Sex steroid regulation and identification of different transcription units of the $S_A$ gene in mouse kidney

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Abstract

Although the $S_A$ gene was first identified as a putative candidate gene to understand the molecular basis of hypertension in rat and humans, the concept has not been supported in recently generated $S_A$-null mice. We had first identified the mouse $S_A$ gene on the basis of its strong androgenic regulation in mouse kidney and further characterized its genomic organization, transcription start site and chromosomal location. Northern blot, RT-PCR and in situ hybridization assays determined mouse strain, tissue distribution, sex-hormone dependence and cell expression of the $S_A$ mRNA. Kidney and liver constitute the main expression sites of the $S_A$ gene; in particular it is expressed in epithelial proximal tubule cells in the presence of androgens. This androgen-dependent expression is abrogated when estrogens are also present. By using the sensitive RT-PCR technique, minor $S_A$ expression sites, corresponding to testes, stomach, heart and lung, have also appeared. Like in kidney, expression of the $S_A$ gene in heart and lung is androgen-dependent. Production of rabbit antibodies against $S_A$-synthetic peptides identified the $S_A$ protein, a moiety of unknown function, which has been defined as a member of the acyl-CoA synthetase family. We have determined that the $S_A$ protein follows the same distribution and regulation as its corresponding mRNA. Transient transfection assays followed by confocal microscopy identified the mitochondria of proximal tubule-derived PCT3 cells as the subcellular location of the $S_A$ protein. Different transcriptional units produced by splicing events, occurring before the translation initiation site, have been identified from mouse kidney. This work provides the basis to further understand the molecular mechanisms that control the sex-steroid-dependent expression of the $S_A$ gene in mouse kidney, heart and lung, where $S_A$ is also expressed in an androgen-dependent manner.


Introduction

Understanding the processes by which extracellular stimuli modulate the expression of specific genes in a temporal and/or tissue-specific manner is crucial for unravelling the molecular mechanisms underlying cellular growth, homeostasis, differentiation and development. The molecular nature of tissue-specific gene regulation by androgens has not been well defined, partly as a result of the variable expression and incomplete regulation of currently available gene models. To overcome this problem we aimed to establish more informative models by identifying alternative genes whose expression would be tightly and co-ordinately regulated by androgens. By means of the subtractive hybridization techniques of Random arbitrarily printed (RAP)-PCR and representative differential analysis of cDNA (cDNARDA) we isolated differentially expressed genes from kidneys of female C57BL/6 mice dosed with dihydrotestosterone (DHT). In addition to well-characterized androgen-regulated genes (e.g. kidney androgen related protein), we demonstrated the differential expression of other genes previously not known to be under androgen control (Melià et al. 1998). Among them, and because the physiological significance of androgen-inducible gene expression in the kidney is quite unknown, we were particularly interested in the $S_A$ gene, since it was initially identified on the basis of its marked overexpression in kidneys of spontaneously hypertensive rats compared with the normotensive Wistar–Kyoto rats (Iwai & Inagami 1991). $S_A$ gene markers were subsequently shown to co-segregate with blood pressure (BP) in F2 cohorts of different rat crossings (Iwai and Inagami 1992, Iwai et al. 1992, Harris et al. 1993, Lindpaintner et al. 1993). Other authors not only demonstrated co-segregation of the gene with BP, but also that genotype at the $S_A$ locus determined the level of expression of the $S_A$ mRNA in kidney (Samani et al. 1993, Kaiser et al. 1994). Further evidence of its putative
association with BP was provided by mapping the $S_A$ locus on to the chromosome 1 linkage group (Lindpaintner et al. 1993), a well-characterized chromosomal region that contains several genes of potential relevance to cardiovascular function that is synthetic to the human chromosome 16, where the $S_A$ gene was also mapped (Samani et al. 1994). Although association between a polymorphism at the $S_A$ locus and hypertension has remained controversial (Iwai et al. 1994, Harrap et al. 1995), it has recently been reported that different alleles of the $S_A$ gene are associated with multiple risk factors including hypertriglyceridemia, hypercholesterolemia, obesity and hypertension (Iwai et al. 2002). A genetic polymorphism in the $S_A$ gene has also been related to BP and prognosis of renal function in patients with immunoglobulin A nephropathy (Narita et al. 2002). Finally, the $S_A$ locus has been found positively linked with loci regulating water and sodium metabolism and membrane ion transport in essential hypertension (Chu et al. 2002). Different reports on chromosome-transfer studies in congenic strains, to isolate chromosome regions that contain the BP quantitative trait locus (QTL) in the region around the $S_A$ gene from different rat strains, have also provided controversial data (St Lezin et al. 1997, Frantz et al. 1998, Iwai et al. 1998, Hübner et al. 1999, Saad et al. 1999, St Lezin et al. 2000, Frantz et al. 2001). In a recent paper, Walsh et al. (2003) have shown direct evidence of the lack of $S_A$ involvement in the regulation of either basal or salt-related BP in $S_A$–null mice, demonstrating that the absence of different BP in these animals is not the consequence of compensatory activation of the renin–angiotensin system.

The $S_A$-encoded protein is significantly homologous to bovine xenobiotic-metabolizing medium-chain fatty acid-CoA ligase (Vessey & Kelley 1997) and recent studies have identified the $S_A$ protein as a medium-chain acyl-CoA synthetase (MACS; Fujino et al. 2001a, 2001b). Acetyl-CoA synthetase (ACS; also called acetate-CoA ligase) is an enzyme of energy metabolism known to be present in mitochondria and responsible for acetate production accompanied by ATP generation. The $S_A$ and ACS genes probably derived from duplication of an ancestral gene, but acquired different functions (Karan et al. 2001) which are not completely understood for the $S_A$ gene. It remains important to understand the physiological function of this highly restricted tissue-specific gene to gain insight into the physiological effects of sexual steroids in the kidney. In this report, we further explored the tissue distribution and sex-steroid regulation of the mouse $S_A$ gene and compared them with the protein profile obtained using specific antibodies raised against $S_A$–derived synthetic peptides. Moreover, we determined the genomic organization of the mouse $S_A$ gene and identified its transcription start units. This work forms the basis for further study of molecular mechanisms that control the androgen-dependent and kidney-restricted expression of the mouse $S_A$ gene.

### Materials and Methods

#### Animals and treatments

C57BL/6, BALB/c and 129/SvJ mice were obtained from IFFA CREDO (L’Arbescle, France) at 6 weeks of age and housed in animal facilities as described elsewhere (Melìa et al. 1998). Male mice were castrated at the age of 8 weeks under droperidol and midazolam anesthesia and allowed to recover for 1 week post-surgery. Male and castrated mice were treated for 6 weeks with DHT and 17-β-estradiol (Sigma) with subcutaneous injections of 120 and 240 µg/day, respectively. Control mice received vehicle alone (95% sesame oil/5% ethanol). After treatment, animals were killed by cervical dislocation. Several tissues were collected and immediately frozen in liquid N$_2$.

#### RNA extraction and Northern blot analysis

Total RNA was extracted from different tissues using the guanidium thiocyanate/acid phenol method (Chomczynski & Sacchi 1987). Total RNA (15 µg) was electrophoresed in 6.5%-formaldehyde/1.4%-agarose gels, transferred to ZetaProbe membranes (Bio-Rad) and hybridized at 42 °C overnight with random-primed [$α$-32P]dCTP (Amersham Pharmacia Biotech)-labeled cDNA probes, washed following the membrane manufacturer’s instructions and exposed to Hyperfilm (Amersham Pharmacia Biotech). A probe corresponding to cDNA of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control on each hybridization. Where noted, band intensity was measured by densitometric scanning of the resultant autoradiograph using the Bio-Rad GS700 image densitometer and Molecular Analyst 1·40 program.

#### RT-PCR and Southern blotting

Total RNA from various tissues of male 129/SvJ mice was isolated using the total RNA preparation kit (Qiagen) and subjected to RT-PCR analysis. A total amount of 500 ng of each tissue was reverse-transcribed using specific primers (see Table 1) and the SuperScript One-Step system (Invitrogen) following the manufacturer’s instructions. RT-PCRs were performed under linear conditions with respect to RNA input and the number of amplification cycles. PCRs using SA1 and SA2 primers were determined as linear for 25 cycles and those performed with primers E1’, E1, E2, E3 and E4 were determined as linear for 35 cycles. Cyclophilin A was amplified as a control for RNA amount and integrity. Amplification products were separated on 2% agarose gel and transferred to ZetaProbe membranes (Bio-Rad). The blots were probed with specific random primed [$α$-32P]dCTP-labeled cDNA. Hybridization, washes and exposure were performed as above.
Amplified products were subcloned into the TopoTA cloning pCR2·1 vector (Invitrogen) and sequenced in both directions.

**Synthesis of riboprobes and in situ hybridization histochemistry**

$^{35}$S-labeled sense and anti-sense transcripts from a Bluescript plasmid containing 148 bp fragment of the mouse $S_A$ cDNA were prepared as previously described (Melià et al. 1998). Preparation of renal sections, hybridization protocol and autoradiographic analysis were all performed as reported (Meseguer & Catterall 1990, 1992).

**Primers**

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<th>Sequences</th>
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<tr>
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<tr>
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<td>Lower</td>
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<td>SA2</td>
<td>Lower</td>
<td>5’-gctgacAGTGATTAGTGGAGG-3’</td>
</tr>
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**Intron/exon mapping**

A 129/SvJ mouse genomic library Lambda FIX II vector (Stratagene) was screened with a 2·0 kb probe corresponding to mouse $S_A$ full-length cDNA. Briefly, approximately 500 000 independent clones were plated and transferred to nitrocellulose membranes (Duralose-UV, Stratagene, Saint Quentin en Yvelines, France). Prehybridization was carried out for a minimum of 2 h at 42 °C in the same hybridization buffer consisting of 50% formamide, 2 × Pipes, 0·5% SDS and salmon sperm DNA (100 µg/ml). $^{32}$P-random primed labeled probe was added to the hybridization solution (1 × 10$^6$ c.p.m./ml) and incubated overnight at 42 °C. The next day, filters were washed twice in 1 × SSC/0·1% SDS for 5 min at room temperature, followed by three high-stringency washes in 0·1 × SSC/0·1% SDS for 15 min at 65 °C. Plaque filters were then exposed to autoradiographic films (X-Omat; Kodak) at −70 °C for approximately 20 h. Positive plaques were identified and after four further rounds of purification of phage DNA, five genomic clones – designated $\lambda$SA1, $\lambda$SA2, $\lambda$SA3, $\lambda$SA4 and $\lambda$SA5 – were isolated. Genomic DNA from positive clones was isolated with the QIAGEN Lambda MiniKit and Maxi Kit. Double-stranded DNA was sequenced using ABI Prism Big Dye terminator chemistry (PE Applied Biosystems). Exon sizes were determined by nucleotide sequencing and intron sizes determined by either nucleotide sequencing or estimation from the size of the corresponding PCR-generated DNA fragments using exon-specific primers.

**Production of anti-$S_A$ polyclonal antibodies**

A short peptide, pSA, corresponding to amino acids NH$_2$-CGNFKGMIKPGSMGK-COOH from position 380 to 395, was selected on the basis of its putative immunogenicity and synthesized in the Servei de Síntesi
Western blot analysis

Tissues were homogenized by N2 cavitation in RIPA buffer (0.5% Na-deoxycholate, 1% Nonidet P-40, 0.1% SDS and protease inhibitors in 1× PBS). For Western blot analysis, samples were normalized for protein concentration using the Bradford assay (Bio-Rad), adjusted for equal protein levels, and separated on 10% polyacrylamide gel electrophoresis under denaturing conditions. Proteins were transferred to PVDF (Shleicher & Schuell) membranes and blots blocked overnight at 4 °C in 5% non-fat dried milk in PBS. Primary polyclonal antibodies were tested at different concentrations; the best results were obtained with the 261 antiserum diluted at 1:350 in blocking buffer. Washes were performed following the membrane manufacturer’s instructions and secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit; Dako A/S), diluted 1:5000, incubated for 1 h at room temperature. After washing, bands were detected using the ECL+ chemiluminescence detection method (Amersham Pharmacia Biotech) and exposed to Hyperfilm.

Results

Androgen-dependent expression and cell specificity of the S_A gene in different mouse strains

An initial report from our laboratory first described the mouse counterpart of human and rat S_A genes (GenBank accession number AF068246) on the basis of their profound androgenic regulation, at the mRNA level in mouse kidney (Melià et al. 1998). Since S_A gene expression in rat kidney was not completely prevented by castration (MJ Melià & A Meseguer, unpublished observations), we wondered whether the strong androgenic control observed in mice was a general phenomenon in mice or an isolated event occurring in the C57BL/6 strain used. Northern blot assays of kidney RNA from castrated and control C57BL/6, 129/SvJ and Balb/c male mice showed that S_A expression is not completely abolished in castrated males but perhaps only undetectable using Northern blot analysis (Fig. 1A). Moreover, in situ hybridization of frozen kidney sections using sense- and antisense-specific probes demonstrated that this gene is expressed in epithelial cells of the early (S1 and S2) and late (S3) segments of proximal convoluted tubules (Fig. 1B), as determined by periodic acid shift counterstaining (results not shown). S_A mRNA was first located in the rat proximal tubule by Samani’s group using in situ hybridization (Patel et al. 1994) and by Yang et al. (1996) using RT-PCR in cDNAs prepared from microdissected nephron segments. While in intact mice the first report on S_A mRNA location was made by Takanaka et al. (1998), we demonstrate here that castration prevents expression in all segments of the tubules and DHT replacement restores the expression in castrated mice.

Distribution and androgen regulation of S_A mRNA in mouse tissues

Although tissue specificity of S_A mRNA has previously been stated (Melià et al. 1998), the more-sensitive
RT-PCR/Southern blot technique was used and distribution of SA mRNA determined in a wider panel of tissues including kidney, liver, brain, stomach, prepucial gland, duodenum, spleen, testis, lung and heart. Results in Fig. 2A, showing a saturated image on X-ray film after 3 h exposure (left-hand panel), corroborate the concept that kidney and liver tissues are those where the SA gene is expressed preferentially. Moreover, expression in stomach, testis, lung and heart was also detected on overnight exposure of the autoradiographic film (Fig. 2A, right-hand panel), and was completely neglected in brain, duodenum and spleen. While Northern blot assays were sensitive enough to detect expression in liver, testes and brain in rat tissues (Iwai and Inagami 1991, Kaiser et al. 1994), no expression in brain was observed in mice by RT-PCR. Although kidney and liver remain the preferential expression sites for the SA gene, our results demonstrate that it exhibits tissue distribution wider than that reported previously in mice (Melià et al. 1998). Some assays performed in castrated male mice indicate that the SA gene appears to be under androgenic control not only in kidney but also in heart and lung. The liver and stomach appear to express the gene in an androgen-independent manner (Fig. 2B).

Estrogenic effects on SA expression in kidney and liver

We aimed to determine whether estrogens could also exert an effect on SA gene expression. To do so, we performed Northern blot assays using total RNA from kidneys and livers of mice treated with DHT, estrogens or both hormones simultaneously and compared SA expression levels with those obtained in untreated control animals. Results were normalized with the endogenous

Figure 1 (A) Northern blot analysis of SA mRNA in kidneys of different inbred mouse strains. Total RNA (15 μg) from intact (N) and castrated (Cx) male kidneys of C57BL/6, 129/SvJ and BALB/c strains was electrophoresed and transferred to nylon membranes. Hybridization was performed with mouse SA cDNA under the conditions described in the Materials and Methods section. A mouse cyclophilin A probe was used as an internal control for loading and integrity of RNA. A single 3·0 kb transcript was significantly detected only in intact males in all three mouse strains tested. (B) In situ hybridization analysis of SA mRNA in mouse kidney. Longitudinal kidney sections from castrated males treated with pharmacological doses of DHT (a and b) and castrated males (c and d) were hybridized with strand-specific 35S-labeled RNA probes (antisense, a and c; sense, b and d). After hybridization, sections were exposed to photographic emulsion for 4 weeks. After developing, slides were mounted and examined under a light microscope. The magnification chosen (× 25) allows visualization of the three major compartments of the kidney (c, cortex; o, outer medulla; m, inner medulla; see A) and determination of the spatial location of SA mRNA. Kidney sections shown in the panels were each analyzed in a single experiment. Consequently, slides were exposed to the same conditions throughout the procedure, and levels of SA mRNA expression on different slides were comparable. Photomicrographs shown in this figure were selected from similar results obtained in different experiments using animals from different litters.

Figure 2 Tissue distribution of SA mRNA expression. (A) Expression profile of SA in intact male mouse tissues analyzed by RT-PCR. Total RNA (500 ng) from various murine tissues (indicated at the top) was reverse-transcribed and PCR-amplified using specific SA primers (SA1 and SA2; see Table 1). As an internal control, mouse cyclophilin A (CypA) primers were used in (B). Expression profile of SA in castrated male mouse tissues analyzed by RT-PCR, under the same experimental conditions described in (A).
control GAPDH gene and densitometric analysis performed in non-saturated X-ray films. The SA/GAPDH ratios expressed in arbitrary units are depicted at the bottom of Fig. 3A. The same treatments and assays were also made in castrated male mice (Fig. 3A). Results from these experiments revealed that pharmacological doses of DHT can induce further expression of the SA gene in untreated control male mice and that levels in castrated males are restored upon treatment, which indicates that the gene responds to androgens in a dose-dependent manner. Estrogenic treatment of control males or DHT-induced intact male mice resulted in a very drastic down-regulating effect on SA mRNA expression, even in the presence of pharmacological doses of androgens (Fig. 3A). Expression of SA mRNA in liver was completely independent of steroid hormones, as neither castration nor induction with pharmacological doses of DHT and/or estrogens modified the levels attained by control male mice (Fig. 3B). Since estrogens exerted a powerful negative effect on SA kidney expression, we wondered whether an estrogenic-dependent repression was responsible for the lack of expression in female kidney (Melià et al. 1998).

Ovariectomized females failed to express the SA gene in kidney (Fig. 3C) and respond to DHT stimulation (Fig. 3D), indicating that although estrogens can repress the gene, its kidney expression is fully androgen-dependent. Previous data from our laboratory demonstrated that the effects are mediated by the androgen receptor since...
flutamide-treated mice do not express the gene (Melià et al. 1998). These results indicate that there are tissue-specific mechanisms underlying SA expression and, therefore, that this gene constitutes an excellent model for understanding the basis of androgen regulation of specific gene expression in kidney.

**Genomic organization of the mouse SA gene and identification of different SA transcriptional units**

As an initial approach to elucidating the mechanisms regulating SA gene expression, we identified genomic clones through screening a mouse genomic library, using its full-length cDNA as a probe and characterized transcription units of the mouse SA gene. Several positive clones were isolated, cloned until homogeneity and sequenced with specific primers derived from the cDNA sequence of the gene. The genomic structure and intron/exon organization of the mouse SA gene are shown in Fig. 4 and Table 2, respectively. The gene spans approximately 23 kb and consists of 16 exons and 15 introns (Fig. 4) and has been annotated at mouse chromosome 7 (ENSMUSG00000030935) at the Ensemble Genome Browser. The translation initiation site is present in exon 4. Exon sizes range from 69 to 274 bp, with the exception of exon 16, which is 616 bp and contains the TAG stop codon and 3′ untranslated region, including the polyadenylation signal. The size of the introns was determined by either direct DNA sequencing or long-distance PCR with exon-specific primers; in some cases, alignment with mouse genomic traces from the mouse genome sequencing database was also used to verify and determine the length of some introns. All exon/intron boundaries conform to canonical splice donor and acceptor consensus AG–GT sequences (Mount 1982). The transcription initiation site of the SA gene was mapped by primer extension and 5′-RACE (rapid amplification of cDNA ends). For primer extension, a pair of reverse primers was tested (E3 and E4; see Table 1), complementary to the third and fourth exons, respectively. Primer E3, situated 89 nucleotides from the translation initiation ATG codon, rendered three products of 132, 167 and 259 nt which indicated the existence of three transcription start sites, with the smallest being the most prominent, mainly in the 129/SvJ strain (Fig. 5A). Results were confirmed using primer E4, which gave a single product of 240 bp corresponding to the 132 site obtained with E3. Results were the same in both mouse strains. From these experiments we located three major potential transcription start sites at 221, 256 and 348 bp upstream from the translation initiation ATG codon. The right-hand panel of Fig. 5A depicts the transcriptional units obtained by primer extension that were further confirmed by 5′-RACE. Sequencing of the products revealed that the previously cloned cDNA (Accession number AF068246) corresponds to the 167 product; the 259 band includes 35 bp from the 5′ site of

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**Table 2 Genomic organization of murine SA gene**

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**Figure 4 Genomic structure of the murine SA gene.** Schematic representation of the SA gene. Exons (numbered 1–16) are represented by boxes and oblique-horizontal lines indicate intronic regions (numbered I–XV). White and black boxes represent untranslated and protein-coding regions, respectively.
Figure 5  Identification of the transcriptional start sites of the mouse $S_A$ gene. (A) The nucleotide sequence flanking the transcription initiation sites is illustrated on the left, with transcription initiation sites obtained by primer extension (arrowheads). The arrows represent the major transcription start sites common to C57BL/6 and 129/SvJ mouse strains as mapped by primer extension, obtained using primers E3 and E4 and mouse kidney total RNA as a template. The sizes of the extended bands are indicated. For further details see the Materials and Methods section. The right-hand side of the figure shows the three major forms and the nature of the exons included. (B) RT-PCR amplification of the mRNAs of mouse kidney $S_A$. PCR products were obtained using six sets of primers, E1–E2, E1–E3, E1–E4, E1–E2, E1–E3 and E1–E4. Representative agarose gels of PCR products obtained with the six sets of primers are shown at the top of the figure. M indicates the molecular DNA markers used (M is 100 bp and #M is 1 kb ladder markers from Invitrogen). A schematic representation of the possible origin of PCR products appears at the bottom. Arrows indicate the annealing regions of the PCR primers, lines above indicate the sizes for the different moieties obtained upon cloning and sequencing of the PCR products. (C) Schematic representation of the splicing cryptic sites found in exons 1 and 2 of the $S_A$ gene.
exon 1 (also present in the 167 and named for convenience 1') and an additional 92 bp situated further down from 1' that complete the entire exon 1. This new form was also deposited in the GenBank database under accession number AY064696.

Apart from the transcripts obtained in our laboratory, other authors identified a new unit of S_A cDNA, which included 208 bp of exon 2 and no sequences from exon 1 (accession number BC015248). At that point, we aimed to determine whether other S_A mRNA products could also exist in our kidney mRNA samples. By performing RT-PCR assays using different sets of primers, indicated at the top of each gel (Fig. 5B, upper panel), we obtained a variety of products that upon cloning and sequencing revealed an even more complex organization of the 5' untranslated region of the S_A gene. There are two forms on the lower panel of Fig. 5B that correspond to the 167 and 259 fragments of Fig. 5A (marked with an asterisk). Both forms have skipped exon 2 and contain either the full exon 1 (1'-1'), or exon 1' Apart from these forms, we obtained four new products which show different combinations of exons 1, 2, 1' and 2' (the latter corresponds to the 63 bp from the most 3' part of exon 2), followed by exons 3 and 4. These splicing variants are generated from canonical splice donor and acceptor consensus AG–GT sequences located in exons 1 and 2 of the S_A gene that define the boundaries between exon 1'-1 and exon 2-2' (Fig. 5C). By comparing the genomic structure and intron/exon organization of the mouse S_A gene (see Fig. 4 and Table 2) with rat S_A genomic sequences (GenBank accession number AY456695) we have observed as the main difference that the rat gene is lacking mouse exon 2. It contains, therefore, 15 exons instead of 16, which share 86–98% homology with the mouse sequence. The translation initiation site and the stop codon are located at exons 3 and 15, respectively. Moreover, rat exon 1 lacks what we have named exon 1' in mouse; the remaining 65 bp of rat exon 1 share 95% homology with the 3' end of mouse exon 1. The main differences between the rat and mouse S_A genes correspond to the 5' region, before the translation initiation site, which indicates that both proteins will be very similar.

Location and hormonal control of the S_A protein

Polyclonal antibodies raised against S_A-specific peptides revealed the appearance of four different molecular species by Western blot assays (Fig. 6). From them, the 62 and 118 kDa products disappeared in castrated males and appeared in DHT-treated castrated mice, indicating that their expression is androgen-dependent (Fig. 6A, left-hand panel). Assays performed in the presence of specific S_A-blocking peptide showed that the 62 kDa protein disappears, while the other three remain under this condition (Fig. 6A, right-hand panel). We postulate that the protein of apparent molecular mass 62 kDa corresponds to S_A because (i) 62 kDa is close to the expected size of the deduced S_A protein, taking the ATG codon in exon 4 as the translation initiation codon, (ii) it disappears in the presence of the specific blocking peptide, (iii) its expression is androgen-dependent and (iv) the anti-S_A antibody recognizes the same moiety as the anti-FLAG antibody in cells transiently expressing the S_A–FLAG fusion protein (results not shown).

To further explore whether expression of the protein parallels that of the mRNA, we studied the effects of estrogen treatment on S_A protein levels. As shown in Fig. 6B, estrogens inhibit expression at physiological and pharmacological doses of androgens, i.e. in control males and in DHT-induced castrated males. In conclusion, the protein follows the same sex steroid-dependent expression pattern as its corresponding mRNA. The nature of the 118 kDa product which follows an androgen-dependent but estrogen-independent pattern of expression is unknown and might not necessarily be related to the S_A protein. The mouse S_A protein presents high homology with the human and rat S_A, 86 and 94%, respectively. Alignment of the three sequences (Fig. 7) reveals several functional motifs that include 29 amino acids on the N-terminal region, which corresponds to a mitochondrial translocation signal (see boxed sequence in Fig. 7) and an AMP-binding domain, according to the PROSITE

![Figure 6](image-url) Western blot analysis of crude homogenates from mouse kidney. (A) Crude kidney homogenates (100 μg/lane) from intact non-treated males (NT), castrated (Cx) or castrated males treated with pharmacological doses of DHT (Cx+DHT) were analyzed by Western blot using rabbit antiserum raised against S_A synthetic peptide (left-hand panel). Preabsorbing the antibody with an excess of the antigenic peptide proved the specificity of the reactions (right-hand panel). Arrow points to the 62 kDa apparent-molecular-mass product that corresponds to the S_A protein. (B) In this experiment, crude kidney homogenates from intact or castrated males treated with estrogens (E2) or with estrogens and DHT (DHT+E2) were included. Non-treated female kidney homogenates were also studied using the experimental approach described for (A).
program (see underlined sequence in Fig. 7). The differences between the 65.5 kDa predicted molecular mass of the mouse SA protein and the estimated 62 kDa size found in Western blot assays suggested that the predicted mitochondrial targeting signal is cleared during transportation of the enzyme into the mitochondrial matrix. SA-FLAG expression vectors were transfected into PCT3 cells and location of the fusion protein was determined by immunocytochemistry using anti-FLAG antibodies. By performing double-labeling confocal fluorescence imaging, using the mitochondrial marker dye MitoTracker Red in transfected SA-FLAG cells, we were able to co-locate both fluorescence signals, demonstrating the location of the SA protein in the mitochondria (Fig. 8).

Discussion

The mouse $S_A$ gene was first described in our laboratory as a kidney-restricted androgen-dependent gene (Melià et al. 1998). By using the subtractive technique of representative differential analysis of cDNA, we aimed to find informative models for studying the nature of tissue-specific gene regulation in kidney. Furthermore, we were also hoping that new target genes might provide insight into the largely unknown physiological significance of androgen-inducible gene expression in the kidney. Apart from $S_A$, other coordinately expressed genes belonging to the organic-anion-transporting (OATP) and cytochrome (Cyp4) families were also identified and characterized (Isern et al. 2001, Isern & Meseguer 2003). As mentioned above, the $S_A$ gene has been characterized as a proposed candidate gene for essential hypertension in rat and humans (Iwai & Inagami 1991, Harris et al. 1993, Lindpaintner et al. 1993, Iwai et al. 1994, Harrap et al. 1995, Nabika et al. 1995). Its highly restricted and abundant expression in kidney, a key organ in BP regulation, suggested that the $S_A$ gene might be involved in a
metabolic pathway with regulatory effects on renal vascular resistance, impaired renal hemodynamics and hypertension. A large subset of human hypertension is sexually dimorphic, i.e. more severe in males than in females, with the differences being minimized after menopause (Mantzoros et al. 1995, Chen 1996, August 1999, Garbers & Dubois 1999, Reckelhoff & Granger 1999). In the spontaneously hypertensive rat model, a sexual dimorphism in BP has also been observed (Chen & Meng 1991, Turner et al. 1991, Phillips et al. 1997, Reckelhoff et al. 1999). Despite the scarce information on sex-related BP differences in mice, a recent report from Holla et al. (2001) described lower BPs in female than in age-matched male mice in controls and in the hypertensive phenotype of Cyp4a14-knockout mice, which these authors have developed (Walsh et al. 2003). Despite this evidence Samani’s group have clearly shown that SA is not involved in the regulation of either basal or salt–related BP using SA-null mouse.

Predictions based on its amino acid sequence similarity included the SA protein in the acyl/acetyl-CoA synthetase family (Karan et al. 2001). Later studies confirmed a medium-chain acyl-CoA synthetase nature for the SA protein by means of enzymatic assays using a purified recombinant mouse SA protein heterologously expressed in COS cells. Two reports demonstrated that the SA protein plays a role in the degradation of medium-chain fatty acids for the production of energy. While Fujino et al. (2001b) concluded that isobutyrate constitutes a specific substrate for SA, Iwai et al. (2002) described octanoate as the preferred substrate for CO2 and ATP production. While these reports address a putative function for the SA protein, it remains to be determined what the real substrates and function of this protein are in vivo.

In this report we confirm previous data referring to kidney as the main site for SA mRNA synthesis followed by liver, but also other sites for minor SA production including stomach, testis, lung and heart. Of these, kidney, heart and lung express SA mRNA in an androgen-dependent fashion, indicating that SA constitutes a specific male enzyme for most of the tissues in which it is expressed and that its function necessarily be important for males. Interestingly, we found a profound negative effect of estrogens on SA kidney mRNA levels since they block the action of androgens at physiological and pharmacological doses. Since ovariectomy in females does not permit SA expression in kidney, and DHT-induction triggers the SA gene in females, we conclude that it is a

Figure 8 Subcellular location of the recombinant SA protein. Transfected SA-FLAG fusion protein in PCT3 cells was labeled with anti-FLAG antibodies and recombinant protein visualized with FITC-conjugated goat anti-mouse secondary antibody (SA-Flag). MitoTracker Red CMXRos was used to stain PCT3 mitochondria (MitoTracker) and analyzed using a confocal microscope. Green fluorescence signals for the recombinant SA protein were overlapped with the red fluorescence signals for mitochondria (overlay).
truly androgen-dependent gene. In order to explain the inhibitory role of estrogens in $S_A$ expression, we might speculate as to the presence of a common co-activator for sex steroid receptors, in proximal tubule cells, which becomes unavailable to the androgen receptor in our experimental conditions; alternatively, a newly synthesized estrogen-dependent repressor might be interfering with the mechanisms triggered by androgens, precluding expression. In any event, isolation and functional assays of the proximal promoter of the $S_A$ gene will provide insight into the elements and mechanisms governing the sex steroid-controlled expression of the $S_A$ gene in kidney and those that permit constitutive expression of the same gene in liver. To this end, we first determined the transcription initiation site by primer-extension analysis, 5'-RACE and RT-PCR, and found multiple forms of $S_A$ mRNAs which upon cloning and sequencing appeared to be the result of complex alternative-splicing events which included usage of 19 cryptic internal sites in exons 1 and 2. Although we cannot rule out trans-splicing events, there is no exon repetition that could indicate that this phenomenon is occurring in the mouse $S_A$ gene, as has been described for its rat orthologue (Frantz et al. 1999) and the rat carnitine octanoyltransferase gene (Caudevilla et al. 1998). As for the rat $S_A$ gene, Frantz et al. (1999) reported exon 2 and exon 2–4 repetition in kidney mRNA from Wistar–Kyoto rats not present in the spontaneously hypertensive strain, which was shown not to correspond to duplications of these specific exons or to the entire gene in the Wistar–Kyoto germ line. Exon 2 is located upstream of the putative translation start site and therefore the presence of the duplication would not be expected to alter the protein product. However, the exon 2–4 duplication would alter the reading frame, resulting in a truncated, altered product of 157 amino acids. Although the physiological significance of these modified transcripts has not been established, the transcripts have also been detected in Milan hypertensive and Dahl salt-sensitive rat strains (Frantz et al. 1999). In mouse, we found no alternative transcript of the $S_A$ gene compromising the putative translation initiation site which has been predicted to be in exon 4; Western blot analyses of mouse kidney extracts show that the single moiety that disappears upon blocking the antibody with the specific peptide corresponds to a product with the expected $S_A$ protein size. This result indicates that the splicing events occurring further up exon 4 have no impact on the correct synthesis of the $S_A$ Protein. The biological role, if any, of our findings is unknown but might relate to the use of alternative promoters which might be located on the intronic sequences before exon 4 and upwards, which in turn could contribute to the differential regulation of the gene in kidney and liver. Studies currently being conducted in our laboratory using different reporter gene constructs in transient transfection assays may aid understanding of the complexity of $S_A$ gene expression in mouse tissues.

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