Ribozyme mediated cleavage of acute phase serum amyloid A (A-SAA) mRNA in vitro

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Abstract The 1000-fold induction of acute phase serum amyloid A (A-SAA) in the liver during inflammation indicates that this protein plays an important, though ill-defined, role in host defence. Paradoxically, prolonged overproduction of A-SAA is a causative factor in secondary amyloidosis and possibly other diseases such as atherosclerosis; the ability to down-regulate A-SAA synthesis is therefore of considerable clinical importance. We have successfully generated anti-SAA hammerhead ribozymes and we report that they are capable of cleaving A-SAA mRNA in vitro.

Key words: Acute phase response; Hammerhead ribozyme; Inflammation; Serum amyloid A

1. Introduction

The serum amyloid A (SAA) protein family is comprised of a number of differentially expressed polymorphic apolipoproteins which are associated predominantly with HDL3 [1,2]. During the acute phase response a subset of the SAA's, the acute phase SAA's (A-SAA's), increase dramatically in concentration [3]. There are two acute phase genes, *A-SAA1* and *A-SAA2*, the products of which are 92% identical at the amino acid level. The association of A-SAA with HDL3 displaces apolipoprotein A1 from the HDL particle and remodels it for an ill-defined short-term role in host defense [4,5]. However, in the long-term, high sustained levels of A-SAA may have detrimental effects. The association of A-SAA with HDL3 may comprimise its capacity to mediate reverse cholesterol transport [6] and may contribute to the pathogenesis of atherosclerosis in patients with chronic inflammatory diseases [7]. Also, the principal component of amyloid deposits formed during secondary amyloidosis, a progressive fatal disease, is amyloid A, a proteolytic derivative of A-SAA [8], the generation of which appears to be dependent, at least in part, on the maintenance of high circulating levels of A-SAA.

The capacity to down-regulate A-SAA synthesis is potentially of great benefit when the detrimental clinical consequences of prolonged over-production of A-SAA are considered. There are several possible approaches to the therapeutic down-regulation of A-SAA, one of which is to interfere with the inductive pathway(s) of A-SAA biosynthesis mediated by inflammatory cytokines and hormones such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and by glucocorticoids [9,10,11]. Reagents that inhibit the action of cytokines, however, act non-specifically and could abrogate important and beneficial general immune functions if used over long periods of time. A more specific down-regulation could be achieved using antisense RNA or DNA molecules, which bind to the target mRNA and inhibit its translation. However, high levels of antisense RNA relative to target mRNA would likely be required and given the magnitude of A-SAA synthesis during inflammation this poses practical limitations. Hammerhead ribozymes (or RNA enzymes) are potentially a more effective alternative to antisense RNA in that, in addition to binding to the target RNA specifically, they cleave the RNA, dissociate, and participate in further cleavages in a catalytic manner. Originally identified as *cis*-acting (self-cleaving) RNAs in plant viruses, they have subsequently been exploited for their ability to effect cleavage *in trans* [12]. So-called because they adopt a secondary structure that resembles a hammerhead on binding to the substrate, they can be designed to base-pair with their complementary target mRNA on either side of a GUX (X = A, C or U) triplet, and cleave the target immediately 3′ to the triplet in a magnesium-dependent, protein-independent manner. In this paper we report the design, synthesis and function of several hammerhead ribozymes that give specific and efficient cleavage of full-length A-SAA mRNA in vitro.

2. Materials and methods

2.1. Computer-assisted prediction of RNA secondary structure

Prediction of A-SAA mRNA secondary structure was carried out by using the RNAPlotFold program (based on the algorithm of Zuker and Stiegler [13]) of the GCG package.

2.2. Substrate mRNA

Plasmid pGEMSAA, used as a template for A-SAA mRNA transcription, was generated as follows: A-SAA2 cDNA was PCR amplified, gel-purified and cloned into the ApaI and NsiI sites of pGEM 5zf(+) (Promega, Madison, WI).The plasmid was linearized with MscI (which cuts at the natural site of polyadenylation for A-SAA mRNA) prior to in vitro transcription.

The A-SAA mRNA substrate was generated as follows: a 555-base A-SAA mRNA product, comprising the entire 542 bases of the mature A-SAA2 mRNA and a 13-base 5′ vector-derived element, was transcribed as detailed below and gel-purified on a 4% polyacrylamide gel. The RNA band was eluted overnight in 2 volumes of RNA elution buffer (0.3 M sodium acetate, 0.2% SDS). The supernatant was phenol extracted once to remove residual SDS and the RNA was ethanol precipitated and redissolved in DEPC (diethylpyrocarbonate) treated dH_2O.

2.3. Ribozyme plasmid construction

Ribozyme sequences were synthesised as sense and antisense oligonucleotides using an Applied Biosystems 391 DNA synthesiser. The basic A-SAA ribozyme plasmid (RpSAA) was synthesised by annealing the oligonucleotides RBasF (5′-GAAAAGCTTGCCAAGGCTGATGAGTCCGTGAGGACGAAACGAAAAGTCTAGAGC-3′) and

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Abbreviations: A-SAA, acute-phase serum amyloid A; HDL, high density lipoprotein; SAA, serum amyloid A.

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RBasR (5′-GCTCTAGACTTTTCGTTTCGTCCTCACGGACTCA-TCAGCCTTGGCAAGCTTTTC-3′) which contain the restriction sites *Hin*dIII and *Xba*I.

The autocatalytic ribozyme plasmid (RpSAA3′) was synthesised by annealing the oligonucleotides Rib3′F (5′-ATTCTAGAGTCGACG-GACTCGAGTCCGTCCTGATGAGTCCGTGAGGACGAAACT-CTAGGGCCCTA-3′) and Rib3′R (5′-TAGGGCCCTAGAGTTTC-GTCCTGACGGACTCATCAGGACGGACTCGAGTCCGTCGA-CTCTAGAAT-3′) which contain the restriction sites *Xba*I and *Apa*I.

Sense and antisense oligonucleotide pairs were annealed by heating to 95°C for 3 min and cooling to room-temperature overnight, digested with appropriate restriction enzymes, and cloned into pcDNA 3 (Invitrogen, Leek, The Netherlands).

The sequences of all constructs were confirmed by dideoxy sequencing using the Sequenase Version 2.0 kit (US Biochemical, Cleveland, OH).

2.4. In vitro transcription of ribozyme and SAA mRNA

Transcription of RNA's from plasmid templates that contained the T7 RNA polymerase promoter were carried out using the Ribomax transcription kit (Promega, Madison, WI).

A typical 100 μl reaction mixture contained 5 μg linearised template DNA (see below), 80 mM HEPES-KCl, pH 7.5, 24 mM $MgCl_2$, 2 mM spermidine, 40 mM DTT, 5 mM rATP, rGTP and rCTP, 2 mM rUTP, 120 μCi [α-^{32}P]UTP (specific activity 800 Ci/mmol) (Amersham, Bucks, UK), 3000 U T7 RNA polymerase and 0.15 U/μl RNAsin ribonuclease inhibitor (Boehringer Mannheim, Lewes, UK).

The reactions were incubated at 37°C for 3–4 h. Following transcription RNAse-free DNAse (Promega) was added at a concentration of 1 U/μg DNA template and incubated at 37°C for 15 min. This treatment was followed by phenol/chloroform extraction and ammonium acetate ethanol precipitation of the RNA.

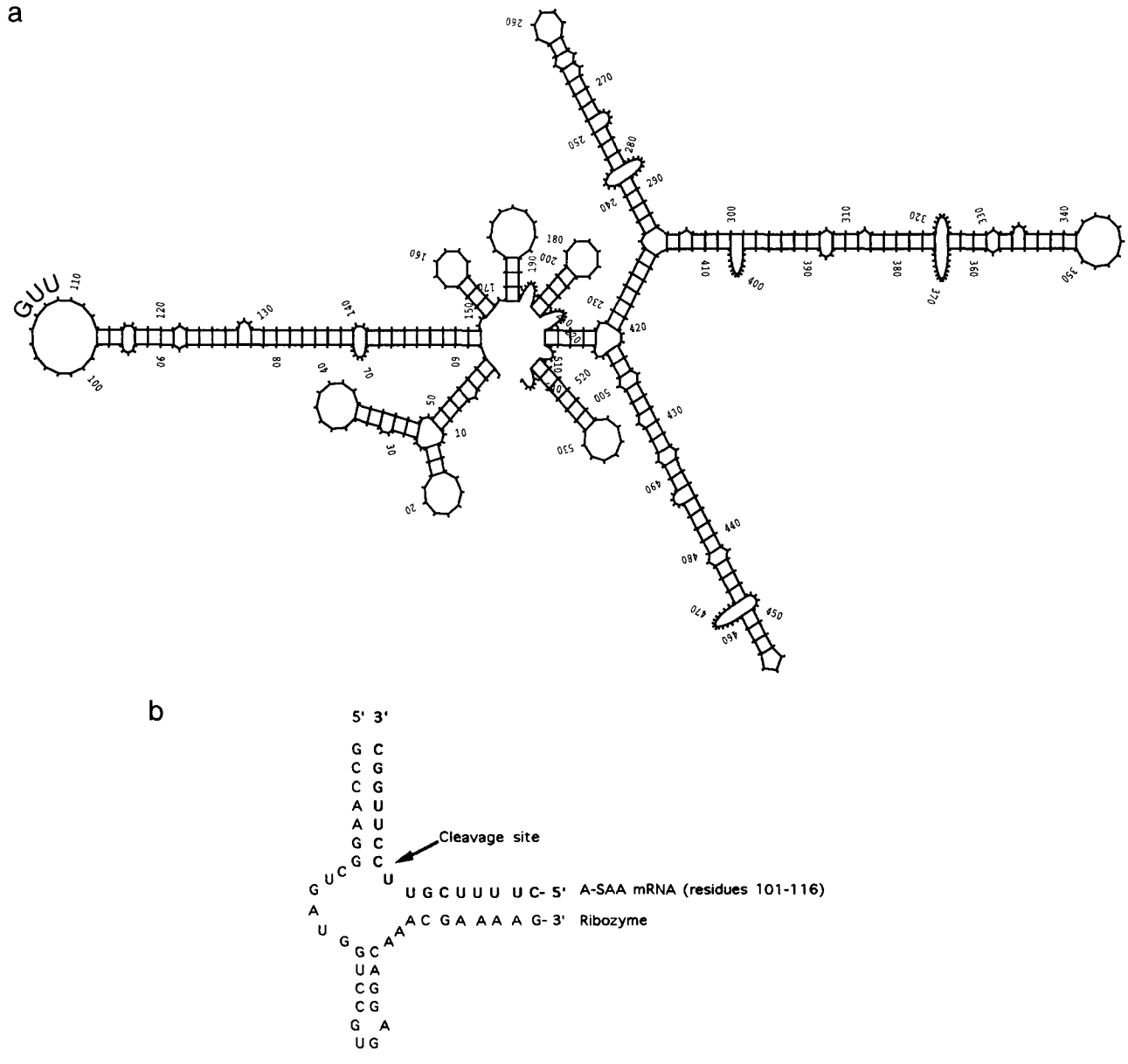

Fig. 1. Hammerhead ribozyme targetted against A-SAA mRNA. (a) A-SAA2 mRNA secondary structure predicted using the RNAPlotFold program; the GUU target site (residue 107–109) on an exposed loop is indicated on the left-hand side of the structure. (b) The hammerhead structure of RzSAA is formed from residues 101–116 on the A-SAA mRNA target and the catalytic strand containing the conserved bases of the ribozyme. The cleavage site immediately 3′ to the GUU triplet (residues 107–109) is indicated. Note the U-rich content of the target sequence.

2.5. Ribozyme cleavage reactions

Ribozyme and substrate were mixed and heated at 90°C in a solution of 50 mM Tris-HCl and 5–15 mM $MgCl_2$ in the presence of 10 U RNAse inhibitor. The reactions were incubated at 37°C for various times. After incubation the reactions were stopped by adding an equal volume of stop solution (1 mM EDTA, pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol in deionised formamide), heated at 95°C for 3 min; products were size fractionated on a 5% polyacrylamide gel in TBE and autoradiographed.

3. Results and discussion

Hammerhead ribozymes can be designed to cleave virtually any mRNA since the only requirement for cleavage activity is the presence of a GUX triplet on the target mRNA. The A-SAA2 mRNA sequence was searched for potential GUX cleavage sites and seven were identified. Several factors that increase the effectiveness of a ribozyme were taken into consideration when selecting the most suitable GUX target site for the anti-SAA ribozyme. These are: (a) the accessability of an mRNA target site for the ribozyme, (b) the strength of ribozyme–substrate base-pairing, and (c) the stability of the ribozyme.

The most accessible target site on an mRNA substrate can in theory be identified using computer-generated predictions of mRNA secondary structures and previous ribozyme studies have established the importance of using such secondary structure analyses in the selection of a ribozyme target site [14,15]. For example, Hullier et al. [15] used a predicted secondary structure of α-lac mRNA when choosing an α-lac ribozyme target site; two ribozymes targetted to an 'unstructured' region of α-lac mRNA were much more effective in cultured cells than a ribozyme targetted to a central region predicted to have a complex secondary structure. We used the RNAPlotFold program (GCG) to predict the A-SAA2 mRNA secondary structure and identified a GUU triplet (nucleotides 107–109) in an open-loop region (Fig. 1a) which should be very accessable to a ribozyme in vivo.

The length of the region flanking the target site determines the specificity of a ribozyme; however strong interactions between a ribozyme and substrate mRNA can prevent rapid dissociation of the ribozyme following cleavage of the target [16]. This could significantly reduce the catalytic activity of the ribozyme. In theory a ribozyme will cleave with an optimal K_{cat}/K_m an RNA that is base-paired with a free-energy of less than −16 kcal/mol [17]. Calculations have shown that a GC-rich ribozyme reaches a ΔG of <-16 with only 8 base-pairs. An AU-rich ribozyme is therefore more desirable than a GC-rich ribozyme in order to have the longest possible RNA binding sequence (and hence highest specificity) while maintaining ΔG <-16. An A-rich ribozyme also eliminates the problem of wobble UG pairing between the ribozyme and target mRNA and consequently further increases the specificity of the ribozyme. As well as being in a relatively unstructured region of the A-SAA mRNA, the region surrounding the GUU triplet at bases 107–109 is very U-rich; thus this site was chosen as the target site around which an anti-SAA ribozyme was designed.

The anti-A-SAA hammerhead ribozyme designed to cleave A-SAA mRNA was modelled on the design of Haselhoff and Gerlach [12] and is shown in Fig. 1b. The hammerhead ribozyme reported here has been designed to cleave both A-SAA1 and A-SAA2 mRNA; as a result of the high degree of sequence homology between both genes, the target sequence flanking the GUX at 107–109 is identical in both. This will allow for the eventual targetting of both acute-phase mRNAs in cell culture and in vivo studies in which both species will be present.

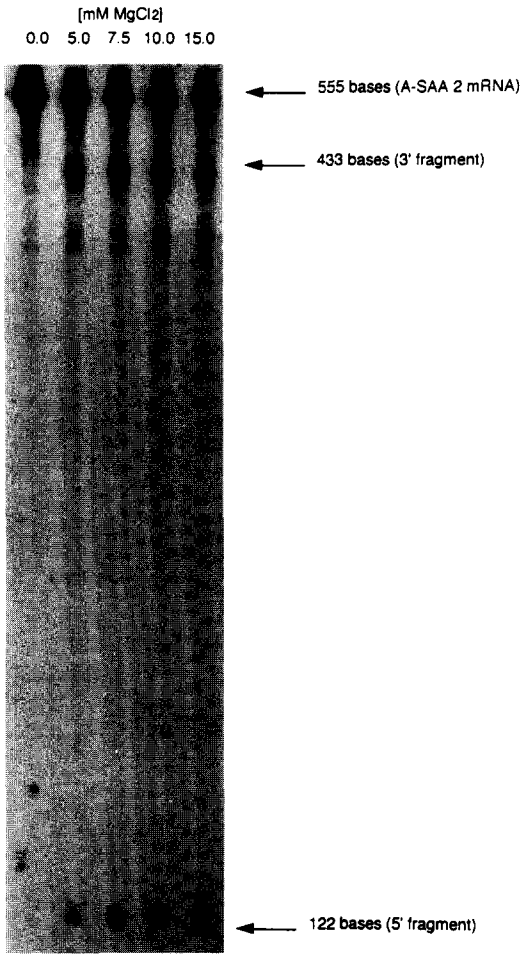

Fig. 2. Magnesium-dependent cleavage of A-SAA mRNA by RzSAA in vitro. In vitro transcribed A-SAA2 mRNA (555 bases) and RzSAA ribozyme (derived from RpSAA) were incubated for 4 h with 50 mM Tris-HCl and increasing concentrations of $MgCl_2$ (indicated along the top of each lane). The sizes of the A-SAA2 cleavage fragments (433 and 122 bases) are indicated. Note the absence of cleavage fragments in lane 1, due to the absence of $MgCl_2$.

The in vitro cleavage reactions were carried out using target A-SAA2 mRNA, which is full-length (542 bases plus 13 bases of vector derived sequence at the 5′ end), and therefore likely to fold into the most authentic representation of the A-SAA2 mRNA secondary structure found in vivo. Incubation of in vitro transcribed A-SAA2 mRNA and ribozyme resulted in magnesium-dependent cleavage of the A-SAA2 mRNA to the expected fragments of 122 (109 bases of A-SAA2 mRNA plus 13 vector-derived bases) and 433 bases (Fig. 2). The magnesium dependence of the ribozyme cleavage reaction is in concordance with previous observations that such hammerhead cleavage reactions have an absolute requirement for divalent metal ions [18].

The activity of a ribozyme also depends on the in vivo stability of the ribozyme. The poly(A) tail and 5′ cap structure both

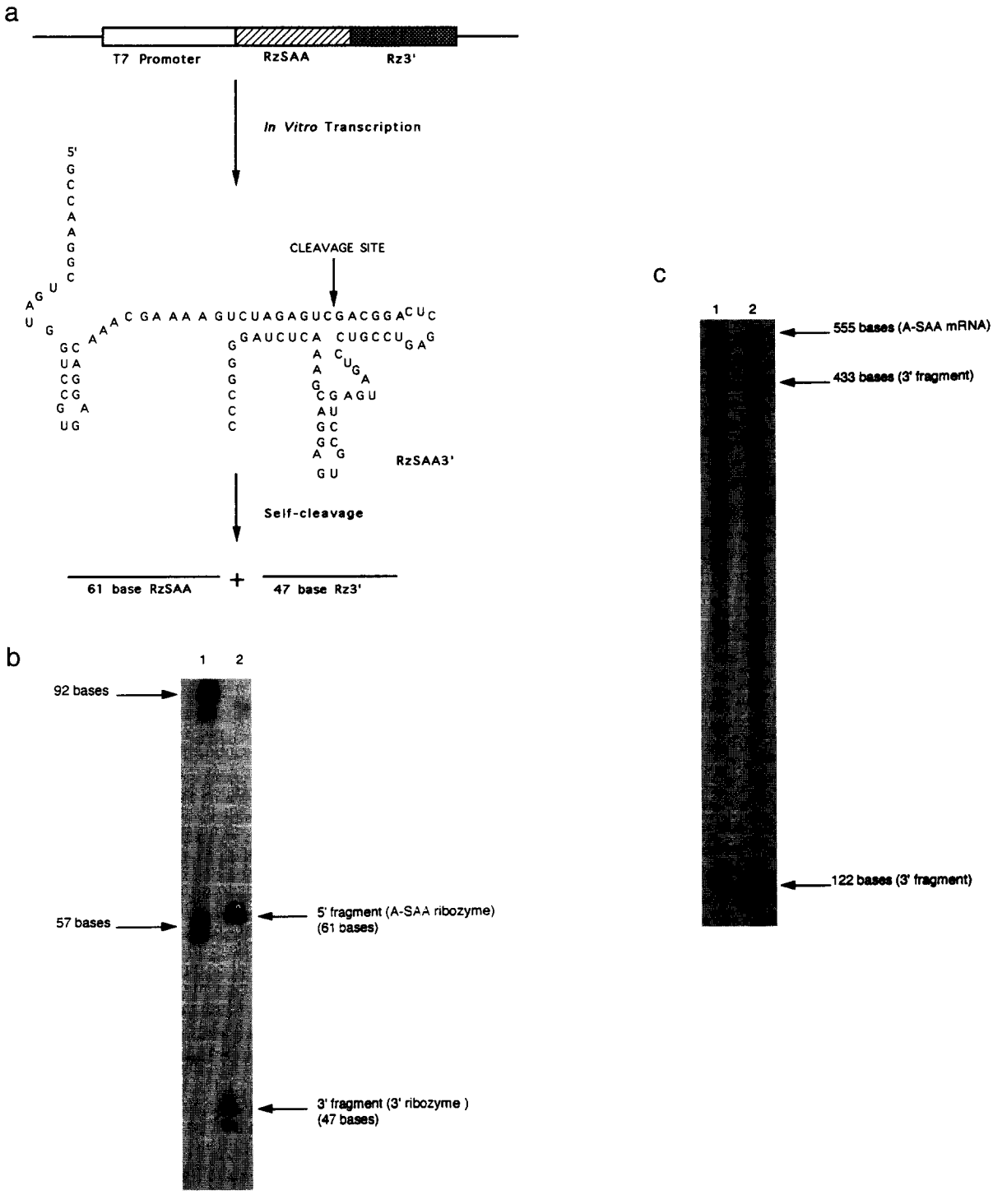

Fig. 3. Autocatalytic cassette for the generation of anti-A-SAA ribozymes with defined 3′-termini. (a) The autocatalytic cassette used to generate an anti-A-SAA ribozyme with a defined 3′-terminus (RzSAA3′). The second, autocatalytic, ribozyme (Rz3′) forms a hammerhead structure (details in text) and cleaves the A-SAA ribozyme at the 3′ end at the position indicated. (b) In vitro transcription from the autocatalytic cassette: lane 1 = size markers; lane 2 = product following transcription of RzSAA3′. The autocatalytic cassette has completely self-cleaved into the expected size fragements (61 and 47 bases). Note the additional bands at 46 and 48 bases which are extraneous cleaved transcription products due to transcription from a template with a 3′ overhang (the template was linearised with ApaI prior to transcription). (c) A-SAA2 mRNA incubated with RzSAA transcribed directly from RpSAA (lane 1) and the gel purified 5′ fragment released by the autocatalytyic cleavage of RzSAA3′ (lane 2). The expected A-SAA2 mRNA cleavage products are present in lane 2, indicating that the 5′ fragment generated from the autocatalytic ribozyme is a functional anti-A-SAA ribozyme.

play a well-documented role in the stability of many eukaryotic mRNAs [19,20]. Due to the presence of polyadenylation signal sequences and a poly(A) tail at the 3′ end, the A-SAA ribozyme (RzSAA) transcribed in vivo will be 200–300 bases larger than the 60 base ribozyme produced in vitro. This increase in size may, however, prevent access of the ribozyme to the target site. *cis*-Cleaving autocatalytic ribozymes have been previously used to generate RNA transcripts with defined 5′- and 3′-termini [21,22]. We have therefore designed an autocatalytic cassette (RpSAA3′) that contains RzSAA immediately upstream of another ribozyme and is capable of folding back and forming a hammerhead ribozyme using the 3′ end of RzSAA as the target substrate and cleaving between the two (Fig. 3a). The resulting ribozyme generated from this construct (RzSAA3′) will have a defined 3′ end lacking a poly(A) tail and thus is likely to more efficiently access the target site on A-SAA mRNA. This autocatalytic cassette, tested in vitro, self-cleaves completely during transcription to produce two fragments; the 61 base RzSAA and the 47-base 3′ ribozyme (Fig. 3b). The 61-base fragment was gel purified and shown to cleave A-SAA2 mRNA into the expected fragment sizes, thus indicating that it is a fully functional anti-A-SAA ribozyme (Fig. 3c). It will be possible to transcribe each of the above anti-A-SAA ribozymes in vivo with the guanosine cap structure at the 5′ end which will protect the ribozyme from 5′ to 3′ exonucleotic degradation.

The aim of this in vitro study was to design and test a functional anti-A-SAA ribozyme which can be adapted for future in vivo studies of the applicability of ribozyme technology to the specific down-regulation of A-SAA synthesis under pro-inflammatory conditions. We have successfully generated a hammerhead ribozyme that cleaves full-length A-SAA mRNA in vitro in a magnesium-dependent, protein-independent manner. A second *cis*-cleaving ribozyme, when positioned downstream from the A-SAA ribozyme, has been shown to be fully active in vitro, releasing a functional 61-base anti-A-SAA ribozyme; this 61-base ribozyme is considerably smaller than the polyadenylated A-SAA ribozyme that would be synthesised by the 'basic' ribozyme. As a result this ribozyme may be able to target the substrate more efficiently and may prove to be a more effective catalyst in vivo.

The ultimate goal of this work is to achieve anti-A-SAA ribozyme mediated down-regulation of A-SAA synthesis in a transgenic animal carrying a human A-SAA transgene. In addition to the use of such an experimental model to determine the general effectiveness of ribozymes as therapeutic agents under inflammatory conditions, it could also be used as a model to obtain information regarding the 'normal' protective function of the A-SAAs during the acute phase response, the pathogenic consequences of chronic concentrations of A-SAA, and the therapeutic potential of anti-A-SAA ribozymes in the treatment of A-SAA dependent diseases such as secondary amyloidosis and possibly atherosclerosis.

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