Molecular Cloning, Expression, Functional Characterization, Chromosomal Localization, and Gene Structure of Junctate, a Novel Integral Calcium Binding Protein of Sarco(endo)plasmic Reticulum Membrane*

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Screening a cDNA library from human skeletal muscle and cardiac muscle with a cDNA probe derived from junctin led to the isolation of two groups of cDNA clones. The first group displayed a deduced amino acid sequence that is 84% identical to that of dog heart junctin, whereas the second group had a single open reading frame that encoded a polypeptide with a predicted mass of 33 kDa, whose first 78 NH2-terminal residues are identical to junctin whereas its COOH terminus domain is identical to aspartyl β-hydroxylase, a member of the α-ketoglutarate-dependent dioxygenase family. We named the latter amino acid sequence junctate. Northern blot analysis indicates that junctate is expressed in a variety of human tissues including heart, pancreas, brain, lung, liver, kidney, and skeletal muscle. Fluorescence in situ hybridization analysis revealed that the genetic loci of junctin and junctate map to the same cytogenetic band on human chromosome 8. Analysis of intron/exon boundaries of the genomic BAC clones demonstrate that junctin, junctate, and aspartyl β-hydroxylase result from alternative splicing of the same gene.

The predicted luminal portion of junctate is enriched in negatively charged residues and is able to bind calcium. Scatchard analysis of equilibrium 45Ca2+ binding in the presence of a physiological concentration of KCl demonstrates that junctate binds 21.0 mol of Ca2+/mol protein with a KD of 217 ± 20 μM (n = 5). Tagging recombinant junctate with green fluorescent protein and expressing the chimeric polypeptide in COS-7-transfected cells indicates that junctate is located in endoplasmic reticulum membranes and that its presence increases the peak amplitude and transient calcium released by activation of surface membrane receptors coupled to InsP3 receptor activation.

Our study shows that alternative splicing of the same gene generates the following functionally distinct proteins: an enzyme (aspartyl β-hydroxylase), a structural protein of SR (junctin), and a membrane-bound calcium binding protein (junctate).

The sarcoplasmic reticulum (SR) is an intracellular membrane compartment that controls the intracellular Ca2+ concentration thereby playing an important role in the excitation-contraction coupling mechanism (for review see Refs. 1–3). The anatomical site of excitation-contraction coupling is the triad, a unique intracellular synapsis that is formed by the association of the following membrane compartments: transverse tubules, which are an invagination of the sarcolemma, and the SR terminal cisternae (3). The portion of terminal cisternae facing the transverse tubules is referred to as junctional face membrane SR (4). Ordered arrays of junctional feet (3, 4), referable to as rymodine-sensitive Ca2+ release channels (RYR) (5–9), bridge the gap of 90–120 Å that separates the membrane of the transverse tubules from the junctional face membrane. The dihydropyridine-sensitive calcium channel of the transverse tubules acts as the voltage sensor for excitation-contraction coupling and plays a crucial role in the regulation of the RYR calcium channel (10–14). In addition to the RYR, the junctional face membrane contains several proteins including the histidine-rich calcium binding protein, triadin, calsequestrin, and junctin (15–18). During the past decades numerous studies have appeared concerning the biochemical characterization of the protein constituents of the junctional face membrane (19–29). The most abundant polypeptide appears to be calsequestrin, the SR calcium storage protein (30), which might also be involved in the regulation of the RYR (31, 32). Whether this effect is mediated by a direct interaction between the two proteins or via bridging calsequestrin binding proteins such as triadin or junctin is still controversial (17, 21, 29, 33). The junctional face membrane is endowed with numerous other less abundant proteins having a molecular mass ranging from 20 kDa up to 120 kDa, which have yet to be identified and characterized at the molecular level (4). Because of their localiza-

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1 The abbreviations used are: SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; RYR, ryanodine receptor; EGFP, enhanced green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; TE-MED, N,N,N',N'-tetramethylethylenediamine; FISH, fluorescence in situ hybridization; PCR(s), polymerase chain reaction(s); GST, glutathione S-transferase; bp, base pair(s); TM, transmembrane; Ab(s), antibody(ies).
tion, the proteins that are present on the junctional face membrane are deemed to be involved in the excitation-contraction coupling mechanism, and a defect in the function of these proteins could potentially lead to alterations of the Ca$^{2+}$ release process and/or intracellular Ca$^{2+}$ homeostasis. Thus the characterization of all the molecular components of the junctional face membrane is important not only for our understanding of the basic mechanism of Ca$^{2+}$ storage and release in muscle and non-muscle cells but also in view of the enormous effort aimed at identifying novel genes which, upon mutation, may be linked to neuro/muscular diseases (34, 35).

In the present report, we demonstrate the existence of junctionate, a novel integral Ca$^{2+}$ binding protein of sarco(endo)plasmic reticulum membranes. The Ca$^{2+}$ binding properties of junctionate indicate that the protein might have an active role in the Ca$^{2+}$ storage/release process in ER membranes in a variety of tissues including heart, brain, pancreas, lung, liver, kidney, and skeletal muscle. In addition, we report that an enzyme (aspartyl $\beta$-hydroxylase), a structural protein of sarco(endo)-plasmic reticulum membranes (junction), and a calcium binding protein (junctionate) belong to a family of single membrane-spanning proteins that result from alternative splicing events of the same gene located in human chromosome 8.

**EXPERIMENTAL PROCEDURES**

**Materials**

Nitrocellulose was from Amershams Pharmacia Biotech; isopropyl-$\beta$-D-thiogalactoside, restriction enzymes, the chemiluminescence kit, synthetic primers, DNA-modifying enzymes, the DNA-digoxigenin labeling triplex, 0.1% SDS; positive plaques were identified and purified, and the DNA was prepared according to the manufacturer's recommendations. Templates for sequencing were prepared in the Bluescript cloning vector.

To obtain the full-length sequence we carried out nested exonuclease III/mung bean nuclease deletions according to a previously described procedure (39). Random oligodeoxythymidylate-primed Agt10 human skeletal muscle and cardiac muscle libraries using a cDNA-cloned cDNA probe obtained using a RT-PCR of human skeletal muscle using forward and reverse primers obtained from the protein sequence of peptides 1 and 3; the sequences were 5'-AGA ATT CAC AGA GCA AAG AGA G-3' and 5'-AGA ATT CTA ACA CTA GGA TCA TTT-3', respectively. Amplification conditions were as follows: 45° annealing at 45 °C, 30° extension at 72 °C, and 30° denaturation at 95 °C for a total of 35 cycles. The addition of an EcoRI restriction enzyme sequence was used to facilitate subcloning and sequencing of the amplified cDNA. After overnight hybridization of the filters at 42 °C with the labeled probe, the filters were washed at high stringency at 65 °C in 0.2× SSC, 0.1% SDS; positive plaques were identified and purified, and the DNA was prepared according to the manufacturer's recommendations. Templates for sequencing were prepared in the Bluescript cloning vector.

To obtain the full-length sequence we carried out nested exonuclease III/mung bean nuclease deletions according to a previously described procedure (39). Random oligodeoxythymidylate-primed Agt10 human skeletal muscle and cardiac muscle libraries using a cDNA 32P-labeled probe were obtained from a tripex library clone. The primary sequence of this clone was obtained with an automated DNA sequencer using the dye deoxy method. Human BAC genomic libraries were screened as described previously (40). To identify BAC clones, colonies were lifted onto nylon membrane for hybridization probe analysis with radioactively labeled cDNA probes encompassing the 3' end of both human junction and junctionate. FISH analysis was performed by Genomics Inc. (St. Louis, MO).

**Extra Long PCR and Sequence Analysis of BAC Clones and Genomic DNA—**Extra long PCR products were obtained using the GeneAmp XL PCR kit (Applied Biosystems, Foster City, CA) from 1 μg of BAC or genomic DNA with either 28 or 37 amplification cycles using the PCR primers reported in Table I. PCR products were carried out according to the manufacturer's instructions; the products were purified with Microcon-100 (Millipore) and sequenced with the ABI PRISM Big Dye terminator cycle sequencing ready reaction kit using the ABI PRISM 377 DNA sequencer (PE Applied Biosystems, Foster City, CA).

**Northern Blot Analysis and Southern Blot Analysis—**2 μg of poly(A)$^+$ RNA from eight different human tissues (CLONTECH) and 20 μg of total human genomic DNA digested with EcoRI and PvuII and blotted onto nylon membranes were hybridized with the appropriate cDNA probes as described previously (38). Hybridization was performed twice with two distinct membranes. Blots were washed under high stringency (0.2× SSC, 0.1%SDS at 65 °C for 60 min), and autoradiography was performed for 1 week (Northern blot) or 3 weeks (Southern blots) at -80 °C with an intensifying screen. Northern blots were also probed with digoxigenin-labeled $\beta$-actin probe and washed under high stringency and the tissue distribution of $\beta$-actin was revealed using an anti-digoxigenin peroxidase-conjugated (1:10,000) antibody followed by chemiluminescence.

**Expression of the Recombinant Protein—**A GST fusion protein containing the COOH-terminal domain of junctionate was constructed by fusing the 610-bp PCR-amplified cDNA (amino acids residues 98-298) in frame into the multiple cloning site of pGex5× 3. The forward and reverse primers were reported in Table II. PCRs were carried out according to the manufacturer's instructions; the products were purified with Microcon-100 (Millipore) and sequenced with the ABI PRISM Big Dye terminator cycle sequencing ready reaction kit using the ABI PRISM 377 DNA sequencer (PE Applied Biosystems, Foster City, CA).
reverse primers were, respectively, 5′-TCT CGA GGGGCA GTC TTT TTG AA-3′ and 5′-AGA ATT CTA CTT CAG ACG CAG CA-3′. Amplification conditions were as described for cDNA cloning except that the annealing temperature was 60 °C; the addition of the XhoI/EcoRI restriction enzyme sequence was used to facilitate cloning and sequencing of the amplified fragment. The GST fusion protein was purified from the bacteria using glutathione-Sepharose 4B as described by the manufacturer.

Expression of Recombinant junctate in Eukaryotic Cells and Intracellular Distribution—To monitor the intracellular distribution of junctate, we cloned it into the pEGFPC1 mammalian expression vector and monitored expression of the recombinant protein after transfection into COS-7 cells. Two NH2-terminal green fluorescent protein-tagged constructs were made; one encompassed the whole coding sequence of junctate (nucleotides 1–979; EGFP-junctate), whereas the other encompassed the putative hydrophobic transmembrane domain between nucleotides 137 and 254 (EGFP-TM-junctate). PCR amplification conditions were as described for cDNA cloning except that the annealing temperature was 58 °C. The EcoRI/BamHI restriction enzyme sequence was used to facilitate cloning and sequencing of the amplified fragment into the pEGFPC1 plasmid. Forward and reverse primers for the EGFP-junctate construct were 5′-AGA ATT CAC AAA TGG CTG AAG-3′ and 5′-GAA GGT TTT AGG ATC CTG GTG-3′, respectively; and forward and reverse primers for the EGFP-TM-junctate construct were 5′-GGA ATT CCA CCA TGA GGA ACG GGC GAC TCT CA-3′, and 5′-GGG ATC CCT TTG CTT TGG CTA GA, respectively. COS-7 cells grown on glass coverslips were transfected using Lipofectin as described previously (42). 24 or 48 h after transfection, cells were washed with 0.6 M KCl. Biochemical analyses of kidney microsomes were performed using the Origin computer program (Microcal Software, Northampton, MA).

Preparation of Kidney Microsomes—Kidneys from 3.0–3.5-kg male New Zealand rabbits were homogenized (10% w/v) in a buffer containing 10 mM HEPES, pH 7.2, 150 mM KCl plus a mixture of anti-proteolytic agents ("Heparin-Agarose Chromatography"). The homogenate was centrifuged at 3,000 × g max for 10 min, and the resulting supernatant was centrifuged at 10,000 × g max for 15 min. The 10,000 × g max supernatant was then filtered through 10 layers of cheesecloth and centrifuged at 150,000 × g max for 60 min. The pellet was resuspended in a solution containing 10 mM HEPES, pH 7.2. 0.6 mM KCl plus anti-proteolytic agents and was centrifuged at 150,000 × g max for 60 min. The KCl-washed membranes were resuspended in a solution containing 10 mM HEPES, pH 7.2, 150 mM KCl at a final concentration of 1–2 mg/ml. The micromolar suspension was then incubated for 30 min at 4 °C in the presence of 100 mM Na2CO3, pH 11. The membrane fraction pellet was obtained by centrifugation at 150,000 × g max for 60 min and washed with 0.6 mM KCl. Biochemical analyses of kidney microsomes were carried out with three different preparations with microsomes isolated from two different animals.

Preparation of Rabbit Heart Microsomes—Preparation of rabbit heart microsomes was essentially as described by Pessah et al. (43), except that the total microsomal fraction was washed with 0.6 mM KCl before being layered onto the sucrose gradient to remove cytoskeletal proteins.

Preparation of Antibodies—Polyclonal Abs were raised by immunizing mice with the proteins present in the fraction eluting at 500 mM NaCl from the heparin-agarose column. To affinity purify anti-junctin Ab we blotted the fraction eluting at 500 mM NaCl from the heparin-agarose column onto nitrocellulose; to visualize the proteins, membranes were stained with a solution containing 5% (w/v) trichloroacetic acid, 0.1% (w/v) Ponceau red. The band that corresponds to junctin as revealed by NH2-terminal amino acid sequencing data was cut out of the membrane and used to affinity purify the Abs as described previously (38). To raise anti-junctate antibodies, the glutathione-Sepharose purified GST fusion protein was used to immunize rabbits as described previously (38); the serum was tested for the presence of antibodies, and the IgG fraction was purified through a protein A-Sepharose column. Antibodies reacting with the bacterial apo-protein were removed by batch extraction on the purified GST protein.

Peptide Synthesis—A peptide corresponding to the first 10 amino acids of rabbit skeletal muscle junctin was synthesized as described previously (44). Quality control was carried out by mass spectrometry analysis.

RESULTS

The biochemical and functional characterization of the molecular components present in SR membranes is important for understanding the fine mechanisms underlying Ca2+ homeostasis not only in skeletal muscle but also in other tissues. To analyze novel components involved in Ca2+ homeostasis, we identified the proteins present in terminal cisternae that co-purify with the ryanoxin receptor. We obtained a fraction eluting at 500 mM NaCl from a heparin-agarose column that was particularly enriched in a 27-kDa protein (Fig. 1A), as well as other proteins, including the RYR and triadin as determined by immunostaining (not shown). The NH2-terminal amino acid sequence of the 27-kDa protein did not match that of any protein present in the Swiss-Prot and NCBI BLAST data bases. Internal sequencing subsequently revealed the protein as junctin (Fig. 1B), a protein previously identified as a calsequestrin binding protein expressed in both skeletal and cardiac muscles (15, 16, 29).

cDNA Sequence Determination—The divergence of the NH2-terminal sequence between rabbit skeletal muscle and dog heart junctin could be due to tissue-specific or species-specific differences. We addressed this issue by determining and comparing the primary sequences of skeletal muscle and cardiac junctin deduced from cDNA clones pulled out from human skeletal and cardiac muscle libraries. A 98-bp cDNA probe was obtained by RT-PCR on human skeletal muscle RNA using the forward and reverse primers indicated under "Experimental Procedures." The PCR product was sequenced to confirm its identity and then used to screen a Atriplex human skeletal muscle library. After screening approximately 1 × 106 plaque-forming units we obtained a strong positive signal; single plaque purification was carried out, and a cDNA insert of approximately 750 bp was obtained and subjected to nucleotide sequencing. The insert contained a 150-bp 5′ untranslated region; the initial methionine residue was followed by an open reading frame of approximately 500 nucleotides, after which the sequence was terminated by a series of (A)10. Because the Atriplex human skeletal muscle library was oligo(dT)-primed, the stretch of poly(A) introduced a second priming site that prevented the isolation of overlapping clones containing the 3′ end of the cDNA. Thus, we screened random/oligo(dT)-primed λgt10 human skeletal and cardiac muscle libraries using (i) a cDNA 32P-labeled probe obtained from the whole 750-bp segment (probe A) of the Atriplex library or (ii) a 200-bp cDNA segment obtained from the most 3′ end (probe B) of the Atriplex insert. Several clones were pulled out, and those larger than 800 bp were characterized. Partial nucleotide sequence, restriction enzyme digestion, and hybridization probe analyses revealed that the cDNA clones fell into two groups. The prototype of the first group, clone 16, pulled out from both cardiac and skeletal muscle libraries with both probes (A and B), was similar to the published sequence of dog heart junctin (not shown). Comparison of the amino acid sequence deduced from the only open reading frame within the cDNA of human skeletal muscle...
Fig. 1. Heparin-agarose fraction eluting at 500 mM NaCl. Panel A, fractions eluting at 500 mM NaCl were pooled, concentrated, and resuspended in 100 μL of Laemmli buffer. 10 μL of resuspended proteins were loaded on a 10% SDS polyacrylamide gel. The arrow indicates the protein band on which NH₂-terminal sequencing was carried out. The amino acid sequence is given in single letter code; X indicates an unknown residue. Panel B, the band of 27 kDa was cut from the 10% SDS polyacrylamide gel and digested with CNBr. The proteolytic fragments were separated on a 15% SDS polyacrylamide gel and blotted onto polyvinylidene difluoride membrane. The proteins were stained with Coomassie Brilliant Blue, cut off the membrane, and used for microsequencing. The amino acid sequence is given in single letter code; X indicates an unknown residue. Sequences obtained from peptides 2 and 3 are similar to dog heart junctin.

Table I
Comparison of amino acid sequences of junctin from dog heart, rabbit, and human skeletal muscle

| Peptide Number | Amino Acid Sequence          | Mammal          |
|----------------|------------------------------|-----------------|
| 1.27 kDa protein | TEEKPHGHROXNK                 | rabbit skeletal |
| 5.27 kDa protein | APLKRDKEKESKRESRENLTK         | human skeletal  |
| 5.27 kDa protein | VADVGEYTVAVVVDVVEVYEV         | dog heart      |
|                |                              |                 |

a Deduced from cDNA (15).
b Deduced from cDNA; present work.

The prototype of the second class of clones is represented by clone 58, which was pulled out from the human cardiac Agt10 library, because a nearly identical clone was also pulled out of the skeletal muscle Agt10 library (not shown), a result that would also exclude possible cloning artifacts. Restriction enzyme mapping, Southern blot analysis, and partial sequencing of the latter clone obtained from the skeletal muscle library revealed it to be identical to cardiac clone 58, except that it was missing a sequence, AAQKDFRYNLSEVLQ, defined by residues 56–70 of cardiac clone 58. Comparison of the deduced amino acid sequences of junctate and aspartyl β-hydroxylase reveals that the two molecules were identical between residues 6–55 and 35-85 and between residues 71–298 (Fig. 3, panel A for comparison and alignment of the sequences).

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Expression of junctate and junctin in Human Tissues—The identification of junctate cDNA was rather intriguing and prompted us to investigate the expression pattern of the pro-
FIG. 2. Cloning of junctate cDNA. The upper part shows the restriction map of junctate cDNA. Solid bars indicate the open reading frame, and arrows indicate sequencing reactions performed in both orientations. The lower part of the figure shows nucleotide and deduced amino acid sequences. The nucleotides are numbered positively. The first initiator codon is located at position 89. A polyadenylation signal is located at position 1991–1998.
FIG. 3. Comparison of the amino acid sequence deduced from the cDNAs of human junctate, human aspartyl β-hydroxylase, human junctin, and human cardiac junctin isoform 1. Panel A, multiple sequence alignment was carried out with ClustalW computer program, which is available at the Swiss node of the European Molecular Biology network. Black boxes indicate identical residues; gray boxes indicate conserved residues. Underlined residues indicate the amino acid sequence obtained from protein microsequencing (Table 1). Human junctate and junctin sequences were from the present work. Human cardiac junctin isoform 1 and human aspartyl β-hydroxylase sequences were downloaded from the NCBI data bank. Note that junctin isoform 1 contains a 15-amino acid residue insert at position 56, which was not found in
and consensus sequence for glycosylation, and junctate-specific cDNA probes. Total genomic DNA was isolated from human peripheral blood leukocytes; 20 µg of DNA were digested with EcoRI (lanes 1) or PvuII (lanes 2), and the fragments were separated on a 0.8% agarose gel and then blotted onto a nylon membrane. The blots were then probed with either a radiolabeled XhoI-BamHI (nucleotides 850–1237) fragment obtained from junctate cDNA (Fig. 5, panel A) or a 0.2-kb SacI fragment from junctin cDNA (amino acid residues 100 to 165) (Fig. 5, panel B). Digestion with EcoRI (Fig. 5A, lane 1) yielded either a single 4-kb hybridizing band with a junctate-specific cDNA probe or two bands of about 2.4 and 5.0 kb with a junctin-specific cDNA probe (Fig. 5B, lane 1). Similarly, different hybridization patterns were obtained when the genomic DNA was digested with PvuII (Fig. 5B, lane 2). These results are compatible with the existence of either two independent single copy genes or a single copy of one gene encoding both proteins. In the latter case one would expect a large gene, and the hybridization with different cDNA probes would pick up different regions of the gene. We investigated this possibility by analyzing the gene(s) encoding junctin and junctate. We first isolated two BAC genomic clones by using the unique 3′ end cDNA sequences of junctin and junctate as probes. The identity of the BAC clones was confirmed by nucleotide sequencing. The two BAC clones cover up to 200 kb of human genomic sequence and contain overlapping sequences as revealed by Southern blot analysis (not shown). FISH analysis revealed that the loci of both junctin and junctate map to the cytogenetic band 1q12.1 on human chromosome 8 (Fig. 6). Interestingly, the aspartyl β-hydroxylase locus was also mapped to the same region of chromosome 8 (45). These data indicate that either the genes for aspartyl β-hydroxylase, junctin, and junctate are very close to each other or that the three proteins result from alternative splicing events of the same gene. We addressed this issue by determining the intron/exon boundaries in correspondence with the 5′ end of the gene (Fig. 7). The two BAC clones were cut with EcoRI, BglII, or PstI, and some of the fragments were cloned and partially sequenced. To amplify both BAC clones and human genomic DNA by extra long PCR, we designed a set of primers on the basis of sequences we obtained from BAC clones and cDNA (see Table II). The nucleotide sequences we obtained were then compared with those of the cDNAs of junctate, junctin, and aspartyl β-hydroxylase. The combination of data obtained by sequencing, PCR amplification, and Southern blotting allowed us to define the structure of the 5′ region of the locus of aspartyl β-hydroxylase, junctin, and junctate (see Fig. 7 and Table III). The data obtained indicate that the first exon of aspartyl β-hydroxylase is located more than 20 kb upstream from the first exon of junctin/junctate. The second and the third

FIG. 4. Tissue distribution of junctin and junctate. 2 µg of poly(A)+ RNA per lane from eight human tissues were separated in a denaturing agarose gel. Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas. Panel A, hybridization with junctate cDNA probe. Panel B, hybridization with junctin cDNA probe. Panel C, the blot used in panels A and B was reprobed with digoxigenin-labeled β-actin probe.

FIG. 5. Southern blot analysis of genomic DNA with junctin- and junctate-specific cDNA probes. Total genomic DNA was isolated from human peripheral blood leukocytes; 20 µg of DNA were digested with EcoRI (lanes 1) or PvuII (lanes 2), and the fragments were separated on a 0.8% agarose gel and then blotted onto a nylon membrane. The blots were then probed with either a radiolabeled XhoI-BamHI (nucleotides 850–1237) fragment obtained from junctate cDNA (Fig. 5, panel A) or a 0.2-kb SacI fragment from junctin cDNA (amino acid residues 100 to 165) (Fig. 5, panel B). Digestion with EcoRI (Fig. 5A, lane 1) yielded either a single 4-kb hybridizing band with a junctate-specific cDNA probe or two bands of about 2.4 and 5.0 kb with a junctin-specific cDNA probe (Fig. 5B, lane 1). Similarly, different hybridization patterns were obtained when the genomic DNA was digested with PvuII (Fig. 5B, lane 2). These results are compatible with the existence of either two independent single copy genes or a single copy of one gene encoding both proteins. In the latter case one would expect a large gene, and the hybridization with different cDNA probes would pick up different regions of the gene. We investigated this possibility by analyzing the gene(s) encoding junctin and junctate. We first isolated two BAC genomic clones by using the unique 3′ end cDNA sequences of junctin and junctate as probes. The identity of the BAC clones was confirmed by nucleotide sequencing. The two BAC clones cover up to 200 kb of human genomic sequence and contain overlapping sequences as revealed by Southern blot analysis (not shown). FISH analysis revealed that the loci of both junctin and junctate map to the cytogenetic band 1q12.1 on human chromosome 8 (Fig. 6). Interestingly, the aspartyl β-hydroxylase locus was also mapped to the same region of chromosome 8 (45). These data indicate that either the genes for aspartyl β-hydroxylase, junctin, and junctate are very close to each other or that the three proteins result from alternative splicing events of the same gene. We addressed this issue by determining the intron/exon boundaries in correspondence with the 5′ end of the gene (Fig. 7). The two BAC clones were cut with EcoRI, BglII, or PstI, and some of the fragments were cloned and partially sequenced. To amplify both BAC clones and human genomic DNA by extra long PCR, we designed a set of primers on the basis of sequences we obtained from BAC clones and cDNA (see Table II). The nucleotide sequences we obtained were then compared with those of the cDNAs of junctate, junctin, and aspartyl β-hydroxylase. The combination of data obtained by sequencing, PCR amplification, and Southern blotting allowed us to define the structure of the 5′ region of the locus of aspartyl β-hydroxylase, junctin, and junctate (see Fig. 7 and Table III). The data obtained indicate that the first exon of aspartyl β-hydroxylase is located more than 20 kb upstream from the first exon of junctin/junctate. The second and the third

both junctin and aspartyl β-hydroxylase. Junctin and junctin isoform 1 have a predicted molecular mass of 26 and 24 kDa. Panel B, schematic representing junctin and junctate. The cylinder represents the putative transmembrane segment. The small NH2-terminal domain faces the myoplasm whereas the major portion of the protein faces the lumen of the SR. + indicates the prevalence of positively charged residues; - indicates the prevalence of negatively charged residues. The gray line indicates the region of divergence between junctin and junctate. NLS indicates the consensus sequence for glycosylation, and Cys indicates the unique cysteine residue in the luminal loop of junctate.
exons, which are common to the three proteins and encompass the amino acid sequence of the predicted transmembrane segment, as well a the cytoplasmic loop of junctin and junctate, are located 5 to 6 kb downstream the first exon of junctin. Interestingly, the sequence AKAKDFRYNLSEVLQ, defined by residues 56–70 of cardiac clone 58, is encoded by a small exon (number 4) located between the third and fifth exons, which are common to the three proteins. We found a rare GCAAG splice donor site at the 5' end of the fifth intron. Such a splice site has been also described for other genes (46–48). The use of differential splice donors has been shown to be involved in the generation of protein diversity by alternative splicing (47). Taken together these data clearly show that three distinct proteins, i.e. junctin, junctate, and aspartyl b-hydroxylase result from alternative splicing events of the same gene localized in the human chromosome 8 (40).

Targeting of junctate to the ER Membranes of COS-7 Cells and Its Functional Characterization—Junctate contains a hydrophobic sequence that has been predicted to form a membrane-spanning segment, i.e. the structure necessary to anchor
the protein to sarco(endo)plasmic reticulum membrane. To verify this we expressed the full-length junctate cDNA in COS-7 cells. To visualize the cells expressing the junctate cDNA, we fused EGFP to the NH2-terminal portion of junctate. As expected cells expressing the full-length EGFP-junctate fusion protein display granular perinuclear fluorescence (Fig. 8, panel A). This type of fluorescence is consistent with localization of the recombinant protein into the ER membranes. To define in greater detail the minimal sequence necessary for targeting of junctate we synthesized by PCR the cDNA sequence encoding the membrane-spanning segment of junctate and fused it in frame to the COOH terminus of EGFP. As can be seen the fluorescence pattern of the EGFP-TM-junctate is similar if not identical to its full-length counterpart (Fig. 10, panel A). In fact COS-7 cells transfected with the cDNA encoding EGFP-junctate showed a 55% increase (student’s t test for paired samples, p < 0.000001) in peak [Ca2+]i, compared with those transfected with the cDNA encoding TM-junctate (Fig. 10, panel A). The expression of the recombinant proteins displays different functional properties from junctin. Fusion protein display granular perinuclear fluorescence (Fig. 8, panel A). This type of fluorescence is consistent with localization of the recombinant fusion protein into the ER membranes. To define in greater detail the minimal sequence necessary for targeting of junctate we synthesized by PCR the cDNA sequence encoding the membrane-spanning segment of junctate and fused it in frame to the COOH terminus of EGFP. As can be seen the fluorescence pattern of the EGFP-TM-junctate is similar if not identical to its full-length counterpart (Fig. 10, panel A).

The COOH terminus of junctate differs from that of junctin because of the prevalence of negatively charged residues (Fig. 3). This observation led us to investigate whether junctate displays different functional properties from junctin. Fusion proteins consisting of the COOH-terminal region of junctate were constructed. The expression of the recombinant proteins was monitored by SDS-PAGE, and a protein of the expected size is present only in the soluble fraction of the induced Escherichia coli culture (not shown). Because of the prevalence of negatively charged residues in the COOH terminus domain of junctate, we investigated whether such a domain is able to bind Ca2+. Fig. 9A shows an autoradiogram of a 45Ca2+ ligand overlay with bacterially expressed, recombinant COOH-terminal junctate (GST-C-junctate), which is able to bind calcium (Fig. 9A, lane 2). This result was confirmed by staining the proteins with Stains All, a carbocyanine cationic dye that metachromatically stains calcium binding proteins blue (Fig. 9B, lane 1). Finally Scatchard analysis (Fig. 9C) shows that the COOH-terminal portion of junctate binds 650 μmol of Ca2+/mg of protein (21.0 mol of Ca2+/mol of protein) with a Kd of 217 ± 20 μM (n = 5).

We next examined the effect of transiently overexpressing junctate on the [Ca2+]i, elevations in response to ATP, an agonist that has been shown to release Ca2+ from intracellular stores via InsP3 production (49). Cells were transfected either with the full-length pEGFP-junctate construct or with the truncated version, pEGFP-TM-junctate, as control; 48 h post transfection cells were loaded with indo-1, and the [Ca2+]i was monitored. Both the peak amplitude and the total amount of calcium released by ATP were significantly higher in cells transfected with the cDNA encoding full-length junctate, compared with those transfected with the cDNA encoding TM-junctate (Fig. 10, panel A). In fact COS-7 cells transfected with the cDNA encoding EGFP-junctate showed a 55% increase (student’s t test for paired samples, p < 0.000001) in peak [Ca2+]i, compared with those transfected the pEGFP-TM-alone (Fig. 10, panel B). When the total amount of Ca2+ released by 10 μM ATP was examined (by calculating the integral), the difference between the two constructs was even greater (increase of 116%; student’s t test for paired samples, p < 0.000001) (Fig. 10, panel C). No difference was observed either in peak amplitude or total calcium released, between mock-transfected cells and cells transfected with the pEGFP-TM cDNA (not shown).

### Identification of the Protein Product of the junctate Transcript in Kidney and Cardiac Microsomes

Based on the deduced amino acid sequence and analysis of the expressed recombinant protein, it is clear that junctate is (i) an integral membrane protein; (ii) has a molecular mass of 33 kDa; and is (iii) structurally related to junctin. To confirm the existence of a protein product encoded by the junctate mRNA transcript, we looked for a protein having the above biochemical properties in the membrane fraction of kidneys, an organ expressing junctate mRNA. The total microsomal fraction isolated from rabbit kidney was washed with 0.6M KCl (Fig. 11, lanes 1 and 5) and then extrinsic proteins were removed from membrane vesicles by treatment with 100 mM Na2CO3 at alkaline pH (Fig. 11, lanes 2 and 6). The microsomal fraction resulting from the treatment with sodium carbonate was also washed with 0.6 M KCl (Fig. 11, lanes 3 and 7). Integral proteins were separated on a 10% SDS-PAGE and blotted onto nitrocellulose. To establish whether kidney membranes are endowed with an integral membrane protein structurally related to junctin we carried out Western blot analysis with affinity-purified anti-rabbit skeletal muscle junctin Ab. The affinity-purified Ab immunodecorated a protein of approximately 27 kDa in rabbit terminal cisternae (Fig. 11, lane 4; **), as well as a protein of slightly slower mobility (Fig. 11, lanes 5–7; *), as expected from the cDNA and Northern blot analysis, which is present in kidney microsomal membranes. Analysis of the second exon indicates that junctin and junctate share the first 6 NH2-terminal amino acid residues. To confirm the identity of the immunopositive band we carried out a Western blot in the presence of a competing peptide encompassing amino acids encoded by the second exon plus the first 4 amino acids of exon 3. The immunological reactivity of the anti-junctin Ab with the 32-kDa protein was abolished by the addition of the competing peptide. Thus the 32-kDa protein present in kidney microsomal membranes contains an amino acid sequence that is identical to the NH2-terminal sequence of rabbit skeletal muscle junctin and junctate. This 32-kDa protein is enriched 2–3-fold in the microsomal fraction.
Junctate: a Novel Integral Membrane Calcium Binding Protein

**TABLE III**

| Exon-intron boundary | Donor site       | Intron-exon boundary | Acceptor site  |
|----------------------|------------------|----------------------|---------------|
| exon 1/intron I      | GAGGAGgtcaga     | intron II/exon 3     | aaacagAGACAA  |
| exon 3/intron III    | TTCTAGgttaaga    | intron III/exon 4    | atgagCCAAAG   |
| exon 5/intron V      | TATTAGcgaagt     | intron IV/exon 5     | ttatagGACCTA  |
| exon 8/intron VIII   | AGGCAGgttaagc    | intron V/exon 6      | cacaagAGACCC  |
| exon 9/intron IX     | ATCAGGgtatga     | intron VI/exon 7     | gacagTTAAGG   |
| exon 11/intron XI    | ATGCCAGgtttaa    | intron VII/exon 8    | ttttagAAGCTA  |
| exon 12/intron XII   | ATACAGgtatatta   | intron VIII/exon 9   | ttttagAACCAG  |
| exon 13/intron XIII  | ACAAAGgtttgc     | intron IX/exon 10    | ttttagATTCGA  |
| exon 14/intron XIV   | TCACAAGgttaag    | intron X/exon 11     | ttttagATGATG  |
| exon 15/intron XV    | TAGAACGgttaaga   | intron XI/exon 12    | ttttagAAGCTA  |
| exon 16/intron XVI   | CACCGAGgtatga    | intron XII/exon 13   | ttttagAACCAG  |

| Exon-intron boundary | Donor site       | Intron-exon boundary | Acceptor site  |
|----------------------|------------------|----------------------|---------------|
| exon 2/intron II     | ATAAAGgttatat    | intron III/exon 3    | aaacagAGACAA  |
| exon 3/intron III    | TTCTAGgttaaga    | intron IV/exon 5     | ttttagAAGCTA  |
| exon 5/intron V      | TATTAGcgaagt     | intron V/exon 6      | cacaagAGACCC  |
| exon 6/intron VI     | CTAAAAGgttat    | intron VI/exon 7     | gacagTTAAGG   |

**Fig. 8. Intracellular distribution of green fluorescent protein-junctate.**

COS-7 cells were transfected with the pEGFP-C1 plasmid alone (panel A), pEGFP full-length junctate (panel B), or pEGFP-TM-junctate (panel C). Cells were fixed in 3.7% formaldehyde 48 h post-transfection, and the fluorescence was monitored as described under “Experimental Procedures” (magnification, × 2,500).

We also analyzed the presence and the distribution of junctate in cardiac SR membranes. The total microsomal fraction was isolated from rabbit hearts and fractionated according to the procedure described under “Experimental Procedures” (43). The very same blot affinity-purified as-junctin Abs that were used to immunodetect the proteins present in kidney microsomes were used to immunostain the proteins of cardiac SR fractions. Western blotting revealed the presence of two immunopositive proteins in cardiac microsomes; one has the expected molecular mass of 27 kDa and is referable to as junctin (Fig. 12, *), and the other band has a higher apparent molecular mass of approximately 32 kDa (Fig. 12, **). The distribution of the two proteins roughly overlapped; they are mainly distributed in the fractions collected from the 32–34 and 34–38% sucrose interfaces. The 32-kDa protein also appears to be present, though to a lower extent, in the fraction collected from the 27–32% sucrose interface. We could not discern gross differences in their apparent abundance (Fig. 12, panel C). The proteins present in the cardiac SR membranes were also stained with a polyclonal Ab raised against the C-terminal domain of junctate, i.e. the portion of protein that is in common with the central non-catalytic domain of aspartyl-β-hydroxylase. As expected, when we used the latter Ab, two bands having a molecular mass of 32 and 90 kDa, respectively, were immunopositive (Fig. 12, panel B). The component having a higher molecular mass displays a distribution clearly distinct from the 32-kDa band. The 90-kDa protein is referable to as the...
active form of aspartyl β-hydroxylase and is partitioned in the light membranes and collected from the 27–32% sucrose interface (Fig. 12, panel B, lane 2). The 32-kDa immunopositive band, on the other hand, exhibits a distribution similar, if not identical, to the 32-kDa protein stained with anti-junctin Ab, a result consistent with the notion that such a protein represents junctate.

**DISCUSSION**

In the present report we describe for the first time the existence of junctate, a protein that, because of its biochemical properties and tissue distribution, is deemed to play an important role in cardiac homeostasis. Junctate is made up of 298 amino acids and contains one membrane-spanning segment; the first 23 residues of the protein are predicted to be in the cytoplasm whereas the bulk of the molecule is located in the lumen of sarco(endo)plasmic reticulum membranes. The NH2-terminal portion of junctate displays an amino acid sequence that is identical to that of junctin, a component of the calcium-releasing site of sarcoplasmic reticulum. The membrane-spanning segment of junctin also shows a high degree of similarity with that of triadin, another protein selectively localized to the junctional face membrane of skeletal and cardiac muscles, i.e., the calcium-releasing site of sarcoplasmatic reticulum. The membrane-spanning segment of junctin also shows a high degree of similarity with that of triadin, another protein selectively localized to the junctional sarcoplasmic reticulum (20, 29). Although aspartyl β-hydroxylase and junctin share their transmembrane segment, the distribution of these two proteins within the cardiac SR membranes is distinct. Western blot analysis of cardiac SR fractions clearly shows that two proteins within the cardiac SR membranes is distinct.

**FIG. 9.** Ca2+ binding of GST-C-junctate. Panel A, [35Ca2+] ligand overlay. Proteins contained in 15 µl of a total bacterial extract from an isopropyl-β-D-thiogalactoside-induced culture of E. coli was transformed with pGEX (lanes 1 and 2), and the glutathione-Sepharose 4B-purified GST-C-junctate (lanes 3 and 4) were electrophoretically separated in a 10% SDS gel and blotted onto nitrocellulose. Lanes 1 and 3, Ponceau red staining of the blot; lanes 2 and 4, autoradiogram of [35Ca2+] overlay. Panel B, Stains All staining. Lane 1, 3 µg of GST-C-junctate, the arrow indicates the GST-C-junctate band that metachromatically stained blue. Lane 2, 15 µl of isopropyl-β-D-thiogalactoside-induced total bacterial culture; the arrow indicates the GST protein band that did not stain blue. Panel C, Scatchard analysis. Calcium binding was carried out by microflow dialysis as described under “Experimental Procedures.” Values represent the mean ± S.E. of five experiments carried out in duplicate.

up to residue 93 (Fig. 3, panel A). On the other hand, the COOH-terminal domain of junctate is identical to the central region of aspartyl β-hydroxylase, a widely distributed protein responsible for the post-translational hydroxylation of aspartic acid/ asparagine residues. However, because junctate lacks the domain endowed with enzymatic activity it must play a different role from amino acid hydroxylation within the cell.

This result was rather intriguing and prompted us to investigate the matter in greater detail; FISH, gene structure, and cDNA analysis clearly revealed that via an alternative splicing event of the same gene, which is located at position q12.1 of human chromosome 8, three functionally distinct proteins could be generated. As to the regulation of the expression of this gene, we think that it is rather complex most likely under the control of different promoters and transcriptional factors. In fact, Northern blot analysis of human tissues demonstrated that two transcripts of 2.6 and 4.3 kb encoding aspartyl β-hydroxylase are approximately equally distributed in the heart, placenta, skeletal muscle, and kidney, whereas the most enriched tissue appeared to be the lung (50). On the contrary, junctate mRNA is particularly abundant in pancreas and heart followed by brain and kidney, whereas skeletal muscle exhibits the least amount of transcription. The tissue distribution of junctin does not overlap either with that of junctate or with that of aspartyl β-hydroxylase (50).

The presence of mRNA strongly suggests, but does not necessarily prove, the existence of a protein product. We approached this issue by performing biochemical analyses on the microsomal fraction of kidney, a tissue enriched in the junctate transcript. Western blot analysis using an affinity-purified anti-junctin antibody revealed the presence of a 32-kDa integral membrane protein. Competition experiments unambiguously show that the binding of the anti-junctin Ab to the rabbit kidney 32-kDa integral membrane protein occurs through the first 10 NH2-terminal residues. Because (i) such a sequence is unique for junctin and for junctate, (ii) Northern blot analysis shows that there is no detectable amount of junctin mRNA expressed in kidney, and (iii) the molecular mass (32 kDa) of the immunopositive integral protein is compatible with that of junctate, we provide unequivocal evidence for the existence of the protein junctate.

The primary sequences predicted from junctate and aspartyl β-hydroxylase cDNAs reveal that these two proteins share the membrane-spanning sequence with junctin, a protein selectively localized in the junctional face membrane of skeletal and cardiac muscles, i.e., the calcium-releasing site of sarcoplasmatic reticulum. The membrane-spanning segment of junctin also shows a high degree of similarity with that of triadin, another protein selectively localized to the junctional sarcoplasmic reticulum (20, 29). Although aspartyl β-hydroxylase and junctin share their transmembrane segment, the distribution of these two proteins within the cardiac SR membranes is distinct. Western blot analysis of cardiac SR fractions clearly shows that the 90-kDa component, which represents the enzymatically active form of aspartyl β-hydroxylase, is mainly present in the light fraction collected from 27–32% sucrose interface, whereas junctin and junctate are enriched in the 32–34% and 34–38% sucrose interface fractions, the same fractions that have been shown to be enriched by [3H]ryanodine binding activity (43). To our knowledge this is the first time that the protein aspartyl β-hydroxylase has been shown to exist in cardiac SR membranes, and for the time being we have no clear cut answers as to its exact role in cardiac physiology. On the other hand, junctin and junctate have a similar distribution that is distinct from that of aspartyl β-hydroxylase; thus the transmembrane segment is not sufficient to target proteins to their ultimate
Fig. 10. [Ca\(^{2+}\)]\(_i\) increases in response to Ca\(^{2+}\) release from intracellular stores. Cells were either transfected with the cDNA-encoding full-length junctate or that encoding the truncated version encoding the transmembrane domain portion (pEGFP-TM) fused in frame with pEGFP as described for Fig. 7. Cells were loaded with the fluorescent Ca\(^{2+}\) indicator indo-1 and stimulated with 10 µM ATP to elicit calcium release from intracellular stores; the fluorescence ratio (410/480 nm) of individual cells was measured as described under "Experimental Procedures." Panel A, trace representing typical response of cells transfected for 48 h with the indicated construct. As can be seen both the peak amplitude and integral calcium increases are elevated when cells overexpressing full-length junctate were examined. The calcium increase elicited by ATP in mock-transfected cells or cells transfected with the truncated junctate were not significantly different. The peak amplitude (panel B) and integral calcium (panel C) released by ATP in the presence or absence of extracellular calcium in cells transfected with the cDNA encoding the full-length junctate or the transmembrane portion (pEGFP-TM) fused in-frame with pEGFP was calculated. Results are expressed as the mean peak increase in fluorescence ratio or integral of the total calcium released (calculated with Origin software) in at least four separate experiments and the indicated number of cells.
Junctate: a Novel Integral Membrane Calcium Binding Protein

A peculiar feature of junctate is the net negative charge in its calcium-free form, whereby it would be available to interact directly with other components of the sarcoplasmic reticulum (SR). The capacity of junctate to bind calcium could be interpreted as an adaptive response of the protein to a high calcium environment, such as that found in the lumen of SR/ER (54). If this is the case, whatever function junctate might have would be protected by neutralizing the high calcium concentration in the lumen of SR. Thus, under these conditions the calcium binding sites of junctate would be saturated by their ligand, and the protein would not be available for electrostatic interactions with junctin and/or other proteins. Immediately after calcium release and before refilling of the calcium stores, however, the intralumenal calcium concentration is lower than the \( k_d \) of junctate for calcium. In the latter condition, junctate would be in its calcium-free form, whereby it would be available to interact directly with other components of junctional SR, including junctin and triadin.

Thus, by analogy with its muscle membrane counterpart, it is possible that the calcium bound to junctate could increase the local calcium concentration in microdomains that are adjacent to the lumenal mouth of the calcium release channel of ER membranes. The prevalent \( Ca^{2+} \) release channel of the ER is the InsP_3 receptor (53), and further studies are needed to clarify whether the COOH terminus of junctate interacts and/or co-localizes with the InsP_3 receptor. Alternatively, the capacity of junctate to bind calcium could be interpreted as an adaptive response of the protein to a high \( Ca^{2+} \) environment, such as that found in the lumen of SR/ER (54). If this is the case, whatever function junctate might have would be protected by neutralizing the high \( Ca^{2+} \) by binding this ion, as has been recently suggested to occur for other ER proteins including calnexin, protein disulfide isomerase, and calreticulin (54).

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Junctate: a Novel Integral Membrane Calcium Binding Protein

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