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Serum amyloid P component controls chromatin degradation and prevents antinuclear autoimmunity

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Serum amyloid P component (SAP), a highly conserved plasma protein named for its universal presence in amyloid deposits[1], is the single normal circulating protein that shows specific calcium-dependent binding to DNA and chromatin in physiological conditions[2,3]. The avid binding of SAP displaces H1-type histones and thereby solubilizes native long chromatin, which is otherwise profoundly insoluble at the physiological ionic strength of extracellular fluids[4]. Furthermore, SAP binds *in vivo* both to apoptotic cells[5], the surface blebs of which bear chromatin fragments[6], and to nuclear debris released by necrosis[7]. SAP may therefore participate in handling of chromatin exposed by cell death[2–4,7]. Here we show that mice with targeted deletion of the SAP gene[8] spontaneously develop antinuclear autoimmunity and severe glomerulonephritis, a phenotype resembling human systemic lupus erythematosus, a serious autoimmune disease. The SAP$^{-/-}$ mice also have enhanced anti-DNA responses to immunization with extrinsic chromatin, and we demonstrate that degradation of long chromatin is retarded in the presence of SAP both *in vitro* and *in vivo*. These findings indicate that SAP has an important physiological role, inhibiting the formation of pathogenic autoantibodies against chromatin and DNA, probably by binding to chromatin and regulating its degradation.

In preliminary experiments with native long chromatin incubated with human monocytes or neutrophils *in vitro*, the presence of human SAP considerably retarded internucleosomal cleavage of the DNA, whereas the very closely related pentraxin protein, human C-reactive protein[9] (CRP), had no effect. There was no appreciable internalization of the chromatin substrate, the DNA of which was rapidly cleaved in the culture medium. We also incubated long chromatin with mouse peritoneal exudate cells in the presence of SAP-sufficient serum from wild-type mice or SAP-deficient serum from SAP$^{-/-}$ mice. DNA cleavage was appreciably faster in the absence of SAP, indicating that mouse SAP has the same protective effect as human SAP. For example, after 3 hours of incubation at 37 °C, cleavage into DNA fragments corresponding to monosomes was 400% greater in the absence of SAP. The protection of DNA by SAP resembles the protection against proteolysis conferred by the binding of SAP to amyloid fibrils[10], indicating that SAP may have a general capacity to stabilize structures to which it binds. In support of this, mice lacking SAP through targeted deletion of the SAP gene were relatively resistant to induction of reactive systemic AA amyloid[8].

Although the SAP-deficient mice developed and bred normally, they spontaneously produced high titers of antinuclear antibodies and autoantibodies against chromatin, DNA and histones, starting after 3 months of age and then increasing progressively (Tables 1 and 2). There is a significant, genetically determined, background of antinuclear autoimmunity in the [129/Sv x C57BL/6]F2 mice used in these studies[11], but the frequency and titer of autoantibodies were considerably greater in the SAP knockout mice than in wild-type control mice (Table 1). Furthermore, the females, in particular, suffered from a high incidence of severe proliferative glomerulonephritis, with typical immune complex deposition (Fig. 1), and some mortality (Tables 1 and 2). This is reminiscent of the 9:1 female predominance in human systemic lupus erythematosus (SLE).

Deficiency of the C1q component of complement strongly predisposes to SLE in man, and C1q knockout mice also develop glomerulonephritis, associated with antinuclear and antihistone antibodies, but no increase in antibodies against chromatin or DNA (ref. 11). In a further contrast, C1q knockout mice accumulate multiple apoptotic bodies in their normal glomeruli[11], yet there was no such accumulation in the SAP-deficient mice. C1q binds to apoptotic cells and apparently contributes to their clearance[11], whereas SAP evidently protects against breakdown of tolerance by a different mechanism. The SAP-deficient mice also did not produce antibodies against extractable nuclear antigens, nor

Table 1 Spontaneous autoantibodies and glomerulonephritis in SAP-deficient and wild-type [129/Sv × C57BL/6]F2 mice

	Males % positive			Females % positive		
	SAP$^{+/+}$ $n = 54$	SAP$^{-/-}$ $n = 49$	χ^2 test	SAP$^{+/+}$ $n = 56$	SAP$^{-/-}$ $n = 50$	χ^2 test
Antinuclear antibody	17.3	63.3	$P < 0.00001$	50.9	82	$P = 0.0007$
Anti-chromatin	28.3	59.2	$P = 0.00165$	46.4	74	$P = 0.0039$
Anti-histone	5.7	30.6	$P = 0.001$	14.5	54	$P < 0.00001$
Anti-single stranded DNA	9.4	51.0	$P < 0.00001$	28.6	74	$P < 0.00001$
Anti-double stranded DNA	7.5	24.5	$P = 0.0187$	10.7	46	$P < 0.00001$
Glomerulonephritis	1.9	3.9	ns	9.3	41.8	$P = 0.0001$

Surviving mice from contemporary cohorts of 113 SAP$^{-/-}$ mice (56 male, 57 female) and 112 age-matched wild-type SAP$^{+/+}$ control mice (55 male, 57 female), all of the same (129/Sv × C57BL/6) strain and were housed identically in pathogen-free conditions at age 8 months. The histological severity of glomerulonephritis was significantly greater in the female SAP$^{-/-}$ mice than in the wild-type control mice, mean (s.d.) grade 1.35 (1.68) compared with 0.33 (1.08), $P = 0.0039$, by Mann Whitney U test. At 3 months, antinuclear antibodies were present in 6% of both SAP$^{-/-}$ and wild-type groups, but at 5 months, were present in 30.4% of male and 44.4% of female SAP$^{-/-}$ mice compared with 7.7% of male ($P = 0.0029$) and 26.8% of females wild-type mice ($P = 0.0529$).

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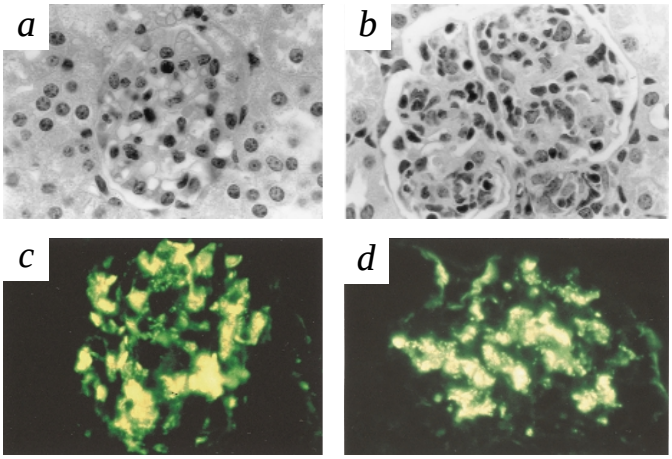

Fig. 1 Glomerulonephritis in SAP-deficient mice. ***a***, Glomerulus with normal morphology from a wild-type $SAP^{+/+}$ mouse. Hematoxylin and eosin staining. ***b***, Enlarged glomerulus from $SAP^{-/-}$ mouse, showing proliferative glomerulonephritis with increased mesangial matrix and marked hypercellularity with mononuclear cells in capillary loops. Hematoxylin and eosin staining. ***c*** and ***d***, Immunofluorescence staining with anti-mouse IgG (*c*) and anti-mouse C3 (*d*), showing the granular appearance typical of immune complex deposition. Original magnifications, ×680.

any tissue- or organ-specific autoantibodies; thus, SAP has an important, selective, *in vivo* role in the normal handling of chromatin but not other autoantigens.

Immunization with native long chromatin, in either complete or incomplete Freund's adjuvant, induced antibodies against chromatin, histones, single-stranded DNA and double-stranded DNA in both wild-type and $SAP^{-/-}$ mice. However the number of responders and the titers tended to be higher in the $SAP^{-/-}$ mice, and for antibody against double stranded DNA, the difference from wild-type mice was statistically highly significant (Table 3), confirming the spontaneous age-related autoantibody phenotype associated with SAP deficiency. The titer of antibodies against double-stranded DNA correlates most closely with pathogenesis of human SLE.

The rate of chromatin degradation, monitored by measurements of radioactivity in the whole body after intraperitoneal injection of native long chromatin in which the histones had been radio-iodinated, was significantly faster in $SAP^{-/-}$ than in wild-type control mice or $C1q^{-/-}$ mice (Fig. 2). The range of values in each group probably reflects the genetic heterogeneity of these F2-generation mice, but the robust, statistically highly significant differences between the $SAP^{-/-}$ and control mice were reproducible in each of six separate experiments. These results suggest that SAP stabilizes chromatin *in vivo*, perhaps by binding and solubilizing it, as happens *in vitro*[4], and that in the absence of SAP the more aggressive degradation of insoluble chromatin may enhance its immunogenicity. In addition, as parenchymal hepatocytes are the only cells in which both SAP and CRP are catabolized *in vivo*[12], pentraxin binding may be a mechanism for directing the catabolism of potential autoantigens to this non-antigen-presenting organ. Indeed, studies of the clearance of circulating nucleosome core particles support this and have identified the liver as a chief site of uptake[13,14].

Although supraphysiological concentrations of aggregated SAP alone can activate the classical complement pathway *in vitro*, the binding of SAP to DNA or chromatin both prevents SAP aggregation and inhibits this complement activation[4]. The rate of chromatin degradation was normal in C1q knockout mice (Fig. 2*b*), showing that the classical complement pathway is not involved. Thus, SAP does not act as an opsonin for chromatin uptake; instead contrast, SAP acts powerfully to inhibit and control inappropriate, immunogenic processing of this ubiquitous autoantigen.

We have shown here that $SAP^{-/-}$ mice spontaneously develop antinuclear antibodies and autoantibodies against chromatin, DNA and histones, associated with a high incidence, especially in females, of severe immune complex proliferative glomerulonephritis. The knockout mice also have an enhanced anti-DNA antibody response to immunization with extrinsic chromatin. Furthermore, the degradation of long chromatin by leukocytes *in vitro* is slowed in the presence of SAP, and $SAP^{-/-}$ mice degrade long chromatin more rapidly *in vivo* than do control wild-type mice. Binding of SAP may help to maintain tolerance to chromatin epitopes by solubilizing and stabilizing long chromatin, and possibly also by targeting it for catabolism in the liver. Controlled and less-aggressive degradation of chromatin is likely to reduce its immunogenicity, —and the liver, the sole site of SAP catabolism *in vivo*[12], is specifically equipped for non-immunogenic processing.

Our results here were obtained in [129/Sv x C57BL/6]F2 mice, but we have just examined the first cohort of inbred C57BL/6 mice into which the deleted SAP gene has been backcrossed for six generations. At 6 months of age, 45 of 51 $SAP^{-/-}$ C57BL/6 mice (88%) have antinuclear antibodies: 16 at a titer of 1/80, 12 at 1/160, 12 at 1/320, and 5 at 1/640. In contrast only 5 of 56 control wild-type $SAP^{+/+}$ C57BL/6 mice (9%) had antinuclear antibodies: 4 at 1/80 and 1 at 1/320, whereas in the $SAP^{+/-}$ cohort heterozygous for the deleted gene and with about 50% of normal SAP levels, 20 of 60 mice (33%) had the antibodies: 12 at 1/80, 5 at 1/160, 1 at 1/320 and 2 at 1/640. These salient findings sup-

Table 2 Titers of spontaneous autoantibodies in SAP-deficient and wild-type [129/Sv × C57BL/6]F2 mice at 8 months of age

	Anti-nuclear antibody		Anti-chromatin		Anti-histone		Anti-single stranded DNA		Anti-double stranded DNA	
	$SAP^{+/+}$	$SAP^{-/-}$	$SAP^{+/+}$	$SAP^{-/-}$	$SAP^{+/+}$	SAP–/–	$SAP^{+/+}$	$SAP^{-/-}$	$SAP^{+/+}$	$SAP^{-/-}$
Males (*n*)	54	49	54	49	54	49	54	49	54	49
Median	0	80	0	33	0	0	0	7	0	0
Range	0–1280	0–5120	0–299	0–708	0–36	0–32	0–23	0–86	0–80	0–640
	$P < 0.0001$		$P = 0.0001$		$P = 0.0283$		$P = 0.0002$		ns	
Females (*n*)	56	50	56	50	56	50	56	50	56	50
Median	80	320	0	56	0	8	0	20	0	0
Range	0-2560	0-5120	0-517	0-856	0–120	0–520	0–635	0–1074	0–5120	0–1280
	$P = 0.0045$		$P = 0.0019$		$P = 0.0003$		$P < 0.0001$		$P = 0.0025$	

Significant differences between $SAP^{-/-}$ and $SAP^{+/+}$ groups by Mann-Whitney U test. ns, not significant.

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Table 3 Antibody against double-stranded DNA in SAP-deficient and wild type mice immunized with long chromatin

Immunization with 100 μg long chromatin in	Day	Number positive/total in group		χ^2 test	Median titer (range)		Mann-Whitney U test
		$SAP^{+/+}$	$SAP^{-/-}$		$SAP^{+/+}$	$SAP^{-/-}$	
Females							
Complete adjuvant	26	2/15 (13.3%)	10/17 (58.8%)	$P = 0.008$	0 (0-160)	20 (0–640)	$P = 0.0266$
Incomplete adjuvant	28	3/11 (27.3%)	7/12 (58.3%)	ns	0 (0-40)	60 (0–160)	ns
Incomplete adjuvant	44	3/11 (27.3%)	9/12 (75.0%)	$P = 0.022$	0 (0-80)	60 (0–320)	$P = 0.0197$
Males							
Incomplete adjuvant	28	0/9 (0%)	3/9 (33.3%)	ns	0 (0-0)	0 (0–160)	ns
Incomplete adjuvant	44	0/9 (0%)	8/9 (88.9%)	$P = 0.0002$	0 (0-0)	80 (0–160)	$P = 0.0004$

ns, not significant.

port the association between SAP deficiency and antinuclear autoimmunity.

Inbred wild-type 129/Sv mice do not develop autoimmunity[11], but they might have a gene located immediately adjacent to the deleted SAP gene conferring susceptibility to antinuclear autoimmunity when expressed in the C57BL/6 background. To eliminate this remote possibility, we are now producing mouse $SAP^{-/-}$ C57BL/6 mice transgenic for human SAP. The presence of human SAP should prevent development of anti-chromatin autoimmunity; results will be available in 18 months.

The results here, with previous observations of avid specific binding of SAP to DNA and chromatin in physiological conditions *in vitro*[2], solubilization of native long chromatin by SAP *in vitro*[4], and binding of SAP to apoptotic cells[5] and exposed chromatin *in vivo*[7], indicate that SAP is important in reducing the immunogenicity of chromatin and preventing autoimmunity. This is the first physiological *in vivo* function of a pentraxin to be identified, to our knowledge, and it is probably sufficient to explain the stable phylogenetic conservation of SAP and the absence of any convincing evidence of deficiency or even polymorphism of SAP in man[1]. Furthermore, the observations here elucidate aspects of physiological processing of chromatin after cell death and show that failure of appropriate autoantigen handling may contribute to the pathogenesis of autoimmune disease.

The patterns of autoimmunity and nephritis in $SAP^{-/-}$ mice resemble aspects of human SLE. Although SLE patients have apparently normal circulating levels of SAP, it has long been known that the acute phase response of CRP, the classical human nonspecific acute-phase protein, is deficient during 'flares' of SLE (ref. 15). CRP and SAP are very similar molecules[16,17], sharing substantial sequence homology, the same tertiary fold and oligomeric assembly; together they constitute the pentraxin family. In the mouse, in which SAP rather than CRP is the main acute phase protein[18], the NZB/W strain that spontaneously develops antinuclear autoimmunity and glomerulonephritis also fails to mount an acute-phase response to disease activity[19]. These defects might contribute to the lupus diathesis[3,15,19], a view that is now supported by the development of antinuclear autoimmunity in the $SAP^{-/-}$ mice.

SAP is the single principal DNA- and chromatin-binding protein of human plasma in physiological conditions[2], but human CRP binds specifically to small nuclear ribonucleoprotein particles[3,20]. Epitopes in these particles may contribute to the immunogenic drive in SLE, as autoantibodies against double-stranded DNA, the specificity that correlates most closely with tissue damage in SLE, show cross-reactivity with small nuclear ribonucleoprotein particle proteins[21], which are exposed on the surface blebs of apoptotic cells[6]. CRP reportedly reduces the immune response to ligands to which it binds[22], as we have demonstrated here for mouse SAP and chromatin, and human CRP may therefore play a comparable part in handling small nuclear ribonucleoprotein particle epitopes and controlling their immunogenicity. The relative failure of patients with SLE to mount appropriate acute-phase CRP responses to autologous tissue damage thus might indeed contribute to the development and/or persistence of antinuclear autoimmunity. Furthermore, cardiolipin, a mitochondrial membrane phospholipid, is recognized both by CRP and by the pathogenetic autoantibodies in the anti-phospholipid (Hughes) syndrome[23] that overlaps with SLE and is also characterized by defective CRP responses to autologous tissue damage.

Further work is required to determine whether patients with SLE and their relatives may have defects in the structure, regulation, metabolism or function of SAP. However, given the results here in $SAP^{-/-}$ mice and the known properties of human CRP, an important function of both SAP and CRP may be to bind to autologous ligands from dead or damaged cells and facilitate their safe, non-immunogenic, disposal.

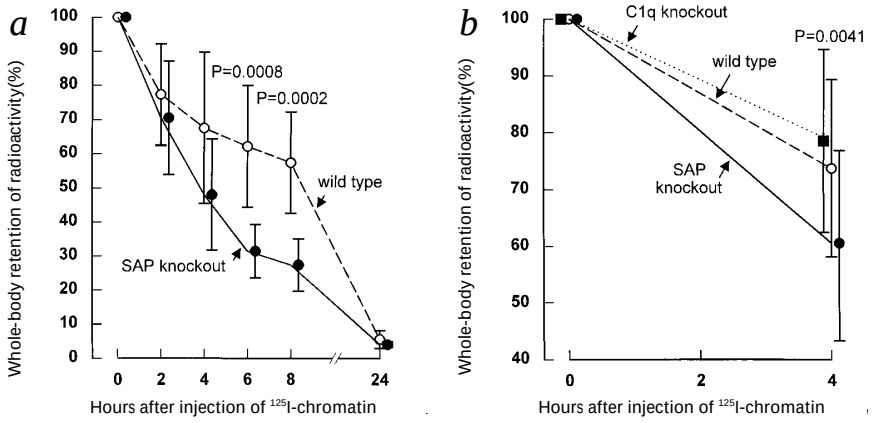

Fig. 2 Accelerated chromatin degradation in SAP-deficient mice, shown by whole-body clearance of radioactivity after injection of radio-iodinated long chromatin. Each point represents the mean (s.d.) of all mice in each group. ***a***, SAP knockout mice ($n = 7$) compared with wild-type control mice ($n = 14$). *P* values, Bonferroni *t*-tests. ***b***, SAP knockout mice ($n = 22$) compared with C1q knockout mice ($n = 13$) and wild-type control mice ($n = 15$). *P* value in Fig., by one-way ANOVA. $SAP^{-/-}$ compared with wild-type, $P = 0.0197$; $SAP^{-/-}$ compared with C1q–/–, $P = 0.0026$, by *t*-tests. The values in all groups were at baseline at 24 h, as shown in *a*.

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Methods

Chromatin degradation *in vitro*. Aliquots (35 μg) of native avian erythrocyte long chromatin[4] in 10 mM Tris, pH 8.0, were incubated at 37 °C with 2 × 10^6 peritoneal exudate cells, typically about 43% macrophages, 43% polymorphs, 14% lymphocytes, collected from C57BL/6 mice after elicitation with oyster glycogen (type II; Sigma). Chromatin and cells were suspended in Iscove's medium supplemented with $CaCl_2$ to 2 mM and 50% v/v mouse serum derived from either wild-type or $SAP^{-/-}$ mice. After incubations as long as 3 hours, the cells were spun down, DNA was extracted from the supernatant, separated by 1.2% agarose gel electrophoresis; the intensities of bands corresponding to monosomes and higher-order structures were quantified.

SAP knockout and control mice. SAP and C1q knockout mice in the [129/Sv × C57BL/6]F2 genetic background were produced as described[8,11]. Well-matched, contemporary cohorts, with wild-type littermate control mice, were bred in standard pathogen-free conditions. Blood samples were obtained at 3 and 5 months, and then mice were exsanguinated at age 8 months.

Antibody assays. Antinuclear antibodies were sought by indirect immunofluorescence on Hep-2 cells[24]. All positive staining was in a homogeneous pattern. Antibodies against double-stranded DNA were detected by indirect immunofluorescence on *Crithidia luciliae*[25]. Antibodies against chromatin (avian erythrocyte native long chromatin[4]), single-stranded DNA (calf thymus) and histones (calf thymus) were assayed by ELISA, each sample in duplicate with a non-antigen-coated well to control for nonspecific binding[26]. Results are expressed relative to the ELISA absorbance produced by a standard of pooled, high-titer MRL/Mp-lpr/lpr serum, which was assigned an arbitrary value of 100 units, and were considered positive at 7.0 units or more (3 s.d. above the lower limit of detection). Antibodies against extractable nuclear antigen (Sm) were sought by ELISA (Diastat; Shield Diagnostics, Dundee, Scotland). Tissue- and organ-specific autoantibodies were sought by standard indirect immunofluorescence using 'snap frozen' heart, spleen, skeletal muscle, kidney, liver, intestine and lung tissue from normal adult CBA/Ca mice as substrate and fluoresceinated sheep anti-mouse immunoglobulin (Dako, Carpinteria, California). Immune complexes in the glomeruli of $SAP^{-/-}$ mice were sought by direct immunofluorescence using sheep anti-mouse total IgG (Dako, Carpinteria, California) and goat anti-mouse C3 (Cappel/Organon Teknika, Turnout, Belgium). Specificity was established by abolition of all staining when the labeled antibodies were absorbed before use with their respective specific antigens[27].

Histology. Kidneys were fixed in 10% buffered formalin and stained with hematoxylin and eosin. Glomerulonephritis and apoptotic bodies were scored in coded slides precisely as reported[11]. As assignment between 0 and grade I glomerulonephritis was marginal and few tissues were in grade I, only grade II and above were considered positive.

Immunization. Groups of age- and sex-matched $SAP^{-/-}$ and control wild-type mice received 100 μg of avian erythrocyte native long chromatin[4] emulsified in either complete or incomplete Freund's adjuvant by intramuscular injection in the right thigh. Blood samples were obtained from all mice the day before immunization and at approximately weekly intervals thereafter.

Chromatin degradation *in vivo*. Native long chromatin from avian erythrocytes in 10 mM Tris, pH 8.0, was iodinated with $Na^{-125}I$ using N-bromosuccinimide[28] at a final concentration of 1 mg/ml for 20 s before dilution and extensive dialysis against the same buffer. Specific activity was as much as 844 μCi/mg and was all in protein not DNA, and 84% was precipitated by 150 mM NaCl, 2 mM $CaCl_2$, indicating that it was in histones associated with native long chromatin. Mice 8 weeks old, before substantial expression of spontaneous antinuclear autoimmunity, were given water containing KI to block thyroid uptake of radioiodine and blood was obtained from these mice to check for absence of pre-existing autoantibodies. Labeled chromatin (1.5–13 μg in various experiments, corresponding to 2 × 10^6 cpm) was then injected intraperitoneally and radioactivity in the whole body was measured immediately and at intervals up to 24 h. In the experiments here (Fig. 2), none of the mice had antibodies against histone, but 3 of 32 $SAP^{-/-}$ mice and 1 of 29 wild-type mice had very low titers of antibodies against chromatin or DNA and were excluded from the analysis, although their chromatin degradation rates were well within the range of the seronegative mice. The results in Fig. 2 are all from females but were the same in separate experiments with male mice.

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