-kDa protein and C2 are shown in Fig. 6 at each step of the purification as analyzed by SDS-PAGE with reducing conditions and Coomassie Blue staining. As indicated earlier, the iC4/C4b-Sepharose step allowed removal of 99.9% of the applied protein and resulted in recovery of two distinct components comprising almost all of the preparation (lane 2 versus lane 3). The subsequent DEAE-Sephacel step cleanly separated the two proteins from each other (lane 4, drop-through containing C2 versus lane 5, eluate containing the coisolating protein). By comparison to the molecular mass standards the proteins were assigned masses of 95 kDa for C2 and 120 kDa for the copurifying protein. Under nonreducing conditions identical patterns of electrophoresis were obtained for both proteins. Repeat affinity chromatography resulted in the final purification for both C2 (lane 6) and 120 kDa (lane 7). Western blot analysis not shown here demonstrated that the 120-kDa protein was isolated from plasma in an intact form as judged by antigen distribution.

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TABLE II
Functional purity of 120-kDa protein following DEAE-Sephacel chromatography

| Component assayed | Titer in 120-kDa protein | Titer in human serum |
|-------------------|--------------------------|----------------------|
|                   | units/ml                 | units/ml             |
| C1                | <1                       | 70,000               |
| C2                | <1                       | 2,000                |
| C3                | 70                       | 81,000               |
| C4                | 2                        | 71,000               |
| C5                | 40                       | 167,000              |
| C6                | 230                      | 204,000              |
| C7                | 5                        | 100,000              |
| C8                | 26                       | 151,000              |
| C9                | 90                       | 85,000               |
| 120 kDa C1 Inh    | 4                        | 270,000              |
| 120 kDa           | 440 μg/ml                | 300 μg/ml            |

*The concentration of 120-kDa protein was determined by Mancini radial immunodiffusion.

Fig. 8. IEP analysis of purified and fresh serum containing 120-kDa protein and rabbit anti-120-kDa serum. The immunoreactivity of serum versus anti-WHS and anti-C2 is shown for reference. The concentration for each of the antigens used was: WHS, neat; C2, 400 μg/ml; and 120-kDa protein, 440 μg/ml.

Production of Monospecific Antisera and IEP Analyses—Neither the anti-WHS or any of the 23 monospecific antisera immunoprecipitated with the 120-kDa protein at concentrations up to 600 μg/ml. The lack of reactivity of anti-WHS to the 120-kDa protein shown in Fig. 8 as well as the failure to produce an antisem to the 120-kDa protein by immunization of a goat suggests that this protein may not be very immunogenic. The goat was stimulated with repeated injections of 50–100 μg of protein to 250 μg of total protein. A rabbit subsequently produced a monospecific antisem to the 120-kDa protein (Fig. 8) which does not immunoprecipitate C2 and reacts poorly with its antigen in comparison for example to that obtained with C2 versus anti-C2. Quantitative Mancini radial diffusion with rabbit antisem and purified 120-kDa protein for a standard indicates that this protein is present in serum at a concentration of about 300 μg/ml.

**Western Blot Analysis of Isolated 120-kDa Protein and C2**—As shown in Fig. 8, antisem to the 120-kDa protein does not immunoprecipitate C2; similarly antibody to C2 does not precipitate this protein. However, by sensitive Western blot technique anti-120-kDa protein antisem reacts with 120-kDa protein as expected but as well cross-reacts with C2 (Fig. 9). Reactivity to bovine serum albumin, far left lane, as a control at 1000 times as much protein input or in the absence of the first antibody (not shown) was negative. Similarly with polyclonal C2 antisem weak cross-reactivity to the 120-kDa protein could be demonstrated.

**Time Course of 120-kDa Protein Cleavage in Plasma at 4 °C**—The results of the IEP analysis shown earlier for the 120-kDa antigen distribution demonstrated a time- and concentration-dependent conversion of this protein into at least two antigenically dissimilar components of α and β mobility. This cleavage could arise from the action of a protease during purification. To study this question more closely, under the conditions for preparation and storage at 4 °C, the kinetics of 120-kDa protein conversion in serum as well as in NPGB/EDTA plasma was followed by IEP analyses over an 11-day period. The results of this study shown in Fig. 10 demonstrate the slow conversion of the 120-kDa protein in serum or inhibitor-treated plasma at 4 °C. Obvious cleavage is seen by day 5 and is complete by day 11. Neither NPGB (25 μM) nor EDTA (10 mM) were effective in preventing 120-kDa protein fragmentation.

**Effect of Protease Inhibitors on the Stability of the 120-kDa Protein**—A more thorough search for an inhibitor of 120-kDa protein cleavage as detected by immunoelectrophoresis was performed using the protease inhibitors and protein modifying agents indicated under "Materials and Methods." A number of protease inhibitors slowed or prevented the cleavage of the 120-kDa protein. The order of their activity was: SBTI > aprotinin > NaN₃ > leupeptin = pepstatin. No cleavage was observed in solutions with these inhibitors for periods of up to 21 days at 4 °C, and SBTI prevented full cleavage for up to 67 days. Polybrene and EACA showed no effect, and iodoacetamide significantly accelerated cleavage of the 120-kDa protein in serum.

**Fig. 9. SDS-PAGE protein blot analysis of isolated 120-kDa and C2 proteins.** Twenty-five and 100-ng amounts of 120-kDa protein and C2 protein were electrophoresed on SDS-PAGE (8% gel), transferred to nitrocellulose, and developed using monospecific rabbit antisem to C2 against. Reactivity to bovine serum albumin for the far left lane at 1000 times as much protein or in the absence of first antibody was negative. Lane 1, bovine serum albumin; lane 2, C2 (25 ng); lanes 3 and 4, two preparations of 120-kDa protein (25 ng); lane 5, C2 (100 ng); lanes 6 and 7, two preparations of 120-kDa protein (100 ng).
A New C4b-binding Control Protein of the Complement System

**Fig. 10. IEP analysis of the kinetics of conversion of the 120-kDa protein.** The stability of the 120-kDa protein in fresh serum and in EDTA/NPGB fresh serum was followed over an 11-day period at 4 °C. In both samples there was a time-dependent cleavage of the 120-kDa protein into at least two distinct components, one of which was detected by Coomassie Blue staining. Excluding the 120-kDa protein as a site-specific ligand.

**Requirement for C4 Fragments on Sepharose.—** To prove the specificity of this protein for C4 fragments on the Sepharose matrix, we compared 120-kDa protein binding on iC4/C4b-Sepharose with a control HSA-Sepharose column. NPGB/EDTA plasma was fractionated as before to obtain the 7.6-21.4% PEG cut containing the 120-kDa protein and C2. The PEG fraction was divided, and a portion was applied to each affinity adsorbent column at 5.6 mS in GVBSM. Each column was washed in the same buffer and then eluted with GVB, μ = 15 mS, at 60 ml/h. The fractions collected were assayed for BCA protein, 120-kDa antigen, and C2 function. The results for each column are shown in Fig. 11. As expected, over 5 mg of protein were recovered from the elution of the iC4/C4b-Sepharose column. Analysis of the protein profile showed direct correlation of protein with 120-kDa protein antigen; the pool of protein containing all the 120-kDa antigen also contained all the C2 activity. SDS-PAGE of 30 μg of this pool showed the presence of both the 120-kDa and C2 proteins at the appropriate molecular weights and ratio (inset). However, only 0.15 mg of protein was recovered and no 120-kDa antigen nor C2 activity was detected in the pool prepared from the Sepharose column. The SDS-PAGE shown in the inset clearly demonstrates that the major protein constituent of this pool is HSA, most likely released from the Sepharose matrix. No C2 antigen at 95-kDa or 120-kDa protein is detectable by Coomassie Blue staining. Excluding the HSA component, the level of protein bound to the HSA-Sepharose column was less than 0.05% of that bound to the iC4/C4b-Sepharose column. Thus, both the 120-kDa protein as well as C2 required "C4" fragments on the Sepharose matrix as a site-specific ligand.

**Inhibition of C1 Site Formation.—** The specific binding of the 120-kDa protein to iC4/C4b residues suggested that initial attempts to delineate a functional role for this protein be directed to the early events of the classical complement pathway. Preliminary experiments were performed with highly purified 120-kDa protein separated from C2 by DEAE-Sephasel chromatography to assess the effect of 120-kDa protein incubation with EAC4b. The results of a series of experiments examining kinetic and dosage effects are shown in Table III. Experiment 1 shows that with a 10 μg/ml pretreatment of EAC4b with 120-kDa protein almost half the potential C1-fixing sites are inhibited. Inhibition was noted with both short (5 min) or extended (30 min) periods of incubation of the 120-kDa protein with EAC4b at 30 °C. Subsequent experiments (2-4) consistently show a dose-dependent inhibition of sites with almost complete inhibition at the highest dose of 120-kDa protein offered of about 160 μg/ml. Experiment 5 shows that a single wash of EAC4b/120-kDa protein reduces the inhibitory effect of the 120-kDa protein. The fact that some inhibition remains suggested that the 120-kDa protein may bind to EAC4b and compete with C1 sterically to prevent C2 binding. This effect was fully reversible, however, since further washes eliminated the inhibition.

**Fig. 11. Requirement for Sepharose-bound iC4/C4b in the isolation of 120-kDa and C2 proteins.** NPGB/EDTA plasma was fractionated to obtain the 7.6-21.4% PEG cut containing the 120-kDa and C2 proteins. A portion was applied to the affinity column prepared with C4 fragments, and a portion was applied to a control column prepared with HSA, both equilibrated to 5.6 mS. Following wash, both were eluted as described in the text for the isolation of 120-kDa and C2 protein. These proteins were recovered from the C4 affinity column ( — — ) and neither was recovered from the HSA-Sepharose (— — ). The SDS-PAGE of the recovered protein shown in the inset for each profile confirms the functional and antigenic analyses performed on the respective pools. Thus, C4 fragments are required for the affinity purification of 120-kDa and C2 proteins reported here.

**Table III**

| Protein pretreatment of EAC4b: % inhibition of C1 site formation | 120-kDa protein (μg/ml) | Time (min), 30 °C | 5  | 15  | 30  |
|---|---|---|---|---|---|
| 1 | 10.3 | 44 | 48 |
| 2 | 1.4 | 1.5 |
|   | 6.8 | 14.9 |
|   | 25.4 | 46.7 |
| 3 | 1.3 | 0  |
|   | 8.8 | 15.6 |
|   | 32.8 | 53.8 |
| 4 | 39.6 | 59.1 |
|   | 79.2 | 76.5 |
|   | 158.4 | 87.0 |
| 5* | 1.8 | 5.5 |
|   | 8.8 | 16.1 |
|   | 32.8 | 28.6 |

*120-kDa-treated EAC4b washed prior to C1 addition.
A newly developed isolation procedure for human C2 was highlighted by the isolation and identification of a previously unidentified plasma protein of Mr, 120,000 similar to C2 but with many distinct features (34). The isolation of this protein as presented required five steps and yielded about 1 mg of pure protein. Key to its discovery was the use of the affinity adsorbent IC4/C4b-Sepharose designed after the method of Mayer et al. (35) for the final purification of guinea pig C2. Later Kerr and Porter (36, 37) and recently Schultz and Arnold (38) described the use of "aged" CNBr-Sepharose without C4b for the isolation of C2. Factor B and other proteins as well bind to this adsorbent (36, 37) but none with the characteristics of the 120-kDa protein described here. Like others (5, 33), we (15) initially used the affinity step following crude fractionation and some form of ion exchange chromatography to purify C2. However, it was noted that the low ionic strength of the elution buffer for the subsequent anion exchange step was detrimental to C2 stability. To improve the protocol we incorporated the affinity step prior to DEAE-Sephal chromatography and found not one but two relatively pure proteins (Fig. 6). The efficiency and speed at this step, such that 99.9% of the contaminating proteins are removed within a few hours of the start of the preparation, is critical to the stability of the 120-kDa protein (Fig. 5). IEP analysis of the 120-kDa protein (Fig. 2) and functional test of the C2 from the crude 7.6–21.4% PEG cut just hours after preparation demonstrated rapid inactivation of both proteins. Because of their differing charge and purity the proteins could be completely separated by gradient elution from DEAE-Sephal (Fig. 4). Binding of the 120-kDa protein to the affinity matrix independently of C2 is suggested by the fact that one can rechromatograph the protein on the affinity matrix in the absence of C2 (Fig. 5). The requirement of IC4/C4b for 120-kDa protein binding was clearly shown by the inability to purify 120-kDa protein with HSA-Sepharose (Fig. 11). Absence of fragments on IEP, SDS-PAGE, and Western blot analysis (not shown) demonstrated that the purified 120-kDa protein was isolated from plasma in an intact form. However, the bulk of the 120-kDa antigen was not recovered by affinity isolation since repeated passes of the first drop-through produced diminishing recovery of the protein. Lack of stability of the 120-kDa protein may explain the inability to pass the column drop-through over the column a second time to significantly improve its yield. This concept is supported by the rapid cleavage of 120-kDa protein shown by IEP analysis (Fig. 2) in the concentrated 7.6–21.4% PEG cut. Further studies of the stability of this protein in the PEG concentrate are in order.

The purity of the final stage 120-kDa protein is evident from the results of physical (Figs. 6 and 7), functional (Table II), and immunochemical (Fig. 8) tests. A similar molecular mass was obtained with reduced and unreduced proteins suggesting that the 120-kDa protein is not part of a higher molecular mass disulfide-linked complex. This finding does not exclude, however, noncovalent association in plasma. The immunochemical findings that the 120-kDa protein does not react with several anti-WHS antibodies (Fig. 8) and the relative difficulty in producing antisera upon immunization suggests that this protein is a poor immunogen. This may account for the lack of its identification to date. The failure of all 23 monospecific test antisera as well as anti-WHS to immunoprecipitate the 120-kDa protein also attests to its purity and suggests that this protein is a newly recognized plasma protein. This is supported by the fact that standard tables of plasma proteins do not list a protein with the physicochemical properties of the 120-kDa protein (39, 40).

Some evidence presented here suggests that the 120-kDa protein and C2 are similar. They are similar in molecular weight and both are single-chain molecules. There is no change in molecular weight of either protein on SDS-PAGE with reduction. By Western blotting they have been shown to contain common antigenic domains. Both proteins radiolabel to a similar extent using two different methods of iodination and both bind to IC4/C4b-Sepharose. Further studies should clarify this similarity. It is evident, however, from the data in this paper that the 120-kDa protein is not C2. Evidence for this includes lack of C2 functional activity, a serum concentration 15 × higher than C2, lack of cross-precipitation with potent polyclonal antisera, different molecular weight, and a different fragmentation pattern. In particular the protein is present in normal concentration in genetically C2-deficient individuals and is not cleaved when complement is activated as is C2.

The data presented raise the issue of whether the 120-kDa protein is a "pro"-C2 or a nonfunctional, C2-related variant of the molecule. While the concept of a pro-C2 is attractive in that such precursor molecules for C3, C4, and C5, which undergo post-translational proteolytic processing to the active forms, have been described (41–43), evidence available at this time suggests that this is not the case. The 120-kDa protein is present in plasma at 15 × the concentration of C2 (Table II); in comparison precursor forms of C3, C4, and C5 are present at only about 1/20th the concentration of the active components. Precipitating antibody to C2 does not immunoprecipitate the 120-kDa protein and the converse is also true (Fig. 8), suggesting no major and/or multiple determinant sites in common and the protein is present in C2-deficient individuals. Unlike C2, the protein is not cleaved when complement is activated. The fact that antibodies to C2 and to the 120-kDa protein recognize the other protein on sensitive Western blot analysis but did not precipitate with the opposite protein suggest to us that these separate proteins may have similar domains which bind to C4b. We believe that it is unlikely that this represents trace contamination of the opposite protein in the immunizing antigen since the 120-kDa protein appears to be a weak immunogen. Furthermore, the 120-kDa protein used as an immunogen was affinity adsorbed for trace contamination with C2, making formation of anti-C4b highly unlikely.

Recent structural studies have identified a family of complement proteins which are responsible for the regulation of the classical and alternative complement pathways and are functionally identified as C3b/C4b-binding proteins (44). Included in this class are also the enzymatic elements of the C3 convertases: C2, factor B, C1r and C1s as well as the noncomplement proteins β2-glycoprotein, the interleukin 2 receptor, and the β chain of factor XIII. Some of the complement proteins from this family, factor H, C4BP, CR1, decay-accelerating factor, and gp45-70 have been classified as well based on genetic linkage and functional similarities (45). Studies of the primary structures of a number of these proteins have revealed the presence of a 60-residue repeat sequence (44). It will be of interest to determine whether this protein is a member of the C4b-binding protein family. 

Preliminary studies to ascribe a functional role for the 120-kDa protein have demonstrated its ability to inhibit C1 activity. It will be of interest and importance to determine the biochemical and biological effects of its presence in plasma.

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