

PURIFICATION AND CHARACTERIZATION OF MOUSE SERUM PROTEIN WITH SPECIFIC BINDING AFFINITY FOR C4 (Ss PROTEIN)*

By ARTURO FERREIRA, MORINOBU TAKAHASHI, AND VICTOR NUSSENZWEIG

(From the Department of Pathology, New York University Medical Center, New York 10016)

The *Ss* locus in mice controls the levels of a serum globulin, the *Ss* protein, which is detected with a heterologous antiserum (1, 2). This locus maps in the *S* region of *H-2*, the major histocompatibility complex (MHC)¹ of this species (2). Two main alleles have been described, *Ss-H* (high) and *Ss-L* (low). The amount of *Ss* protein in serum of *Ss-H* mice is 10-20-fold higher than those of *Ss-L* mice, and all mice which are phenotypically *Ss-L* bear the *k* allele in the *S* region of *H-2* (3).

Injection of serum from *Ss-H* mice into *Ss-L* mice yielded antisera which detected a variant of *Ss* whose expression was sex-limited. This variant was named *Slp* (sex-limited protein) and it was found only in the serum of males carrying the dominant allele (3).

The mechanism of control of the serum levels of the *Ss* and the function of this protein is still incompletely understood. The observations of Démant et al. (4), confirmed and extended by Hansen et al. (5), demonstrated that the *Ss* protein was part of the hemolytic complement system. Recent evidence showed an immunochemical homology between *Ss* and human C4 (6, 7). However, the functional homology between these two proteins has been more difficult to establish since in no instance did the C4 hemolytic activity in sera of *Ss-H* mice come close to being 20-fold higher than in *Ss-L* mice (8, 9). Furthermore, although in EDTA-plasma the *Ss* protein has an apparent mol wt of about 180,000 daltons, which is close to the molecular weight of human C4, in serum the *Ss* protein appears to be very heterogeneous. For example, upon Sepharose-4B filtration of serum of *Ss-H* mice, the *Ss*-protein is eluted over a range of mol wt between 180,000 and 1,000,000 daltons (3).

While attempting to resolve these inconsistencies, we found that mouse serum contains a relatively large concentration of a previously undescribed protein with a high affinity for the *Ss* protein. This *Ss*-binding protein (*Ss-bp*) has been purified and characterized. Its properties, which differ from all known complement (C) components, are described in the present paper.

Materials and Methods

Reagents. The sources of the reagents were as follows: Sephadex G-200, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.; polyethylene glycol (PEG, mol wt 6,000-7,500), J. T. Baker Co., Philipsburg, N. J.; phenylmethylsulfonyl fluoride (PMSF), Sigma Chemical Co., St. Louis,

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¹ *Abbreviations used in this paper:* BCL, barbital calcium lactate; C, complement; CIE, cross immunoelectrophoresis; EAC, sheep erythrocytes sensitized with IgG antibody and complement; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride; RIE, rocket immunoelectrophoresis; *Slp*, sex-limited protein; *Ss-bp*, *Ss* or C4-binding protein; *Ss-H*, *Ss-high*; *Ss-L*, *Ss-low*.

Mo.; Pevikon C-870, Stockholm Superfosfat Fabriks A.-B., Stockholm, Sweden; agarose and human transferrin, Behring Diagnostics, American Hoechst Corp., Somerville, N. J.; DEAE-cellulose (DE-52), Whatman Chemicals, Div. W & R Balston, Maidstone, Kent, England.

C4 purified from human serum was a gift from Dr. B. F. Tack. Its sp act was 8.4×10^6 CH₅₀ U/mg protein and 178 times higher than in the original plasma. In gel electrophoresis a single band was obtained with the unreduced material, while reduced C4 gave three bands (10).

Buffers. Phosphate-buffered saline (PBS) (Dulbecco's PBS), Grand Island Biological Co., Grand Island, N. Y.

PBS-EDTA. PBS containing 2 mM of Na₃H EDTA, pH 7.55.

Barbital calcium lactate (BCL). BCL containing 0.023 M sodium barbital, 0.037 M barbituric acid, and 0.0009 M calcium lactate.

Animals. Mice: 4- to 6-mo-old mice, both sexes, from the following strains, were used: DBA/2J, AKR/J, the F2 generation originated from these two strains, A.CA, C3H.OH, C3H.OL, A/Sn, A.AL ØC3H, B10.D2, and B10.BR. The F2 generations were obtained in our animal facilities from AKR/J and DBA/2J parentals purchased from The Jackson Laboratory, Bar Harbor, Maine. The congenic and congenic recombinant pairs were donated by Doctors D. C. Shreffler and J. Stimpfling. Mice were bled from the axillary vein and artery into tubes containing Na₃H EDTA (0.01 M final concentration in plasma) or allowed to clot in ice for 1 h. Sera and plasma were used fresh or after storage at -70°C.

C4-deficient and normal guinea pigs were bred in our animal facilities.

Antisera. Rabbit anti-mouse C3 and anti-mouse Ig were prepared as described in (11-13). Goat anti-human C4 was purchased from Meloy Laboratories, Inc., Springfield, Va. The IgG fraction of a rabbit anti-human transferrin was obtained from Dakopatts A/S, Copenhagen, Denmark. The IgG fractions of rabbit and goat antisera to mouse serum were purchased from Cappel Laboratories, Inc., Downingtown, Pa. Rabbit antiserum to Ss and mouse antibodies to SIp were a gift from Dr. D. C. Shreffler. A rabbit antiserum specific for guinea pig C4 was a gift from Dr. M. Frank. Antiserum to Ss-bp was obtained as follows: pooled EDTA-plasma from AKR/J male mice was passed through a Sephadex G-200 column. Ss-bp was contained in the first protein peak. 1.5 mg protein from the pooled fractions of the ascending limb of this peak was emulsified in complete Freund's adjuvant and injected in the four footpads of 5- to 6-mo-old female rabbits. The animals were boosted on day 14 by injecting 0.75 mg of protein intraperitoneally. Bleedings were done on days 21 and 24. By cross immunoelectrophoresis of whole mouse plasma, the presence of antibodies to Ss-bp and to several contaminants was detected in these antisera. Because female mice have only about 30% of the Ss-bp levels present in males (see text) their sera were used for absorption of the contaminant antibodies from the oligo-specific antisera. In addition, antisera were also raised against partially purified Ss-bp (see below) by the same method.

Anti-H-2 antisera, serum designation D1 and D4, directed against private specificities of K and D regions, respectively, were obtained from the National Institutes of Health, Bethesda, Md.

Radiolabeling of Ss-bp. This was performed by the method of Hunter and Greenwood (14). 25 µl of Tris-HCl, 0.8 M, pH 7.6, was added to 2 mCi of ¹²⁵I, aqueous solution in a V vial (New England Nuclear, Boston, Mass.) followed by 50 µl of purified Ss-bp (0.7 mg/ml), 10 µl of chloramine T (0.5 mg/ml), 10 µl of sodium metabisulfite (0.5 mg/ml), and 0.7 ml of PBS. The V vial and all reagents were kept in crushed ice. The labeled protein was dialyzed for 48 h against PBS. After removal from the dialysis bag, 2 mg of bovine serum albumin in 0.1 ml of PBS was added and the mixture stored at 4°C.

Analytical Procedures. Cross immunoelectrophoresis (CIE) and rocket immunoelectrophoresis (RIE) were done as described (15-17), by using 5 × 5-cm glass plates and the BCL buffer described above. For CIE, the electrophoresis in the first dimension was performed at 10 V/cm and 5°C. Human transferrin was mixed with the antigen in every plate to serve as a marker. The second dimension took 16 h, at 10°C and 2 V/cm, in agar containing the required antiserum and antibodies to human transferrin. The electrophoretic mobility of Ss-bp was compared in each plate with the mobility of the transferrin/anti-transferrin peak. The Rf of Ss-bp was defined as the ratio between the distances to the origin of the Ss-bp and transferrin peaks. In CIE or RIE, the levels of antigen were evaluated by measurement of the peaks after staining. Serial dilutions of standards containing each protein were included in each experiment to calculate the relative concentrations of the unknowns. RIE was run at 2 V/cm and 10°C for 16 h.

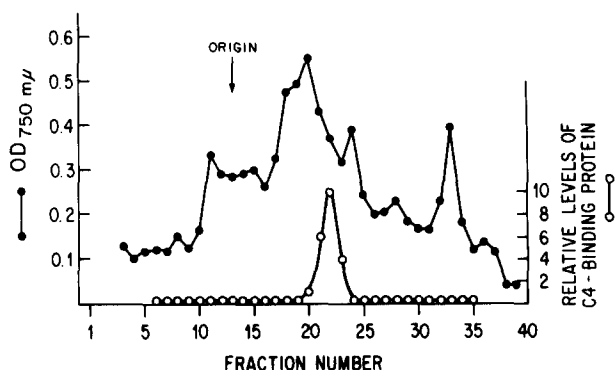


FIG. 1. Pevikon-block electrophoresis profile of a 0-5% PEG precipitate of mouse (AKR/J, male) EDTA-plasma. The precipitate was dissolved in PBS containing 0.002 M EDTA, pH 7.5, and run at 4°C for 18 h at 5 V/cm. Pevikon fractions were eluted with PBS containing 2 mM EDTA, pH 7.5. The presence of Ss-bp (C4-binding protein) was monitored by RIE.

Linear gradients of 10-40% sucrose in 0.0005 M phosphate buffer, pH 7.4 containing 0.15 M NaCl, 50 mM ϵ -aminocaproic acid, and 5 mM EDTA were used for sucrose density ultracentrifugation. Centrifugation was performed for 18 h at 36,000 rpm at 4°C by using an SW 50.1 rotor. The positions of 19s Ig and albumin were determined by inspection of the optical density pattern of the eluted fractions. The positions of Ss-bp and mouse C3 were determined by RIE. The position of IgG was monitored by single radial immunodiffusion. The separation of mouse serum into a lipoprotein-rich and a depleted fraction by ultracentrifugation in KBr (sp gr 1.25) was performed by Dr. H. Kayden. The centrifugation was done at 40,000 rpm, 4°C, in a Beckman ultracentrifuge (Beckman Instruments, Inc., Cedar Grove, N. J.).

Pevikon block electrophoresis was carried out as in (18). The separation was carried out for 18 h at 4°C, in barbital buffer pH 8.6, $\Gamma/2 = 0.05$, at 6.3 V/cm.

Statistical Analysis. The significance between or among means in different experiments was assessed by two-tailed *t* tests or by analysis of variance. The least squares method was used for the regression analysis.

Results

Purification and Properties of Ss-bp. The purification of Ss-bp was monitored by RIE with an antiserum monospecific to Ss-bp. The starting material was EDTA-plasma of 8- to 12-wk-old AKR/J male mice. 90 ml of plasma, collected in about 10 ml of 0.1 M Na₃H EDTA, pH 7.55, were mixed at 0°C with a solution of PMSF. The PMSF was previously dissolved in anhydrous ethanol to a concentration of 10-20 mg/ml and added slowly to the plasma to a final concentration of 0.5 mM. Subsequently, 50 ml of a solution of 15% PEG-6,000 prepared in PBS containing 15 mM Na₃H EDTA, was added dropwise, with constant stirring. After 20 min at 0°C, the precipitate was collected by centrifugation, dissolved in 25 ml of PBS containing 2 mM Na₃H EDTA, pH 7.5 and submitted to electrophoresis in a Pevikon block. As shown in Fig. 1, Ss-bp moved as a sharp band with a β_1 mobility. The positive fractions, eluted in PBS containing 2 mM Na₃H EDTA pH 7.5, were concentrated to 8 ml in an Amicon stirring cell, (Amicon Corp., Lexington, Mass.) by using a PM-30 membrane. The final step in the purification consisted of filtration through a Sephadex G-200 column equilibrated in the same PBS-EDTA buffer. Ss-bp was eluted in the exclusion volume, concentrated to 5 ml, and frozen in aliquots at -70°C. Assuming an

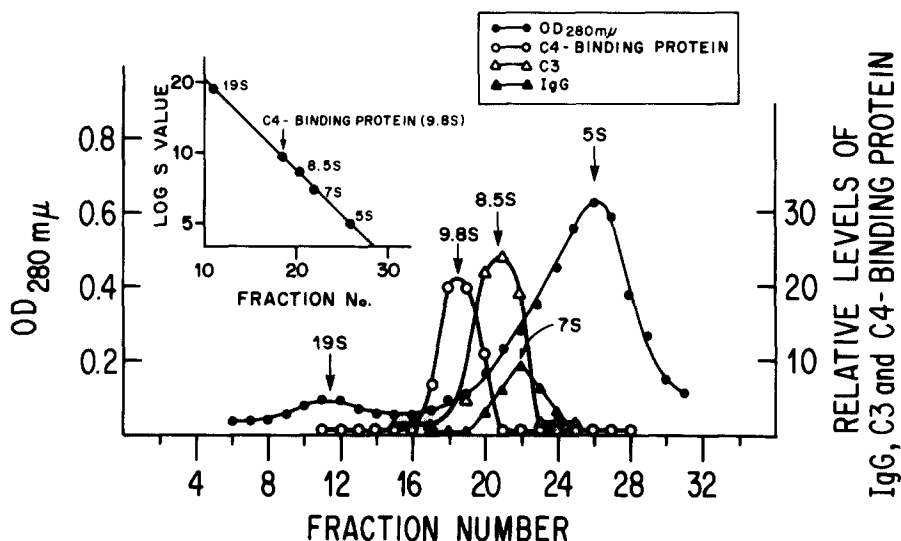


FIG. 2. Sucrose gradient (10–40%) ultracentrifugation of mouse (AKR/J, male) EDTA-plasma. The positions of the 5s and 19s proteins were determined by the OD profile of the fractions. The concentrations of IgG (7s) were determined by single radial immunodiffusion and those of Ss-bp and C3 were determined by RIE.

absorption coefficient value of $E_{280\text{nm}}^{1\text{cm}} = 1$ for all serum proteins at a concentration of 1 mg/ml, the final recovery of Ss-bp was between 10 and 20%, and the purification factor about 200 times. After the first step, PEG precipitation, recoveries were greater than 95% and the purification factor close to 30. The final preparation contained at least 35% of Ss-bp as shown by specific precipitation of the radiolabeled material with a monospecific antiserum. Ss-bp retained its antigenicity as well as its affinity for the Ss protein and human C4 after heating at 56°C for 30 min. The sedimentation coefficient of Ss-bp was determined by subjecting samples of plasma from AKR/J male mice to sucrose gradient ultracentrifugation (Fig. 2). A mean value of 10 ± 0.35 was obtained from three separate runs. In other determinations with the purified Ss-bp at a concentration of 1 mg/ml in PBS, the *s* values were not significantly different.

Effects of H-2 Type and Sex of Mice on the Levels of Ss-bp. The H-2 complex as well as sex hormones influence the levels of several C components in mice (19–23). Fig. 3 shows the relative levels of Ss-bp in male and female mice of an F2 generation of mice derived from crosses between AKR/J and DBA/2J. The differences in levels of Ss-bp in serum of mice with different H-2 types are not significant. However, the concentration of Ss-bp in serum of males is two and one half times that of females. This difference is so large and reproducible that it permits the determination of the sex of an adult mouse with almost complete accuracy by inspection of the CIE pattern of its serum (Fig. 4). The absolute values for the serum levels of Ss-bp could be calculated assuming that our partially purified preparations contained 35% Ss-bp (see above) and that $E_{280\text{nm}}^{1\text{cm}} = 1$ at a concentration of Ss-bp of 1 mg/ml. Based on these assumptions, and on results of RIE with the purified Ss-bp as a standard, we calculated that the levels of Ss-bp in the plasma from 8- to 10-wk-old male and female C3H.OH mice were 160 and 60 $\mu\text{g/ml}$, respectively.

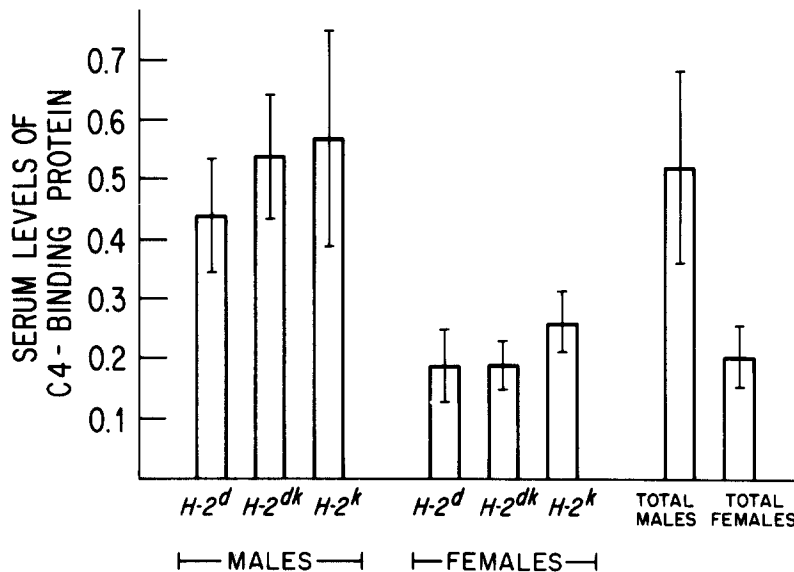


FIG. 3. Serum levels of Ss-bp (C4-binding protein) in 8 wk old mice. These animals were the F2 generation derived from AKR/J and DBA/2J parentals (AKD2F1 \times AKD2F1). Their H-2 type was determined by hemagglutination by using specific antisera against the products of the K and D regions (serum designation D1 and D4, respectively). As estimated by analysis of variance, the difference between males and females is highly significant ($P < 0.0005$). However, the differences among males or females bearing different H-2 types are not significant.

Affinity of Ss-bp for Ss. In plasma collected in EDTA, to prevent C activation, Ss and Ss-bp are not associated, and can be almost completely separated by filtration in Sephadex G-200 (Fig. 5A). However, as described below, several lines of evidence demonstrate the formation of the Ss/Ss-bp complexes in serum.

(a) An antiserum to human C4 which cross-reacts with the Ss protein, but not with Ss-bp, simultaneously precipitates Ss and Ss-bp from mouse serum. This was demonstrated by a combination of RIE and CIE of a B10.A serum (H-2^a, Ss-H). In the first part of the experiment, the serum was mixed with human transferrin and subjected to RIE, in the presence of various amounts of the above mentioned IgG anti-human C4, to precipitate the Ss protein. Then the plates were subjected to a second electrophoresis (CIE), perpendicular to the first one, into agarose-containing antibodies to Ss-bp and transferrin. The presence of antibodies to C4 in the first dimension (RIE) completely abrogated the Ss-bp peaks in the second dimension (Fig. 6A) but did not affect the areas of the transferrin-anti-transferrin peaks. The same result was obtained by incorporating an antiserum to Ss (instead of anti-human C4) in the first dimension (not shown).

To rule out the possibility that Ss-bp had bound nonspecifically to the Ss-anti-Ss precipitate formed in the first dimension, the reciprocal experiment was performed. An antiserum to transferrin rather than to C4 was present in the first dimension. Now (Fig. 6B) the peak of transferrin in the second dimension was abrogated, but Ss-bp was not affected.

The same experiments were repeated with an Ss-L serum (C3H.OL) and as

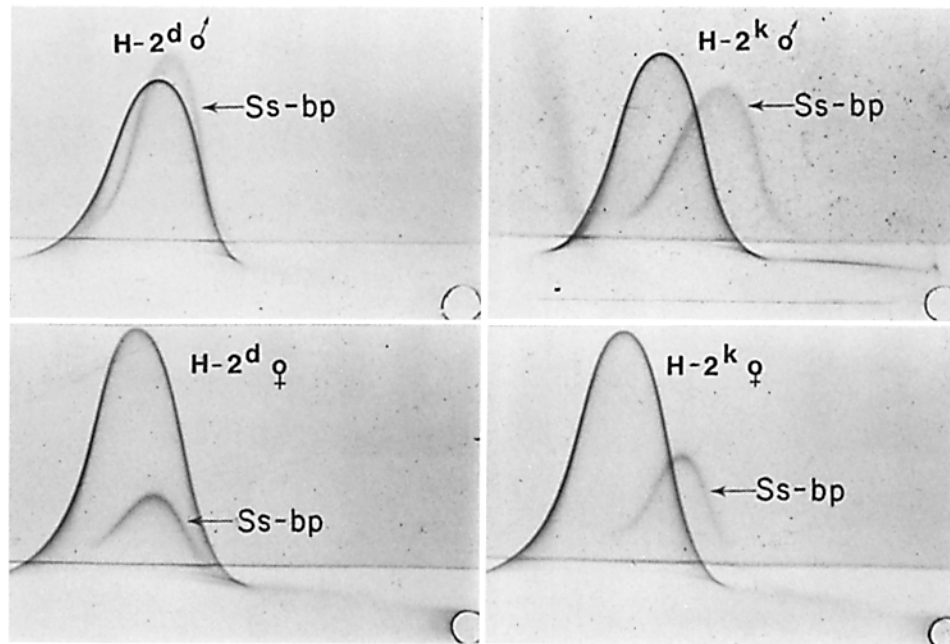


FIG. 4. CIE patterns of individual sera from adult male and female mice obtained from a cross between AKD2F1 \times AKD2F1. The *H-2* types were determined as explained in the legend of Fig. 3. Antisera against Ss-bp and human transferrin were used in the second dimension. A standard amount of human transferrin was added to each mouse serum as an internal marker. The peak of transferrin is easily recognized (stronger line). Comparing the positions of the Ss-bp peaks with that of the internal marker, it is clear that Ss-bp moves slower towards the anode in *H-2^k* mice. Also shown is the striking difference in Ss-bp levels in sera from males and females irrespective of *H-2* type. The anode is on the left.

predicted, the presence of antiserum to human C4 in the first dimension barely affected the Ss-bp peak. On close inspection only a small part of the descending limb of the Ss-bp peak was abrogated (not shown). This represents the fast moving Ss/Ss-bp complexes formed with the small amount of Ss present in this serum and which have a faster electrophoretic mobility (see below). In short, these experiments demonstrate that in Ss-H serum all of Ss-bp is bound to Ss, while in Ss-L serum most of Ss-bp is not associated with Ss.

(b) In serum, part of Ss appears to have higher molecular weight than in plasma (Fig. 5 B). As mentioned in the Introduction section, the patterns of elution of the Ss protein, after filtration of EDTA plasma or serum in Sephadex G-200 columns, are markedly different. If EDTA plasma is used, Ss appears in the ascending limb of the peak which contains 7s Ig (Fig. 5 A). If serum is used, most of the Ss is found in the exclusion volume (Fig. 5 B). In the latter case, as shown by experiments analogous to those described in Fig. 6, all high molecular weight Ss could be precipitated by an antiserum specific for Ss-bp. However, the S1p was found only in the second peak.

Another important point shown in these experiments is that once formed, the Ss/Ss-bp complexes cannot be dissociated by chelation of divalent cations. The pattern of elution of Ss when serum is passed through Sephadex G-200 columns is exactly the same whether the eluting buffer does or does not contain free Ca

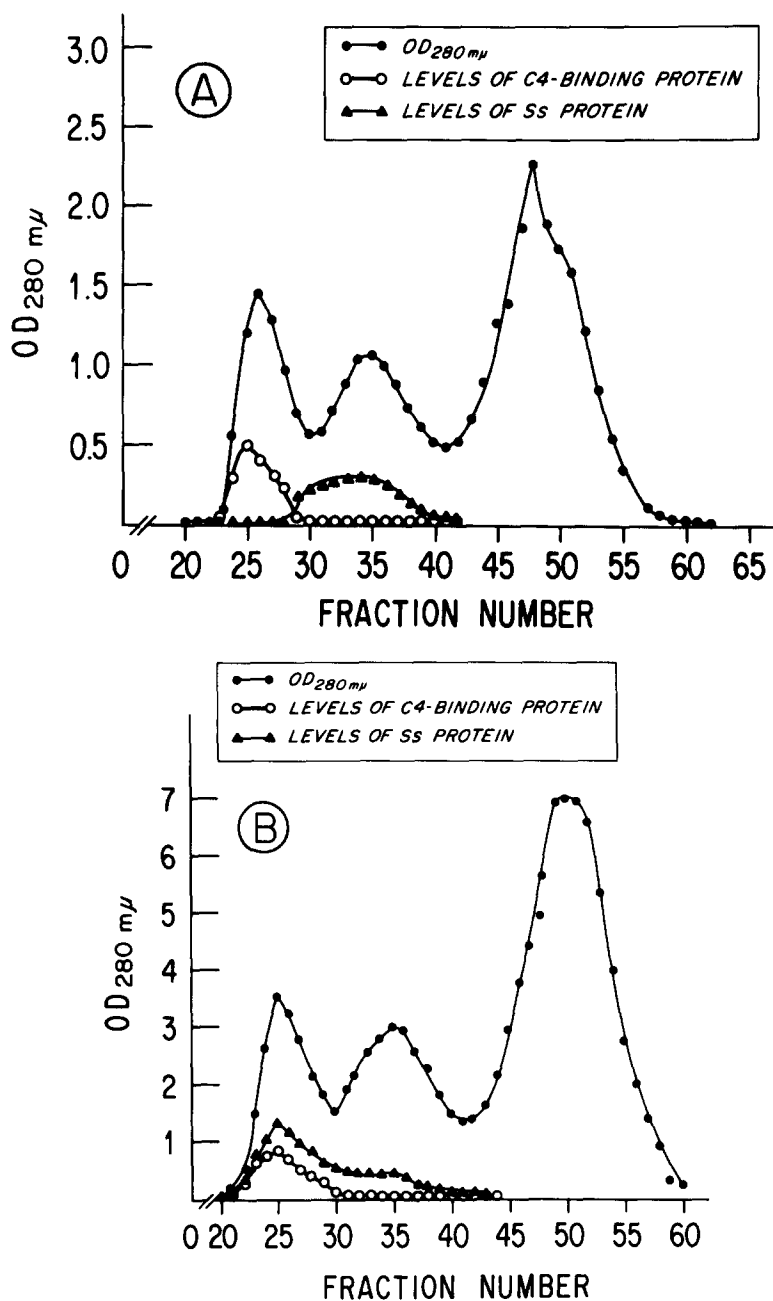


FIG. 5. (A) Sephadex G-200 gel filtration of pooled EDTA plasma from adult B10.D2 ($H-2^a$, Ss-H, Slp-positive) male mice. Buffer: PBS pH 7.4 EDTA 0.01 M. The relative concentrations of Ss-bp (C4-binding protein) and Ss were determined by RIE of each fraction by using monospecific antisera. The eluting buffer was PBS-EDTA. (B) Sephadex G-200 gel filtration pattern of pooled serum from B10.D2 mice. The eluting buffer was PBS-EDTA. The relative concentrations of Ss-bp and Ss (C4) were determined by RIE of each fraction by using monospecific antisera. Slp-positive fractions were found only in the second peak. All Ss in the first peak, but not in the second peak, could be specifically precipitated by anti-Ss-bp.

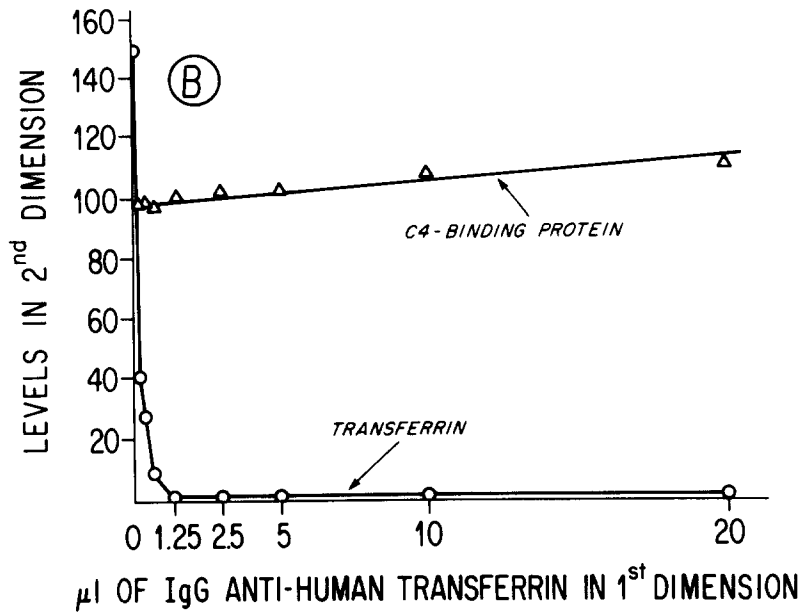
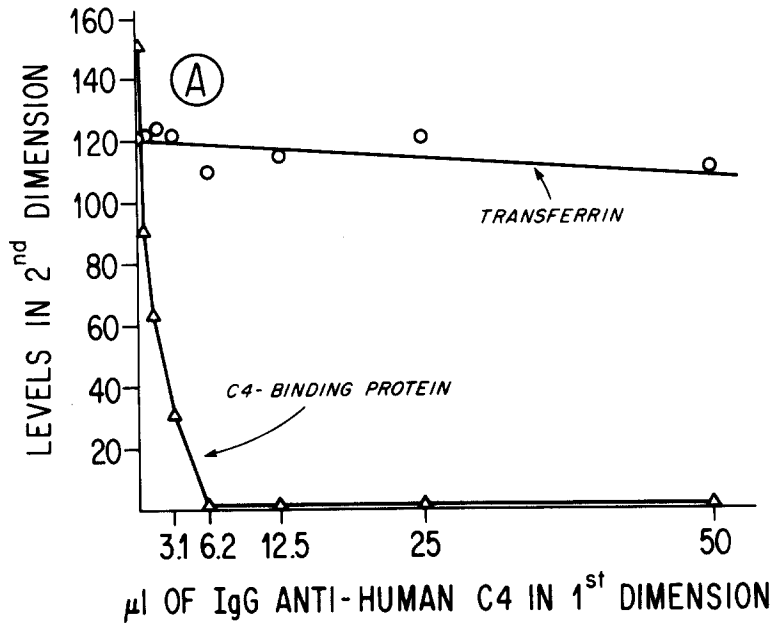


FIG. 6 A and B.

and Mg ions. Under both conditions, high molecular weight Ss determinants are found in the exclusion volume in association with Ss-bp.

(c) The formation of Ss/Ss-bp complexes in serum could also be detected by electrophoresis. The electrophoretic mobility of Ss-bp is not different in EDTA-plasma of Ss-H or Ss-L mice. In serum, however, marked differences in mobility of Ss-bp have been observed. In Ss-H mice, the Ss-bp moves faster toward the anode than in Ss-L mice (Fig. 4). These differences are a consequence of the formation of Ss/Ss-bp complexes. When Ss is high, all Ss-bp is bound to Ss and the complexes are more negatively charged than either protein in EDTA-plasma. When levels of Ss are low, most of Ss-bp remains free in the serum, and its real mobility is apparent. Indeed, as will be shown in the section below, the electrophoretic mobility of Ss-bp in serum of Ss-L mice can be shifted by the addition to mouse serum of human or guinea pig C4.

One interesting consequence of the differences in electrophoretic mobility between Ss-bp and the Ss/Ss-bp complexes is that Ss-bp appears to be a polymorphic protein in serum. A detailed genetic analysis demonstrating this pseudopolymorphism in a series of congenic mice and among the progeny of an F2 generation obtained by breeding AKR/J (Ss-L) and DBA/2J (Ss-H) is shown in Fig. 7. Without exception, the sera from mice which were Ss-L had the slow variety of Ss-bp. All Ss-H had fast Ss-bp. If the Ss/Ss-bp interactions had been unknown, these results would have indicated the presence in the *S* region of *H-2* of a structural gene controlling the electrophoretic mobility of Ss-bp, with two alleles, one of them (slow Ss-bp) linked with the Ss-L gene, and the other (fast Ss-bp) linked with the Ss-H gene.

Interaction of Ss-bp with Human C4 and Guinea Pig C4. Fig. 8 and Table I show that the electrophoretic mobility of Ss-bp changes when serum from a C3H.OL mouse (Ss-L) is preincubated at 37°C for 10 min with purified human C4. The new position of Ss-bp is similar to that found in serum of Ss-H mice (Fig. 4). The physical association between activated or native human C4 with Ss-bp in Ss-L serum was shown by the complete abrogation of the Ss-bp peak in the second dimension of a CIE if anti-human C4 was present in the agarose during the electrophoresis in the first dimension.

Ss-bp in the serum of Ss-L mice can also bind to guinea pig C4. In Fig. 9 we

FIG. 6. Simultaneous precipitation of Ss (C4) and Ss-bp from mouse serum (B10.A, *H-2^a*, Ss-H) by an antiserum specific for human C4. This antiserum cross-reacts with Ss (C4) but not with Ss-bp. Mouse serum was mixed with a standard amount of human transferrin and many samples were subjected to CIE under different conditions described below. In all cases, the second dimension was developed with a mixture of antisera to Ss-bp and to human transferrin. (A) In these plates, increasing amounts of antibodies to human C4 were present in the agarose during electrophoresis in the first dimension. As seen, the Ss-bp peak in the second dimension was eliminated. However, the transferrin peak was not affected. (B) In this series of control plates, increasing amounts of antibodies to human transferrin were present in the agarose during the electrophoresis in the first dimension. The transferrin peak, but not the Ss-bp peak, was abrogated. The slope for the regression line for Ss-bp levels is not different from zero. The rationale for choosing transferrin-anti-transferrin as a control precipitating system was based on the observation that transferrin moves slightly ahead of Ss-bp during the electrophoresis. Therefore, Ss-bp had to migrate through the transferrin-anti-transferrin lattice.

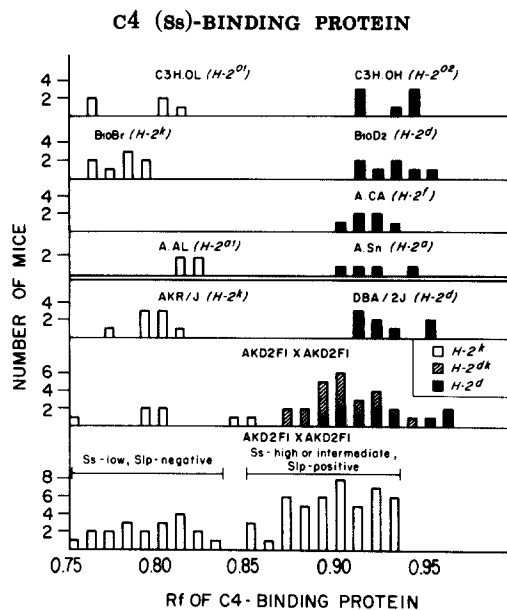


FIG. 7. Pseudopolymorphism of Ss-bp protein in the serum of congenic mice (top part of figure) or in sera from parentals and F2 mice obtained from crosses between AKD2F1 \times AKD2F1. All mice were adults, between 7 and 8 wk of age. Rf of the C4-binding protein (Ss-bp) were determined by CIE and defined as the ratio of the distances from the origin of the Ss-bp peak and transferrin peaks. The $H-2$ types of F2 mice were determined either by hemagglutination (see legend of Fig. 3) or by measuring the levels of the Ss and Slp proteins. Mice which are Ss-L and Slp⁰ are classified as $H-2^k$. The others are either $H-2^d$ or $H-2^{dk}$. As shown, the mobility of Ss-bp was strictly controlled by $H-2$ and the levels of Ss. The slow variety of Ss-bp, without exception, was observed in mice carrying the k allele in the $H-2$ region. The congenic pair C3H.OH and C3H.OL differ only in the S region of $H-2$. C3H.OH carries the d allele (Ss-H) and C3H.OL carries the k allele (Ss-L). B10.Br, A.AL, and AKR/J also carry the k allele in the S region. The A.CA ($H-2^f$, Ss-H) strain, as expected, has Ss-bp of the fast variety.

show the progressive change in the Rf of Ss-bp after the incubation of serum from C3H.OL mice (Ss-L) with increasing volumes of normal guinea pig serum. No change was observed in the presence of C4-deficient guinea pig serum. Therefore, the change in mobility of Ss-bp was C4-dependent, and, as in the experiment above with human C4, Ss-bp shifted from slow to fast electrophoretic mobility. With the highest doses of guinea pig serum, the mobility of Ss-bp was similar to that found in Ss-H serum. That in these serum mixtures mouse Ss-bp was bound to native or activated guinea pig C4, was demonstrated by the complete precipitation of Ss-bp with antibodies specific to guinea pig C4 (Table II).

Discussion

We describe here a protein of mouse serum which specifically binds to C4 of man and guinea pig, as well as to the autologous Ss (C4) protein. Its properties differ from any other known C components. In view of its high affinity for Ss and C4, we provisionally named it Ss(C4)-binding protein, or Ss(C4)-bp.

Ss-bp is a heat stable (56°C, 1 h) β -globulin, with a sedimentation coefficient in sucrose density ultracentrifugation of 10s, which is excluded on gel filtration

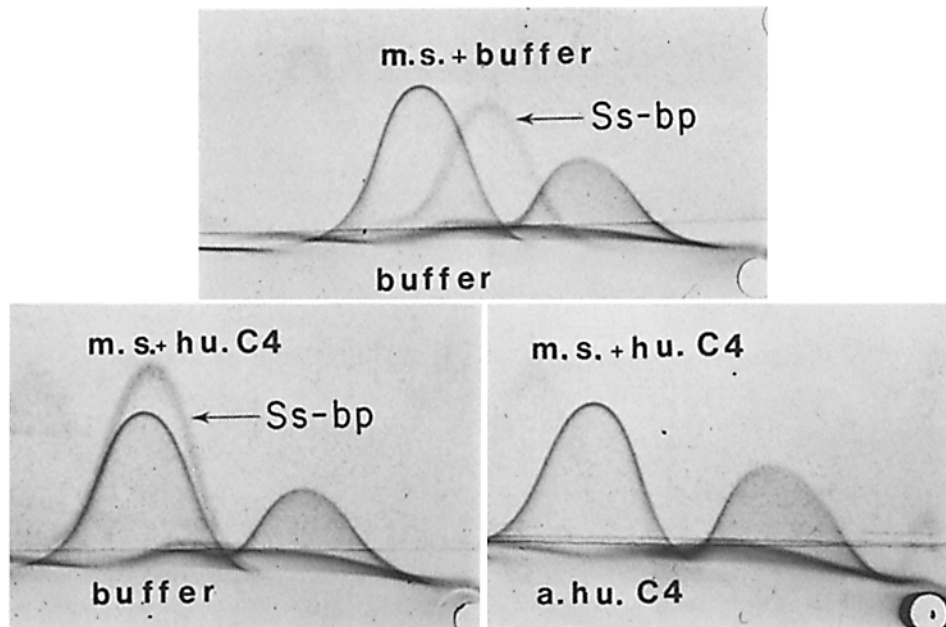


FIG. 8. Human C4-mediated shift of the electrophoretic mobility of Ss-bp in serum of C3H.OL (Ss-L) male mice. 30- μ l samples of mouse serum were mixed with human transferrin and with an equal volume either of PBS or of purified human C4 (10 μ g). The mixtures were incubated at 37°C for 10 min and then subjected to CIE. The second dimension contained antisera to human transferrin and an oligo-specific antiserum containing antibodies to Ss-bp. The top figure shows the pattern obtained in the absence of human C4 in the first run. The Ss-bp peak is the weak one in the center of the figure between the transferrin peak (left) and a contaminant (right). The figure on the bottom left shows the pattern obtained in the presence of human C4. The Ss-bp peak is now shifted and its position coincides with the transferrin peak. These patterns should be compared with those of Fig. 4. It is clear that the new position of Ss-bp coincides with that of Ss-bp in serum from Ss-H mice. The figure at the bottom right should be compared with the one at the left. It shows the complete abrogation of the Ss-bp peak when antibodies to human C4 (10 μ l) were present during the electrophoresis in the first dimension. Otherwise, conditions were identical in all plates.

in Sephadex G-200. On the basis of its ultracentrifugation in potassium bromide it is not a lipoprotein. Ss-bp is found in relatively large amounts in mouse plasma, and as is the case for other components of the C system in mice, such as C4 and C5 (3, 19) it is found in higher concentration in the serum of males (160 μ g/ml) versus females (60 μ g/ml). No significant differences in Ss-bp levels have been found among mice of *H-2^d* (Ss-H) and *H-2^k* (Ss-L) haplotypes.

Ss-bp has been purified about 200 times from plasma of Ss-L mice, by a combination of PEG-precipitation, Pevikon-block electrophoresis, and Sephadex G-200 gel filtration, with yields between 10 and 20%. The final preparation contains at least 35% of pure Ss-bp as judged from specific precipitation with a monospecific antiserum to Ss-bp. Purified Ss-bp does not react with antisera to mouse Ig, Ss, or C3. By using polyvalent antisera to mouse serum from different sources, only two unidentified contaminants have been detected in this purified Ss-bp preparation.

TABLE I
*Association between Human C4 and Ss-bp in Serum of Ss-L Mice
 Demonstrated by Cross Immunoelectrophoresis**

Loaded in anti- gen well	Antibody present during electrophoresis in first di- mension	C4-binding protein peak in the second dimension	
		Levels‡	Rf§
Mouse serum plus: Human C4	None	130 ± 3.46	0.98 ± 0.01
	Anti-human C4	Zero	—
Buffer	None	113 ± 0.00	0.79 ± 0.00
	Anti-human C4	100 ± 3.76	0.71 ± 0.01

* The experimental conditions were described in the legend of Fig. 8. Note that the formation of human C4/Ss-bp complexes caused a change in mobility of Ss-bp. The formation of the complexes is shown by the complete abrogation of the Ss-bp peak by the antibodies to human C4 in the first dimension. The numbers represent the means ± SD of triplicate plates.

‡ Levels are obtained by measuring the areas under the peaks and comparing them to the areas of different dilutions of standards.

§ Ratio between distances from the origin of the transferrin and Ss-bp peaks.

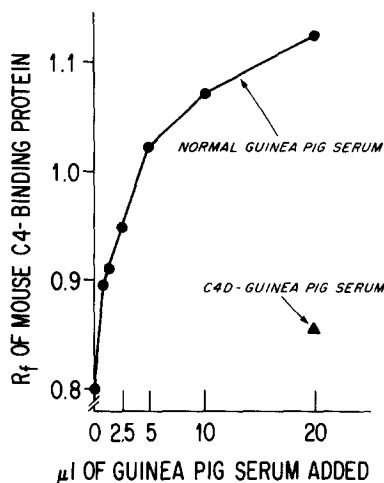


FIG. 9. Guinea pig C4-mediated shift of Ss-bp in serum of C3H.OL (Ss-L) mice. 30 μ l samples of mouse serum were mixed with increasing volumes of normal guinea pig serum and incubated at 37°C for 10 min. Subsequently, they were mixed with human transferrin and subjected to CIE. Antisera to Ss-bp and human transferrin were used in the second dimension. The figure shows a progressive shift of the electrophoretic mobility of Ss-bp (Rf) caused by addition of normal guinea pig serum, but not of C4-deficient guinea pig serum. In all cases a single symmetrical peak of Ss-bp was observed.

In mouse EDTA-plasma, Ss and Ss-bp do not seem to be associated since they can be almost completely separated by a single passage through a Sephadex G-200 column. In contrast, in serum of Ss-H mice, most or all of Ss-bp is combined to a portion of the Ss protein.

Although the formation of the Ss-bp/Ss complexes depends on the presence of Ca^{++} and/or Mg^{++} ions, once formed the bonds between these proteins cannot be

TABLE II
*Association between Guinea Pig C4 and Ss-bp in Serum of Ss-L Mice Demonstrated by Cross Immunoelectrophoresis**

Loaded in antigen well	Antibody present during electrophoresis in first dimension	C4-binding protein peak in the second dimension	
		Levels [‡]	Rf [§]
Mouse serum plus:			
Normal guinea pig serum	None	112 ± 2.89	1.03 ± 0.01
	Anti-guinea pig C4	Zero	—
C4-deficient guinea pig serum	None	151 ± 4.90	0.84 ± 0.00
	Anti-guinea pig C4	148 ± 6.03	0.83 ± 0.01

* The experimental conditions were described in the legend of Fig. 9. Note that formation of guinea pig C4/Ss-bp complexes caused a shift in the mobility of Ss-bp. The formation of the complexes is demonstrated by the complete abrogation of the Ss-bp peak when antibodies to guinea pig C4 were present during electrophoresis in the first dimension. Numbers are means ± SD of triplicate plates.

[‡] Levels are obtained by measuring the areas under the peaks and comparing them to the areas of different dilutions of standards.

[§] Ratio between distances from the origin of the transferrin and Ss-bp peaks.

reversed by chelation of divalent cations. It seems likely that the initial requirement of divalent cations for the formation of the Ss/Ss-bp bonds is related to the complement activation which follows clotting of mouse plasma. The C cascade of the mouse is known to be particularly sensitive to activation during clotting. Even after the serum is separated from the clot, the total hemolytic activity decays extremely rapidly even if the serum is maintained at low temperatures (9).

Evidence that all Ss-bp in serum of Ss-H mice is associated with Ss derives from experiments showing that specific precipitation of Ss leads to coprecipitation of all of Ss-bp (Fig. 6). A monospecific antiserum to human C4, which cross-reacted with mouse Ss, was used to precipitate Ss. Identical results were obtained by using rabbit antisera to Ss (not shown). Neither antiserum cross-reacted with Ss-bp. Control experiments showed that Ss-bp did not bind nonspecifically to unrelated antigen-antibody precipitates. In sera from Ss-L mice most of Ss-bp is free. However, these sera do contain small amounts of Ss/Ss-bp complexes, since about 10% of Ss-bp could be precipitated by antibodies to Ss. Therefore, the Ss protein from Ss-L serum is not defective in its capacity to bind Ss-bp.

As a consequence of the formation of the Ss/Ss-bp complexes, the Ss protein appears to be heterogeneous in size as determined by passage of mouse serum through Sephadex G-200 columns (Fig. 5B). The molecular weight heterogeneity of Ss in serum had been previously observed by Shreffler and his co-workers (3), and attributed to a spontaneous aggregation of Ss. We show here that all Ss found in the exclusion volume of the Sephadex G-200 column is in reality associated with Ss-bp. In view of the classical observations of Müller-Eberhard et al. (24) showing that human C4 and C2 form complexes during C activation, the possibility that Ss-bp is mouse C2 has to be considered. However, this is

unlikely for several reasons. First, C2 is a heat labile trace constituent of serum, with a sedimentation rate of approximately 4.5s (25), while Ss-bp is heat stable, present in large amounts in serum, and has a sedimentation rate of 10s. Furthermore, purified preparations of Ss-bp do not have C2 hemolytic activity.²

The association between Ss and Ss-bp can also be demonstrated by high voltage electrophoresis in agarose at alkaline pH. When Ss binds to Ss-bp, the complex becomes more negatively charged than either protein alone. Consequently, in the serum of Ss-L mice (in which Ss levels are very low and Ss-bp is mostly free) Ss-bp appears to have a slower electrophoretic mobility than in the serum of Ss-H mice (Fig. 4). The strict dependency of the mobility of Ss-bp on the levels of Ss was demonstrated both in congenic mice and in the progeny of a cross between Ss-H (DBA/2J) and Ss-L mice (AKR/J). All mice which had the *k* allele in the *S* region and therefore low levels of Ss, had the slower variety of Ss-bp protein in their serum. All other mice had the fast Ss-bp (Fig. 7). In EDTA plasma, no differences in mobilities of Ss-bp have been observed among mice of different *H-2* haplotypes.

This striking pseudopolymorphism of Ss-bp in serum highlights its specificity for an activated form of mouse C4. This specificity is also shown by the observation that the slow variety of Ss-bp can be transformed in vitro into the fast variety simply by the addition to the Ss-L serum of pure human C4, C4-sufficient guinea pig serum, but not of C4-deficient guinea pig serum. Although many enzymes and complement components are activated during clotting of mouse blood (9) all Ss-bp becomes associated only with an activated form of C4, presumably fluid phase C4b. In a subsequent publication it will be shown that, indeed, Ss-bp binds to sheep erythrocytes sensitized with IgG antibody and complement (EAC1,4b) or EAC4b, but not to EAC1 or sheep erythrocytes sensitized with IgG antibody, and that the binding is directly proportional to the number of C4b sites on the erythrocyte.²

The specific association of C4b and Ss-bp indicates a functional role for Ss-bp in the classical C system. The stoichiometry of the reaction, as well as the formation of relatively tight bonds between Ss-bp and C4b² is reminiscent of the interaction of proteinase inhibitors with serum proteinases (26). Although no proteinase activity has been associated with C4b, it participates in the assembly and probably modulates the activity of the two key C enzymes, C4b,2a and C4b,2a,3b, whose substrates are, respectively, C3 and C5. The important role of C4b in these complex enzymes is suggested by the observation that although their catalytic site resides in C2a (27), after dissociation from C4b, C2a retains its capacity to hydrolyze synthetic esters but does not have C3-convertase activity.

Three regulators of the complement cascade have been previously characterized, the C1-inactivator (28-30), C3b-inactivator, presumably identical to C4b-inactivator (31, 32) and β 1H (33, 34). C1-inactivator is a glycoprotein with a mol wt close to 100,000 which stoichiometrically inhibits the activity of C1s. Although its spectrum of activity is large, since it also inhibits enzymes of the clotting, fibrinolysis, and kallikrein systems, no interaction with C4b has been reported. C3b-inactivator is a 5.5S β 2 globulin with a mol wt of approximately 100,000 daltons, present in trace amounts in serum which splits C3b (or C4b)

² I. Gigli, A. Ferreira, and V. Nussenzweig. Manuscript in preparation.

into two fragments. Unlike Ss-bp, C3b-inactivator does not combine stoichiometrically with the substrate. β 1H is a heat stable 6.4s globulin of human serum which potentiates the inactivation of C3b by C3b-inactivator. It has been reported that β 1H binds stoichiometrically to EAC4b and EAC4b3b cells. β 1H has a modulating activity on the formation of cell-bound alternative pathway C3-convertase enzymes $\overline{\text{EAC4b3bB}}$ and $\overline{\text{EAC4b3bBP}}$ by increasing their rate of decay. Although the mechanism of action of β 1H is not fully understood, preliminary evidence suggests that in contrast to Ss-bp, its primary affinity is for C3b (33) rather than C4b. In short, the characteristics of Ss-bp are distinct from other known C components. It should be pointed out that its properties bear some resemblance to an incompletely characterized 10s globulin in human serum which enhances the destruction of C4b by C4b-inactivator (35). This 10s globulin has not been isolated, and its similarity with Ss-bp remains to be established.

The present results also establish a functional analogy between the Ss protein and C4 from two different species, man and guinea pig. Other investigators attempted to demonstrate that Ss functions as C4 in the hemolytic sequence, but met with only partial success (8, 9). The difficulties stemmed from technical problems encountered in the titration of mouse C in general, and in the titration of C4 in particular. It remains to be established whether these difficulties are related to the presence of high concentrations of Ss-bp in mouse serum.

Finally, our results may contribute to the understanding of the relation between Ss and the Slp described by Passmore and Shreffler (36, 37). Slp is a serum protein, with a mol wt of 180,000 daltons, found in some strains of Ss-H mice, but not in Ss-L mice. The interesting observation has been made that, with one exception (38, 39) Slp is absent in females or castrated males. Several lines of evidence show that Ss and Slp bear common antigenic determinants. It appears that all Slp molecules have Ss determinants, but only some Ss molecules react with the alloantiserum to Slp. For this and other reasons it has suggested that Slp constitutes a subclass of Ss (39). Genetic studies have also shown that the Slp locus is localized in the *S* region of *H-2*, very closely linked to Ss (36). The present results suggest a functional difference between Ss and Slp molecules. Indeed, after Sephadex G-200 filtration of serum from B10.D2 male mice (Ss-H, Slp-positive), most Ss molecules, but no Slp determinants, were found in the exclusion volume associated with Ss-bp (legend of Fig. 5B). In contrast, as also shown by Hansen et al. (40) Slp determinants were found almost exclusively in the second peak, which contains low molecular weight Ss.

The most likely explanation for these findings is that Slp has a lower affinity for Ss-bp than Ss. Perhaps only Ss is activated during clotting, or alternatively, both Ss and Slp may be activated, but only Ss has an affective binding site for Ss-bp. Because of the current interest in the interaction between products of the major histocompatibility complex, the intriguing relationship between Ss, Slp, and Ss-bp, and their role in the C system, is under investigation in our laboratory.

Summary

A new component of the complement (C) system, with a specific binding affinity for the activated Ss-protein (C4) has been identified in mouse serum.

This protein, named Ss- (or C4)-binding protein (Ss-bp), was purified about 200 times from mouse plasma.

Ss-bp is a heat stable (56°C, 60 min) β -globulin with a sedimentation coefficient in sucrose density ultracentrifugation of 10s. Its concentration in serum of adult male and female mice is 160 and 60 $\mu\text{g/ml}$, respectively.

In EDTA-plasma, Ss and Ss-bp are not associated and can be separated by chromatography in Sephadex G-200. However, in serum Ss-bp binds tightly to Ss. The bonds between these proteins cannot be reversed by chelation of divalent cations.

As a consequence of the formation of Ss/Ss-bp complexes, the properties of Ss-bp appear to be quite different in serum of mice with high (Ss-H) or low (Ss-L) levels of Ss-protein. In Ss-H serum, all of Ss-bp is bound to Ss. In Ss-L serum, Ss-bp is mostly free. Because the electrophoretic mobilities of free and complexed Ss-bp are quite different, Ss-bp appears to be polymorphic in serum (but not in EDTA-plasma). The strict dependency of the apparent electrophoretic mobility of Ss-bp on the levels of Ss in serum was demonstrated in a series of congenic mice and among the progeny of a cross between Ss-H and Ss-L strains of mice. Without exception, the slow and fast varieties of Ss-bp were associated with the Ss-L and Ss-H traits.

Ss-bp of the slow variety can be transformed into the fast variety by addition of pure human C4, or C4-sufficient guinea pig serum, to Ss-L serum. In both instances Ss-bp formed stable complexes with C4 or a C4-derived peptide. These findings highlight the binding specificity of Ss-bp for the fourth component of the complement system, and in addition they demonstrate a functional homology between the Ss-protein and C4 from two different species.

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