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Mouse serum amyloid A protein

Complete amino acid sequence and mRNA analysis of a new isoform

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Four serum amyloid A protein (*SAA*) genes and two gene products, apo-SAA_1 and apo-SAA_2 were identified in BALB/c mice (type A). SJL/J mice (type B) are thought to be defective in apo-SAA_2 expression. A unique variant of mouse apo-SAA was identified in SJL/J mice by isoelectric-focusing analysis of high-density lipoprotein from endotoxin-treated mice. Complete amino-acid-sequence analysis of this quantitatively major form of SJL/J apo-SAA (pI 5.9) showed it to be identical with the apo-SAA_2 isoform from BALB/c mice, except for the substitution of aspartic acid for alanine at position 101. Isoform-specific analysis of mRNA from liver of BALB/c and SJL/J mice and their F_1 hybrid progeny (CSJLF1/J) mice revealed further differences in the 3′ untranslated regions of the genes, not only encoding apo-SAA_2 and apo-SAA pI 5.9, but also apo-SAA_1. The *SAA* genes of SJL/J mice thus differ from BALB/c in exon 4. Additional minor isoforms corresponding to apo-SAA_2 (pI 6.3) in SJL/J mice and apo-SAA (pI 5.9) in BALB/c mice were identified. We propose that, when analysing a multigene family such as *SAA*, thorough analysis at the protein level should complement molecular-biological approaches where the use of a too-limited repertoire of probes can obscure complexities.

INTRODUCTION

The genomic structure of the mouse serum amyloid A protein (*SAA*) gene family has been most extensively studied in BALB/c mice [1]. Studies have thus far identified three active genes and a pseudogene with deletions of exons 1 and 2 and a portion of exon 3 resulting in an in-frame stop codon [1,2]. The 3200 bp SAA_1 and SAA_2 genes are 96% identical and code for the apolipoproteins apo-SAA_1 and apo-SAA_2 [1,2]. Both apo-SAA_1 and apo-SAA_2 associate with the high-density-lipoprotein 3 subclass (HDL_3) in the plasma and are rapidly transported to peripheral tissues [3]. The third active gene, SAA_3, is co-ordinately transcribed in the liver with the SAA_1 and SAA_2 genes, but a corresponding protein has not been found [4,5].

Taylor & Rowe [6] surveyed 48 mouse strains and sub-strains for restriction-fragment-length polymorphisms (RFLP) associated with the *SAA* gene family. BALB/c mice, which along with 32 other inbred mouse strains exhibited a common RFLP pattern, were designated type A. SJL/J mice were distinct from type A mice and along with seven other inbred strains of mice were designated type B on the basis of common RFLPs. It was not possible to determine whether the differences resided within the coding regions of the SAA genes.

In the present study we compared the apo-SAA phenotype of SJL/J mice with BALB/c mice and their F1 progeny CSJLF1/J mice. SJL/J mice were chosen as they were originally thought to be resistant to amyloid A protein (AA) amyloidosis, owing to a defect in apo-SAA_2 production [2], but recently were found to develop amyloidosis of the AA type using amyloid-enhancing factor (AEF) [7]. In addition to the expected apo-SAA_1 and apo-SAA_2 isoforms, novel minor apo-SAA isoforms of pI 6.15 and 5.9 were detected in BALB/c (type A) mice. In SJL/J mice (type B), minor isoforms isoelectrically corresponding to apo-SAA_2 and apo-SAA pI 6.15 were detected. However, in SJL/J mice the pI 5.9 isoform was the second quantitatively major isoform rather than apo-SAA_2. F_1 hybrid mice (CSJLF1/J) responded to LPS with codominant expression of three quantitatively equivalent apo-SAA isoforms, apo-SAA_1 (pI 6.45), apo-SAA_2 (pI 6.3) and apo-SAA (pI 5.9). The minor apo-SAA isoforms of pI 6.15 and pI 5.9 in BALB/c mice and apo-SAA_2 of pI 6.15 in SJL/J mice were analysed by *N*-terminal sequencing, two-dimensional gels and immunoblotting (results not shown). These studies suggest that the minor isoforms could represent novel intact apo-SAA molecules.

To define whether the mechanism for the alternate display of dominance between apo-SAA_2 (pI 6.3) and the pI 5.9 isoform in type A and B mice resides at the genomic level or is the result of post-translational modification(s), we determined the complete amino acid sequence of the pI 5.9 isoform of SJL/J mice by using microsequencing techniques. Additionally we compared SAA expression in type B (SJL/J) mice with that of type A (BALB/c) mice using seven different oligonucleotide probes corresponding to the three known active genes in the BALB/c family as determined by Lowell *et al.* [1].

Our protein sequence and mRNA hybridization data indicate that the pI 5.9 isoform is the protein product of an *SAA* gene differing from the SAA_2 gene in exon 4. Our Northern-hybridization analysis indicates that additional differences reside at the SAA_1 locus since, although the apo-SAA_1 of BALB/c and SJL/J mice are isoelectrically indistinguishable, the untranslated 3′ portion of exon 4 does differ between these strains.

MATERIALS AND METHODS

BALB/cbyJ, SJL/J and CSJLF1/J mice, 6–8 weeks old, male or female, were obtained from Jackson Laboratories, Bar Harbor, ME, U.S.A. An acute-phase response was elicited by

Abbreviations used: AA, amyloid A protein; apo-SAA, serum amyloid A protein; AEF, amyloid-enhancing factor; BCIP, 5-bromo-4-chloroindol-3-ylphosphate *p*-toluidine salt; HDL, high-density lipoprotein; LPS, lipopolysaccharide; NBT, Nitroblue Tetrazolium chloride; PVDF, poly(vinylidene difluoride) membrane; RFLP, restriction-fragment-length polymorphism; Cl_3Ac, trichloroacetic acid; F_3Ac, trifluoroacetic acid; PBS, phosphate-buffered saline.

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intraperitoneal injection of 100 μg of lipopolysaccharide (LPS) from *Salmonella typhosa* type W (Difco Laboratories, Detroit, MI, U.S.A.). EDTA-anti-coagulated blood was collected 20 h later by cardiac puncture of CO_2-anaesthetized animals. Control animals received no LPS. Livers were frozen and stored at −85 °C for RNA extraction.

Preparation of HDL

High-density lipoprotein (HDL) was isolated from plasma essentially as described [8]. Plasma density was adjusted to 1.09 g/ml with solid KBr and centrifuged for 5.3 h at 55000 rev./min (VTi80 rotor, Beckman Instruments, Palo Alto, CA, U.S.A.) at 10 °C. The density of the infranatants, which contained the HDL, was adjusted to 1.21 g/ml with solid KBr and re-centrifuged for 9.4 h at 55000 rev./min in a VTi80 rotor at 10 °C. The pellicles containing HDL were extensively dialysed against 0.15 M-NaCl/0.1% (w/v) EDTA, pH 7.4.

Electrofocusing

Aliquots (200 μg) of mouse HDL (normal or acute-phase) or 20 μl of serum were freeze-dried and delipidated with 0.5 ml of chloroform/methanol (2:1, v/v) [9]. The delipidated proteins were resuspended in sample buffer consisting of 7 M-urea, 1% (w/v) decyl sodium sulphate (Eastman Kodak Company, Rochester, NY, U.S.A.), and 5% (v/v) 2-mercaptoethanol. Samples were electrofocused on 0.3 mm-thick polyacrylamide gels containing 7 M-urea and 20% (v/v) Ampholines pH 3–10, 40% (v/v) Ampholines pH 4–6.5 and 40% (v/v) Ampholines pH 7—9 (Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.) as described in [10,11].

Immunochemical analysis

Samples on electrofocused gels were pressure-blotted on to 0.2 μm-pore-size nitrocellulose membranes (Schleicher and Schuell, Keene, NH, U.S.A.) for 20 h at room temperature [8]. The membrane was wetted with 25 mM-Tris/HCl (pH 8.3)/192 mM-glycine/15% (v/v) methanol. After pressure blotting the membrane was blocked overnight at 4 °C with 5% (w/v) non-fat dry milk in phosphate-buffered saline (PBS; 0.01 M-sodium phosphate/0.137 M-NaCl, pH 7.4) containing 2% (w/v) BSA. The apo-SAA isoforms were detected with our standard rabbit anti-(mouse AA) antibody [7], as primary antibody, and an alkaline phosphatase-conjugated goat anti-rabbit IgG as secondary antibody (A8025; lot no. 39F-88961, Sigma Chemical Co., St. Louis, MO, U.S.A.). The chromogenic substrates for alkaline phosphatase, 5-bromo-4-chloroindol-3-ylphosphate *p*-toluidine salt (BCIP) and Nitroblue Tetrazolium chloride (NBT) were applied according to the manufacturer's (Bethesda Research Laboratories Life Technologies, Gaithersburg, MD, U.S.A.) instructions.

Electroblotting

Acute-phase HDL (3.6 mg) from SJL/J mice was separated in aliquots of 200 μg by electrofocusing. The pI 5.9 bands, which constituted approx. 30% of the total apo-SAA, were excised. Quantification was by pyridine extraction of Coomassie Blue-stained bands as described in [12]. Nine bands were pooled, boiled in SDS sample buffer, loaded into a single well and resolved in a second-dimension 5–20%-(w/v) acrylamide/SDS (0.1%, w/v) gel with a 3% acrylamide stacking gel [13]. Subsequently the isoform was electroblotted [14] for 2.5 h at 200 mA on to poly(vinylidene difluoride) (PVDF) membranes (Millipore, Bedford, MA, U.S.A.) using 25 mM-Tris/HCl (pH 8.3)/192 mM-glycine/10% (v/v) methanol/0.05% SDS as transfer buffer. Electroblotted protein was identified by staining the membranes with Amido Black.

Proteinase cleavage of apo-SAA *in situ*

For each proteinase cleavage *in situ*, nine excised electrofocused bands of apo-SAA isoform pI 5.9 were electroblotted on to PVDF membrane and subjected to enzymic degradation as described in [15]. Briefly the membrane-protein-binding sites were blocked with polyvinylpyrrolidone (M_r 24000; Aldrich Chemical Company, Milwaukee, MI, U.S.A.) and then the blotted protein was digested with either 1 μg of trypsin (Cooper Biomedical, Malvern, PA, U.S.A.) or 0.5 μg of Endoproteinase Lys-C (Boehringer Mannheim, Indianapolis, IN, U.S.A.) for 15 h at 37 °C on a vertical rotating platform. The digestion buffers, formic acid and water washes were pooled and frozen at −20 °C or loaded immediately on to an h.p.l.c. column.

Electroelution

The pI 5.9 isoform was electroeluted from 18 electrofocused tracks, each containing 200 μg of acute-phase HDL from SJL/J mice. The desired bands were excised and equilibrated in elution buffer [25 mM-Tris/HCl (pH 8.3)/192 mM-glycine/0.01% SDS] before electroelution in a model UEA unidirectional electroelutor (International Biotechnologies, New Haven, CT, U.S.A.) according to the manufacturer's instructions. Electroelution was carried out for 45 min at 125 V, and the electroeluted protein was trapped in 7.5 M-ammonium acetate. The pooled eluates were dialysed against 20 mM-Tris-HCl (pH 8.4)/1 mM-EDTA/150 mM-NaCl and the protein was precipitated overnight at 4 °C with a final concentration of 20% (w/v) trichloroacetic acid (Cl_3Ac). Total initial amount of pI 5.9 apo-SAA was approx. 324 μg, yielding 190 μg on electroelution.

CNBr fragmentation of electroeluted protein

An estimated 2 nmol of electroeluted apo-SAA pI 5.9 was subjected to CNBr fragmentation [16]. The precipitated protein was made 3.7 M in CNBr in 78% formic acid and incubated at room temperature for 16 h. After drying the sample by vacuum centrifugation, 20 μl of ethanolamine was added, and, after 10 min, the sample was dried again. The protein fragments were dissolved in 25 μl of 95% formic acid plus 85 μl of 0.06% trifluoroacetic acid (F_3Ac) and the solution subjected to reverse-phase h.p.l.c.

Reverse-phase h.p.l.c. of peptides

Peptides generated by enzymic or chemical cleavage were desalted and separated by reverse-phase h.p.l.c. on a 4.6 mm × 250 mm Vydac (Hesperia, CA, U.S.A.) C_{18} column (TP silica). Elution was with a linear gradient of acetonitrile in 0.06% F_3Ac at a flow rate of 1 ml/min. Absorbance was monitored at 214 nm. U.v.-absorbing peaks were collected and subjected to amino-acid-sequence analysis. A Hewlett–Packard 1050 h.p.l.c. apparatus, consisting of a quartenary pump, autosampler, variable-wavelength detector and a model 3396 integrator was used for all chromatography.

Amino acid sequencing

All protein sequence analysis was performed at the Macromolecular Structure Analysis Facility at the University of Kentucky. *N*-Terminal sequencing of apo-SAA pI 5.9 electroblotted on to PVDF membrane was carried out essentially as described [17] by using a pre-cycled Polybrene-coated glass-fibre disc and sequencer cycles modified for use with PVDF on an Applied Biosystems (Foster City, CA, U.S.A.) model

477A protein sequencer with on-line phenylthiohydantoin identification.

RNA isolation and Northern hybridization analysis

Polyadenylated RNA was extracted from individual and pooled liver samples from nine BALB/c and nine SJL/J mice and their F_1 hybrids by using the Mini RiboSep mRNA isolation kit (Collaborative Research, Bedford, MA, U.S.A.) according to the manufacturer's instructions. RNA was denatured by glyoxylation, size-fractionated by electrophoresis through 1.5% agarose, and transferred to Genescreen Plus nylon membranes (du Pont/New England Nuclear, Boston, MA, U.S.A.). The composition of SAA mRNAs was analysed by using oligonucleotides synthesized in the Core Facility of the Department of Biochemistry, Boston University School of Medicine, Boston, MA, U.S.A., and purchased from Oligos Etc. (Guilford, CT, U.S.A.). The 18-mer exon 2 probes were designed to distinguish between SAA_1, SAA_2 and SAA_3 transcripts in the regions encoding residues 4–9 [2]. The 17-mer exon 3 probe corresponds to residues 37–42 common to the mouse SAA_1, SAA_2 and SAA_3 genes. The set of exon 4 probes was designed by Meek & Benditt [18] to distinguish between SAA_1, SAA_2 and SAA_3 transcripts in an 18-base region in the 3′ untranslated region starting 14 bases beyond the termination codon. Oligonucleotides were end-labelled with T_4 polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.) in the presence of [γ-^{32}P]ATP. Unincorporated ATP was removed from the reaction mixture through the use of Nensorb columns (du Pont New England Nuclear). After overnight hybridization at 45 °C, filters were washed as previously described [19] and exposed to X-ray film at −70 °C for 2–24 h. After hybridization of SAA mRNAs with radiolabelled oligonucleotide probes and autoradiography, the oligonucleotides were stripped from the blots and the quantity of actin mRNA in each sample was determined by hybridization with rat β-actin cDNA [22] radiolabelled with [α-^{32}P]dCTP using a random priming kit (Bethesda Research Laboratories). The total mRNA content of each lane was normalized on the basis of its β-actin hybridization signal.

The relative amounts of exon-specific SAA mRNA and β-actin mRNA were determined by densitometric scanning of autoradiographs using an Electrophoresis Data Center (Helena Laboratories, Beaumont, TX, U.S.A.). Both quantity of RNA loaded and length of exposure time were varied to assure linear response between quantity of mRNA and intensity of hybridization signal.

RESULTS

Analytical electrofocusing and immunoblotting of mouse apo-SAA isoforms

The apo-SAA isoform distribution of BALB/c mice (type A) was compared with that of SJL/J mice (type B) (Figs. 1*a* and 1*b*). Both express apo-SAA_1 (pI 6.45) as a major isoform. Apo-SAA_2 (pI 6.3), a major isoform in BALB/c mice, is only minimally expressed in SJL/J mice. In this strain an apo-SAA isoform of pI 5.9, only minimally expressed in BALB/c mice, becomes the second major apo-SAA isoform. The F_1 progeny of these two strains (CSJLF1/J) exhibit co-dominant expression of the three major apo-SAA isoforms present in the parent strains, with the minor pI 6.15 isoform of the parent strains clearly present in each individual F_1 mouse (Fig. 2).

Primary structure of mouse apo-SAA pI 5.9

The primary structure of mouse apo-SAA pI 5.9 was determined from the sequences of overlapping peptides generated by trypsin, Endoproteinase Lys-C and CNBr cleavage of the protein as well as by *N*-terminal sequencing (Fig. 3).

The reverse-phase h.p.l.c. separation of peptides generated by trypsin digestion of apo-SAA (pI 5.9) yielded the profile in Fig. 4(*a*). Peaks T1–T8 provided the sequences shown in Fig. 3. Peak T5, containing amino acids 29–34 represents an incomplete digest. Peak T4, also an incomplete digest, was sequenced for 14 cycles, starting at amino acid 71. Peak T7 contained two peptides, namely a major peptide representing amino acids 39–46, also sequenced in peak T2, as well as a minor peptide, representing amino acids 19–24.

Endoproteinase-Lys-C-generated peptides of apo-SAA (pI 5.9), separated by h.p.l.c., produced the chromatogram shown in Fig. 4(*b*). Peaks L1, L4 and L5 yielded peptides identical with amino acid segments 25–29, 57–77 and 57–80 of mouse SAA_2 [20] respectively. Peak L3 yielded the *C*-terminal part of the molecule which was similar to mouse apo-SAA_2 [20], except at position 101, where aspartic acid was substituted for alanine. Peak L2 contained two peptides, one identical with amino acids 34–56 of

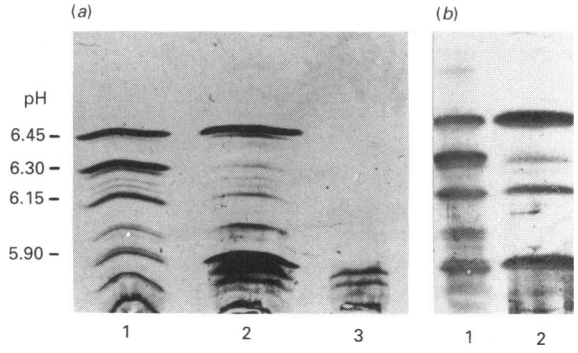

Fig. 1. Isoelectric-focusing analysis of mouse HDL

(*a*) Coomassie Blue staining of electrofocused mouse HDL. Lane 1, electrofocused HDL (200 μg) from LPS-injected BALB/c mice (type A); lane 2, electrofocused HDL (200 μg) from LPS-injected SJL/J mice (type B); lane 3, electrofocused HDL (200 μg) from control SJL/J mice (type B). The Figure shows the presence of major apo-SAA isoforms with pI 6.45 and 6.3 in type A mice, whereas apo-SAAs with pI values of 6.45 and 5.9 are major isoforms in type B mice. (*b*) Immunochemical staining of apo-SAA isoforms from BALB/c and SJL/J mice with rabbit anti-mouse AA. Lane 1, LPS-injected BALB/c mice; lane 2, LPS-injected SJL/J mice.

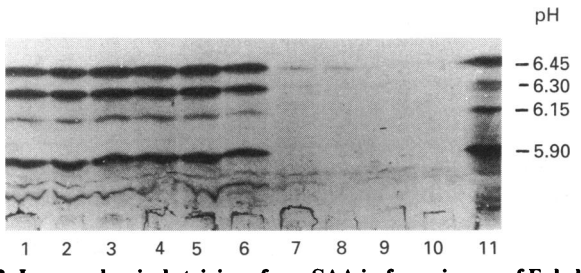

Fig. 2. Immunochemical staining of apo-SAA isoforms in sera of F_1 hybrid mice

Sera from individual F1 hybrid mice was subjected to electrofocusing and the apo-SAA isoforms identified with a rabbit anti-mouse AA antibody. Lanes 1–6, LPS-injected CSJLF1/J mice; lanes 7–10, control CSJLF1/J mice; lane 11, 50 μg of HDL from LPS-injected SJL/J mice. CSJLF1/J mice show co-dominant expression of apo-SAA isoforms pI 6.45 (SAA_1), pI 6.3 (SAA_2) and pI 5.9, the major apo-SAA isoforms present in the parent strains. A minor isoform (pI 6.15) is evident.

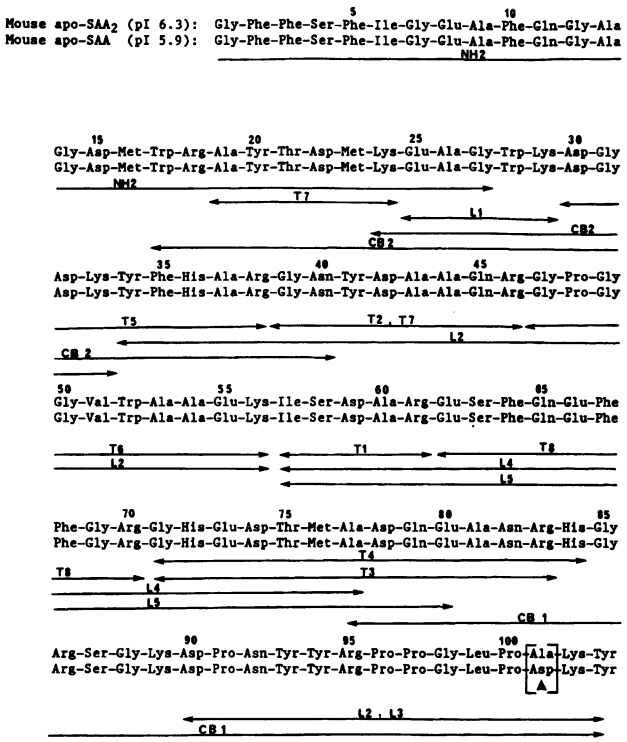

Fig. 3. Amino acid sequence of SJL/J apo-SAA (pI 5.9)

The amino acid sequence was obtained from *N*-terminal sequencing (NH2), and overlapping peptides generated by fragmentation with trypsin (T), Endoproteinase-Lys-C (L) and CNBr (CB).

mouse apo-SAA_2 [20] and one consisting of amino acids 90–103, confirming the single amino acid substitution at position 101.

Fragments generated by CNBr cleavage of electroeluted apo-SAA (pI 5.9) and separated by h.p.l.c. gave the profile in Fig. 4(*c*). CNBr cleavage could have been performed on the PVDF membrane, but we were concerned that the long peptides generated might not easily dissociate from the membrane. Two of the peaks chosen for analysis provided the data necessary to complete the primary structure of the pI 5.9 protein. Peak CB1 represents amino acids 77–103, again confirming the amino acid substitution at position 101. This fragment also provided the sequence of the five amino acids not identified by the microsequencing strategies involving enzymic digestion of the protein electroblotted on to PVDF membrane. Two peptides were contained in peak CB2, namely those representing amino acids 17–76 and 24–76. The first peptide represents an incomplete fragmentation. Both peptides were sequenced for 17 cycles.

N-Terminal sequencing of the protein electroblotted on to PVDF membrane provided the first 27 amino acids (Fig. 3).

The primary structure of apo-SAA (pI 5.9) differs from that of apo-SAA_2 only at position 101 (Fig. 3), where an aspartic acid is substituted for alanine.

RNA hybridization analysis

We find that, on the basis of hybridization with the exon 2 and exon 3 probes, BALB/c, SJL/J and their F_1 hybrids express *SAA* genes to an equivalent extent after LPS administration. These data are reflected in Fig. 5, with the densitometric analyses being given in Table 1. When the 17-mer exon 3 probe common to SAA_1, SAA_2 and SAA_3 is used, it is evident that comparable amounts of mRNA were loaded for analysis (Fig. 5; Table 1). The

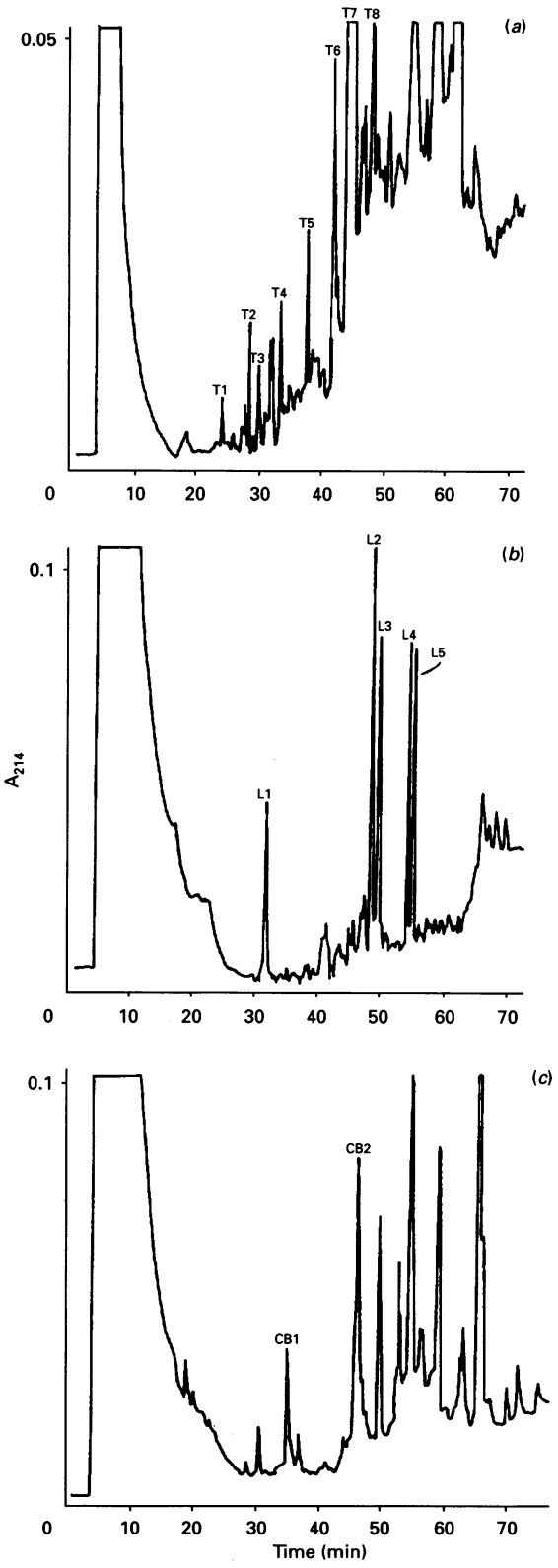

Fig. 4. Separation of peptides by reverse-phase h.p.l.c.

Peptides generated by enzymic or chemical cleavage of mouse apo-SAA (pI 5.9) were separated by reverse-phase h.p.l.c. on a 4.6 mm × 250 mm Vydac C_{18} column with a linear gradient of acetonitrile in 0.06% F_3Ac at a flow rate of 1 ml/min. Absorbance was monitored at 214 nm. (*a*) Peptides from trypsin digestion of apo-SAA (pI 5.9) electroblotted on to PVDF membrane; (*b*) peptides from Endoproteinase Lys-C digestion of apo-SAA (pI 5.9) electroblotted on to PVDF membrane; (*c*) peptides generated by CNBr cleavage of electroeluted mouse apo-SAA (pI 5.9).

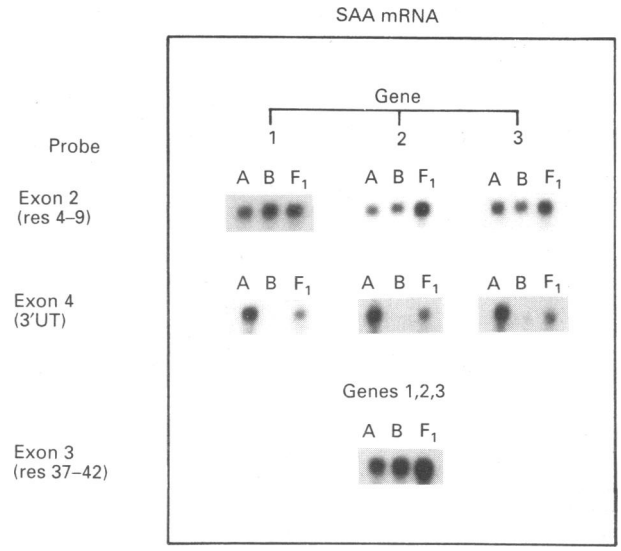

Fig. 5. Gene-specific hybridization analysis of SAA mRNA in type A and B mice and their F_1 hybrids

BALB/c (A), SJL/J (B) and CSJLF1/J (F_1) mice were injected intraperitoneally with 100 μg of LPS type W from *S. typhosa*. Livers were removed 16 h later and polyadenylated RNA was extracted, denatured, size-fractionated by electrophoresis (2 μg/lane) and transferred to nylon filters. Hybridization of identical blots was carried out with oligonucleotide probes corresponding to BALB/c *SAA* genes 1, 2 and 3 [4]. The set of 18-mer exon 2 probes corresponds to the region of exon 2 encoding residues 4–9; a single 17-mer probe corresponds to the portion of exon 3 encoding residues 37–42 identical in SAA_1, SAA_2 and SAA_3 gene transcripts, and the set of probes designed for the 3' untranslated region [18] corresponds to 18 bases located 14 bases beyond the termination codons. A, B and F_1 are the haplotypes.

18-mer exon 2 probe, directed to distinguish transcripts encoding residues 4–9 from the three mouse genes, again shows comparable amounts of mRNA in haplotype A, B and F_1 hybrid mice.

However, when the set of exon 4 probes designed to distinguish the three genes over an 18-base region of the 3' untranslated region of BALB/c mice was used, neither the SAA_1 nor SAA_2 probe showed any hybridization signal in SJL/J mice. The SAA_3 probe gave a reduced signal in this strain that was less than 25% of the signal of BALB/c mice. The exon 4 probe gives an intermediate signal in F_1 mice for both the SAA_1 and SAA_2 genes. These data confirm divergence between type A and type B mice in exon 4 of all three known mouse genes. No size differences between the various mRNAs could be detected by their migration in Northern-blot-hybridization analysis.

DISCUSSION

Members of the *SAA* gene family are about 3200 bp in length. They consist of four exons, the first of which encodes the 5' untranslated region. In the present study we demonstrate that the structure of the *SAA* gene family of SJL/J mice differs from that of BALB/c mice. We demonstrate by amino-acid-sequence analysis the existence of a previously unrecognized variant of the apo-SAA family with an isoelectric point of 5.9. This isoform appears to be qualitatively similar in BALB/c and SJL/J mice, but quantitative differences are apparent during LPS-induced inflammation. In primary structure it is very similar to apo-SAA_2, differing only at position 101, where an aspartic acid residue has been substituted for an alanine. The amino acid substitution at position 101 is most likely the result of a single base change in the translated region of exon 4 where GCC → GAC. This substitution of a negatively charged amino acid for a neutral amino acid is reponsible for the acidic shift of the pI. Theoretical calculation of the pI of the pI 5.9 isoform, as we described for human apo-SAA isoforms [11], corresponds to the measured pI of 5.9. This pI 5.9 isoform is most likely the substrate for amyloid fibrils in SJL/J mice, owing to its identity with SAA_2 in the 76 *N*-terminal amino acids that constitute AA.

The most likely reason why the pI 5.9 isoform was not previously detected in SJL/J mice is that, when analysed by the 6.4% urea/SDS/PAGE system, commonly utilized to separate apo-SAA_1 from apo-SAA_2, the pI 5.9 isoform co-migrates in a single band with apo-SAA_1 [23].

Northern analyses reveal that exon 4 of the pI 5.9 isoform differs further from SAA_2 in the 3' untranslated region where an SAA_2-specific probe for this region fails to detect any SAA_2 mRNA, whereas the mRNA for the major pI 5.9 isoform is abundant (Fig. 5). Similarly, the BALB/c-specific SAA_1 probe modelled on the 3' untranslated region fails to hybridize in the case of SJL/J mice. This leaves the interesting situation where isoelectrically identical SAA_1 molecules in the two strains are translated from mRNAs that differ in exon 4. It is possible that exon 4 of pI 5.9 and SAA_1 of SJL/J mice are identical in that the aspartic acid substitution at position 101 changes the protein-coding region of exon 4 of pI 5.9 to that of SAA_1. The weak hybridization of the SAA_3 probe for this region in SJL/J mice suggests that even this gene's exon 4 differs from BALB/c.

It has been estimated that the mouse *SAA* genes were derived from an ancestral gene which, through gene duplication, gave rise to the $SAA_{1/2}$ and SAA_3 subfamilies over 85 million years ago, and that, through gene conversion, the $SAA_{1/2}$ subfamily gave rise to the SAA_1 and SAA_2 genes [1,21]. Type A and B mice have qualitatively the same isoforms. The fact that type A mice express the pI 5.9 isoform and type B mice the apo-SAA_2 isoform, albeit in a minor way, suggests the possibility that the pI 5.9

Table 1. Relative abundance of *SAA* exon expression in type A and B mice and their F_1 hybrids

Seven different oligonucleotide probes corresponding to the three known active genes in BALB/c (type A mice) were hybridized with identical sets of polyadenylated liver RNA from LPS-treated mice (Fig. 5). After autoradiography, the bound SAA oligonucleotide probe was removed and the blots were hybridized with the β-actin cDNA probe [22]. The ratio percentage of SAA signal/percentage of actin signal in each lane is presented. Standard deviations are in parentheses.

		SAA_1		SAA_2		SAA_3		$SAA_{1,2,3}$
Type	Exon...	2	4	2	4	2	4	3 (common)
A		1.2 (0.2)	2.6 (0.5)	0.9 (0.3)	3.9 (1.2)	1.0 (0.2)	1.9 (0.8)	0.8 (0.1)
B		1.0 (0.1)	0	1.1 (0.3)	0	0.9 (0.1)	0.3 (0.1)	1.1 (0.2)
F_1		0.8 (0.2)	1.8 (0.8)	0.9 (0.2)	1.8 (0.6)	1.1 (0.2)	1.6 (0.4)	1.1 (0.1)

isoform is not the result of mutation(s) in exon 4 at the SAA_2 locus but is, in fact, the product of a new gene that differs in quantitative acute-phase expression between the strains. It is conceivable that the control regions responsible for the prolific expression of the respective *SAA* genes have converted to a lesser extent than the coding regions and segregated with different *SAA*-gene conversion copies in BALB/c and SJL/J mouse strains. Our isoelectric-focusing data are compatible with the hypothesis that there are more $SAA_{1/2}$-gene conversion copies than previously found. We have proved it for the pI 5.9 isoform. The pI 6.15 isoform (Fig. 1) could conceivably represent the protein product of yet another undiscovered gene. The fact that these putative conversion copies are so similar has precluded their prior detection by molecular-biological techniques.

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REFERENCES

1. Lowell, C. A., Potter, D. A., Stearman, R. S. & Morrow, J. F. (1986) J. Biol. Chem. **261**, 8442–8452
2. Yamamoto, K., Shiroo, M. & Migita, S. (1986) Science **232**, 227–229
3. Hoffman, J. S. & Benditt, E. P. (1982) J. Biol. Chem. **257**, 10510–10517
4. Lowell, C. A., Stearman, R. S. & Morrow, J. F. (1966) J. Biol Chem. **261**, 8453–8461
5. Stearman, R. S., Lowell, C. A., Peltzman, C. G. & Morrow, J. F. (1986) Nucleic Acids Res. **14**, 797–809
6. Taylor, B. A. & Rowe, L. (1984) Mol. Gen Genet. **159**, 491–499
7. Rokita, H., Shirahama, T., Cohen, A. S. & Sipe, J. D. (1989) Br. J. Exp. Pathol. **70**, 327–335
8. Strachan, A. F., de Beer, F. C., Van der Westhuyzen, D. R. & Coetzee, G. A. (1988) Biochem. J. **250**, 203–207
9. Folch, J., Lees, M. & Sloane Stanley, G. H. (1957) J. Biol. Chem. **226**, 497–509
10. Strachan, A. F., Shephard, E. G., Bellstedt, D. U., Coetzee, G. A., Van der Westhuyzen, D. R. & de Beer, F. C. (1989) Biochem. J. **263**, 365–370
11. Strachan, A. F., Brandt, W. F., Woo, P., Van der Westhuyzen, D. R., Coetzee, G. A., de Beer, M. C., Shephard, E. G. and de Beer, F. C. (1989) J. Biol. Chem. **264**, 18368–18373
12. Godenir, N. L., Jeenah, M. S., Coetzee, G. A., Van der Westhuyzen, D. R., Strachan, A. F. & de Beer, F. C. (1985) J. Immunol. Methods. **83**, 217–225
13. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
14. Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
15. Bauw, G., Van Damme, J., Puype, M., Vandekerckhove, J., Gesser, B., Ratz, G. P., Lauridson, J. B. & Celis, J. E. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 7701–7705
16. Gross, E. (1967) Methods Enzymol. **11**, 238–255
17. Speicher, D. W. (1989) Techniques in Protein Chemistry (Hugli, T., ed.), pp. 24–35, Academic Press, San Diego
18. Meek, R. L. and Benditt, E. P. (1986) J. Exp. Med. **164**, 2006–2017
19. Rokita, H., Shirahama, T., Cohen, A. S., Meek, R. L., Benditt, E. P. & Sipe, J. D. (1987) J. Immunol. **139**, 3849–3853
20. Yamamoto, K. & Migita, S. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 2915–2919
21. Yamamoto, K., Goto, N., Kosaka, J., Shiroo, M., Yeul, Y. D. & Migita, S. (1987) J. Immunol. **139**, 1683–1688
22. Farmer, S. R. (1986) Cell and Molecular Biology of the Cytoskeleton (Shay, J. W., ed.), pp. 131–149, Plenum Publishing Corp., New York
23. De Beer, F. C., De Beer, M. C. and Sipe, J. D. (1990) in Amyloid and Amyloidosis (1990) (Natvig, J. B., Forre, O., Husby, G., Husebekk, A., Skogen, B., Sletten, K. & Westermark, P., eds.), pp. 890–893, Kluwer Academic Publishers, Dordrecht

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