α and β isoforms of ryanodine receptor from chicken skeletal muscle are the homologues of mammalian RyR1 and RyR3

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To define the relationship between the two ryanodine receptor (RyR) isoforms present in chicken skeletal muscle, we cloned two groups of cDNAs encoding the chicken homologues of mammalian RyR1 and RyR3. Equivalent amounts of the two chicken isoform mRNAs were detected in thigh and pectoral skeletal muscles. RyR1 and RyR3 mRNAs were co-expressed in testis and cerebellum whereas RyR3 mRNA was expressed also in cerebrum and heart. The full-length sequence of the chicken RyR3 cDNA was established. The RyR3 receptor from chicken had the same general structure as mammalian and amphibian RyRs. The 15089 nt cDNA encoded a 4869-amino-acid-long protein with a molecular mass of 552445. The predicted amino acid sequence of the chicken RyR3 showed 86.9 % identity to mammalian RyR3 and 85.6 % to frog RyR3. Antibodies specific for chicken RyR1 and RyR3 recognized two different proteins with an apparent molecular mass of about 500 kDa. The two proteins differ slightly in their apparent molecular mass on SDS/PAGE: the protein recognized by antibodies against RyR3 had a higher mobility than the protein recognized by the antiserum against RyR1. Antibodies against RyR1 detected a protein already present in chicken skeletal muscle from 12-day-old embryos and older, while antibodies against RyR3 isoform detected a protein in muscle from only 18-day-old embryos and older. The expression patterns of RyR1 and RyR3 superimpose with those previously reported for the α and the β isoforms respectively. We conclude that α and β isoforms present in chicken skeletal muscle are the homologues of mammalian RyR1 and RyR3.

INTRODUCTION

Ryanodine receptors (RyRs) are intracellular proteins that embody intracellular calcium-release channels present in both muscle and non-muscle tissues [1,2]. Three RyR genes are known in mammals. The first two are expressed mainly in muscle: RyR1 at high levels in skeletal muscle fibres and a different isoform, RyR2, in cardiac myocytes. In addition to their preferential expression in skeletal and cardiac muscles, mammalian RyR1 and RyR2 have also been detected in the central nervous system and other peripheral tissues, where they may participate in mechanisms of signal transduction [3–5]. In muscle fibres, where they have been studied most, RyR1 and RyR2 are responsible for releasing calcium from the sarcoplasmic reticulum (SR) following depolarizing signals on the plasma membrane, a process referred to as excitation–contraction coupling [6]. In skeletal muscle fibres, calcium release from the SR seems to require a direct contact between the voltage sensor on the T-tubule, the dihydropyridine receptor (DHPR) and the RyR/calcium-release channel, located on the SR [7,8], without any obvious requirement for a calcium influx through the DHPR.

A third RyR (RyR3), cloned from a mink lung epithelial cell line and from rabbit brain, appears to be expressed in several mammalian tissues [4,9,10]. In mammals, it is expressed also in skeletal muscle, although at levels that are 20- to 50-fold lower than those of the RyR1 [4,9]. In contrast with this unbalanced ratio between RyR1 and RyR3 in skeletal muscle of mammals, several studies have demonstrated that two different RyRs, named α and β, are present at approximately the same levels in skeletal muscles of fish, amphibians and birds [11–15]. On this basis we reasoned that it was likely that in avian skeletal muscle one of the two isoforms was the homologue of the mammalian RyR1, which in mammals represents the major skeletal muscle isoform, while the second isoform could correspond to the third RyR isoform, RyR3, as it had been already detected, although at very low levels, in mammalian skeletal muscle. To verify this hypothesis and to conclusively determine the relationship between the α and β isoforms expressed in avian skeletal muscle, we screened a chicken skeletal muscle cDNA library with mammalian RyR probes and isolated several clones corresponding to the chicken RyR1 and RyR3. The expression of the mRNAs and of the proteins encoded by these genes was analysed in both adult and embryo chicken tissues. The full-length nucleotide sequence of the chicken RyR3 was determined.

EXPERIMENTAL

cDNA cloning and sequencing

An oligod(T)-primed chicken skeletal muscle cDNA library prepared from thigh muscle (Stratagene) was screened. Phages were plated and screened according to standard protocols [16]. Duplicate plaque lifts were made on nitrocellulose filters. For the screening, the cDNA library was prehybridized in 0.15 M NaCl/0.015 M sodium citrate (SSC) at 55 °C for 2 h as described previously [17]. After prehybridization, filters and probe were combined and hybridization was carried out for 16 h at 55 °C for low-stringency conditions and 65 °C for normal-stringency conditions. Filters were initially washed several times at 55 °C with SSC; further washes were performed at 60 °C, and eventually at 65 °C, in 0.1 SSC. For the first screening, cDNA fragments correponding to the 3′ end of mouse, mink and human RyR3 cDNAs were used as probes. Labelling of cDNA probes was carried out with [α-^{32}P]dCTP using a protocol for random-primed DNA labelling. All cDNA inserts were subcloned into the multiple cloning site of Bluescript SK (+) according to

Abbreviations used: RyR, ryanodine receptor; DHPR, dihydropyridine receptor; SR, sarcoplasmic reticulum.

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standard procedures. DNA sequencing was performed by the dideoxy chain-termination method.

RNase protection analysis

Total RNA from adult and embryonic chicken tissues was purified by the guanidine isothiocyanate/CsCl method [18]. To prepare the antisense RyR1 and RyR3, several probes derived from the isolated clones were used. RyR1 and RyR3 antisense cRNA probes were obtained by transcription of the plasmid, in the presence of [α-^{32}P]UTP (800 Ci/mmol) (Amersham), with a T3/T7 RNA polymerase Stratagene Transcription Kit, according to the manufacturer's instructions. After purification on an 8 % sequencing gel, 2.5×10^5 c.p.m. of the specific cRNA probe was hybridized to 50 μg of total RNA from the chicken tissues in 30 μl of 40 mM Pipes (pH 6.4)/1 mM EDTA/0.4 M NaCl/80 % formamide overnight at 55 °C. The hybrids were treated with RNase A (40 μg/ml; Sigma) and RNase T1 (2 μg/ml; Boehringer Mannheim) for 1 h at 33 °C. Samples were further treated with proteinase K (50 μg; Boehringer Mannheim), in the presence of 0.4 % SDS, at 37 °C for 30 min, phenol/chloroform extracted, ethanol precipitated and analysed on an 8 % sequencing gel. tRNA (50 μg) was hybridized to each probe as a negative control.

Microsomal membranes preparation

Fertilized chicken eggs were incubated until the desired age. The tissues were then removed from decapitated embryos and homogenized in 5 ml per g of solution A containing 0.32 M sucrose, 10 mM Hepes, pH 7.4, 0.23 mM PMSF and 230 μM leupeptin for 3×30 min using a Polytron apparatus. Homogenates were rapidly frozen in liquid nitrogen and stored at -80 ° C. Three-week-old chicks were killed, and tissues were rapidly excised and immediately frozen in liquid nitrogen. Neuronal tissues were homogenized in 5 ml of solution A per g of wet tissue at 0–4 °C using 3×30 min bursts of the Polytron apparatus at setting 4. Homogenates were centrifuged at 9000 ***g*** for 30 min. Microsomes were collected from the resulting supernatant by centrifugation at 100000 ***g*** for 60 min. Muscular tissues were homogenized in the same conditions, but required 3 bursts of 1 min each with the Polytron apparatus at setting 7. The homogenate was centrifuged at 8000 ***g*** for 14 min. The supernatant was discarded, and the pellet was homogenized again and centrifuged as above. Microsomal membranes were pelleted from the second supernatant by centrifugation at 130000 ***g*** for 90 min, resuspended in solution A, rapidly frozen in liquid nitrogen and stored at -80 °C. Membrane protein concentration was measured using the Bradford method and BSA as a standard.

PAGE and Western blot analysis

Proteins were separated by 5 % SDS/PAGE at 80 V–130 V (25 mA constant) at 4 °C. The electrophoretic transfer of the resolved proteins onto nitrocellulose was achieved at 100 mA, 4 °C, overnight in 192 mM glycine/25 mM Tris/0.01 % SDS/10 % methanol. The blotted proteins identified by specific antibodies, as described below, were revealed using the Amplified Alkaline Phosphatase Immunoblot reagents supplied by Bio-Rad.

Fusion proteins and polyclonal antisera

A specific region corresponding to the region of lowest similarity (divergent region 1, or D1) (see [19]) of the RyR1 cDNA was amplified using oligonucleotides from the upstream (5′-CTCCCAGCTGAGCAGCGA) and downstream (5′-TCTG-CACCTCCAACTCCGT) regions. The equivalent region of the RyR3 gene was amplified using oligonucleotides from the upstream (5′-CAGTTTGGAATCCATGAT) and downstream (5′-TTGCCAGGTAGTGAAGTAT) regions. The fragments were digested with *Eco*RI and *Bam*HI and subcloned in the pGEX2T plasmid (Pharmacia). The resulting plasmids were transformed in the JM101 strain of *Escherichia coli*. Recombinant proteins were induced with isopropyl-β-δ-thiogalacto-pyranoside as described previously [20]. Cell pellets were washed, resuspended in PBS/1 % Triton X-100/100 mM EDTA and sonicated. The bacterial lysate was cleared by centrifugation. Glutathione–agarose beads (Pharmacia) were then added and incubated with the bacterial lysate for 10 min at 4 °C. After several washes with PBS, the recombinant proteins were eluted in 50 mM Tris (pH 8)/10 mM reduced glutathione. These GST fusion proteins were used to generate polyclonal antisera in rabbits according to standard protocols [21]. Specificity of the antisera was tested in competition experiments with homologous and heterologous proteins, as described previously [4]. For Western-blot analysis, primary antisera were diluted 1:1000. For competition experiments, 6 μl of each undiluted antiserum was preincubated with 50 μg of recombinant protein in 500 μl of blocking buffer. After 4 h the adsorbed antisera were diluted and blotted as described previously.

RESULTS

Two different RyR genes are expressed in avian skeletal muscle

Isolation of cDNA sequences was performed as follows. A chicken skeletal muscle cDNA library (Stratagene) was screened, under low-stringency conditions, using as probes three DNA fragments corresponding to the 3′ end of mouse, human and mink RyR3 cDNAs [21]. This region was chosen because it encodes the C-terminal part of the RyRs, i.e. a region highly conserved between different RyR isoforms and across species. We used a mix of fragments from different species to increase the chances of successful hybridization. The low-stringency conditions of hybridization were such to allow detection of more than one isoform, should more than one RyR gene be expressed [17,22].

Out of 1×10^6 phages screened, 20 positive clones were isolated. The cDNA inserts were subcloned and sequenced. All clones represented independent isolates, and all contained a poly(A)$^+$ tail at their 3′ ends. Alignment of the nucleotide sequence of these cDNAs allowed their subdivision into two distinct groups of overlapping clones. The nucleotide sequences of a first group of five cDNAs, overlapping each other for a total of 2011 bp before the poly(A)$^+$ tail, were assembled. The resulting sequence, when aligned against known RyR sequences, presented 79.1 and 75.2 % identity with mammalian and frog RyR1 nucleotide sequences, and 70.1 and 72.6 % identity with mammalian RyR2 and RyR3 sequences respectively. Thus this nucleotide sequence corresponds to the 3′ end of the chicken RyR1 cDNA (EMBL accession no. X95266). It encodes 628 amino acid residues that exhibit 86.8 and 84.2 % identity to the amino acid sequences at the C-termini of the mammalian and frog RyR1s respectively. The open reading frame ends with a termination codon followed by a 122-bp-long 3′ untranslated region and by a poly(A)$^+$ tail. The assembly of the nucleotide sequences of the 15 cDNAs of the second group yielded a 2940-nt-long common sequence which showed 75.6 and 75.0 % identity to the 3′ end of mammalian and frog RyR3 nucleotide sequences respectively. The above-described identification of RyR1 and RyR3 cDNAs in a cDNA

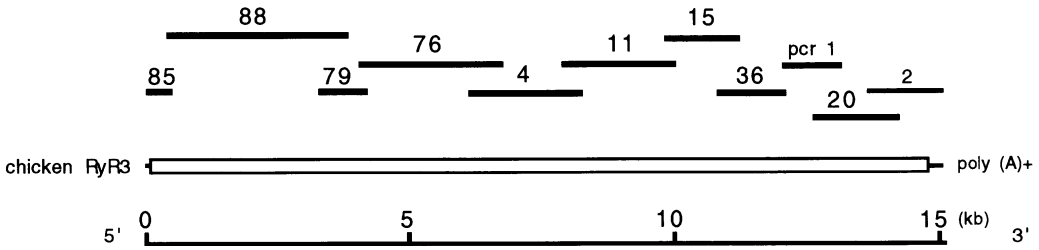


Figure 1 Structure of the chicken RyR3 mRNA

The protein-coding region of chicken RyR3 is represented by an open box, and 5′ and 3′ untranslated regions by a solid line. The original clones isolated and sequenced are reproduced above.

library prepared from chicken skeletal muscle is in line with the recent cloning of RyR1 and RyR3 cDNAs from a frog skeletal muscle cDNA library [23]. Extensive screening of this cDNA library did not reveal the presence of any additional isoforms of RyR or related sequences, in agreement with published results indicating that no more than two RyR isoforms are present in chicken skeletal muscle [13].

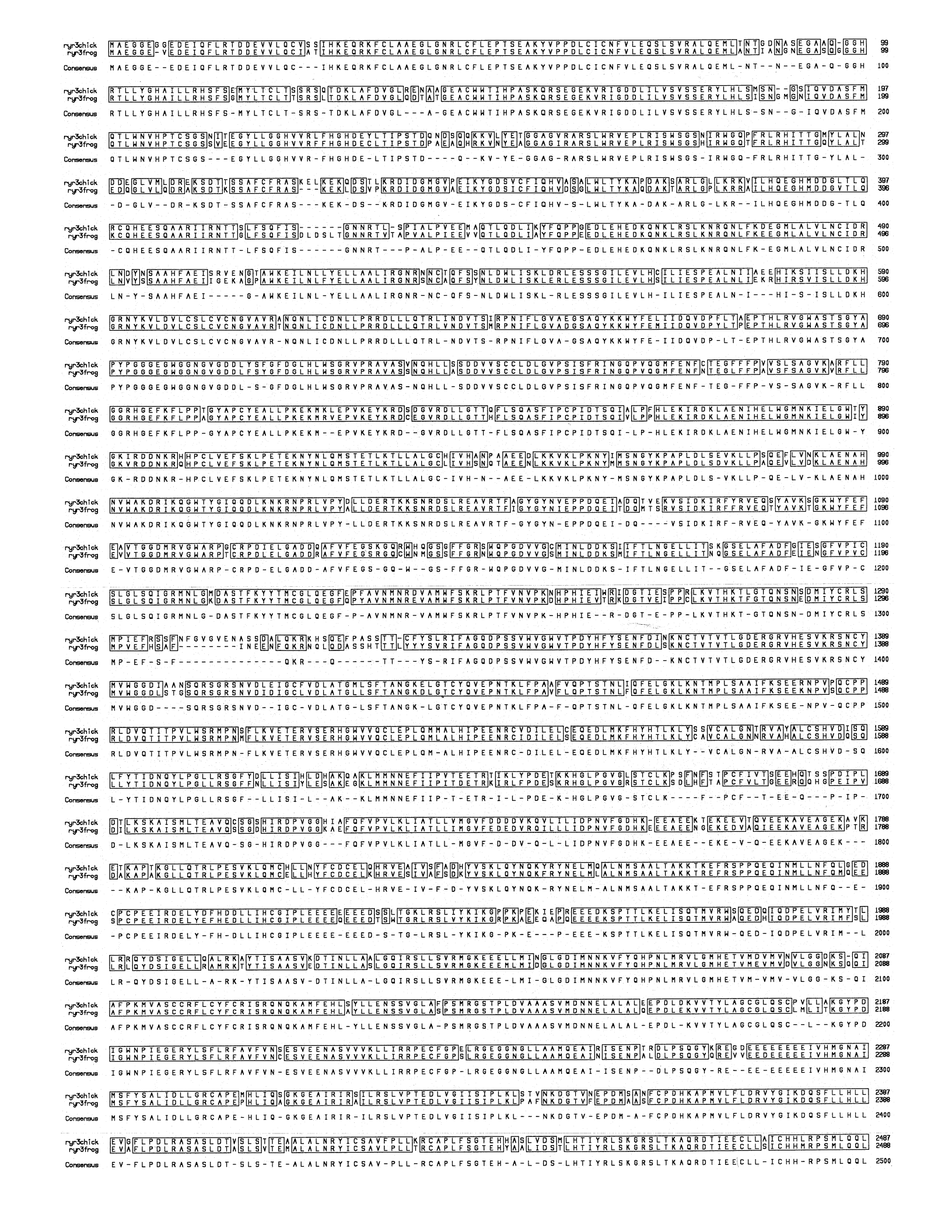
Cloning and sequence determination of the full length chicken RyR3 cDNA

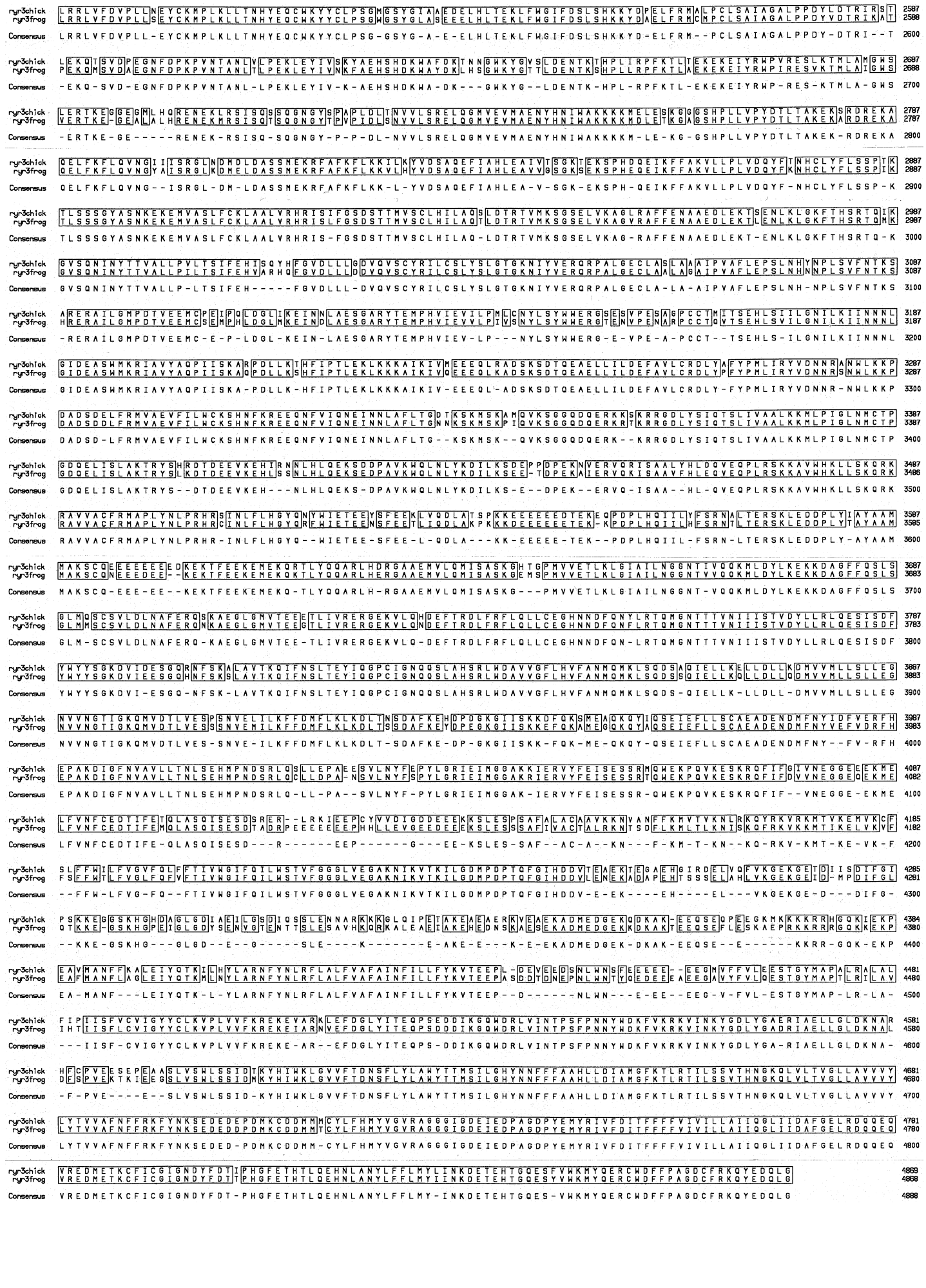
Having defined that two RyR genes were expressed in chicken skeletal muscle we concentrated our attention on the chicken RyR3. In order to isolate a full-length chicken RyR3 gene, we screened the same chicken muscle library with a probe corresponding to the upstream sequence of mink RyR3. This screening yielded clone 76 (Figure 1). Clone 79 was identified using a probe derived from the upstream region of clone 76. Clones 85 and 88 were isolated with the same strategy. In a similar way clone 4 was identified using a probe derived from the downstream region of clone 76. Clones 11, 15 and 36 were isolated with the same strategy. We then cloned by PCR the sequence corresponding to residues 10863–12149, using oligonucleotides derived from the downstream sequence of clone 36 and from the upstream sequence of clone 20 (see Figure 1). This clone (pcr1) allowed the joining of a consecutive 15089 nt sequence containing the entire coding sequence of the chicken RyR3 cDNA (EMBL accession no. X95267). In this sequence, the first ATG is found after 77 bp of the 5′ untranslated region. A termination codon, TGA, is located upstream of the suggested initiator codon, and other termination codons are found in the other two potential reading frames. The GAGCCATGG nucleotide sequence surrounding the translation initiation codon is in agreement with the consensus sequence for eukaryotic initiation sites. The termination codon TAA was found 14604 bp after the first ATG. The 3′ non-coding region is 405 bases long. All clones contained a poly(A)$^+$ tail.

Structural analysis of chicken RyR3 protein

The open reading frame of the chicken RyR3 is 4869 amino acids long, corresponding to a protein with a molecular mass of 552445. The amino acid sequence, deduced from the nucleotide sequence of the chicken RyR3 full-length cDNA, is shown in Figure 2 together with the frog RyR3 homologue. The predicted amino acid sequence of the chicken RyR3 shows 86.9% identity to mammalian RyR3 and 85.6% to frog RyR3 [10,23]. The amino acid sequence of the chicken RyR3 was compared with those of rabbit RyR1 and RyR2, revealing an overall sequence identity of 67.8% and 69.6% respectively [24–27]. Several binding sites for calcium, ATP and calmodulin have been observed in previously cloned RyRs. The nucleotide-binding consensus sequence GXGXXG was found five times in chicken RyR3 (696–701, 698–703, 1133–1138, 2237–2242 and 2525–2530); the fourth of these (2237–2242) is common to all known RyR1, RyR2 and RyR3 sequences. Putative Ca^{2+}/calmodulin-dependent protein kinase phosphorylation sites, 21 in total, were found using the consensus sequence RXX(S/T); three of these (126–129, 286–289 and 2708–2711) are also present in RyR1 and RyR2, as well as in RyR3 from other species, whereas four other sites were conserved only between RyR3s (258–261, 411–414, 550–553, 2197–2200 and 3353–3356). Furthermore the chicken RyR3 contains two potential cyclic AMP-dependent phosphorylation sites [KRXX(S/T)] (1238–1242 and 1315–1319), the first one of which is conserved in all RyR3s. Of several potential glycosylation sites found using the consensus sequence NX(S/T), the site between residues 4696–4698 was conserved in all RyRs. In the chicken RyR3 sequence, four internal repeats (848–933, 964–1048, 2600–2685 and 2717–2798) are present, as has also been observed in other RyRs.

The C-terminus of the chicken RyR3 contains 12 stretches rich in hydrophobic amino acids, with the potential to form transmembrane domains, as also observed in the sequence of other RyRs (2988–3008, 3052–3070, 3839–3858, 3877–3895, 4131–4154, 4196–4216, 4410–4431, 4481–4504, 4621–4652, 4669–4688, 4711–4730 and 4746–4769). Based on the 12 domains (named M′, M″ and M1–M10), two models, based on structure-prediction algorithms, have been proposed, one containing four transmembrane domains (4410–4431, 4481–4504, 4669–4688 and 4746–4769) that have a high probability of passing through the membrane [26], whereas the second, in addition to the previous four, includes six more regions (3839–3858, 3877–3895, 4131–4154, 4196–4216, 4621–4652 and 4711–4730) for a total of 10 (M1–M10) [27]. However, whether all 10 segments are likely to span the membrane is not clear, since some of them, such as segments 2 and 9, present only a weak membrane score, according to computer programs that evaluate transmembrane regions [27,28]. On the other hand, data from proteolytic fragmentation of the channel [29] indicate that the region following Arg-4475 in RyR1 still possesses calcium-channel activity, thus suggesting that only those transmembrane segments present in this region of the protein (which still contains the four transmembrane domains of the first model, but only the last six of the second model) may be required for assembly of the calcium pore.


ryr3chick
ryr3frog
Consensus


ryr3chick
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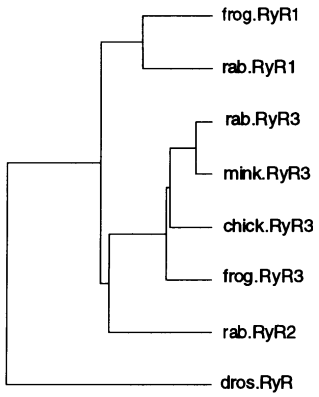

Figure 3 Phylogenetic classification of RyR isoforms

Data were from available sequences, including *Drosophila* (dros) [59], rabbit (rab) [10,25,26], frog [23], chicken and mink (G. Marziali, D. Rossi, G. Giannini, A. Charlesworth and V. Sorrentino, unpublished work). The analysis was performed with the pileup program of the GCG sequence analysis software.

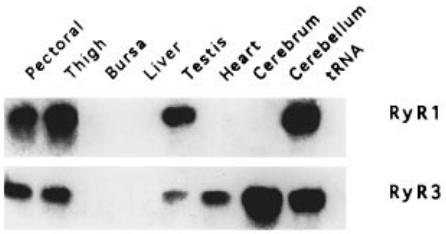

Figure 4 RNase protection of RyR1 and RyR3 mRNA in chicken tissues

Total RNA (50 μg) was used for each protection assay, except for RNA from pectoral and thigh muscles, where only 5 μg of total RNA was used.

The relationship between the different RyR isoforms cloned from different species has been analysed. A phylogenetic classification of all these isoforms indicates that the *Drosphila* isoform is distinct from the three isoforms observed in vertebrates, which probably evolved from a common precursor very early in the evolution of vertebrates (Figure 3). In the phylogenetic tree reported in Figure 3, it is evident that the chicken RyR3 isoform is more closely related to the RyR3 from frog, mink and rabbit, than to RyR1 and RyR2 isoforms.

Tissue-specific expression of the two chicken RyR1 and RyR3

To analyse the tissue-specific expression of the RyR1 and RyR3 genes, RNase protection assays were performed on total RNA isolated from several chicken tissues using chicken RyR1 and RyR3 cDNA-specific probes (Figure 4). In these RNase protection experiments, 10-fold less total RNA was loaded for skeletal muscle preparations (i.e. 5 μg) compared with all other tissues (i.e. 50 μg). As shown in Figure 4, chicken RyR1-protected fragments were observed in RNAs extracted from skeletal muscle (pectoral and thigh), testis and cerebellum, but not in RNA from cerebrum, bursa of Fabricius, liver and heart. The chicken RyR3 probe detected expression of RyR3 mRNA in total RNA prepared from pectoral and thigh muscles, as well as from cerebrum, cerebellum, testis and heart, but not in RNA prepared from bursa and liver. In rodents, RyR3 expression in heart has been traced to the conduction system ([31]; L. Gorza, S. Vettore, A. Tessaro, A. Martini, P. Volpe and V. Sorrentino, unpublished work).

These results indicate that RyR1 and RyR3 are expressed at different levels in various chicken tissues. In some tissues they are actually co-expressed. In contrast with the pattern observed in mammals, where RyR1 mRNA levels are on average 20- to 50-fold higher than those of the RyR3 mRNA, in RNA prepared from chicken pectoral and thigh muscles both RyR1 and RyR3 are expressed at high and equivalent levels. Expression of RyR1 and RyR3 genes was observed in testis and cerebellum, whereas only RyR3 was expressed in chick cerebrum. The absence of the RyR1 transcript in chicken cerebrum differs from data obtained in mice and rabbit [4,10]. No detectable levels of RyR1 and RyR3 mRNAs were observed in RNA prepared from the bursa of Fabricius and liver.

Identification of RyR1 and RyR3 proteins

The existence of two distinct RyR isoforms in chicken skeletal muscle has been based on the observation of two high-molecular-mass proteins with different mobilities in SDS/PAGE, both able to bind labelled ryanodine and to react with antibodies against RyRs [12–15]. The two proteins have been since referred to as α, the one with the higher molecular mass, and β, the one with apparent lower molecular mass. To verify the relationship of the α and β proteins with the RyR1 and RyR3 mRNAs, specific antibodies against chicken RyR1 and RyR3 were produced. Based on the two predicted amino acid sequences, regions with poor similarity were chosen and subcloned in expression vectors to obtain fusion proteins. Immune antisera, obtained by immunization of rabbits with recombinant proteins, were used for Western-blot analysis of microsomal proteins prepared from chicken skeletal muscle. These antisera recognized mainly two distinct proteins with a high molecular mass (in the range of 500 kDa), compatible with that expected for RyR isoforms (Figure 5, upper panel, lanes 1 and 4). The specificity of these two antisera was tested in competition experiments. The recombinant RyR1 protein (Figure 5, upper panel, lane 2), but not the recombinant RyR3 protein (Figure 5, upper panel, lane 3), was able to compete the RyR1 antiserum. In a similar way the RyR3 protein competed the anti RyR3 antiserum (Figure 5, upper panel, lane 6), which was not competed by the recombinant RyR1 protein (Figure 5, upper panel, lane 5). Interestingly, of the two proteins recognized by these antisera, the antiserum against RyR1 recognized the one with higher molecular mass, probably the α-RyR isoform (Figure 5, lower panel, lane A), whereas the antiserum against RyR3 recognized the protein with a slightly lower molecular mass, probably the β-RyR (Figure 5, lower panel, lane B). Both isoforms were detected only when the two antisera were used together (Figure 5, lower panel, lane C).

Figure 2 Amino acid sequence of the chicken RyR3

The amino acid sequence of the chicken RyR3 (top line), as deduced from the cDNA sequence, is aligned with the frog RyR3 (bottom line) sequence reported by Oyamada et al. [23]. Amino acids are shown in single letter code. Identical amino acids or conservative substitutions are boxed. Gaps in the amino acid sequence are indicated by dashes. Alignment was obtained with the use of the GCG sequence-analysis software. Conserved amino acids are shown in the consensus sequence.

Figure 5 Western-blot analysis of microsomal fractions from chicken pectoral muscle

Aliquots of 3 μg of protein each were loaded in each lane. (Upper panel) Antibodies against RyR1 and RyR3 recognized a single band in microsomes prepared from chicken skeletal muscle (lanes 1 and 4). In competition experiments, 6 μl of antiserum against RyR1 was preincubated with 50 μg of recombinant RyR1 (lane 2), or with 50 μg of recombinant RyR3 protein (lane 3). Otherwise, 6 μl of antiserum against RyR3 was preincubated with 50 μg of recombinant RyR1 (lane 5), or with 50 μg of recombinant RyR3 protein (lane 6). (Lower panel) Three equivalent lanes were then processed with antisera against the RyR1 isoform (lane A), antisera against the RyR3 isoform (lane B) or both antisera (lane C).

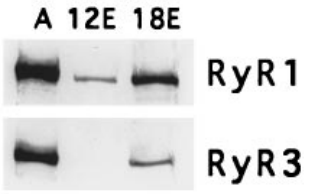

Figure 6 Western-blot analysis of proteins

Proteins were prepared from pectoral muscles of adult chicken (A), day 12 chicken embryo (E12) and day 18 chicken embryo (E18). Protein (15 μg) from pectoral embryo muscle lysates from day 12 and day 18 chicks and 3 μg of microsomal proteins from adult chicken pectoral muscle were resolved by PAGE. After blotting, two equivalent filters were incubated with antisera against RyR1 and RyR3 respectively.

RyR1 and RyR3 proteins are differentially expressed during embryonic chick skeletal muscle development

Previous observations had shown a distinct pattern of expression for α and β isoforms in embryonic chicken muscle, with the α-RyR being expressed at day 10 and the β-RyR first appearing after day 15 and then gradually increasing in amount until hatching [32]. To further clarify the correspondence between the polypeptides encoded by RyR1 and RyR3 and the α and β isoforms of chicken RyRs, homogenates were prepared from embryonic pectoral and thigh skeletal muscle at day 12 (E12) and at day 18 (E18) of development. Whole homogenates of embryonic muscle were prepared, since it is not known which subcellular fraction of immature muscle may contain the RyRs. These preparations were then analysed, along with microsomal membranes prepared from the pectoral muscle of adult chicken (to avoid the large amount of myosin present in adult muscle homogenates), on Western blots using the polyclonal antisera prepared against chicken RyR1 and RyR3 recombinant proteins. As shown in Figure 6, the isoform detected by anti-RyR1 sera is present in all three different stages of development, as expected for the α isoform. In the same samples, the RyR3 antiserum detected only the isoform with lower molecular mass in homogenates from E18 and in microsomes from adult muscle, which is in agreement with the developmental profile reported previously for the β isoform [32]. A similar pattern was observed also in thigh muscle preparation of the same embryos (results not shown). These data further strengthen the relationship between RyR1 and RyR3 and α and β isoforms. They also confirm previous data indicating that the two isoforms are expressed during different periods of embryonic development, where they may regulate different or complementary activities [32]. While this work was being completed, two cDNAs, which encode proteins homologous to mammalian RyR1 and RyR3, have been cloned from a frog skeletal muscle cDNA library. The proteins encoded by the two cDNAs have been shown to correspond to the α and β RyR isoforms, based on comparison of the amino acid sequences of tryptic fragments of the purified α and β frog isoforms [23]. Thus it appears that expression of RyR1 and RyR3 genes in skeletal muscle and their relationship to α and β RyR isoforms is maintained in both frogs and chicken. Our results are also in agreement with data on the relationship between the chicken α-RyR and RyR1 determined by Dr. J. Sutko and co-workers [33].

Expression of RyR1 and RyR3 proteins in chicken tissues

In chicken, RyRs have been identified not only in muscle tissues but also in brain. Monoclonal antibodies developed against avian skeletal muscle RyR recognize two proteins in avian central nervous system [34–36]. Availability of specific anti-(chicken RyR1) and anti-(chicken RyR3) antibodies allowed us to investigate the expression of these proteins in chicken tissues. In previous experiments we found that antibodies against murine RyR2 were able to recognize the chicken RyR2 isoform but did not cross-react with chicken RyR1 and RyR3 (results not shown). Therefore antibodies against murine RyR2 were used to localize RyR2 in chicken tissues. Western-blot analysis was performed on microsomal membrane preparations from muscular and nervous tissues. Both RyR1 and RyR3 isoforms are present in chicken skeletal muscle, whereas only RyR2 can be detected in chicken cardiac muscle under these experimental conditions (Figure 7). In the central nervous system the distribution of the three isoforms differs between cerebellum and cerebrum. All three RyRs can be observed in cerebellum, but only RyR2 and RyR3 are present in cerebrum. When comparing the amount of RyR1 and RyR3 between pectoral muscle and other tissues, note that the total amount of pectoral microsomal protein loaded on the gel is 20-fold lower than that of microsomal protein of other tissues. Similarly in experiments with RyR2 antibodies the amount of microsomal protein from heart is 20-fold lower than

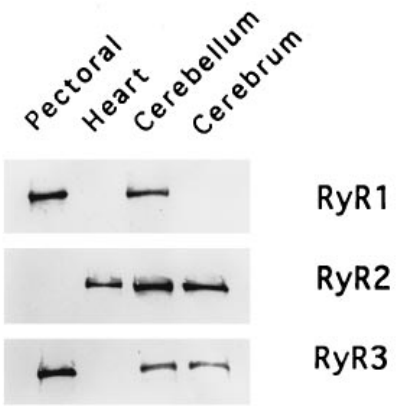

Figure 7 Western-blot analysis of microsomal proteins from adult chicken pectoral muscle, heart, cerebellum and cerebrum

Aliquots of 5 μg of microsomal proteins from pectoral muscle, 100 μg of microsomal proteins from heart and 100 μg of microsomal proteins from cerebellum and cerebrum were separated by PAGE, then blotted on nitrocellulose paper, and the resulting membrane was tested with an antiserum against RyR1. Aliquots of 100 μg of microsomal proteins from pectoral muscle, 5 μg of microsomal proteins from heart and 100 μg of microsomal proteins from cerebellum and cerebrum were separated by PAGE, then blotted on nitrocellulose paper, and the resulting membrane was tested with an antiserum against RyR2. Aliquots of 100 μg of microsomal proteins from pectoral muscle, 100 μg of microsomal proteins from heart and 100 μg of microsomal proteins from cerebellum and cerebrum were separated by PAGE, then blotted on nitrocellulose paper, and the resulting membrane was tested with an antiserum against RyR3.

that of other tissues. The lack of detection of RyR3 in heart in Figure 7 contrasts with the results reported in Figure 4, probably as a consequence of the lower sensitivity of Western blots compared to RNase mapping.

DISCUSSION

Over the past years, several groups have reported that two RyR isoforms, referred to as α and β, are present in most skeletal muscles of non-mammalian vertebrates [11–15,37]. In this report we present evidence demonstrating that these isoforms are encoded by two distinct genes that, based on sequence homology, were identified as the chicken homologues of mammalian RyR1 and RyR3. RNase protection experiments indicated that the two genes are expressed at approximately the same levels in chicken thigh and pectoral muscles and, similar to the situation reported for mammalian RyRs [4,9], are also expressed in several other tissues. In agreement, the results obtained with isoform-specific antibodies raised against chicken RyR1- and RyR3-specific sequences support the finding that the proteins encoded by RyR1 and RyR3 correspond to the α and β isoforms respectively.

The presence of RyR1 and RyR3 isoforms in chicken and frog skeletal muscles [23] mirrors the recent identification of low levels of RyR3 mRNA expressed in mammalian skeletal muscle [4,9], where so far only RyR1 was known to be expressed. Identification of a second RyR isoform was initially based on detection of RyR3 mRNA transcripts in mink and mouse skeletal muscle preparations, and has received support from the more recent detection of the RyR3 protein, by Western-blot analysis of SR prepared from bovine skeletal muscle with RyR3-specific antibodies (A. Conti, L. Gorza and V. Sorrentino, unpublished work). Functional evidence for the presence of a second RyR in mammalian skeletal muscle comes from data showing that caffeine can induce calcium release from the SR of skeletal muscle of skrr^{m1} mice, which are homozygous for a non-functional RYR1 allele [39]. Recent results reported by Takeshima and co-workers suggest that the RyR3 gene product is likely to be responsible for the ability of skrr^{m1} mice to release calcium following caffeine treatment [40]. Interestingly, in spite of the low levels of RyR3 mRNA detected in mammalian skeletal muscle, these authors observe that stimulation with caffeine of muscle prepared from skrr^{m1} mice can induce a calcium release that is only about 1/10 of those induced in normal mice. In their report, they also found that the calcium-induced calcium-release mechanism present in skrr^{m1} myocytes is apparently about 10 times less sensitive to calcium than is RyR1. Should the lower sensitivity to calcium of the calcium-release mechanism observed in skrr^{m1} myocytes be due to properties of the mammalian RyR3 isoform, this would contrast with the relative sensitivity to calcium of chicken RyR isoforms [41]. A more complete analysis of the properties of the mammalian RyR3 isoform is awaited to verify the basis of this apparent discrepancy.

At variance with the situation in mammals, the role of RyR3 cannot be ignored in non-mammalian vertebrates, where it contributes up to 50% of total RyR molecules present. It is generally accepted that either a direct or an indirect contact with DHPR, which acts as the voltage sensor on the T-tubule, is the first step in mediating the internalization of the signal from the T-tubule to the SR, in skeletal muscles [1,42,43]. Biochemical support for this hypothesis comes from reports describing co-precipitation of RyR1 and DHPR [7,8]. However, while some authors consider the interaction between DHPR and RyR to be sufficient to completely activate calcium release from the SR, other authors have proposed that the same calcium, initially released from the SR, may contribute, via a calcium-induced calcium-release mechanism, to the stimulation of bulk calcium release from SR [43–46]. In this second model, it is not clear whether the calcium-induced calcium-release mechanism stimulates the same channels that are also activated by the DHPR, or whether it stimulates a different set of RyRs not directly coupled to DHPR. Data from Block et al. [47] have shown the existence of an alternative juxtaposition of RyRs and DHPR in the swimbladder muscle triads, where only one of two RyRs is faced by a DHPR tetrad. Although these observations have been made in a muscle subsequently shown to contain only the α isoform [37], they have provided a structural basis for a model where only one RyR of two is directly activated via interaction with the DHPR. This model postulates that the other RyR, not directly coupled to the DHPR, is activated by the calcium initially released by the RyR operated by the DHPR.

Known properties of α/RyR1 and β/RyR3 revealed different properties for the two molecules that fit well with such a model. Biochemical characterization of chicken α and β RyRs has revealed the existence of significant differences between the two proteins; for example, the α isoform binds calmodulin more than does the β isoform, and, on the contrary, the β isoform is phosphorylated by the calmodulin-dependent protein kinase more than is the α isoform [13]. More recently, the ion-channel properties of two RyR isoforms expressed in chicken skeletal muscle have been described [41]. The two channels appear to differ in their gating behaviour. Interestingly the α isoform (RyR1) is less sensitive to calcium activation than is the β isoform (RyR3). In addition, the α/RyR1 isoform is activated by perchlorate ions, while the β/RyR3 is not, suggesting that only the former may interact with the DHPR, as it has been suggested that perchlorate ions favour interaction between DHPR and RyR [48,49]. Therefore, as initially suggested by Sutko and co-workers [41], it is conceivable that the two RyR isoforms contribute differently to the mechanism of excitation–contraction

coupling in skeletal muscle, with the chicken α/RyR1 behaving similarly to the mammalian skeletal RyR1 in association with the DHPR, and the chicken β/RyR3 seeming apparently to fit better into the role of being activated by calcium, in a calcium-induced calcium-release fashion. Furthermore, physiological studies of SR preparation from frog and fish skeletal muscles indicated that the two RyRs present in skeletal muscles of these species have distinct properties comparable with those of chicken α/RyR1 and β/RyR3 [50,51]. Also, the Crooked Neck Dwarf (*cn*/*cn*) chicken mutant does not express the α/RyR1 but does express the β/RyR3 [52,53]. In this mutant β/RyR3 is apparently not able to substitute for the α/RyR1 function, once more indicating that the two channels are linked to a distinct mechanism of activation [33].

In spite of differences in expression levels and eventual relevance to the mechanism of excitation contraction, RyR3 isoforms are strongly conserved between frogs, chicks and mammals. The full-length amino acid sequence of the chicken RyR3 has been deduced from the nucleotide sequence of the RyR3 cDNA. The amino acid sequence of the chicken RyR3 is 85.6% identical to the sequence reported for the frog RyR3. Frog and chicken RyR1 and RyR3 share an 86% overall homology with the respective mammalian isoforms. The identity between the amino acid sequences of RyR3 from mammals, frogs and avian species can be as high as 91% in regions that are well conserved also among different isoforms (i.e. RyR1, RyR2 and RyR3). Interestingly, the part of the protein with the major differences in the amino acid sequence between chicken and frog RyR3 (amino acids 4248–4371) coincides with the region where the three different isoforms of RyRs exhibit the lowest level of sequence conservation, called divergent (D) region 1 [22]. This region contains some of the potential transmembrane domains as well as some of the domains that regulate calcium-channel activity, at least in the RyR1 [54–58]. This region may also be important in regulating the specific properties of each isoform. In this part of the protein (amino acids 4248–4371), the amino acid sequences of chick and frog RyR3 are only 57.0% identical. Although this value is significantly higher than values of about 30% identity observed comparing the very same region between the three RyR isoforms in mammals, it is still significantly lower than values as high as 90% observed in other parts of the RyR molecule. These differences could reflect changes in the functional properties of the specific isoforms during evolution, or identify those residues that are less critical to regulation of calcium release. Alternatively, those amino acids, in this sequence, that have been conserved during evolution may be important for maintaining the properties specific to each isoform. These hypothesis should be verified experimentally in the future.

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