Identification of Novel Members of the Serum Amyloid A Protein Superfamily as Constitutive Apolipoproteins of High Density Lipoprotein*


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A novel serum amyloid A protein (SAA) has been identified as a normal apolipoprotein component of non-acute phase high density lipoprotein. This novel SAA has been designated "constitutive" SAA (C-SAA) to distinguish it from "acute phase" SAA (A-SAA). C-SAA was partially sequenced, and immunochemical analyses indicated that it constitutes a distinct subclass of apolipoproteins within the SAA superfamily. A C-SAA cDNA clone was isolated from a human liver library and sequenced. The clone predicts a pre-C-SAA molecule of 130 residues from which an 18-residue leader peptide is cleaved. The 112-residue mature molecule is 8 residues longer than human A-SAA; the size difference is due to the presence of an octapeptide between positions 70 and 77 that is not found in the corresponding region of human A-SAA. Paradoxically, octapeptides of similar composition are found at similar positions in the A-SAAs of a number of other species. The C-SAA octapeptide specifies the first two residues of a NSS tripeptide, the only potential glycosylation site in the molecule. Studies indicate that approximately 50% of these sites are glycosylated, thereby giving rise to two size classes, 14 and 19 kDa, of C-SAA in vivo. Human acute phase liver contains little C-SAA mRNA relative to the levels of A-SAA mRNA, and the treatment of PLC/PRF/5 hepatoma cells with monocyte-conditioned medium does not induce C-SAA mRNA concentrations to detectable levels, in contrast to the massive induction of A-SAA mRNA observed. C-SAA is therefore not a major acute phase reactant.

The acute phase response encompasses a spectrum of physiological changes as a consequence of an inflammatory stimulus (reviewed in Ref. 1). Among the many systemic manifestations is a dramatic increase in the circulating concentration of a number of serum proteins (known as acute phase reactants, APRs) during the 24-48 h post-stimulus. In all mammalian species studies to date, serum amyloid A protein (SAA) is a major APR in that the magnitude of its induction can be as high as a 1000-fold (2, 3). The dramatic nature of its induction suggests that SAA plays an important role in host defense during inflammation; the precise nature of its function has not, however, been determined.

SAA is a small apolipoprotein (104 amino acids in human and, like most other APRs, is synthesized principally by the liver. During the acute phase response it associates with high density lipoprotein (HDL), in particular HDL, on which it can become the predominant apolipoprotein, exceeding apolipoprotein A-I (apoA-I) in molar ratio (6). An occasional consequence of chronic inflammation is secondary amyloidosis, a progressive, fatal condition in which insoluble deposits, composed predominantly of amyloid A, occur in the major organs (reviewed in Ref. 7). Amyloid A is derived from SAA by a putative proteolytic cleavage event (8) that generates a 76-residue amino-terminal fragment with a β-sheet conformation that determines the fibrillar nature of the amyloid deposits.

SAA is the product of multiple genes in several species. In humans, two acute phase SAA (A-SAA) genes have been described, both of which are allelic (9-12). Recently, however, Steinkasserer et al. (13) have isolated five distinct SAA cDNAs from a library constructed using hepatic mRNA from a single individual, thereby establishing that there are at least three transcribed human genes. Our own studies (14) have defined five distinct SAA cDNAs in a dog acute phase liver library indicating that there are at least three transcribed canine genes. Three genes, SAA1, SAA2, and SAA3, are transcribed in the mouse (15), the first two giving rise to dramatically elevated levels of hepatic mRNA and circulating protein during inflammation and the last giving rise to moderate hepatic mRNA induction but no detectable translated product. In addition, a number of SAA-like genes and products has been described in a number of species although these are as yet ill defined. A human SAA-like gene that could encode...
a protein with 80% identity to A-SAA has been reported by Sack and Talbot (16); however, no mRNA or protein product corresponding to this gene have been identified. Brinckerhoff and co-workers (17) have described a molecule that is a product of rabbit fibroblasts and appears to act as an autocrine collagenase inducer that shares 78% identity with human A-SAA. In the mouse, Ramadori et al. (18) have identified several putative SAA-like molecules electrophoretically.

In this report, we define a novel member of the SAA superfamily. This molecule has 55% identity with A-SAA and appears both as mRNA and mature apolipoprotein on HDL. It is unique among SAA molecules in that its amino acid sequence contains a potential N-linked glycosylation site that is occupied by a carbohydrate moiety in about half of the mature molecules that result in an apparent M, 5000 increase on SDS-PAGE. In addition, it is expressed constitutively on normal HDL. We have therefore named this class of SAA molecules constitutive SAA (C-SAA). The significance of the structure and expression of C-SAA is discussed in terms of the evolution and possible function of the various members of the SAA superfamily.

MATERIALS AND METHODS

Preparation of HDL—Blood was obtained with informed consent from healthy individuals and patients in acute phase. HDL was isolated from plasma essentially as described (6, 19). Briefly, plasma density was adjusted to 1.09 g/ml with solid KBr and centrifuged for 5.3 h at 55,000 rpm (VTi80 rotor, Beckman Instruments, Palo Alto, CA) at 10°C. The density of the infranatants, which contained the HDL, was adjusted to 1.21 g/ml with solid KBr and recentrifuged for 9.4 h under the same conditions. The pellicles containing HDL were extensively dialyzed against 0.15 M NaCl, 0.1% (w/v) EDTA, pH 7.4.

Electrofocusing—Aliquots of HDL were lysophilized and delipidated with 0.5 ml of chloroform:methanol (2:1, v/v) (20). The delipidated proteins were resuspended in 7 M urea, 1% (v/v) decyl sodium sulfate (Eastman Kodak Co., Rochester, NY), 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 (Pharmacia LKB Biotechnology, Inc.). Electrofocused gels were fixed and stained with Coomassie Brilliant Blue (19).

Immunochemical Analysis—The SAA isoform distribution in HDL samples was determined by immunochemical analysis. Samples on electrofocused gels were not fixed and stained but were immediately pressure-blotted onto 0.2-μm nitrocellulose membranes (Schleicher and Schuell) for 20 h at room temperature (19). The membranes were wetted with 25 mM Tris-HCl, pH 8.3, 122 mM MgCl₂, and 15% (v/v) methanol prior to blotting. Following pressure blotting, the nonspecific binding sites on the membranes were blocked by overnight incubation at 4°C with 5% (v/v) nonfat dry milk in phosphate-buffered saline containing 2% (w/v) bovine serum albumin. A variety of antibodies was used to detect SAA isoforms with an alkaline phosphatase-conjugated goat anti-rabbit IgG (A8025, lot 50H 8878, Sigma) as the secondary detection reagent. The immunochromatographic strips were electroblotted onto nitrocellulose and the 14- and 19-kDa species were excised for sequencing (26).

In Situ Trypsin Digestion—For the in situ trypsin digestion of the C-SAA molecules, 5.0 mg of HDL from normal individuals was electrofocused in aliquots of 300 μg. The pl 7.9 and 8.1 isoforms were electroblotted onto nitrocellulose and the 14- and 19-kDa species subjected to enzymatic degradation as described (26, 27), omitting the NaOH wash to minimize protein loss. Peptides were separated by narrow-bore reverse phase HPLC on a Hewlett-Packard 1090 using a Vydac 2.1 × 150-mm C18 column. The gradient employed was essentially that described by Stone et al. (27). Absorbance was monitored at 210 nm and UV-absorbing peaks collected for sequencing.

Amino Acid Sequence Analysis—Tryptic fragments for amino acid sequence analysis were applied to a Polybrene precoated glass fiber filter in an ABI model 477A protein sequenator; C-SAA samples electroblotted onto polyvinylidene fluoride were likewise applied to an ABI model 477A protein sequenator. The resultant phenylthiohydantoin amino acids were manually identified using an on-line ABI model 120A HPLC and Shimadzu CR4A integrator.

Human cDNA Synthesis and Library Construction—A human acute phase liver cDNA library was constructed essentially by the method of Caput et al. (28), with modifications as previously described (29). The cDNA was synthesized HuSAA, a 30-mer (5'TCTCATGTCAGAGTAGGCGTTTCCAAATAAATAGTAGTCTAT-3') complementary to the amino-terminal sequence (ESWRSFFKEALQGVG) of A-SAA and HCSins, a 36-mer (5'TACAGTGCTGTCATTTTTCAAGGAGGCATTACAGGGAGTCGGA-3') corresponding to the amino-terminal sequence (PSWRSFFKEALQGVG) of C-SAA protein was synthesized.

Sequence Analysis—The insert of CS1 was directly sequenced using oligonucleotide primers and a Sequenase kit (U. S. Biochemical Corp.) via the dye-decor chain termination method of Sanger et al. (31), as modified by the manufacturer for sequencing from double-stranded templates.

Oligonucleotides—All oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. For screening the cDNA library, a best guess oligonucleotide KHSAA2 (5'GAAAGCTGGCGTTACATTTCTCAAGGAGGCATTACAGGGAGTCGGA-3') complementary to the amino-terminal sequence (PSWRSFFKEALQGVG) of C-SAA protein was synthesized.

After generation of sequence corresponding to the 3'-untranslated region using the universal sequencing primer, a total of seven 18-base-long oligonucleotides was synthesized for use as directed sequencing primers. The entire antisense strand and 90% of the sense strand (which is preceded by a stretch of oligo G generated as a result of the cloning method used) was sequenced.

To analyze A-SAA and C-SAA mRNA levels in the PLC/PRF/5 human hepatoma cell line RNA samples two specific oligonucleotides were synthesized: HuSAA, a 30-mer (5'-TCTTGATGTCAGAGTAGGCGTTTCCAAATAAATAGTAGTCTAT-3') complementary to the mRNA sequence encoding residues 16–25 of A-SAA and HCSins, a 36-mer (5'-TACAGTGCTGTCATTTTTCAAGGAGGCATTACAGGGAGTCGGA-3') complementary to the structure and expression of C-SAA is discussed in terms of the evolution and possible function of the various members of the SAA superfamily.

A Novel Constitutive SAA
mRNA sequence encoding residues 69-80 of C-SAA.

Cell Culture and Induction of Acute Phase Reactants—Human PLC/PRF/5 hepatoma cells were maintained in a 5% CO₂ atmosphere at 37 °C in Eagle’s minimal essential medium (MA Bioproducts, Walkersville, MD) supplemented with bovine calf serum (HyClone, Logan, UT), 0.01 mM sodium pyruvate, 0.01 M Hepes, 50 μg/ml gentamycin (MA Bioproducts), and 0.01 mM nonessential amino acids (Sigma).

Mononuclear cells were purified from whole human blood using a Ficoll-Paque gradient (Pharmacia LKB Biotechnology Inc.). Cells were resuspended in Eagle’s minimal essential medium supplemented with 10% (v/v) autologous human serum and allowed to adhere to 100-mm tissue culture dishes for 5 h at 37 °C. Nonadherent cells were removed and the adherent cells washed 3 times with Hanks’ buffered salt solution (MA Bioproducts). Cells were then incubated with 10 ml per dish of Eagle’s minimal essential medium supplemented with gentamycin (MA Bioproducts), and 0.01 mM nonessential amino acids salt solution (MA Bioproducts). Cells were then incubated with 10% PRF/5 cells were harvested at timed intervals for isolation of RNA.

RESULTS AND DISCUSSION

During the acute phase response, the cytokine-induced hepatic synthesis of A-SAA (33) is followed by secretion and a rapid association with HDL particles on which it may become the major apolipoprotein, exceeding the molar quantity of apoA-I (4, 5). A-SAA incorporation into existing normal HDL particles results in a remodeling of their surfaces to yield larger particles with higher hydrated densities and less apolipoprotein (6). The plasma clearance for A-SAA from acute phase HDL is more rapid than that of any of the other HDL apolipoproteins suggesting either that A-SAA is associated with a HDL subpopulation that turns over more rapidly or that A-SAA dissociates from HDL before clearance (3, 34).

We have identified a class of novel SAA molecules (C-SAA) that, unlike A-SAA, is the major form of these apolipoproteins present on normal HDL₃. Based on limited amino acid sequencing and by immunodetection with some, but not all, antisera directed against epitopes on A-SAA, these constitute a distinct subgroup within the SAA superfamily.

C-SAAs were initially discovered when electrophoresed normal and acute phase HDL were immunoblotted with a polyvalent rabbit anti-human A-SAA antibody (Fig. 1A). Two pools of normal HDL₃ surprisingly showed predominant staining of basic bands (pI 8.1, 7.9, and 7.3) even when staining of the major A-SAA isoform pair (pI 6.4 and 6.0) is, as expected in these normal samples, hardly detectable (Fig. 1A, lanes 1 and 2). These immunoreactive basic bands are clearly visible in a Coomassie stain of electrophoresed normal HDL (Fig. 1B, lanes 1 and 2). In contrast, when two acute phase HDL₃ samples were immunoblotted with the same antibody (Fig. 1A, lanes 3 and 4) staining of the expected three isoform pairs of A-SAA was observed. These are the primary products and post-translational modifications of three SAA genes, two being allelic variants at a single locus (35). When a monoclonal antibody specific for A-SAA or rabbit antiserum raised against synthetic peptides 58-69 and 95-104 of human A-SAA was used, the immunoblots of the two acute phase HDL₃ samples were identical to that obtained with the polyvalent rabbit anti-human A-SAA (Fig. 1A, lanes 3 and 4). However, the two normal HDL₃ pools showed only very weak staining of the predominant pI 6.0/6.4 isoform pair with the basic bands (pI 8.1, 7.9, and 7.3) undetectable (Fig. 1A, lanes 1 and 2).

Two-dimensional SDS-PAGE of the C-SAA isoforms showed that they exist as two molecular weight species (Fig. 2): 14 kDa derived from the pI 8.1 and 7.9 bands, and 19 kDa derived from the pI 7.9 and 7.3 bands.

The C-SAA isoforms (14- and 19-kDa species) were electroblotted onto polyvinylidene fluoride membranes and the
two species of 19 and 14 kDa molecular weight standards with molecular sizes as indicated. SAA. C-SAA with PI 8.1 represents a single species with a molecular SAA.

man novel C-SAA. The nucleotide sequence of the insert of clone sequence The residues not identified by direct protein sequencing of C-SAA the tryptic peptide present in the chromatograms of the 14-kDa to those comprising the additional octapeptide present in the C-SAA in the the amino-terminal sequence determined by standard procedures (25, 25). All had identical amino-terminal sequences throughout the first eight cycles of Edman degradation (Fig. 3). This confirmed that the C-SAA molecules were related to, but distinct from, A-SAA. An additional amino acid sequence was obtained from peptides generated by in situ trypsin digestion of the C-SAA molecules electrobotted onto nitrocellulose and separated by narrow bore reverse phase HPLC (Fig. 3). The chromatograms of the tryptic digest of the 14-kDa pl 8.1 and 7.9 molecules were identical (data not shown) and contained a peptide peak not present in the chromatogram of the tryptic digest of the 19-kDa pl 7.9 molecule. This peptide peak had the sequence VYLQLGIDYYLFGNSSTVLED which contained a NSS tripeptide, a putative glycosylation site which could explain the size difference between the C-SAA molecules. We propose that the glycosylation of this peptide (see below) alters its hydrophobicity precluding detection in the chromatogram of the tryptic digest. To determine whether C-SAA is glycosylated, we stained SDS-PAGE gels carrying C-SAA with the carbohydrate-specific detection agent periodic acid-Schiff (Fig. 4A). The 19-kDa form of C-SAA (track 3) is positively stained, whereas the 14-kDa form (track 4) is not. Little staining is apparent in the normal HDL sample (track 2). The positive control transferrin (track 5) is stained; the negative control bovine serum albumin (track 1) is not. Overstaining of the same SDS-PAGE gel with Coomassie (Fig. 4B) confirmed that equivalent amounts of the 19- and 14-kDa C-SAA species had been analyzed and established that the 14-kDa species was not detected using the carbohydrate-specific reagent and is therefore unglycosylated. The carbohydrate carried by the 19-kDa C-SAA species is N-linked was confirmed by treatment with endoglycosidase F which removes only N-linked carbohydrate moieties and effects a reduction in size of the 19-kDa band (Fig. 5, track 2) to 14 kDa (Fig. 5, track 1). This is the first report of a member of the SAA superfamily that is glycosylated.

An acute phase human liver cDNA library was screened using a best guess oligonucleotide corresponding to the amino-terminal 15 residues of the pl 8.1 C-SAA species. A full-length clone, CS1 was isolated and analyzed (Fig. 3). CS1 contains 614 nucleotides specifying 75 residues of mRNA 5'-untranslated region and 146 residues of 3'-untranslated region bounding 390 residues of coding sequence and a stop codon. The coding sequence predicts a C-SAA premolecule of 130 amino acids. Computer analysis (using the PSIGNAL program) offers alternative sites for the cleavage of a leader peptide from the mature molecule between residues Leu-15 and Val-16, and between residues Leu-18 and Glu-19. Although both sites conform to the "-3, -1 rule" governing the cleavage of leader
peptides (36), the identification of the amino-terminal residue of mature C-SAA as glutamic acid confirms the latter putative cleavage site as that which is used in vivo. CS1, therefore, predicts a mature C-SAA molecule of 112 amino acids. This is 8 residues longer than intact A-SAA and 9 residues longer than the modified des-Arg form of A-SAA generated by removal of the amino-terminal arginine. The alignment of the predicted CS1 protein sequence with that of an A-SAA (Fig. 3) indicates that the size difference is due to the presence of an additional 8-amino acid peptide (residues 70-77) in the C-SAA sequence “inserted” relative to the A-SAA sequence between residues 69 and 70. This octapeptide is, therefore, in the same position as the corresponding peptides found in the A-SAA of dog (13, 38), cat (38), horse (39), cow (40), and mink (41) that render the A-SAA superfamily members of these species 8 (9 in the case of cow) residues larger than the A-SAA of mouse (14), rabbit (42), and human (9-11). There is, therefore, an evolutionary paradox regarding the presence/absence of the octapeptide and the acute phase/nonacute phase nature of the C-SAA molecules in the different species. The relatedness between the octapeptide in human C-SAA and in the A-SAA of other species is confirmed by sequence similarities. All of the octapeptides are bounded by an aminoterminal aspartic acid and a carboxyl-terminal serine; in addition, the similarity often extends to other residues, for example, the human novel SAA octapeptide DYYLFGNS; shares four identities with the dog A-SAA octapeptide DLLRFGD. The C-SAA octapeptide contains the first two residues of a NSS tripeptide that constitutes the N-linked glycosylation site in the molecule.

The essentially constant low levels of C-SAA associated with normal HDL strongly suggest that the expression of C-SAA is constitutive. The cloning of C-SAA provided the means to determine the level of C-SAA mRNA in human acute phase liver and in human hepatoma cells given an in vitro inflammatory stimulus. Oligonucleotide probes specific for C-SAA mRNA and for A-SAA mRNA were synthesized and used in the Northern blot analysis of total RNA extracted from human acute phase liver and from human PLC/PRF/5 hepatoma cells harvested at timed intervals following treatment with monocyte-conditioned medium (Fig. 6). Acute phase liver RNA contains very high concentrations of A-SAA mRNA (Fig. 6, panel A, track 1) but contains very low concentrations of C-SAA mRNA (Fig. 6, panel B, track 1). The latter could only be detected by very long autoradiographic exposure of the Northern blot despite the C-SAA oligonucleotide being labeled to a similar specific activity as the A-SAA oligonucleotide and despite these oligonucleotides giving signals of similar intensity when hybridized to C-SAA- and A-SAA-specific DNAs, respectively, in Southern blot analyses (data not shown). We, therefore, conclude that C-SAA mRNA (and by implication C-SAA protein) is not a major product of acute phase liver. This finding was not unexpected given the small number of clones isolated from the cDNA library which was constructed using RNA from the same source. Although it is possible that C-SAA is the product, for example, of Kupffer cells, we consider it likely to be synthesized by hepatocytes which are the known source of A-SAA in addition to many other APRs and apolipoproteins.

Accordingly, we stimulated cultures of the human hepatoma cell line PLC/PRF/5, which we have previously shown to synthesize a range of APRs (43), with monocyte-conditioned medium to produce an in vitro acute phase response. Northern blot analysis of RNA extracted from these cells at various time points poststimulus revealed the appearance of A-SAA mRNA at 6 h. The A-SAA mRNA concentration peaks at 24 h and is reduced by 48 h at which time point the mRNA size is smaller due to the well characterized reduction in polyadenylation that has been demonstrated in mouse (44, 45), dog (13), and human between A-SAA mRNA present immediately postinduction and A-SAA mRNA present at later times (Fig. 6, panel A, tracks 2-6). In contrast, C-SAA mRNA is absent or is below the level of detection in Northern blot analysis of the same RNA samples (Fig. 6, panel B, tracks 2-6). We, therefore, conclude that C-SAA does not behave as a major APR in stimulated PLC/PRF/5 hepatoma cells and that its presence in normal HDL likely reflects a constitutive functional requirement for this apolipoprotein.

Elevated HDL levels correlate inversely with susceptibility to atherosclerosis (46). HDL is central to the process of reverse cholesterol transport, and there is a significant decrease in plasma HDL cholesterol during inflammation (6). It is likely that during inflammation A-SAA association with HDL modifies the particle and equips it for a protective host defense role for which there is an overriding short term requirement. We would further speculate that C-SAA on normal HDL contributes to its normal physiological role in reverse cholesterol transport. The chronic persistence of A-SAA on HDL in chronic inflammatory diseases could compromise the function of HDL over significant periods of time. Together with the concomitant sustained decrease in total HDL this would constitute a major risk factor for the development of atherosclerosis and could provide a molecular explanation for the increased mortality from cardiovascular disease observed in patients with active systemic rheumatoid arthritis (47). A thorough examination of the structure, expression, and molecular genetics of all of the members of the SAA superfamily is likely, therefore, to be of considerable clinical, as well as biological, importance.

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REFERENCES

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