

# STRUCTURAL AND FUNCTIONAL DIFFERENCES BETWEEN THE H-2 CONTROLLED Ss AND Slp PROTEINS

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The S region of the H-2 complex controls the serum levels of two proteins, Ss and Slp, which are closely related antigenically (1-4). Several observations suggest that the Ss protein may be part of the complement system and homologous to C4. F(ab')<sub>2</sub> fragments of antibody to Ss inhibit the hemolytic activity of mouse serum (5) and Ss antigenic determinants are incorporated into immune complexes after complement activation (6). Furthermore, Ss shares some physicochemical properties with C4 of other species (7, 8) and some antisera to human C4 cross-react with Ss (9). As for the Slp protein, its function is unknown.

The present report demonstrates that Ss and Slp can be distinguished functionally by (a) their role in the hemolytic complement system, (b) their susceptibility to C1s enzymatic activity, and (c) their affinity for the C4-binding protein (C4-bp)<sup>1</sup> (10, 11). In addition, new information regarding structural characteristics of these two proteins is presented.

## Materials and Methods

**Reagents.** Agarose was obtained from Behring Diagnostics, American Hoechst Corp., Somerville, N. J.; N,N'-methylene-bisacrylamide, N,N,N',N'-tetramethylethylenediamine, ammonium persulphate, and sodium dodecyl sulfate (SDS), were obtained from Bio-Rad Laboratories, Richmond, Calif.; diisopropylfluorophosphate (DFP) from Calbiochem, San Diego, Calif.; bovine serum albumin (BSA) from Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.; *Staphylococcus aureus*, Cowan I, (IgG-sorb), New England Enzyme Center, Boston, Mass.; Nonidet P-40 (NP-40), Shell Chemicals Co., London, England; ovalbumin,  $\beta$ -galactosidase (from *Escherichia coli*), catalase (from beef liver), myoglobin (from horse heart), alcohol dehydrogenase (from yeast), and phosphorilase A (from rabbit muscle) were obtained from Sigma Chemical Corp., St. Louis, Mo.; ferritin albumin, and Sephadex G-200 from Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.; DEAE-cellulose (DE-52) from Whatman Chemicals, Div. W & R Balston, Maidstone, Kent, England. Pevikon G-870 was obtained from Stockholm Superfosfat Fabriks A.-B., Stockholm, Sweden; ammonium sulphate was obtained from Schwarz/Mann Div., Becton Dickinson & Co., Orangeburg, N. Y.

**Buffers.** Phosphate-buffered saline (Dulbecco's PBS), Grand Island Biological Co., Grand Island, N. Y. PBS-EDTA: PBS containing 2 mM Na<sub>2</sub>H EDTA, pH 7.55. Barbitol buffer, pH

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<sup>1</sup> *Abbreviations used in this paper:* BSA, bovine serum albumin; CIE, crossed immunoelectrophoresis; C4-bp, C4-binding protein; DFP, diisopropylfluorophosphate; DGVB<sup>++</sup>, GVB mixed with an equal volume of 5% dextrose in water containing 0.00015 M MgCl<sub>2</sub> and 0.00015 M CaCl<sub>2</sub>; DOC, deoxycollate; GVB<sup>++</sup>, VBS containing 1% gelatin, 0.005 M MgCl<sub>2</sub>, and 0.00015 M CaCl<sub>2</sub>; NET, sodium chloride-EDTA-Tris buffer; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RIE, rocket immunoelectrophoresis; SDS, sodium dodecyl sulfate; VBS, isotonic veronal-buffered saline.

8.6, containing 0.023 M sodium barbital, 0.037 M barbituric acid, and 0.002 M Na<sub>3</sub>H EDTA. Isotonic veronal-buffered saline (VBS<sup>-</sup>), pH 7.4, containing 0.025 M barbital, NaCl added to achieve a conductivity of 7.4 mS at 0°C, and 0.002 M Na<sub>3</sub>H EDTA, (VBS-EDTA). GVB<sup>++</sup>: VBS containing 0.1% gelatin, 0.005 M MgCl<sub>2</sub>, and 0.00015 M CaCl<sub>2</sub>. DGVB<sup>++</sup>: GVB<sup>++</sup> mixed with an equal volume of 5% dextrose in water containing 0.001 M MgCl<sub>2</sub> and 0.00015 M CaCl<sub>2</sub>. Sodium chloride-EDTA-Tris buffer (NET), containing 0.15 M NaCl, 0.005 M Tris, 0.002 M Na<sub>3</sub>H EDTA, and 0.02% wt/vol sodium azide, pH 7.4. NET-0.5% NP-40: NET containing 0.5% NP-40 vol/vol. NET-0.05% NP-40: NET containing 0.05% vol/vol NP-40. Deoxycollate buffer (DOC buffer): containing 0.02 M Tris-HCl, pH 7.5, 0.05 M NaCl, and 0.05% wt/vol deoxycollate.

*Animals.* 4-8-mo-old mice were used. DBA/2J, DBA/1J, C57B1/10, AKR/J male and female mice were purchased from Jackson Laboratory, Bar Harbor, Maine. C3H.OH, C3H.OL, and C3H.WSlp, both sexes, were kindly supplied by Dr. D. C. Shreffler, Washington University, St. Louis, Mo., and subsequently bred in our animal facilities. MP males were donated by Dr. G. Biozzi, Fondation Curie, Paris, France. Mice were bled from the axillary vein and artery into tubes containing Na<sub>3</sub>H EDTA to a final concentration of 0.01 M, or allowed to clot for 10 min at room temperature and 1 h in the ice. Immediately after centrifugation, 0.005 M ε-aminocaproic acid and 0.004 M DFP were added to both serum and plasma, which were used on the same day.

*Antisera.* Rabbit anti-mouse Ss donated by Dr. H. Passmore, Rutgers University, New Brunswick, N. J. (12) was used. This antiserum reacted against a single component of mouse serum by crossed immunoelectrophoresis. Mouse antisera to mouse Slp and C5 were obtained as previously described (12, 13). Goat anti-human C4, strongly cross-reactive with mouse Ss and weakly cross-reactive with mouse Slp, was purchased from Meloy Laboratories Inc., Springfield, Va. A rabbit antiserum to human C3, strongly cross-reactive with mouse C3, was prepared by injecting 100 μg of pure human C3 incorporated in complete Freund's adjuvant, into rabbits. A rabbit antiserum to mouse C4-bp was prepared as described (10). The IgG fractions of normal mouse serum or mouse antiserum to mouse C5 and Slp were prepared by passage of the sera through a Sephadex G-200 column. The second protein peak containing IgG was pooled and an equal volume of saturated ammonium sulphate was added. The precipitate was dissolved in PBS and further purified by Pevikon block electrophoresis (Mercer Consolidated Corp., Yonkers, N. Y.) (14). Anti-C5 and anti-Slp containing fractions were detected by rocket immunoelectrophoresis, pooled, and dialyzed against veronal-buffered saline.

The IgG fractions of goat anti-human C4, and of rabbit anti-human C3, cross-reactive with mouse C3, were obtained by fractionation of whole antisera in DEAE-cellulose columns.

Immunoprecipitation of radiolabeled Ss and Slp: fractions containing Ss and Slp were radiolabeled according to a modification of a previously described method (15): 25 μl of 0.8 M Tris-HCl buffer, pH 7.5, were added to an aqueous solution of 2 mC <sup>125</sup>I, New England Nuclear, Boston, Mass., followed by 50 μl of the Ss/Slp containing sample and 25 μl of chloramine T (0.5 mg/ml). The mixture was incubated for 2 min at room temperature; 25 μl of sodium metabisulfite (0.5 mg/ml) and 0.5 ml PBS were added to stop the reaction. The labeled material was dialyzed against PBS for 24 h and mixed with 0.1 ml of 10 mg/ml BSA.

Immunoprecipitation was performed as follows: ≈10<sup>7</sup> cpm of the radiolabeled samples containing Ss and Slp were treated with the IgG fractions of the following antisera: mouse anti-mouse Slp, rabbit anti-mouse Ss, or goat anti-human C4. As controls, samples were treated with IgG fractions of normal rabbit serum, of rabbit anti-human C3, or normal mouse serum. The samples were incubated 2 min in the ice and immune complexes removed by addition of 100 μl of a 50% vol/vol *Staphylococcus aureus* suspension in PBS-EDTA (16) followed by 2 min incubation in the ice, with mixing every 30 s. The tubes were centrifuged at 2,000 g for 15 min and the pellets washed 5 times with 1.5 ml DOC buffer. The specific radioactivity bound to the bacteria was calculated, and the bound complexes were eluted by incubation at 90°C for 3 min with Tris-HCl buffer, 0.08 M, pH 6.8, containing 2% SDS, 6 M urea, and 10% glycerol. After centrifugation, the supernates were counted and specific recoveries calculated. The eluates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) slab electrophoresis under reducing and nonreducing conditions. The gels were stained, dried, and radioautographed for 24-48 h at -70°C, using Kodak X-omat R film (Eastman Kodak, Rochester, N. Y.).

*Immunoelectrophoresis.* Crossed-(CIE) and rocket-(RIE) immunoelectrophoresis were per-

formed as previously described (17, 18), using 5 × 5 cm glass plates. The levels of antigen measured by RIE were calculated by comparing the height of the peaks generated by the samples with that of serially diluted standards which were included in each plate.

*Polyacrylamide Gel Electrophoresis.* SDS-PAGE was performed as previously described (19). In all cases, a 3% stacking gel and 6% and 7.5% running gels were used for nonreducing and reducing conditions, respectively. Approximate molecular weight determinations under reducing conditions were calculated using the following markers as standards: yeast alcohol dehydrogenase (41,000), beef liver catalase (60,000), BSA (68,000), rabbit muscle phosphorilase (94,000) and *E. coli*  $\beta$ -galactosidase (130,000). When nonreducing conditions were necessary, three molecular weight markers were used, catalase, albumin, and ferritin (half U:220,000 daltons).

*C4 hemolytic titration.* Sheep or ox erythrocytes were coated with anti-sheep or anti-ox hemolysin made in rabbits (EA). EAC $\bar{1}$  cells were prepared by mixing EA in DGVB<sup>++</sup> with an equal volume of partially purified guinea pig C1, diluted to provide 250 effective molecules/cell. Effective molecular titrations of mouse C4 either in mouse serum, plasma, or pools of fractions obtained from Sephadex G-200 columns were performed by incubating 0.5 ml EAC $\bar{1}$ ,  $1 \times 10^8$ /ml, with an equal volume of serial dilutions of the material to be tested. After incubation for 15 min at 30°C the reaction was brought to completion by the addition of a 1:100 dilution of C4-deficient guinea pig complement, to which oxydized human C2 had been added to overcome species incompatibility between mouse C4 and guinea pig C2. After 60 min at 37°C, the hemoglobin released was measured in a spectrophotometer at E/415 nm. Titers were calculated by plotting serum dilutions vs.  $y/1 - y$  ( $y = \% \text{ lysis}/100$ ), and determining the dilution giving  $y/1 - y = 1$ .

*C $\bar{1}$  treatment of Ss and SIp.* 10  $\mu$ l of purified C $\bar{1}$  (1.4 mg/ml in DGVB<sup>++</sup>), prepared as described in reference 20, were added to 100  $\mu$ l of the Ss/SIp-containing sample. A control tube received an equal volume of buffer. Both tubes were incubated 1 h at 37°C. The effect of C $\bar{1}$  on Ss or SIp was assessed functionally, in CIE and by SDS-PAGE under reducing and nonreducing conditions.

*Statistical Analysis.* The effect on C4 hemolytic activity of removal of Ss or SIp antigens was assessed by determining the correlation coefficient ( $r$ ) between two variables (depletion of antigen vs. decrease in hemolytic activity) after regression analysis by the least squares method.

## Results

*Pattern of Elution from Sephadex G-200 Columns of Ss and SIp Antigens and C4 Hemolytic Activity.* Fig. 1 shows the pattern of elution of the Ss and SIp antigens and C4 hemolytic activity. In plasma (Fig. 1 A) the bulk of Ss antigen and C4 hemolytic activity (shaded area) appear in the ascending portion of the second protein peak. In serum, most of the hemolytic activity is lost and the Ss antigen is now found in the first peak, superimposed with C4-bp. SIp elutes in the second peak in both serum and plasma.

Those plasma fractions which contained C4 hemolytic activity were pooled and used as a source of Ss and SIp in most of the experiments described. To remove contaminating IgG, the pool was treated with 10% vol/vol *S. aureus* for 1 h in ice. The pretreatment did not affect either the C4 hemolytic activity or the amount of Ss or SIp antigens.

*Relationship between the Ss Protein and C4 Hemolytic Activity.* To determine whether there was a relationship between the Ss or SIp proteins and C4 hemolytic activity, the Ss/SIp positive pooled fractions from the chromatographic separation of plasma from male and female mice were treated with IgG preparations from mouse anti-mouse SIp and goat anti-human C4 which cross-reacted with mouse Ss. This cross-reactive antiserum (rather than rabbit anti-Ss) was used because most antisera to Ss are polyspecific, and after the necessary absorptions, they may contain soluble immune complexes. However, the depletion of Ss antigen by the cross-reactive antiserum was

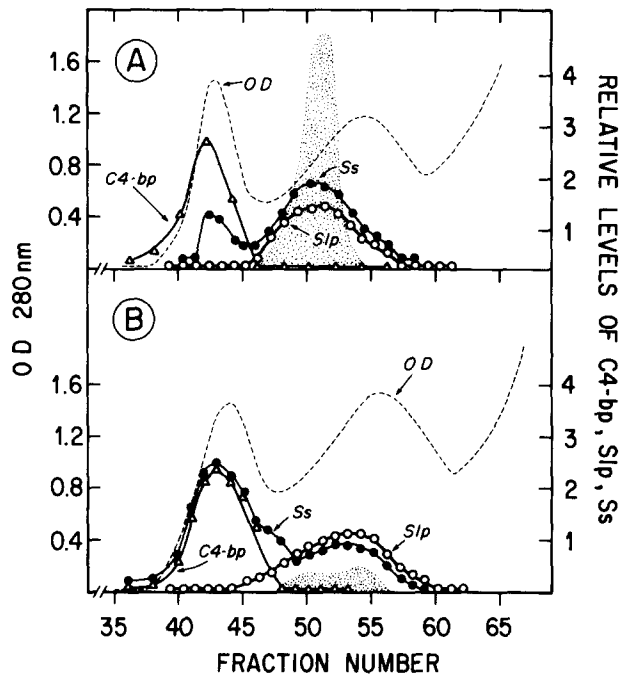


FIG. 1. Sephadex G-200 gel filtration of pooled EDTA-plasma (A) or serum (B) from adult DBA/2J (H-2<sup>d</sup>, SsH-SIp positive) male mice. Buffer VBS<sup>-</sup>, pH 7.4, 7.4 mS at 0°C, containing 0.002 M NH<sub>3</sub> EDTA and 0.005 M ε-amino-caproic acid. Relative concentrations of C4-bp, Ss, and SIp were determined by RIE. C4 hemolytic activity is shown in the shaded area.

C4-bp was found in the first peak in serum and plasma. SIp and C4 hemolytic activity were found in the ascending limb of the second peak. After fractionation serum contained only 7-8% of the C4 hemolytic activity of plasma. In plasma, the bulk of the Ss antigen was found in the second protein peak, but in serum, it was found in the first peak together with C4-bp.

Similar results were obtained with serum and plasma from C3H.WSlp and MP males.

evaluated by RIE using monospecific rabbit anti-Ss. Control samples received either IgG from rabbit anti-human C3, which cross-reacted with mouse C3, or from mouse anti-mouse C5. After removal of the immune complexes (by treatment with *S. aureus*), Ss or SIp levels, and C4 hemolytic activity were measured in the supernates. Fig. 2 shows that specific removal of Ss, correlated almost perfectly ( $r = 0.99$ ,  $P < 0.001$ ), with the decrease in C4 hemolytic activity. Fig. 3 shows the results of hemolytic assays in SIp-depleted fractions. In contrast with the previous results, we found no correlation between SIp levels and C4 hemolytic activity. Removal of unrelated antigens by anti-C3 (Fig. 2) and anti-C5 (not shown) did not affect the levels of Ss antigen or C4 hemolytic activity.

To further demonstrate the correlation between Ss levels and C4 hemolytic activity, these two parameters were measured in plasma and serum from phenotypically negative SIp mice from different strains. As shown in Fig. 4, the correlation coefficient ( $r$ ) between these two variables was 0.94 ( $P < 0.001$ ). When sera or plasma from SIp-positive strains were included,  $r = 0.74$ ,  $0.01 > P > 0.001$ . This decrease in significance is to be expected, because SIp does not have hemolytic activity although it is recognized by antisera to Ss.

*Structure of Ss and SIp and Susceptibility to the Enzymatic Activity of C1̄.* Next, we evaluated the effect of C1̄ on the function of mouse C4. Duplicate samples of the pool

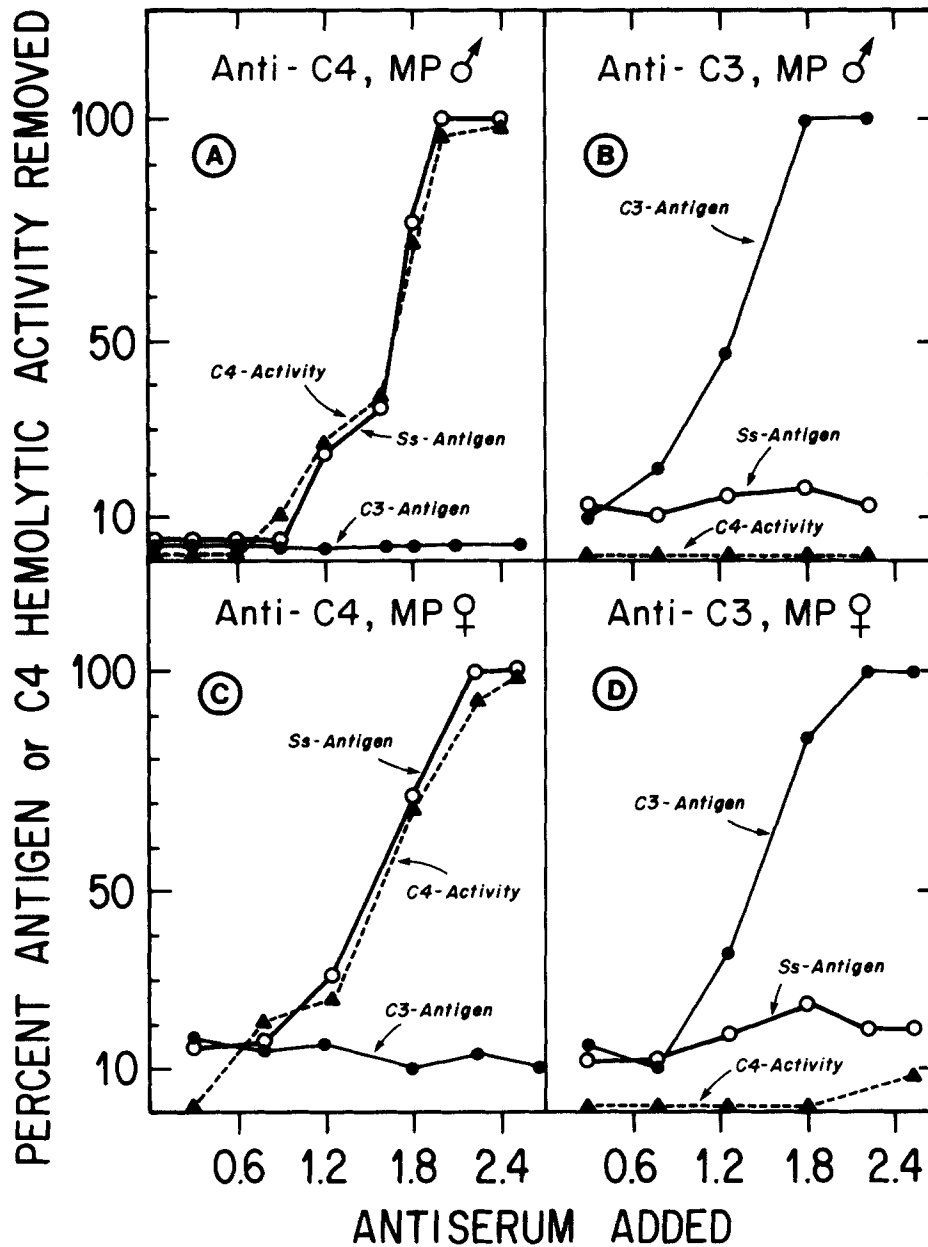


FIG. 2. Correlation between Ss antigen and C4 hemolytic activity. Tubes from the ascending limb of the second protein peak of Sephadex chromatography (Fig. 1 A) were pooled and 100- $\mu$ l samples were incubated with IgG anti-human C4, which is cross-reactive with Ss (A and C), and IgG anti-human C3, which is cross-reactive with mouse C3 (B and D). After removal of the immune complexes by treatment with *S. aureus*, the levels of Ss and C3 antigens were measured by RIE and the degree of C4 hemolytic activity determined.

The correlation coefficient between the levels of Ss antigen and C4 hemolytic activity is 0.99 for males (A) and 0.98 for females (C), for seven and four degrees of freedom, respectively ( $P < 0.001$  in both cases).

Removal of an unrelated antigen (B and D) did not affect the levels of Ss nor the C4 hemolytic activity. Similar results were obtained with DBA/2J and C3H.WSlp male mice.

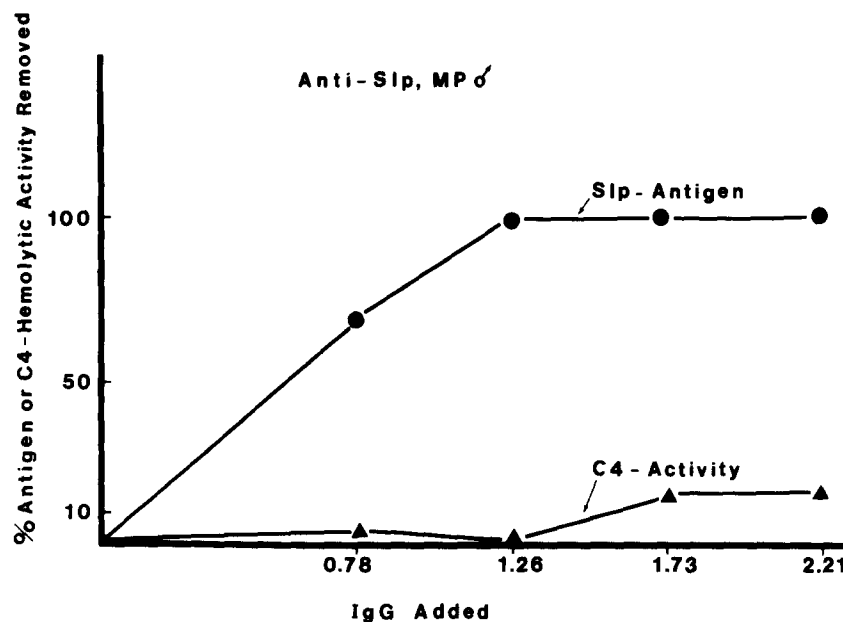


FIG. 3. Lack of correlation between the Slp antigen and C4 hemolytic activity. The source of Ss/Slp positive fractions was described in the legend of Fig. 2. 100- $\mu$ l samples were incubated with IgG anti-Slp. After removal of the immune complexes with *S. aureus*, the levels of Ss and Slp antigens were determined by RIE and C4 activity measured. 100% of the Slp antigen could be removed without affecting the C4 hemolytic activity.

from the G-200 column, containing both proteins, and which had a C4 hemolytic titer of 3,000 U/ml, were treated with purified C $\bar{1}$  or with buffer. After C $\bar{1}$  treatment, C4 hemolytic activity was totally abolished.

To determine the effect of C $\bar{1}$  on the structure of Ss and Slp, samples were radiolabeled and immunoprecipitated with either anti-human C4, which cross-reacts strongly with Ss and weakly with Slp (see below), or with anti-Slp. The immune-complexes were bound to *S. aureus*. After repeated washings, the radiolabeled Ss and/or Slp molecules, which remained bound to the bacteria, were eluted and analyzed by slab SDS-PAGE and radioautography.

Under nonreducing conditions (Fig. 5) native Ss and Slp have a mol wt of 205,000 daltons (tracks 1 and 3). C $\bar{1}$ -treated Ss and Slp molecules are shown in tracks 2 and 4. It is clear that C $\bar{1}$  cleaved the Ss molecules, resulting in the loss of a fragment of 7,000–8,000 daltons, although it had no effect on Slp. Tracks 5 and 6 are controls for nonspecific precipitation with normal rabbit and normal mouse serum.

Fig. 6 shows the polypeptide chains of Ss and Slp after reduction and alkylation. Native Slp has three chains, with apparent mol wt of 105,000, 74,500, and 32,000 daltons, respectively (track 3). The calculated sum of the mol wt of these three chains is 211,500 in agreement with that observed for unreduced Slp (Fig. 5). Native Ss, immunoprecipitated by goat anti-human C4, is shown in track 2. Similarly to Slp, it has three chains but their mol wt are different: 98,000, 77,000, and 34,000 daltons. This antiserum to C4 appears not to have recognized the Slp molecules. In reality, the antiserum to human C4 does cross-react with Slp, and the immunocomplexes bind to *S. aureus*. However, after multiple washings, the Slp molecules dissociate from the

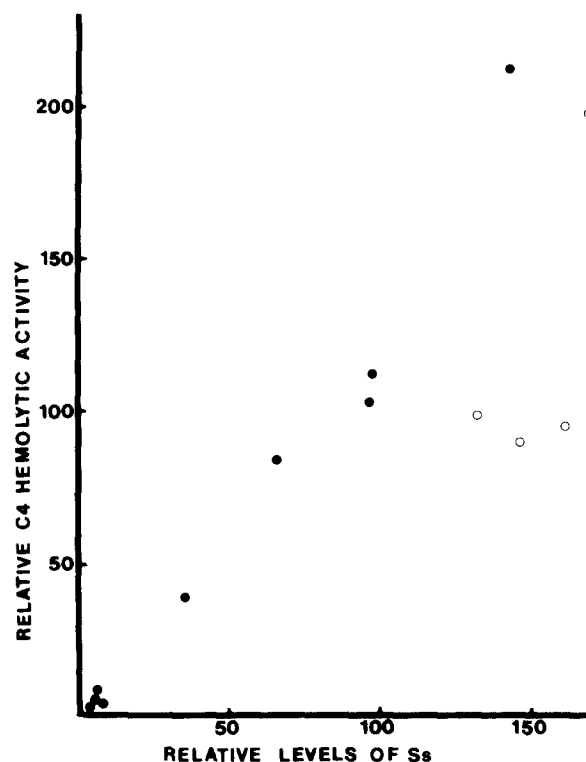


FIG. 4. Correlation between the levels of S<sub>s</sub> and C4 hemolytic activity in plasma from different strains of mice. Bleedings were done into plastic tubes containing 0.005 M  $\epsilon$ -aminocaproic acid and 0.010 M NaH<sub>3</sub>EDTA. After separating the plasma, DFP was added to a concentration of 0.004 M. C4 hemolytic activity and levels of S<sub>s</sub> were determined in pools of plasma from three mice per strain.

The closed circles represent plasma from mice which do not contain S<sub>lp</sub> antigen. The four closed circles near the origin are from two S<sub>s</sub>-low strains (AKR/J and C3H.OL), males and females. The other five closed circles represent, in the order of increasing hemolytic activity, the plasma from C57B1/10Sn males, C3H.OH females, and MP females. The open circles represent plasma from S<sub>lp</sub>-positive mice: C3H.WSlp, males and females, C3H.OH males, and MP males. The correlation coefficient between the levels of S<sub>s</sub> and C4 hemolytic activity in the S<sub>lp</sub> negative samples was 0.94 ( $P < 0.001$  for seven degrees of freedom).

When the four S<sub>lp</sub> positive samples were included, the correlation coefficient decreased to 0.74 ( $0.01 > P > 0.001$ , for 11 degrees of freedom).

Similar levels of C4 hemolytic activity were obtained in serum samples from the same animals, provided  $\epsilon$ -aminocaproic acid, diisopropyl-fluorophosphate and NaH<sub>3</sub>EDTA were added immediately after clotting.

antibodies although the S<sub>s</sub> molecules remain firmly bound.

The effect of C1 on S<sub>s</sub> and S<sub>lp</sub> is also shown in Fig. 7, tracks 2 and 6. No changes in the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chains of S<sub>lp</sub> were detected. In contrast, the  $\alpha$ -chain of the S<sub>s</sub> protein was cleaved, and a 90,000-mol wt fragment ( $\alpha'$ ) was observed. The  $\alpha$ -,  $\beta$ - and  $\gamma$ -chains of both S<sub>s</sub> and S<sub>lp</sub> molecules which were immunoprecipitated with an antiserum to S<sub>s</sub>, are shown in tracks 3 and 4. Antisera to S<sub>s</sub> do not distinguish between S<sub>s</sub> and S<sub>lp</sub> molecules, thus six distinct polypeptide chains are seen. Again, it is clear that only the  $\alpha$ -chain of S<sub>s</sub> is cleaved by C1 (track 4).

Finally, we asked whether genotypically S<sub>lp</sub>-negative mice have S<sub>lp</sub>-like molecules in their serum. Until recently, the presence or absence of S<sub>lp</sub> could only be ascertained

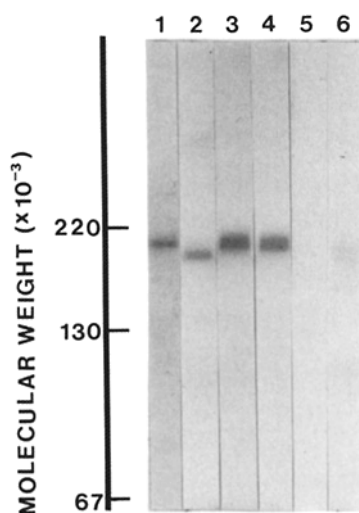


FIG. 5. Effect of  $C\bar{1}$  on Ss and Slp molecules as detected by SDS-PAGE. Tubes from the ascending limb of the second protein peak of Sephadex G-200 chromatography (Fig. 1 A) were pooled. A sample was radiolabeled with  $^{125}I$  and 100  $\mu$ l incubated with 14  $\mu$ g (10  $\mu$ l) of purified human  $C\bar{1}$ . Control was incubated with an equal volume of buffer. After incubation, each sample was divided in four aliquots and treated with IgG obtained from anti-human C4 (cross-reactive with Ss), anti Slp, normal rabbit serum, and normal mouse serum. Immune complexes were bound to *S. aureus*, eluted with SDS-urea, and subjected to SDS-PAGE under nonreducing conditions.

Tracks 1-6 show, respectively: native Ss, Ss treated with  $C\bar{1}$ , Slp, Slp treated with  $C\bar{1}$ , and controls with normal mouse serum and normal rabbit serum. It is clear that  $C\bar{1}$  splits Ss but not Slp.

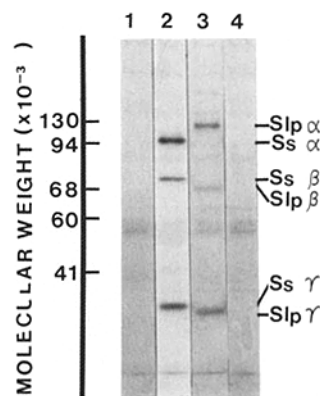


FIG. 6. Structure of Ss and Slp molecules as determined in SDS-PAGE. Experimental conditions were as described in Fig. 5, except that  $C\bar{1}$  treatment was omitted and a 7% SDS-PAGE was run under reducing conditions. Tracks 1 and 4 show the results of controls with normal rabbit serum and normal mouse serum, respectively. Tracks 2 and 3 show the results of immunoprecipitation with IgG anti-human C4 and anti-Slp, respectively. The  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chains of Ss and Slp have different molecular weights. It also appears that the antiserum to human C4 did not immunoprecipitate Slp molecules.

by reactivity with the alloantiserum. No alloantiserum has been prepared which recognizes the allelic product which might be present in Slp-negative mice. The present results show an additional way of identifying Slp-molecules, because they are structurally different from Ss molecules. We attempted to immunoprecipitate Slp-like molecules from serum of C57 B1/10 and A.CA mice. (Ss-high, Slp negative) with



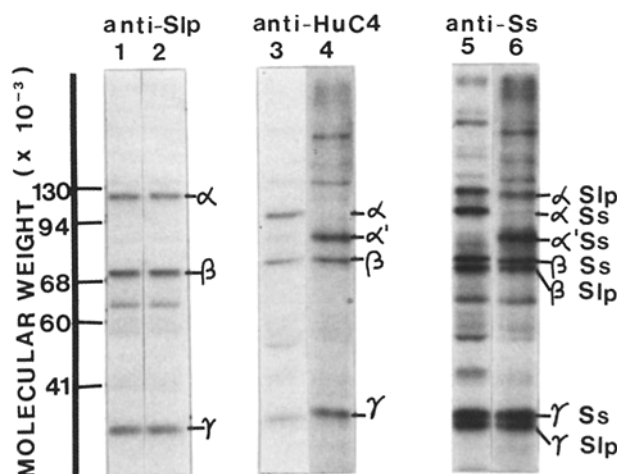


FIG. 7. Assessment of C $\bar{1}$  effect on the polypeptide chains of Ss and Slp. Experimental conditions were as in Fig. 5. A 7% SDS-PAGE was run under reducing conditions. Immunoprecipitation was performed with antisera to human C4, Slp, and Ss. Anti-human C4 (track 3) did not precipitate Slp. Anti-Ss (track 5) recognized Ss and Slp. Only the specific bands,  $\alpha$ ,  $\beta$ , and  $\gamma$ , are indicated. C $\bar{1}$  treatment did not affect the Slp chains, as shown in tracks 2 and 6, but cleaved the  $\alpha$ -chain of Ss with loss of an 8,000-dalton fragment (tracks 4 and 6). Track 6 also shows that, out of six specific bands, the only affected by C $\bar{1}$  treatment was Ss- $\alpha$ . Track 5 clearly shows the differences in molecular weight between the  $\alpha$ -,  $\beta$ - and  $\gamma$ - chains from Ss and Slp. The same results were obtained with two other SsH-Slp positive strains (C3H.WSlp and C3H.OH). When anti-Ss was used to immunoprecipitate Slp-negative strain (C57 B1/10 and A.CA), only three specific bands were seen, and their apparent molecular weights coincided with the bands precipitated with anti-human C4.

antiserum to Ss. By SDS-PAGE, only three bands were seen, and their molecular weight coincided precisely with those obtained with antiserum to human C4. Therefore, Slp appears indeed to be absent in these mice, or it cannot be separated from Ss molecules by SDS-PAGE.

### Discussion

The results presented in this paper deal with structural and functional differences between the Ss and Slp proteins and clarify a number of contradictory observations as to the relationship between these proteins and the hemolytic activity of mouse complement.

The different elution patterns of the Ss and Slp proteins and C4 hemolytic activity obtained when mouse plasma and serum are filtered through a Sephadex G-200 column are shown in Fig. 1. In EDTA-plasma, most of the Ss antigen was found in the ascending limb of the second protein peak, although in serum it was present primarily in the first peak, associated with C4-bp. (10). In contrast, Slp eluted in the second protein peak, whether it was fractionated with plasma or serum. Identical results were obtained using plasma and serum from several Ss high, Slp positive strains of mice (C3H.WSlp, C3H.OH, DBA/2J, MP). In contrast with the observations of Goldman et al. (21), C4 hemolytic activity was invariably found only in the ascending limb of the second protein peak. No hemolytic activity was associated with the Ss/C4-bp complexes (10) present in the first peak.

C4 activity in serum represented only 10% of that found in plasma. (Fig. 1). It is very likely that this decrease in C4 titers reflects the activation of C1 during the

fractionation of serum, as reported by Sjöholm and Laurell (22) rather than inactivation of C4 by enzymes generated during clotting. Indeed, measurement of C4 hemolytic activity in whole serum and plasma yielded identical C4 titers provided  $\epsilon$ -aminocaproic acid and DFP were added to the serum after separation of the clot.

Direct evidence that Ss protein mediates C4 hemolytic activity was obtained in experiments in which different proportions of Ss antigen were specifically removed from plasma fractions. C4 activity was measured in these fractions and compared with the levels of remaining Ss protein (Fig. 2). The two variables correlated with a high degree of significance ( $r = 0.99$ ,  $P < 0.001$ ). In contrast, no significant decrease in C4 hemolytic activity was observed in samples in which up to 100% of the Slp antigen was removed (Fig. 3).

Next, we compared Ss levels and C4 hemolytic activity in plasma and/or serum from phenotypically Slp negative mice (Fig. 4). A highly significant correlation was obtained ( $P < 0.001$ ). This degree of significance decreased if serum or plasma from Slp positive mice were included. This was expected, because Slp does not have hemolytic activity but is recognized by the antiserum to Ss.

These results explain some of the previously reported discrepancies between Ss levels and C4 hemolytic activity (21), because the contribution of Slp to the measurable Ss antigenic levels were not taken into consideration. Additional difficulties are that unless precautions are taken to prevent activation of serum proteases, the Ss protein is rapidly cleaved during fractionation and binds to C4-bp forming complexes with high molecular weight (10) which are hemolytically inactive. Finally, most techniques used for detection of mouse C4 do not provide the optimal conditions for its measurement. The use of sensitized ox erythrocytes and C4 deficient guinea pig serum to which oxidized human C2 (23) was added, to overcome species incompatibilities (24) in the formation of the C4<sub>2</sub> enzyme, increased the sensitivity of C4 titration in mouse plasma or serum.

Because the Ss protein expresses the functional activity of C4, it should be susceptible to the enzymatic effect of C1. Indeed, treatment with C1 cleaved Ss with loss of a 7,000–8,000 dalton fragment although Slp was not altered in its molecular size. (Figs. 5 and 7). By CIE, C1-treated Ss showed a faster moving component consistent with conversion of C4 to C4b (not shown). In addition, cleavage of the Ss protein was associated with complete loss of C4 hemolytic activity.

Ss and Slp differ also structurally. As shown in Fig. 6 (track 2) Ss, the same as human C4, consists of three covalently linked polypeptide chains with apparent mol wt of 98,000, 77,000, and 34,000 daltons. The Slp protein displays the same molecular weight as Ss when subjected to SDS-PAGE in the absence of reducing agents (Fig. 5) and, upon reduction, three specific bands  $\alpha$ ,  $\beta$  and  $\gamma$  are also seen. However, their mol wt, 105,000, 74,000, and 32,000 daltons (Fig. 6, track 3) differ respectively from those of Ss (track 2). Previous studies on the structure of the Slp protein have indicated that it consisted of three chains covalently linked, differing somewhat in the mol wt of  $\alpha$ - and  $\gamma$ -chains (7). Our results confirm these observations and show, in addition, differences in the  $\beta$ -chain.

Based on the functional and structural data summarized in Table I, we conclude that the Ss protein (but not Slp) represents the fourth component of complement in mouse serum. Recently Carrol and Capra (25) have partially isolated Ss from mouse ascitis fluid. Although the ascitis fluid contained functional C4 there was no correlation between the protein concentration and the functional activity, as compared to

TABLE I  
*Differences between Ss and Slp Molecules*

Property	Ss	Slp
Recognition by anti-human C4	Yes	Poor
Binding to C4-bp	Yes	No
Activable by C $\bar{1}$	Yes	No
C4 hemolytic activity	Yes	No
Molecular weight of chains		
$\alpha$	98,000	105,000
$\beta$	77,000	74,500
$\gamma$	34,000	32,000

plasma. When the partially purified Ss was analyzed by SDS-PAGE, three major bands and one minor band of estimated mol wt of 190,000, 160,000, 106,000 and 90,000 were seen. These molecular weights are in disagreement with ours for the Ss protein and those reported for native human C4. The 190,000 mol wt material probably contains primarily C4b, and the 160,000 may represent the C4c fragment generated by the action of C4-bp and C3b inactivator on Ss.<sup>2</sup> In addition, Carrol and Capra showed that treatment of mouse plasma with F(ab')<sub>2</sub> fragments of immunoglobulins from an antiserum to Ss abrogated the C4 hemolytic titer.

### Summary

Based on functional and structural data, it is concluded that the Ss protein in the mouse expresses the activity of the fourth component of complement. Removal of the Ss, but not of Slp, antigen correlates with a high degree of significance ( $P < 0.001$ ) with decrease of C4 hemolytic activity. In phenotypically Slp negative mice the plasma/serum levels of Ss correlate with the C4 activity ( $P < 0.001$ ).

Structurally, Ss is a 209,000-mol wt protein, consisting of three covalently linked polypeptide chains ( $\alpha$ ,  $\beta$ ,  $\gamma$ ). Treatment of Ss with C $\bar{1}$  cleaves a 7,000–8,000-mol wt fragment from the  $\alpha$ -chain. Slp is also a three chain covalently linked protein of 209,000 daltons, however its three chains differ in size from those of the Ss protein. Slp does not express hemolytic activity and its  $\alpha$ -chain is not cleaved by C $\bar{1}$ .

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