Molecular and Cellular Endocrinology 332 (2011) 228–233

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Functional expression of angiotensinogen depends on splicing enhancers in exon 2

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ARTICLE INFO

Article history:
Received 25 August 2010
Received in revised form 21 October 2010
Accepted 21 October 2010

Keywords:
Angiotensinogen
Exonic splicing enhancer
Alternative splicing

ABSTRACT

Angiotensinogen belongs to the family of serpins and is the only precursor of the potent cardiovascular peptide, angiotensin II, the main effector of the renin–angiotensin system. The gene coding for this protein carries an internal exon (exon 2), the length of which (859 bp) by far exceeds the mean length of internal exons in vertebrates (<300 bp). Here, we show that this essential exon is skipped in about 20% of all transcripts in liver, brain, and kidney of rats and mice. Deletion mutants of exon 2 revealed a 62 bp region located at its 5′-end which is important for its inclusion in the mature angiotensinogen mRNA in transfected COS7 cells. Using an artificial minigene, we defined sequences inside this region as exonic splicing enhancers. These data reveal a novel molecular mechanism important for the renin–angiotensin system with implications in the basic understanding and the therapeutical assessment of cardiovascular diseases.

1. Introduction

Splicing is an essential process in eukaryotic gene expression which starts in the cell nucleus with the transcription from DNA to an mRNA precursor (pre-mRNA). During transcription, introns are removed while exons are joined together (Matlin et al., 2010; Licatalosi and Darnell, 2010). For this process, consensus sequences at the 5′ end (donor splice site, AG/GURAGU) and 3′ end (acceptor splice site, CAG/G) of introns, as well as a preserved polypyrimidine tract and a degenerate branchpoint sequence located at about 20–50 nucleotides (nt) preceding the 3′ splice site are identified by the spliceosome machinery composed of 5 small nuclear (sn)RNAs (U1, U2 and U4–U6) and a multitude of proteins. Alternative splicing of exons generates different mRNAs from the same pre-mRNA, consequently often also leading to multiple protein isoforms (Matlin et al., 2010). Regulatory elements for splicing which select exons for inclusion in the final mature mRNA can be secondary structures around splice sites, specific sequences so called exonic (ESE) or intronic (ISE) splicing enhancers and exon length (Cartegni et al., 2002; Singh and Valcarcel, 2005). Serine/arginine-rich (SR) proteins bind to such elements to facilitate exon identification and promote splicing (Wahl et al., 2009).

Mammalian exons show a wide variety of lengths but while the first and ultimate exon can easily contain several kbs, internal exons are very limited in their maximal size (Robberson et al., 1990). Very few of the mammalian pre-mRNAs contain internal exons which exceed the length of 300 nt. Abnormal length of an exon can hamper its inclusion in the mature mRNA (Berget 1995; Humphrey et al., 1995).

One of the very few examples of an mRNA with a very long internal exon is the transcript coding for angiotensinogen. Angiotensinogen is a glycoprotein of the serpin family mainly produced in the liver. It is the natural substrate for renin required for the production of all active peptides of the renin–angiotensin system. The renin–angiotensin system is one of the most important cardiovascular hormone systems and a major target for drugs against cardiovascular diseases (Bader, 2010). Furthermore, it has been shown that the local production of angiotensins is involved in numerous other physiological processes in tissues, such as the pancreas (Leung, 2007a,b) and in brain (Bader, 2010).

The gene coding for angiotensinogen is composed of 5 exons and 4 introns (Fig. 1). Exon 2 is the longest exon (859 nt) and contains the translational start codon, the signal peptide and the angiotensin I coding sequences (Clouston et al., 1988). Notably, nearly all serpin genes carry internal exons, the length of which by far exceed

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doi:10.1016/j.mce.2010.10.016

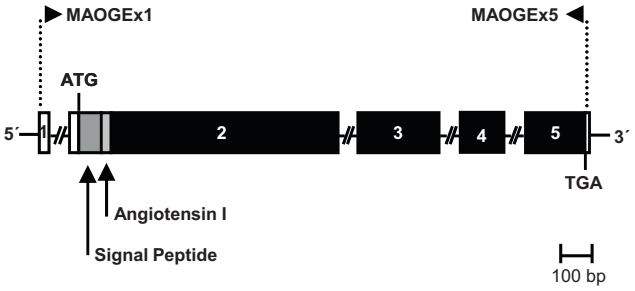

Fig. 1. Schematic drawing of the mouse angiotensinogen gene. Numbers denote the exons. The location of the start and stop codons as well as the signal peptide and the angiotensin I encoding sequences are indicated. Filled and open boxes refer to the coding and non-coding sequences, respectively. Thin broken lines between the boxes represent the intronic regions. Primers mapping to exon 1 (MAOGEx1, ATAGCTGTGCTTGTCTAGGTT) and exon 5 (MAOGEx5, CACCGGCCTTGTCTCCATGGC) were used to amplify the mouse angiotensinogen gene by long-range PCR.

the normal length of internal exons in other mammalian genes (50–300 bp) (Van Gent et al., 2003; Ragg et al., 2009).

Here, we show that a splice enhancer sequence in exon 2 of the angiotensinogen gene is important for its inclusion into mature mRNA.

2. Materials and methods

2.1. Cloning of the mouse angiotensinogen gene

The whole mouse angiotensinogen gene was amplified by long range-polymerase chain reaction (LR-PCR) from mouse genomic DNA. The reaction was carried out using primers mapping to exon 1 (MAOGEx1, ATAGCTGTGCTTGTCTAGGTT) and exon 5 (MAOGEx5, CACCGGCCTTGTCTCCATGGC) in the presence of the thermostable Taq- and Pwo-polymerases and buffer 3 provided in the Expand Long Template PCR System kit (Roche Diagnostics, Mannheim, Germany). The PCR conditions were as previously described (Cayla et al., 2002) with an annealing temperature of 58 °C. The fragment was cloned into an expression vector (pcDNA 3.1 (+), Invitrogen). The final construct was verified by sequencing and named pmAOG.

2.2. Generation of deletions in pmAOG

The construct pmAOG was linearized with Sfi I for 1 h at 50 °C. Two micrograms of DNA were digested at 37 °C for 10 min using the endo-/exonuclease Bal 31 (1 U/μl). After inactivation of the enzyme with EDTA (50 mM), the DNA was precipitated by sodium-acetate. The DNA endings were filled with T4 polymerase in the presence of dNTPs, creating blunt ends. By this method, it was possible to generate deletions of different size at the same site in exon 2 of the angiotensinogen gene. After re-ligation of the blunt ends, bacteria were transformed with the different size constructs; colonies of bacteria were picked and grown in LB medium at 37 °C for 12 h. DNA extraction from 2 μl of each bacterial colony was performed by adding TE buffer and incubating at 95 °C for 5 min. The length of the deletions in exon 2 was analyzed by PCR with specific primers, (MMANG25, GGACACACAGAAGCAAATGC) and (MMANGI23, CCACTCCGTTTGTGAACCCA) and Taq polymerase (Invitrogen, Germany). The PCR was performed with 36 cycles of denaturation at 95 °C (45 s), annealing at 55 °C (30 s) and extension at 72 °C (1 min). The PCR products were visualized on a 1% agarose gel. After sequencing, 7 different clones with deletions between 56 and 677 bp in exon 2 were selected for further analysis.

2.3. Splicing assay

COS7 cells (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 1 mg/ml glucose (Invitrogen, Germany) supplemented with 10% fetal calf serum, 100 IU/ml penicillin/streptomycin in an incubator with constant temperature (37 °C) and CO_2 level (5%). When the cells reached 80% confluence they were transfected with 20 μg of DNA using the calcium-phosphate co-precipitation method (Pesquero et al., 1994). 72 h later, total RNA was extracted from the cells using TRIzol reagent (Invitrogen) followed by chloroform–isopropanol extraction as previously described (Pesquero et al., 1994). The samples were resuspended in RNase-free water and kept at −80 °C until use.

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

cDNA was generated using 2 μg of total RNA, random hexamer primers and the MMULV reverse transcriptase enzyme (Invitrogen) as described (Silva et al., 2000). The PCR was performed using 10 μl of the RT reaction with Taq polymerase and the primers MAOGEx1 and MAOGEx4 (ACTCGGGGGTTGGTGTCACC) for both, mouse and rat angiotensinogen mRNA.

2.5. Ribonuclease protection assay (RPA)

A mouse angiotensinogen cDNA probe containing 11 nt of exon 1, the whole exon 3 and two nucleotides of exon 4 was amplified by RT-PCR from mouse liver RNA resulting in a 281 bp cDNA that was cloned into a pGEM-T vector (Promega). The plasmid was linearized with SpeI and transcribed in vitro with T7 polymerase in the presence of [α^{32}P]-UTP using a RNA-transcription kit (Stratagene, La Jolla, USA). The probe was used for RPA in an RPA II kit (Ambion, Austin, USA) as previously described (Schinke et al., 1999). Briefly, 20 μg of total RNA from the transfected cells were hybridized with 40,000 cpm of the radiolabeled probe. Fragments protected from RNase A and T1 digestion were separated on a 5% acrylamide/8 M urea gel. The dried gel was exposed to a radiosensitive plate and analyzed using a Fujix BAS 2000 phosphoimager system (Fuji, Düsseldorf, Germany). The expression ratio between spliced and full-length protected fragments was calculated according to the band intensity values obtained by image analysis using the software program TINA version 2.08c.

2.6. Human β-globin minigene construct

Two fragments of the human β-globin gene were amplified by PCR in order to generate a minigene construct for in vivo splicing assays (Xu et al.,

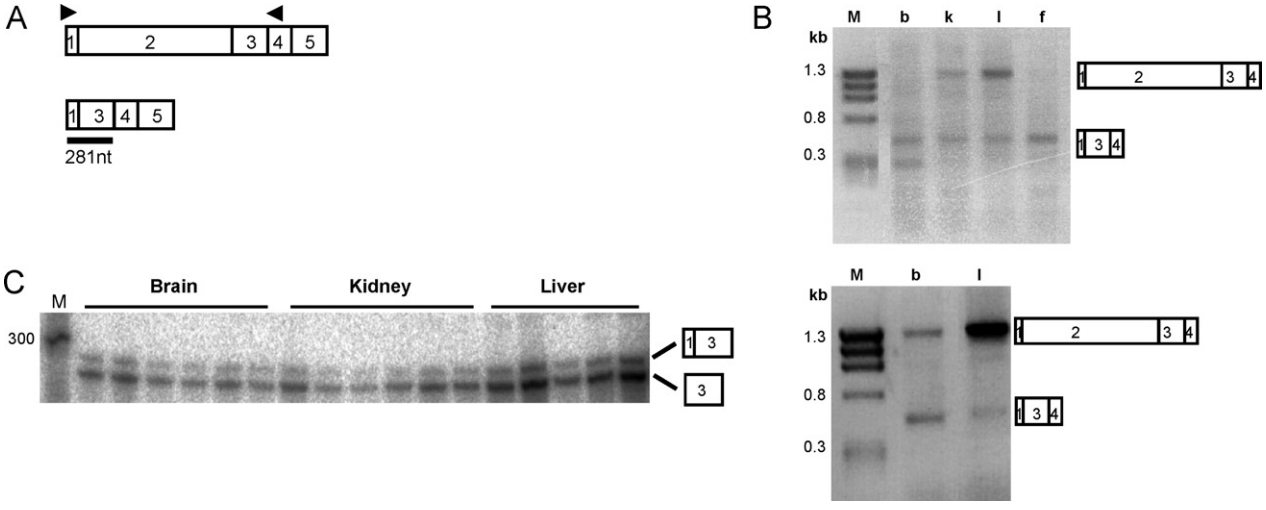

Fig. 2. Angiotensinogen expression analysis in mouse and rat tissues. (A) Scheme of the mouse angiotensinogen cDNAs with and without exon 2. Arrow heads indicate sense and antisense primers used in the RT-PCR. The probe of 281 nt for the ribonuclease protection assay (bar) maps to exons 1 and 3. (B) Reverse transcriptase-polymerase chain reaction on total RNA from different rat (upper panel) and mouse (lower panel) tissues. Marker (M, ΦX174/HaeIII). (C) Ribonuclease protection assay of total RNA from different mouse tissues (n = 5–6) with the 281 nt probe. Marker (M), brain (b), kidney (k), liver (l), fat (f).