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Immunology

Amino acid sequence homologies and glycosylation differences between the fourth component of murine complement and sex-limited protein

(major histocompatibility complex/gene duplication)

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ABSTRACT **Limited primary sequence data have been obtained for all three subunits of the fourth component of murine complement (C4) and its related homologue, the sex-limited protein (Slp). These data show a high degree of NH_2-terminal homology between C4 and Slp: four of the six residues identified for the α chain, seven of eight for the β chain, and four of four for the γ chain. This suggests that apparent molecular weight differences between C4 and Slp subunits are *not*, as previously suggested, due to a shift in the proteolytic processing sites in the pro-Slp polypeptide molecule. Chemical deglycosylation (apparently complete) of the C4 and Slp α chains with trifluoromethanesulfonic acid removes the molecular weight difference between them, suggesting that acquisition of extra glycosylation sites in the latter is responsible for this difference.**

Sex-limited protein (Slp) is a homologue of the fourth component of murine complement (C4). Both Slp and C4 are synthesized as antigenically distinct, $M_r \approx 200{,}000$, single-chain precursors that are subsequently cleaved to the α, β, and γ chains of the native molecules (1, 2). Slp synthesis is inducible (androgen dependent) in certain strains (3) and constitutive (androgen independent) in others (4, 5). Based on pulse–chase studies and peptide mapping, it has been shown that C4 and Slp are encoded by separate loci in the *S* region of *H-2*, the murine major histocompatibility complex (2).

The major structural differences between Slp and C4 are in the apparent molecular weights of the subunits on Na-DodSO$_4$/polyacrylamide gel electrophoresis. The α, β, and γ chains of Slp have M_rs of $\approx$105,000, 72,000, and 32,000, respectively. The corresponding C4 subunits have M_rs of 94,000–98,000, 74,000, and 34,000 (2). Most mouse strains have the M_r 98,000 C4 α chain, while those having the $H\text{-}2^{w7}$, $H\text{-}2^{w16}$, and $H\text{-}2^{w19}$ haplotypes have the M_r 94,000 α chain. This has been shown to be due to differential glycosylation of the carboxyl-terminal portion of the subunit (6). Partial amino acid sequences of pro-C4 and its subunits showed that the β chain is amino terminal in the precursor (7). Carboxypeptidase Y digestion showed that the γ chain is carboxyl terminal in pro-C4, thus establishing the subunit order as β-α-γ (8). Assuming that the subunit order is the same in pro-Slp and given the differences in subunit molecular weight, Roos *et al.* (1) hypothesized that the β-α and α-γ cleavage sites in pro-Slp had been mutationally shifted (1). We report here that this explanation is not correct and that the α-chain size difference is related to differential glycosylation.

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MATERIALS AND METHODS

Mice. All mice were obtained from the animal colony of the Department of Genetics, Washington University School of Medicine. Mice bearing the S^{w7} region were the F_2 and F_3 generations of a mating of $H\text{-}2^{w7}$ congeneic strains B10.WR and C3H.WSlp.

Antisera. Rabbit anti-murine serum substance (anti-Ss) was prepared as described (9) and reacts with both C4 and Slp. Anti-Slp was produced as described (7) using SWR/J mice as recipients and DBA/2J male mice as donors. Murine C4 and its associated fragments are the major proteins immunoprecipitated from plasma by anti-Ss after clearance by anti-Slp.

Cultures. C4 and Slp were biosynthetically radiolabeled and purified by direct immunoprecipitation as described (7). [2,3-^{3}H]Valine (15 Ci/mmol; ICN; 1 Ci = 3.7×10^{10} becquerels), [4,5-^{3}H]leucine (135 Ci/mmol), and [4,5-^{3}H]lysine (75 Ci/mmol) (both Amersham) were added at an activity of 5 mCi/ml to RPMI 1640 medium deficient in the appropriate amino acid (Select-Amine, GIBCO). [^{35}S]Methionine (1,500 Ci/mmol, Amersham) was used at an activity of 200–800 μCi/ml.

Amino-Terminal Sequence Analyses. Automated microanalyses were performed as before (7). To obtain reproducible sequences of Slp subunits, it was necessary to preclear the culture supernatants with an irrelevant antiserum (8).

Deglycosylation of C4 and Slp. Carbohydrate was removed from immunoprecipitated C4 and Slp by a modification (6) of the method of Edge *et al.* (10). Briefly, the precipitated proteins were treated with anhydrous trifluoromethanesulfonic acid containing 10% anisole. After 2 hr on ice, the proteins were recovered by neutralization with pyridine/diethyl ether. Pyridine and the acid salt were removed by dialysis against 0.1 M ammonium bicarbonate, and the proteins were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (11).

RESULTS

Partial Amino-Terminal Sequence of Slp. The known amino acid sequences of the murine C4 and Slp subunits and human C4 subunits are compared in Table 1. Overall, there is a high degree of homology. Certain differences do exist. For example, position 8 of the Slp β chain differs from that of the C4 β chain and positions 5 and 8 of the C4 and Slp α chains differ from one another and from those of the human C4 α chain. Each of the amino acid differences can be explained by single-base-pair

Abbreviations: C4, fourth component of complement; Ss, murine serum substance; Slp, sex-limited protein.

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Table 1. Partial amino acid sequence of murine C4 and Slp and human C4

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Human C4β*	Lys	Pro	Gly	Leu	Leu	Leu	Phe		Pro								
Murine																	
C4β†	Lys			Leu	Leu	Leu	Phe	Val			Val	Val	Val				
Pro-C4†	Lys			Leu	Leu	Leu	Phe	Val			Val	Val	Val				
Slpβ	Lys			Leu	Leu	Leu		—			Val	Val	Val				
Pro-Slp	Lys			Leu	Leu	Leu		—			Val	Val	Val				
Human C4α	Asn	Val	Asn	Phe	Gln	Lys	Ala	Ile	Asn	Glu	Lys	Leu	Gly	Gln	Tyr	Ala	Ser
Murine																	
C4α†		Val		Phe	—	Lys		Val			Lys	Leu					
C4α-γ‡		Val			—	Lys		Val			Lys	Leu					
Slpα		Val			Leu	Lys		Leu			Lys	Leu					
Slpα-γ		Val			Leu	Lys		Leu			Lys	Leu					
Human C4γ	Glu	Ala	Pro	Lys	Val	Val	Glu	Glu	Gln	Glu	Ser	Arg	Val	His	Tyr	Thr	Val
Murine																	
C4γ†				Lys	Val								Val				Val
Slpγ				Lys	Val								Val				Val

Peritoneal macrophages from mice bearing the S^{w7} region were cultured in the presence of the indicated radioactive amino acid. C4 and Slp were isolated by immunoprecipitation, subunits were purified by gel electrophoresis, and subunit sequences were determined by automated microanalysis. Blank spaces indicate amino acids not tested or identified. Dashes indicate the absence of a residue found in some, but not all, murine C4 or Slp chains.

* Human C4 sequences are from Gigli *et al.* (12), Bolotin *et al.* (13), Moon *et al.* (14), and our unpublished data.

† Parker *et al.* (7).

‡ Karp *et al.* (15).

transversions in the DNA sequence. These differences were confirmed in two ways. First, the sequences of at least two different protein preparations were determined. Second, in addition to the subunits, the sequences of two other related polypeptides were determined. The C4 β chain is amino terminal within pro-C4 and, as expected, the same is true for the Slp β chain and pro-Slp. Therefore, the differences between the β chain are confirmed by the precursor sequences. Likewise, the α-chain sequences were confirmed by analyzing the unprocessed α-γ fragment of pro-C4 and pro-Slp that is present in most strains (15). These data suggest that the major differences between the C4 and Slp subunits, particularly the α and γ chains, are not due to shifts in the processing sites in the precursors.

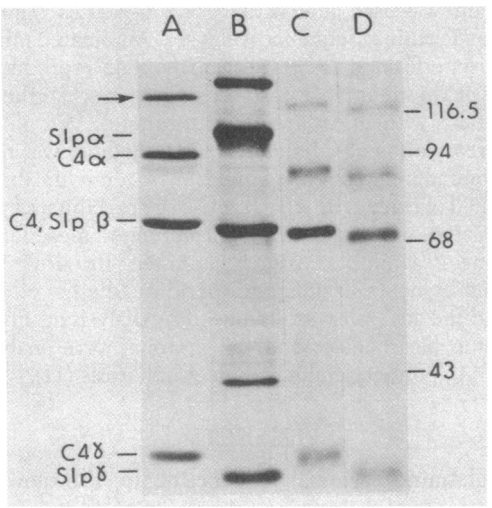

FIG. 1. Deglycosylation of murine C4 and Slp. Peritoneal macrophages were cultured in the presence of [^{35}S]methionine. C4 and Slp were immunoprecipitated from the culture medium and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions. Prior to electrophoresis, some immunoprecipitates were treated with anhydrous trifluoromethanesulfonic acid to remove carbohydrate. Tracks: A, untreated murine C4; B, untreated Slp; C, deglycosylated murine C4; D, deglycosylated Slp. The arrow (left) indicates the α-γ fragments of pro-C4 or pro-Slp (15). Relative molecular weights ($\times 10^{-3}$) of marker proteins are given on the right. Exposure times were 1 day (track A), 2 days (tracks B and C), and 7 days (track D).

Removal of Carbohydrate Eliminates the Molecular Weight Difference Between C4 and Slp α Chains. An autoradiograph of a polyacrylamide gel containing [^{35}S]methionine-labeled Slp and C4 isolated from peritoneal macrophage cultures is shown in Fig. 1. Lanes A and B contain intact (untreated) C4 and Slp, respectively. Lanes C and D contain C4 and Slp that have been chemically deglycosylated with trifluoromethanesulfonic acid. The large difference in mobility between the Slp and C4 α chains is abrogated by removal of carbohydrate. This suggests that most, if not all, of the apparent molecular weight difference between the Slp and C4 α chains is due to differential glycosylation. The β chains, which are also glycosylated (ref. 16; unpublished data), have lower molecular weights after acid treatment. However the slight difference between the C4 β chains and the Slp β chains is maintained.

DISCUSSION

This paper reports amino acid sequence data from murine Slp. Early attempts using the methodology developed for microanalysis of the sequence of murine C4 were hampered by high background radioactivity. This problem was solved by first clearing the culture supernatants with goat anti-human factor B, which crossreacts with murine factor B. The sequences obtained provide detailed evidence for the hypothesis that murine C4 and Slp chains arose by gene duplication and underscore the evolutionary conservation of C4.

The demonstration that the Slp β chain is amino terminal in pro-Slp and the existence of Slp α-γ fragments (15) points to similarities in the biosynthesis and processing of C4 and Slp. Both precursor molecules appear to be first cleaved at the β-α site, creating molecules with a (β, α-γ) composition. Some of these molecules are secreted, as shown by the appearance of

α-γ fragments on polyacrylamide gels. The rest are cleaved at the α-γ site to form the nascent three-chain structures.

The data in this paper support the thesis that differential glycosylation is responsible for the M_r difference in the α chains of C4 and Slp. In another paper (6), it is reported that the decrease in α-chain M_r of $\approx$4,000 seen in certain wild-derived strains is due to the loss of a "complex type" oligosaccharide on the carboxyl-terminal portion of the protein. This was also found to correlate with loss of 75% of the C4 hemolytic efficiency (17).

The majority of the apparent molecular weight differences between C4 and Slp α chains also localize to the carboxyl-terminal portion of the subunit (unpublished data). We have found allotypic molecular weight variants of the Slp α chain in two wild-derived congeneic strains. Preliminary experiments suggest that this variation is also due to glycosylation differences in the carboxyl terminal portion of the subunit. It may be that this portion of the α chain is especially susceptible to mutational changes that affect the ability of the protein to be glycosylated. The synthesis of N-linked oligosaccharide involves transfer of the sugar from a lipid carrier to the asparagine in the sequence Asn-X-Ser(Thr) (18). Any change in this sequence can cause the loss or gain of a glycosylation site. However, not all potential sites are glycosylated (18, 19). This implies that other features of primary, secondary, or tertiary protein structure are important. Only when the relevant amino acid sequences of C4 and Slp are obtained will the mechanisms of differential glycosylation be determined.

The hypothesis that the apparent molecular weight differences between C4 and Slp subunits represent differential processing of a common-sized precursor is untenable in light of these data. One alternative is that multiple mutations in the Slp gene(s) have led to acquisition of glycosylation sites in the α chain and conformational changes or deletions in the β and γ chains. However, it is still impossible to determine the molecular basis for the lack of hemolytic activity seen in the Slp protein.

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