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Slp is an essential component of an EDTA-resistant activation pathway of mouse complement

(complement component C3 bypass/hemolysis)

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ABSTRACT **Slp (sex-limited protein) is a mouse serum protein encoded by a major histocompatibility complex class III gene. It is considered to be a product of a duplicated complement component C4 gene, but without functional activity. Originally it has been found expressed only in adult males with the *S* region of the *H-2*^{d} or *H-2*^{s} haplotype. In this report we present evidence that Slp is involved in a form of mouse complement activation that occurs after fractionation of serum by polyethylene glycol precipitation. This activation pathway is EDTA-resistant (i.e., independent of classical and alternative pathway activation), is regulated by C1 inhibitor, and leads to the generation of hemolytically active membrane attack complexes. A positive correlation between this EDTA-resistant mouse complement activity and reported Slp levels was found. Direct evidence for a functional role of Slp came from substitution experiments in which purified Slp induced hemolytic activity in polyethylene glycol-fractionated, Slp-deficient mouse serum. Selective depletion of other complement components suggested a role for C1$\bar{s}$, C2, and C5, but not C3, in the Slp-dependent complement activation. A model for this type of mouse complement activation is presented.**

The class III region of the major histocompatibility complex (MHC) in the mouse, also known as the *S*-region, encodes serum proteins including factor B, complement components C2 and C4, and Slp (1–4). Slp expression is testosterone-dependent (5, 6) and is detected in adult male mice with the *S* region of the *H-2*^{d} or *H-2*^{s} haplotype only but is constitutively expressed in some wild-type-derived mice (1–4). Although the *Slp* gene is thought to be a duplication of the *C4* gene with about 95% homology to *C4* at both the DNA and the protein level (7, 8), the product of this gene is so far considered to be nonfunctional. This is based on experiments in which Slp could not restore the hemolytic activity of C4-deficient guinea pig serum (9) and in which Slp, unlike C4, showed resistance to cleavage by activated human C1s (8, 9). A mutation downstream from the C1$\bar{s}$ cleavage site was thought to be the cause of the hemolytic inactivity of Slp (10). A more likely explanation for the apparent nonfunctional behavior of Slp, however, is incompatibility between mouse Slp and heterologous C1s. Similar species incompatibility has been described for mouse C4 and guinea pig C1s and C2 (11).

Earlier studies demonstrated that a fraction of mouse serum prepared by precipitation with a critical concentration of polyethylene glycol [11% (wt/vol) PEG 6000] underwent "spontaneous" activation, resulting in the generation of functional membrane attack complexes (MACs) that were hemolytic for a broad panel of heterologous erythrocytes, including those from rabbits and sheep (12, 13). The generation of functional MACs by this pathway was found to be C5-dependent, EDTA-resistant (i.e., Ca^{2+}/Mg^{2+}-independent; ref. 12), and regulated in serum by C1 inhibitor (C1-INH; ref. 14). Thus far, this type of activation seems to be restricted to the mouse system, since guinea pig, human, rabbit, and rat sera do not become hemolytically active upon similar treatment.

This paper presents a model of this EDTA-resistant complement activation pathway in mouse serum. The strong correlation between the MHC haplotype and the hemolytic activity suggested an involvement of Slp. Evidence is presented that Slp plays an essential role in this activation pathway, and the role other C components play is evaluated and discussed.

MATERIALS AND METHODS

Sera. Male mice (age, 10–15 weeks) were obtained from the animal facilities of Utrecht University [The Netherlands; C3H/FeJ, Swiss, (BALB/c × Swiss)F_1], Iffa Credo (St. Germain sur l'Arbresle, France; BALB/c, SJL, CBA/J, DBA/2, C57BL/6), Jackson ImmunoResearch (B10.D2), Charles River Breeding Laboratories (AKR), and Harlan (Harlan Olac, Oxon, U.K.; SWR). All other mice were bred at the animal facilities of the Netherlands Cancer Institute (Amsterdam). Mouse blood was obtained by retroorbital puncture. Serum was obtained after clotting of the blood for 2 hr at room temperature and stored at −70°C until use.

Male mice were castrated at the age of 10 weeks and bled for serum 3 weeks later. Six weeks after castration, (BALB/c × Swiss)F_1 mice were supplemented with testosterone (Organon, The Netherlands) by daily 400-μg subcutaneous injections for 2 weeks. Mice were bled 1 day after the last injection.

Buffers. Veronal-buffered (25 mM) saline (750 mM), pH 7.35 ± 0.05, served as a 5× concentrated stock solution for the preparation of Veronal saline buffer (VSB) containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (VSB^{++}) or VSB containing 10 mM EDTA (VSB/EDTA).

Rabbit Erythrocytes. Blood withdrawn from the ear artery of a rabbit was diluted 1:2 with Alsever's old solution (114 mM citrate/27 mM glucose/72 mM NaCl, pH 6.1) and used as a source of erythrocytes. Before use, the rabbit erythrocytes were washed three times with 0.16 M NaI to elute possibly adsorbed proteins.

Purified Components and Chemicals. Slp was isolated from male DBA/2 serum by a recently developed FPLC procedure [4–10% PEG precipitation, followed by fractionation on heparin-Sepharose, Mono Q, and Superose 12 (Pharmacia)

Abbreviations: CVF, cobra venom factor; VSB, Veronal saline buffer; MAC, membrane attack complex; MHC, major histocompatibility complex; C1-INH, C1 inhibitor.

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columns] to be described elsewhere. C5 was purified from male (BALB/c × Swiss)F_1 mice as described (15). Cobra venom factors (CVFs) were purified from the venoms of the Egyptian cobra *Naja haje* (V 4254; Sigma) and the common Indian Cobra *Naja naja* (V 4378; Sigma) by a one-step Mono Q anion-exchange procedure (16). CVF activities were assessed by using undiluted mouse serum as target for CVF activation (17). PEG 6000 was obtained from Fluka.

PEG-Induced Complement Activation. PEG precipitation-induced complement activity was determined by using the optimal conditions described by Van Dijk *et al.* (12). Sera were diluted 1:10 in ice-cold VSB/EDTA containing 12.1% (wt/vol) PEG 6000 and for "direct" assay spun at 4000 × *g* and 4°C for 60 min or, for "indirect assay," first mixed with an equal volume of Slp-deficient mouse serum (C3H/FeJ serum) and subsequently diluted and spun. Precipitates were reconstituted to the original serum volume in VSB/EDTA and, after incubation at 41°C for 45 min, diluted in VSB/EDTA (100 μl). Fifty microliters of a 2% suspension of rabbit erythrocytes (7.5 × 10^6 per well) was added. After incubation at 39°C for 60 min, unlysed cells were spun and hemolysis was read by measuring the absorbance of the supernatant at 405 nm. One hemolytic unit was defined as the amount of serum giving rise to 50% hemolysis of 7.5 × 10^6 rabbit erythrocytes in a total volume of 150 μl.

Complement Depletion. Selective depletion of complement components was carried out as follows. For depletion of C1, 9 volumes of mouse serum was mixed with 1 volume of 38.5% PEG 6000 (wt/vol in VSB^{++}) and centrifuged 1 hr at 4000 × *g* and 4°C. The supernatant was used as a source of selectively C1-depleted serum (18). To deplete C2/factor B, mouse serum was heat-inactivated for 30 min at 48°C (19); after this treatment classical and alternative pathway activities were undetectable, whereas 60% of functional Slp activity was retained. To deplete C3 or C3 and C5, mouse serum was treated with CVF from *N. haje* or *N. naja*, respectively (5 μg/ml of serum; 30 min at 37°C; ref. 17). C3 depletion was complete after treatment with either CVF; C5 was undetectable after *N. naja* CVF treatment, but no reduction in C5 activity was observed after treatment with CVF from *N. haje* (17). Slp was depleted by heat-inactivation for 30 min at 52°C (2). C3/C4 depletion was performed by methylamine treatment (20). No classical or alternative pathway activities were detectable after methylamine treatment. Depletion of the components was checked by hemolytic assays for total complement activity (21), C1 (18), and C3 and C5 (15).

RESULTS

MHC Class III Dependence. Sera of male mice with different MHC class III haplotypes were tested for hemolytic activity after precipitation with 10% PEG. Only sera of male C5-sufficient mice carrying the *H-2*^{d} or *H-2*^{s} haplotype expressed direct hemolytic activity in the PEG precipitation-induced lysis assay (Table 1, panel 1, direct assay). C3H/FeJ serum, which was shown to be inactive in the PEG fractionation-induced hemolytic assay, was used as a source of C5 to study the intrinsic capacity of C5-deficient mouse sera to generate hemolytic activity after PEG precipitation. Use of C3H/FeJ serum also diminished the effect of differences in non-MHC-linked complement components on the hemolytic activity. Only sera of mice carrying the *H-2*^{d} or *H-2*^{s} haplotype were hemolytically active after PEG precipitation in the indirect assay (Table 1).

Congenic Mouse Strains and Strains with Natural Low or High Slp Levels. To diminish the effect of non-MHC-linked differences in genetic background of mice on the PEG-induced hemolytic activity, several congenic mice were used. Two pairs of congenic Slp-positive and Slp-negative mouse strains [B10.A(2R) vs. B10.A(4R), and C3H.OH vs. C3H.OL] were used, as well as the O20/A mouse strain, which expresses very high Slp levels, and mice with low plasma Slp levels due to trans regulation (recombinant inbred strains BxA-10 and AxB-2; refs. 22 and 23). Of congenic mice differing only in the central MHC region [B10.A(2R) vs. B10.A(4R), and C3H.OH vs. C3H.OL], solely those expressing Slp exhibited hemolytic activity (Table 1, panel 2). The O20 mice showed the highest hemolytic activity, whereas mice with low plasma Slp levels (BxA-10 and AxB-2) correspondingly exhibited poor hemolytic activity.

Table 1. PEG precipitation-induced hemolytic complement activities of various mouse sera

Mouse strain	Hemolytic activity, units/ml				Phenotype			
	Direct	Indirect N	Indirect C	Indirect T	H-2	Slp	C4	C5
Panel 1								
C57BL/6	—	—			b	−	H	+
BALB/c	130	121			d	+	H	+
DBA/2	—	46			d	+	H	−
B10.D2/OSn*	—	36			d	+	H	−
B10.D2/NSn*	79	96			d	+	H	+
AKR	—	—			k	−	L	−
CBA/J	—	—			k	−	L	+
C3H/FeJ	—	—			k	−	L	+
SWR	—	—			q	−	H	−
SJL	100	96			s	+	H	+
Swiss (inbred)	101	98			s	+	H	+
(BALB/c × Swiss)F_1	134	118	—	167	d+s	+	H	+
Panel 2								
B10.A.2R†	115	105	20		h2	+	H	+
B10.A.4R†	—	—			h4	−	H	+
C3H.OH‡	103	149	23		o2	+	H	+
C3H.OL‡	—	—			o1	−	L	+
O20	—	175	44		pz	+(H)	H	−
BxA-10	—	14			a	+(L)	H	−
AxB-2	—	58			a	+(L)	H	−

Serum from at least three male mice was pooled and tested for PEG precipitation-inducible hemolytic activity in a direct assay (without addition of Slp-deficient serum) or an indirect assay (with addition of Slp-deficient serum as a source of other complement components). Relative standard errors were <12% of the mean. N, sera of normal mice; C, sera of animals castrated at 10 weeks and bled at 13 weeks; T, sera of animals castrated at 10 weeks and testosterone-supplemented; H, high level; L, low level.
*†‡Pairs of congenic strains are indicated by identical footnote symbols.

Castration and Testosterone Treatment. To study the sex-hormone dependency of the PEG precipitation-induced complement activation, male mice were castrated at the age of 10 weeks. Three weeks later mice were bled and serum was assayed in the indirect activation assay. Six weeks after castration, mice received daily injections of testosterone for 2 weeks. One day after the last injection the mice were bled for serum. Castration abolished or largely diminished the PEG precipitation-induced hemolytic activity, and testosterone injections reversed this effect of castration (Table 1).

Age Dependency. As shown above, the expression of the PEG precipitation-induced hemolytic activity is dependent on the presence of the male sex hormone testosterone. To study the relation between the PEG precipitation-induced hemolytic activity and maturity, mice of different ages were bled for serum and tested in the direct and the indirect assay. Hemolytic activity was first detectable 6 weeks after birth and reached maximal values at 10 weeks of age (Fig. 1).

Purified Slp. The correlation of hemolytic activity with the *Slp* allele of the *H-2*^{d} and *H-2*^{s} haplotypes—and, within these haplotypes, with testosterone levels and age—gives strong evidence that Slp is an essential component in this EDTA-resistant, PEG precipitation-induced complement activation.

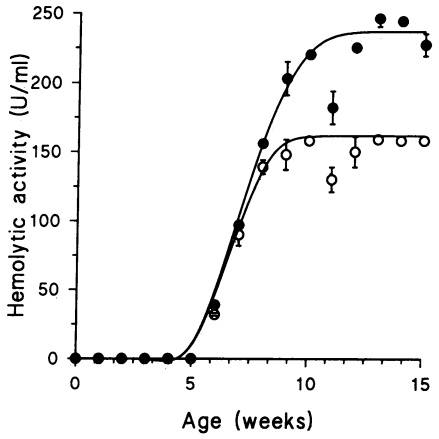

FIG. 1. PEG precipitation-induced hemolytic activities of sera from male (BALB/c × Swiss)F_1 mice at various ages. Sera were tested either in the direct assay (○) or after mixture with an equal volume of C3H/FeJ serum as a source of Slp-deficient serum (indirect assay; ●). Sera from five mice were pooled and tested in duplicate (mean ± SEM). U, units.

Final proof was given by induction of hemolytic activity in an 11% PEG precipitate of Slp-deficient C3H/FeJ serum after addition of FPLC-purified Slp (specific activity, 23 U/μg; Fig. 2*A*).

Role of Other Complement Components. The role of other complement components in the Slp-dependent complement activation pathway was studied by addition of purified complement components or selective depletion of individual components. Purified mouse C5 added to C5-deficient DBA/2 serum restored the PEG precipitation-inducible activity (Fig. 2*A*), as well as total hemolytic activity (15).

Depletion of mouse serum of C1 (by precipitation with PEG), C2 and factor B or Slp (by heat inactivation), or C3/C5 (by CVF treatment) abrogated the PEG precipitation-induced complement activity (Fig. 2*B*). C3/C4 inactivation by methylamine slightly reduced the hemolytic activity, but selective C3 depletion by CVF of *N. haje* did not affect the PEG precipitation-induced hemolytic activity (Fig. 2*B*).

DISCUSSION

The first evidence that Slp could be a functional component in a pathway of complement activation was obtained from *in vitro* experiments dealing with the PEG precipitation-induced activation of sera from different inbred mouse strains. Only sera of male C5-sufficient mice carrying the *H-2*^{d} or *H-2*^{s} haplotype expressed direct hemolytic activity in the PEG precipitation-induced lysis assay (Table 1, panel 1). Sera of female animals never showed hemolytic activity under similar conditions. This suggests a critical role for Slp, whose expression in *H-2*^{d} and *H-2*^{s} mice is controlled by male sex hormones, and confirms the involvement of C5 in the generation of MACs via the PEG precipitation-induced pathway (12). A further conclusion may be that C4 is not limiting in the PEG precipitation-induced complement activation, since sera from C57BL/6 males, which combine C5 sufficiency with the C4-high allele, were nevertheless nonhemolytic. With respect to a role of C2 and factor B, no conclusion can be drawn since these components are not polymorphic in these mice (3). In the indirect assay, which was performed after mixing test serum with an equal volume of C5-sufficient, Slp-deficient serum, a total concurrence between hemolytic activity and a documented presence of antigenic Slp was observed (Table 1, panel 1). This sustained our hypothesis that Slp was necessarily involved in the PEG precipitation-induced complement activation.

To diminish the effect of non-MHC-related differences in genetic background of mice on their PEG-induced hemolytic activities, several congenic mice and mice with high or low plasma Slp levels were tested. Of congenic mice differing only in the central MHC region, only those expressing Slp exhibited hemolytic activity, and their reported plasma Slp levels corresponded with levels of hemolytic activity (Table 1, panel 2, refs. 22 and 23).

Castration of serum donors diminished or abolished, and testosterone treatment virtually restored, PEG precipitation-induced hemolytic activities. These observations are completely in line with the reported control of Slp expression by male sex hormones (5, 6). Furthermore, the late appearance (at 6 weeks) of the PEG precipitation-induced hemolytic activity and its increase with the age of serum donors parallel with the reported levels of antigenic Slp (Slp appears in plasma when mice are about 6 weeks old and reaches maximal levels by 12 weeks; ref. 24) but not with levels of C4 (maximal levels are already reached at an age of 5 weeks; ref. 24). All these results strongly suggest that Slp, and not C4, is the limiting factor in the PEG precipitation-induced complement activation.

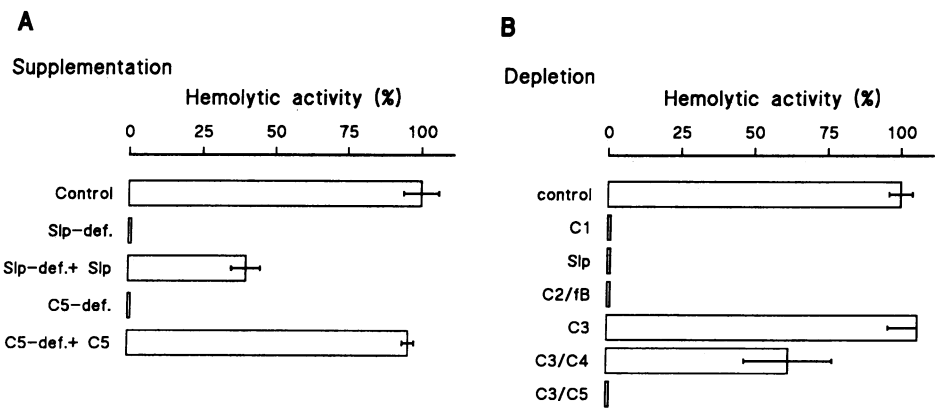

FIG. 2. PEG precipitation-induced hemolytic activities of pooled mouse sera after supplementation with (*A*) or depletion of (*B*) complement components. (*A*) Eleven percent PEG precipitates of Slp-deficient C3H/FeJ and C5-deficient DBA/2 serum were supplemented with purified Slp (3 μg/ml) and C5 (50 μg/ml), respectively, and tested for hemolytic activity. (*B*) Sera from male (BALB/c × Swiss)F_1 mice were selectively depleted of complement components and tested for PEG precipitation-induced hemolytic activity in the direct assay. Depletions: C1, prior precipitation with 3.5% PEG 6000; C2/factor B, heat inactivation at 48°C for 30 min; C3, treatment with CVF from *N. haje* (selectively C3-depleting); C3/C5, treatment with CVF from *N. naja* (C3- and C5-depleting); Slp, heat inactivated at 52°C for 30 min (activity was measured in the indirect assay using C3H/FeJ serum as Slp-deficient serum); C3/C4, methylamine-treated serum. Assays were carried out in duplicate and values are expressed as percentages (mean ± SEM; n = 2) of corresponding activities in untreated serum.

Definite evidence for the essential role of Slp in the PEG precipitation-induced mouse complement activation came from the substitution experiment, which showed that FPLC-purified Slp rendered a PEG precipitate of Slp-negative C3H/FeJ serum hemolytic (Fig. 2*A*).

The involvement of other complement components in the Slp-dependent activation pathway was studied by addition or selective depletion of different complement components in serum before testing. Fig. 2 indicates that C1, C2 and/or factor B, and C5, but not C3 and C4, are essential in the induction of Slp-dependent hemolytic activity. The reduction of activity after methylamine treatment might be explained by partial inactivation of Slp, which, like C3 and C4, has a reactive thioester bond. Moreover, the reactive thioester bond may be only partially essential for this type of activation, but binding to the target cell may enhance the efficiency of lysis by targeting activated C5 to the cell membrane. The functional activity of Slp, as tested with Slp-deficient serum, is lost by heating mouse serum at 52°C, which is in line with a reported loss of Slp antigenicity at that temperature, whereas C4 loses its antigenicity and hemolytic activity only after heating at 70°C (2).

Fig. 3 presents a model for the Slp-dependent activation of mouse complement. Fractionation of mouse serum with 11% PEG results in the precipitation of complement components necessary for activation and MAC formation, leaving C1-INH in the supernatant. Upon reconstitution of the PEG precipitate with EDTA-containing buffer and subsequent incubation at 41°C, C1s becomes spontaneously activated to $\overline{C1s}$ in the absence of C1-INH. Since intact C1 is not required for the autoactivation of C1s, this step is EDTA-resistant. $\overline{C1s}$ subsequently activates Slp in a manner analogous to $\overline{C1s}$-induced C4 activation (either with or without the release of a small peptide). Subsequently, C2 may bind. Binding of factor B seems less likely but cannot be excluded at this stage. While binding of C2 to C4 requires Mg^{2+}, binding of C2 to Slp appears to be also relatively EDTA-resistant (the activation proceeds in the presence of up to 30 mM EDTA; data not shown). $\overline{C1s}$ may now additionally activate C2, which, in the activated Slp–C2 complex, is capable of activating C5 directly, thereby bypassing C3. Activation of C5 and subsequent binding of C6 result in the formation of $\overline{C56}$. This complex, in concert with complement components C7 through C9, gives rise to lysis of heterologous erythrocytes by MAC formation. In human serum, a form of C3-bypass activation has been described in which a C4b–C4b dimer instead of a C4b–C3b complex participates in the C5 convertase (25). It is possible that in our model a similar Slp dimer or an Slp–C4 complex, rather than a single Slp molecule, is an essential part of the C5 convertase.

As yet there is no direct indication that the Slp-induced complement activation observed after PEG-precipitation is of any biological significance. There are, however, important indications that a similar type of activation may take place at inflammatory sites *in vivo*. At these sites, C1-INH may be inactivated by mediators of inflammation such as reactive oxygen species and proteinases released (26, 27) in response to, for example, microbial infections. Local C1-INH consumption could in this way lead to spontaneous C1s activation and indirectly result in Slp-dependent, C3-bypass activation. A function of the putative Slp-dependent complement activation *in vivo* may be the mobilization of additional polymorphonuclear leukocytes to sites of inflammation.

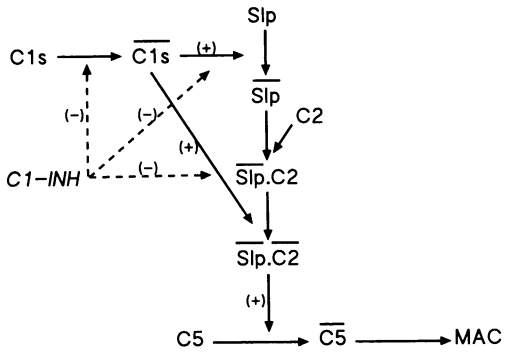

FIG. 3. Model of Slp-dependent mouse complement activation. Activated components are marked with overbars. The figure is further explained in the text.

Slp and mouse C4 may be considered to be analogues of human C4A and C4B, which are also the products of two genes but are more homologous than their mouse counterparts (3). C4A and C4B differ significantly in their activity, resulting from a divergent substrate specificity (28) and also with respect to C3 conversion. Deficiency of human C4A has been associated with immune complex-mediated disease, especially systemic lupus erythematosus (SLE) (28). The role of Slp in preventing SLE-like syndromes in mice will have to be investigated.

So far, the PEG precipitation-induced hemolytic activity has been found only in Slp-positive mouse strains. Some other mammalian species express only a C4B-like C4, whereas others, including sheep and cattle, express both types (29). It is possible that C4A molecules in other species have Slp-like properties and are capable of instituting direct activation of C5. However, differences in precipitation patterns and concentrations of natural regulators of this PEG precipitation-induced complement activation may make the recognition of this type of activation more difficult. Furthermore, the Slp-dependent activation seems not to be completely resistant to EDTA, and stability of the Slp–C2 complex may require another cation. Variations in sensitivity to EDTA of the Slp-like C4–C2 complex from different species may exist. Evidence that complement activation via pathways bypassing C3 is not restricted to mice has been provided by studies using purified human components in which it was shown that the C4b–C2a complex is able to directly activate C5 in the absence of C3 (30).

In conclusion, our results indicate that Slp, which has long been considered to be a nonfunctional mouse serum protein, is in fact an active complement component involved in the generation of hemolytic MACs via an activation pathway that bypasses C3. The putative importance of this type of activation at sites of inflammation and in immune-complex handling in man as well as in mouse is intriguing.

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