Expression of recombinant human serum amyloid A in mammalian cells and demonstration of the region necessary for high-density lipoprotein binding and amyloid fibril formation by site-directed mutagenesis

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Site-directed mutagenesis of the acute-phase human serum amyloid A (SAA1 α) protein was used to evaluate the importance of the N-terminal amino acid residues, namely RSFFSFLGEAF. The full-length cDNA clone of SAA1 α (pA1.mod.) was used to create two mutations, namely Gly-8 to Asp-8 and an 11 amino acid truncation between Arg-1 and Phe-11 respectively. Wild-type and mutant cDNAs were expressed in Chinese hamster ovary (CHO) cells under the control of the human cytomegalovirus promoter, which resulted in the secretion of the processed proteins into the culture media. Wild-type recombinant human SAA (rSAA) protein was shown to have pI values of 6.0 and 6.4, similar to the human SAA isoform SAA1 α and SAA1 α desArg found in acute-phase plasma. N-terminal sequencing of 56 residues confirmed its identity with human SAA1 α . The total yield of wild-type rSAA measured by ELISA was between 3.5 and 30 mg/l. The two mutations resulted in reduced expression levels of the mutant SAA proteins (3–10 mg/l). Further measurements of rSAA concentration in lipid fractions of culture medium collected at a density of 1.21 g/ml (high-density lipoprotein;

HDL) and 1.063–1.18 g/ml (very-low-density lipoprotein/low-density lipoprotein; VLDL/LDL) showed that 76% of the wild-type protein was found in the HDL fraction and the remaining 24% in the infranant non-lipid fraction. In contrast the relative concentration of mutant rSAA in HDL and infranant fractions was reversed. This is consistent with the previously proposed involvement of the 11 amino acid peptide in anchoring SAA protein on to HDL₃ [Turnell, Sarra, Glover, Baum, Caspi, Baltz and Pepys (1986) *Mol. Biol. Med.* 3, 387–407]. Wild-type rSAA protein was shown to form amyloid fibrils *in vitro* under acidic conditions as shown by electron microscopy, and stained positive with Congo Red and exhibited apple-green birefringence when viewed under polarized light. Under the same conditions mutSAA(G8D) and mutSAA Δ 1–11 did not form amyloid fibrils. In conclusion, replacement of Gly-8 by Asp-8 or deletion of the first 11 amino acid residues at the N-terminus of rSAA diminishes its capacity to bind to HDL and decreases amyloid fibril formation.

INTRODUCTION

Human serum amyloid A (ApoSAA) proteins are a group of highly similar 12–14 kDa apolipoproteins found predominantly in the high-density lipoprotein (HDL) fraction of plasma. Some behave as acute-phase proteins and can be found as pairs: SAA1 α (pI 6.0/6.4), SAA1 β (pI 5.6/6.1), SAA2 α (pI 7.0/7.5) and SAA2 β (pI 7.4/8.0) [2,3]. Each pair of proteins has identical amino acid sequences, but one member of each pair lacks the N-terminal arginine residue. Baba et al. [4] have reported a novel allelic variant of SAA1, SAA1 γ , which differs at residues 52 and 57 compared with SAA1 α and SAA1 β respectively. There is also a constitutively expressed protein, cSAA4 (pI 7.3/7.9 and 8.1); unlike other human ApoSAA proteins, cSAA4 is glycosylated [5–7]. During the acute phase, ApoSAA levels can increase 1000-fold in response to injury, infection or inflammation. The high serum levels of ApoSAA are necessary in the pathogenesis of reactive amyloidosis and have been shown to be the precursor of the 8 kDa amyloid protein (AA) [8,9], which is the major fibrillar component of amyloid deposits. ApoSAA protein has been

shown to be partly degraded at the C-terminus; the remaining N-terminal part is essential for fibril formation [10,11]. By definition, amyloid fibrils stain positive with Congo Red and exhibit green birefringence when viewed under polarized light [12]. This characteristic has been proposed by Turnell and Finch [13] to be a function of an anti-parallel β -pleated sheet conformation with a globular structure interacting via hydrogen bonds [13,14]. During experimentally induced amyloidosis in CBA mice, amyloid deposits were found to be preferentially derived from mouse ApoSAA2 rather than mouse ApoSAA1 [15,16]. Two structural differences within the N-terminus of the mouse ApoSAA isoforms were found at positions 7 and 8, where ApoSAA1 has Val-His and ApoSAA2 has Ile-Gly [16,17]. In support of this hypothesis the amyloid-resistant CE/J mouse strain has been shown to have a unique ApoSAA isoform that is a hybrid protein with features of both mouse ApoSAA1 and ApoSAA2. The N-terminal sequence was found to be identical with the non-fibril-forming mouse ApoSAA1 containing Val-His at positions 7 and 8 [18]. No N-terminal variation at positions 7 and 8 (Leu-Gly) has been observed between the different human ApoSAA1 and ApoSAA2;

Abbreviations used: AA, 8 kDa amyloid protein; AD units, arbitrary densitometric units; ApoSAA, serum amyloid A protein; BCIP, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt; CHO, Chinese hamster ovary; E.M., electron microscope; FCS, foetal calf serum; HDL, high-density lipoprotein; LDL, low-density lipoprotein; RFLP, restriction fragment length polymorphism; rSAA, recombinant human SAA; VLDL, very-low-density lipoprotein; BCA, bicinchoninic acid.

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both these proteins are found in reactive amyloid deposits [19]. However, Liepnieks et al. [19] found that the predominant form in amyloid deposits isolated from patients with Familial Mediterranean Fever was ApoSAA1, and SAA1 γ was the predominant fibril protein found in Japanese subjects [4], which suggest that allelic variations at this locus may be a risk factor in AA amyloidosis. Furthermore Yamada et al. [20] have demonstrated that human recombinant SAA1 (rSAA1) was more fibrillogenic than rSAA2 *in vitro*. Studies of spontaneous fibril formation *in vitro* with synthetic peptide and cleavage fragments from human ApoSAA have suggested that the amyloidogenic region is located within the first 10–15 amino acid residues [10]. Yamada et al. [10] have also demonstrated that cleavage of the nine residues at the N-terminus of rSAA rendered it non-fibrillogenic. These structural differences at the N-terminus might be important factors in determining the amyloidogenicity of ApoSAA.

Using one-dimensional Fourier analysis of the amino acid sequence of human SAA1 α together with sequence matching to known secondary structural motifs, Turnell et al. [20a] predicted an extremely hydrophobic lipid-binding N-terminus consisting of 11 amino acid residues (RSFFSFLGEAF), two α -helices and a putative calcium-binding site. The sequence between Ser-2 and Gly-8 forms two turns of a hydrophobic but weakly amphipathic helix, which was postulated to act as an anchor for the ApoSAA by inserting the molecule into the 11 Å thick hydrophobic region of the lipid monolayer that forms the coat of the HDL₃ particle [21,22]. Antibodies directed against the N-terminal region of ApoSAA do not recognize ApoSAA–HDL complexes [23]. This supports the hypothesis that ApoSAA binds via a peptide inserted into the HDL particle.

The function of ApoSAA is still unclear, but it is thought to influence lipid metabolism and reverse cholesterol transport [24]. It has been shown to lower lecithin:cholesterol acyltransferase activity indirectly [25] and increase levels of cholesterol during the acute phase by displacing apolipoprotein AI (ApoAI) from HDL, resulting in an HDL particle with a higher hydrated density, greater size and relatively less ApoAI [26–28]. At inflammatory sites ApoSAA mRNA and protein have been demonstrated in macrophage, smooth-muscle, adipose and endothelial cells, suggesting probable local production at these sites [29,30]. Purified ApoSAA or recombinant ApoSAA seem to have different properties from ApoSAA bound to HDL. Recombinant ApoSAA has been shown to bind cholesterol *in vitro* and enhances cholesterol uptake by HepG2 cells [31]. Purified and recombinant ApoSAA-like molecules have been shown to inhibit oxygen burst from neutrophils [32], and rSAA has recently been reported to induce adhesion and chemotaxis of human monocytes and polymorphonuclear neutrophils *in vitro* [33]. Recombinant SAA has been shown to induce directional migration of T-cells *in vitro* [34], indicating an important role in recruiting T-lymphocytes, neutrophils and monocytes into inflammatory regions. However, HDL inhibits these effects and because HDL is prevalent in high concentration in the plasma and tissue fluids, it is unclear whether these pro-inflammatory effects have physiological/pathological importance.

In this study we describe a mammalian expression system for human ApoSAA1 and the use of site-directed mutagenesis to investigate the amyloidogenicity and lipid-binding properties of the 11 amino residues at the N-terminus of human ApoSAA1 α .

MATERIALS AND METHODS

Materials

All tissue-culture media and their ingredients were obtained from Gibco BRL Life Technologies (Renfrewshire, Scotland, U.K.).

Oligonucleotides were synthesized by Genosys (Cambridge, U.K.). The radioactive nucleotide [α -³²P]dCTP (3000 Ci/mM), Hybond N, enhanced chemiluminescence kit and oligonucleotide-directed *in vitro* mutagenesis kit (version 2.0) were obtained from Amersham (Bucks., U.K.), human actin cDNA probe was purchased from Clontech, Cambridge Bioscience (Cambridge, U.K.) and mycophenolic acid from ICN (Thame, Oxfordshire, U.K.). Restriction endonucleases and DNA-modifying enzymes were supplied by Promega (Southampton, Hants., U.K.) or Gibco BRL, 0.22 μ m Millipore GS sterilizing filters from Millipore (Watford, Herts., U.K.), 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) and Nitro Blue tetrazolium chloride by BRL (Bethesda, MD, U.S.A.) and poly(vinylidene difluoride) membrane from Applied Biosystems (Warrington, Cheshire, U.K.). Alkaline phosphatase-conjugated goat anti-rabbit IgG, BCA (bicinchoninic acid) total protein assay, xanthine, hypoxanthine and thymidine were purchased from Sigma (Poole, Dorset, U.K.). Protein A, CNBr-activated Sepharose beads, Ampholines (pH 3–10) and 'ready to go' DNA labelling kit was from Pharmacia, Biotech (St. Albans, Herts., U.K.). TRI-Reagent RT-LS[®] was supplied by Molecular Research Center (Cincinnati, OH, U.S.A.). Pooled plasma was obtained from the blood transfusion service at Northwick Park Hospital (Harrow, U.K.), and 0.2 μ m nitrocellulose membranes were purchased from Schleicher and Schuell (Dassel, Germany). Goat anti-rabbit IgG conjugated with horseradish peroxidase was obtained from Bio-Rad (Hemel Hempstead, Herts., U.K.).

Bacterial strains, mammalian cell lines, plasmids and oligonucleotides

Escherichia coli strain DH-5 α was used for plasmid construction. M13mp19-based phage vectors were propagated by using bacterial strain TG1. Chinese hamster ovary (CHO) cells and the expression vector pGWHlg were gifts from Dr. J. Divine (British Biotechnology, Oxford, U.K.). The vector carries the human constitutive promoter and enhancer of the human cytomegalovirus and the *E. coli* xanthine/guanine phosphoribosyl transferase gene (*gpt*) for the selection of transfectants in mycophenolic acid-containing medium. Oligonucleotides pw143 (5'-GGACCAAGGAGCAGAAGCAAGCTTGGCGTA-3'), pw144 (5'-CGAAAAGAACTTCGGCT-3') and pw145 (5'-CC-AAGGAGCAGAAAACCAGGCCCGTGAGCAGCTTCA-TGGTGGCAAGCTTGGCG-3') were used for site-directed mutagenesis to create a full-length human SAA1 α cDNA, pA1.mod. Oligonucleotides pw160 (5'-CTGGGTGTACGACGATGGGGCTCGGGAC-3') and pw155 (5'-TCGTCTTGACGAGGCTTTTGATG-3') were used for the construction of the SAA1 α mutants. Oligonucleotides pwRT3 (5'-CAGCCAGC-GAGTCCTCCGCACCAT-3') and pwRT5 (5'-GCCACCAT-GAAGCTGCTCAC-3') were used for reverse transcriptase-PCR.

Site-directed mutagenesis to create mutSAA(G8D) and mutSAA1–11

Site-directed mutagenesis of the cDNA clone pA1 [35] was used to construct a full-length SAA1 α cDNA (pA1.mod) in M13mp19 [36]. The single-stranded DNA of the pA1.mod was used as a template to create two mutants by using the oligonucleotide-directed *in vitro* mutagenesis kit version 2.0, in accordance with the manufacturer's protocol. Oligonucleotide pw155 created a single nucleotide G $\rightarrow$ A substitution; this changed the wild-type amino acid sequence from ⁺⁵SFLGEAFD⁺¹² to ⁺⁵SFLDEAFD⁺¹² to produce M13mp19Asp8. Oligonucleotide pw160 was used to delete the wild-type sequence ⁺¹RSFFSFLGEAF⁺¹¹, resulting in

the deletion of these 11 residues to produce M13mp19(Δ 1–11). Phage M13 subclones containing the mutated cDNA inserts were selected by plaque hybridization with the oligonucleotide used for mutagenesis, plaque-purified and verified by nucleotide sequencing with the Sequenase kit version 2.0 in accordance with the manufacturer's protocol. The double-stranded replicative forms of the wild-type and mutation-positive phage clones were isolated and cleaved with *Hind*III/*Eco*R1 and inserted into the expression vector pGWHig to create final expression constructs, pSAACHO, pSAAmut Δ 1–11 and pSAAmutAsp8 carrying the wild-type and mutant SAA1 α cDNAs.

Cell culture and transfection

Untransfected CHO cells were maintained in Dulbecco's modified Eagle's Medium containing 10% (v/v) foetal calf serum (FCS). CHO cells were transfected with 40 μ g of *Not*I linearized wild-type or mutant cDNA by electroporation at 800 V, 25 μ F, in PBS, pH 7.4, with the Bio-Rad gene pulser. A limiting dilution (1:5000) of transfected cells was made into 500 ml of DMEM selection medium containing 10% FCS, 2.5 μ g/ml mycophenolic acid, 12.5 μ g/ml xanthine, 6.8 μ g/ml hypoxanthine, 1.9 μ g/ml thymidine and 3 mM NaOH [36]. These cells were plated into microtitre plates and grown on selection medium for 2 weeks. Stable CHO transfectants expressing the SAA1 α cDNA species pSAACHO, pSAAmut Δ 1–11 and pSAAmutAsp8 were designated wild type, mutSAA Δ 1–11 and mutSAA(G8D) respectively. For harvesting of recombinant SAA (rSAA) proteins, CHO transfectant cells were grown in serum-free selection medium containing 0.1% (v/v) purified human HDL, designated as HDL medium.

Genomic restriction fragment length polymorphism (RFLP) analysis from transfected cells

Genomic DNA isolated from untransfected and transfected CHO cells expressing human wild-type or mutant SAA1 α cDNA were digested with *Hind*III to cut an internal site within the SAA1 α leader sequence and thereby determine copy number. These digests were fractionated on to 1% (w/v) agarose gel and transferred to Hybond N membrane by using Southern analysis [37]. The filter was probed with the radiolabelled *Hind*III/*Eco*R1 fragment of the pA1.mod cDNA and lambda *Hind*III DNA markers with [α -³²P]dCTP by the 'ready to go' DNA labelling kit in accordance with instructions supplied by the manufacturer. As a positive control, human genomic DNA was digested with *Bgl*II to confirm homologous binding of the probe pA1.mod to the human SAA1 α sequence as described by Betts et al. [5]. The membrane was washed to a high stringency [0.1% SDS; 0.2 $\times$ SSC (where SSC is 0.15 M NaCl/0.015 M sodium citrate)] at 65 °C and exposed to Kodak (X-OMAT) film for autoradiography.

Isolation and analysis of mRNA

Total cellular RNA was isolated with Tri-Reagent RT-LS[®] by the protocol of Chomczynski [38]. RNA samples (20 μ g each) were separated by electrophoresis through a denaturing formaldehyde/agarose gel and transferred to Hybond-N nylon membrane by the method of Sambrook et al. [37]. The filter was probed with the radiolabelled *Hind*III/*Eco*R1 fragment of pA1.mod cDNA and human β -actin cDNA probe by using [α -³²P]dCTP. Quantification of autoradiographs was performed on a Bio-Rad GS-670 imaging densitometer equipped with Molecular Analyst software version 1.2. Relative amounts of SAA

mRNA were normalized against human β -actin mRNA. Arbitrary densitometric units (AD units) were expressed as a ratio of SAA to actin mRNA transcript.

Preparation of HDL

HDL was isolated from pooled human blood plasma by flotation ultracentrifugation as previously described [39]. Plasma density was adjusted to $d = 1.063$ g/ml [low-density lipoprotein/very-low-density lipoprotein (LDL/VLDL) fraction] with solid KBr and the mixture was centrifuged at 200 000 g for 25 h at 15 °C in a Ti70 rotor. After centrifugation, the top layer containing the LDL/VLDL was removed. To obtain HDL, the density of the supernatant was adjusted to $d = 1.21$ g/ml (HDL fraction) and the mixture was centrifuged as above. Excess KBr was removed by dialysing HDL against 150 mM NaCl, 0.1% EDTA, pH 7.4. The HDL was then assayed for protein content with the BCA protein assay. HDL was sterilized by filtration through a 0.22 μ m Millipore GS filter and stored at 4 °C.

Western analysis

Protein samples were electrophoretically separated by the procedure of Laemmli [40] by SDS/PAGE (12% gel) and then transferred to nitrocellulose membrane by the method of Towbin et al. [41]. The nitrocellulose was probed with rabbit anti-(human ApoSAA) as the primary antibody, a gift from F. C. De Beer. (Department of Biochemistry, University of Kentucky Medical Center, Lexington, KY, U.S.A.) [39]. Goat anti-(rabbit IgG) conjugated with horseradish peroxidase was used as a secondary reagent. To test for antibody reaction, the enhanced chemiluminescence detection kit was used in accordance with the manufacturer's protocol. Autoradiograms were quantified by densitometry as described above. Relative rSAA protein expression was expressed as AD units.

Electrofocusing

Aliquots of wild-type rSAA containing HDL, purified by flotation ultracentrifugation as described previously [42], were freeze-dried and delipidated with 0.5 ml of chloroform/methanol (2:1, v/v) [43]. The delipidated proteins were resuspended in a solution containing 7 M urea, 1% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. Samples were electrofocused on 0.3 mm polyacrylamide gels containing 7 M urea and an Ampholine gradient consisting of 20% (v/v) Ampholines, pH 3–10, 40% (v/v) Ampholines, pH 4–6.5, and 40% (v/v) Ampholines, pH 7–9. Samples were electrofocused in accordance with the protocol of Strachan et al. [42]. Samples on electrofocused gels were blotted on to 0.2 μ m nitrocellulose membranes [37]. rSAA isoforms present in refloated rSAA–HDL samples were identified with rabbit anti-(human ApoSAA) [36], followed by alkaline phosphatase-conjugated goat anti-(rabbit IgG). The chromogenic substrates for alkaline phosphatase, BCIP and Nitro Blue tetrazolium chloride were applied in accordance with the manufacturer's instructions [42,44].

ELISA for ApoSAA

Reagents for the assay of human SAA1 α by ELISA with a monoclonal antibody were kindly donated by Dr. P. Limburg (Department of Rheumatology, University of Groningen, Netherlands). The assay was performed as described previously [45]. The assay had a sensitivity of 1.1 ng/ml. Briefly, all samples were tested in a series of eight 2-fold dilutions. Only those samples

corresponding to rSAA concentrations falling on the accurate section of the standard curve (slope greater than 0.1) were deemed acceptable. The mean of these values was taken as the best estimate of rSAA concentration.

Calculation of wild-type rSAA half-life in serum-free medium and 10% FCS medium

Wild-type CHO cells were grown to confluence in T75 tissue-culture flasks containing selection medium with 10% FCS. Confluent layers were rinsed with fresh medium (either serum-free or with 10% FCS) and then incubated for a further 24 h in the presence of 10 ml of fresh medium, either with or without FCS, until the maximum rSAA concentration was reached. Aliquots (2 ml) of each 10 ml batch of medium were transferred to confluent layers of untransfected CHO cells for periods of up to 72 h. Five replicates of each experiment were performed although different time points were used in some replicates to increase the number of time points analysed. After each time point, the media were harvested and analysed by ELISA. The half-life of rSAA in each of these conditions was calculated by plotting the time against the natural logarithm of the rSAA concentration. Maximum Likelihood Program (MLP) software was used to plot the best-fit straight line and calculate the half-life (the slope of the line) and 95% confidence limits [46].

Affinity chromatography

Protein A-purified polyclonal or monoclonal anti-human ApoSAA antibody was coupled to CNBr-activated Sepharose beads in accordance with the manufacturer's protocol. Selection medium was passed over the column, unbound material was removed by washing with PBS and the bound material was eluted with 7 M urea containing 20 mM Tris/HCl, 1 mM EDTA and 500 mM NaCl, pH 8.4 (10 ml/h). The rSAA was dialysed against 15 mM NaCl, 0.1 mM EDTA, 2 mM Tris, pH 8.4, and then freeze-dried on an Edwards Modulo freeze drier until the volume was reduced 10-fold.

Purification of ApoSAA for N-terminal sequencing

Recombinant SAA protein was immunoprecipitated from selection medium by using polyclonal rabbit anti-(human ApoSAA) antibody [39] in accordance with the protocol previously outlined [47]. The immunoprecipitate was run on SDS/PAGE and transferred to a poly(vinylidene difluoride) membrane by electroblotting. The membrane was stained with Coomassie Brilliant Blue and the rSAA band was excised and used for solid-phase sequencing on an automated sequencer at the MRC Clinical Science Centre, Hammersmith Hospital (London, U.K.).

Amyloid fibril formation

Freeze-dried recombinant wild-type and mutant rSAA proteins were resuspended at room temperature in 10% (v/v) acetic acid at a concentration of 10 mg/ml for 24 h. The solutions were then neutralized with concentrated NH_4OH at room temperature for 24 h [11]. The samples were applied to glass slides, air-dried overnight, stained with Congo Red and examined for green birefringence under polarized light [12]. For examination under the electron microscope (E.M.) the samples were diluted 1:20 and negatively stained with 3% (w/v) phosphotungstic acid. Briefly, one drop of the protein solution was applied with a Pasteur pipette on to the surface of a carbonized Formvar-coated copper and rhodium grid (400 mesh per inch). After approx. 30 s, negative staining was performed with 3% phospho-

tungstic acid by holding the grid at an angle of 45° and allowing three or four droplets of the stain solution to fall across the grid surface. Residual liquid was drained from the grid on to Whatman 3MM filter paper. The preparation was then allowed to dry in air at room temperature. Samples were examined under a Philips CM120 E.M. at 80 kV; images were recorded on Kodak E.M. film 4489 at magnifications of $\times 70000$ – 187500 .

RESULTS

Characterization of wild-type rSAA protein in CHO cells

The N-terminal sequencing of wild-type rSAA showed that the first 56 residues from the N-terminus of the rSAA were identical with the human ApoSAA1 α sequence. Isoelectric focusing of the purified rSAA protein demonstrated the presence of two isoforms with pI values of 6.0 and 6.4. (Figure 1).

Calculation of wild-type rSAA half-life in serum-free medium and 10% FCS medium

The wild-type rSAA reached concentrations of approx. 30 $\mu\text{g/ml}$ after incubation with medium with 10% FCS. In serum-free medium the maximal concentration achieved was 4 $\mu\text{g/ml}$. For wild-type rSAA, the half-life in 10% FCS was 34.8 h (95% confidence limits 26.2–51.6 h) and in serum-free medium it was 8.5 h (95% confidence limits 7.5–9.9 h) (Figure 2).

Integration of the wild-type and mutant SAA1 α cDNA into the genome of CHO cells

Southern analysis of genomic DNA isolated from transfected and non-transfected CHO cells showed that wild-type and mutant cDNA plasmids were integrated within the genome of CHO cells

Figure 1 Isoelectric focusing and immunochemical analysis of wild-type rSAA protein

(a) Coomassie Blue staining of isoelectric focusing gel of delipidated and purified wild-type rSAA-HDL. Lane 1, 100 μg of human ApoSAA-HDL; lane 2, 100 μg of HDL-rSAA from wild-type CHO transfectant, showing two isoforms (pI values 6.4 and 6.0). (b) Immunochemical analysis of isoelectric focusing gel of delipidated and purified wild-type rSAA-HDL. The nitrocellulose blot of electrofocused delipidated wild-type rSAA protein isolated from culture media was identified by using rabbit anti-(human ApoSAA) antibody [39]. Lane 1, 100 μg of HDL-rSAA from CHO transfectants; lane 2, 100 μg of human ApoSAA-HDL, showing two isoforms (pI values 6.4 and 6.0).

Figure 2 Half-life of wild-type rSAA in the presence of CHO cells in different media

(a) Half-life of wild-type rSAA in 10% FCS medium. Culture medium containing rSAA and 10% FCS was harvested after 24 h and incubated with untransfected CHO cells for up to 72 h. rSAA was estimated from aliquots taken at 2, 6, 12, 24, 48 and 72 h by ELISA. The half-life of wild-type rSAA was calculated by plotting the time against the natural logarithm of the rSAA concentration. Maximum Likelihood Program (MLP) software was used to plot the best-fit straight line and calculate the half-life (the slope of the line) and 95% confidence limits of the results. (b) Half-life of wild-type rSAA in serum-free medium. Culture medium containing rSAA alone was harvested after 24 h and incubated with untransfected CHO cells for up to 24 h. rSAA was estimated from aliquots taken at 4, 8, 16 and 24 h by ELISA. The calculation of half-life was as in (a).

Figure 3 RFLP analysis of genomic DNA isolated from transfected CHO cells

Genomic DNA isolated from untransfected CHO cells and transfected CHO cells expressing wild-type and mutant rSAA were digested with *Hind*III to cut an internal site within the SAA1 α leader sequence, and thereby determine the copy number of the transfectants. Southern analysis was performed with [α - 32 P]dCTP-labelled pA1.mod cDNA and lambda *Hind*III DNA markers as described by Sambrook et al. [37]. Human genomic DNA was digested with *Bgl*II to confirm homologous binding of the probe pA1.mod as described by Betts et al. [5]. Lanes 1 and 7, lambda *Hind*III DNA markers; lane 2, human genomic DNA; lane 3, genomic DNA from transfectant wild type; lane 4, genomic DNA from transfectant mutSAA Δ 1–11; lane 5, genomic DNA from transfectant mutSAA(G8D); lane 6, genomic DNA from untransfected CHO.

at random positions (Figure 3). The genomic DNA from CHO cells containing wild-type rSAA cDNA showed up to three integration sites of the plasmid pSAACHO (*Hind*III RFLP fragments of 6.9, 6.0 and 5.5 kb). Genomic DNA from mutSAA(G8D) transfectant showed at least two integration sites of the plasmid pSAAmutAsp8 on the *Hind*III fragments (21 and 7.8 kb). Genomic DNA from mutSAA Δ 1–11 transfectant showed at least three integration sites of plasmid pSAAmut Δ 1–11 (7.8, 4.8 and 3.7 kb). The human genomic DNA control showed a *Bgl*II RFLP fragment of 4.2 kb carrying the SAA1 α gene as observed by Betts et al. [5]. This demonstrates the specific homologous binding of the probe pA1.mod to SAA1 α DNA sequence.

Figure 4 Northern analysis of rSAA mRNA levels in transfected cells

(a) Northern analysis of total RNA (20 μ g per lane) with a [α - 32 P]dCTP-radiolabelled *Hind* III/*Eco*RI fragment of pA1.mod cDNA and a human β -actin cDNA. Lane 1, RNA from wild-type transfectant; lanes 2 and 3, RNA from untransfected CHO cells; lane 4, RNA from transfectant mutSAA(G8D); lane 5, RNA from transfectant mutSAA Δ 1–11. (b) Relative amounts of transcripts were quantified by densitometric scanning and expressed as AD units.

Northern and reverse transcriptase-PCR analysis

Northern analysis and densitometry of RNA isolated from transfected CHO cells, mutSAA(G8D) (5.2 AD units) and mutSAA Δ 1–11 (8.7 AD units) showed a 1.3–2.2-fold increase in rSAA mRNA expression compared with wild-type rSAA mRNA (3.9 AD units) when normalized against human β -actin mRNA in two separate experiments (Figure 4). RNA isolated from untransfected CHO cells did not show any rSAA mRNA transcripts. Total RNA isolated from the transfected CHO cells

Figure 5 Immunodetection of ApoSAA in selection media of CHO transfectants

(a) Aliquots (10–20 μ l) of media containing 10% FCS isolated from 24 h cultures of transfected and untransfected cell cultures were subjected to Western analysis [40,41]. The blot was probed with rabbit anti-(human ApoSAA1) antibody. Lane 1, human ApoSAA protein; lane 2, wild-type transfectant; lanes 3 and 4, untransfected CHO; lane 5, mutSAA(G8D) transfectant; lane 6, mutSAA Δ 1–11 transfectant. (b) Relative rSAA protein expression was quantified by densitometric scanning and expressed as AD units.

expressing the wild-type and mutant rSAA transcripts were used as the template for random-primed oligonucleotide synthesis of single-stranded cDNA, which was then employed as the template for PCR with primers pwRT3 and pwRT5. Nucleotide sequencing of the PCR products confirmed the mutations introduced within the respective SAA cDNA (results not shown).

Western analysis

Immunoblots of selection media isolated from transfected CHO cells containing recombinant wild-type, mutSAA(G8D) and

Table 2 ELISA measurement of rSAA distribution in HDL medium with 0.1% (v/v) HDL after flotation ultracentrifugation

The mean values are derived from four separate experiments. Experimental results are expressed as means $\pm$ S.D. ($n = 4$).

Transfectant	Total rSAA in HDL medium (μ g)	rSAA in HDL fraction (μ g)	% of total rSAA in HDL fraction (range)	rSAA in infranant fraction (μ g)	% of total rSAA in infranant fraction (range)
Wild-type	247.7 $\pm$ 54.9	190.9 $\pm$ 24.5	69–82	56.9 $\pm$ 16.5	17–30
mutSAA(G8D)	53.3 $\pm$ 13.4	12.2 $\pm$ 4.4	23–32	38.2 $\pm$ 11.4	68–77
mutSAA Δ 1–11	51.8 $\pm$ 14.2	14.7 $\pm$ 1.0	20–40	37.1 $\pm$ 14.6	60–80

mutSAA Δ 1–11 proteins and untransfected CHO cells were analysed for the presence of rSAA protein by using rabbit anti-(human ApoSAA) antibody. Both mutant forms of rSAA protein were shown to have a molecular mass (as determined by electrophoretic mobility) of 14 kDa, which was identical with that of the wild-type rSAA. This band also co-migrated with the ApoSAA isolated from human sera (Figure 5). Densitometry of the Western blot showed that mutants mutSAA Δ 1–11 (0.8 AD units) and mutSAA(G8D) (2.5 AD units) contained one-seventh to one-tenth of the protein in the wild type (9.5 AD units).

Table 3 ELISA measurement rSAA in lysates of CHO transfectants

Cells (10^7) were lysed in 1 ml of lysing solution containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA and 1 mM PMSF, and assayed for rSAA by ELISA in duplicate experiments. Experimental results are expressed as means $\pm$ S.D. ($n = 4$) for four separate experiments.

Transfectant	rSAA (μ g per 10^7 cells)	
	Lysates of transfectants in medium with 0.1% HDL	Lysates of transfectants in medium with 10% FCS
Wild-type rSAA	11.1 $\pm$ 4.5	10.9 $\pm$ 0.2
mutSAA(G8D)	2.5 $\pm$ 1.2	6.4 $\pm$ 2.3
mutSAA Δ 1–11	3.7 $\pm$ 1.9	5.1 $\pm$ 0.9

Table 1 ELISA measurements of rSAA distribution in selection media with 10% (v/v) FCS after flotation ultracentrifugation

The mean values are derived from four separate experiments. Experimental results are expressed as means $\pm$ S.D. ($n = 4$).

Transfectant	Total rSAA in FCS medium (μ g)	rSAA in HDL fraction (μ g)	% of total rSAA in HDL fraction (range)	rSAA in VLDL/LDL fraction (μ g)	% of total rSAA in VLDL/LDL fraction (range)	rSAA in infranant fraction (μ g)	% of total rSAA in infranant fraction (range)
Wild-type	31.9 $\pm$ 5.3	18.0 $\pm$ 2.1	52–64	1.3 $\pm$ 0.3	3.0–5.4	12.6 $\pm$ 3.7	30–44
mutSAA(G8D)	11.0 $\pm$ 0.4	3.9 $\pm$ 0.6	28–40	0.4 $\pm$ 0.1	2.7–5.5	6.8 $\pm$ 1.1	54–68
mutSAA Δ 1–11	11.9 $\pm$ 0.4	3.8 $\pm$ 0.7	25–38	0.6 $\pm$ 0.2	3.5–6.3	7.5 $\pm$ 0.8	57–71

Figure 6 Light and E.M. pictures of rSAA proteins isolated from wild-type and mutant CHO transfectants expressing ApoSAA1 cDNA

Recombinant wild-type and mutant rSAA proteins were resuspended at room temperature in 10% acetic acid at a concentration of 10 mg/ml for 24 h. The solutions were then neutralized with concentrated NH_4OH at room temperature for 24 h [11]. The samples were either stained with Congo Red and examined for green birefringence under polarized light, or examined under the E.M. after negative staining with phosphotungstic acid. The arrow indicates the region of protein stained with Congo Red which changes to apple green when viewed under polarized light. Magnification $\times 8.4$. **(a)** Wild-type rSAA protein observed by light microscopy. Wild-type rSAA protein was stained with Congo Red to reveal the presence of green birefringence characteristic of amyloid when viewed under the light microscope using polarized light (magnification $\times 8.4$). **(b)** Wild-type rSAA protein observed by electron microscopy. Negative staining of wild-type rSAA protein reveals amyloid fibrils when viewed under the E.M. **(c)** Mutant mutSAA $\Delta 1-11$ protein observed by electron microscopy. Negative staining of mutSAA $\Delta 1-11$ protein reveals the presence of numerous microaggregates when viewed under the E.M. **(d)** Mutant mutSAA(G8D) protein observed by electron microscopy. Negative staining of mutSAA(G8D) protein reveals the presence of numerous microaggregates when viewed under the E.M.

Analysis of rSAA distribution in the different compartments of selection and HDL media

Selection media (containing 10% FCS) and serum-free selection media with 0.1% (v/v) HDL (HDL media) were collected from wild-type and mutant transfectants. The VLDL/LDL and HDL fractions were separately isolated from selection media by using flotation ultracentrifugation. ApoSAA ELISA was used to measure the total amount of rSAA protein in these fractions. Tables 1 and 2 show the distribution of wild-type and mutant rSAA in different fractions of selection media containing FCS or HDL. Approx. 52–64% of rSAA wild-type protein was harvested as an rSAA–HDL complex in the HDL part of FCS when using selection media (Table 1) compared with 69–82% when using HDL media, suggesting that the wild-type protein associates with HDL at high affinity. The remaining wild-type rSAA protein was found in the infranatant (17–44%) and VLDL/LDL (3.0–5.4%) fractions respectively (Tables 1 and 2). Only 28–40% of both types of mutant rSAA proteins was harvested as an rSAA–HDL complex in the HDL fraction when using FCS or HDL media. The bulk of mutant proteins were found to be present in the infranatant fractions (54–80%) and a small proportion in the VLDL/LDL (2.7–6.4%) (Tables 1 and 2). Thus the ratio of rSAA in the HDL and infranatant fractions was reversed for the mutants when compared to the wild-type rSAA, but binding to VLDL/LDL was low and unchanged. The total yield of mutant rSAA proteins decreased to 40% when using FCS media and to 20% when using HDL media when compared with wild-type total yield. Table 3 shows that the expression of mutated rSAA protein levels in whole-cell lysates from mutant cells grown in either FCS or HDL media decreased to between one-half and one-quarter compared with the wild-type cell lysate. Thus less protein was found in the cells containing the mutant cDNAs in spite of higher mRNA levels than in the wild type (Figure 4).

Amyloid fibril formation and Congo Red staining

After treatment with acetic acid (described in the Materials and methods section) wild-type rSAA protein stained positive with Congo Red and showed green birefringence when viewed under polarized light (Figure 6a). Further fibrils were detected by viewing under the E.M. (Figure 6b). These fibrils ranged from 3 to 7 nm in width. MutSAA(G8D) and the mutSAAΔ1–11 proteins did not stain with Congo Red and lacked the characteristic green birefringence of the wild-type proteins. Under the same E.M. conditions, both mutants showed spherical non-fibrillar precipitates with a diameter range of 16–27 nm (Figures 6c and 6d). Media isolated from untransfected CHO cells when viewed under the E.M showed clear areas with occasional stained areas of debris.

DISCUSSION

Previous workers have described the expression of various ApoSAA alleles in *E. coli* [20] and the baculovirus system [48]. The bacterial expression system has produced high yields of ApoSAA protein (5–10 mg/l), but cell lysis and plasmid loss due to toxicity of the product was found. In addition, post-translational processing of the N-terminal arginine residue was not demonstrated in the bacterial system. The baculovirus system proved unsatisfactory for the expression of mouse ApoSAA2 in the pBluebac system because a mixture of correctly and incorrectly processed peptides was expressed and not secreted into culture media.

In the present study we have demonstrated the expression of

wild-type and mutant rSAA cDNAs in CHO cells as stable transfectants with the production of 3.5–30 mg/l wild-type rSAA and 3–10 mg/l mutant rSAA protein. These expression levels are comparable to those of other human genes expressed in mammalian cells by using the human cytomegalovirus promoter [49,50]. Protein sequencing and characterization of the isoelectric point of the wild-type rSAA protein showed that there were two products with pI values of 6.0 and 6.4, corresponding to the human ApoSAA1 isoforms ApoSAA1 α and ApoSAA1 α desArg found in acute-phase sera. This indicates that the correct processing of the leader sequence of the human SAA1 α cDNA and post-translational processing have been achieved in our expression system. Between 52% and 64% of the total yield of wild-type rSAA1 protein was isolated as an rSAA–HDL complex. In contrast, when Forte et al. [51] expressed ApoAI in CHO cells, only 16% of the secreted ApoAI associated with the HDL fraction of FCS. Therefore ApoSAA probably has a higher affinity for HDL than ApoAI.

Only 28–40% of the total yield of mutant rSAA proteins was found in the HDL fraction, 58–80% was predominantly in the infranatant, and 2.7–6.3% was in the VLDL fraction. Thus both mutant rSAAs bind less well to HDL. The deletion of 11 N-terminal residues or the substitution of one amino acid residue, changing the charge and disrupting the tertiary structure of rSAA, result in the significant decrease in the lipid-binding property of mutant rSAA proteins. Both mutants have two residual amphipathic helices and are still partly able to form a lipid complex with HDL. This would account for the approx. 30% of mutant rSAA protein found complexed to the HDL (Table 2).

The decrease in the total yield of mutant rSAA proteins compared with the wild type could be explained by a lower affinity for HDL. We have shown that free rSAA is degraded faster than rSAA bound to HDL in FCS, as the half-life of rSAA was markedly reduced to a few hours when FCS was omitted from the culture medium: therefore HDL protects ApoSAA from proteolytic enzymes. Thus we have confirmed the report of Skogen et al. [52], who demonstrated that ApoSAA was protected against degradation from serine proteases when bound to HDL. Because the N-terminal mutant proteins have an impaired ability to bind to HDL, a higher rate of proteolytic degradation is likely. This might explain the lower levels of the mutant rSAA protein in the culture medium shown in the immunoblot (Figure 5) and the ELISA (Table 2). Other explanations could be that the mutant mRNA species are less stable, or translation and secretion of the mutant proteins are less efficient, because mRNA levels are the same as in the wild type but the cytosolic concentration of mutant proteins is lower than that of wild-type rSAA.

Because AA peptides of the fibrils have variable C-termini but all retain the N-terminal region [8], this region might contribute to the amyloidogenic properties of the protein. The positive staining of wild-type rSAA protein with Congo Red and the formation of fibrils *in vitro*, shown under light and electron microscopy in Figures 6(a) and 6(b), demonstrate that the wild-type rSAA behaves like the native human ApoSAA1 α [13]. These fibrillar structures were absent when the mutSAAΔ1–11 and mutSAA(G8D) proteins were examined under the same conditions. Thus our results corroborate the findings of other workers showing the importance of the N-terminus [10,11]. The N-terminal region is also important for the interaction of AA dimers to form a lattice of AA molecules capable of binding to Congo Red, a model proposed by Turnell et al. [14], who furthermore predicted that mutation at Gly-8 would disrupt fibril formation. It is interesting that this is the only difference between mouse non-fibril-forming ApoSAA1 (His-8) and mouse

fibril-forming ApoSAA2 (Gly-8), and Gly-8 is present in the N-terminus of the human acute-phase ApoSAAs [15]. Our E.M. examination showed that mutSAA(G8D) protein failed to form AA fibrils *in vitro*, demonstrating the importance of this Gly-8 residue in amyloidogenesis. No N-terminal variation has been observed between the different human ApoSAA1 and ApoSAA2 species or their alleles, indicating that other factors must be involved in amyloidogenicity. The deposition of ApoSAA1 might be the result of its being the major isoform during the acute-phase response because it has been demonstrated experimentally in human serum and amyloid fibrils [19,53]. Thus the amyloidogenicity might be directly attributable to the regulation of ApoSAA genes during the acute phase. Of interest is the fact that the cSAA4 protein does not have a glycine residue at position 8 [7], and human recombinant cSAA4 does not form amyloid fibrils *in vitro* [20]. Our findings suggest that the primary and secondary structures of ApoSAA proteins are a few of the several factors contributing to reactive amyloidosis.

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