Proc. Natl. Acad. Sci. USA
Vol. 76, No. 11, pp. 5853–5857, November 1979
Immunology

Structural characterization of the murine fourth component of complement and sex-limited protein and their precursors: Evidence for two loci in the *S* region of the *H-2* complex

(major histocompatibility complex/gene duplication)

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Communicated by Ray D. Owen, August 17, 1979

ABSTRACT **The *S* region of the murine major histocompatibility complex controls the expression of two related, serum substance-positive proteins; one (C4) has functional complement activity, whereas the other, the sex-limited protein (Slp), is hemolytically nonfunctional. The structural relationships of these molecules to each other and to their putative intracellular precursors have been examined. Radiolabeled intracellular C4 and Slp precursors were isolated from lysates of cultured peritoneal cells. The C4 and Slp precursors and their processed subunits were purified by immunoprecipitation and sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Antigenically distinct precursors for C4 and Slp were demonstrated by sequential immunoprecipitation experiments in which anti-Slp-reactive molecules were precleared by exhaustive immunoprecipitation and residual C4 molecules were precipitated by antibody to serum substance. Both molecules had apparent molecular weights of 185,000. Their molecular identities as precursors of the mature C4 and Slp proteins were established in pulse–chase studies and by comparisons of their tryptic peptide profiles with those of isolated subunits from the processed proteins. When isolated α- or β-subunits from C4 and Slp proteins were compared by peptide mapping, it was possible to detect multiple distinct and multiple shared peptides. This evidence indicates that the C4 and Slp proteins derive from distinct precursor polypeptides and suggests that the primary structures of the C4 and Slp α- and β-subunits are different. These results support the postulate that the *S* region contains two discrete structural loci that specify discrete C4 and Slp proteins.**

The *S* region of the murine major histocompatibility complex was originally defined by the serum substance (Ss) marker (1). It was noted that quantitative levels of a serum β-globulin, termed Ss, assayed by immunodiffusion with a specific rabbit antiserum, were controlled by a gene closely linked to the H-2 major transplantation antigen genes. In an attempt to detect allelic forms of Ss by alloimmunization, a new variation in the Ss protein was detected. It was shown that males of certain strains possess an antigenically distinct subclass of Ss. This protein was termed the sex-limited protein, or Slp (2). Extensive mapping studies have revealed no recombinants between the gene controlling levels of Ss and the gene determining presence or absence of the Slp variant. It was therefore not established whether the Slp-positive and Slp-negative subclasses of Ss protein represent the products of two distinct but closely linked and highly homologous structural genes in the *S* region, or whether the gene controlling the Slp variant might act *via* modification of the product of the *Ss* structural gene.

Major progress in characterization of the Ss protein was made when it was demonstrated that it is antigenically (3, 4), structurally (3, 4), and functionally (5) homologous to the fourth component of human complement (C4). These observations were extended by studies in this laboratory (6) and subsequently by others (7), showing that the molecular weights of the isolated α-, β-, and γ-chains of the Ss protein (approximately 100,000, 75,000, and 34,000, respectively) are very similar to those reported for humans (8) and guinea pigs (9) C4. Differences were demonstrated in the apparent molecular weights of both the α- and γ-chains between the Slp-negative and Slp-positive subclasses of Ss (6, 7). It was therefore proposed (6) that the Slp-positive and Slp-negative Ss molecules have different primary structures and represent the products of two discrete genes.

Roos *et al.* (6) found an intracellular molecule with an apparent molecular weight of 185,000 which reacted with anti-Ss antibody—a putative C4 precursor. In accord with an earlier finding by Hall and Colten (10), in which an apparent single-chain precursor for guinea pig C4 was synthesized in a cell-free translation system, these findings suggested that a single structural gene might determine all three subunits of the C4 molecule.

Recent data have also shown functional differences between Slp-positive and Slp-negative Ss molecules. Ferreira *et al.* (7) showed that the action of complement component C1 on Slp-negative molecules resulted in the cleavage of a small fragment (presumably C4a) from the α-chain, whereas no effect of complement component C1 on Slp-positive molecules was noted. Furthermore, functional studies with a hemolytic assay employing C4-deficient guinea pig serum indicated that only the Slp-negative Ss molecules possess C4 activity (ref. 7; J. P. Atkinson and D. C. Shreffler, unpublished results).

The goals of the present study were 2-fold. First, we sought to demonstrate conclusively a precursor–product relationship between the 185,000-dalton intracellular molecules and the processed Slp-positive and Slp-negative Ss molecules. To this end, a combination of kinetic pulse–chase studies and structural comparisons by tryptic peptide mapping has been performed which confirms the suggestion (6) that the putative precursors give rise to the processed extracellular molecules. Second, the structural differences between Slp-positive and Slp-negative Ss molecules were further examined. Evidence is presented which demonstrates that Slp-positive and Slp-negative Ss molecules are derived from distinct precursors; the uniqueness of the two precursors is shown by their differential reactivity with anti-Slp antibody. Peptide map comparisons indicate that the two populations of molecules also have distinct amino acid

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Abbreviations: C4, fourth component of complement; $NaDodSO_4$, sodium dodecyl sulfate; Slp, murine sex-limited protein variant; Ss, murine serum substance; P-185; 185,000-dalton precursor protein.

sequences; these studies reveal a number of distinct as well as shared peptides.

The data presented strongly suggest that the Slp-positive and Slp-negative subclasses of Ss are the products of two discrete structural genes located in the S region. Consequently, in order to simplify the designation of these proteins, we suggest an alteration in the previous nomenclature. We propose that the protein reactive with anti-Slp, which lacks functional C4 activity, continue to be termed Slp, but that the Slp-negative subclass of molecules, which has functional C4 activity, be referred to as C4. This terminology will be followed in the remainder of this paper.

MATERIALS AND METHODS

Synthesis of Radiolabeled Proteins. Normal peritoneal wash cells were obtained and cultured *in vitro* as described (6). For the experiments described in this paper, mice with the S^{w7} haplotype were used, because this is the only strain from which radiolabeled Slp-positive molecules have been obtained in culture. Short-term cultures of peritoneal cells were incubated at 37°C in methionine-free RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine, and antibiotics. Radiolabeled methionine, either [^{35}S]methionine (400 μCi/ml, 1 Ci = 3.7×10^{10} becquerels) (Amersham) or [*methyl*-^{3}H]methionine (500 μCi/ml, Amersham), was added. After incubation for 6–10 hr, the medium was harvested and processed and the adherent cells were lysed as described (6).

Pulse–Chase Studies. For pulse–chase experiments, the cells were cultured for 1 hr in the presence of [^{35}S]methionine (400 μCi/ml) as described above. They were washed once with phosphate-buffered saline, and 3 ml of fresh medium containing 1 mM unlabeled methionine was added. The cells were cultured for 0, 40, 90, 180, or 240 min in this medium before harvesting of medium and lysis of cells. Immunoprecipitations of lysates and medium were then performed as described below.

Immunoprecipitation. Direct immunoprecipitations of radiolabeled proteins from culture medium and cell lysates were carried out as described (6). Rabbit anti-Ss and mouse alloanti-Slp sera {[C3H.Q × B10.T(6R)]F_1 anti-B10.WR} were the same as those employed previously (6). To separate the putative precursors for Slp and C4, it was necessary first to deplete the cell lysates of all Slp-reactive molecules. Two different methods were employed. The first involved multiple, direct immunoprecipitations with anti-Slp and carrier plasma at equivalence (determined by Ouchterlony analysis). Alternatively, after initial treatment with anti-Slp and carrier plasma at equivalence, an excess of anti-Slp was added to the supernatant and allowed to react at 4°C for 6 hr. This supernatant was then absorbed with 150 μl of *Staphylococcus aureus* strain Cowan I as described (11), and the pellet was discarded. The supernatant remaining after either of these procedures was then immunoprecipitated with anti-Ss antibody at equivalence. The resulting precipitates were dissolved by boiling for 5 min in 10 μl of buffer containing 0.25 M Tris·HCl (pH 6.8), 2% sodium dodecyl sulfate ($NaDodSO_4$), 10% glycerol, 0.003% bromphenol blue, and 5% 2-mercaptoethanol. The solubilized precipitates were then electrophoresed.

Electrophoresis. Separation of the individual subunits from the immunoprecipitates was accomplished by electrophoresis in 9% polyacrylamide/0.1% $NaDodSO_4$ gels as described (6). The gels were stained in 0.1% Coomassie blue/50% methanol/7% acetic acid and destained in 30% methanol/7% acetic acid. The stained bands were cut from the gel, rinsed in distilled water, and homogenized in a Dounce homogenizer in 0.125 M Tris·HCl, pH 6.8/0.1% $NaDodSO_4$. Proteins were allowed to elute from the gel for 12 hr at 37°C.

For preparative purification of precursor molecules, immunoprecipitates from cell lysates were subjected to electrophoresis on 6% polyacrylamide/0.1% $NaDodSO_4$ gels. When the radiolabeled precursors contained [^{35}S]methionine, the gels were stained to visualize molecular weight standards and were then dried and autoradiographed. The appropriate bands of labeled protein were then cut from the gel, using the developed autoradiogram as a reference; the gel slices were minced and allowed to elute as described above. The positions of the [^{3}H]-methionine-labeled precursors were located on reference tracks from the same gel by the fluorography procedure of Laskey and Mills (12). The corresponding region of the fixed gel was cut, homogenized, and eluted as described above.

For the demonstration of antigenically distinct precursor molecules and for the pulse–chase experiments, electrophoresis of immunoprecipitates was done on 0.1% $NaDodSO_4$ slab gels

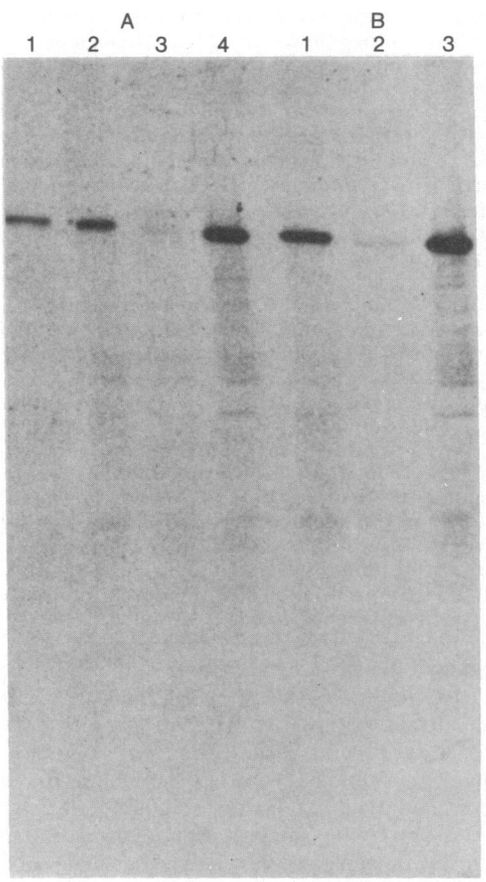

FIG. 1. Gradient gel separation of antigenically distinct intracellular precursors for Slp and C4. Lysates from cells labeled with [^{35}S]methionine were subjected to one of two methods (below) to remove all Slp-positive molecules. The supernatant was then mixed with fresh plasma and anti-Slp antibody to demonstrate that no anti-Slp-reactive molecules remained. C4 present in the supernatant was then immunoprecipitated with anti-Ss. (Gel A) Clearance of residual Slp molecules via multiple direct immunoprecipitations with anti-Slp. Lanes: 1, anti-Slp immunoprecipitate; 2, anti-Slp immunoprecipitate of supernatant from sample 1 after addition of fresh plasma; 3, anti-Slp immunoprecipitate of supernatant from sample 2 after addition of fresh plasma; 4, anti-Ss immunoprecipitate of supernatant from sample 3 after last anti-Slp precipitation. (Gel B) Clearance of residual Slp molecules via treatment with excess anti-Slp and *S. aureus* strain Cowan I. Lanes: 1, anti-Slp immunoprecipitate; 2, anti-Slp immunoprecipitate with fresh plasma after reaction of supernatant from sample 1 with excess anti-Slp and *S. aureus* strain Cowan I; 3, anti-Ss immunoprecipitate of supernatant from sample 2 after *S. aureus* strain Cowan I treatment.

with a linear gradient from 5 to 18% acrylamide as described (6).

Tryptic Peptide Mapping. Differentially labeled proteins to be compared were isolated from polyacrylamide gels and mixed. Samples were adjusted to pH 8 by addition of 0.5 M Tris·HCl and bovine gamma globulin (1 mg) was added as carrier. After reduction in the presence of dithiothreitol at 3.5 mg/ml (37°C, 30 min) and alkylation with iodoacetamide at 9 mg/ml (0°C, 60 min), the proteins were precipitated by addition of 100% trichloroacetic acid to a final concentration of 15%. The precipitate was washed once with cold 5% trichloroacetic acid and twice with cold acetone (−20°C) and then dispersed in 1.0 ml of 0.1 M NH_4HCO_3. The precipitate was digested for 1 hr at 37°C with tosylphenylalanyl chloromethyl ketone-treated trypsin (Worthington) at a 1:10 weight ratio of trypsin to carrier protein and for another 12 hr after an identical addition of fresh trypsin. Glacial acetic acid (1 ml) was added, and the filtered digest was applied to a 0.32 × 30 cm column of Durrum DC-4a cation-exchange resin. Recovery of radioactivity, from time of mixing of radiolabeled proteins to application of sample to the column, was consistently 65–85%. The chromatogram was developed at 51°C with a five-chamber linear gradient as described by Brown *et al.* (13). Samples were dried at 80°C and assayed for radioactivity. Recovery of radioactivity loaded on the column was 80–90%. The percentage of total counts loaded that was recovered in the first five fractions was 20–30%.

RESULTS

Intracellular Precursors for Slp and C4 are Antigenically Distinct. Preliminary experiments suggested that anti-Slp antibody reacts with a distinct subclass of anti-Ss-reactive molecules. Difficulty was encountered in these experiments in removing all Slp-positive putative precursor in a single treatment with anti-Slp antibody at equivalence for carrier protein. It was important to exclude the possibility that this apparent antigenic difference represented a lower affinity of anti-Slp serum for a single class of precursor that was reactive with *both* anti-Slp and anti-Ss antibodies. To demonstrate antigenically distinct subclasses of putative precursors for Slp and C4, it was necessary to devise a preparative method that would allow complete separation of the two proteins. To address these questions, control immunoprecipitations were performed. The results, shown in Fig. 1, demonstrate that the putative precursors for Slp and C4 are antigenically distinct. It was possible, through exhaustive immunoprecipitation by either repetitive immunoprecipitation or *S. aureus* strain Cowan I treatment, to deplete virtually all Slp-reactive putative precursor molecules from the cell lysate (lanes A3 and B2). When Slp-depleted supernatants were then mixed with anti-Ss antibody, residual C4 molecules were precipitated (lanes A4 and B3). The existence of distinct intracellular precursors, as discriminated by differential reactivity with anti-Slp antibody, strongly suggests that the presence of discrete Slp-positive and Slp-negative subclasses of the mature protein does not result from differential processing of a common precursor.

Precursors and Isolated Subunits Show a Reciprocal Relationship in Pulse–Chase Analyses. Pulse–chase studies were conducted to provide kinetic evidence that the intracellular molecules containing Slp and C4 determinants were precursors of the processed Slp and C4 molecules found in the culture medium. After incorporation of [^{35}S]methionine for 1 hr, radiolabel was chased for various intervals prior to harvesting of medium and preparation of cell lysates (Fig. 2). At 0 min there was a heavily labeled band in the lysate that precipitated with anti-Slp antibody and a separate C4 precursor that precipitated with anti-Ss antibody but not with anti-Slp antibody. Both precursors have an apparent size of 185,000 daltons (P-185). As the length of chase was increased, there was a progressive decrease in the amount of label in the precursor bands for both Slp and C4 and a corresponding increase in the amount of label in the subunit bands from the medium. This inverse relationship between the amount of label in the intracellular Slp and C4 precursor molecules and that found in the processed subunits strongly suggests a precursor–product relationship between the putative precursors and the mature proteins.

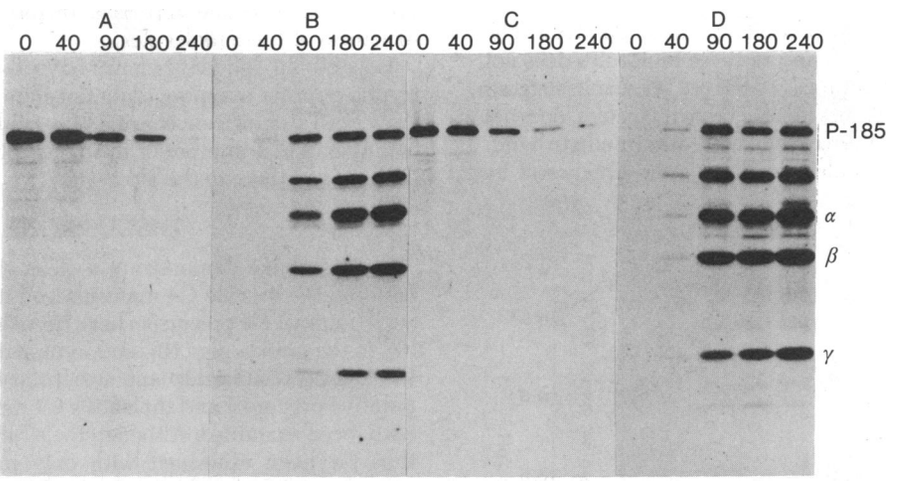

FIG. 2. Pulse–chase analysis of intracellular Slp and C4 precursors and of Slp and C4 subunits in medium. Cells were cultured in RPMI 1640 medium containing [^{35}S]methionine (400 μCi/ml) for 1 hr and washed once with phosphate-buffered saline. Fresh medium containing 1 mM unlabeled methionine was added. The cultures were chased in this medium for the indicated times (0, 40, 90, 180, or 240 min) before the medium was collected and the cells were lysed. Immunoprecipitation of the lysates and medium, first with anti-Slp and then with anti-Ss antibodies, was performed. The immunoprecipitates, after dissociation with $NaDodSO_4$ and reduction with 2-mercaptoethanol, were analyzed on 5–18% polyacrylamide/$NaDodSO_4$ gradient gels. Fluorographs of the gels were obtained after impregnation of gels with 2,5-diphenyloxazole. (Gel A) Slp from lysate: Pool of two sequential anti-Slp immunoprecipitates of lysates collected after the indicated length of chase. (Gel B) Slp from medium: Anti-Slp immunoprecipitate of medium collected after the indicated length of chase. (Gel C) C4 from lysate: Anti-Ss immunoprecipitate of the lysate after exhaustive immunoprecipitation with anti-Slp antibody. (Gel D) C4 from medium: Anti-Ss immunoprecipitate of the medium after immunoprecipitation with anti-Slp antibody.

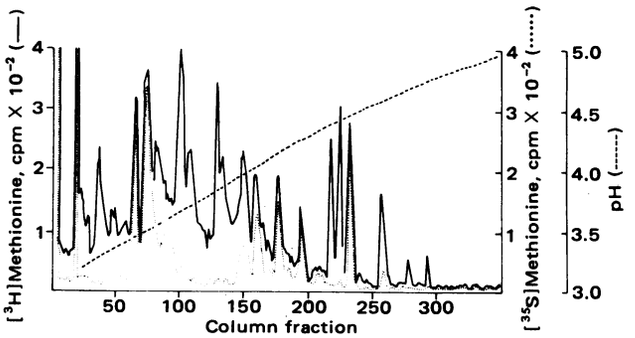

FIG. 3. Comparison of methionine-labeled tryptic peptides of C4 P-185 and C4 β-chain. [^{3}H]Methionine-labeled C4 P-185 and [^{35}S]methionine-labeled C4 β-chain were prepared by immunoprecipitation and gel electrophoresis. The purified proteins were reduced and alkylated and then digested with trypsin. Tryptic peptides were chromatographed on a column of Durrum DC-4a cation-exchange resin and assayed for radioactivity by scintillation counting. —, [^{3}H]Methionine-labeled tryptic peptides of C4 P-185; ·····, [^{35}S]-methionine-labeled tryptic peptides of C4 β-chain.

Immunoprecipitates of the culture medium contain several bands in addition to radiolabeled α-, β-, and γ-chains. A number of minor components may represent degradation products of Slp and C4 molecules. Two other major components are consistently present in the immunoprecipitates. One of these, with a molecular weight of 185,000, possesses a mobility on $NaDodSO_4$ gels identical to that of the putative intracellular precursor. Distinct subpopulations of the extracellular precursor were detected; both Slp and C4 precursor molecules were present in the culture medium. The failure of the radioactivity found in extracellular precursor to diminish with increasing time suggests that this molecule is not processed extracellularly into the mature protein.

The other major radiolabeled band seen in the culture medium immunoprecipitates possesses a molecular weight of approximately 130,000, which is between the molecular weight of the putative precursor and that of the processed α-subunit. Again, separate Slp and C4 subclasses of this protein are found. It is possible that these molecules represent processing intermediates between putative precursors and mature proteins. The fact that the radioactivity found in these molecules does not chase into the extracellular processed Slp or C4 subunits suggests that such processing, if it does involve these molecules, does not occur extracellularly. Alternatively, these intermediate bands may reflect extracellular degradation of the precursors by proteases.

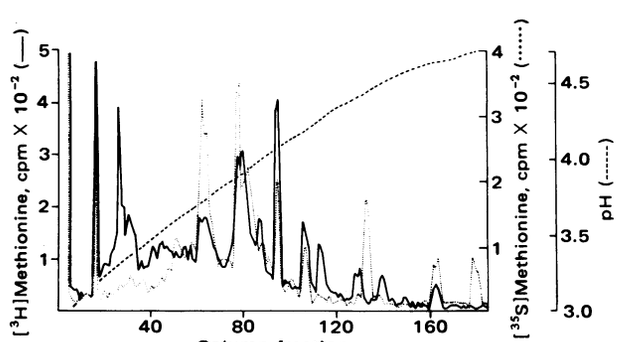

FIG. 4. Comparison of methionine-labeled tryptic peptides of Slp α-chain and C4 α-chain. Samples were prepared and chromatographed as described in the legend for Fig. 3. —, [^{3}H]Methionine-labeled tryptic peptides of Slp α-chain; ·····, [^{35}S]methionine-labeled tryptic peptides of C4 α-chain.

Tryptic Peptide Comparisons of Precursor Molecules and Isolated Subunits Show Sequence Homologies. To define the degree of structural homology between putative precursors and processed C4 molecules, tryptic peptide comparisons were performed between the precursor and the individual subunits. A representative map is shown in Fig. 3, in which C4 precursor is compared with the isolated β-chain. It is evident that a high degree of similarity exists in the peptide profiles; the profile of C4 P-185 contains all of the tryptic peptides present in the β-chain. Similar results were obtained when the tryptic peptides of isolated C4 α- or γ-chains were compared with those of C4 P-185 and when Slp α-chain was compared with Slp P-185 (data not shown). This identity of tryptic peptides, in conjunction with the pulse–chase data presented above, indicates that the putative Slp and C4 precursors are indeed precursors for the processed molecules and not related but structurally distinct molecules.

Tryptic Peptide Comparisons of Isolated Subunits from Slp and C4 Show Structural Differences. The degree of homology between isolated α- and β-subunits of Slp and C4 was analyzed by comparison of their tryptic peptide profiles. A representative peptide map is shown in Fig. 4, in which the [^{3}H]methionine-labeled tryptic peptides of the Slp α-chain are compared with those of the [^{35}S]methionine-labeled C4 α-chain. It is apparent that the two molecules share a number of peptides, revealing a significant degree of homology in their primary structures. However, peptides that are not shared are also present for each molecule. Similar results were obtained when a second comparison of Slp and C4 α-chains was performed in which the radiolabels in the two chains were reversed (data not shown). In the tryptic peptide map shown in Fig. 4, seven peptides are shared (fractions 17, 62, 64, 78, 95, 106, and 163); the Slp α-chain possesses an additional seven distinct peptides (fractions 26, 31, 80, 87, 113, 130, and 140) and the C4 α-chain has at least four unshared peptides (fractions 51, 82, 133, and 180). Incompletely resolved differences may also occur in fractions 40–50. These distinct peptides can not result from differential glycosylation, because the glycopeptides for the α- and β-chains of both Slp and C4 are found in the first five fractions eluted from the column (K. Parker, unpublished data). They must therefore represent differences in the primary structures of these molecules.

Isolated Slp and C4 β-chains have also been compared by tryptic peptide mapping (data not shown). The results again show both a significant sharing of peptides (seven peptides in common) and a number of distinct peptides (three in the C4 β-chain and three in the Slp β-chain).

DISCUSSION

The data above demonstrate a clear structural relationship between the murine C4 molecule and its putative precursor. Such putative C4 precursors have been described in the mouse (6), in the guinea pig (10), and in humans (14, 15). However, the kinetic relationship and structural identity between the putative precursor and the native C4 molecule had not previously been examined. Although the structural relationship has thus far been examined with only methionine-containing peptides and with only one method of peptide resolution, the total identity of peptides from isolated subunits with the precursor leaves little doubt of their structural correspondence. This relationship is further evidenced by the pulse–chase kinetics presented. Similar peptide map and pulse–chase analyses of hamster C3 and its precursor have been reported (16).

Our observation in the pulse–chase experiments of two major supernatant bands with higher molecular weights than the α-chain raises some questions concerning the nature of the

processing mechanism. The failure of these bands to chase into subunits implies that they are secreted in parallel with the subunits. Whether this represents an escape of unprocessed (or partially processed) molecules or the active secretion of proteins that might have some other, as yet undefined, function(s) remains to be determined. The band at approximately 130,000 daltons is antigenically and, because it shares peptides with the isolated α-chain (K. Parker, unpublished data), also structurally related to C4. Whether it represents an intermediate step in processing or is the result of proteolytic degradation of the precursor is not yet established.

It was first postulated in 1971 (17) that two discrete structural genes in the *S* region control Slp-positive and Slp-negative molecules, but structural evidence on this point has heretofore been difficult to obtain. The data presented in this paper demonstrate a clear antigenic difference between Slp and C4 precursors and multiple peptide map differences between the subunits of these molecules. Thus it seems clear that they have different amino acid sequences, because the peptide map differences cannot be due to differences in glycosylation. This finding extends the previous demonstration of molecular weight differences between the individual subunits of the two molecules (6, 7). The peptide map differences are not, however, due simply to the differences in molecular weights of the subunits, because each α-chain has multiple unique peptides. Thus, barring some mechanism in which a single transcript could be processed into two different messages under the influence of androgen, the data indicate that there must be two discrete structural genes for the two precursors.

It is difficult to evaluate the degree of homology between C4 and Slp molecules, because only methionine-containing peptides have been examined. In the α-chains, about 50% of peptides are shared; similar levels of sharing have been reported between products of other discrete loci in the major histocompatibility complex (13). It seems clear that the two molecules are structurally very similar and almost certainly represent products of duplicate structural genes (6, 17). Recent evidence also suggests duplication of the major histocompatibility complex-linked human C4 genes (18).

It is likely that the two murine genes are located in the *S* region of the *H-2* gene complex. We reported previously (6) that the C4 α-subunit determined by the $H\text{-}2^{w7}$ haplotype differs in molecular weight from that controlled by other *H-2* haplotypes. Evidence presented elsewhere (19) shows a peptide map difference between the C4 β-chains determined by the *S* regions of the $H\text{-}2^{w7}$ and $H\text{-}2^{d}$ haplotypes. Carroll and Capra (20) have recently reported peptide map differences, using limited proteolysis mapping, in C4 β-chains controlled by different *H-2* haplotypes. We have also observed an *S*-region-controlled difference in C4 hemolytic efficiency (J. P. Atkinson, and D. C. Shreffler, unpublished results), apparently due to a difference in molecular structure. All of this evidence indicates that the *S* region gene specifying C4 is indeed a *structural* rather than a *regulatory* gene that controls a structural gene located elsewhere. Structural differences in Slp molecules determined by different *H-2* haplotypes, which would define the *Slp* structural gene, have not yet been identified, but it is likely that the *Slp* structural gene is adjacent to the *C4* structural gene in the *S* region. The methodologies now developed for detailed structural analyses of the C4 and Slp molecules should permit the definition of this and other genetic, functional, and evolutionary aspects of these proteins.

We thank Drs. Lynne Brown and James Wedner for technical advice, Drs. John Atkinson, Carol Cowing, Susan Cullen, Paul Levine, and Benjamin Schwartz for helpful comments on the manuscript, Mr. Ronald Jackson and Mr. John Peterein for technical assistance, and Mrs. Carol Jones and Mrs. Karen Perks for preparation of the manuscript. This work was supported by Research Grant AI12734 and Training Grant GM07200 from the National Institutes of Health.

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