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Characterization of rat serum amyloid A4 (SAA4): A novel member of the SAA superfamily

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ABSTRACT

The serum amyloid A (SAA) family of proteins is encoded by multiple genes, which display allelic variation and a high degree of homology in mammals. The *SAA1/2* genes code for non-glycosylated acute-phase SAA1/2 proteins, that may increase up to 1000-fold during inflammation. The *SAA4* gene, well characterized in humans (*hSAA4*) and mice (*mSaa4*) codes for a SAA4 protein that is glycosylated only in humans. We here report on a previously uncharacterized *SAA4* gene (*rSAA4*) and its product in *Rattus norvegicus*, the only mammalian species known not to express acute-phase SAA. The exon/intron organization of *rSAA4* is similar to that reported for *hSAA4* and *mSaa4*. By performing 5′- and 3′RACE, we identified a 1830-bases containing *rSAA4* mRNA (including a GA-dinucleotide tandem repeat). Highest *rSAA4* mRNA expression was detected in rat liver. In McA-RH7777 rat hepatoma cells, *rSAA4* transcription was significantly upregulated in response to LPS and IL-6 while IL-1α/β and TNFα were without effect. Luciferase assays with promoter-truncation constructs identified three proximal C/EBP-elements that mediate expression of *rSAA4* in McA-RH7777 cells. In line with sequence prediction a 14-kDa non-glycosylated SAA4 protein is abundantly expressed in rat liver. Fluorescence microscopy revealed predominant localization of rSAA4-GFP-tagged fusion protein in the ER.

1. Introduction

The synthesis of acute-phase proteins is largely regulated by inflammation-associated cytokines, peptide hormone signals produced by endothelial cells, lymphocytes, and activated monocytes/macrophages. Serum amyloid A (SAA) is a generic term for a family of acute-phase proteins coded for by different genes with a high degree of homology in mammals [1,2].

In humans and mice, *SAA1* and *SAA2* genes are best characterized sharing approx. 95% overall sequence identity [3,4]. The different alleles of the *SAA1/2* loci encode non-glycosylated acute-phase SAA (A-SAA) 1/2 proteins (104-amino acids [*aa*], 12-kDa). A-SAA may reach plasma levels 1000-fold greater than that found in the non-inflammatory state, thus representing an ideal marker for clinical use [5]. A-SAA acts as precursor protein for reactive systemic amyloid A (AA) amyloidosis [6] and modulates the atheroprotective properties of high-density lipoproteins (HDL) during inflammation [7]. Other activities, e.g. interaction with various receptors that modulate cytokine release and intracellular signalling pathways, activation of transcription factors and production of matrix metalloproteinases, underline a potential role of A-SAA in atherogenesis, coronary syndrome, and tumor pathogenesis [8–10]. While human *SAA3* (*hSAA3*) is considered a pseudogene, the murine *Saa3* (*mSaa3*) homolog codes for mSAA3 protein.

In humans and mice, the *SAA4* locus contains the gene encoding for a unique SAA family member assumed to be constitutively expressed [11,12]. The *SAA4* gene differs from *SAA1/2*, whose four exons and three introns share in between 93 and 100% and 87 and 92% identity. The hSAA4 protein (14-kDa) shares approx. 55% identity with SAA1/2. Due to an octapeptide insertion, hSAA4 consists of 112-*aa*. A N-linked glycosylation site in this octapeptide

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may lead to an additional 19-kDa protein [11]. Under physiological conditions hSAA4 constitutes more than 90% of total SAA [13]; it associates with very low-density lipoproteins and HDL [13]. In contrast to A-SAA, SAA4 does basically not act as the precursor molecule for AA fibrils, and levels do not change dramatically during the acute-phase response [14].

Contrary to all mammalian species studied, the rat lacks expression of A-SAA proteins [15]. Consequently, rats do not develop AA amyloidosis [15], a disease in which fibrils formed from the 76-*aa* N-terminal portion of A-SAA induce severe organ dysfunction.

We here present first evidence on characterization of mRNAs for rat *SAA4* (*rSAA4*), abundant expression of rSAA4 in the liver on mRNA and protein level, and its cellular localization.

2. Materials and methods

2.1. Rapid amplification of cDNA ends (RACE)

Rat brain Marathon-Ready cDNA (Clontech Laboratories, Takara Bio Company, Mountain View, CA) was used as template to amplify full-length *rSAA4* cDNA (Supplemental Table I). Primers used for the amplification of the 5′ and 3′cDNA ends are given in Supplemental Table II. RACE products were cloned into a pCR®2.1-Topo® vector (Life-Technologies Ltd., Invitrogen, Paisley, UK) and transformed into TOP10 One Shot® Cells (Life-Technologie). After transformation plasmid DNA was isolated using Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany) and sequenced with vector-specific M13 and *rSAA4*-specific primers (Supplemental Table II). After dye terminator removal with Centri-Sep spin columns (Princeton Separations, Freehold, NJ) sequencing reactions were analyzed with a 3100 Genetic Analyzer (Life-Technologies™, Foster City, CA).

2.2. RNA isolation, DNase-I digestion and reverse-transcription

Sprague–Dawley rats (200–300 g body weight; Himberg, Austria) received an i.p. injection of 2 mg/kg LPS (*Escherichia coli*, Serotype 055:B5; Sigma–Aldrich, St. Louis, MO) or vehicle (PBS). The study was approved by the ethics committee of the Federal Ministry of Science, Austria [GZ 66.010/9-Pr/4/2002] carried out in line with the European Communities Council Directive. After 48 h rats were killed with an overdose of sodium pentobarbital and tissues were kept either in N_2 or at 25 °C (in case of pancreas, colon, and lungs) in 1 ml RNA Later Reagent (Life-Technologies). Using Trizol reagent (Life-Technologies) or RNeasy-Minikit (Qiagen) total RNA was isolated from rat tissues and McA-RH7777 cells. One μg of RNA was digested with DNase-I and reverse-transcribed using random hexamer primers and SuperScript® II Reverse Transcriptase (Life-Technologies) [16].

2.3. qPCR-analysis

qPCR-assays were performed using Power SybrGreen Mastermix, ABI Prism 7000 SDS instrument (Life-Technologies) and respective primers (Supplemental Table III). Fold-change in gene expression of the target gene compared to *rGAPDH* was calculated by the $2^{-\Delta\Delta CT}$ method and absolute mRNA expression (copy number) was determined by the equation of a calibration curve.

2.4. Protein isolation and Western blot analysis

Frozen rat tissues were immersed in extraction buffer (Supplemental Table IV) and homogenized with Ultraturrax (Ika GmbH, Staufen, Germany). Lysates were centrifuged and supernatants were subjected to Western-blot analysis [16]. Membranes were incubated with anti-human SAA4 peptide (*aa*-position 94–112) rabbit antiserum (dilution 1:2500) [17]. As positive controls, hSAA4 [18] and tagged-hSAA4 [17] (both expressed in *E. coli*) were used.

2.5. Cell culture experiments

McA-RH7777 hepatoma cells (ATCC) were grown in DMEM (Supplemental Table IV) at 37 °C in 5% CO_2. Cells, seeded in 6-well plates, were incubated in DMEM containing 10 ng/ml IL-1α/β, IL-6, TNFα (R&D Systems Inc., Minneapolis, MN) or 1 μg/ml LPS (Sigma–Aldrich) for 12–60 h.

2.6. Cloning of rSAA4pcDNA3.1/CT-GFP-TOPO vector and transfection of McA-RH7777 cells

To obtain a full-length rSAA4-GFP fusion protein a PCR was performed using *rSAA4*-cloning primers including the native Kozak-sequence (Supplemental Table V) and rat liver cDNA as template (Supplemental Table VI). The PCR-product was separated by gel electrophoresis and purified using Wizard SV Gel- and PCR-Clean-Up System (Promega, Madison, WI).

cDNA for *rSAA4* was cloned into pcDNA3.1/CT-GFP-TOPO vector (Life-Technologies) and McA-RH7777 cells (seeded on glass plates in 6-well-dishes, 70% confluence) were transfected using ExGen-500 in vitro Transfection Reagent (Fermentas, St. Leon-Rot, Germany). After 6 h, 1 ml of culture medium was added and incubated for 20 h.

2.7. Confocal imaging

Cells were incubated with 1 ml of a loading solution (Supplemental Table IV) containing 0.5 μM ER-Tracker® Red or 0.1 μM Mito-Tracker® Red (Life Technologies Ltd.) for 5 or 40 min. GFP signals were imaged together with ER-Tracker® Red or Mito-Tracker® Red using a *Nipkow*-disk-based array confocal laser scanning microscope (Supplemental material section).

2.8. Luciferase assay

In silico analysis of *rSAA4* promoters was performed with the Genomatix software package version 7.0. Gene2promoter was used to search for vertebrate promoter sequences of *SAA4* and MatInspector to identify transcription factor binding sites and their position and orientation towards each other.

2.8.1. Mutagenesis of the full-length rSAA4 promoter fragment (fl-rSAA4)

The sequence +44 to −3293 bp relative to the transcription start codon of *rSAA4* was cloned in pGL3 Basic vector (Promega) to create fl-*rSAA4*. Primers were designed with 15 bp homologous to the multiple cloning site of pGL3 with restriction enzyme binding sites to enable cloning of the *rSAA4* promoter fragment into pGL3 (Supplemental Table VII).

PCR-amplification of the fl-*rSAA4* promoter fragment was performed using the BAC clone CH230-458A11 (BACPAC resources center, Oakland, CA) as template (Supplemental Table VI). The PCR-products were purified using the Nucleospin II kit (Macherey & Nagel GmbH & Co. KG, Düren, Germany) and ligated with the In-Fusion Dry-Down PCR-Cloning Kit (Clontech-Laboratories) to create the recombinant fl-*rSAA4* vector. After transformation positive clones were identified with colony-PCR, restriction analysis and DNA sequencing.

To test the activity of the three C/EBP-elements most proximal to the start codon a deletion of the BOX (C/EBP-element 1–3 flanked by FAST and HOXF binding sites, −889 to −1166 bp) was performed to create construct A. Mutagenesis was performed with the Phusion Site-Directed Mutagenesis Kit (Thermo-Fisher-

Scientific, Rochester, NY). Linearized vector A was created via PCR (Supplemental Tables VI and VIII). The PCR-product was separated from unmutated, non-linearized template vector by agarose gel electrophoresis and isolated with the Nucleospin II Kit. For circularization, 20 ng linearized vector was ligated with T4 DNA-Ligase (Fermentas), transformed into *E. coli* Fusion-Blue cells (Clontech-Laboratories). Positive clones were identified by colony-PCR and DNA-sequencing.

2.8.2. Cloning of rSAA4 promoter fragments

Deletions constructs were created to further investigate the three proximal C/EBP- elements: B (+44 to −1084), C (+44 to −1113), D (+44 to −2449), and E (+44 to −932). Promoter fragments of *rSAA4* were PCR-amplified using the respective synthetic oligonucleotides (Supplemental Table VII) and the BAC-clone CH230-458A11 (BACPAC resources center) as template.

2.8.3. Luciferase reporter assay

Cells (80% confluence) were transfected with 2 μg of the respective pGL3 construct (expressing Firefly Luciferase) and 4 ng of the control plasmid pRL-TK (expressing Renilla Luciferase) using ExGen-500 in vitro Transfection Reagent (Fermentas) according to manufacturer's suggestion. Cells were lysed and Luciferase activity was measured using the dual-Luciferase reporter assay (Promega) on a luminometer (Lumat-LB9501, Berthold-Technologies GmbH & Co. KG, Bad Wildbad, Germany). To account for non-specific effects on reporter plasmids, experimental results were expressed as normalized ratios. The ratio of Renilla and Firefly-Luciferase activity of each experiment was normalized against cells transfected with the empty Luciferase vector.

2.9. Statistical analysis

Statistical analysis of qPCR data was performed by one-way ANOVA and of Luciferase-Assay by Student's *t*-test (unpaired, two-tailed). Means were considered as significantly different at $p < 0.05$.

3. Results

To determine the genomic structure of the *rSAA4* gene and the corresponding full-length *rSAA4* cDNA, we performed 5′- and 3′RACE using a rat cDNA library as template. These experiments showed an exon/intron organization for *rSAA4* consisting of one untranslated 5′exon followed by three translated exons (Fig. 1). The exon/intron organization of *rSAA4* is similar to that reported for *hSAA4* and *mSaa4* [12,19]. The 5′RACE experiments (Fig. 1C) in combination with reported ESTs (e.g. FQ107900, Fig. 1B) revealed that the maximal 5′UTR exceeds the reported RefSeq for *rSAA4* (NM_001009478.1) by 90-bases (Supplemental Table IA–C). Within this region, we found a GA-dinucleotide tandem repeat with 23 repeats in the RefSeq sequence for *rSAA4* (NM_001009478.1) (Fig. 1E and F). The sequence of this accession number is based on the cDNA clone MGC:108857.

Performing 3′RACE, we were able to reveal a maximum size of the 3′UTR of 1214-bases. This observation parallels the current version of *rSAA4* mRNA (*BC088188*-NM_001009478.1). We conclude that the full-length sequence of *rSAA4* mRNA consists of 1830-bases (Supplemental Table IC).

To test the possibility of a read-through transcription between *rSAA4* and the neighboring *HPS5* gene (NM_001135612.1) indi-

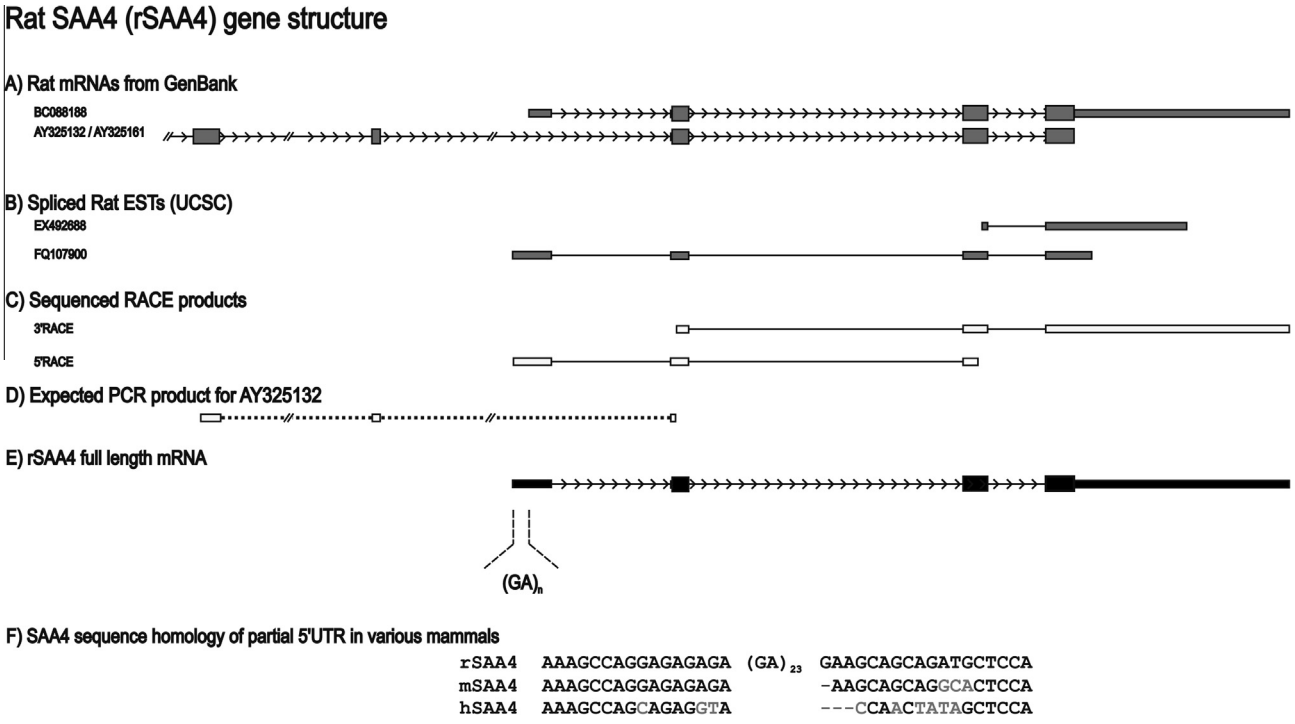

Fig. 1. *Gene structure of rSAA4.* (A) Rat *SAA4* mRNAs (*BC088188*, *AY325132*, *AY325161*) from GenBank® (http://www.ncbi.nlm.nih.gov/genbank/) are shown; thin grey squares (▭) represent untranslated regions, broad grey squares (▭) represent translated regions; intronic sequences are shown as thin black lines. Arrowheads indicate orientation of the gene. (B) Exon/intron structure of spliced ESTs published in the UCSC Genome Browser with the most extended 5′UTR (FQ107900) and 3′UTR region (EX492688) of *rSAA4* are shown. (C) Schematic representation of results obtained by RACE: 3′RACE product extends the 5′UTR of *EX492688* with 574 bases; that confirms the 5′UTR of *BC088188*; 5′RACE product extends the 3′-region of *BC088188* and confirms the 3′UTR represented by the spliced EST FQ107900. (D) The predicted PCR-product for primers spanning from the first coding exon of *rSAA4* reaching to a primer located 2 exons upstream of *rSAA4* within a conserved exonic region of *AY325132*. PCR-amplification of the 190 bp product was unsuccessful using rat Marathon cDNA as template and standard PCR programs. (E) Deduced full-length *rSAA4* from RACE is shown: thin black squares (▬) represent untranslated regions, broad black squares (■) represent translated regions, intronic sequences are shown as thin black lines. The location of the GA-dinucleotide repeat $(GA)_n$ within the 5′UTR of *rSAA4* is indicated below. (F) Partial 5′UTR of the *SAA4* gene in different mammalian species (i.e. *rSAA4*, *mSAA4*, and *hSAA4*): note the GA-dinucleotide tandem repeat is only present in the rat genome. The UCSC Genome Browser [29] was used.

cated by the rat mRNA sequences *AY325132* and *AY325161* (Fig. 1A), a PCR spanning from the coding region of *rSAA4* to a highly conserved exon of *AY325132* (Fig. 1D) was performed using the Marathon rat brain cDNA as template. We observed no PCR-product indicating that these read-through mRNAs are not expressed in rat brain.

Next we analyzed *rSAA4* mRNA expression in various organs/tissues revealing highest expression in liver, lung, heart, spleen and kidney (Fig. 2A). This is comparable to expression patterns in the mouse [11]. Injection of LPS did not alter *rSAA4* mRNA expression levels in all organs/tissues investigated (Fig. 2A).

Due to the striking homology of rSAA4 with hSAA4/mSAA4 (Supplemental Fig. I), an antibody raised against the C-terminal portion (that differs from SAA1/2) of hSAA4 was used. The antiserum recognizes non-glycosylated hSAA4 (14-kDa [18], Fig. 2B [lane 1]) and non-glycosylated tagged hSAA4 (17-kDa [17], Fig. 2B [lane 2], Fig. 2C [lane 1]), and a 14-kDa band in rat liver (Fig. 2C [lane 2]). This finding parallels data obtained in murine liver where no glycosylated mSAA4 protein is present. No immunoreactive bands for a 14-kDa protein were detected in other organs/tissues used for mRNA expression profiling (Fig. 2A).

We then investigated whether cytokine-treatment could modulate *rSAA4* transcription in McA-RH7777 cells. Highest induction (up to 9-fold) was found in IL-6-treated cells between 24 and 36 h. Other cytokines did not significantly alter *rSAA4* mRNA expression within 60 h (Fig. 3A). In contrast to the in vivo situation (Fig. 2A), where *rSAA4* mRNA seems to be constitutively expressed, LPS-treatment significantly induced *rSAA4* mRNA in McA-RH7777 cells (up to 4-fold) at almost all time points.

As basal levels of *rSAA4* mRNA in McA-RH7777 cells are low, we failed to detect rSAA4 by Western-blots. Therefore a full-length rSAA4-GFP fusion protein was overexpressed. Fluorescence microscopy revealed predominant localization of this fusion protein in the ER (Fig. 3B).

Previous studies have shown that the C/EBP-family of transcription factors plays important roles in the expression of *rSAA1* [20,21]. As disruption of hepatic C/EBPα in mice is paralleled by massive down-regulation of *mSaa4* mRNA [22] we hypothesized that a C/EBP-element could be responsible for *SAA4* mRNA expression in rats. To investigate the regulation of the *rSAA4* gene we performed in silico promoter analysis of a region approx. 3000 bp upstream of the start codon using the Genomatix software [23], predicting five C/EBP-elements (Fig. 4A); two are far distal from the start codon, whereas three others are located more proximal (<1.200 bp). To test the activity of these elements we cloned a series of promoter constructs in a Luciferase-reporter vector (Fig. 4A). Deleting the three most proximal C/EBP-elements between −888 and −1167 bp (construct A) leads to reduced Luciferase-activity by approx. 90% (Fig. 4B). This suggests that the BOX (containing C/EBP-element-1/-2/-3 flanked by FAST and HOXF sites) is required for maximal promoter activity. To investigate the proximal elements C/EBP-element-1/-2/-3 within the BOX in more detail we surveyed truncation constructs for their capacity to regulate Luciferase activity. A proximal segment containing a single

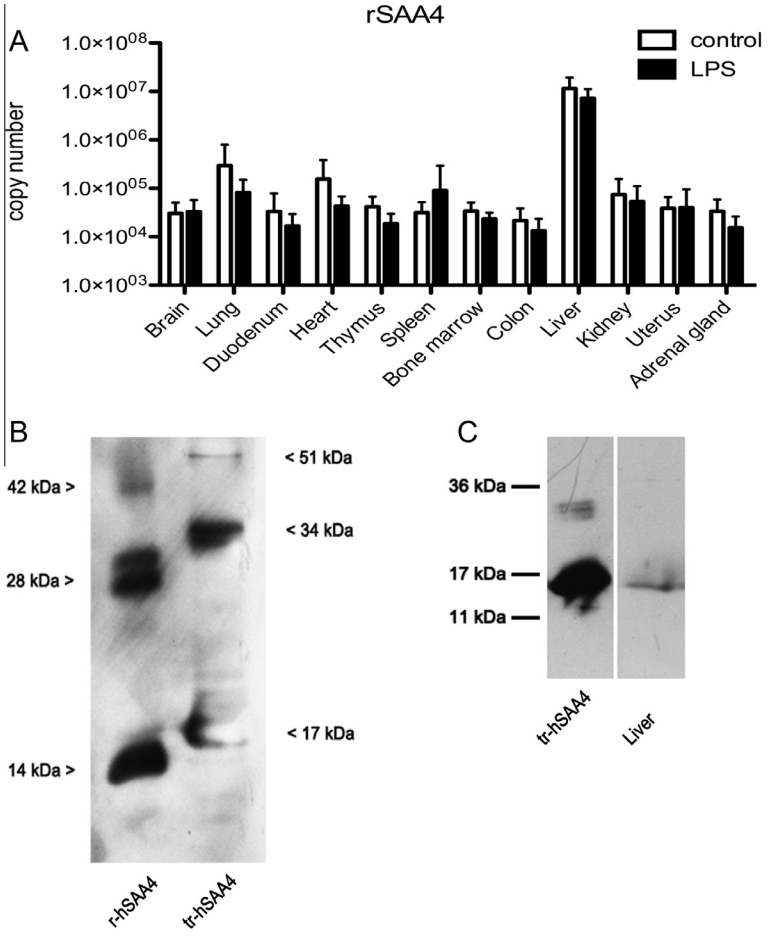

Fig. 2. *Expression of rat and human SAA4.* (A) Organs/tissues of control and LPS-treated rats (48 h) were excised, RNA was isolated, reverse transcribed and subjected to qPCR. Absolute *rSAA4* mRNA expression was determined with a calibration curve. Non-treated (controls, $n = 5$) and LPS-treated rats ($n = 7$; except for lung, heart, thymus, kidney [$n = 6$] and liver [$n = 5$]). Equal amounts of non-glycosylated recombinant hSAA4 (r-hSAA4) (B) and tagged-r-hSAA4 (tr-hSAA4) (B/C) or protein lysate (65 μg/lane) from rat liver (C) was subjected to Western-blot analysis using anti-human SAA4-peptide antiserum as a primary antibody. One representative experiment is shown.

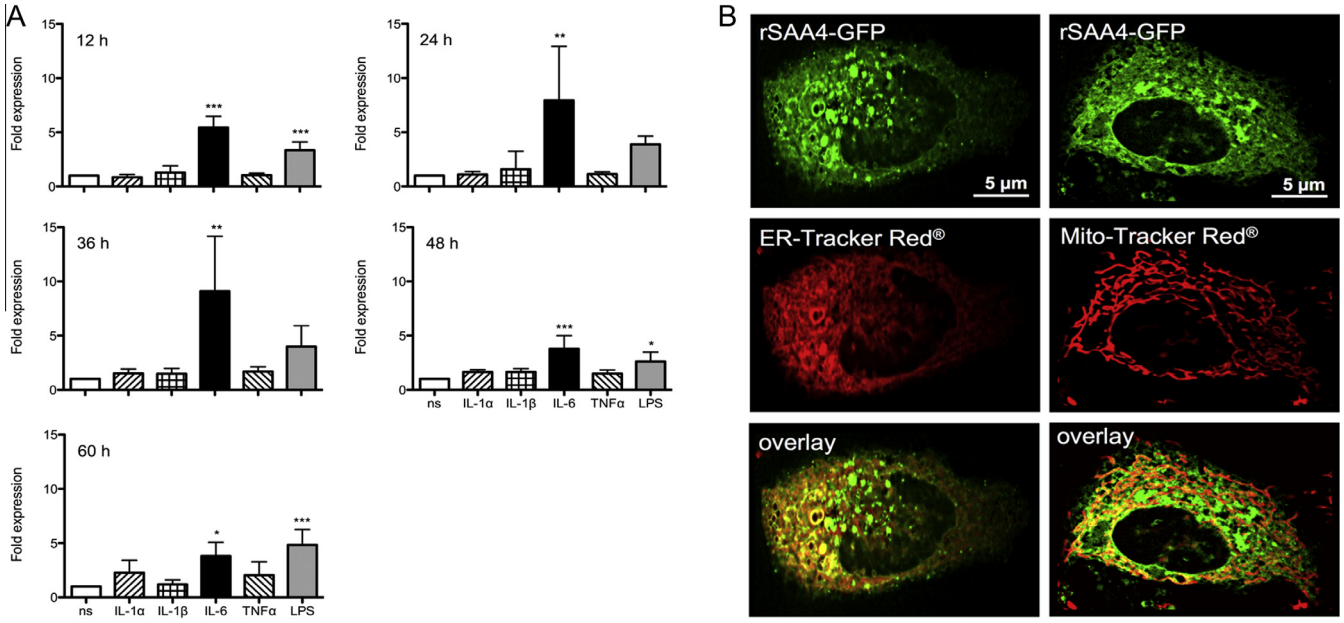

Fig. 3. *Cytokine- and LPS-induced expression of rSAA4 in McA-RH7777 cells and intracellular localization of rSAA4-GFP-fusion protein.* (A) Cells were treated with cytokines or LPS for indicated time periods. RNA was isolated, reverse transcribed and subjected to qPCR. *rSAA4* expression levels were normalized to *rGAPDH* ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Results are derived from two independent experiments performed in duplicate. ns, non-stimulated cells. (B) Intracellular localization of the GFP-tagged rSAA4 protein by confocal laser-scanning microscopy: green (GFP), red (ER or Mito-Tracker®), yellow (colocalization). The bar represents 5 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

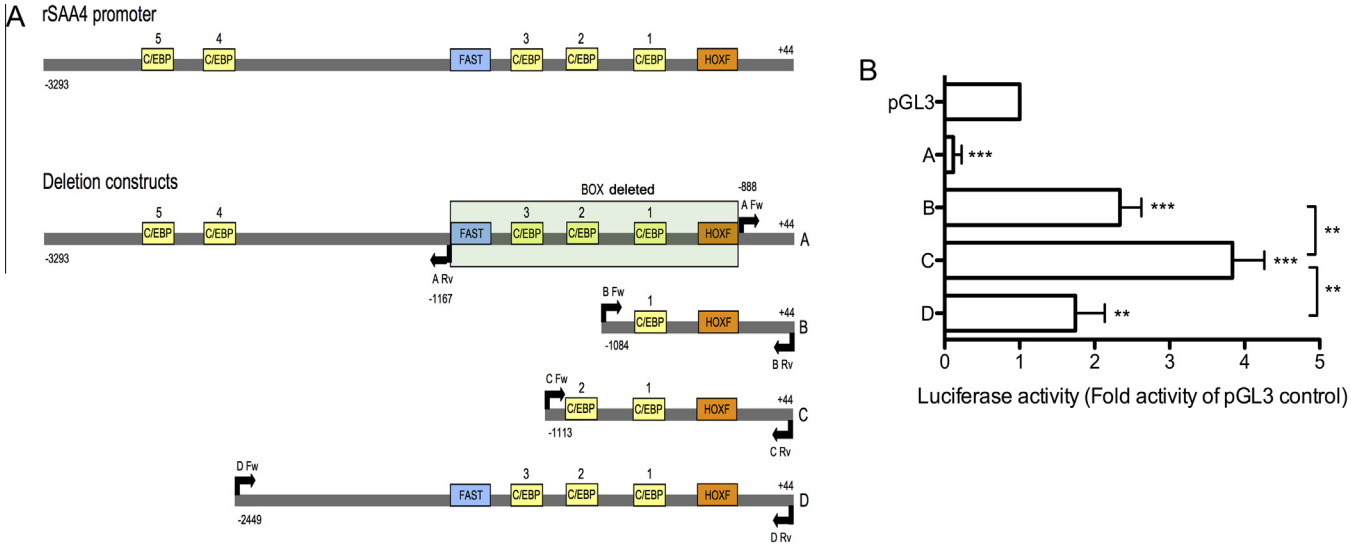

Fig. 4. *Luciferase assay of rSAA4 promoter constructs in McA-RH7777 cells.* (A) Overview of the *rSAA4* promoter (+44 and −3293 bp relative to the start codon) and the *rSAA4* promoter deletion constructs. Deletion of the BOX (between −888 and −1167 bp; containing the three proximal C/EBP response elements and flanked by FAST and HOXF binding sites) resulted in the generation of construct A. Construct B–D contained the one, two, and three most proximal C/EBP binding sites, yielding starting points at −1084 (construct B), −1113 (construct C), and −2449 (construct D), respectively. (B) Cells were transfected with 2 µg of the respective *rSAA4* promoter construct and with 4 ng of the control plasmid pRL-TK. Twenty-four hours after transfection cells were lysed and Firefly and Renilla luminescence was quantified. Results are derived from four (construct B/D) or three (construct A/C) independent experiments performed in triplicate. Results were normalized to the empty Luciferase vector (pGL3; $^{**}p < 0.01$, $^{***}p < 0.001$).

C/EBP-element-1 (+44 to −1084 bp, construct B) was able to evoke increased Luciferase activity >2-fold in comparison to the control vector (Fig. 4B). Of note, we detected an additional increase in Luciferase activity (approx. 50%) in an even shorter proximal promoter fragment containing HOXF but no predicted C/EBP-element-1 (+44 to −932 bp, construct E, data not shown). The promoter fragment containing C/EBP-element-1 and -2 (+44 to −1113 bp, construct C) exhibited a significant increase over construct B (approx. 3.9-fold over pGL3). A promoter fragment containing all three proximal C/EBP-elements plus additional ~1000 bp (+44 to −2449 bp, construct D) showed increased Luciferase-activity compared to pGL3 but decreased activity compared to construct B/C, indicating binding of potential repressive factor(s) between −1114 and −2449 bp. In summary, Luciferase assays suggest a strict requirement of the BOX-sequence for *rSAA4* promoter activity and argue for a positive effect of the predicted proximal C/EBP-elements in McA-RH7777 cells.

4. Discussion

In contrast to humans, mice and other mammals, no A-SAA protein has been found to be associated with acute-phase HDL isolated from rats [24]. Meek and Benditt [24] reported two *SAA*-related genes (*rSAA1*, *rSAA2*) with striking homology to human and murine *SAA1/2*. Both genes were upregulated in the liver but not in extrahepatic tissues in response to LPS. However, *rSAA1/2* mRNA lack a sequence coding for ~50-*aa* of the N-terminus of the predicted A-rSAA protein. Thus, it is obvious that rats, lacking A-SAA, do not develop secondary reactive AA amyloidosis [24]. Although *rSAA3* mRNA has been identified [24], it is still unknown whether rats do express SAA4 mRNA and protein. We here characterized a novel *SAA4* gene in the rat.

Basically, the exon/intron structure of *rSAA4* is similar to *hSAA4* and *mSaa4*. In addition to the 5′UTR and signal peptide, the complete sequence encoding 1–112-*aa* (as found in humans and mice) was determined. However, observations that the maximal 5′UTR exceeds the published rat RefSeq for *rSAA4* (NM_001009478.1) by 90 bases (containing a GA-dinucleotide tandem repeat with 23-repeats) is unique. This tandem repeat is only present in the rat genome. Different repeat numbers found in rat ESTs (18- and 22-repeats) and also in our cloned 5′RACE products (9- and 10-repeats) indicate that this tandem repeat might be a result of different allelic variations. Whether the length of this repeat modulates rSAA4 expression remains to be elucidated.

The regulation of acute-phase genes in the rat by inflammatory mediators represents an excellent model to study the interplay between transactivators and transrepressors on inducible gene control [25]. The control of eukaryotic gene transcription involves positive/negative regulatory mechanisms, and various transcription factors (primarily C/EBPs) have been reported to modulate mRNA expression of *A-SAA* [1]. Several lines of evidence prompted us to study expression of *rSAA4* as well as its potential transcriptional inducers, namely C/EBP. First, when treated with LPS, *Saa1* and *Saa2* mRNA levels were not upregulated in the liver of C/EBPα-deficient compared to wild-type mice [26]. Second, specific disruption of hepatic C/EBPα in mice led to an almost complete reduction of *Saa4* mRNA compared with that observed in floxed-mice [22]. Third, NF-κB- and C/EBP-like transcription factors act as primary inducers of *rSAA1* mRNA in the liver [20,21].

Although NF-κB binding sites are present in the promoter region of *rSAA4*, we focussed on C/EBP-binding sites. Using specific deletions in the promoter region, we identified three most proximal C/EBP-elements (between −888 and −1167 bp) that mediate expression of *rSAA4* in McA-RH7777 cells. Members of the C/EBP family act as pivotal regulators of cellular differentiation, terminal function, and response to inflammatory insults due to cytokine activation. Although IL-6 (in contrast to other cytokines) promotes *rSAA4* expression in rat hepatoma cells even at early time points (Fig. 3A), mRNA levels of individual members of the C/EBP-family (*C/EBPα* and *C/EBPβ*) were not affected (Supplemental Fig. II). This raises the possibility that NF-κB could act in concert with C/EBP to promote *rSAA4* activation. Alternatively, transcription factor YY1 may modulate *rSAA4* transcription by binding to a site overlapping with the NF-κB-binding site [27]. AP-2 acts as a negative regulator in extrahepatic tissues but promotes liver-specific *rSAA1* expression [28]; in addition, a proximal AP-2 site in combination with the NF-κB-binding site is essential for *rSAA1* promoter activity [25]. Whether these different modes of action, as previously reported for *rSAA1* [27], are also operative for *rSAA4* is currently unknown. However, our data showing even stronger Luciferase activation with a short construct devoid of the three proximal C/EBP-sites (not shown) and decreased activity with construct D (containing all three C/EBP-sites plus additional ~1000 bp) compared to construct C (containing only two C/EBP-sites) argue for the involvement of additional activators and repressors, respectively.

To sum up, we demonstrate for the first time the presence of a novel member of the SAA4 family on mRNA and protein level, previously not identified in the rat. The rSAA4 protein is expressed in the liver as a non-glycosylated protein, comparable to mice.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.054.

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