STUDIES ON THE MURINE Ss PROTEIN

I. Purification, Molecular Weight, and Subunit Structure*

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The serologically detectable serum substance, Ss, is a protein variant controlled by the Ss locus in the S region of the murine H-2 complex (1). The Ss protein, which is usually detected by a rabbit antiserum produced against a globulin fraction of normal mouse serum, is present in different quantities in the sera of all mice. Mice carrying the H-2^k haplotype and some of its genetic derivatives have a low level of the Ss protein in their sera (Ss-L, allele Ss^l), whereas mice of all other inbred strains tested have a high level of the protein (Ss-H, allele Ss^h, cf. 2). The difference between the Ss level in Ss-L and Ss-H animals is approximately 24-fold (3). More recent refined quantitative determinations have revealed additional interstrain variations in the level of the Ss protein (4).

Our interest in the Ss protein has been motivated by the following three considerations. First, the Ss locus is borne by the major histocompatibility complex of the mouse and the question must therefore be asked what relationship, if any, it has to the other loci in the complex. Second, recent data suggest an involvement of the Ss protein in complement (C) function (5). Third, the finding that genes linked to the HL-A system of man influence C activity suggests that an Ss homologue exists in the HL-A complex (6). In addition, a recent report presented evidence for a genetic linkage between serum levels of the third component of C and the H-2 complex (7). In this communication we describe the isolation of Ss protein from serum of DBA/2 mice and report its molecular weight, amino acid composition, and subunit structure.

Materials and Methods

Antiserum. Normal serum from adult DBA/2 males was fractionated on a Sephadex G-200 column and the fractions were tested with anti-Ss serum kindly provided by Dr. H. C. Passmore, Department of Zoology, Rutgers University, New Brunswick, N. J. The Ss-positive fractions were pooled and concentrated to the original volume by lyophilization. The concentrate was mixed with an equal amount of complete Freund's adjuvant and injected subcutaneously into rabbits (1 ml of antigen/animal). This was followed 1 wk later by an intraperitoneal injection and by intraveneous injection of the concentrate without Freund's adjuvant on the next day. The rabbit was bled 1 wk after the last injection. The antiserum was mixed with normal serum from B10.BR mice (the optimal proportion of antiserum and normal serum was determined empirically) and incubated for

24 h at 4°C. The precipitate was removed by centrifugation and the absorbed serum was stored at −75°C.

Immunodiffusion Tests. Ss testing was done by Ouchterlony microslide technique modified from the method described by Shreffler and Owen (1). Microslides (2.5 × 7.5 mm) were layered with 2.5 ml agar medium (0.9% Ionagar no. 2 [Oxoid Division, Consolidated Laboratories, Chicago Heights, Ill.], 0.43% NaCl, 0.36% Na citrate, and 0.001% Na azide). Wells in the agar were arranged in hexagonal patterns with the antiserum well in the center. The wells were 2.5 mm in diameter and were 9 mm apart. The slides were incubated for 24 h at 37°C. The control wells on each slide contained DBA/2 male (Ss-H) and B10.BR (Ss-L) normal sera. Reactions were grossly quantitated on a 0–4+ scale.

Gel Filtration Chromatography. Normal serum from adult DBA/2J males was dialyzed against 0.5 M Tris buffer, pH 8.2, for 24 h and centrifuged at 15,000 g for 20 min. After filtration through a Nalgene filter unit (0.45 μm grid; Nalge Co., Nalgene Labware Div., Rochester, N. Y.) it was applied to a 5 x 200 cm G-200 Sephadex column equilibrated with the same buffer. At a later stage in the purification a similar column was employed but with dimensions of 2.5 x 130 cm.

Ion-Exchange Chromatography. After dialysis against 0.05 M phosphate buffer, pH 7.85, for 24 h, fractions containing Ss material were applied to either a 2.5 x 90 cm or a 1.5 x 10 cm column of DEAE-Sephadex equilibrated with the same buffer. Various sodium chloride gradients, in the same buffer, were applied and the effluent was monitered at 280 nm.

Amino Acid Analysis. Analyses were performed on a Durrum D-500 amino acid analyzer equipped with a 440 channel (Durrum Instrument Corp., Palo Alto, Calif.). Hydrolysis was in 6 N HCl in vacuo for 18 h at 120°C.

Radioiodination and Immunoprecipitation of the Purified Ss Protein. The partially purified Ss protein was dialyzed for 24 h at 4°C against 500 volumes of phosphate-buffered saline (PBS),[1] pH 7.3. 50 μg of the dialyzed material was enzymatically radioiodinated (8) and exhaustively dialyzed against PBS. After dialysis, aggregated material was removed by centrifugation at 100,000 g for 60 min at 4°C. Protein-associated radioactivity was determined by precipitation of a small aliquot in 10% TCA. Aliquots of the labeled protein were treated with 50 μl of rabbit antiserum against either Ss, mouse Ig, or bacteriophage φX 174 (9). (The latter two were used as controls.) The immune complexes were precipitated by a slight excess of goat antirabbit Ig and washed four times with PBS. After the last wash, precipitates were transferred to new tubes in small volumes of PBS, pelleted by centrifugation, counted in a Beckman γ-counter (Beckman Instruments, Inc., Fullerton, Calif.), and stored at 4°C.

Sodium dodecyl sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE). Before electrophoresis, precipitates were dissolved by boiling for 3 min in 0.1 M Tris buffer, pH 8.6, containing 1% SDS and 8 M urea. Aliquots of the dissolved precipitate were reduced with 0.1 M 2-mercaptoethanol (2-ME) either by boiling or by incubation at 56°C. Samples were mixed with 10% the number of counts per minute of ^{125}I-labeled-human IgG and/or with fivefold the number of counts per minute of ^{3}H-labeled μ-and L-chains (10). Samples were electrophoresed on 7.5% SDS gels for 17 h at 5 mA/gel. Gels were fractionated (9) and counted either in a Beckman γ-counter or in a Beckman LS 350 liquid scintillation counter with appropriated discriminators (10).

Re-Electrophoresis of Eluted Material. Fractions from the acrylamide gels were pooled and the gel removed by filtration through a 200 mesh stainless steel screen. When necessary, the pooled fractions were concentrated by pervaporation. Samples were again adjusted to a final concentration of 1% SDS—8 M urea (with or without 2-ME), boiled, and electrophoresed as described above.

Results

Purification of Ss Protein. The strategy employed in the purification of Ss protein consisted of alternate gel filtration and ion-exchange chromatographic procedures and is illustrated in Fig. 1. Initially 20 ml, but later 50 ml of dialyzed DBA/2 male mouse serum was applied to a large G-200 Sephadex column (Fig. 1

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[1] *Abbreviations used in this paper:* 2-ME, 2-mercaptoethanol, PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, SDS, sodium dodecyl sulfate.
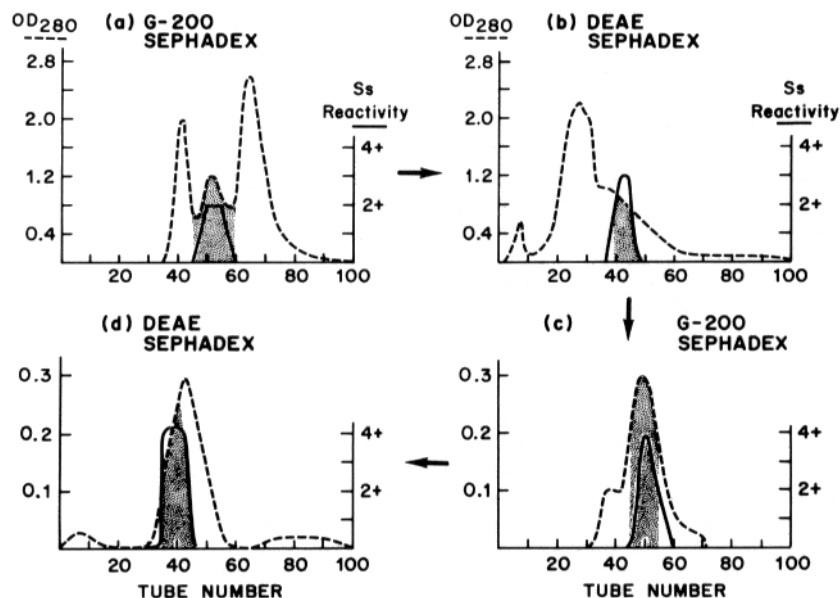
Fig. 1.   Scheme for the isolation of Ss protein. (a) G-200 Sephadex gel filtration chromatography of 50 cc of DBA/2J male serum on a 5 × 200 cm column. Protein contained in the middle peak from two such columns was pooled and applied to a 2.5 × 100 cm DEAE-Sephadex column (b). After thoroughly washing with starting buffer (0.05 M phosphate buffer, pH 7.85), a NaCl gradient was applied (0–1 M). Ss protein was detected by antiserum late in the gradient and the material indicated was pooled, concentrated to 3 ml, and applied to a 2.5 × 130 cm G-200 Sephadex column (c). Material from two of these columns was then applied to a 1.5 × 10 cm DEAE-Sephadex column for the final isolation (d). 50 mg of protein were applied and a shallow gradient utilized (0.2 to 0.8 M NaCl). 24 mg of Ss protein were recovered from the leading edge of the major protein peak.

a). As has been previously reported (3), Ss protein was detected in the middle peak which was pooled, concentrated by pressure filtration, dialyzed against 0.05 M phosphate buffer, and applied to a DEAE-Sephadex column. As shown in Fig. 1 b Ss protein emerged from the column well away from the bulk of the serum proteins, being eluted only at high salt concentrations. The next step in the purification has been attempted with G-150 and G-200 Sephadex as well as with Sepharose 4B, all in the same buffer as in previous experiments, but with smaller column dimensions than the original purification step. No more than a two- to threefold purification was achieved by this step, but it was essential before the final ion-exchange chromatography step. As shown in Fig. 1 c, on a G-200 column Ss material is slightly retarded relative to the main protein material. The material indicated by the hatching was pooled and applied to a second, smaller DEAE-Sephadex column and a shallower NaCl gradient was applied. The Ss material used in this study was obtained from this chromatography step.

Immunization with Purified Ss.   0.2 mg of purified Ss material in complete Freund's adjuvant was injected weekly into a rabbit. After the third injection, the rabbit was bled and the resulting antiserum was monospecific for Ss substance not requiring absorption with Ss-L sera. On Ouchterlony analysis the

unabsorbed antiserum reacted with the same protein in DBA/2 sera as the previously described heavily absorbed antisera. This antiserum has been useful in establishing that the murine Ss protein is an early complement component.[2]

Molecular Weight and Subunit Structure. As seen in Table I, in a typical

TABLE I

Immunoprecipitation of ^{125}I-Labeled Ss Protein from Partially Purified Material

Exp.[*]	Acid precipitable radioactivity/μg protein	Acid precipitable radioactivity immunoprecipitated with rabbit antibody[‡] against:		
		Ss	Mouse Ig	ϕX 174
	$cpm \times 10^6$	%	%	%
1	6.37	40.6	7.7	7.0
2	6.73	41.0	—	7.5

[*] Two different batches of material were radioiodinated in experiments 1 and 2.

[‡] Complexes were precipitated with an excess of goat antirabbit Ig.

experiment approximately 40% of the protein-associated radioactivity from the radioiodinated Ss material was immunoprecipitated with anti-Ss serum. When the unreduced precipitates were electrophoresed (Fig. 2), the anti-Ss precipitate gave a single peak of 120,000 daltons. No peaks were seen with either anti-Ig (Fig. 2) or anti-ϕX 174 (not shown). The reduced Ss precipitate gave four peaks of 46,000, 35,000, 23,000, and 14,000 daltons (Fig. 3). When the 120,000 dalton peak from the unreduced immunoprecipitate was eluted from the gel and re-electrophoresed similar results were obtained. However, after reduction, in addition to the four peaks noted above, a small peak of 80,000 daltons was seen.

Further experiments designed to investigate the relationship between the subunits (using various reducing conditions) suggested that the 23,000 and 14,000 dalton subunits tended to aggregate after even more vigorous reduction (presumably by disulfide exchange) into the 46,000 and 35,000 dalton peaks, respectively. In several experiments when the immunoprecipitate was boiled, reduced, and reboiled, only 23,000 and 14,000 dalton subunits were detected.

Amino Acid Composition. Native and reduced and carboxymethylated Ss protein were subjected to amino acid analysis and the results are displayed in Table II. Tryptophan was not analyzed. In addition, after reduction and alkylation, Ss protein was applied to a G-100 Sephadex column in 5 M guanidine HCl. While the yield of the smaller molecular weight subunits was low, on SDS-PAGE the four subunits were identified and their amino acid compositions determined. The composition of the 46,000 dalton subunit was identical to the 23,000 dalton subunit and the composition of the 35,000 dalton subunit was identical to the 14,000 dalton subunit. The 14,000 dalton subunit contained 2.0 carboxymethylcysteine residues per 100 residues.

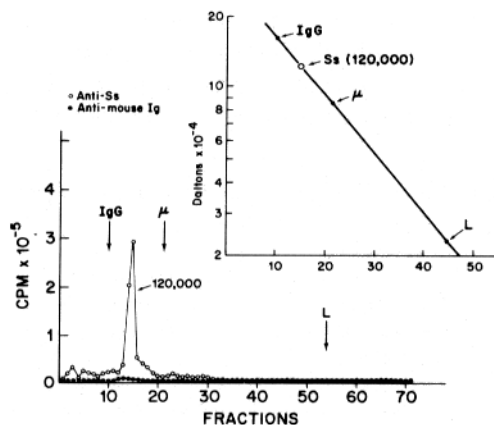[2] Capra, J. D., H. Jasin, and J. Klein. Manuscript in preparation.

FIG. 2.   Radioiodinated Ss immunprecipitated from partially purified material. The precipitate was boiled for 3 min in SDS-urea and electrophoresed with markers in a 7.5% SDS gel.
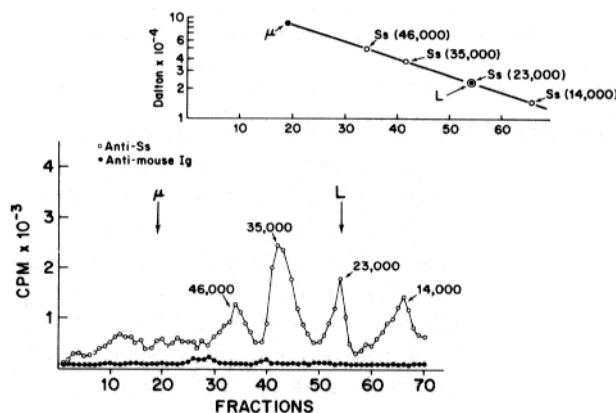

FIG. 3.   Radioiodinated Ss immunoprecipitated and reduced before electrophoresis on a 7.5% SDS gel with molecular weight markers.

Discussion

This paper describes the purification, molecular weight, and subunit composition of Ss protein, a molecule which is genetically controlled by the central region of the murine H-2 complex. The molecular weight of certain of its subunits is similar in some respects to certain surface proteins also controlled by genes on chromosome 17 of the mouse, namely H-2, Tla, Ia, and T/t.

The strategy employed in the isolation of Ss protein consisted of alternating gel filtration and ion-exchange chromatographic procedures. By this technique, 24 mg of relatively pure Ss protein were isolated from 200 ml of mouse serum. Considering an approximate loss of 50% with each chromatographic procedure this would indicate that Ss protein is present in concentrations approximating 1 mg/ml in DBA/2 male mouse sera. A radioimmunoassay is currently being developed to more accurately quantitate the amount of this protein in various strains.

TABLE II
Amino Acid Analysis of the Ss Protein

Amino Acid	Residues per 100
Lysine	7.0
Histidine	2.4
Arginine	5.7
Aspartic acid	10.5
Threonine	4.0
Serine	3.2
Glutamic acid	10.1
Proline	3.9
Glycine	6.4
Alanine	8.3
Valine	7.2
Methionine	2.9
Isoleucine	3.9
Leucine	12.9
Tyrosine	3.9
Phenylalanine	5.6
Cysteine*	2.1

* Measured as carboxymethylcysteine.

By SDS-PAGE the mol wt of Ss protein is approximately 120,000 ± 10,000. This is consistent with its emergence from a G-200 column in the middle peak along with IgG immunoglobulin (mol wt approximately 150,000). The subunit composition of Ss protein is more difficult to unambiguously assign. When the purified preparation of Ss material was electrophoresed on SDS-PAGE several bands were detected. A major band of 120,000 daltons (comprising over 60% of the stained material) was always seen, but varying amounts of material with molecular weights of approximately 80k, 46k, 35k, 23k, and 14k were alternately seen. In four separate experiments when the ^{125}I-labeled purified Ss protein was immunoprecipitated and electrophoresed by SDS-PAGE gels, only the 120,000 dalton material was seen (see Fig. 2). When the band corresponding to 120,000 mol wt was eluted from the gel and re-electrophoresed both reduced and unreduced, the unreduced material remained at 120,000 while the reduced material produced subunits with mol wt of approximately 46,000, 35,000, 23,000, and 14,000 (Fig. 3). Occassionally a small 80,000 dalton peak was detected. In other experiments, however, when the 120,000 mol wt peak was eluted from an SDS acrylamide gel, reduced, and rerun, only the 23,000 and 14,000 mol wt species were apparent. Several experiments indicated that the 23,000 mol wt subunit and the 46,000 subunit were interconvertible. Similarly, the 14,000 mol wt subunit could be interconverted to the 35,000 mol wt subunit.

These results indicate that a high degree of noncovalent interaction occurs between the various subunits of the Ss protein. It is likely that a combination of boiling in urea, reduction, and reboiling will be necessary in order to achieve maximal yields of the smallest subunits (14,000 and 23,000). Indeed, in recent experiments these two lower molecular weight subunits appear to be the only

constituents of the molecule. The exact molecular weight of the smaller subunit is presently being investigated. In several experiments it was determined to be between 12,000 and 14,000 daltons.

It is noteworthy that the molecular weight of human C-2, the second component of complement, is very close to the molecular weight of the murine Ss protein (11). Since it has been demonstrated that C-2 deficiency in man is linked to the HL-A system (7) it would not be unreasonable to assume that this same protein in man is also controlled by an Ss-like region in the human HL-A complex. Experiments are currently underway to further define this relationship.[2]

The subunit composition of Ss protein in many ways resembles the subunit composition of several membrane antigens controlled by chromosome 17. Thus, evidence has been presented that a 12,000 mol wt subunit can be isolated from the *H-2* (12–15), TL (12, 16), and F9 (a product of the *T/t* locus (17) antigens. It is probable that this subunit represents the murine equivalent of human β_2-microglobulin, a carbohydrate-free protein with a mol wt of 11,700 daltons which has been demonstrated to have considerable homology with IgG (18, 19). Unlike β_2-microglobulin associated with *H-2*, TL, and F9, the 14,000 dalton subunit is clearly covalently linked to the Ss protein, but its lack of carbohydrate, amino acid composition, and molecular weight suggest it may be similar if not identical to β_2-microglobulin.

The higher mol wt subunit of 23,000 is of course reminiscent of immunoglobulin light chains and a subunit of this size is also found in *H-2* and F-9 (17). In addition, in some experiments we detected subunits of 35,000 and 46,000 daltons. The 46,000 subunit is similar to that reported for the murine *H-2* (12–14), TL (12, 16), and F9 (17) antigens, and Ia is reported to have a mol wt of 35,000 (20, 21).

The Ss protein may provide a crucial link between the *H-2* complex, a complement component, β_2-microglobulin, and immunoglobulins. It would be of fundamental interest if the products of the 17th chromosome of mice and its equivalent in man, represent a series of gene duplications — the products of which have homologies to β_2-microglobulin and accordingly to immunoglobulin. Its presence in relatively large quantities in certain mouse sera, the ease of its isolation, and the relatively small molecular weights of its two major subunits, make the Ss protein an ideal candidate for amino acid sequence analysis.

Summary

The murine Ss protein has been isolated and purified. Using specific antisera, the radiolabeled protein has a mol wt of 120,000 in sodium dodecyl sulfate-polyacrylamide gels. It is composed of two basic subunits of 23,000 and 14,000 daltons. The smaller molecular weight subunit contains a single disulfide bridge, is devoid of carbohydrate, and may represent the murine equivalent of β_2-microglobulin.

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