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Important Role of Junctophilin in Nematode Motor Function

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Junctional complexes between the plasma membrane and endoplasmic/sarcoplasmic reticulum are shared by excitable cells and seem to be the structural ground for cross-talk between cell-surface and intracellular ionic channels. Our current studies have identified junctophilins (JPs) as members of a novel transmembrane protein family in the junctional membrane complex. Biochemical and gene-knockout studies have suggested that JPs contribute to the formation of the junctional membrane complex by spanning the intracellular store membrane and interacting with the plasma membrane. We report here invertebrate JPs in fruit fly and nematode. Three distinct JP subtype genes are found in the mammalian genome, while a single JP gene exists in either invertebrate genome. Mammalian and invertebrate JPs share characteristic structural features, although some intervening sequences are found in invertebrate JPs. A reporter assay indicated that the JP gene is predominantly activated in muscle cells in nematode. Nematodes, in which expression of JP was inhibited by RNA-mediated interference (RNAi), showed hypolocomotion. Taking account of the cell-type-specific expression and data from previous reports, the hypolocomotion is likely to be due to the deficiency of junctional membrane structures and the resulting reduction of Ca^{2+} signaling during excitation-contraction coupling in muscle cells.

Key Words: **Ca^{2+} release; endoplasmic reticulum; intracellular Ca^{2+} store; junctophilin; sarcoplasmic reticulum; subsurface cisternae.**

The junctional membrane complex between the plasma membrane (PM) and the endoplasmic/sarcoplasmic reticulum (ER/SR) is thought to be the structural foundation for functional cross-talk between cell-surface and intracellular ionic channels (1–3). The junctional membrane structure has been best characterized in skeletal muscle as the "triad junction," in which the transverse (T-) tubule of the invaginated PM and the SR membrane are closely associated (4). During excitation–contraction (E–C) coupling, the dihydropyridine receptor (DHPR) and the ryanodine receptor (RyR) function as the cell-surface voltage sensor and the SR Ca^{2+} release channel, respectively, and the proposed direct coupling between them in the triad junction converts the depolarization signal to intracellular Ca^{2+} signaling for muscle contraction (5–7). Junctional membrane complexes are shared by excitable cell types, and they are designated as "diad" in cardiac myocytes, "peripheral coupling" in smooth muscle and immature striated muscle cells, and "subsurface cisternae" in neurons.

Our current studies have identified members of a novel transmembrane-protein family, namely junctophilin (JP) subtypes, that are localized in junctional membrane complexes (8). JP is composed of a carboxyl-terminal hydrophobic segment spanning the junctional SR/ER membrane, and the remaining cytoplasmic region that enables binding the PM. Moreover, junctional membrane complexes are produced by injection of JP cRNA in amphibian embryonic cells (8). Three JP subtypes derived from different genes have been found in mammalian excitable tissues: JP-1 is found predominantly in skeletal muscle, JP-2 is expressed throughout skeletal, cardiac and smooth muscle cells, and JP-3 is expressed specifically in the brain (8, 9). Mutant mice lacking JP-2 exhibit embryonic lethality due to cardiac failure. Mutant cardiac myocytes lacking JP-2 show deficiency of peripheral couplings and abnormal Ca^{2+} transients, indicating that the JP-2-mediated formation of the junctional membrane complex is essential for physiological coupling between DHPR and RyR

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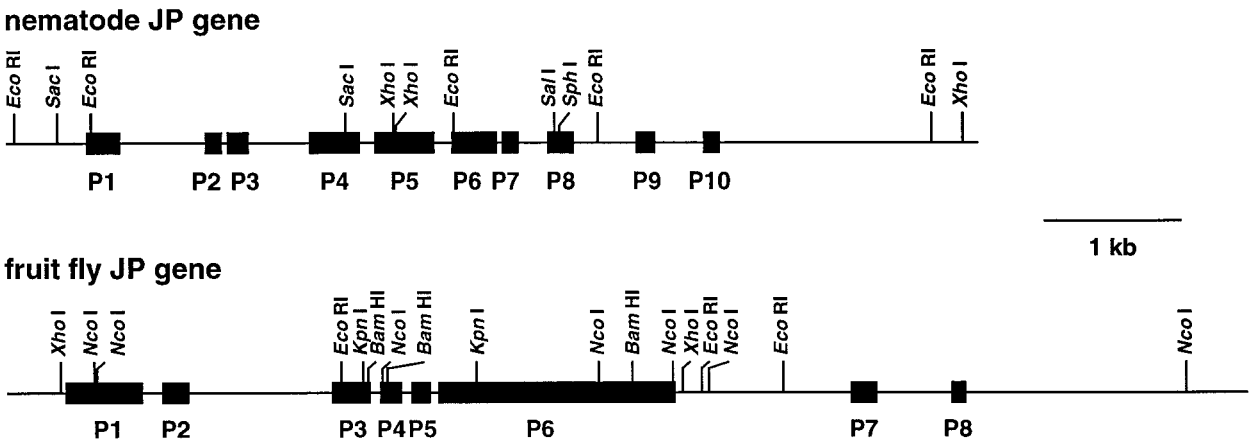

FIG. 1. Structures of JP genes in nematode and fruit fly. The protein-coding sequences (P1–P10 indicated by filled boxes) were determined by cDNA cloning, and are mapped in the reported genomic sequences. The sequence data for JP cDNAs from *C. elegans* and *D. melanogaster* will appear in the DDBJ/GenBank/EMBL Databases under Accession Nos. AB069909 and AB069908, respectively.

(8). Mutant mice lacking JP-1 exhibit perinatal lethality and suckling failure. Mutant skeletal muscle cells lacking JP-1 show deficiency of triad junctions and insufficient contraction, indicating that the JP-1-mediated formation of the triad junction is essential for efficient coupling between DHPR and RyR (10).

Excitatory cholinergic inputs increase cytoplasmic Ca^{2+} levels of muscle cells and therefore induces contraction in nematode (11). Previous genetic studies have demonstrated that functional coupling between DHPR and RyR counterparts controls Ca^{2+} signaling for contraction. Ca^{2+} influx through the DHPR homologue has been identified as an essential component for E–C coupling in nematode muscle cells (12). The complete structure of the RyR gene has been defined (13) and its contribution to E–C coupling in body-wall muscle cells has been established in nematode (14, 15). As in the case of mammalian muscle cells, the junctional membrane complexes might be an important structure for the functional coupling between DHPR and RyR in invertebrate muscles. We report here the structures of invertebrate JPs and abnormal behavior in JP-deficient nematodes.

METHODS

DNA cloning of invertebrate JPs. A fruit fly cDNA library was screened using ^{32}P-labeled rabbit JP-1 cDNA fragments as probes under low-stringency conditions (16), and several overlapping clones thus isolated covered the entire protein-coding sequence of fruit fly JP. To amplify nematode JP cDNA, a set of synthetic primers was designed based on the reported genomic DNA sequence (sense primer: CGGAATTCAGCAGTACGGCAAATGGG; antisense primer: CGGAATTCTAAAATAACCACATAATCCC). Polymerase chain reaction using a nematode cDNA library as the template was carried out to yield the full-length coding sequence. Nucleotide sequences of the cDNAs were determined by an automatic DNA sequencer (ALF express, Amersham Pharmacia Biotech.), and primary structures of invertebrate JPs were deduced using sequences of mammalian JP subtypes. Total RNA preparations from fruit fly and nematode were analyzed by Northern blot hybridization as described previously (8).

JP promoter assay in nematodes. The nematode genomic segment preceding the JP-coding sequence was amplified by polymerase chain reaction using synthetic primers (forward primer: GGACTAGTTGTTCTGCCATTACCAGCCCG; and reverse primer: TCCCCCGGGTTCCCATTTGCCGTACTGCTG), and was digested with *Spe*I and *Sma*I. The resulting 4.5-kb fragment was cloned into pPD95.67 cleaved with *Xba*I and *Sma*I (17); the putative JP promoter is linked to the coding sequence of green fluorescent protein (GFP) in this constructed vector. Three independent clones were injected into adult hermaphrodites (N2 wild type) as described previously (18), to yield the transgenic nematode lines that produced indistinguishable GFP-expression patterns under fluorescence microscopic observation (Olympus, Fluoview). As control experiments, nematodes carrying the *myo*-3 promoter-GFP transgene were examined.

RNAi and nematode locomotion. A partial cDNA fragment encoding the amino acid residues 101–388 of nematode JP was cloned into the *Eco*RI site of pBluescript SK(−) (Stratagene). The sense and antisense cRNAs were prepared in T3 and T7 RNA polymerase reactions and were mixed to generate double-stranded cRNA. The cRNA (1 mg/ml) was injected into gonad of 17 nematodes as described previously (19), and mutant F1 nematodes were obtained from 16 animals. Locomotion activity of the adult nematodes was monitored through a digital camera mounted on a binocular microscope.

RESULTS AND DISCUSSION

cDNA Cloning of Invertebrate JPs

Homology search of mammalian JP cDNA sequences in the databases revealed that JP homologues are found in the fruit fly (chromosome 2L, region 30B) and nematode (chromosome I, region T22C1) genomes; however, exon–intron boundaries have not been defined in the proposed genes. By cloning and sequencing the cDNAs, we deduced the primary structures of invertebrate JPs, and the organizations of the JP genes were determined by comparing genomic and cDNA sequences (Fig. 1). The protein-coding sequences are interrupted by 7 and 9 introns in the *Drosophila* and *C. elegans* genes, respectively, and all of the exon–intron boundaries follow the GT-AG rule (20). *Drosophila* and

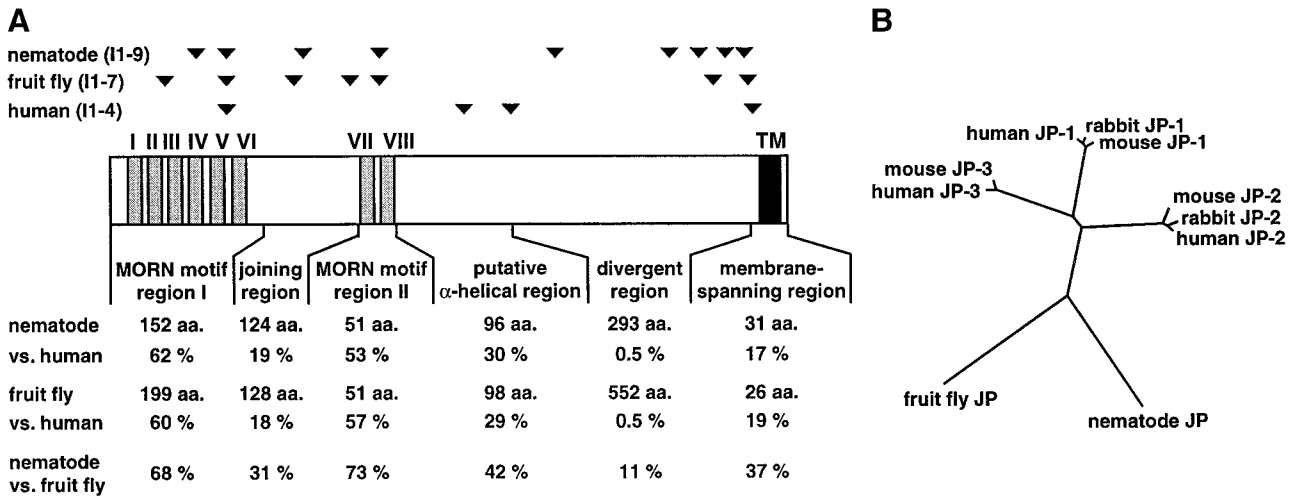

FIG. 2. Structural comparison between invertebrate and mammalian JPs (A). Each primary sequence for proposed six functional domains was compared among nematode JP, fruit fly JP and three human JP subtypes (JP-1, -2, and -3); the amino-acid residue number and sequence identity are given. The locations of introns interrupting the protein-coding sequences (arrowheads numbered I1–I9) are also shown. Phylogenetic tree of JP family (B). The sequences of JP family members were analyzed using Clustal W in the DDBJ Database. The sequence data for mammalian JP subtypes were taken from previous reports (8, 9).

C. elegans JPs are composed of 1054 and 747 amino-acid residues and show 27 and 28% overall sequence identities with human JP subtypes, respectively. The proposed genes are transcribed in the invertebrate tissues because hybridization probes from the cDNAs detected mRNAs of ~6.3 and ~3.2 kilobases in fruit fly and nematode, respectively (data not shown).

Based on local sequence conservation and characteristic structures in mammalian JP subtypes, six functional domains were proposed in the molecule (9), and Fig. 2A shows a schematic representation of defined invertebrate JP structures. The MORN motif regions are responsible for the interaction of JP with the cell-surface membrane, although binding partners on the membrane are still unclear (8). High sequence identities are observed among invertebrate and mammalian JPs in the regions, suggesting that invertebrate JPs retain the biological activity for specific interaction with PM. On the other hand, in the divergent region no significant similarities are detected among JPs, and intervening insertions are often observed in invertebrate JPs. It is interesting to note that invertebrate JPs carry additive sequences in the carboxyl-terminal end when compared with mammalian JP subtypes.

The determined introns are mapped on the primary structures of JP (Fig. 2A). Between the fruit fly and nematode genomes only two introns separate same positions of the aligned JP structure, and the JP gene organizations are relatively divergent among animal species. On the basis of sequence alignment, a phylogenetic tree exhibiting the evolutionary relationships among JP family members structurally defined was inferred (Fig. 2B). The tree indicates that three mammalian subtypes diverged at about the same time after the divergence between invertebrate and vertebrate JPs. A quite similar branching pattern was observed in the RyR family and IP3 receptor family (21), and both families have a single gene in invertebrates and three genes for tissue-specific subtypes in mammals. Therefore, these Ca^{2+}-store-related proteins including JP seem to share similar transition in molecular evolution.

Cell-Type-Specific Expression of JP in Nematode

We utilized the 4.5-kb DNA segment containing the 5′-noncoding and flanking sequences of the nematode JP gene for the construction of an expression vector, in which the transcription of GFP mRNA was controlled under the putative JP promoter sequence. To examine cell-type-specific expression of JP, the expression vector was microinjected into nematode gonad and the resulting transgenic animals were observed by fluorescence microscopy (Fig. 3). Transgenic nematodes carrying the *myo*-3 promoter-GFP fusion gene were used as controls; the *myo*-3 gene was specifically expressed in body-wall and vulval muscle cells (22). The *myo*-3-GFP and JP-GFP transgenes produced similar overall fluorescence images. Major discrepancies were observed in head regions; terminal bulb muscle cells of the pharynx and some neurons of the nerve ring were fluorescence positive in the JP-GFP transgenic nematodes, but not in animals expressing the *myo*-3-GFP transgene. Thus, the results suggest that JP is predominantly expressed in all muscle cells and certain neurons of the nerve ring. The cell-type specific expression of RyR in nematode has been reported previously (13–15), and the expression patterns of the RyR and JP genes are thought to be essentially the same.

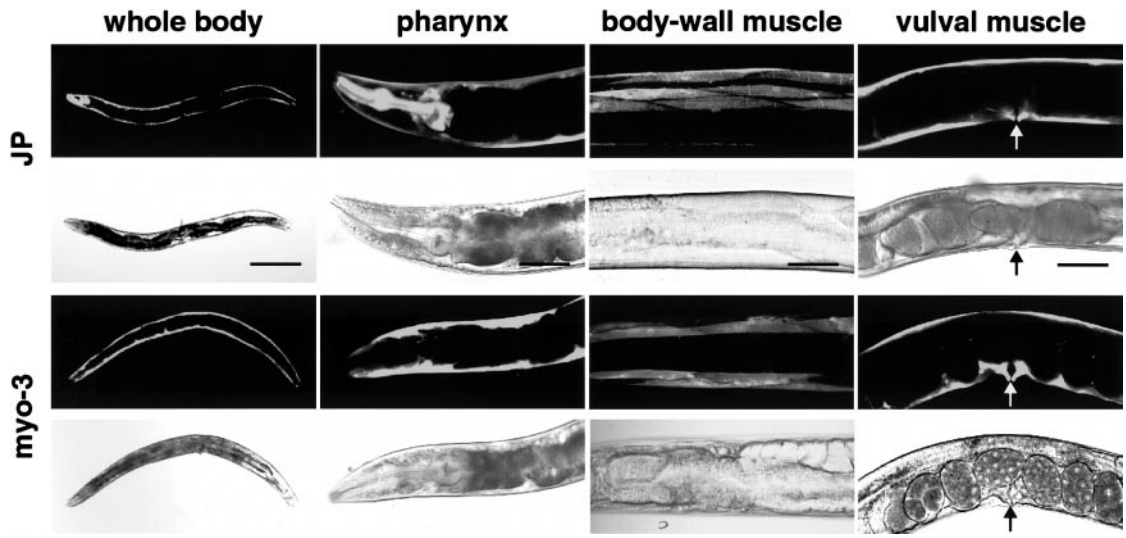

FIG. 3. JP promoter assay in nematode. Adult nematodes carrying the JP-GFP or *myo*-3-GFP transgene were analyzed under a confocal microscope, and fluorescence (upper panels) and bright-field images (lower panels) are shown. In the JP-GFP transgenic nematodes, body-wall muscle, vulval muscle, and pharynx muscle cells were fluorescence-positive. A similar fluorescence pattern was observed in the *myo*-3-GFP nematodes, but pharynx bulb muscle cells were fluorescence-negative. Arrows indicate the vulva. Scale bars: 200 μm in the whole-animal image and 50 μm in the other images.

Hypolocomotion in JP-Deficient Nematode

To gain an insight to the physiological role of invertebrate JP, we performed a conventional gene knockout experiment using the RNAi technique. Nearly 90% of the predicted genes on nematode chromosome I have been examined using the RNAi technique in current experiments (23). Although the JP gene is mapped on chromosome I, the RNAi phenotype on the JP gene has not been reported yet. Nematodes derived from gonadal cells injected with double-stranded JP cRNA survived, developed normally and showed no gross anatomical abnormalities. Normal nematodes move by propagating sinusoidal contraction waves in strips of muscle cells attached to the cuticle along the body length. Contraction waves of the RNAi nematodes propagated more slowly than those of controls, and thus the RNAi animals traveled a shorter averaged distance than wild-type animals (Fig. 4). The results indicate that deficiency of JP results in the "*unc*"-like phenotype. The motility defect, together with the tissue distribution of JP, suggests that the function of body-wall muscle cells is partly impaired when JP expression is disturbed in nematode.

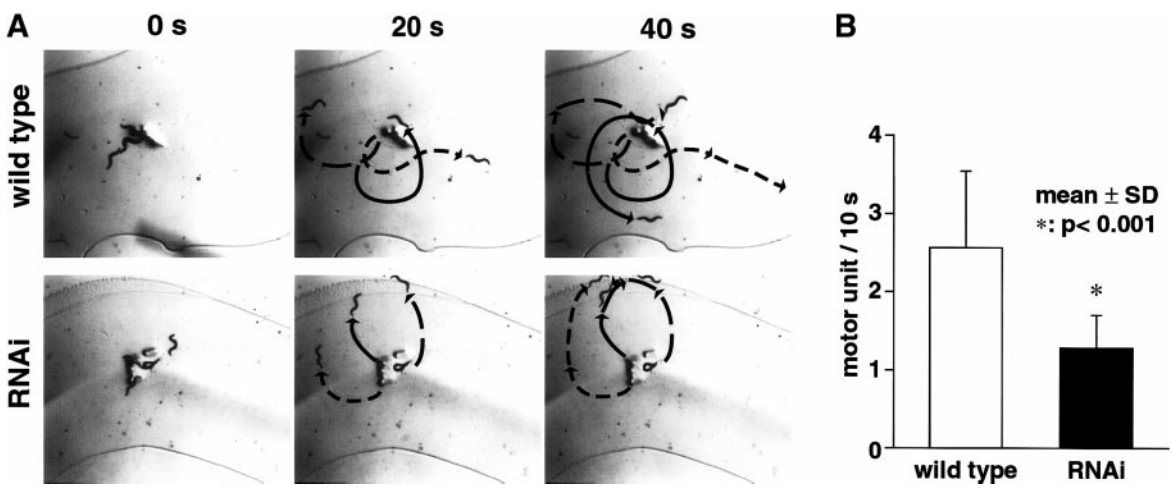

FIG. 4. Hypolocomotion induced by JP-RNAi in nematode. Locomotion of nematode monitored under a stereo microscope (A). Tracks of nematodes on agar plates are shown by arrows. Reduced locomotion in JP-RNAi nematodes (B). A total of 16 nematodes were injected with double-stranded JP cRNA, and resulting F1 adult nematodes were examined in terms of locomotion activity. The motor unit determined under the microscopic observation is in proportion to travel distance.

In *C. elegans*, the *egl-19* locus encodes the voltage-gated Ca^{2+} channel expressing predominantly in muscle cells, and the study of several *egl-19* mutants has indicated that Ca^{2+} influx via the channel is essential for muscle contraction (12). A single copy of the RyR gene in the nematode genome is predominantly expressed in muscle cells, and mutant nematodes lacking RyR (*unc*-68 mutants) move poorly and slowly (14, 15). These results clearly indicate that E-C coupling in nematode muscle cells is similar to that of mammalian cardiac type, in which Ca^{2+} flowing via DHPR binds to open RyR and triggers Ca^{2+} release from the intracellular store to produce amplified Ca^{2+} signaling for efficient contraction. Because Ca^{2+} mobility is highly limited in the cytoplasm, the junctional membrane complex between the cell-surface membrane and intracellular Ca^{2+} store is thought to be the structural basis for functional coupling between DHPR and RyR. In mammalian striated muscle cells, JP subtypes contribute to the formation of the junctional membrane complex, and the conserved structural characteristics (Fig. 2) indicate the shared biological role in mammalian and invertebrate JPs. In nematode muscle cells, inhibition of JP expression may result in deficiency of the junctional membrane complexes and thus may interfere with close association and functional coupling between *egl-19* and *unc-68* products. Mutant nematodes lacking either RyR or JP share an essentially similar phenotype of hypolocomotion. It may be likely that in the JP-deficient nematode muscle cells Ca^{2+} influx cannot trigger the full activation of RyR for Ca^{2+} signal amplification during E-C coupling. Therefore, our present results suggest that JP-mediated formation of the junctional membrane structure is essential for physiological functions in not only mammalian but also invertebrate muscle cells.

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