Murine complement component C4 and sex-limited protein: Identification of amino acid residues essential for C4 function

(activated form of complement component C1s protease/site-specific mutagenesis/protein structure)

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ABSTRACT Murine sex-limited protein (Slp) is an isotype of murine complement component C4 that shares 95% sequence identity with C4 as well as the intramolecular thioester necessary for C4 function but has no complement activity. Slp is nonfunctional at least in part because it is not cleaved by the activated form of complement protease C1s (C1s), which proteolytically activates C4 in the classical complement pathway. Slp is also distinct from C4 in that its expression in some mouse strains is under testosterone control. In the present studies, we used site-directed mutagenesis of C4 and expression of the mutant proteins in cultured cells to identify the amino acid substitutions in Slp that are responsible for resistance to C1s cleavage. We focused on sequence changes immediately downstream of the cleavage site in C4 because the arginine at that site is conserved in Slp, but the downstream sequences diverge substantially, with six differences in the first 7 residues followed by a 3-residue deletion in Slp. We found that a C4 mutant carrying only the 3-residue deletion is not cleaved by C1s and has essentially no hemolytic activity, whereas a mutant carrying only the six replacement changes is cleaved by C1s and has normal hemolytic activity. Both mutants have intact thioesters. A third mutant in which two acidic residues in the segment deleted in Slp were replaced by aliphatic residues is also cleaved by C1s, has an intact thioester group, and has normal hemolytic activity. These results indicate that the downstream mutations are responsible for the resistance of Slp to C1s cleavage and suggest that the length rather than the specific sequence of this segment is critical in determining susceptibility to the protease.

Twenty-six years ago, Shreffler and Owen (1) reported that mice have a serum protein present at distinctive levels among different inbred strains and that the genetic locus responsible for this quantitative trait is very closely linked to the genes for serologically defined murine histocompatibility (H-2) antigens. They named this then unidentified serum protein substance (Ss) and designated as S the H-2-linked genetic locus controlling the serum Ss level. This and subsequent studies (for review, see ref. 2) that mapped S between the K and D loci of H-2 provided conclusive evidence that the histocompatibility antigens are encoded in a gene complex rather than a single genetic locus.

In a later search for allotypic variants of Ss that was aimed at determining whether a regulatory gene controlling Ss expression or the Ss structural gene itself lay in the S locus, Passmore and Shreffler (3, 4) found a Ss variant that was expressed only in males of a subset of strains expressing high Ss levels. Because of its sex-limited expression, this Ss variant was named sex-limited protein (Slp). More recent studies (for review, see refs. 2, 5, and 6) have established that Ss is murine complement component C4 and that Ss (C4) and Slp are isotypic rather than allotypic variants with distinct structural genes that lie closely linked within the S region of the H-2 complex.

The biological role of Slp is somewhat obscure. In addition to its sex-limited expression, it also appears to be totally absent in all mouse strains (3, 4) and may, therefore, be unessential. Furthermore, Ferreira et al. (7) have shown that Slp is devoid of C4 activity in spite of a primary structure that has been shown (8-11) to be 95% identical to murine C4. Slp does, however, contain the internal thioester that is crucial to C4 function (12), and it binds to sensitized erythrocytes under the same conditions that lead to C4 binding (5). Most studies of Slp have, therefore, been aimed at identifying the structural difference(s) between C4 and Slp that renders Slp nonfunctional, at determining the biological function of this protein, and at understanding the biological significance and molecular mechanism of sex-limited expression. These are still essentially unresolved issues although recent sequence comparisons suggest that Slp has no biological function, as there appears to be little or no selective pressure to maintain a unique Slp amino acid sequence (13). In addition, sequencing of a region several kilobases upstream of the Slp gene (14) suggests that testosterone regulation of Slp expression is due to a provirus inserted in this region.

The present studies were undertaken to identify the amino acid changes that render Slp nonfunctional. We have shown (15) that transfection of cultured mammalian cells with a mouse C4 cDNA under the transcriptional control of a simian virus 40 promoter results in the biosynthesis and secretion of mature active C4. In the present studies we have used site-directed mutagenesis of C4 to construct C4 mutants carrying Slp sequences. These mutants have been used to identify Slp sequences that render C4 inactive.

MATERIALS AND METHODS
cDNAs. Construction of plasmid pC427A, which consists of a full-length mouse C4 cDNA cloned into the pC vector (16), and the sequence of the cDNA have been described (8). All plasmid DNAs were prepared by a preparative alkaline lysis procedure (17) (excluding the CsC1 equilibrium centrifugation procedure) followed by extraction with phenol, treatment with RNase A at 50 μg/ml, precipitation twice from 5.5% (wt/vol) polyethylene glycol (PEG 6000) to remove RNA (18), and ethanol precipitation.

Oligonucleotides. Oligonucleotides synthesized on an Applied Biosystems automated synthesizer or purchased from Genetic Designs (Houston, TX), were purified by electrophoresis on a 15% or 20% polyacrylamide gel in Tris/borate/EDTA (TBE) buffer (17), visualized (19), and eluted into Tris/EDTA (TE) buffer (17). Oligonucleotides used in these studies were as follows: 102, TCTATCAACTG-

Abbreviations: Slp, murine sex-limited protein; C1s, proteolytically active form of complement component C1s; Ss, serum substance.
Base-pair shock coding on EN3HANCE CrTs routinely coli polyacrylamide Washington University mice radiolabeled C4 that the preparatively G418 DNA with the gel in annealed and (22) phenol, 105 pmol of the desired mutation primer; this was by mutating oligonucleotide with that of the component C1s; see ref. 8); (iii) 0.2 pmol of a 380-bp EcoRI-Fsp I fragment that was derived from the 900-bp EcoRI-Kpn I fragment described above by digesting that fragment with Fsp I, separating the two resulting fragments on a 6% polyacrylamide gel in TBE buffer, and eluting the 380-bp fragment by diffusion (17); and (iv) 40 pmol of the kinase-treated mutating oligonucleotide. The annealed and filled-in plasmids were introduced into Escherichia coli DH5α (Bethesda Research Laboratories, and clones carrying mutant plasmids were identified by hybridization (22) with the mutating oligonucleotide that had been 5'-end-labeled with 32P.

Mutant plasmids were purified by removing a hybridizing colony from an agar plate with a toothpick, suspending the bacteria in 100 μl of TE/50 mM NaCl, extracting three times with phenol, precipitating with ethanol, taking up the nucleic acid in TE, and retransforming DH5α with this total nucleic acid extract. Candidate clones were again identified by hybridization with the mutating oligonucleotide. To confirm that the desired mutation was introduced, plasmid DNA was isolated preparatively or from minipreps (23) and sequenced (24) by the dideoxynucleotide method using oligonucleotide 105 as a primer; this oligonucleotide is complementary to the coding strand at a position about 100 bp downstream of the C Ts cleavage site. Sequences extending approximately 60 bp on either side of the C Ts cleavage site were examined.

Transfections. Mouse L cells and monkey COS-1 cells (25) were obtained from the American Type Culture Collection and routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) fetal calf serum. Transient transfections were carried out as described (26, 27) by treating cells in 35-mm dishes with 2 μg of DNA in 0.7 ml of DEAE-dextran at 200 μg/ml followed by a 15% (vol/vol) glycerol shock after 6 hr. Stable transfections were carried out by the calcium phosphate method (28) with 1 μg of plasmid DNA cotransfected with 0.5 μg of the neomycin-resistance plasmid pSV2neo (29); clones resistant to antibiotic G418 (GIBCO) were cultured individually and tested for C4 expression by immunoprecipitation of cell supernatants and gel electrophoresis as described below.

C4 Assays. Transfected cells were cultured with [35S]methionine in methionine-free medium (GIBCO) and radiolabeled C4 was immunoprecipitated from 100 μl of culture supernatants by adding carrier plasma from B10.WR mice and anti-Ss antisera (a gift from D. C. Shreffler, Washington University School of Medicine), as described (15, 30). Immunoprecipitated products were fractionated on SDS/8.5% polyacrylamide gels, which were treated with ENHANCE (DuPont/NEN) and autoradiographed. Transiently transfected COS cells were radiolabeled for 8 hr, 2 or 3 days after infection.

Autolytic cleavage of radiolabeled C4 and mutant C4 α-chains from COS-1 culture supernatants was carried out as described by Karp et al. (12). Cleavage with C Ts was carried out by adding human C Ts, prepared as described (31), to culture supernatants to give a final concentration of 5 μg/ml and incubating at 37°C for 2 hr. The reaction mixture was immunoprecipitated, separated on a SDS/polyacrylamide gel, and autoradiographed as described above.

C4 hemolytic activity was measured as described (15) with supernatants from stably transfected murine L cells that were cultured for 4 days in methylamine-inactivated fetal calf serum (32), [35S]methionine, and methionine-free medium. Relative amounts of total C4 was estimated by comparing the autoradiographic intensities of immunoprecipitated C4 γ-chains from each culture supernatant.

RESULTS

A 3-Amino Acid Deletion Is Adequate to Block C Ts Cleavage. Complement component C4 is a heterotrimer with α, β, and γ subunits having apparent molecular weights on SDS/polyacrylamide gels of 94,000, 78,000, and 33,000, respectively. Slp is an essentially identical heterotrimer with subunit molecular weights of 104,000, 78,000, and 31,000; the α-chain difference between C4 and Slp is due primarily to additional glycosylation in Slp and the γ-chain difference appears to be a gel artifact since both C4 and Slp γ-chains are identical in length with 98% amino acid sequence identity (8–11). Activation of C4 in the complement pathway involves cleavage of the α-chain to yield a small C4a peptide (M, = 9000), which has weak anaphylatoxin activity, and activated C4b, which is composed of α' (M, ≈ 85,000), β-, and γ-chains (for review, see ref. 33).

Ferreira et al. (7) have demonstrated that Slp lacks C4 function at least in part because it is not cleaved by C Ts and hence cannot undergo proteolytic activation. Comparisons of the complete amino acid sequences of C4 and Slp have revealed that the required arginine at the C Ts cleavage site in C4 is present in Slp (10, 11). However, the two sequences diverge substantially for about 10 residues immediately downstream of the cleavage site, including 6 amino acid changes and a deletion of 3 amino acids 7 residues from the cleavage site (Table 1). This striking divergence between sequences that are 95% identical overall suggested that the amino acid changes result in a conformational change in Slp, at the cleavage site, that prevents C Ts recognition or cleavage (35). One of us later proposed (10) that the deletion is likely to be the most important barrier to C Ts cleavage since the amino acid sequences of murine and human C4 also differ substantially in this region (except for the deletion) and both are cleaved by human C Ts.

To test this proposal as well as the assumption that the mutations proximal to the cleavage site are indeed responsible for blocking C Ts cleavage, we constructed two mutant mouse C4 cDNAs that would allow us to examine separately the effects of the deletion and the six amino acid replacements on C4 function. Only the deletion was introduced into the wild-type C4 sequence in the first mutant, designated CMD3; and only the replacements were introduced into the second, designated CM235 (see Table 1).

Plasmids carrying the mutant C4 sequences were transiently transfected into monkey COS-1 cells and mutant C4 proteins from cell culture supernatants were then tested for susceptibility to C Ts cleavage and for the presence of the internal thioester by autolytic cleavage (12, 36). The thioester is a sensitive indicator of the native structure (37) and hence we infer that mutant proteins that harbor the thioester have the native C4 conformation.
Table 1. Sequence of mutant C4 proteins at the C1S cleavage site

<table>
<thead>
<tr>
<th>Description</th>
<th>Plasmid</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type murine C4</td>
<td>pC427A</td>
<td>HLAHHMMLDEQNEW1DDED</td>
</tr>
<tr>
<td>Human C4A*</td>
<td></td>
<td>G-Q-AL1E10-----------</td>
</tr>
<tr>
<td>Slp&lt;sup&gt;W1.1&lt;/sup&gt;</td>
<td>pSlp20B</td>
<td>P-KV1RDN000----------</td>
</tr>
<tr>
<td>Slp-like deletion in C4</td>
<td>pCMD3</td>
<td></td>
</tr>
<tr>
<td>Slp-type amino acid replacements</td>
<td>pCM235</td>
<td></td>
</tr>
<tr>
<td>Substitution at deletion site</td>
<td>pCMD6</td>
<td>LI</td>
</tr>
<tr>
<td>Revert pCMD3 to wild-type C4</td>
<td>pRM236</td>
<td></td>
</tr>
</tbody>
</table>

Only the amino acid residues proximal to the C1S cleavage site (at the underlined arginine) are shown; these are residues 750–769 in the pre-pro-C4 sequence (8). Flanking sequences are identical in C4 and Slp for over 100 residues in either direction except for an Asp→Glu substitution 7 residues downstream of the sequence shown. Dashes indicate identity with the wild-type C4<sup>W7</sup> sequence and 0 denotes a deleted amino acid.

*Ref. 34.

†Three complete Slp cDNA sequences have been reported; two, designated Slp<sup>W1.1</sup> and Slp<sup>W7.2</sup>, are from distinct genes in mouse strain B10.WR (10, 13) and the third, Slp<sup>FM</sup>, is from mouse strain FM (11). The proline at the N-terminal end of the segment shown for Slp<sup>W1.1</sup> is replaced by histidine in Slp<sup>W7.2</sup> and Slp<sup>FM</sup> and hence is not distinctive of Slp.

Fig. 1 is an autoradiogram of an SDS/polyacrylamide gel showing the results of C1S and autolytic cleavage of radio-labeled culture supernatants from COS-1 cells transiently transfected with plasmids pC427A, pCMD3, or pCM235, which encode wild-type, deleted, or substituted C4, respectively. These results show clearly that C1S does cleave the α-chain of mutant CM235 to yield an α'-chain but does not cleave the α-chain of CMD3. There is no evidence of cleavage of CMD3 even after 6 hr in C1S at 10 μg/ml (data not shown). Hence the 3-amino acid deletion but not the 6-amino acid replacement is sufficient to block C1S cleavage. All three proteins show comparable autolytic cleavage patterns, indicating that both mutant proteins have overall conformations very similar to that of native C4; thus the effect of the mutation in CMD3 cannot be explained by a general deterioration of the native structure.

A fortuitous by-product of the construction of CMD3 was the related mutant CMD6 (Table 1) in which two charged glutamyl residues, in the segment deleted in Slp, are replaced by aliphatic leucyl and isoleucyl residues. Fig. 2 shows that this replacement mutant is also cleaved by C1S and thus provides additional evidence that C1S cleavage is not very dependent on the specific amino acid sequence of this region.

Although both CM235 and CMD6 are cleaved by C1S, they are somewhat less efficiently cleaved than wild-type C4. This is illustrated in the time dependence of the cleavage shown in Fig. 3. For both mutants cleavage was complete after 3–6 hr in C1S at 10 μg/ml (data not shown). Hence the changes in CM235 and CMD6 are not sufficient to block C1S cleavage, but they do result in poorer substrates for C1S.

Reversion of the Deletion Mutation Restores C4 Function.

Close examination of Figs. 1 and 2 reveals that the α-chains of CMD3 and CMD6 have much greater mobilities relative to the wild-type C4<sup>W7</sup> α-chain than would be expected from the anticipated mutations. These electrophoretic mobilities are consistent with CMD3 and CMD6 α-chains having a molecular weight of 91,000, suggestive of a loss of about 30 amino acid residues. The molecular change responsible for this increased mobility appears to lie in the N-terminal portion of the α'-chain since the αN autolytic fragments of both CMD3 and CMD6, and the α'-chain of CMD6 show a similar increase in mobility (Figs. 1–3). Disproportionately large mobility shifts on SDS/polyacrylamide gels have been ob-
observed, of course, in particular with the murine C4 β - and γ-chains (15) and with the α-chains of human C4A and C4B (34, 38). Nevertheless, the large shift observed with CMD3 and CMD6 raised the possibility that other unanticipated changes in the cDNA sequence may have been introduced during the mutagenesis procedure. To eliminate this doubt, we used the same mutagenesis procedure to revert CMD3 back to the original wild-type C4W7 sequence by using oligonucleotide 236. The reverted sequence, in plasmid pRM236 directs the expression of a protein with an α-chain that is indistinguishable on SDS/polyacrylamide gels from that of wild-type C4 and is also indistinguishable from wild-type C4 in its reactivity with C Ts and in autolysis (data not shown). We conclude that the properties of CMD3, including the unexpectedly large shift in α-chain mobility are due only to the anticipated mutation and assume that this is the case with CMD6 as well.

C Ts Cleavage Reflects Hemolytic Activity. Hemolytic activities of the mutant C4 proteins were assayed to determine the effects of each mutation on C4 functional activity. Stably transformed murine L cells were used for these studies because they express higher levels of C4 than transiently transfected COS-1 cells (15). Cultures were radiolabeled with [35S]methionine and both C4 quantitation and hemolytic assays were carried out on the same sample of culture supernatant. Our results are given in Table 2; they demonstrate that mutants CM235 and CMD6 have approximately normal hemolytic activities whereas CMD3 has negligible activity. The small residual activity found with CMD3 is reproducible, suggesting that this mutant is cleaved by C Ts at a very slow rate.

Table 2. Hemolytic activities of C4 mutants

<table>
<thead>
<tr>
<th>L-cell line/mutant</th>
<th>Activity</th>
<th>Relative total C4</th>
<th>Relative specific activity</th>
</tr>
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<tbody>
<tr>
<td>AJ4.41/wild-type</td>
<td>659</td>
<td>1</td>
<td>700</td>
</tr>
<tr>
<td>CMD3-26/CMD3</td>
<td>≤1</td>
<td>0.03</td>
<td>≤30</td>
</tr>
<tr>
<td>CM235F-3/CMD23</td>
<td>10</td>
<td>0.02</td>
<td>500</td>
</tr>
<tr>
<td>CMD6-19/CMD6</td>
<td>26</td>
<td>0.03</td>
<td>900</td>
</tr>
<tr>
<td>L cells</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND = not detectable. Activity is defined as the reciprocal of the dilution factor giving one effective “hit” per cell (Z = 1) as described by the one-hit theory (15, 39, 40). The relative total amount of C4 in culture supernatants with respect to wild-type AJ4.41 culture supernatant was estimated from autoradiographic intensities of γ-chains of immunoprecipitated proteins. For the relative specific activity of C4 mutants the activity was divided by the relative total C4.

FIG. 3. Time course of cleavage with C Ts. C4, CM235, and CMD6 are samples from cell culture supernatants of COS-1 cells transiently transfected with the corresponding plasmid (Table 1). Samples were treated with C Ts at 5 μg/ml for 0, 0.5, 1, and 3 hr, immunoprecipitated, separated on an SDS/polyacrylamide gel, and autoradiographed. Other labels are as in Fig. 1.

DISCUSSION

The present studies have used site-specific mutagenesis of murine C4 cDNA to alter the encoded amino acid sequence and DNA transfection to biosynthesize these specifically mutated proteins. Mutations introduced into murine C4 were based on the sequence of Slp and were designed to identify those amino acid differences that are primarily responsible for the absence of C4 function in Slp. Our results indicate that sequence changes downstream of the C Ts cleavage site are responsible for the resistance of Slp to C Ts cleavage and that a 3-amino acid residue deletion is the most important of these mutations. The latter result suggests that the length, rather than the amino acid sequence, of this segment dictates susceptibility to C Ts. It is of interest that alignment of the mouse and human C4 sequences in this region shows a single-residue deletion in mouse C4 about 10 residues upstream of the cleavage site and a single-residue insertion into the mouse sequence two residues downstream of the cleavage site; thus the overall length of this segment is equal in the mouse and human proteins.

A simple interpretation (10) of our results proposes that the peptide segment containing the C Ts cleavage site constitutes a relatively exposed linker connecting two more rigid structural domains (C4a and a domain of the α'-chain). Thus the amino acid sequence of the linker can be quite variable without affecting function, but a deletion in this segment would decrease the distance between these domains and might render the cleavage site sterically inaccessible to the protease. Our autolytic cleavage results are consistent with this picture as they indicate that the mutants we constructed all have internal thioesters with normal stabilities. Thus the mutations in this segment apparently affect only the local protein structure and do not cause long-range conformational changes.

Although our studies were carried out with C4 rather than Slp, we infer that they apply to Slp. We cannot, of course, exclude the possibility that the 3-residue deletion has a very different effect within the context of the entire Slp sequence; but we feel this is quite unlikely. We also cannot exclude the more likely possibility of other structural defects in Slp that would render it inactive even if C Ts cleavage were to occur. We have tried to address this question by placing into the pcD expression vector the wild-type Slp cDNA (10, 15) and a Slp mutant in which the three deleted residues have been restored (unpublished data). Expression of both wild-type and mutant Slp proteins is very poor, however (e.g., ref. 15), and our preliminary results suggest that both proteins, from both COS-1 and L cells, are not in the native conformation because they do not appear to have intact internal thioester groups. These results are equivocal, however, because the levels of expression we observed were very low and C4 expression at similar low levels also yields apparently non-native proteins (unpublished observations).

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