cDNA cloning and sequencing of the human ryanodine receptor type 3 (RYR3) reveals a novel alternative splice site in the RYR3 gene

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Abstract The human ryanodine receptor type 3 (RYR3) was cloned from a fetal brain cDNA library and its complete sequence was determined (EMBL accession number AJ001515). The sequenced cDNA spanned 15 564 bp and contained an open reading frame of 14 613 bp. The corresponding protein consisted of 4870 amino acids with a calculated molecular mass of 552 kDa. Amino acid sequence identities to the RYR3 proteins from rabbit, mink, and chicken were 96%, 95%, and 83% respectively. A previously unidentified alternative splice site was detected generating a transcript that lacked bases 11 569–11 650 and encoded a truncated protein.

Key words: Ryanodine receptor type 3; Ryanodine receptor; Calcium release channel; Sequence; Alternative splicing; Human fetal brain

1. Introduction

The release of calcium ions from internal stores such as the endoplasmic reticulum into the cytoplasm is an important response to a variety of extracellular stimuli. Two families of calcium channels have been identified that mediate this calcium release: the inositol-1,4,5-trisphosphate receptors and the ryanodine receptors (RYRs) [1–4]. So far three different RYR genes have been isolated. RYR1 is the major calcium release channel of the sarcoplasmic reticulum in skeletal muscle while RYR2 is the corresponding molecule in cardiac muscle. RYR3, also termed brain-type RYR, is expressed in specific regions of the brain, i.e. hippocampus, corpus striatum and thalamus. It should be noted that RYR1 and RYR2 are also found in the brain but the expression patterns are different for the three subtypes [5]. RYR3 is also expressed in skeletal muscle but its protein amount is less than 1% of the amount of RYR1 [6]. So far, the function of RYR3 is unclear. RYR3 deficient mice are viable and fertile and show only slight behavioral abnormalities [7,8]. The analysis of the protein function is hampered by the fact that the RYRs are extremely large proteins which contain approximately 5000 amino acids. Functional calcium release channels are formed by RYR tetramers and have a molecular mass of more than 2000 kDa.

From the amino acid sequence two models of the protein structure have been proposed which contain either 4 or 10 transmembrane domains, a large cytoplasmic domain (foot region) and a very small luminal domain [9,10]. Recent advances in electron microscopy of 2D crystals have provided an image of RYR1 molecules at 3 nm resolution. These data are in favor of the 10 transmembrane model [11].

Full-length RYR3 has been cloned from rabbit, mink, and very recently from man [12–15]. Beside these mammalian sequences the homologous sequences from chicken and bullfrog have also been determined [16,17]. The channel properties of rabbit RYR3 were analyzed using native receptors from brain [18] or recombinant receptors expressed in HEK 293 cells [13].

The diversity of the RYRs is extended by the existence of multiple splice variants. In RYR3 three alternative splice sites ASI, ASII, and ASIII have been described which affect a potential modulatory region between amino acids 3335 and 3710 of the mink sequence [14]. Another alternative splice site is located in the channel forming region and leads to a truncated protein that lacks the C-terminal 250 amino acids [19]. The distribution of the splice variants of the RYR3 gene seems to be tissue-specific [14,19].

To investigate the properties of the human RYR3 we have cloned and sequenced the complete RYR3 cDNA. We were able to determine a number of polymorphisms and found a novel alternative splice site.

2. Materials and methods

2.1. Isolation of RYR3 cDNA clones

A random primed fetal brain cDNA library constructed in λZAPII (Stratagene) was used for the screening of RYR3 clones. The library was screened six times according to [20] to isolate a total of 20 overlapping cDNA clones. For the initial screen we used the following probes: TIGR/ATCC human cDNA clone HE9MN66 (ATCC 102029, accession number T28322, nucleotide positions 14 386–15 564, +1 is the A of the start codon) and a RT-PCR product from pig RNA corresponding to nucleotide positions 1518–2006 [21,22]. All subsequent probes were derived from end fragments of the isolated clones. In detail, fragments corresponding to the following nucleotide positions were used: 3343–3956 and 12 458–12 800 (second screen), 9378–9789 (third screen), 7898–8884 (fourth screen), 4922–5430 (fifth screen), and 4132–5360 (sixth screen).

2.2. Sequencing

Sequencing was performed on plasmid templates either with the T7 Sequencing kit (Pharmacia) and [α-^{35}S]thiodATP as label or with the Thermo Sequenase kit (Amersham) and a LICOR 4000L automated sequencer. Both strands of all RYR3 clones were completely sequenced using either appropriate subclones or a primer walking strategy. The sequences were analyzed with Sequencher 3.0 and MacDNASIS 3.6.

2.3. RT-PCR

Human fetal brain total RNA pooled from 24 individuals was obtained from Clontech (Cat. No. 64019-1). 75 pmol of 5′-$(T)_{24}$V-3′ was annealed to 2 μg of RNA and reverse transcribed with 200 U of SuperscriptII reverse transcriptase (Gibco-BRL) according to the manufacturer's protocol. 2 μl of this cDNA was used in a 100 μl PCR reaction containing 100 pmol of each primer, 200 μM dNTPs, 2 U Taq polymerase (Pharmacia) and the supplied reaction buffer. After an initial denaturation of 5 min at 94°C, 35 cycles of 1 min

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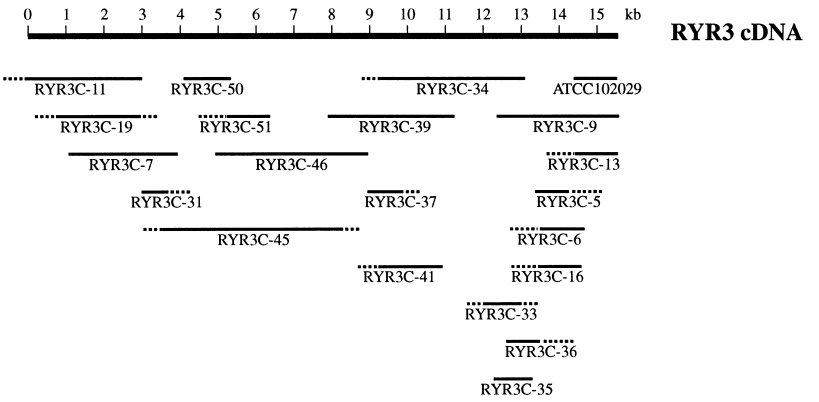

Fig. 1. Overview of the isolated RYR3 cDNA clones. The bold line indicates the complete RYR3 cDNA. Thin lines represent isolated RYR3 clones. The clone ATCC 102029 was obtained from ATCC and served as probe for the initial screening of the library. Chimeric parts of the isolated phages are shown as dotted lines.

94°C, 1 min 55°C and 2 min 72°C were performed in a Hybaid Omnigene thermocycler. RT-PCR products were separated on 1% agarose gels and cloned into pGEM-4Z for sequencing. Densitometric analysis of agarose gels was performed with Eagle Sight 3.0 on an Eagle Eye II (Stratagene) documentation system.

2.4. Primer sequences

The names of the primers indicate their positions on the human RYR3 cDNA sequence, V: forward, R: reverse. RYR3V2014, 5′-TTC CTA ACA GCA GAG CC-3′; RYR3R2307, 5′-GAA GTT CTC AAA CAT CCC-3′; RYR3V9984, 5′-TGG AGA CAG CAA AAG CAA G-3′; RYR3R11668, 5′-TGC CAT TTA CCA CAT TCC C-3′; RYR3V11217, 5′-ACA GTT ACT TTG TGA GGG-3′; RYR3R11799, 5′-TGG GTC ATA TTC TTT GAA GG-3′.

3. Results and discussion

3.1. Cloning and sequencing of the human RYR3 cDNA

A random primed fetal brain cDNA library was chosen to isolate overlapping RYR3 clones. This strategy was employed instead of RT-PCR to minimize the chance of artifacts due to polymerase errors during amplification. To accelerate the screening of the library we initially used a probe near the 5′ end and a probe containing the 3′ end of the RYR3 cDNA. The 3′ probe was available from the TIGR/ATCC special collection of human cDNA clones, while for the 5′ region a heterologous probe was obtained by RT-PCR on pig RNA. The first screening of the library resulted in the isolation of three phage clones from the 5′ end of the RYR3 gene (Fig. 1) and five independent phage clones from the 3′ end. The clone RYR3C-11 contained the start codon and at least 70 bp of 5′ untranslated sequence as was determined by comparison with the rabbit RYR3 sequence. As this clone contained a chimeric insert it was not possible to specify the exact 5′ end of the RYR3 cDNA. After the first screening of the library, end fragments of isolated clones were used as probes for further screenings of the library. The procedure was repeated five times until a total of 20 RYR3 phages had been isolated which harbored the complete RYR3 cDNA. The positions of these clones are indicated in Fig. 1. All the RYR3 containing phages were completely sequenced on both strands. RYR3 containing fragments of chimeric inserts were identified by comparison to the rabbit RYR3 sequence.

3.2. Analysis of polymorphisms

Most of the RYR3 cDNA was present on more than one phage clone. During the sequencing of these clones a number of nucleotide differences became apparent. A summary of all these sites is given in Table 1. Remarkably, several of these nucleotide substitutions also resulted in amino acid exchanges. This phenomenon could either be due to cloning artifacts or on the other hand these mutations could represent truly different alleles. In the latter case one can assume that these amino acid changes do not severely affect protein function. To test for the possibility of cloning artifacts, a cDNA fragment located at nucleotides 2014–2307 was amplified by RT-PCR on human fetal brain RNA pooled from 24 individuals. The amplification product was cloned and several clones were sequenced. At both polymorphic sites, i.e. positions 2078 and 2191, both alleles were represented in several clones. Therefore, it is conceivable that the polymorphisms found are most likely authentic reflections of the haplotypes present in the

Table 1
Nucleotide polymorphisms of the human RYR3 cDNA

Nucleotide position	Nucleotide exchange	Amino acid exchange
783	T→G	$S^{261} \rightarrow R^{261}$
1110	C→T	silent
1480	A→G	$I^{494} \rightarrow V^{494}$
2078	G→A	$C^{693} \rightarrow Y^{693}$
2191	G→A	$V^{731} \rightarrow I^{731}$
4139	A→G	$E^{1380} \rightarrow I^{1380}$
6803–6805	Deletion of GCA	Deletion of S^{2268}
9111	G→A	Silent
14 939–14 942	Deletion of ACTC	Silent (3′-UTR)
15 356–15 364	$(A)_9 \rightarrow (A)_8$	Silent (3′-UTR)
15 453	A→G	Silent (3′-UTR)

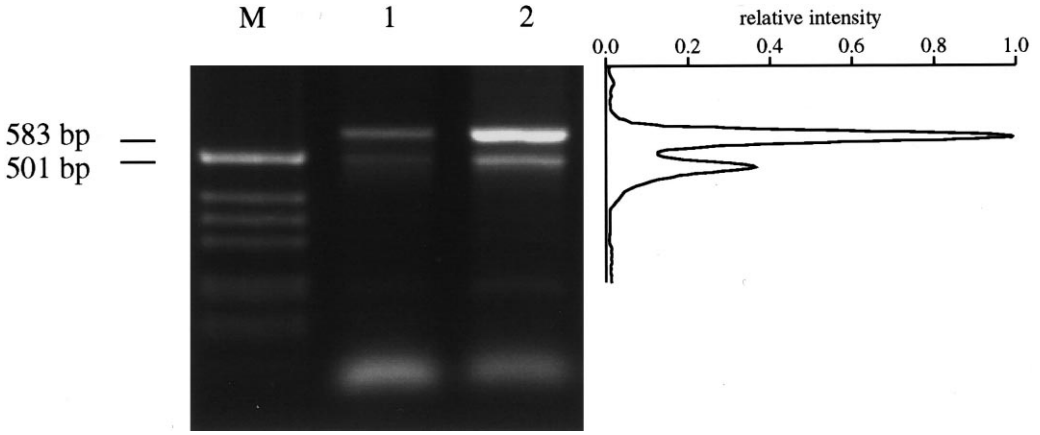

Fig. 2. RT-PCR with primers RYR3V11217 and RYR3R11799. Human fetal brain total RNA was reverse transcribed using an oligo-d(T) primer and subjected to 35 cycles of PCR. PCR products were separated on a 1% agarose gel. The experiment was performed with two independently synthesized cDNAs (1, 2). M: size standard (1 kb ladder). On the right side of the gel the densitometric profile of lane 2 is shown. The molar ratio of the 583 bp band to the 501 bp band is 7:3.

population. All the mutations were located in the N-terminal part of the protein. In the C-terminal part of RYR3, which contains the channel forming region, no mutation was found although several independent phage clones from the cDNA library were sequenced. Apparently in this region of the protein mutations are less tolerable than in the N-terminal region. In the 3′ untranslated region further nucleotide polymorphisms were observed (Table 1). The comparison of our RYR3 sequence with the human RYR3 sequence of Nakashima et al. [15] revealed several differences. Most strikingly, their sequence [15] contained insertions of single G residues after nucleotides 2224, 2248, and 2298 which led to a frame shift in this region so that amino acids 742–766 were completely different from our sequence which is identical to the sequences of rabbit, mink and chicken in this region. The sequence of Nakashima et al. [15] also lacked the 15 bp of alternative splice site ASI from 10 012–10 026. In addition to these two differences, 24 nucleotide substitutions were detected that resulted in 15 amino acid exchanges.

3.3. Analysis of splice variants

Alternative splice sites were named according to the nomenclature of Marziali et al. [14] who described alternative splice sites ASI, ASII, and ASIII. The alternative splice site found by Miyatake et al. [19] was named ASIV.

ASI, ASII, and ASIII are located relatively close together between positions 10 012 and 11 163 on the human RYR3 cDNA. The use of these alternative splice sites affects the potential modulatory region of the channel between amino acid residues 3338 and 3721. To investigate the different splice variants within the whole region of ASI, ASII, and ASIII, we designed PCR primers to amplify this part of the cDNA from position 9984 to 11 668 by RT-PCR. The amplification product was cloned and several clones were sequenced. The use of ASI was confirmed as approximately 50% of the sequenced clones lacked the 15 bp at 10 012–10 026. ASII and ASIII, however, did not seem to be used in human fetal brain as all sequenced clones contained the 18 bp exon of ASII and exon B of ASIII.

The sequencing of the RT-PCR products revealed several clones lacking nucleotides 11 569–11 650. In order to confirm the presence of this novel alternative splice site designated ASV, a new PCR primer pair was designed that produced a 583 bp product or a 501 bp product depending on the presence or absence of nucleotides 11 569–11 650. RT-PCR was performed in two separate experiments and showed that a significant portion of the transcripts indeed lacked the 82 bp region. From the intensity of the RT-PCR bands the proportion of the shorter transcript ASV(−) was densitometrically determined to be 30% of the total RYR3 transcripts (Fig. 2). The relative amount of the two PCR bands should be an accurate approximation of the relative amount of the RNAs present in the sample as both templates were amplified under identical conditions within the same tube. Sequence analysis of the cloned PCR products confirmed that both bands were indeed derived from RYR3 transcripts.

The physiological significance of the ASV(−) transcript remains to be determined as the deletion of the 82 nucleotides of ASV leads to a frame shift that introduces a stop codon only 10 nucleotides downstream of the splice acceptor site (Fig. 3). This would therefore lead to a protein lacking 9 out of 10 channel forming transmembrane domains. The truncated RYR3 ASV(−) protein might however have a regulatory effect on calcium release by competing for effectors or inhibitors

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RYR3 ASV(+)    11563  TCT CAG GAT TCC ... CTG GAA GGG AAT GTG GTA AAT  11664
                3855   S   Q   D   S  ...  L   E   G   N   V   V   N   3888

RYR3 ASV(-)    11563  TCT CAG --- --- --- --- --- -GGA ATG TGG TAA AT  11582
                3855   S   Q                       G   M   W  STOP     3859
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Fig. 3. Nucleotide and amino acid sequence of the human RYR3 in the region of ASV. Only the boundaries of the alternative splice site are shown. The central part of ASV contains 69 bp and is represented by dots in the ASV(+) sequence. Missing bases in the ASV(−) sequence are indicated by dashes.

Table 2
Alternative splice sites of the RYR3 gene

Name	Nucleotide pos.	Type	Species / ref.	Observed in human fetal brain (% alt. spliced transcripts)
ASI	10 012–10 026	Removal of 15 nucleotides	Mink [14]	Yes (approx. 50%)
ASII	11 146–11 163	Removal of 18 nucleotides	Mink [14]	No
ASIII	10 503–10 599	Alternative use of exons A and B (encoding 31 and 32 amino acids respectively)	Mink [14]	No
ASIV	13 799–13 800	341 bp intron is not removed and leads to truncated protein	Mouse [19]	No
ASV	11 569–11 650	Removal of 82 nucleotides leads to truncated protein	Human	Yes (30%)

of full-length RYR3. Table 2 summarizes the analyzed splice variants in comparison with all published alternative splice sites of the RYR3 gene.

3.4. Analysis of the RYR3 protein sequence

Translation of the 14 613 bp open reading frame of the human RYR3 cDNA led to a protein with 4870 amino acids and a calculated molecular mass of 552 kDa. Like all other known RYR sequences the human RYR3 contains a sequence motif of approximately 85 amino acids which is repeated four times. The positions of this motif are 849–934, 965–1049, 2598–2683, and 2715–2795. The hydrophobic domains M′, M″, M1–M10 originally proposed by Zorzato et al. [10] can also be found in the human RYR3 at positions 2985–3005, 3049–3057, 3836–3852, 3874–3852, 4138–4157, 4198–4223, 4410–4431, 4482–4505, 4632–4653, 4670–4692, 4712–4731, and 4751–4774. The two domains M3 and M4 are quite divergent from RYR1 and RYR2 sequences but they still contain a high percentage of hydrophobic residues. Recently, a new protein domain called SPRY was identified in RYR1 and certain kinases [23]. Like RYR1 the human RYR3 contains three of these SPRY domains at positions 657–795, 1084–1207, and 1324–1475. An ATP/GTP binding site consensus sequence AIVSSGKT is located at positions 2840–2847. Three potential calmodulin binding sites can be found at positions 2903–2919, 3471–3489, and 4391–4408.

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