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STRUCTURAL STUDIES ON THE MURINE FOURTH COMPONENT OF COMPLEMENT (C4)

IV. Demonstration that C4 and Slp are Encoded by Separate Loci[1]

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The *S* region of the murine major histocompatibility complex (MHC) encodes the structural gene for the fourth complement component (C4) and controls C4 serum levels and expression of the sex-limited protein (Slp). Although Slp was first thought to be an allele of C4, more recent studies have suggested that Slp is either a modified product of the *C4* locus or is encoded at a separate locus. By using a sensitive peptide analysis technique, each of the three constitutive polypeptide chains of C4 and Slp was compared, and multiple distinct peptides as well as several shared peptides were detected. These results demonstrate that although C4 and Slp undoubtedly share a common genetic origin, they are encoded by separate structural genes.

The *S* region of the murine major histocompatibility complex (MHC)[2] controls the expression of at least one functionally active complement component (C4) and the closely related sex-limited protein (Slp) (1). In a previous study, we demonstrated that within the *S* region, the *C4* locus encodes the structural gene for the fourth component of complement (C) and two alleles, *C4.1* and *C4.2*, were mapped to this locus (2). A second locus within the *S* region controls the phenotypic expression of the sex-limited serum protein variant Slp (3) whose expression is under the direct control of testosterone and with the exception of the mutant S^{w7} allele is only present in the serum of males of Slp-positive strains (4). The *S* region also encodes a gene controlling the expression of the serum substance (Ss) high and low serum level phenotypes (3, 5). A large body of experimental data suggests that the Ss protein is both structurally (6–8) and functionally (9–12) the murine homologue of C4. Therefore, C4 will be used in all references to the Ss protein in this paper.

The Slp protein was first described by Passmore and Shreffler after alloimmunization of inbred mice with partially pure C4 protein (3). Since the expression of C4 and Slp mapped to the *S* region and the two molecules cross-reacted immunochemically, initially it appeared that they were alleles. However, more recent immunoprecipitation studies indicate that two distinct populations of molecules exist in the serum of C4-high, Slp-positive strains (2, 6, 12). Although similar in overall molecular weight and gross polypeptide chain structure, these two serum proteins migrate distinctly on sodium dodecyl sulfate (SDS) polyacrylamide gels (2, 6, 12). Together, these results suggest the alternative possibility that Slp represents either a modified *C4* locus product or the product of a separate locus.

This paper addresses the question of whether Slp is a product of the *C4* locus or is encoded by a separate locus. By using a sensitive peptide analysis technique, each of the three constitutive polypeptide chains of C4 and Slp was compared. Although the γ-chains of C4 and Slp appear quite similar, clear structural differences exist between the α- and β-polypeptide chains. These results suggest that although C4 and Slp share a common genetic origin, they are encoded by separate structural genes.

MATERIALS AND METHODS

Materials. The sources of materials used in this study have been described in previous reports (2, 13). Electrophoresis chemicals and apparatus were purchased from Bio-Rad Laboratories, Richmond, Calif. Rabbit anti-murine C4 antisera donated by Drs. Arturo Ferreira and Victor Nussensweig, Department of Pathology, New York University School of Medicine (12), gave identical results as anti-murine C4 antisera prepared as described previously (3, 11). Mouse anti-Slp was prepared as described previously (3).

Isolation of individual polypeptide subunits of C4 and Slp. Each of the subunits of C4 and Slp was isolated by a modification of the procedure described by Roos *et al.* (6) and reported in a previous report (2). By using specific antisera, mouse C4 was directly precipitated from mouse plasma drawn fresh on ice and made 25 mM EDTA (see Abbreviations) and 10 mM diisopropyl fluorophosphate (iPr_2P-f). The immune complexes were separated on Laemmli SDS polyacrylamide gels under reducing conditions, fixed, stained, and appropriate bands were cut out for peptide mapping.

Peptide analysis on SDS slab gels. The technique used was modified from that described by Cleveland *et al.* (14) and described in detail previously. Gel slices were dialyzed into Laemmli SDS sample buffer and loaded on 25 cm 15% SDS slab gels. After overlaying as many as five gel slices with 15 μl of staph V-8 enzyme (200 μg/ml), both enzyme and substrate

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[2] Abbreviations used in this paper: iPr_2F-f, diisopropyl fluorophosphate; C4, the fourth component of complement; MHC, major histocompatibility complex; Slp, sex-limited protein; Ss, serum substance; HPLC, high pressure liquid chromatography.

were electrophoresed into the stacking gel, then power was turned off for approximately 15 min. Subsequently, the peptides were separated by electrophoresis into the resolving gel, then fixed and stained.

Labeling and enzymatic digestion of polypeptides for HPLC comparison. Methanol-dehydrated gel slices were labeled with either ^{3}H or ^{14}C by a modification of the procedure described by Jentoft and Dearborn (15). Gel slices were incubated overnight at 22°C in approximately 25 μl of a solution (per gel slice) containing either 1×10^{6} dpm of ^{3}H-formaldehyde (New England Nuclear, Boston, Mass.; specific activity: 85 mCi/mmole) or ^{14}C-formaldehyde (specific activity: 50 mCi/mmole) and 10 mM sodium cyanoborohydride in 0.5 M sodium phosphate buffer, pH 7.5. Subsequently, gel slices were washed extensively in 10% methanol, then dehydrated in ascending concentrations of methanol. The labeled C4 subunits were then enzymatically digested in the gel slice by using the procedure of Elder *et al.* (16). After overnight incubation at 37°C in 1 ml of 100 mM ammonium bicarbonate solution containing 50 μg of either Staph V-8 enzyme or Tos-Phe-Ch_2Cl-trypsin, the gel slices were removed and the peptide containing supernatant was lyophilized for peptide analysis.

High pressure liquid chromatography, HPLC. Peptides were resolved on a high pressure liquid chromatograph equipped with a micro-bondapak C18 column (Waters Associates, Milford, Mass.; column dimensions: 0.4 x 30.0 cm). The aqueous phase was 2% acetic acid and the organic phase, acetonitrile. Over a period of 60 min, a linear gradient of 0 to 25% acetonitrile was generated by using curve 6 of the Model 660 solvent programmer (Waters Associates). Isocratic elution at 25% acetonitrile was continued after the gradient for a period of 10 to 15 min. A constant flow rate of 0.7 ml/min was maintained, and fractions were collected into scintillation vials at 0.5-min intervals. Water (0.5 ml) and scintillation fluid were added to the vials, and the samples were counted.

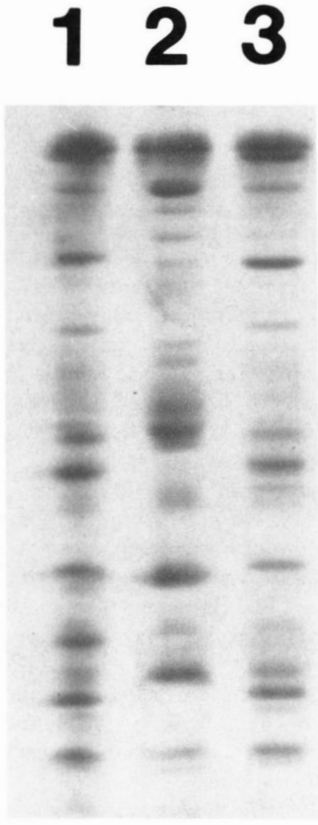

Figure 1. C4.1, C4.2 and Slp α subunits isolated from two inbred strains of mice were digested with V8 protease and the peptides separated on analytical Laemmli SDS slab gels as described in the text. *Lane 1*, C4.1α(C57BL/6); *Lane 2*, Slpα(DBA/2J); *Lane 3*, C4.2α(DBA/2J).

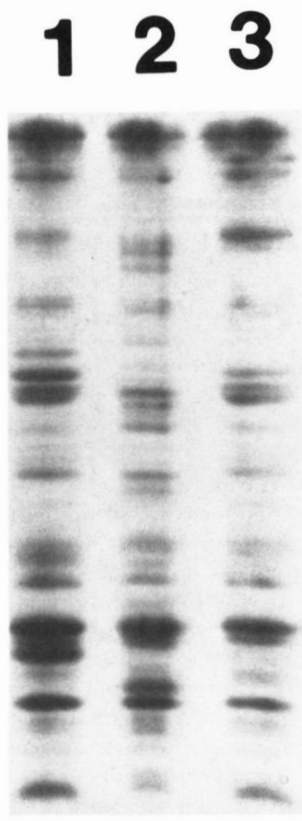

Figure 2. C4.1, C4.2, and Slp β subunits isolated from two inbred strains of mice were digested with V8 protease and the peptides separated on analytical Laemmli SDS slab gels as described in the text. *Lane 1*, C4.1β(C57BL/6); *Lane 2*, Slpβ(DBA/2J); *Lane 3*, C4.2β(DBA/2J).

RESULTS

Isolation of C4 and Slp subunits. The structures of murine C4 and Slp are similar in that both are composed of three distinct polypeptide chains joined together by disulfide bonds (2, 6, 12). The reported apparent m.w. of the α, β, and γ subunits of C4 are approximately 100,000, 75,000, and 34,000 daltons, respectively (2, 6, 12). Although the whole molecules are similar in total apparent m.w., the respective subunits of Slp and C4 migrate distinctly on SDS Laemmli Gels. The Slp α-subunit migrates slower, whereas the Slp β- and γ-subunits migrate slightly ahead of their C4 counterparts (2, 6, 12).

Murine C4 and Slp were isolated from fresh EDTA mouse plasma by direct immunoprecipitation, and the individual polypeptide subunits were isolated on preparative Laemmli SDS gels under reducing conditions. In those strains expressing the Slp phenotype, both C4 and Slp were precipitated with rabbit anti-murine C4 antisera. However, each of the respective subunits was sufficiently resolved on the preparative gel to allow complete separation of C4 and Slp polypeptides. The Slp subunits used for peptide comparison were precipitated with homologous anti-Slp antisera that does not cross-react immunochemically with C4. Thus, only Slp subunits were precipitated with anti-Slp.

Peptide analysis. Each of the three polypeptide chains of

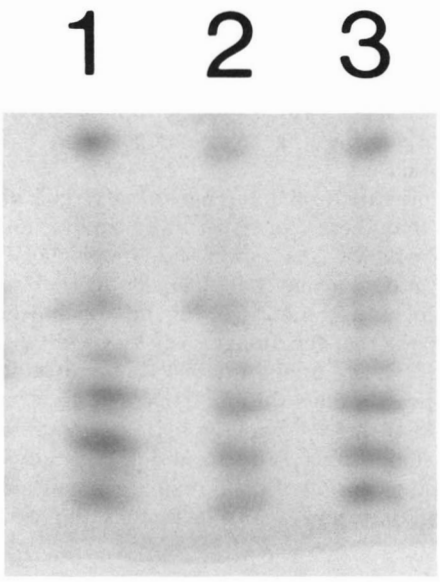

Figure 3. C4.1, C4.2, and Slp γ subunits isolated from two inbred strains of mice were digested with V8 protease and the peptides separated on analytical Laemmli SDS slab gels as described in the text. *Lane 1*, Slpγ(DBA/2J); Lane 2, C4.2γ(C57BL/6); Lane 3, C4.2γ(DBA/2J).

C4.1 and C4.2 was isolated from the respective inbred strains and compared by peptide analysis to the three polypeptide chains of Slp. Figure 1 illustrates a comparison of C4.1 and C4.2 α-subunits with an Slp α-subunit. Of the approximately 20 peptides resolved, half were shared between C4 and Slp, whereas C4.1 and C4.2 α-chains had no consistent differences. Likewise, when the peptide patterns of the C4.1, C4.2, and Slp β-chains were compared as seen in Figure 2, again approximately half of the resolved peptides were common between Slp and C4. The peptide patterns of C4.1 and C4.2 β-chains differed only at the allospecific sites previously demonstrated (2).

In contrast to the extensive variation observed between the α- and β-subunits of C4 and Slp, the γ-subunits appeared quite similar. Figure 3 illustrates the peptide patterns of the γ-polypeptide subunits of C4.1, C4.2, and Slp. In an attempt to further evaluate the degree of homology between the γ-subunits of C4 and Slp, a second method of peptide digestion and separation was employed. C4.1 and Slp γ-chains were labeled with either ^{3}H or ^{14}C, digested with trypsin, and compared by HPLC as described in *Materials and Methods*. The tryptic maps of these two molecules appeared nearly identical, supporting the results observed by peptide separation on SDS analytical gels. Thus, by using two different enzymes and two fundamentally different techniques for separating peptides, the γ-polypeptide chains of C4 and Slp appear very similar in structure. This is in marked contrast to the extensive variation observed for the α- and β-subunits.

DISCUSSION

To assess the structural similarity between C4 and Slp, the subunits of C4.1, C4.2, and Slp were isolated and compared by peptide analysis on SDS gels. The peptide patterns of the two *C4* alleles, i.e., *C4.1* and C4.2, differed only at the allospecific sites in the β-subunit as reported previously (2), whereas the peptide patterns of the Slp α- and β-subunits varied extensively, i.e., only 50% peptides in common, from the patterns observed for the C4 α- and β-subunits. The allospecific peptides that characterize the C4.2 β subunit were not observed in the Slp β-peptide pattern. Although actual structural differences are overstated by this peptide mapping technique, the extent of variation observed suggests that multiple primary structural differences exist between the α- and β-polypeptide chains of C4 and Slp. Thus, the peptide differences observed cannot be explained by mechanisms such as post-translational modifying mechanisms, i.e., glycosylation or enzyme trimming, or post-transcriptional processing of mRNA (17), which might account for polypeptide chain length differences between two otherwise identical molecules. Unlike the α- and β-peptide patterns, the γ-chain peptide patterns of C4 and Slp were quite similar as determined by two separate peptide mapping techniques. The minor differences observed between the γ-chains could be explained by one of the modifying mechanisms discussed above and/or limited differences in primary structure. A tentative working model for the *S* region of the murine MHC is shown in Table I. According to the model, this region of the 17th chromosome encodes at least four loci: two regulatory and two structural. Their order is totally arbitrary at this time.

Locus 1 of the *S* region encodes two alleles, $C4^{h}$ and $C4^{l}$, which control the serum level phenotype of C4. Although the serum level *phenotype* appears to be independent of testosterone, the hormone does affect the serum level since adult males have a higher C4 level than adult females whose serum C4 level is about the same as adolescent $C4^{h}$ males. It is conceivable that this apparent genetic regulation of C4 serum level is the result of primary structural differences in the molecules themselves. However, C4 isolated from a $C4^{h}$ strain, i.e., $H\text{-}2^{b}$, appeared identical by peptide mapping to C4 isolated from a $C4^{l}$ strain, i.e., $H\text{-}2^{k}$ (2).

Locus 2 encodes the structural gene for C4, and we previously mapped two alleles, *C4.1* and *C4.2*, to this locus on the basis of structural variation in the β-subunit (2). A third allele, $C4.S^{w7}$, carried by the B10.WR7 congenic strain, has been tentatively assigned to this locus on the basis of data linking an apparent m.w. difference in the α-subunit to the MHC (6). These results along with recent experiments mapping the C4 γ-subunit to the *S* region (Dr. V. Nussenzweig, personal communication) suggest that the structural genes for all three polypeptide chains of murine C4 are encoded in locus 2 of the *S* region. Further support for assigning all three C4 subunit structural genes to this locus comes from recent evidence for a single polypeptide chain precursor molecule, pro-C4, in the mouse (6) and guinea pig (18). It must be noted, however, that it is not known if the pro-C4 molecule includes *all three* polypeptide chains, since the reported m.w. of the precursor is approximately 30,000 daltons less than the three chain molecule secreted into them. This m.w. variation may be attributed to conformational differences between precursor and product, or may represent the absence of the γ-chain.

Locus 3 encodes the hormone-sensitive *Slp* regulatory locus. The sex limitation phenotype represents one allele of this locus, and the constitutive (testosterone-insensitive) phenotype rep-

TABLE I

Tentative model of the S region

	Locus 1	Locus 2	Locus 3	Locus 4
Alleles	$C4^{h}$	*C4.1*	Sex-limitation	Slp^{a}
	$C4^{l}$	*C4.2*	Constitutive (S^{w7})	
		$C4.S^{w7}$		Slp^{o}
Controls	Serum level of C4	Structural gene for C4	"Regulatory" locus (hormone sensitive)	Structural gene for Slp

resents the second. With the exception of the S^{w7} wild haplotype, only males of Slp-positive strains express Slp in the serum (4). The S^{w7} variation is thought to be insensitive to testosterone due to a defect in the Slp operator gene (4).

Locus 4 of the *S* region encodes the structural gene for the Slp protein. The two alleles assigned to this locus are presence, Slp^a, and absence, Slp^o, of Slp in the serum of males. To date, structural variants of Slp have not been detected, however, the structural gene for Slp is tentatively assigned to this locus on the basis of genetic mapping of the serum deficiency (3) and the structural similarity between C4 and Slp. Like C4, a precursor Slp molecule (6) for Slp has been identified in the mouse. This suggests all three subunits of Slp are encoded within this locus.

At present, the order of the four proposed loci within the *S* region cannot be determined, since recombinants within this region have not been reported. In an extensive survey, Shreffler *et al.* (19) were unsuccessful in detecting recombinants between the $C4^1$ and Slp^o phenotypes. The detection of Slp^a in a $C4^1$ mouse may be complicated by an influence of the $C4^1$ gene on the Slp serum level. Thus, recombinants may not have been detected due to the low level of Slp in the serum. Together, these data imply a linkage disequilibrium between the two genes. Another example of linkage disequilibrium within the *S* region occurs between *C4.2* and Slp^a. In our previous study, each of the haplotypes, i.e., *d*, *p*, and *s*, typed as *C4.2*, were also positive for Slp, whereas the strains negative for Slp were *C4.1* (2). Should a structural polymorphism in Slp-positive strains be found, it will facilitate the search for recombinants and thus ordering of the proposed *S* region loci.

The actual arrangement of the DNA encoding C4 and Slp cannot be determined short of sequencing the DNA; however, the results from this study eliminate certain possibilities and suggest new ones. Recent reports describing the gene arrangement for proteins such as the mammalian immunoglobulin heavy (20) and light (21) chains indicate that the actual gene is divided into discrete coding units, i.e., expressed sequences (exons), separated by noncoding sequences, or intervening sequences (introns). Although the function of these intervening sequences is not well understood, they may participate in the regulation of the expressed sequences (22). One possible effect of exons separated by introns may be that different "domains" of the same molecule may be subjected to varying rates of mutation and recombination at the DNA level. This association of function with the arrangement of the DNA may have implications in understanding the evolution of the C4 and Slp genes. For example, when the third component of complement (C3) is activated and sequentially degraded, each of the products of degradation serve distinct functions, i.e., C3a is an anaphlatoxin, C3b serves as an opsonin as well as forming a trimolecular complex with C2a and C4b to form C5 convertase, C3e, a product of C3c, liberates polymorphonuclear leukocytes from bone marrow, and C3d is known to react with specific receptors on lymphocytes (23). Although many of the functions of C4 are not completely understood, C4 appears to be degraded in a systematic manner similar to C3. It is likely that each of the degraded products of C4 likewise serve independent functions and accordingly the DNA may be organized into discrete exons.

Several genetic models could explain the origin of the C4 and Slp structural loci. The first and simplest is that the Slp locus resulted from a duplication of the C4 locus (24). The Slp α and β "genes" then mutated such that their polypeptides became structurally distinct from C4. Because of certain unknown constraints, the γ "gene" was conserved. A second possibile explanation is that only the α and β "genes" duplicated and subsequently mutated and C4 and Slp share a common γ "gene." As a third possibility, the α and β "genes" of C4 and Slp may be transcribed from the same "gene" but spliced differently for translation. Again the γ "gene" is composed of DNA common to both C4 and Slp.

The multiple structural differences observed between C4 and Slp suggest that these two structurally similar molecules are encoded by separate loci within the *S* region of the murine MHC. This situation may be analogous to that recently observed in man, where genetic (25, 26) and immunochemical (27, 28) data suggest that functional C4 is encoded by two closely linked loci. However, unlike the two C4 products in man, Slp does not appear to have C4 functional activity (12) and, with the exception of the S^{w7} haplotype, is only observed in males (3). These relationships, as well as other questions concerning the origin and evolution of the *S* region, are now approachable since methods are now available to fully characterize these molecules.

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Note added in proof: Recently, using a cation-exchange column to compare tryptic peptides, Parker *et al.* (29) detected multiple distinct and multiple shared peptides between the α and β subunits of C4 and Slp. These results support our conclusion that C4 and Slp are encoded by separate structural loci.

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