Cloning of a novel four repeat protein related to voltage-gated sodium and calcium channels

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Received 14 December 1998; received in revised form 12 January 1999

Abstract Cloning has led to the discovery of more ion channels than predicted by functional studies, yet there remain channels that have not been cloned. We report the cloning of a novel protein that contains the four domain structure found in voltage-gated Ca^{2+} and Na^{+} channels. Phylogenetic relationships suggested that the protein might have diverged from an ancestral four repeat channel before the divergence of Ca^{2+} and Na^{+} channels. Northern blot analysis showed that mRNA transcripts encoding the protein are expressed predominantly in the brain, moderately in the heart, and weakly in the pancreas. Despite extensive expression attempts, currents from the putative channel were not detected. Based on its sequence, we propose that the novel protein might be a voltage-activated cation channel with unique gating properties.

Key words: Amino acid sequence; Calcium channel; Sodium channel; Gene library; Molecular sequence data; Protein conformation; Structure-activity relationship

1. Introduction

Molecular cloning and expression studies have uncovered new aspects about structure, diversity, pharmacology, and biophysical properties of Ca^{2+} channels. The α1 subunits are the pore-forming proteins composed of four homologous domains, each of which contains six putative membrane spanning segments and a pore loop. Significant structural similarity exists between a domain of voltage-gated Ca^{2+} channel α1 subunits and voltage-activated K^{+} channel α subunits [1]. A functional K^{+} channel was found to have a tetrameric stoichiometry [2]. Based on their structural homology, an ancestral Na^{+} and/or Ca^{2+} channel α subunit was proposed to have resulted from two rounds of duplication of a single domain channel like a K^{+} channel [3].

In an attempt to clone novel voltage-activated ion channels, we searched the GenBank using the Basic Local Alignment Search Tool (BLAST) program [4]. Two candidates for novel Ca^{2+} channel sequences were found in *Caenorhabditis elegans* cosmids (C27f2 and C54d2). Both putative channels contained four repeats, each of which contains six putative membrane spanning segments and a pore loop. Cloning of the mammalian homologs of C54d2 led to the identification of three T-type Ca^{2+} channel α1 subunits, α1G [5], α1H [6], and α1I [7]. In this study we report the cloning of a novel four domain protein, analysis of its putative structure, alignment to other four domain channels, and distribution of its mRNA. Despite extensive efforts using two expression systems, we were unable to determine the functional properties of the putative channel.

2. Materials and methods

2.1. Design of PCR primers

A pair of degenerate PCR primers was designed against the fifth putative membrane spanning segment (AGVVLFG) and the pore loop (TGEDWNDI) of domain IV of the *C. elegans* C27f2 sequence. The nucleotide sequences of the primers were: the forward primer, T15, GCC GGN GTN GTN (C/T)TN TT(C/T) GG; and the reverse primer, T16, AT (A/G)TC (A/G)TT CCA (A/G)TC (C/T)TC NCC NGT.

2.2. PCR

Total RNA was purified from neonatal Sprague-Dawley rats using guanidium thiocyanate-phenol extraction [8]. First strand cDNA was synthesized from 0.5 μg of total RNA using MMLV reverse transcriptase. The first strand cDNA reaction was supplemented with 2.5 units of *Taq* polymerase, 100 pM of each primer, 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$. Two different annealing temperatures were used during the PCR: the first cycle (30 s at 94°C, 30 s at 57°C, and 30 s at 72°C) for 10 times and the second cycle (30 s at 94°C, 30 s at 60°C, and 30 s at 72°C) for 25 times. PCR products were separated through a 1% agarose gel, cut out of the gel, and then purified with a Qiaquick gel extraction column (Qiagen, Valencia, CA, USA).

The PCR products were subcloned in pGEM-T (Promega, Madison, WI, USA). Plasmid DNA was isolated from each culture using the alkaline lysis method [9]. Plasmids containing PCR products were sequenced using the Sequenase II DNA sequencing kit (Amersham, Arlington Heights, IL, USA). Nucleotide sequences were read from the autoradiograph using a digitizer, and analyzed using WDNASIS software (Hitachi, San Bruno, CA, USA).

2.3. Cloning of a full-length cDNA

A rat brain library (Clontech, Palo Alto, CA, USA, #RL3005a) was screened to clone the full-length cDNA. All probes were labeled using the RadPrime DNA labeling system (Gibco BRL). Hybridization was performed at 42°C overnight. Membranes were washed up to 60°C in a solution of 0.1×SSC plus 0.1% SDS. Lambda DNAs were purified from positive clones cultured on agarose plates using the lambda midi kit (Qiagen). Inserts in purified λ DNA were digested by *Eco*RI, separated in agarose gels, and purified using gel extraction columns (Qiagen). The inserts were subcloned into pGEM-3Z vectors digested by *Eco*RI and dephosphorylated by calf intestinal alkaline phosphatase [9]. Library screening was repeated three times to isolate positive clones covering the entire coding region of the putative ion channel (called Rb21-channel).

The full-length cDNA of the putative channel was obtained from the following overlapping clones; rbIII7, *Eco*RI (−64)/*Xma*I (1505); f2, *Xma*I (1505)/*Eco*RI (2037); g2, *Eco*RI (2037)/*Nco*I (3394); and d39, *Nco*I (3394)/*Sac*I (polylinker). These four fragments were subcloned into the pGEM-3Z vector and the pTracer (Invitrogen). The full-length cDNA was sequenced on both strands. The sequence was deposited in the GenBank (accession no. AF078779).

2.4. Northern blot analyses

The tissue expression of the putative channel transcript was investigated by Northern blot analyses. Total RNA was purified from lung, heart, skeletal muscle, testis, brain, kidney, and liver of a young male

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PII: S0014-5793(99)00082-4

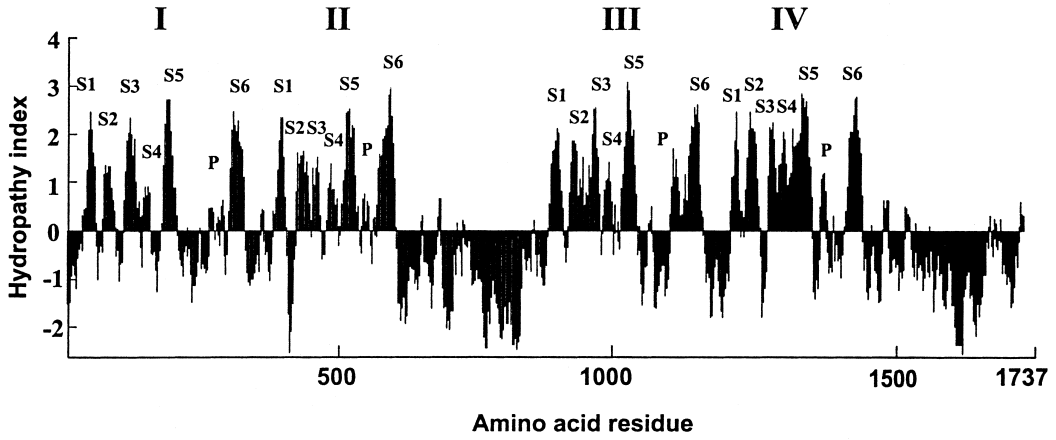

Fig. 1. Hydropathy profile of the novel four domain protein. Hydropathy analysis was performed using the algorithm of Kyte and Doolittle [34]. The amino acid residues of the novel protein are plotted along the *x*-axis, and the calculated hydropathy indexes based on a running average of 14 amino acids are plotted along the *y*-axis. Putative membrane spanning segments of each domain detected as hydrophobic regions are labeled as S1, S2, S3, S4, S5, P (pore), and S6.

Sprague-Dawley rat (200 g) using the guanidium thiocyanate method. RNA samples and size markers were separated on a denaturing agarose gel and transferred to a nylon membrane (Hybond-N, Amersham) [9]. Human tissue blots and brain region blots were purchased from Clontech. The blots were hybridized with a probe synthesized from the d39 clone (nucleotides 2037–6720) using the RadPrime DNA labeling system (Gibco-BRL). Hybridized membranes were washed up to either moderate stringency for the human blots (0.5×SSC and 0.1% SDS solution at 60°C) or high stringency conditions for the rat blot (0.1×SSC and 0.1% SDS solution at 68°C). These membranes were exposed to an X-ray film between two intensifying screens (X-Omatic cassette, Kodak) which was kept at −80°C. Human blots were exposed for 10 days and the rat blot was exposed for 12 days.

2.5. Electrophysiology

For expression in *Xenopus* oocytes, complementary RNA (cRNA) of the putative channel was synthesized using T7 Message Machine (Ambion, Austin, TX, USA) and injected into defolliculated oocytes as described previously [10]. Other cRNAs synthesized by the same method were as follows: α1E from the cDNA encoding the human α1E [11]; β2 from the cDNA encoding the rat β2 [12]; and α2δ from the cDNA encoding the rabbit skeletal α2δ, a gift from Dr. Tsutomu Tanabe (Yale University). Oocytes were voltage clamped using a two-electrode voltage clamp amplifier (OC-725B, Warner Instrument Corp., Hampden, CT, USA). Existence of Na^+ channel currents was assayed in SOS solution (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM pyruvic acid and 50 mg/l gentamicin sulfate). To measure Ca^{2+} channel currents, the bathing solution was exchanged with 40 mM Ba^{2+} solution (40 mM $Ba(OH)_2$, 50 mM NaOH, 1 mM KOH, 5 mM HEPES, adjusted to pH 7.4 with methanesulfonate).

For expression in mammalian cells, cDNA was transfected into tsA201 cells using Lipofectamine reagent (Gibco BRL). Currents were measured 24–72 h after transfection. As a positive control, rat skeletal Na^+ channel α1 [13] was transfected. Pipettes were filled with the following solution: 55 mM CsCl, 75 mM $CsSO_4$, 10 mM $MgCl_2$, 0.1 mM EGTA, 10 mM HEPES (pH 7.2 adjusted with CsOH). Whole cell ruptured patch clamp configuration was applied to measure ionic currents in transfected tsA201 cells using an Axopatch 200A amplifier, a Digidata 1200 A/D converter and pClamp 6 software (Axon Instrument, CA, USA). Na^+ channel currents were measured in Tyrode solution containing 140 mM Na^+. To measure Ca^{2+} channel currents, the external solution was exchanged with a 10 mM Ba^{2+} solution (10 mM $BaCl_2$, 140 mM TEA-Cl, 5 mM CsCl, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES, adjusted to pH 7.4 with TEA-OH).

3. Results and discussion

3.1. Cloning of a novel four domain protein

Low and high voltage-activated Ca^{2+} channels might have diverged from an ancestral four domain channel during evolution [3]. To clone novel channels we aligned the sequences of all known four domain channels, then designed PCR primers based on conserved sequences (S5, pore loop, and S6). Despite considerable effort, only the sequences of known channels were detected [14]. We next used these conserved sequences to screen the GenBank with the program BLAST [15]. This identified two novel sequences from *C. elegans*: one contained in the cosmid C54d2 (U37548) and the other in C27f2 (U40419). Both of these putative proteins contain the canonical four repeat structure of voltage-activated Ca^{2+} and Na^+ channels. Cloning and expression of the mammalian homologs of C54d2 led to the identification of three members of the LVA T-type Ca^{2+} channel family [5–7].

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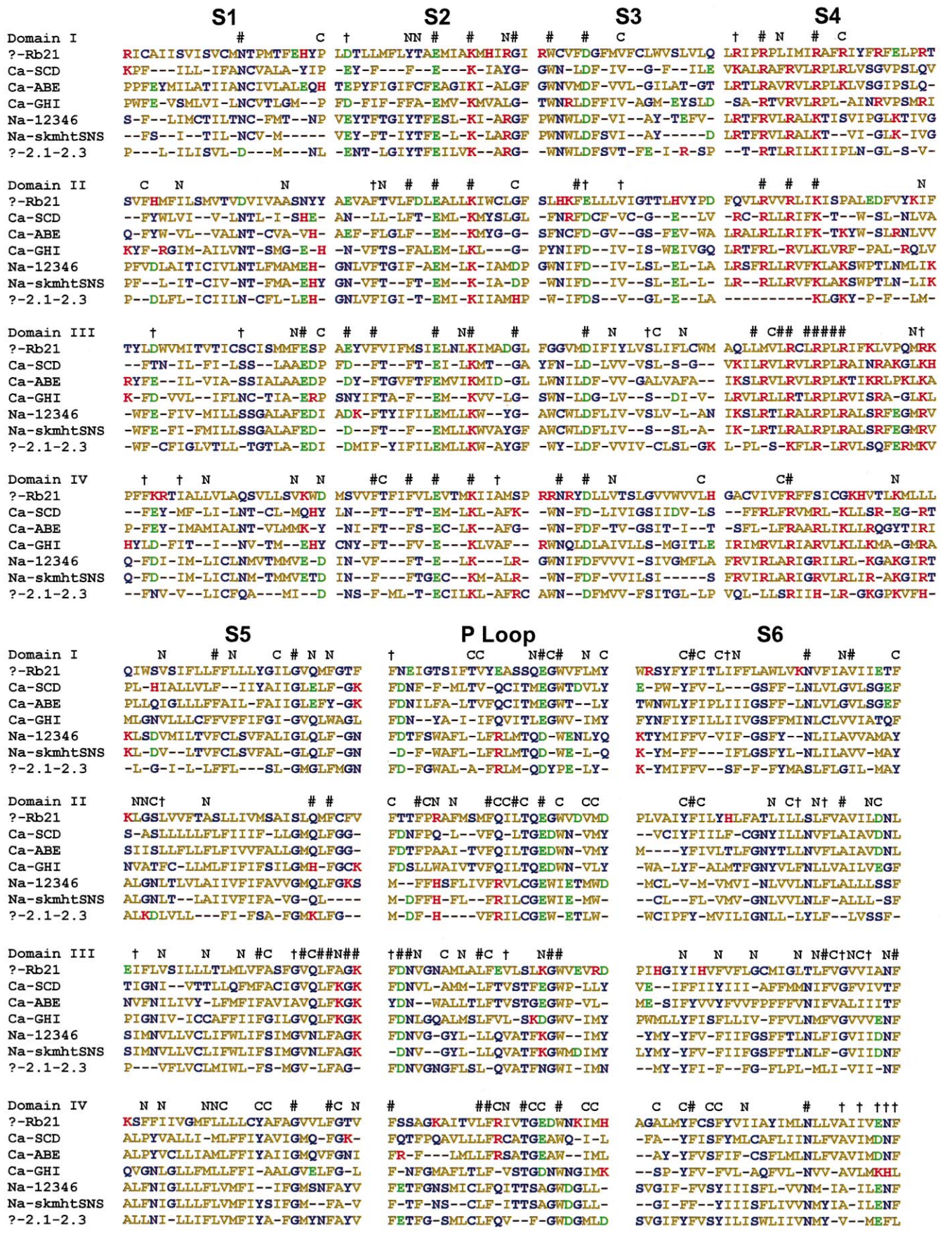
Fig. 2. Alignment of the novel protein to other four domain proteins. The putative transmembrane segments of the novel protein, labeled Rb21, were obtained from hydropathy analyses and sequence comparison to the putative membrane spanning regions of Ca^{2+} and Na^{2+} channels [1,26]. Amino acid residues are colored according to their following properties: positively charged residues are red; negatively charged residues are green; hydrophobic residues are yellow; and uncharged hydrophilic residues are blue. Conserved amino acids found in the novel protein and reported Ca^{2+} and Na^{2+} channels ($Na_v2.1$ and $Na_v2.3$ were not included) are marked as follows: '#' indicates residues found in all 17 channels, '†' indicates Rb21-channel residues that are identical in at least 13 out of 16 channels, 'N' indicates Rb21-channel residues that are identical to all eight Na^+ channels, and 'C' indicates Rb21-channel residues that are identical to all eight Ca^{2+} channels. Sources for Ca^{2+} channel sequences are as follows: Ca-SCD is the consensus sequence for L-type channels using rabbit α1S (M23919), human α1C (L04569), and human α1D (M76558); Ca-ABE is the consensus sequence of rabbit α1A (X57476), human α1B (M94172), and rabbit α1E (X67855); Ca-GHI is the consensus sequence for T-type channels using rat α1G (AF027984), human α1H (AF051946), and rat α1I (AF086827). Sources of Na^+ channels are as follows: Na-12346 is the consensus sequence of Nabr1 (rat brain, X03638), Nabr2 (rat brain, M22254), Nabr3 (rat brain, Y00766), Nabr4/PN1 (human X82835) and Nabr6/rPN4 (rat brain, L39018); Na-skmhtSNS is the consensus of Naskm1 (rat skeletal muscle, M26643), Naht/Skm-2 (rat heart, M29709), and NaSNS/rPN3 (rat sensory neurons, X92184). The consensus sequence, ?-2.1-2.3 is derived from the putative Na^+ channel sequences, $Na_v2.1$ (human heart, M91556) and $Na_v2.3$ (mouse, Y09164).


S1
S2
S3
S4
Domain I
?-Rb21
Ca-SCD
Ca-ABE
Ca-GHI
Na-12346
Na-skmhtSNS
?-2.1-2.3
Domain II
?-Rb21
Ca-SCD
Ca-ABE
Ca-GHI
Na-12346
Na-skmhtSNS
?-2.1-2.3
Domain III
?-Rb21
Ca-SCD
Ca-ABE
Ca-GHI
Na-12346
Na-skmhtSNS
?-2.1-2.3
Domain IV
?-Rb21
Ca-SCD
Ca-ABE
Ca-GHI
Na-12346
Na-skmhtSNS
?-2.1-2.3
S5
P Loop
S6
Domain I
?-Rb21
Ca-SCD
Ca-ABE
Ca-GHI
Na-12346
Na-skmhtSNS
?-2.1-2.3
Domain II
?-Rb21
Ca-SCD
Ca-ABE
Ca-GHI
Na-12346
Na-skmhtSNS
?-2.1-2.3
Domain III
?-Rb21
Ca-SCD
Ca-ABE
Ca-GHI
Na-12346
Na-skmhtSNS
?-2.1-2.3
Domain IV
?-Rb21
Ca-SCD
Ca-ABE
Ca-GHI
Na-12346
Na-skmhtSNS
?-2.1-2.3

To clone a novel mammalian channel, three pairs of PCR primers were designed using the C27f2 sequence. Among the three pairs of degenerate PCR primers, T15 and T16 primers derived from the domain IV S5 and pore loop regions amplified a Ca^{2+} channel-like sequence from rat brain RNA. To clone the full-length cDNA encoding this putative channel, a rat brain cDNA library was screened repeatedly using ends of positive clones as probes. The full-length cDNA contained 6779 bases. The first ATG in the open reading frame was preceded by a stop codon at −66 in the 5′-untranslated region. The flanking DNA sequence (ACACCATGC) of the underlined initiation codon is similar to the consensus initiation sequence CC(A/G)CCATGG identified from mRNAs of diverse eukaryotic cells [16]. The open reading frame encodes 1737 amino acids with a predicted mass of 200 375 Da.

3.2. Structural analysis

The deduced sequence and size of the novel protein showed similarity to those of reported Ca^{2+} and Na^{+} channels, leading to the hypothesis that it may have four homologous domains. This hypothesis was confirmed by matrix homology plots (data not shown). Hydropathy analysis was performed to predict the putative membrane spanning segments and pore loop portions of the putative channel (Fig. 1). The profile indicated that it has four repeats, which are connected by hydrophilic cytoplasmic loops. Each repeat contains six membrane spanning segments, a pore loop, and external and internal connecting loops. S1, S2, S3, S5, and S6 of each domain are detected with distinctive hydrophobic indices, but S4 and pore loop regions have smaller indices. The overall structural properties of the putative channel were similar to those of reported Ca^{2+} and Na^{+} channels, suggesting it may function as a voltage-gated cation channel.

To compare the novel sequence with those of reported Ca^{2+} and Na^{+} channels, we aligned their putative membrane spanning regions (Fig. 2). We searched for residues that were conserved across all four domain channels, and those conserved in either Ca^{2+} or Na^{+} channels. Also, amino acid residues in the alignment colored according to their hydrophobicity and charge, revealing considerable conservation of structure. In general, the S1 regions were the least conserved, followed closely by the S2 and S3 regions. Within the S2 regions conserved residues were spaced every 3 or 4 residues, which is consistent with the protein assuming an α-helical conformation. In the S2 and S3 regions there is striking conservation of negatively charged residues across all four domains (S2, 2nd and 12th residues; S3, 6th residue). These residues may electrostatically interact with positively charged residues in S4 regions, as described for the similarly located residues in *Shaker* K^{+} channels (E283, E293, and D316) and other channels [17–20]. The region of highest sequence identity was found in the S4 sequence of domain III. However, the S4 segments of the novel protein contained less positively charged amino acids compared with those of the Na^{+} and Ca^{2+} channels, especially in domain IV. These differences may endow the channel with unique activation and inactivation properties.

The negatively charged glutamic acid residues in the pores of domain I, II, III, and IV of cloned Ca^{2+} channels (EEEE) and Na^{+} channels (DEKA) have been reported to determine their ion selectivity [21–24]. A site-directed point mutation of E in domain III pore of a Ca^{2+} channel into lysine (EEKE) changed a Ca^{2+}-selective channel into a monovalent cation-selective channel which is not permeable to divalent ions [24]. Putative pore loops of the novel protein contain very conserved amino acids found in Ca^{2+} or Na^{+} channels, including EEKE residues in the corresponding region of its pore loops. These results suggest that the putative channel may have a unique pattern of ion selectivity.

3.3. Evolutionary relationships

BLAST search of the Genbank with the novel sequence identified many putative proteins with considerable homology, including (score in bits): (1172) a second *C. elegans* four domain protein in cosmid C11d2 (AF045640); (1160) the putative C27f2 channel (U40419); and (229) a putative channel from *Shizosaccharomyces pombe* (Z98981). BLAST next identified a number of voltage-gated Ca^{2+} channels as homologous (207–188), followed by voltage-gated Na^{+} channels.

Evolutionary relationships between the four domain proteins were analyzed using the CLUSTAL algorithm [25]. To improve the alignment, only the amino acid sequences of the putative membrane spanning regions (Fig. 2) were used. This phylogenetic analysis grouped the aligned sequences into the three families, Ca^{2+} channel, Na^{+} channel, and Rb21/C11d2/C27f2 (Fig. 3). Alignment of the full-length sequences indicated that the novel protein was 26% identical, and 44% similar on a structural basis to either C11d2 or C27f2. Interestingly, the Ca^{2+} channel family was more closely grouped with the Na^{+} channel family than the Rb21/C11d2/C27f2 family, suggesting that they diverged from an ancestral four domain channel earlier than the divergence of Na^{+} from Ca^{2+} channels.

3.4. Northern analyses

Northern blot analyses were performed to localize the expression of mRNA encoding the putative channel. The probe

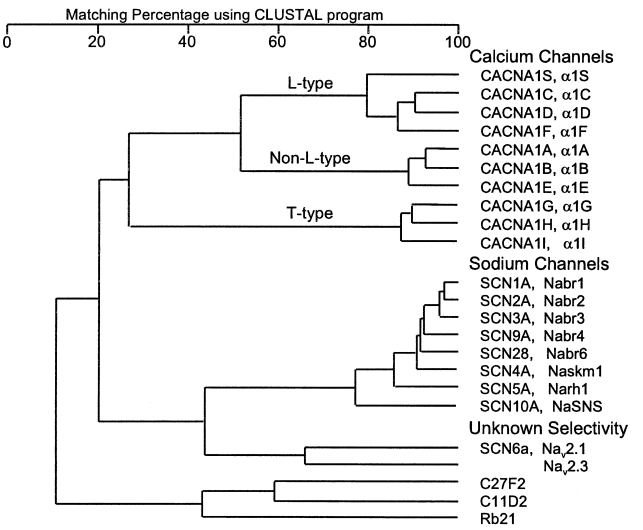


Fig. 3. A phylogenetic tree of the novel protein with Ca^{2+} and Na^{+} channels. To predict evolutionary relationships between the novel protein, and Ca^{2+} and Na^{+} channels, amino acid sequences of only putative membrane spanning regions in Fig. 1 were compared to one another. The sequences are identified by both their gene name and a common alias. Human α1F is a putative L-type channel (AJ224874). Sources of all other Ca^{2+} and Na^{+} channels are given in the Fig. 2 legend.

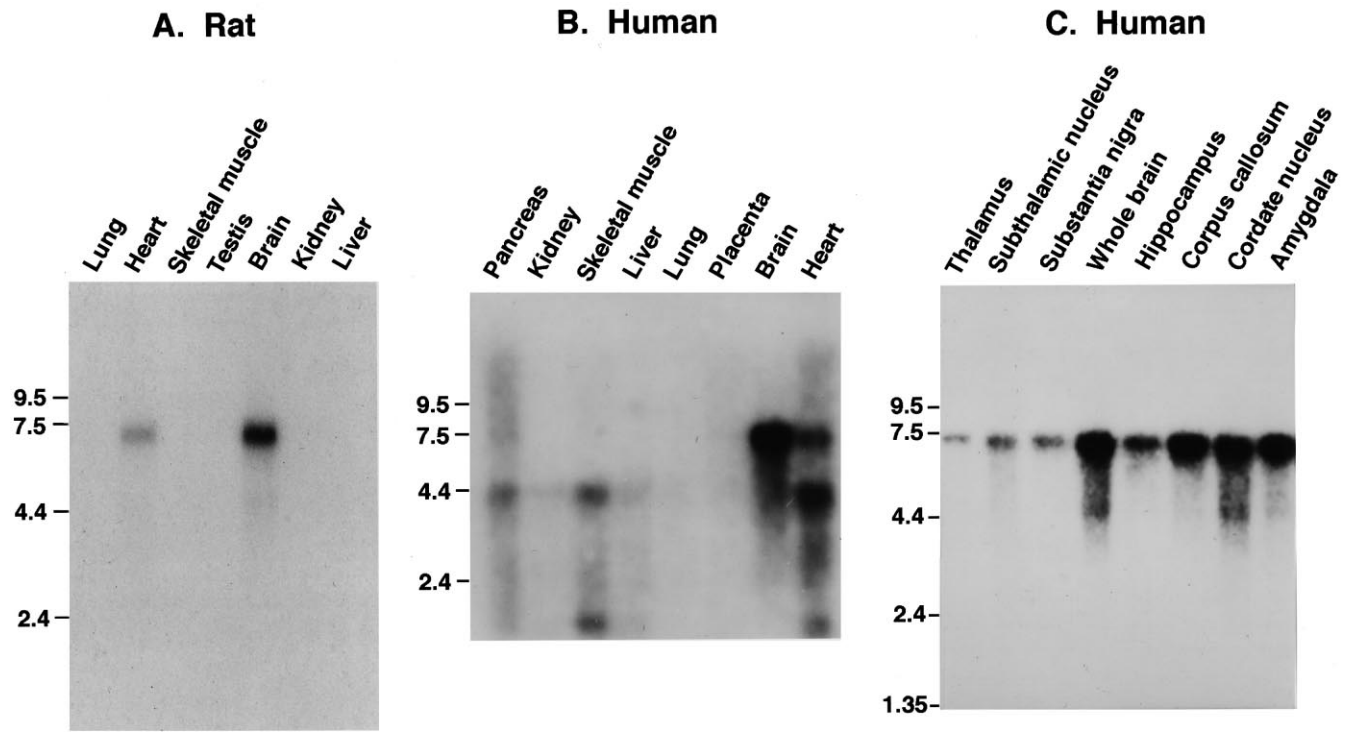


Fig. 4. Expression of RNA transcripts encoding the novel protein in rat (A) and human multiple tissues (B and C). In A, each lane contains 20 μg of total RNA isolated from each rat tissue. Human tissue blots were purchased from Clontech (B and C). Each lane contains 2 μg of poly(A)$^+$ RNA. The small bands were not detected in the rat multiple tissue blot when washed at a higher stringency (68°C at a 0.1×SSC and 0.1% SDS solution), suggesting that these resulted non-specific hybridization to unrelated transcripts.

was synthesized from the cDNA containing the domain III, IV, and 3′-tail. A transcript of 6.9 kilobases was detected in brain, heart, and pancreas (Fig. 4). This size was similar to the cDNA construct of the putative channel, suggesting that the full-length cDNA was cloned. Among brain regions, expression was detected in the following order: amygdala = corpus callosum > caudate nucleus > hippocampus > substantia nigra = subthalamic nucleus > thalamus. BLAST search of the EST database identifies four highly related clones from rat embryo (AI030889), human frontal cortex (AA683293), human pancreas (AA157945), and mouse hypothalamus (AA967995).

3.5. Expression studies

Expression of the putative channel was initially attempted in *Xenopus* oocytes. Significant voltage-activated Ba^{2+} currents were not detected from uninjected control ($n = 27$ from five batches) or Rb21-injected oocytes ($n = 82$ from five batches). On the contrary, robust currents were measured from oocytes injected with α1E cRNA (2.8 μA ± 0.7, mean ± S.D., $n = 13$ from five batches). Expression was also tested in SOS solution, which contains 100 mM Na^+. Again, there was no significant inward current in Rb21-injected oocytes. Oocytes were tested in both solutions with either depolarizing (holding potential of −80 mV, test pulses from −40 to +60 mV) or hyperpolarizing pulses (holding potential of −40 mV, test pulses from −50 to −130 mV).

Expression of cloned Ca^{2+} channels in *Xenopus* oocytes is strongly upregulated by Ca^{2+} channel β subunits and to a lesser degree by $\alpha_2\delta$ subunits [26]. In fact, endogenous β subunits may be sufficient and necessary for expression of exogenous α1 subunits [27]. To test for such a requirement we co-injected β_{2a} and $\alpha_2\delta$ subunits along with the putative channel. Injection of β_{2a} plus $\alpha_2\delta$ in the absence of any α1 subunit caused a small stimulation of endogenous Ca^{2+} channel currents (< 100 nA) as described previously [28]. However, these currents were of similar size and kinetics in oocytes also injected with Rb21-cRNA ($n = 8$). Co-injection of total brain RNA (17 ng) also failed to rescue expression of the putative ion channel ($n = 7$).

Initial studies of the rabbit skeletal muscle Ca^{2+} channel α1 also failed to detect expression in *Xenopus* oocytes (E. Perez-Reyes and R. Moorman, unpublished observations). But the channel was expressed in mouse L-cells and dysgenic myotubes [29,30]. Therefore we attempted to express the putative channel in tsA201 cells after transient transfection. Expression of the channel was assayed for in Tyrode solution containing either 140 mM Na^+ or 10 mM Ba^{2+} as charge carriers. Neither Na^+ nor Ba^{2+} currents were measured from cells transfected with the Rb21-cDNA ($n = 56$ cells out of seven transfections). As a positive control, a rat skeletal Na^+ channel α1 cDNA [13] was cotransfected into tsA201 cells with pTracer vectors. Expression of the Na^+ currents was measured in Tyrode solution from five out of eight cells (306 pA ± 115, mean ± S.D.).

There are putative Na^+ channels which were cloned, but not functionally expressed in *Xenopus* oocytes and other expression systems, such as atypical channels [31], a rabbit Schwann cell channel [32], and a distant jellyfish Na^+ channel [33]. Perhaps these putative channels, and the novel sequence reported here, require auxiliary subunits for proper folding and assembly into ion channels.

In conclusion, we have cloned a novel four domain protein from rat brain. Structural analysis of the protein indicated

that it shared many characteristics with voltage-activated Ca^{2+} and Na^{+} channels. Based on the sequence and structural criteria, we propose that the novel protein might be a voltage-activated cation channel with unique voltage gating properties, such as a non-inactivating Na^{+} channel.

Acknowledgements: This work was supported by the Graduate School of the Loyola University of Chicago.

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