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Cloning and expression of hepatic synaptotagmin 1 in mouse ☆

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ABSTRACT

Mouse hepatic synaptotagmin 1 (SYT1) cDNA was cloned, characterized and compared to the brain one. The hepatic transcript was 1807 bp in length, smaller than the brain, and only encoded by 9 of 11 gene exons. In this regard, 5′-and 3′-untranslated regions were 66 and 476 bp, respectively; the open reading frame of 1266 bp codified for a protein of 421 amino acids, identical to the brain, with a predicted molecular mass of 47.4 kDa and highly conserved across different species. Immunoblotting of protein showed two isoforms of higher molecular masses than the theoretical prediction based on amino acid sequence suggesting posttranslational modifications. Subcellular distribution of protein isoforms corresponded to plasma membrane, lysosomes and microsomes and was identical between the brain and liver. Nonetheless, the highest molecular weight isoform was smaller in the liver, irrespective of subcellular location. Quantitative mRNA tissue distribution showed that it was widely expressed and that the highest values corresponded to the brain, followed by the liver, spleen, abdominal fat, intestine and skeletal muscle. These findings indicate tissue-specific splicing of the gene and posttranslational modification and the variation in expression in the different tissues might suggest a different requirement of SYT1 for the specific function in each organ.

1. Introduction

Synaptotagmins (SYTs) constitute a family of membrane trafficking proteins that are characterized by an N-terminal, a variable linker, and two C-terminal C_2-domains, C_2A and C_2B (Südhof and Rizo, 1996), designed to bind Ca^{2+} and phospholipids (Südhof, 2013), both with a considerable structural similarity with the C_2 domain of protein kinase C (Perin et al., 1991). SYTs are recognized to participate in the docking and fusion of membrane vesicles in neuronal and nonneuronal cells including macrophages (Czibener et al., 2006), insulin-secreting β-cells (Hu et al., 2008), osteoblasts, and osteoclasts (Zhao et al., 2008), and airway and gastric mucin-secreting goblet cells (Davis and Dickey, 2008).

Synaptotagmin 1 (SYT1) was identified using a monoclonal antibody screening for synaptic proteins (Matthew et al., 1981). It is a 65 kDa integral membrane protein of synaptic vesicles and secretory granules in neuronal cells which serves as a Ca^{2+} sensor in synaptic exocytosis (Tucker and Chapman, 2002), it is also involved in the endocytic process, interacting with the adaptor protein 2 complex via the C_2B domain (Zhang et al., 1994; Jarousse and Kelly, 2001) before membrane internalization. The mechanism by which SYT1 performs its function in the brain is known (Kiessling et al., 2013), but recently SYT1 seems to have an important role in other tissues. In the intestine, SYT1 appears to be expressed in the apical membrane and subapical region of intestinal epithelial cells. The role of SYT1 in regulating the apical membrane Na+–H+ exchanger (NHE3) was demonstrated in Caco2BBE cells (Musch et al., 2007) and the cAMP stimulation promotes direct binding of NHE3 with SYT1 directing it to the adaptor protein 2-clathrin complex, which is necessary for regulated endocytosis in intestinal epithelial cells (Musch et al., 2010). Also intestinal *Syt1* was modulated by conjugated linoleic acid (CLA) supplementation that might have a role on mucosal immune responses in early life (Selga et al., 2011). The pancreatic and duodenal homeobox 1 stimulates insulin secretion in response to high glucose through the positive induction of *Syt1* expression (Nakajima-Nagata et al., 2004). SYT1 was found to be localized on the insulin secretory granules and plays an essential role in insulin vesicle exocytosis through its Ca^{2+} dependent phospholipid-binding activity (Jacobsson et al., 1994; Lang et al., 1997). In kidneys, it also appears to participate in the regulation of podocyte homeostasis (Rastaldi et al., 2006). Renal podocytes possess structures similar to synaptic vesicles, glomerular podocytes express *Syt1* gene product and it seems to be associated with the serum creatinine regulatory processes, the most important biomarker for a quick non-invasive assessment of kidney function (Pattaro et al., 2010).

Using nutritional (Western diet enriched in the c9, t11-CLA or t10, c12-CLA isomers or chow diets containing 10% olive oil) and genetic

Abbreviations: RT-PCR, reverse transcriptase-polymerase chain reaction.

☆ The nucleotide sequence reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number FR827897.

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(*Apoe*- and *Cbs*-deficient mice, these last hyperhomocysteinemic) models of hepatic steatosis in mice together with microarray analysis and its confirmation by qPCR, our group found that *Syt1* gene was involved in fatty liver development (Guillén et al., 2009). In this regard, *Apoe*-deficient mice, fed a Western-type diet enriched with linoleic acid isomers, showed significant associations among *Syt1* expression levels and hepatic steatosis. Their involvement was also analyzed in hyperhomocysteinemic mice lacking *Cbs* gene and *Syt1* hepatic expression was also associated with steatosis. *Apoe*-deficient mice consuming an olive oil-enriched diet displayed reduction of the fatty liver, and *Syt1* expression was found increased. Thus, *Syt1* expression was highly associated with hepatic steatosis in a genetic disease such as *Cbs* deficiency and in two common situations such as Western diets containing CLA isomers or a Mediterranean-type diet. The precise molecular mechanism by which SYT1 works in the liver is not known but seems to have an important role in fatty liver disease development, a common disease in Western society. In addition, the possibility that the liver may express a different mRNA to display an organ-specific regulation is an interesting hypothesis to test. Therefore, the aim of this work was to characterize SYT1 in the mouse liver.

2. Materials and methods

2.1. RNA isolation

Hepatic samples of *Apoe*-knockout mouse, fed a 10% olive oil-enriched diet (Acín et al., 2007) preserved at −80 °C, were used for RNA extraction, using Tri Reagent from Ambion® (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. DNA contamination was removed using TURBO DNAse treatment and removal kit from Ambion® (Life Technologies, Carlsbad, CA, USA). RNA was quantified by absorbance at $A_{260/280}$ (ratio was greater than 1.75) with NanoDrop ND-100 Spectrophotometer (Wilmington, DE, USA). Integrity of the 28S and 18S ribosomal RNAs was verified by 1% agarose gel electrophoresis followed by ethidium bromide staining.

2.2. Primer design and synthesis

All primers (Tables 1 and 2) used in this study were designed with Primer Express Applied Biosystems® program (Life Technologies, Carlsbad, CA, USA) from highly conserved regions of brain sequence (NM_009306) and checked by BLAST analysis (National Centre for Biotechnology Information) to verify gene specificity and selective amplification of cDNA vs. genomic DNA. All primers were purchased from Invitrogen (Madrid, Spain).

Table 1
Sequences of polymerase chain reaction (a) and RACE (b) primers used for sequence analysis.

Primer	Primer sequence 5′ → 3′	Length (bp)	T_A (°C)	Use
2SytD	Fw 2: CAGCAGAACATTCCGCTTGA	636	60	a
2SytR	Rv 2: ATTGAGGGTTTTCCGGTGGA			
3SytD	Fw 3: ATAACCAGCTGCTGGTGGGAAT	838	60	a
3SytR	Rv 3: TATGTGGGCAGACGCAGAAA			
4SytD	Fw 4: CTGCTGCTTCTGTGTCTGTAA	672	60	a
4SytR	Rv 4: TTGGCTTCCAGAATGACAACA			
2Xho Syt1	Rv 5: CTCGAGTATGTGGGCAGACGCAGAAA	1352	60	a
3BamHI Syt1	Fw 5: GGATCCAACTGTGTGTGCAGTCTGTGT			
4SytR	Rv outer: TTGGCTTCCAGAATGACAACA	955	60	b
2SytR	Rv inner: ATTGAGGGTTTTCCGGTGGA	675	60	b
D3race	Fw outer: TACAACAGCACCGGCGCAGAGCT	587	65	b
3aDrace	Fw inner: CACACTCTGCAGGTAGAGGA	512	63	b

2.3. Reverse transcriptase and polymerase chain reaction

Total RNA (500 ng) was reverse transcribed using the Super Script™ II RT Kit from Invitrogen (Madrid, Spain). For PCR amplification of the target, *Syt1*, the reaction mixture contained 1 μL of cDNA, 0.625 U TrueStart™ *Taq* DNA Polymerase (Fermentas, USA), 1 X reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTPs, and 0.32 μM of each specific primer in a final volume of 25 μL. PCR was performed in the PTC-100™ Programmable Thermal Controller cycler (MJ Research, Watertown, MA, USA). The thermal cycling profile was 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 40 s, annealing at 60 °C for 1 min and extension at 72 °C for 2 min followed by a final extension at 72 °C for 5 min. Non-template controls were included. PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining, visualized under ultraviolet (UV) light. Molecular size was estimated by using Step Ladder 1Kb, (Fermentas, USA). PCR products were purified using MinElute PCR Purification kit, Qiagen N.V. (Venlo, The Netherlands) according to the manufacturer's instructions and were utilized to sequence both strands by the dideoxy chain termination procedure in an automatic sequencer (GE Healthcare) at Parque Científico de Madrid (Madrid).

2.4. 5′-RACE

To obtain the total coding sequence, FirstChoice® RLM-RACE Kit, Ambion® (Life Technologies, Carlsbad, CA, USA) was used to amplify the cDNA containing the entire 5′end of the *Syt1* hepatic transcript according to the manufacturer. Basically, total liver RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase for 1 h at 42 °C in the presence of the 5′RACE Adapter (5′-GCUGAUGGCGAUGAAUGAAC CUGCGUUUGCUGGCUUUGAUGAAA-3′). By PCR reaction, the 5′ cDNA fragment was generated. This reaction used a specific oligo for the RNA adapter, 5′RACE outer primer (5′-GCTGATGGCGATGAATGAACAC TG-3′) and a gene-specific primer (5′-TTGGCTTCCAGAATGACAACA-3′) corresponding to the *Syt1* transcript. PCR was performed at 94 °C for 20 s, then at 94 °C for 40 s 60 °C for 40 s 72 °C for 1 min for 35 cycles, and finally 72 °C for 3 min. PCR products were visualized with ethidium bromide agarose gel electrophoresis and a nested PCR was required to amplify the discrete previous band seen on the gel. For the nested PCR a specific 5′ inner primer (5′-CGCGGATCCGAACACTGCGTTTGCTGGCTT TGATG-3′) provided with the kit and a gene-specific primer (5′-ATTG AGGGTTTTCCGGTGGA-3′) both inside the first sequence obtained were used. PCR products visualized with ethidium bromide agarose gel electrophoresis were purified and sequenced. The obtained target sequences were verified and analyzed for similarity with other known *Syt1* sequences using BLAST programs at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast).

2.5. 3′-RACE

BD SMART™ RACE, Clontech (BD Biosciences, Madrid, Spain) was used to obtain the 3′ cDNA end of *Syt1* hepatic transcript according to the manufacturer. RNA was reverse transcribed into cDNA using BD PowerScript reverse transcriptase for 1.5 h at 42 °C in the presence of the special oligo (dT) 3′-CDS primer (5′-AAGCAGTGGTATCAA-CGCA GAGTAC$(T)_{30}$V N-3′; where N = A, C, G, or T and V = A, G, or C). This reaction was used an antisense oligo that was specific for the special oligo (dT), BD SMART II A oligo (a mixture of 5′-CTAATACGACTCACTA TAGGGCAAGCA GTGGTATCAACGCAGAGT-3′ and 5′-CTAATACGACTC ACTATAGGGC-3′) and a forward gene-specific primer (5′-TACAACAG CACCGGCGCAGAGCT-3′). PCR was performed at 95 °C for 40 s 65 °C for 40 s and 72 °C for 2 min for 35 cycles. A nested PCR using an inner primer provided with the kit and gene-specific primer was required to obtain 3′ *Syt1* hepatic sequence. PCR products visualized with ethidium bromide agarose gel electrophoresis were purified and sequenced.