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The *C4* and *Slp* genes of the complement region of the murine *H-2* major histocompatibility complex

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Recent analyses, at the protein and DNA levels of structure, of the murine complement component C4 and the closely related sex-limited protein, Slp have led to new insights into the *H-2/S* region-linked *C4* and *Slp* genes and their products. The primary products are 200000 Da precursors which are cleaved, intracellularly and extracellularly, into the mature α–β–γ-subunit molecules of plasma. Precursor order of subunits is β–α–γ; a complementary DNA clone spanning the α–γ junction has been extensively analysed. The C-terminal of the α-chain is of particular interest because of post-secretion processing which differentiates 'secreted' and 'plasma' forms of C4, both apparently functional, and because allelic variants of C4 and the Slp protein, which differ substantially in molecular masses, owe their differences principally to different levels of glycosylation of the α-chain. Allelic variations in rate of C4 synthesis (C4-high compared with C4-low) have been analysed in cultures of hepatocytes and macrophages. Three distinct modes of genetic regulation of the expression of the Slp protein have been identified.

The murine *C4* and *Slp* genes and their products represent a very useful experimental model for the definition of the clinically important *C4* genes and their products in man. The mouse is particularly promising for the potential elucidation of genetic, biochemical and functional aspects of the C4 proteins. These have been the objects of research in our laboratories for a number of years. Understanding of the mouse system has both contributed to and benefited from parallel studies of human C4. Work in our laboratories has been particularly focused on the genetics and biochemistry of the murine C4 protein and its structural homologue, the sex-limited protein (Slp). These two proteins exhibit a variety of genetic variations – in activity, structure and regulation – which make them of particular interest and promise for the molecular genetic definition of the C4 component. In this chapter, we will review the principal features of our recent findings in these areas.

Genetic organization

The *C4* and *Slp* genes were many years ago localized to the *S* region of the major histocompatibility complex, the *H-2* complex, on mouse chromosome 17, by genetic fine

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structure analyses that used polymorphic class I and class II products as markers (Shreffler & David 1975). Separation of *C4* and *Slp* by recombination has not been observed, indicating that the loci are very close together. This is consistent with the recent demonstration that two discrete genomic *C4* DNA sequences are close together (Chaplin *et al.* 1983), although neither has yet been defined as *Slp*.

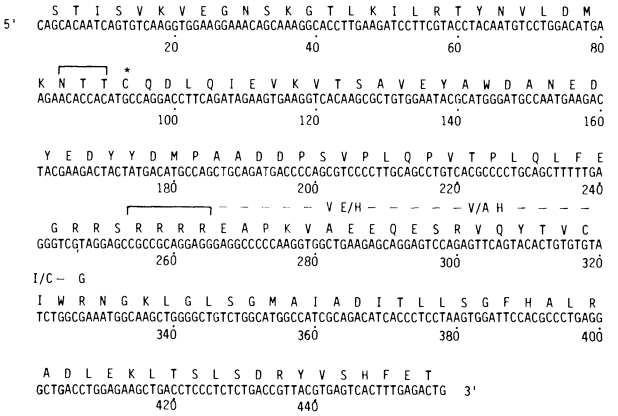

FIGURE 1. Nucleotide sequence of a cDNA clone which spans the α–γ subunit junction in the murine C4 precursor. The square brackets mark a possible glycosylation site on the α-chain and an arginine tetramer which is the probable α–γ cleavage site. The γ-chain sequence begins immediately after the tetramer. The sequence of the human C4 γ-chain is given above the assigned murine sequence; dashes indicate identity, dual assignments indicate different residues reported by two different laboratories. (Reproduced from Ogata *et al.* 1983.)

It has been known for some time that the C4 molecule, which in the mature state has three subunits, α, β and γ, is synthesized as a single precursor that undergoes intracellular processing to yield the α, β and γ-chains (Hall & Colten 1977; Roos *et al.* 1978). The order of chains in the precursor was deduced as β–α–γ from limited amino acid sequence data on the subunits (Parker *et al.* 1980; Karp *et al.* 1981). The α–γ order has been confirmed by the development of a cDNA probe (Ogata *et al.* 1983) which spans the α–γ junction and includes the coding sequences for 84 amino acids at the C-terminal of the α-chain and 64 amino acids at the NH_2-terminal of the γ-chain, plus a tetramer of arginines which are presumed to represent the proteolytic cleavage site (figure 1). The α-chain sequence derived will be particularly useful to the resolution of questions concerning post-secretional processing at the α-chain COOH-terminal (see below). The deduced γ-chain sequence is consistent with assignments based on partial sequencing of the protein. The cysteine at base positions 91–93 is presumed to be the site of the disulphide bridge between the β-chain and the α_4 fragment of the α-chain. A potential glycosylation site has also been recognized.

Although the sequence in figure 1 was identified as a C4 sequence by hybridization to C4 mRNA (Ogata *et al.* 1983), it could also have come from the gene for the structurally similar Slp. However, a new probe has been recently identified as carrying an *Slp* sequence, based upon differential hybridization reactivities with Slp-positive and Slp-negative strains. The new probe differs from the previous probe in a number of base residues, thus it appears that the initial probe defines *C4*, the second *Slp* (R. Ogata, unpublished results).

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Biosynthesis

Our structural studies of the C4 and Slp proteins have been done mainly in the course of studies to define the basis for genetic variations in the proteins. However, a number of interesting features of the biosynthesis of the C4 protein have emerged from these studies. Most of these features have been demonstrated for both murine and human C4.

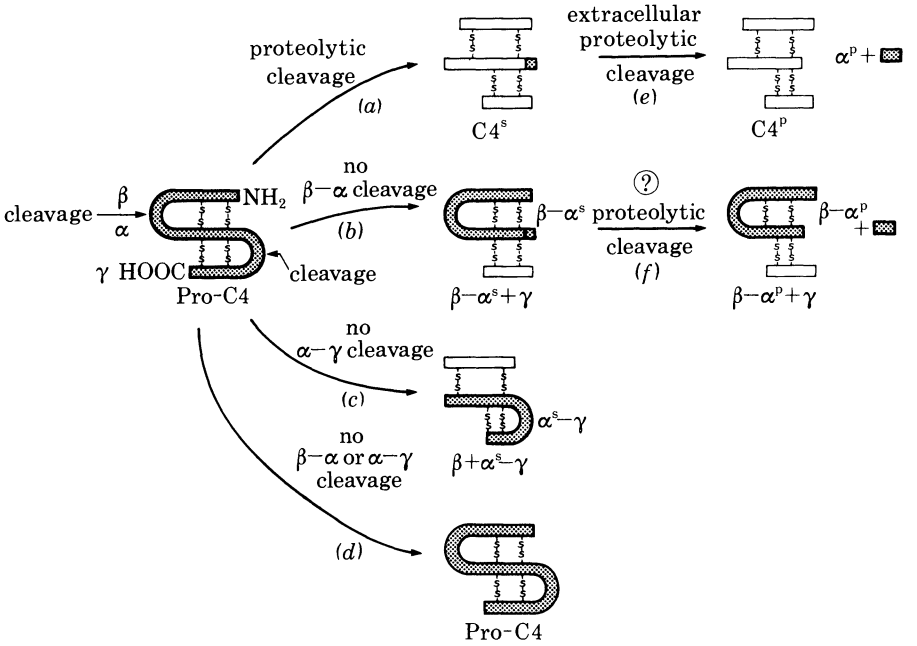

Figure 2. A model of processing of the C4 precursor. (*a*) Cleavage at the β–α and α–γ junctions to yield the predominant, 'secreted' three-subunit molecule. (*b*), (*c*) Cleavage at only one site to yield secreted two-subunit molecules with either uncleaved βα or αγ chains. (*d*) Secretion of uncleaved precursor. (*e*) Extracellular cleavage of a COOH-terminal fragment to yield the predominant, 'plasma' form of C4. (*f*) Extracellular processing of the uncleaved βα chain may occur, but has not be documented. (Reproduced from Chan & Atkinson 1983.)

After the synthesis of the precursor polypeptide, a number of further processing steps occur intracellularly, before secretion (cf. figure 2). The precursor polypeptide is cleaved to release the three subunits. For both human and murine C4, this step does not go to completion, since two-subunit molecules of the βα+γ and β+αγ types have been observed in plasma and in hepatocyte culture media (Karp & Shreffler 1982; Chan & Atkinson 1983). In the mouse, there is a clear cut genetic variation in this step. In mice of the *H-2*^{d} haplotype, a substantial number of molecules carrying the uncleaved βα fragment are observed, while in mice of other haplotypes, much more uncleaved αγ than βα material is secreted (Karp & Shreffler 1982). This difference has been mapped to the *S* region of *H-2* and presumably reflects a structural difference in the C4^{d} precursor molecule which influences the rate of proteolytic cleavage separating the β and α chains. Differences in the relative βα/αγ subunit ratios in different human plasmas have been observed (Chan & Atkinson 1983); these may also reflect genetic differences.

Three additional processing steps occur following synthesis of the C4 precursor polypeptide. Apparently these steps proceed rapidly, that is within about 20 min after synthesis – at least in cultured macrophages (Karp 1983*c*). Processing proceeds more slowly in hepatocytes (Rosa & Shreffler 1983). The three steps are: formation of disulphide bonds, both within and between

chains, formation of the thiol ester group within the sequence Gly–Cys–Gly–Glu–Glx by bonding of the cysteine sulphydryl to the γ-glutamyl group, and glycosylation. The latter process has been shown to be of particular importance to cleavage of the subunits and to secretion (Roos *et al.* 1980). The degree of glycosylation has also been shown to account for a number of genetic variations in molecular masses of C4 and Slp α-chains, as well as for differences in molecular masses of the C4 and Slp proteins.

Two further steps in biosynthesis of the mature molecule have been documented. The first is sulphation of the α-chain (Karp 1983*b*). This step occurs late in the processing of pro-C4, immediately before secretion. The modification involves the sulphation of a single tyrosine residue which has been localized to the segment of α-chain that is COOH-terminal to the thiol ester group. It is not yet known whether this modification may have functional significance.

A final maturation step occurs following the secretion of the three-chain molecule. The secreted form of the murine C4 molecule ($C4^s$) has an α-chain which has an apparent M_r approximately 4000 Da larger than that of the predominant form of C4 found in the plasma ($C4^p$). The processing apparently involves a proteolytic step, since radiolabelled $C4^s$ from macrophage cultures is readily converted to $C4^p$ by incubation with mouse plasma, and this conversion is blocked by protease inhibitors (Karp *et al.* 1982). This modification also occurs in the COOH-terminal segment of the α-chain. Deglycosylation by trifluoromethanesulphonic acid does not alter the M_r difference between α^s and α^p chains, so differential glycosylation is excluded. A parallel difference has been documented between human $C4^s$ from a cultured hepatoma line and the plasma $C4^p$ form (Chan & Atkinson 1983). These authors have also shown that the $C4^s$ form has activity in a functional assay, so this processing step is not required for activation of the molecule. The biological significance of this modification in unclear.

It is worthy of particular note that, although it has been somewhat less rigorously examined to date, the Slp molecule appears to proceed in parallel fashion through all of the processing steps outlined above for C4, with one exception. The α-chain of Slp does not undergo the post-secretion cleavage of a COOH-terminal fragment of the α-chain demonstrated for C4 (Karp *et al.* 1982). Further C4–Slp structural differences are discussed below.

Genetic variations

Since the initial detection (Shreffler & Owen 1963) of MHC-linked quantitative differences among mouse strains in levels of a then unidentified serum protein (Ss), now known to be C4, a variety of forms of genetic variation in this protein have been recognized. These C4 variants may be classified as structural, functional and regulatory. A search for genetic variants of C4 by immunochemical techniques led to the production of alloantisera which defined genetic variation (presence–absence) in a C4-related protein called Slp (sex-limited protein) (Passmore & Shreffler 1970). Subsequently, structural and regulatory variants of Slp were recognized. The known genetic variations in the C4 and Slp proteins are summarized in tables 1 and 2 and further outlined in the following sections.

Structural and functional variants

Because the α-chain is the site of the principal C4 activation and inactivation steps, it might be anticipated that structural changes in this chain could alter functional activity. Although a direct relation has not yet been demonstrated, it is possible that the reduced haemolytic

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efficiency shown in table 1 for the C4 molecules of strains B10.WR ($C4^{w7}$) and B10.KPB ($C4^{w19}$) (Atkinson *et al.* 1980; Karp *et al.* 1982*b*) may reflect the reduced molecular masses of the α-chains of these molecules (Roos *et al.* 1978; Karp *et al.* 1982*b*). The reduced molecule masses reflect a reduced level of glycosylation of the COOH-terminal fragment of the α-chain (Karp *et al.* 1982*b*; Karp 1983*a*). This is illustrated in figure 3. Further studies will be required to establish a relation, but it is clear that proper glycosylation of the C4 α-chain is required for functional activity (Karp 1983*c*). The reduced glycosylation probably reflects a difference in amino acid sequence of the α-chain site or sites for carbohydrate attachment.

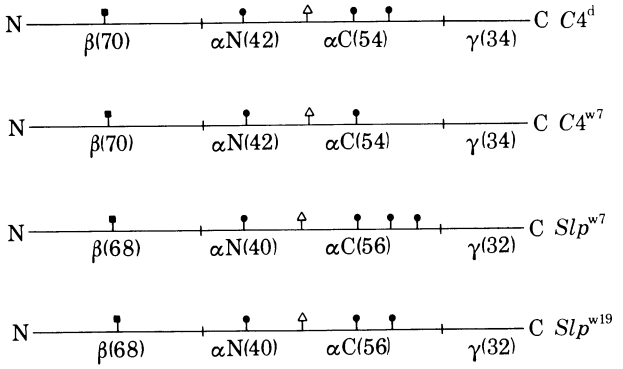

FIGURE 3. A model of the proposed structural differences among *C4* and *Slp* allelic products. Numbers in parentheses are the relative molecular masses of the β and γ-chains and α-chain autolytic fragments. Solid square, high-mannose oligosaccharide; solid circle, complex type oligosaccharide; open triangle, site of internal thiol ester. (Reproduced from Karp 1983*a*.)

TABLE 1. GENETIC VARIANTS OF MURINE C4

		intact C4			α-chain			β-chain		γ		α–γ chain
H-2 haplotype	congenic strain	serum level	haemolytic efficiency	i.e.f variants	molecular mass variants	C4d1 fragment	C4d2 fragment	tryptic peptides	V8 protease peptides	i.e.f variants	molecular mass variants	presence–absence
b	B10	high	1.0	7.5	1	–	+	2	1	2	2	+
d	B10.D2	high	1.0	7.0	1	–	+	1	2	2	2	–
k	B10.K	low	1.0	7.3	1	+	–	1	1	2	2	+
p	B10.P	high	1.0	n.t.	1	+	–	n.t.	2	1	1	+
s	B10.S	high	1.0	7.0	1	+	–	n.t.	2	1	1	+
w7	B10.WR	high	0.15	n.t.	2	–	+	2	n.t.	2	2	+
w19	B10.KPB	high	0.25	n.t.	2	–	+	n.t.	n.t.	1	1	+

Abridged from Atkinson *et al.* 1982.
n.t. Not tested.

Another genetic difference that has been attributed to a difference in level of glycosylation of the COOH-terminal segment of the α-chain occurs in the Slp molecule of B10.KPB ($H\text{-}2^{w19}$) mice (figure 3). It has been demonstrated that the Slp α-chain from this strain has one less complex carbohydrate group than those of other strains (Karp 1983*a*). It is also worthy of note that the Slp α-chains of other strains have one more COOH-terminal carbohydrate group than the typical C4 α-chain, for example of $C4^{d}$ (figure 3; Karp 1983*a*). Whether this has any bearing on the observed lack of functional activity of the Slp molecule in standard haemolytic assays remains to be investigated. It should also be noted that the β- and γ-chains of Slp differ from those of C4 by a few thousand daltons, as do those of the NH_2-terminal and COOH-terminal

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segments of the α-chain. Thus, any of several structural differences could account for the resistance of Slp to human and mouse C1 and consequent lack of functional activity.

Another interesting genetic variation involving the C4 α-chain is seen in the C4d.1 and C4d.2 allotypes. These have been localised to the C4d fragment of the α-chain. They were initially detected serologically. The C4d.1 specificity was defined many years ago as an H-2 specificity, called H-2.7, because it was detected by the haemagglutination assay utilized for detection of the *H-2K* and *H-2D* products and its expression was controlled by an *H-2*-linked gene. However, it was not until 1979 that it was recognized that this specificity is carried by C4d fragments, is detectable because these fragments are associated with erythrocytes, and is controlled by a genetic element in the complement region of the *H-2* complex (Huang & Klein 1979, 1980; Ferreira *et al.* 1980*a*). Shortly thereafter the antithetical specificity, C4d.2, was defined and shown also to be carried by the C4d fragment (Spinella & Passmore 1983). These specificities are somewhat analogous to the Chido and Rodgers specificities of human C4d (Chu *et al.* 1982), however the latter specificities define the products of the two discrete human loci, *C4A* and *C4B*, while C4d.1 and C4d.2 of the mouse define allelic products at the single murine *C4* locus. In addition to the serological difference in murine C4d, it has recently been shown that C4d.1 and C4d.2 fragments have different isoelectric points by isoelectric focusing (C. Killion, unpublished results), suggesting a structural basis for the serological difference. The basis for this molecular charge difference is under investigation.

TABLE 2. GENETIC VARIANTS OF MURINE Slp

H-2 haplotype	congenic strain	serum level		α-chain molecular mass variants	β-chain V-8 protease peptides	γ-chain i.e.f. variants
		males	females			
b	B10	0	0	−‡	1	1
d	B10.D2	1.0	0	1	1	1
p	B10.P	0.04	0	1	1	1
s	B10.S	0.25	0	1	1	1
w7	B10.WR	2.2	1.5	1	2	2
w19	B10.KPB	0.05	0.03	2	n.t.	2
[d	FM†	4.0	1.5	n.t.	n.t.	n.t.]

Abridged from Atkinson *et al.* 1982.
n.t. Not tested.
† Not congenic.
‡ −, Absence of the Slp protein.

As shown in tables 1 and 2, a number of other genetic differences in C4 and Slp structure have been defined. They include peptide map differences in the C4 β-chain (Parker *et al.* 1980; Carroll & Capra 1979) and the Slp β-chain (Parker *et al.* 1981), isoelectric focusing differences in the γ-chains of C4 (Ferreira *et al.* 1980*b*) and of Slp (Ferreira *et al.* 1980*c*), and a small apparent molecular mass difference in the C4 γ-chain (D. Karp, unpublished observations). The isoelectric focusing and molecular mass differences in the C4 γ-chain show the same haplotype distributions and may reflect the same structural difference.

Regulatory variations

In addition to the variety of structural variants available for analysis, the murine C4–Slp system is of great potential interest for molecular genetic studies because of several unique regulatory variations in expression. These are also summarized in tables 1 and 2.

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The genetic variation that first defined the murine C4 system was a 20-fold difference among mouse strains in the level of a specific serum protein, denoted Ss, detected by a specific anti-Ss serum by immunodiffusion (Shreffler & Owen 1963). Ss was eventually shown to be C4. The genetic basis for this large quantitative difference in serum C4 levels has been a subject of particular interest. The two general possibilities for such a difference were: (i) a lower rate of C4 synthesis in C4-low strains, or (ii) a structural difference in the C4 of C4-low mice leading to more rapid degradation of the molecule. This question seems now to have been clearly resolved by three recent papers. Rosa & Shreffler (1983) showed, in primary hepatocyte cultures, that the cells from C4-high strains synthesize C4 more rapidly than those from C4-low strains. Further, there is no evidence for increased catabolism of the C4 molecule synthesized in cultures of C4-low cells. These observations were supported by Ogata *et al.* (1983), who demonstrated, by cell-free translation, higher levels of C4 mRNA in liver cells from C4-high mice than in cells from C4-low mice. The differences in mRNA levels parallel the differences in serum C4. This indicates that the difference in rate of synthesis of C4 reflects a difference in levels of C4 mRNA transcribed. Chaplin *et al.* (1983) demonstrated, by hybridization of C4 mRNA with a C4 cDNA probe, that C4 mRNA levels are significantly higher in C4-high mice than in C4-low mice. Thus it appears that this difference in C4 levels is due to a difference between C4-high and C4-low mice either in the rate of transcription or in the stability of the C4 mRNA.

Another recent observation of interest relating to the C4-high–C4-low variation is that of Newell *et al.* (1982) with cultured peritoneal macrophages. In these studies, no significant difference was found in C4 levels between cells of C4-high and C4-low origins, as measured by either C4 haemolytic activity levels or immunoprecipitable C4 levels. Thus, the regulatory difference appears not to operate in macrophages. Whether this reflects different regulatory elements in macrophages, the absence of enzymes that degrade C4 mRNA, or the expression in macrophages of different *C4* structural genes subject to different regulatory mechanisms remains to be explored. It should be noted that studies reported thus far on C4 products of cultured hepatocytes and macrophages have revealed no structural differences. However, a significant difference in the time scale of C4 biosynthesis and secretion between hepatocytes and macrophages has been observed (P. Rosa, unpublished results).

Finally, there are a number of interesting regulatory variations in Slp expression, for which the molecular genetic bases remain to be determined. The protein was first recognized because some strains express it, some do not. This difference is under the control of the *S* region of the *H-2* complex. Whether failure to express is regulatory or due to absence of the structural gene is a question now approachable at the DNA level. The protein derived its name because, as first detected, its expression was limited to males, in which it is induced by testosterone. The mechanism of testosterone induction is another for which the techniques of molecular genetics offer promising approaches and hopefully new insights in the near future.

The regulation of *Slp* expression became of further interest with the recognition that in some mouse strains there is no sex-limitation, that is, both males and females express the protein without a requirement for testosterone. Two types of genetic control of such 'constitutive' expression of *Slp* have been recognized. One type is directly associated with the *S* region haplotype of the expressing strain (Hansen & Shreffler 1976). Three such haplotypes, all derived from wild mice, have been defined, S^{w7}, S^{w16} and S^{w19}. In mice of these types, expression of the Slp protein is entirely concordant with expression of the C4 protein. This might be most simplistically viewed as the replacement of a testosterone-regulated control element for the *Slp*

gene with the control element regulating C4 expression. Once again, this is a very interesting question which should soon be approachable at the DNA level.

A second type of 'constitutive' expression of *Slp* has been defined, which clearly involves a mechanism distinct from that in the previous paragraph (Brown & Shreffler 1980). In the strains which exhibit this type of expression, for example, strain FM in table 2, expression is mediated by two genetic loci, which are not linked to the *S* region of the *H-2* complex or to each other. These non-*H-2* loci appear to act in females in much the same ways that testosterone does in males. *S* haplotypes capable of Slp expression must be present. A delay in expression of Slp protein until sexual maturity is observed, indicating that some developmental maturation step is required. In males, the genetic induction is superimposed upon the testosterone induction, such that these animals have very high Slp levels. The non-*H-2* genes which mediate this expression appear not to affect hormone levels or levels of expression of other complement proteins. The genetic mechanisms involved in this phenomenon are clearly of great interest, but may be less immediately approachable than those discussed above.

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