Muscle-specific mRNA isoform encodes a protein composed mainly of the N-terminal 175 residues of type 2 Ins(1,4,5) P_3 receptor

Akira FUTATSUGI*†, Goro KUWAJIMA*† and Katsuhiko MIKOSHIBA†‡

*CNS Research Laboratories, Shionogi & Co. Ltd., 2-5-1 Mishima, Settsu-shi, Osaka 566-0022, Japan, †Department of Molecular Neurobiology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan, and ‡Developmental Neurobiology Laboratory, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0106, Japan

We have found a novel isoform of the mouse type 2 Ins(1,4,5) P_3 receptor [Ins(1,4,5) P_3 R] mRNA by reverse transcriptase-mediated PCR analysis. The novel isoform, which was expressed specifically in skeletal muscle and heart, was generated by the inclusion of a novel exon. As this exon contains a stop codon, the isoform encodes a putative protein (designated TIPR) consisting of 175 acid residues of the type 2 Ins(1,4,5) P_3 R and the following six residues derived from this exon. We transfected the cDNA of

this isoform into COS-7 cells; these cells expressed a 24 kDa protein that was recognized by an antibody against TIPR produced in *Escherichia coli*. The isoform encoding TIPR was also found in human skeletal muscle and heart. The N-terminal region of Ins(1,4,5) P_3 R is suggested to have a role in ligand binding and to interact with the C-terminal channel domain of Ins(1,4,5) P_3 R itself. TIPR might regulate the Ins(1,4,5) P_3 signal pathway in both muscles.

INTRODUCTION

Ins(1,4,5) P_3 is a second messenger in the intracellular signal transduction system [1]. Its target, Ins(1,4,5) P_3 receptor [Ins(1,4,5) P_3 R], is a channel for Ins(1,4,5) P_3 -induced release of Ca^{2+} from intracellular stores [2,3]. The increase in Ca^{2+} in cytoplasm mediated by Ins(1,4,5) P_3 R is important for various cellular processes such as neurotransmission, plasticity of synaptic response and smooth-muscle contraction [4].

Ins(1,4,5) P_3 R is composed of approx. 2700 residues. One Ins(1,4,5) P_3 molecule binds to one Ins(1,4,5) P_3 R molecule; four Ins(1,4,5) P_3 R molecules compose a functional channel [5–8]. The primary structure is divided into three domains: the N-terminal domain, the C-terminal transmembrane domain and the regulatory domain [2]. The C-terminal domain has six transmembrane regions and is considered to form the Ca^{2+} channel structure [9]. The N-terminal domain is considered to be responsible for ligand binding [5,6]. Interestingly, Joseph et al. [10] suggest that the N-terminal domain interacts with the C-terminal domain; they think that the N-terminal domain regulates the channel activity of the receptor. The regulatory domain is located between the N- and C-terminal domains and contains the sites for modulators of Ins(1,4,5) P_3 R, such as protein kinase A, ATP and Ca^{2+} , which are thought to regulate the channel activity of Ins(1,4,5) P_3 R [3].

Three subtypes of Ins(1,4,5) P_3 R are known to be encoded by different genes (types I, II and III) [3,11]. These subtypes show approx. 60% identity of their amino acid sequences. Further subtypes are produced by alternative splicing of mRNA [5,12–17]. Because they show different patterns of expression in different tissues, each subtype might have a specific role. In the present study we found a novel alternatively spliced product of mouse type 2 Ins(1,4,5) P_3 R [Ins(1,4,5) P_3 R2] mRNA. We show that this mRNA isoform is produced exclusively in skeletal muscle and

heart, and encodes a putative protein consisting mainly of the N-terminal 175 residues of Ins(1,4,5) P_3 R2. We designate this protein TIPR, from ‘truncated protein of Ins(1,4,5) P_3 receptor’.

EXPERIMENTAL

RNA preparation, cDNA synthesis and reverse transcriptase-mediated PCR (RT–PCR)

All animals were treated ethically in accordance with the rules of the Shionogi Animal Use and Care Committee. ICR mice were anaesthetized and killed by decapitation, and tissues were dissected. The total RNA was isolated by the single-step acid guanidinium thiocyanate phenol/chloroform extraction method [18]. Poly(A)⁺ RNA was purified from total RNA with Oligotex-dT30 (Takara Shuzo). Poly(A)⁺ RNA from human skeletal muscle and heart was purchased from Clontech. Oligo(dT)₁₈-primed synthesis of the cDNA first strand was performed with Moloney murine leukaemia virus reverse transcriptase. PCR primers for the amplification of mouse Ins(1,4,5) P_3 R2 cDNA are listed as follows: P1, 5′-TCACCCTTGCGAGCAGCGGA-3′ [corresponds to sense sequence from nt –54 to –35 of rat Ins(1,4,5) P_3 R2 cDNA, numbered from the initiation codon]; P2, 5′-ACGACTTTATCTCCCACGAC-3′ (anti-sense sequence from nt 529–548); P3, 5′-TACGCGGAGGGCTCGGTCAAT-3′ (sense sequence from nt 52–71); P4, 5′-GCTCCTGGTTC-TACATCCAT-3′ (sense sequence from nt 473–492); P5, 5′-AACAGGCTGTTCCACTGTCC-3′ (anti-sense sequence from nt 889–908). These primers were derived from the sequence of rat Ins(1,4,5) P_3 R2 cDNA (P1, P2, P3 and P5) or from the sequence of the mouse counterpart clarified in this study (P4). The primers PH1 and PH2 were derived from human Ins(1,4,5) P_3 R2 cDNA sequence (DDBJ accession no. D26350): PH1, 5′-ATGCGTG-TGTCCTTGATGC-3′ [sense sequence from nt 439–458 of

Abbreviations used: Ins(1,4,5) P_3 R, Ins(1,4,5) P_3 receptor; Ins(1,4,5) P_3 R2, type 2 Ins(1,4,5) P_3 R; TIPR, truncated protein of Ins(1,4,5) P_3 receptor.

† To whom correspondence should be addressed (e-mail gorou.kuwajima@shionogi.co.jp).

The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers AB012393 and AB012610.

human Ins(1,4,5) P_3 R2 cDNA, numbered from the initiation codon]; and PH2, 5'-GTAGCAGAAGTAGCTGATTG-3' (anti-sense sequence from nt 808–827). For amplification of the Ins(1,4,5) P_3 R2 cDNA fragments, 30–40 cycles of PCR reaction consisting of the following steps were performed: denaturation at 94 °C for 1 min, annealing at 50–55 °C for 2 min, and elongation by AmpliTaq DNA polymerase at 74 °C for 3 min. The PCR products were subcloned into TA plasmids (Invitrogen) and subjected to DNA sequence analysis.

PCR analysis of genome structure

ICR mouse liver was homogenized with a Teflon Potter homogenizer in a solution containing 10 mM Tris/HCl and 100 mM EDTA, pH 8.0, and incubated at 37 °C for 1 h in the same solution supplemented with 0.5 % sarcosyl and 50 μ g/ml RNase A. The mixture was treated overnight with 100 μ g/ml proteinase K at 60 °C. Genomic DNA in the mixture was extracted with phenol, phenol/chloroform and diethyl ether. For PCR amplification of mouse genomic DNA fragments that span the sequence of 57 bp (S57) between nt 525 and 526 of Ins(1,4,5) P_3 R2 cDNA (also see the Results and Discussion section), the following primers were used: P6, 5'-CTGAGAA-GCGAGGGTGATAA-3' [corresponds to the sense sequence from nt 505–524 of mouse Ins(1,4,5) P_3 R2 cDNA] and P7, 5'-TTACAGGCATCAGAACGACT-3' [the anti-sense sequence from nt 543–582 of mouse Ins(1,4,5) P_3 R2 cDNA]. The PCR products were subcloned into TA plasmids (Invitrogen) and their sequences were determined on both strands.

Preparation of an antibody against TIPR

Recombinant TIPR was expressed in *Escherichia coli* under the control of the lacZ promoter, as follows. The 589 bp fragment from nt 2–590 of mouse Ins(1,4,5) P_3 R2 cDNA that includes S57 was amplified by PCR with the following anchor primers: *Hind*III site-including sense primer, 5'-GCAGAAGCTTGTCTGACAAATGT-3'; *Bam*HI site-including anti-sense primer, 5'-GACTGGATCCCCACGACGATCTA-3'. The PCR product was digested with *Hind*III and *Bam*HI, then ligated to the *E. coli* vector pUC119 digested with the *Hind*III and *Bam*HI. The *E. coli* strain XL1 blue transformed with the resultant plasmid was grown in Luria-Bertani medium under the control of isopropyl β -D-thiogalactoside. The harvested cells were sonicated and inclusion bodies were prepared by the method of Marston et al. [19]. TIPR contained in the inclusion bodies was dissolved with 4 M urea and further purified by FPLC on a Mono-S HR 5A (Pharmacia) column; in the resultant solution, any protein other than the recombinant TIPR was not detected by SDS/PAGE followed by staining with silver. Purified TIPR was suspended with Freund's incomplete adjuvant (Difco) and injected into a rabbit (Japanese White; KBL Japan). Immunoglobulins prepared from the antiserum by precipitation with 33 %-(NH₄)₂SO₄ were dissolved in 10 mM phosphate/150 mM NaCl (pH 7.2) and were used as an anti-TIPR antibody.

TIPR expression in COS-7 cells

A PCR product amplified from the S57-including form of mouse Ins(1,4,5) P_3 R2 cDNA with primers P1 and P2, which contained the TIPR-encoding region, was subcloned into plasmid pCRII. A 700 bp fragment of *Eco*RI digests of the resultant plasmid DNA was inserted at the *Eco*RI site of the mammalian expression vector pMIKneo (a gift from Dr. Kazuo Maruyama), which has the 16 S intron of SV40, the multi-cloning site of pBluescript

(Stratagene) and the poly(A)⁺ signal of SV40 at the downstream region of the SR α promoter (K. Maruyama, personal communication); the inserted cDNA would be expressed under the control of SR α promoter [20]. The resultant plasmid was transfected into COS-7 cells with Lipofectamine (Gibco BRL). At 2 days after transfection, cells were harvested, homogenized with a Teflon Potter homogenizer and subjected to SDS/PAGE [15 % (w/v) gel] [21]. After electrophoresis, proteins were transferred to a PVDF membrane (Millipore). The membrane was soaked in 0.5 % skimmed milk in 10 mM phosphate/150 mM NaCl (pH 7.2), incubated with anti-TIPR antibody and then with horseradish peroxidase-conjugated goat immunoglobulin against rabbit IgG. Peroxidase-coupled detection of signals was performed with an enhanced chemiluminescence kit (Amersham).

RESULTS AND DISCUSSION

To amplify the 5'-terminal regions of mouse Ins(1,4,5) P_3 R2 cDNA, we designed three pairs of PCR primers, P1 and P2, P3 and P4, and P4 and P5, from the cDNA sequence of rat Ins(1,4,5) P_3 R2; these sets of primers would span nt –54 to 548, nt 52–548 and nt 473–908 respectively (numbered from the initiation codon) [22]. By RT-PCR for various mouse tissues with these primer sets, we obtained fragments of the expected sizes (Figure 1). DNA sequences and the encoded amino acid sequences showed that the amplified fragments corresponded to the expected region of rat Ins(1,4,5) P_3 R2 cDNA (Figure 2A). Furthermore in each primer set of PCR a larger fragment was also amplified from skeletal muscle and heart (Figure 1). DNA

Figure 1 RT-PCR analysis of splicing patterns at S57

Gel electrophoresis of PCR products with 2% agarose. (A) PCR with three pairs of primers. Arrowheads show the positions of the two amplified fragments. Size markers (indicated at the right) are 1353, 1078, 872, 603, 310, 281/271, 234 and 194 bp from top to bottom. (B) PCR products from various tissues with primers P3 and P2. The positions of S57-including and S57-excluding fragments [S57(+) and S57(–) respectively] are indicated by arrows.

Figure 2 Alternative splicing of Ins(1,4,5) P_3 R2 mRNA generates TIPR

(A) Nucleotide sequence of the 5'-terminal region of mouse Ins(1,4,5) P_3 R2 cDNA and the encoded amino acid sequence. The insertion of S57 is indicated by the box. Asterisks show stop codons. Six amino acid residues derived from S57 are shown by underlining. The absence of amino acid residues from Ins(1,4,5) P_3 R2 is indicated by hyphens. Nucleotide bases of the mRNA that does not include S57 are numbered from the initiation codon. Amino acid residues are numbered from the initiating Met. Nucleotide bases and amino acid residues different from those of rat Ins(1,4,5) P_3 R2 are indicated by dots. (B) Schematic diagram. The initiation codon is indicated by (+). Asterisks show the stop codons. Transmembrane regions of Ins(1,4,5) P_3 R2 (IP₃R2) are indicated by dotted boxes. The additional six amino acid residues derived from S57 are shown by a black box. Primer sets for RT-PCR and amplified regions are shown by the thin lines above the mRNA.

sequence analysis showed that each of the larger fragments corresponded to the expected region of mouse Ins(1,4,5) P_3 R2 cDNA and, in addition, included S57 (Figure 2A). Interestingly, the sequence of S57 had not yet been reported. It has five stop codons in the same frame with that of Ins(1,4,5) P_3 R2. Therefore the S57-including cDNA encodes the sequence of 181 residues consisting of the N-terminal 175 residues of Ins(1,4,5) P_3 R2 and the following six residues derived from S57 (Figures 2A and 2B). We designated the protein of this sequence TIPR. The PCR product containing S57 was amplified only from skeletal muscle and heart (Figure 1). This result shows that TIPR is expressed

Figure 4 Immunoblot analysis of TIPR expressed in COS-7

Homogenate (4 μ g) of COS-7 cells that had pMIKneo containing TIPR (TIPR-transfected) or pMIKneo (control) was subjected to immunoblot analysis with anti-TIPR antibody. The position of TIPR is indicated by an arrowhead. The positions of molecular mass markers are indicated (in kDa) at the right.

specifically in these tissues. Furthermore the PCR product including S57 was present in a lower level than that excluding S57 in skeletal muscle, whereas both PCR products were present at similar levels in heart. Although we did not ensure that the results of PCR in this study were quantitative, these results suggested that the expression level of TIPR was similar to that of Ins(1,4,5) P_3 R2 in heart but lower than that of Ins(1,4,5) P_3 R2 in skeletal muscle.

We examined the genomic structure around S57. PCR with a primer set P6 and P7, which was designed from the mouse cDNA sequence to span S57, generated a single product of 4.3 kbp from the mouse genomic DNA. Comparison of the sequence of this product with the cDNA sequence revealed the exon/intron organization around S57 (Figure 3). S57 corresponded to an exon of 57 bp flanked by introns of 1.3 kbp and 2.9 kbp. The sequences of the 5' and 3' termini of both introns were in accordance with the GT/AG rule [23]. These results show that the inclusion or exclusion of S57 in Ins(1,4,5) P_3 R2 mRNA are mediated by alternative splicing.

We examined whether TIPR was really translated from a novel mRNA isoform that contained S57. We transfected the mouse cDNA fragment encoding TIPR into COS-7 cells under the control of SR α promoter. A band of 24 kDa was detected in the cells carrying the cDNA in an immunoblot analysis with the anti-TIPR antibody (Figure 4). This band was not detected in the cells that did not have the cDNA. Furthermore when the antibody was premixed with the recombinant TIPR, only the band of 24 kDa disappeared. These results show that TIPR can be

Figure 3 Arrangement of exons and introns around S57

Exons are indicated by boxes. Nucleotides at the splice sites fitting the GT/AT rule are underlined.

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human TIPR  ---GGTGACAAATGATGCAAGCTTCTGGATTTAATTGGTTAATAAATTATCTGGTATATCTTCCTTTATTGTT--
              - G D N  D A S F W I * * * * *
mouse TIPR  - G D N  D M G A V I * * * * *

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Figure 5 Nucleotide sequence around human S57

The encoded amino acid sequence, the C-terminus of TIPR, is presented below. The corresponding sequence of mouse TIPR is also aligned. The box shows S57. The six residues derived from S57 are underlined. Asterisks show stop codons. Dots indicate differences in nucleotide bases and amino acid residues between mouse and human.

translated from an $\text{Ins}(1,4,5)P_3R2$ mRNA isoform that has the insertion of S57.

We next investigated whether the $\text{Ins}(1,4,5)P_3R2$ mRNA isoform that encoded TIPR was also expressed in the human. We designed the primers PH1 and PH2 to span the region from nt 439–827 of human $\text{Ins}(1,4,5)P_3R2$ cDNA (also numbered from the initiation codon). Two fragments were amplified from cDNA of human skeletal muscle and heart; both sequences corresponded well to the expected region of human $\text{Ins}(1,4,5)P_3R2$ cDNA. Moreover, the larger fragment had the insertion of 57 bp at the same site as that of S57 in mouse $\text{Ins}(1,4,5)P_3R2$ cDNA. The inserted 57 bp in the human cDNA encodes six residues followed by four stop codons in the same frame as that of $\text{Ins}(1,4,5)P_3R2$ (Figure 5). This result shows that the mRNA encoding TIPR is present also in human. Interestingly, mouse S57 has five stop codons and human S57 conserves the first four of them. This suggests that the expression of TIPR is necessary in skeletal muscle and heart. In contrast, the six residues derived from S57 were not conserved; only two residues were identical between human and mouse. Therefore six residues might not have a role in the function of TIPR. We expressed the TIPR fused with HA in COS-7 cells; anti-HA antibody stained the cytoplasmic region of the cells, suggesting that the expressed HA-TIPR was present in the cytoplasm (results not shown). TIPR is therefore expected to have the same function as that of the N-terminal 175 residues of $\text{Ins}(1,4,5)P_3R2$, which is in the cytoplasmic domain of the receptor.

One of the possible functions of TIPR is $\text{Ins}(1,4,5)P_3$ binding because the N-terminal region of the type 1 $\text{Ins}(1,4,5)P_3R$ is considered to be an $\text{Ins}(1,4,5)P_3$ -binding domain [5,6]. However, the experiment with the type 1 $\text{Ins}(1,4,5)P_3R$ fragments expressed in *E. coli* showed that the region from residues 224–579 of this receptor is essential for the $\text{Ins}(1,4,5)P_3$ binding [24]. Moreover, photolabelled $\text{Ins}(1,4,5)P_3$ bound to the region from residues 406–501 of this receptor [25]. Because the N-terminal regions are quite well conserved between the type 1 and the type 2 receptors [26], a similar region of $\text{Ins}(1,4,5)P_3R2$ is expected to be essential for the ligand binding. Therefore TIPR, which contains only the N-terminal 175 residues of $\text{Ins}(1,4,5)P_3R2$, does not seem to bind to $\text{Ins}(1,4,5)P_3$ directly.

Another possible function is the interaction with the C-terminal region of $\text{Ins}(1,4,5)P_3R$ itself. Joseph et al. [10] digested the cerebellar $\text{Ins}(1,4,5)P_3R$ with trypsin and showed that an N-terminal fragment associates with the C-terminal region. They suggest that the N-terminal $\text{Ins}(1,4,5)P_3$ -binding domain is very close to the C-terminal channel domain and that this topology mediates the $\text{Ins}(1,4,5)P_3$ -gated Ca^{2+} channel activity of the receptor. If this hypothesis is applicable to $\text{Ins}(1,4,5)P_3R2$, TIPR might be able to interact with the C-terminal region of the receptor. Binding of TIPR to the C-terminal region of the type 2 receptor might affect the topology of the receptor and the Ca^{2+} channel activity. We are now investigating whether or not TIPR has such functions.

It is interesting that the mRNA encoding TIPR was detected only in skeletal muscle and heart. The skeletal and cardiac

muscles are special tissues where the ryanodine receptor is the main channel for Ca^{2+} release from intracellular stores; the released Ca^{2+} triggers the contraction of these muscles [27–30]. However, many studies suggest that $\text{Ins}(1,4,5)P_3R$ has roles in these tissues. An $\text{Ins}(1,4,5)P_3$ cascade is certainly present in skeletal muscle and heart. Some of the hormones that activate the $\text{Ins}(1,4,5)P_3$ cascade enhance contraction of the heart [31,32]. $\text{Ins}(1,4,5)P_3R$ is actually expressed in cardiac myocyte and is localized at intercalated discs of the heart [33–35]. We detected the mRNA species for $\text{Ins}(1,4,5)P_3R2$ and the type 1 $\text{Ins}(1,4,5)P_3R$ at similar levels in these muscle tissues (results not shown). The PCR analysis in the present study suggested that mRNA for TIPR was present a similar level to that for $\text{Ins}(1,4,5)P_3R2$ in heart. Therefore, although we were not able to detect TIPR or $\text{Ins}(1,4,5)P_3R2$ in skeletal muscle and heart by Western blot analysis (results not shown), TIPR is expected to be present and to have a role in these tissues. TIPR might regulate $\text{Ins}(1,4,5)P_3R$ -mediated Ca^{2+} release in both muscle types, where the ryanodine receptor-mediated Ca^{2+} release mechanism has a main role in muscle contraction. Elucidation of the function of TIPR will give a new aspect to the mechanism of the $\text{Ins}(1,4,5)P_3$ -induced Ca^{2+} release system in these tissues.

We thank Dr. Masakazu Hatanaka, Dr. Yoriko Hinuma and Dr. Tadahisa Kitamura for encouragement during this work; Dr. Kazuo Maruyama for the gift of a plasmid; and Takeshi Yaoi and Takashi Nakayama for helpful discussions.

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Received 9 April 1998/3 June 1998; accepted 3 July 1998