Alternative splice variants of hTrp4 differentially interact with the C-terminal portion of the inositol 1,4,5-trisphosphate receptors

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Abstract The molecular basis of capacitative (or store-operated) Ca^{2+} entry is still subject to debate. The transient receptor potential proteins have been hypothesized to be structural components of store-operated Ca^{2+} channels and recent evidence suggests that Trp3 and its closely related homolog Trp6 are gated by the N-terminal region of the inositol 1,4,5-triphosphate receptors ($InsP_3R$). In this study, we report the existence of two isoforms of the human Trp4 protein, referred to as α-hTrp4 and β-hTrp4. The shorter variant β-hTrp4 is generated through alternative splicing and lacks the C-terminal amino acids G^{785}–S^{868}. Using a yeast two-hybrid assay and glutathione-*S*-transferase-pulldown experiments, we found that the C-terminus of α-hTrp4, but not of β-hTrp4, associates in vitro with the C-terminal domain of the $InsP_3$ receptors type 1, 2 and 3. Thus, we describe a novel interaction between Trp proteins and $InsP_3R$ and we provide evidence suggesting that the formation of hTrp4–$InsP_3R$ complexes may be regulated by alternative splicing.

Key words: Transient receptor potential protein; Inositol 1,4,5-triphosphate receptor; Alternative splicing; Ca^{2+} entry

1. Introduction

In many non-excitable cells, depletion of internal Ca^{2+} stores causes Ca^{2+} influx from the extracellular space. This mechanism has been referred to as 'store-operated' Ca^{2+} entry and is thought to regulate a variety of important cellular functions ranging from secretion, gene transcription, cell proliferation to apoptosis [1]. The sequence of signaling events leading to the opening of store-operated channels (SOCs) is far from clear and three major hypotheses have been emitted. One involves the release from the empty stores of a soluble messenger that diffuses to the plasma membrane to activate SOCs [2]. The second model suggests a physical coupling between the Ca^{2+} entry channels and the inositol 1,4,5-triphosphate receptor ($InsP_3R$) in the endoplasmic reticulum [3,4]. An alternative proposal assumes exocytotic insertion of vesicular channels or regulatory molecules into the plasma membrane [5,6].

As candidates for SOCs, the transient receptor potential (Trp) and the Trp-like (Trpl) proteins, which mediate light-activated Ca^{2+} entry in *Drosophila* photoreceptors, have been proposed [7]. Since then, seven mammalian Trp homologs have been cloned and expressed functionally in vitro (reviewed in [8]). Trp1, Trp2 and Trp4 appear to be gated by Ca^{2+} store depletion alone whereas Trp3, Trp6 and Trp7 further require a product of receptor-stimulated phospholipase C (PLC) for their activation. Recent findings strongly support the idea that Trp3 is gated by a conformational coupling mechanism. First, in human embryonic kidney (HEK) 293 cells, activation of the overexpressed Trp3 can be blocked by displacing the endoplasmic reticulum from sites of close interaction with the plasma membrane [9]. Secondly, in excised membrane patches, Trp3 channels can be activated by addition of vesicles containing $InsP_3$-liganded $InsP_3R$ [10]. At last, a 289 amino acid sequence just downstream the $InsP_3$ binding site of the $InsP_3R$ has been shown to associate with the C-termini of both Trp3 and its closely related homolog Trp6 [11].

In this study, we report that the human Trp4 protein also binds in vitro to $InsP_3R$. While Trp3 and Trp6 are gated by the N-terminal region of $InsP_3R$, hTrp4 associates with the C-terminal domain of the intracellular Ca^{2+} release channels. The interaction is lost upon removal of 84 amino acids in the hTrp4 C-terminus resulting from alternative RNA splicing.

2. Materials and methods

2.1. Materials

Restriction endonucleases were purchased from Promega. DMEM-F12, fetal calf serum and Geneticin were obtained from Gibco BRL. All other chemicals were obtained from Fluka or Sigma. HEK293 cells were from the American Type Culture Collection (Rockville, MD, USA). 293T cells were a gift from Dr. Didier Trono (Depart-

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Abbreviations: EYFP, enhanced yellow fluorescent protein; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione-*S*-transferase; HEK, human embryonic kidney; $InsP_3$, inositol 1,4,5-triphosphate; $InsP_3R$, inositol 1,4,5-triphosphate receptor; IRES, internal ribosome entry site; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; SOC, store-operated channels; Trp, Transient receptor potential; Trpl, Trp-like

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ment of Genetics and Microbiology, Centre Medical Universitaire, Geneva, Switzerland).

2.2. Amplification by polymerase chain reaction (PCR) of the coding sequence of the hTrp4 cDNA

PolyA$^+$ mRNA was purified from 293T cells using the Fast Track 2.0 kit (Invitrogen). First-strand cDNA was synthesized by using the avian myeloblastosis virus reverse transcriptase (Invitrogen) and either oligo-dT or random hexamers as primers. Three sets of specific oligonucleotides were used to amplify by PCR the entire coding region of the hTrp4 cDNA: FNTrp4 (TAG (C/G)AT GGC (A/C)TG AAC ATG GC(T/G) CAG) and RNTrp4 (CGT ACC AGC GAG ATG CCA GCA GCT GTT GAC), FHTrp4 (GTC AAC AGC TGC TGG CAT CTC GCT GG) and RHTrp4 (CTG CAT GGT CAG CAA TCA GTT GG), FCTrp4 (GAG TTT GTT GGT GCC ACC ATG TTT GGG AC) and RCTrp4 (ATA CGT ATG TGT ATG GTA AAA GCT TCC TCC). The primers were designed according to partial hTrp4 cDNA sequences deposited in the GenBank database under the accession numbers U40983 [12], C21545 and AA629948. The PCR mixture typically contained first-strand cDNA (50 ng), 0.2 mM dNTP, 0.2 μM primers, 1.5–4.5 mM $MgCl_2$ and 1 U Taq polymerase (Qiagen). After an initial step of denaturation (94°C for 1 min), 35–40 cycles of PCR were carried out in a Trio thermoblock thermocycler (Biometra) set to 94°C for 30 s, 50–60°C for 40 s, 72°C for 75 s, followed by a final extension at 72°C for 10 min. The resulting DNA fragments were purified by agarose gel electrophoresis and subcloned into the pGEM-T vector (Promega). For each PCR product, four to six independent clones were sequenced with a Perkin Elmer automated sequencer and the ABIPRISM Dye Terminator Cycle sequencing kit (Perkin Elmer).

2.3. Cloning of the 5′-untranslated region by rapid amplification of cDNA ends (RACE)-PCR

A library for RACE by PCR was prepared, using 1 μg of 293T mRNA, random hexamers as primers, and reagents provided by Clontech in the Marathon cDNA amplification kit. Two sequential RACE steps were conducted using the Advantage Klen Taq polymerase mix (from Clontech). Primary RACE-PCR was performed using the adapter-ligated primer AP1 (5′-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3′, Clontech) in combination with the hTrp4 specific antisense primer R1 (5′-CAG CTC AAC AGC TCC GAC GAC TTC-3′). Nested PCR amplification was then carried out with internal primers AP2 (5′-ACT CAC TAT AGG GCT CGA GCG GC-3′, Clontech) and R2 (5′-GAG TAG TTC GAT GAG CTC CAA G-3′). The thermocycler was programmed as follows: initial denaturation at 94°C for 1 min; 35 cycles: 94°C for 30 s, 68°C for 2 min. The resulting product was gel purified, subcloned into the pGEM-T vector and sequenced.

2.4. Northern blot analysis

Human multiple tissue Northern blot I (Clontech) was prehybridized for 3 h at 65°C in 500 mM $NaHPO_4$ (pH 7), 7% sodium lauryl sulfate, 1% bovine serum albumin and 1 mM EDTA [13] and then hybridized in the same buffer with 4×10^6 cpm/ml ^{32}P-labeled probe for 16 h at 65°C. The filter was washed at room temperature for 15 min twice with 2×SSC/0.1% SDS, once with 1×SSC/0.1% SDS, once with 0.1×SSC/0.1% SDS and at 65°C twice with 0.2×SSC/0.1% SDS for 10 min. The probe was made from a 1 kb restriction fragment encoding the hTrp4 hydrophobic core, labeled by random priming (Promega) with ^{32}P-dCTP. Autoradiography was carried out for 3–8 days with intensifying screens. After washing out of the hTrp4 probe, the filter was rehybridized with a β-actin probe (Clontech) as a control.

2.5. Southern blot analysis

cDNAs (1 ng) from the human multiple tissue cDNA panel I (Clontech) were used as template in PCR reactions in combination with either the FCTrp4 and RCTrp4 oligonucleotides or the control primers (FG3PDH, 5′-TGA AGG TCG GAG CAA CGG ATT TGG T-3′ and RG3PDH, 5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′). 20 to 35 cycles of amplification were carried out with Taq polymerase (Qiagen). Aliquots of the PCR mixture were separated on a 1% agarose gel, capillary transferred to a nylon membrane (Electran, BDH) and then immobilized by UV irradiation. Southern blots were prehybridized at 65°C for 3 h in 6×SSC/5×Denhardt's reagent/1% SDS/1 mM EDTA/50 mM $NaHPO_4$ (pH 7) and then hybridized in the same buffer with a ^{32}P 5′-end-labeled oligonucleotide that recognizes the sequence flanked by the primers used for PCR amplification. Two different probes were used as probes: P1 (5′-GTG AAG CGA TAC GTT GCT GC-3′) served to monitor the expression of the hTrp4 splice variants whereas P2 (5′-CAT CAT CCC TGC CTC TAC TG-3′) allowed the determination of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) transcript levels in each cDNA preparation. The filters were washed at room temperature for 15 min twice with 6×SSC/0.1% SDS, twice with 2×SSC/0.1% SDS and twice with 0.2×SSC/0.1% SDS. Autoradiography was carried out for 1–4 h with intensifying screens.

2.6. Genomic analysis

Genomic DNA was prepared from HEK293 cells using the DNAzol reagent (Gibco BRL). The region of the Trp4 gene encoding the amino acids I^{692} to R^{894} (Fig. 1A) was amplified by PCR using the oligonucleotides SGTrp4 (5′-CAA TAG GGA GGC GAG CTG CTG A-3′) and RGTrp4 (5′-CCC GTG AAG CTA ATC CTC GCA G-3′) as primers. The resulting 3 kb PCR product was subcloned into the pGEM-T vector (Promega) and three independent clones were sequenced.

2.7. Construction of the full length clones and subcloning into the expression vector pIRES-enhanced yellow fluorescent protein (EYFP)

Overlapping PCR fragments (subcloned into the pGEM-T vector) were ligated together to give the full length cDNAs encoding the α- and β-hTrp4 variants. The 5′-untranslated region was removed and both constructs were epitope-tagged at the N-terminus with the c-myc peptide MEQKLISEEDLLR. The first methionine codon was included within the sequence characteristics for translation initiation 5′-GCCGCCATGG-3′ as specified by Kozak [14]. These constructs were controlled by sequencing and then subcloned into the *Not*1 site of the pIRES-EYFP vector (Clontech).

2.8. Yeast two-hybrid assay

The full length cDNA encoding the $InsP_3R$ isoform 2 was cloned from rat liver using the long template PCR system from Boehringer Mannheim (Roche, Basel, Switzerland). The cDNAs encoding the $InsP_3R$ type 1 and 3 were kindly provided by Dr. Mignery and Dr. Bell respectively. Overlapping DNA fragments encompassing the N- and C-terminal domains of the $InsP_3R$ type 2 or the C-terminal regions of the $InsP_3R$ type 1, 2 and 3 were generated by PCR with mutant primers containing appropriate restriction sites. The constructs were confirmed by sequencing and then subcloned into the pACT2 vector (Clontech) which encodes the Gal4-activating domain (Gal4-AD). The complete C-termini of α-hTrp4 (M^{615} to L^{977}) and β-hTrp4 (M^{615} to L^{893}) were fused to the Gal4-DNA binding domain (Gal4-BD) encoded by the pAS1 vector (ATCC). Yeast two-hybrid assays were performed with the AH109 strain (Clontech). Yeast transformations were conducted according to the Matchmaker II user manual (Clontech). Recombinant yeast presenting a blue phenotype when streaked out on agar plates lacking adenine, histidine, leucine and tryptophan and supplemented with α-Gal (Clontech) were considered as positives.

2.9. Glutathione-S-transferase (GST)-pulldown assays

The DNA fragment encoding the C-terminus of the rat $InsP_3$ receptor type 2 (amino acids 2544–2701) was subcloned into the GST fusion protein vector, pGEX-5X-1 (Pharmacia Biotech). The GST-IP_3RCter fusion protein was expressed in BL21 *Escherichia coli* strain and purified according to the manufacturer's instructions. Briefly, IPTG-induced bacteria were harvested and resuspended in PBS containing 1% Triton X-100 and a cocktail of protease inhibitors (Boehringer Mannheim). Cells were lysed by sonication and then centrifuged at 13 000 rpm for 5 min at 4°C. The supernatant was incubated with glutathione-Sepharose beads (Pharmacia Biotech) for 15 min at room temperature. After three washes with the lysis buffer, an aliquot of beads (50 μl of bed volume) was removed: bound proteins were eluted with 10 mM glutathione, analyzed on 12% SDS–PAGE and then stained with Coomassie blue.

HEK293 cells were cultured in DMEM-F12 medium supplemented with 10% heat-inactivated fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin. Transfections were carried out by the calcium

phosphate coprecipitation method as described by Chen and Okayama [15]. Cells were lysed 48 h posttransfection in PBS containing 1% Triton X-100 and a cocktail of protease inhibitors (Boehringer Mannheim). Protein concentrations were measured with the BCA protein assay kit (Pierce). GST-pulldown experiments were performed by mixing 100 μl of cell lysate (300 μg of total protein) with beads charged with either GST or the GST-IP_3RCter fusion protein, for 16 h at 4°C. The beads were then washed three times with the lysis buffer at 4°C. Bound proteins were eluted with 40 μl of 2× Laemli buffer and analyzed by SDS–PAGE and immunoblot. Blots were probed with an anti-c-myc antibody (1/500, clone 9E10, Boehringer Mannheim) and developed using a horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham) and an enhanced chemoluminescence detection system (ECL Amersham).

3. Results and discussion

A PCR-based strategy was used to clone the human Trp4 cDNA as described in experimental procedures. 293T cells were found to coexpress two hTrp4 isoforms referred to as α and β. α-hTrp4 is composed of 977 amino acids (Fig. 1A) and shows 96.7%, 96.6% and 88% sequence identity to bovine [16], mice [17], and rat [18] homologs respectively. The β variant has also been identified in mice tissues [17] and lacks the residues G^{785} to S^{868}. The latter are encoded by a 252 bp sequence (Fig. 1B) flanked by consensus splice donor and splice acceptor dinucleotides (GT and AG respectively). This suggests that the 252 bp cassette is not a true exon but an intron that can be either retained or spliced out during processing of the hTrp4 pre-mRNA. To test this hypothesis, genomic DNA was prepared from HEK293 cells and used as a template in a PCR reaction, in combination with sense and antisense primers to regions surrounding the deletion. As shown in Fig. 1C, the 3 kb PCR product that was obtained is composed of four exons (E1–E4), two invariable introns (I_1 and I_2) and one alternative intron (I_a). Surprisingly, the sites that flanked I_a match the splice donor (AGGTAAGT) and acceptor ($(T/C)_6$NCAG) consensus [19] better than the sequences found at the boundaries of I_1 and I_2 (Fig. 1D). Thus, the fact that I_a is spliced out less efficiently than other introns is not due to mediocre splice acceptor or donor sites.

The occurrence of the hTrp4 C-terminal diversity prompted us to investigate whether any tissue specific regulation of this alternative splicing exists. Northern blot analysis (Fig. 2A) revealed that hTrp4 is strongly expressed in placenta. Intermediate message levels were observed in heart, pancreas, kid-

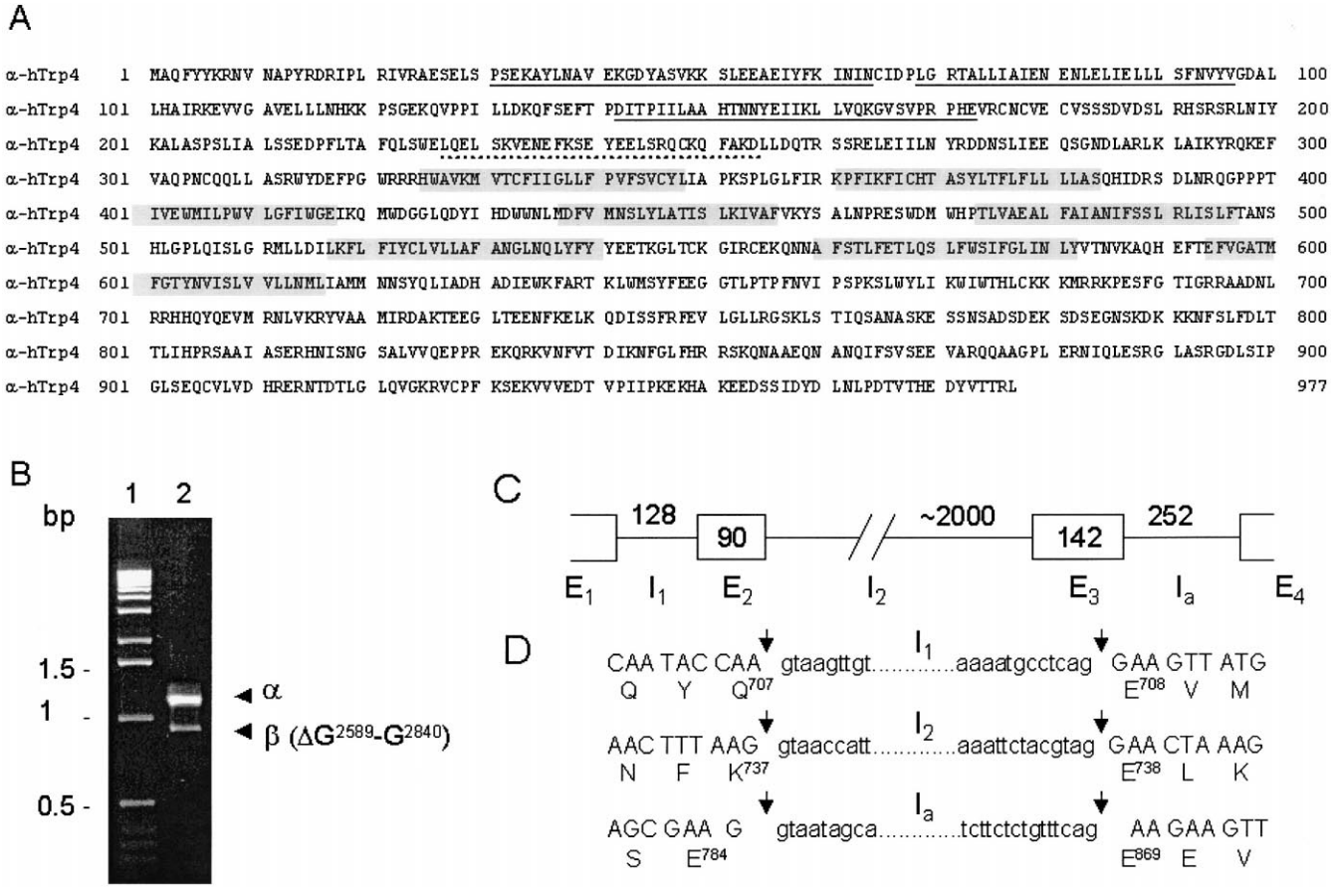


Fig. 1. Identification of the hTrp4 isoforms. A: Primary structure of α-hTrp4. Ankyrin-like motifs, coiled-coil structure and hydrophobic segments are indicated by single underlines, dotted underlines and shadow, respectively. Amino acids are numbered starting from the first methionine. B: Analysis of PCR products generated using 293T cDNA as a template, FCTrp4 and RCTrp4 as primers: molecular weight markers (lane 1) and PCR products (lane 2) were electrophoresed on a 1% agarose gel and visualized with ethidium bromide. Nucleotides that are missing in the β variant are indicated in parentheses. The sequences of α- and β-hTrp4 have been submitted to the GenBank database under the accession numbers AF063822 and AF063823 respectively. C: Structure of the region in the Trp4 gene encoding the amino acids I^{692} to R^{894}. Shaded boxes represent exons and horizontal lines, introns. Length of appropriate units is indicated in the boxes or above lines in bp. The alternative intron is termed I_a. D: Sequence of exon/intron boundaries. Capital letters represent exon sequences and lowercase letters, intron sequences.

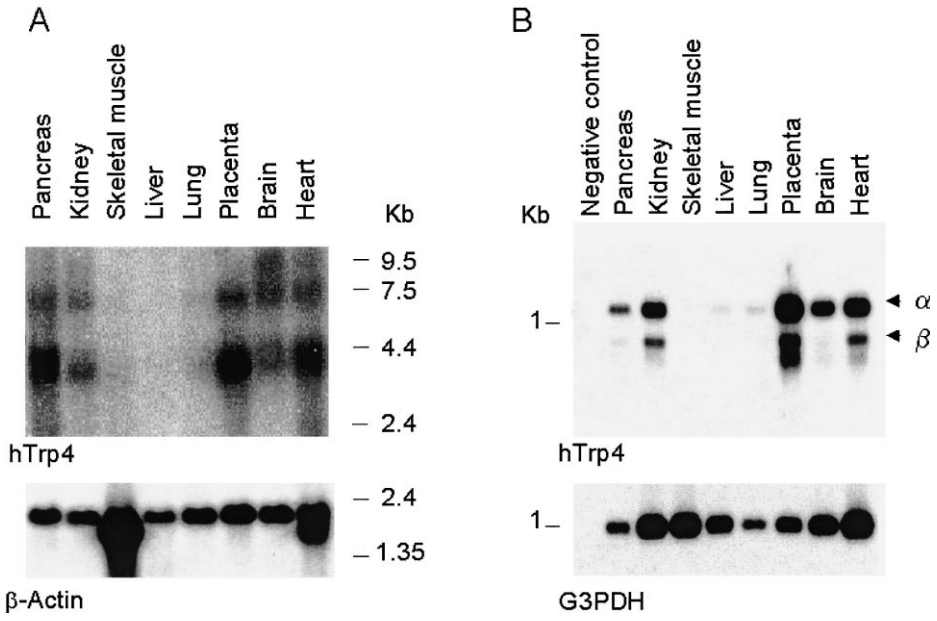
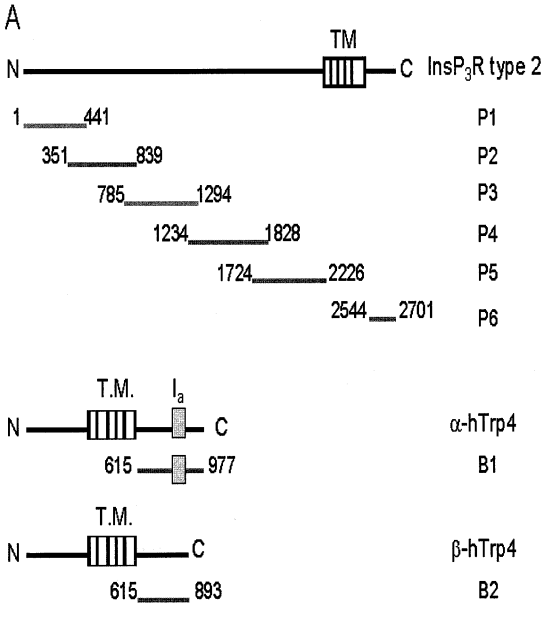


Fig. 2. Tissue distribution of hTrp4 messenger RNAs. A: Northern blot analysis of hTrp4 mRNA expression. Human adult multiple tissue mRNA blot I (Clontech) was hybridized with a ^{32}P-labeled probe encoding the hTrp4 hydrophobic core. The lower panel shows the same filter reprobed with a 2 kb β-actin probe (Clontech). B: Tissue distribution of alternative C-terminal splice variants of hTrp4. First-strand cDNAs of the MTC panel I (Clontech) were used as PCR templates with primers specific for either the C-terminal domain of hTrp4 (upper panel) or the G3PDH (lower panel). PCR products were visualized following Southern blotting, hybridization and autoradiography as described in Section 2. The negative control corresponds to an amplification performed without template. The results shown are representatives of two independent experiments.



B

Preys / Baits	P1	P2	P3	P4	P5	P6
B1	–	–	–	–	–	+++
B2	–	–	–	–	–	–

ney and brain whereas virtually no signal was found in skeletal muscle, liver and lung. Four different transcript sizes were detectable: a doublet at 3.6–3.9 kb and minor species at 6.8 and 8.7 kb. This size heterogeneity was also observed for rat, mice and bovine Trp4 messengers and might be due to either alternative splicing, alternative use of polyadenylation signals or incompletely processed mRNA.

RT-PCR was next performed to estimate the contribution of the α and β isoforms to the total pool of hTrp4 messengers. First-strand cDNAs of the human MTC panel I (Clontech) were used as templates. Amplifications were stopped before reaching the plateau phase and the products were visualized following Southern blotting, hybridization and autoradiography. The expression profile of G3PDH was included as a control for loading and PCR efficiency. As shown in Fig. 2B, α was the predominant isoform in all the hTrp4-positive tissues. The β variant was detected in placenta, kidney and heart but not in brain. Interestingly, we found in placenta an additional product almost as abundant as the β isoform. Note that this C-terminal polymorphism appears to be a specific feature of Trp4. Indeed, other Trp homologs have been shown

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Fig. 3. Interaction between the hTrp4 variants and the $InsP_3$ receptor type 2. A: Schematic description of the preys (P1–P6) and the baits (B1 and B2) used in the yeast two-hybrid assay. The linear diagrams of the $InsP_3R$ type 2 and the hTrp4 isoforms are represented with the same scale. Beginning and ending amino acid numbers of the fragments fused to the Gal4-activating domain (P1–P6) or to the Gal4 binding domain (B1 and B2) are indicated. TM, transmembrane segments; I_a, alternative intron. B: Results of the yeast two-hybrid screen. A positive interaction was found only between the C-terminus of the α variant and the C-terminal domain of the $InsP_3R$ type 2, as shown by growth and acquisition of a blue phenotype on a selective medium devoid of leucine, tryptophan, adenine, histidine and supplemented with α-Gal.

A

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Type1  2556  FADLRSEKQKKEEILKTTCFICGLERDKFDNKTVTFEEHIKEEHNMWHYL  2605
Type2  2544  FADLRSEKQKKEEILKTTCFICGLERDKFDNKTVSFEEHIKSEHNMWHYL  2593
Type3  2520  FADLRSEKQKKEEILKTTCFICGLERDKFDNKTVSFEEHIKLEHNMWNYL  2569
RyaR2  4870  FGELRdqqeqvkEdmeTkCFICGIgnDyFDtvphgFEtHtlqEHNlaNYL  4919

Type1  2606  CFIVLVKVKDSTEYTGPESYVAEMIRERNLDWFPRMRAMSLVSSDSEGEQ  2655
Type2  2594  YFIVLVKVKDPTEYTGPESYVAQMITEKNLDWFPRMRAMSLVSNEGDSEQ  2643
Type3  2570  YFIVLVRVKNKTDYTGPESYVAQMIKNKNLDWFPRMRAMSLVSGEGEGEQ  2619
Ryan2  4920  FFLmyLinKDeTEhTGqESYVwkMyqERcwEFFPagdcfrkqyeDqln    4967

Type1  2656  NELRNLQEKLESTMKLVTNLSGQLSELKDQMTEQRKQKQRIGLLGHPPHM  2705
Type2  2644  NEIRNLQEKLESTMSLVKQLSGQLAELKEQMTEQRKNKQRLGFLGSNTPH  2693
Type3  2620  NEIRILQEKLGSTMKLVSHLTAQLNELKEQMTEQRKRRQRLGFVDVQNCM  2669

Type1  2706  NVNPQQPA   2713
Type2  2694  -ENHHMPPH  2701
Type3  2670  SR        2670
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B

IP_3R isoform	Amino-acids	Growth on His/Ade deficient media	α-Gal assay
Type 1	2556-2713	+++	+++
Type 3	2520-2670	+++	+++

Fig. 4. Binding of the C-terminus of α-hTrp4 to the C-terminal domains of the $InsP_3$ receptors type 1 and 3. A: Sequence alignment of the C-terminal domains of rat $InsP_3R$ type 1 (accession number: J05510), 2 (X61677) and 3 (L06096). The C-terminal sequence of the ryanodine receptor is shown for comparison. Amino acids are numbered starting from the first methionine. Conserved residues are shaded. B: Plasmids encoding the C-terminus of either the $InsP_3R$ type 1 or the $InsP_3R$ type 3 fused to the Gal4-activating domain were cotransfected with the B1-pAS1 construct into the reporter strain AH109. Colony growth on the His/Ade-deficient medium, showing activation of the nutritional reporter genes, was controlled after 2 days. An α-Gal assay was also performed to confirm the interaction.

to exhibit structural variants [20–23]. However, in all those cases it was the N-terminal region or the hydrophobic core that was truncated.

Presently available data suggest that bTrp4 (which closely resembles α-hTrp4) is a component of SOCs. Transfection of bTrp4 in either HEK293 or CHO cells resulted in the formation of Ca^{2+} selective channels activated by $InsP_3$ or thapsigargin-induced store depletion [16,24]. Furthermore, transient expression of bTrp4 in antisense orientation was shown to reduce both the amount of native Trp4 protein and the capacitative Ca^{2+} influx in adrenal cells [25]. As new evidence has recently emerged indicating that $InsP_3R$ interacts with SOCs to control Ca^{2+} entry [9,26] and because the C-terminal tail of Trp proteins has been shown to play a crucial role in channel gating [27], we investigated the association of the α- and β-hTrp4 C-termini with $InsP_3R$.

A yeast two-hybrid assay was first set up to test the formation of Trp4–$InsP_3R$ complexes. Six preys denoted P1–P6 (Fig. 3A) were designed, encompassing the N- and C-terminal domains of the rat $InsP_3R$ type 2 (96% homology to the human $InsP_3R$ type 2). They were subcloned into the pACT2 vector, downstream the Gal4-activating domain. We used as bait the complete C-terminus of either α-hTrp4 or β-hTrp4 fused to the Gal4-DNA binding domain. Yeast strain AH109 was cotransformed with the bait and prey vectors and proper expression of the fusion proteins was controlled by Western blot analysis (data not shown). None of the N-terminal preys associated with the hTrp4 isoforms in the two-hybrid assay. In contrast, an interaction was found between the C-terminal tail of the $InsP_3R$ type 2 and the C-terminus of α- but not β-hTrp4, as shown by growth on selective media and by positive galactosidase assay. The C-terminal domains of the three $InsP_3$ receptor subtypes are closely related (Fig. 4A). To test whether all isoforms could sustain the interaction, we cloned into pACT2 the C-termini of the $InsP_3R$ type 1 and 3. The latter bound to the C-terminus of α-hTrp4 in a similar manner as the $InsP_3R$ isoform 2 (Fig. 4B).

To confirm the interaction found in the yeast two-hybrid assay, we next performed GST-pulldown experiments. N-myc-tagged hTrp4 isoforms were expressed in HEK293 cells and cell extracts were incubated with GST or with the GST-IP_3RCter fusion protein immobilized on glutathione-Sepharose beads. The latter were then extensively washed and the bound proteins were analyzed by immunoblotting. As shown in Fig. 5, N-myc-tagged α-hTrp4 but not β-hTrp4 was able to bind to the GST-P6 fusion protein whereas no significant

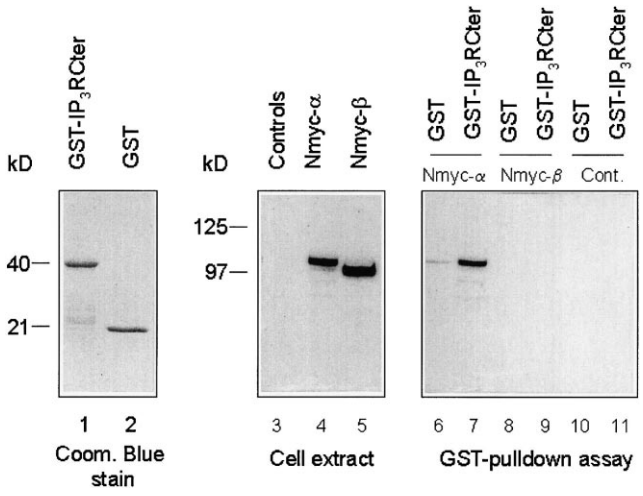

Fig. 5. In vitro binding of full length hTrp4 isoforms to the GST-P6 fusion protein. HEK293 cells were either mock transfected (controls) or transfected with N-myc-tagged α-hTrp4 (Nmyc-α) or N-myc-tagged β-hTrp4 (Nmyc-β). Lysates were prepared from transfected cells and precipitated with either GST or the GST-IP$_3$RCter fusion protein immobilized on Sepharose beads. Proteins were separated on 7% SDS–PAGE, under reducing conditions before Western blotting with anti-c-myc antibody. The left panel represents a Coomassie blue staining of the GST-IP$_3$RCter fusion protein (lane 1) and the GST (lane 2) present in each pulldown. Lanes 3, 4 and 5 contain 20% of the extract used for the binding assay.

interaction was detected between the GST alone and N-myc-tagged α-hTrp4. Protein staining with Coomassie blue (Fig. 5, left panel) demonstrated that equivalent amounts of GST and GST-IP$_3$RCter fusion protein were present in each pulldown.

Taken together, our results suggest that α-hTrp4, like Trp3 and Trp6, may be physically coupled to InsP$_3$ receptors. They also indicate that the formation of hTrp4–InsP$_3$R complexes may be regulated by alternative splicing of the hTrp4 mRNA. To our knowledge, this is the first evidence that alternative splicing may modify the properties of Trp proteins. α-hTrp4 and Trp3 do not preferentially bind to the same domain of the InsP$_3$R. Indeed, no interaction was found in the yeast two-hybrid assay between α-hTrp4 and the P2 prey which contained a sequence (residues 752–822) conserved in the three InsP$_3$R subtypes and that has been proposed to form the signal-transfer domain of the InsP$_3$R to Trp3 [11]. Presently available data suggest that Trp3 is not a store-operated channel in the strictest sense. Indeed, as opposed to SOCs, Trp3 requires InsP$_3$-liganded InsP$_3$R for its activation [10,28] and can be gated by diacylglycerol independently of store depletion [29]. On the other hand, Ma et al. [9] demonstrated that, in HEK293 cells, Trp3 and endogenous SOC activities can be blocked by the same pharmacological agents leading either to a rearrangement of the actin cytoskeleton or to the inhibition of the InsP$_3$R function. We propose that the differential interaction of α-hTrp4 and Trp3 with InsP$_3$R may explain this apparent contradiction.

One of the main arguments against the conformational coupling model is the fact that removal of all three InsP$_3$R isoforms by genetic means in chicken B lymphocytes had no effect on thapsigargin-induced Ca^{2+} influx [30]. However, the C-terminus of InsP$_3$R closely resembles the C-terminal domain of ryanodine receptors (Fig. 4), thus the latter may substitute for InsP$_3$R to activate Ca^{2+} entry in the InsP$_3$R mutants. In support of this hypothesis, depletion of caffeine-sensitive Ca^{2+} stores in some cell types has been shown to promote capacitative Ca^{2+} entry [31,32].

In conclusion, we report the primary sequence and the expression patterns of two isoforms of the human Trp homologue hTrp4. The two variants arise from alternative processing of the mRNA and differ in the length of their C-terminal domains. The C-terminus of the long (α-hTrp4), but not the short (β-hTrp4) variant, is able to bind to the three InsP$_3$R isoforms in vitro. As opposed to Trp3 and Trp6 that interact with an N-terminal region of the InsP$_3$R, α-hTrp4 associates with the C-terminus of the intracellular Ca^{2+} release channel. These results are compatible with recent findings suggesting that store-operated Ca^{2+} channels are regulated by an InsP$_3$R-dependent conformational coupling mechanism. They also suggest that alternative splicing of Trp mRNA may generate channels with distinct properties.

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