

Cloning and functional expression of invertebrate connexins from *Halocynthia pyriformis*

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Abstract Unlike many other ion channels, unrelated gene families encode gap junctions in different animal phyla. Connexin and pannexin genes are found in deuterostomes, while protostomal species use innexin genes. Connexins are often described as vertebrate genes, despite the existence of invertebrate deuterostomes. We have cloned connexin sequences from an invertebrate chordate, *Halocynthia pyriformis*. Invertebrate connexins shared 25–40% sequence identity with human connexins, had extracellular domains containing six invariant cysteine residues, coding regions that were interrupted by introns, and formed functional channels in vitro. These data show that gap junction channels based on connexins are present in animals that predate vertebrate evolution.

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1. Introduction

Cellular life requires a plasma membrane and specialized proteins to move aqueous solutes across it. The majority of these proteins have been highly conserved, like the potassium and chloride channel families whose divergent molecular evolution can be traced from prokaryotes to humans [1–3]. Gap junction channels, which move aqueous solutes between cells, are a notable exception to this pattern. Deuterostome animals, including all vertebrates, utilize two unrelated families of gap junction genes, the connexins and the pannexins [4–8]. The connexin sequences are more numerous and abundant, and their contribution to intercellular communication is supported by a wealth of experimental data [5,9,10]. There are far fewer pannexin genes, but they are also widely expressed and can form functional intercellular channels [7]. In contrast, protostomes like the nematode and fly lack connexins and pannexins, using instead a third family of genes called innexins to make gap junction channels [11,12]. Some investigators have postulated an evolutionary relationship between the pannexins and innexins [6,13], although

it should be noted that the reported sequence identity between these two families is very low [7]. Innexins display only ~16% overall identity when their full-length amino acid sequences are compared to either connexins or pannexins, which may simply reflect that all three gene families encode four transmembrane domain proteins.

Regardless of whether innexins and pannexins are related, there has persisted a widely held belief that the connexins were restricted to vertebrate animals [9,13–15]. This view further assumed that all invertebrate gap junctions would be encoded by innexins, and the name innexin itself was originally defined as the invertebrate analog of connexin [11]. The availability of genomic and cDNA databases from an invertebrate chordate, *Ciona intestinalis* [16,17], has questioned this potential misconception. A recent survey of cellular junction genes in *Ciona* reported evidence for seventeen connexin-like sequences and failed to identify any innexin sequences [8]. However, this cursory analysis did not show any data on sequence alignments, provide any figures of molecular phylogeny, discuss gene structure, or most importantly, demonstrate that the connexin-like sequences encoded proteins that actually functioned as gap junction channels. Thus, while this study suggested that some invertebrate organisms could contain connexins, it also left many important questions unanswered.

The identification of additional connexin genes in species outside the vertebrate phylum and the demonstration that they form bona fide gap junction channels in well characterized functional expression systems would help to end the erroneous belief that connexins are exclusively vertebrate genes [9,13–15]. A good starting point to search for additional invertebrate connexins would be among other basal marine chordates, like the tunicate *C. intestinalis*, which is thought to use connexin-like genes [8]. These tunicates are deuterostomal invertebrate chordates and are thought to share a common ancestor with modern vertebrates [18]. Tunicates are represented in the fossil record 550 million years ago, during the Cambrian explosion [19], and thus date to the same epoch when the protostomal invertebrates such as flies and nematodes were evolving. If tunicates contained connexin-like sequences that formed functional gap junction channels, it would confirm that the connexins were ancient genes that predated vertebrate evolution by hundreds of millions of years. To address this question, we have cloned and functionally expressed connexin-like sequences from a second tunicate species, *Halocynthia pyriformis*.

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2. Materials and methods

2.1. Animal collection

Halocynthia pyriformis tunicates were collected off Ironbound Island in Frenchman Bay, Maine, with the assistance of a commercial dive charter (Diver Ed, www.divered.com, Bar Harbor, ME). Animals were found at depths of 40–60 ft in 52 F water. Tunicates were pried off rocks using a dive knife, collected in nylon mesh bags and immersed in RNAlater on ice (Ambion, Inc. Austin, TX) immediately upon surfacing. Animals were stored in RNAlater at -80°C until use.

2.2. Degenerate RT-PCR

Halocynthia RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA (5 μg) was reverse transcribed with 100 ng of random hexamers and 200 U of RNase H⁻ reverse transcriptase (Superscript II, Invitrogen) in a reaction mixture containing: 20 mM Tris-HCl (pH 8.4 at 25°C), 50 mM KCl, 5 mM MgCl₂, 0.5 mM dNTP mix, 0.01 M dithiothreitol, and 40 U recombinant RNase inhibitor RNaseOUT (Invitrogen). Samples were incubated at 25°C for 10 min and then at 42°C for 50 min. The reaction was terminated by 15 min of incubation at 70°C . After cooling the samples in ice, 1 μl RNase H (2 U) was added and the samples were incubated at 37°C for 20 min. In order to exclude the contribution of genomic DNA to the final PCR step, incubations with reverse transcriptase (RT+) or without reverse transcriptase (RT-) were run simultaneously for each RNA sample.

Degenerate PCR for detection of connexin-like mRNAs was performed by using 48-fold degenerate primers based on the conserved extracellular domain regions of connexins (Sense primer, 5'-ACG TGC GGC CGC GGT TGC VAR AAT GTN TGY TTC AA-3'; Antisense primer, 5'-ACG TGC GGC CGC GGY CTA GAH AYR AAG CAA TCM AC-3'; where V = A + C + G; R = A + G; N = A + G + C + T; Y = C + T; H = A + T + C; and M = A + C). Primers included *Not*I linkers for subcloning (underlined). Two μl of cDNA was amplified with 200 nM primers using the Eppendorf TripleMaster PCR system (Eppendorf AG, Hamburg, DE). After an initial denaturation at 94°C for 5 min, 30 cycles of amplification with a MJ Research DNA engine Dyad (MJ Research, Boston, MA) were performed under the following conditions: 94°C for 30 s; 51°C for 30 s; and 72°C for 1 min. Amplification products were separated by agarose gel electrophoresis and visualized with ethidium bromide. Amplicons were excised from the gel, purified using Qiaquick columns (Qiagen, Germantown, MD), digested with *Not*I, subcloned into the pBlue-scriptII and sequenced on both strands.

2.3. Obtaining a full-length clone of Hp Cx47

The partial sequence of the largest connexin amplicon was used to design primers for 5'- and 3'-RACE. For 5'-RACE, nested primers were 5'-GAT TAT GTG AGT CGCGTAGAG-3' and 5'-ACG TGC GGC CGC TAG ACA CAACCA GTA TCA-3'. For 3'-RACE, nested primers were 5'-GGA TTC CTT GTT GGC CAA TAT TAC-3' and 5'-ACG TGC GGC CGC ACG GTT GGT CAG TTC ATG AAT-3'. In both cases, the second primer contained a *Not*I sequence (underlined) to facilitate cloning. 5'- and 3'-RACE were performed according to the manufacturer's instructions using kits from Invitrogen. RACE products were subcloned into pBluescriptII and sequenced on both strands. The final full-length coding region of the clone was verified by RT-PCR using primers based on the predicted 5'- and 3'-ends and containing *Pst*I linkers (underlined; Sense, 5'-ACG TCT GCA GAT GGC GTG GCA TAT ACT ACA C-3'; Antisense, 5'-ACG TCT GCA GAT TGC TAC GTC ATA TGT TAC G-3'). The full-length clone was subcloned into pBluescriptII and sequenced on both strands.

2.4. Functional expression

The Hp Cx47 coding sequence was subcloned into pCS2+ [20], linearized with *Not*I, gel purified and used as template (1 μg DNA) to produce capped cRNAs using the mMessage mMachine kit (Ambion, Austin, TX). Stage V–VI oocytes were isolated from *Xenopus laevis* (Nasco, Fort Atkinson, WI), defolliculated by collagenase digestion and cultured in Modified Barth's (MB). Cells were injected with a total volume of 40 nl of either an antisense oligonucleotide (5 ng/cell) to suppress endogenous *Xenopus* Cx38, or a mixture of antisense plus Hp Cx47 cRNA, using a Nanoject II Auto/Oocyte injector (Drummond, Broomall, PA). Following overnight incubation, vitelline envelopes

were stripped and oocytes were manually paired. The formation of cell-to-cell channels was assessed by dual voltage clamp [21]. Current and voltage electrodes (1.2 mm diameter, omega dot; Glass Company of America, Millville, NJ) were pulled to a resistance of 1–2 M Ω with a horizontal puller (Narishige, Tokyo, Japan) and filled with 3 M KCl, 10 mM EGTA and 10 mM HEPES, pH 7.4. Voltage clamping of oocyte pairs was performed using two GeneClamp 500 amplifiers (Axon Instruments, Foster City, CA) controlled by a PC-compatible computer through a Digidata 1320A interface (Axon Instruments). pCLAMP 8.0 software (Axon Instruments) was used to program stimulus and data collection paradigms.

2.5. Sequence analysis

Halocynthia pyriformis amplicons were trimmed of primer based sequences and used for translated blast searches (tblastx) of the NCBI non-redundant nucleotide sequence database (<http://www.ncbi.nlm.nih.gov/BLAST/>). Sequences that matched multiple known vertebrate connexins with *E* values $\leq 10^{-7}$ were considered significant connexin-like matches. Pairwise alignment of amino acid sequences from conserved regions of Hp Cx47 and the four *C. intestinalis* connexins with the twenty known human connexin sequences was performed using CLUSTALW and MegAlign software (DNASTAR, Madison, WI). Unrooted phylogenetic trees were generated using TreeView [22].

3. Results

3.1. Molecular cloning of connexins from *H. pyriformis*

We searched for invertebrate connexin genes in the ascidian tunicate *H. pyriformis* (Fig. 1A). Using RT-PCR to amplify *Halocynthia* mRNA with degenerate primers based on conserved extracellular domains of connexins (Fig. 1B), we obtained several amplicons that were subcloned and sequenced (Fig. 1C). After trimming of primer-derived nucleotides, three of these contained sequences that were highly homologous to vertebrate connexins (GenBank Accession Nos. AY386311, AY386312, and AY380580) when analyzed by translated BLAST searches (tblastx, expectation values 10^{-7}). These partial amino acid sequences included the motif C-X₃-P-C-P that is highly conserved in the E2 domain of vertebrate connexins [5,23]. For the largest amplicon, a full-length cDNA encoding *H. pyriformis* connexin47 (Hp Cx47) was obtained using 5'- and 3'-RACE, and RT-PCR using specific primers based on the predicted amino and carboxy termini demonstrated that the Hp Cx47 transcript was expressed in adult tunicates (Fig. 1D). The Hp Cx47 amino acid sequence contained all of the characteristic connexin features, including four predicted transmembrane domains with three conserved cysteine residues in each predicted extracellular domain [5,23]. These data showed that three distinct connexin-like transcripts could be detected in a second chordate organism whose ancestors predated vertebrate evolution and that a bona fide full-length connexin clone could be obtained from tunicate cDNA.

3.2. *Halocynthia pyriformis* Cx47 forms intercellular channels with distinct gating properties

Gap junction channels couple cells ionically and metabolically, and this functional coupling has been demonstrated in vitro for the vertebrate connexins, pannexins and invertebrate innexins [7,11,24]. To determine if the invertebrate connexin genes also encoded functional gap junction channels, Hp Cx47 cRNA was transcribed in vitro and injected into *Xenopus* oocytes pretreated with antisense oligonucleotides to suppress endogenous conductance [25]. Antisense-injected oocyte pairs

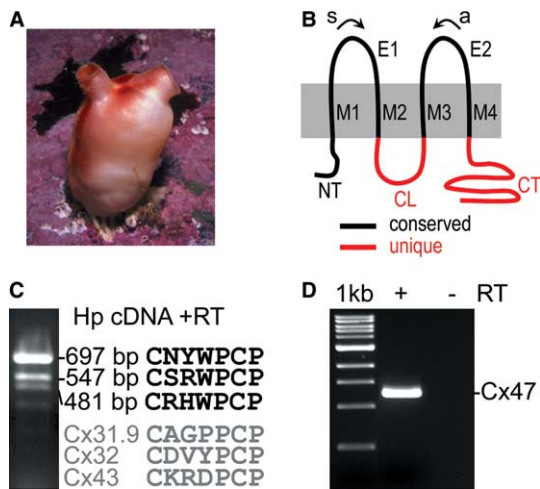


Fig. 1. Cloning of invertebrate connexins from *Halocynthia*. (A) The ascidian tunicate *H. pyriformis*. (B) Connexin schematic showing the pattern of conserved and unique domains, as well as the approximate positions of the sense (s) and antisense (a) primers used for degenerate RT-PCR. (C) RT-PCR of *Halocynthia* mRNA with degenerate primers produced three amplicons encoding connexin like proteins. All partial sequences included a highly conserved portion of the E2 domain, three divergent human connexin sequences are shown for comparison. (D) RT-PCR of adult *Halocynthia* RNA with primers corresponding to Cx47 amplify the proper band in the presence (+), but not in the absence (–) of reverse transcriptase (RT). 1 kb ladder is shown in the first lane.

were poorly coupled when assayed by dual whole cell voltage clamp, yielding a mean junctional conductance (G_j) of $0.04 \mu\text{S}$ ($n = 15$). In contrast, cells injected with Hp Cx47 cRNA were electrically coupled at levels more than 10-fold higher than the water-injected negative controls (mean G_j $0.47 \mu\text{S}$, $n = 15$, Fig. 2A). To further characterize the physiological behavior of gap junction channels composed of Hp Cx47, we analyzed their voltage dependence. A representative family of junctional currents (I_j) evoked by transjunctional voltages (V_j s) of opposite polarities and increasing amplitude (Fig. 2B) showed that I_j decreased modestly in a time- and voltage-dependent manner for V_j s ≥ 60 mV. The rate of channel closure, calculated for V_j s of ± 100 mV, yielded a time constant (τ) on the order of 300 milliseconds. Taken together, these gating properties are most similar to those reported for the Cx35/Cx36 orthologous group of connexins [26–29]. Thus, Hp Cx47 formed functional gap junction channels in a well-characterized expression assay with gating properties similar to some members of the Group III (γ) subfamily of vertebrate connexins.

3.3. Sequence relationships between vertebrate and invertebrate connexin gene families

BLAST searches of the GenBank database using full-length Hp Cx47 showed that it was highly homologous to all of the vertebrate connexins, sharing 25–40% sequence identity, yet pairwise alignment with the twenty known human sequences [5] failed to identify a clear Hp Cx47 ortholog among the vertebrate sequences. Human connexins vary in size from 25 to 62 kDa, with most of the mass difference in the cytoplasmic loop (CL) and carboxy terminus (CT). To eliminate potential alignment artifacts from differences in sequence length, we restricted our analysis to two regions that are highly conserved

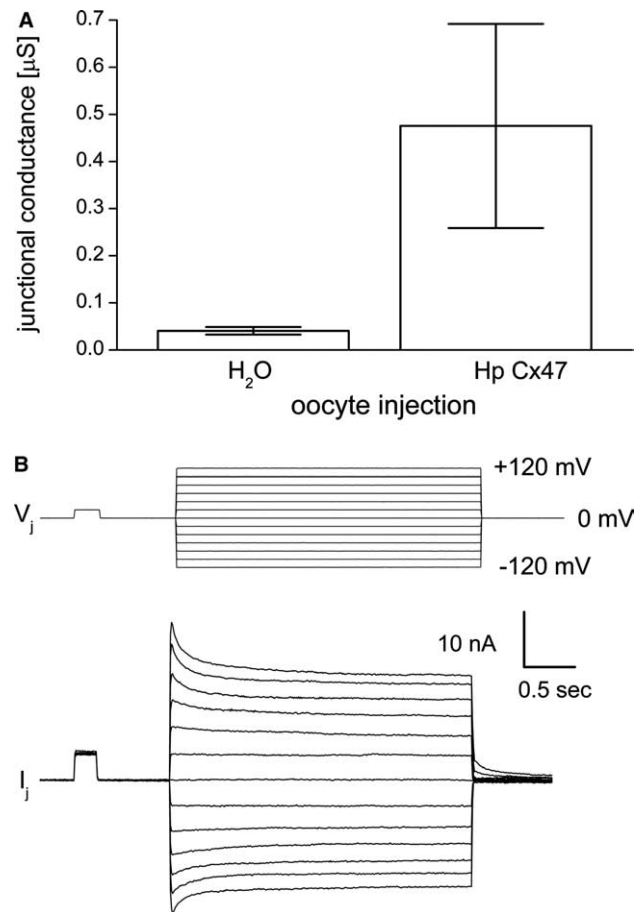


Fig. 2. Hp Cx47 forms gap junction channels. (A) Junctional conductance measured by dual voltage clamp between pairs of *Xenopus* oocytes. Oocytes injected with Hp Cx47 cRNA were coupled at levels 10-fold higher than water-injected controls. Bars show means \pm SE of 15 pairs for each condition. (B) Voltage gating behavior of gap junction channels formed by Hp Cx47. Time dependent decay of junctional currents (I_j) induced by transjunctional voltage (V_j) steps of 3 s duration applied in 20 mV increments. At V_j steps $\geq \pm 60$ mV, I_j decayed weakly and nearly symmetrically over the time course of the voltage step.

in both sequence and size (Fig. 1B). When the sequence alignment was limited to a ~ 100 amino acid region containing the amino terminus through the second transmembrane domain (NT–M2), the pairwise sequence identity increased to 35–45%, but still did not indicate an orthologous human connexin (Fig. 3A). In contrast, alignment of the second conserved ~ 80 amino acid region comprising the third through the fourth transmembrane domains (M3–M4, Fig. 3B) showed a more variable level of homology with the lowest pairwise identity to Cx31 and the greatest to Cx36 (Fig. 3B). When both conserved domains were artificially joined and aligned, Hp Cx47 showed the highest identity with Cx36 (48%) and the lowest with Cx31.1 and Cx31.3 (34%). Thus, while Hp Cx47 did not have a clear vertebrate ortholog, its conserved domains did show the most homology to human Cx36, a vertebrate connexin that has been highly conserved from cartilaginous fish to man [30,31].

Additional BLAST searches of a cDNA database from *C. intestinalis* [17] further revealed that Hp Cx47 was highly homologous to four of the seventeen connexin-like sequences previously identified [8], Ci Cx36.9, Ci Cx37.3, Ci Cx46.7 and

Ci Cx50.6 (GenBank annotated database Accession Nos. BK001247–BK001250). As Sasakura et al. [8] did not publish any sequence alignments, or report if the *Ciona* connexin-like sequences contained the six highly conserved cysteine residues in the extracellular domains, we compared the Hp Cx47 sequence with Ci Cx36.9, Ci Cx37.3, Ci Cx46.7 and Ci Cx50.6. Like Hp Cx47, these *Ciona* connexins contained all of the typical connexin features, including two highly conserved extracellular domains each containing three invariant cysteines (Fig. 3C). Multiple alignment of the full-length Hp Cx47 protein sequence with the four *Ciona* connexins revealed that it was most related to Ci Cx50.6, with which it shared 57% overall amino acid identity.

Human connexins cluster into three major groups based on sequence identity, the previously described groups I & II [23] and a third group composed primarily of sequences that have been identified more recently, and which do not easily fit into either group I or II [26,32]. An unrooted tree based on an alignment of the ~180 amino acids making up the two conserved regions of connexins revealed that all five of the tunicate proteins aligned outside of the two well-defined groups of human connexins (I & II) and that they clustered on a distinct branch among the outlying human connexins within group III (Fig. 4A). These data showed that despite sharing 34–48% identity with the vertebrate connexins within conserved domains, the tunicate sequences appeared to be evolutionarily distant from the vertebrate genes, consistent with vertebrates and modern ascidians descending from a common ancestor.

Further support for this position was provided by an analysis of the tunicate connexin gene structure. Alignment of the *Ciona* cDNA sequences with the draft sequence of the *C. intestinalis* genome [16] revealed that tunicate connexins have a complex gene structure with multiple introns and exons distributed throughout the coding region (Fig. 4B). In marked contrast, vertebrate connexins generally lack introns within the coding region and have a strong 5' bias for introns within untranslated regions [5,9]. It has been argued that such an intron asymmetry arises by preferential loss of 3' introns during evolution [33,34]. This view would argue that ancestral connexin genes would have more frequent and dispersed introns, like the *Ciona* connexins and is consistent with vertebrate connexins having evolved from ancient tunicate precursors. Additional study will be required to fully clarify the meaning of intron loss in vertebrate connexins, and it is also possible that modern tunicates acquired introns within their connexins.

3.4. Sequence comparisons between connexins, pannexins and innexins

Gap junctions are encoded by three distinct gene families, which are unequally distributed among the different animal phyla. The innexin genes are absent from deuterostome genomes, but have been identified in insects, nematodes, annelids, flatworms and molluscs [6,12,35,36], implying that ancestral innexins arose very early in protostomal evolution. As deuterostomes lack innexins, they use instead connexin and pannexin genes to encode gap junction channels [5,7,37]. An evolutionary relationship has been postulated between the pannexins and innexins based on alignment of a small number of heavily truncated sequences [6]. To further evaluate the relationships between these three gap junction gene families,

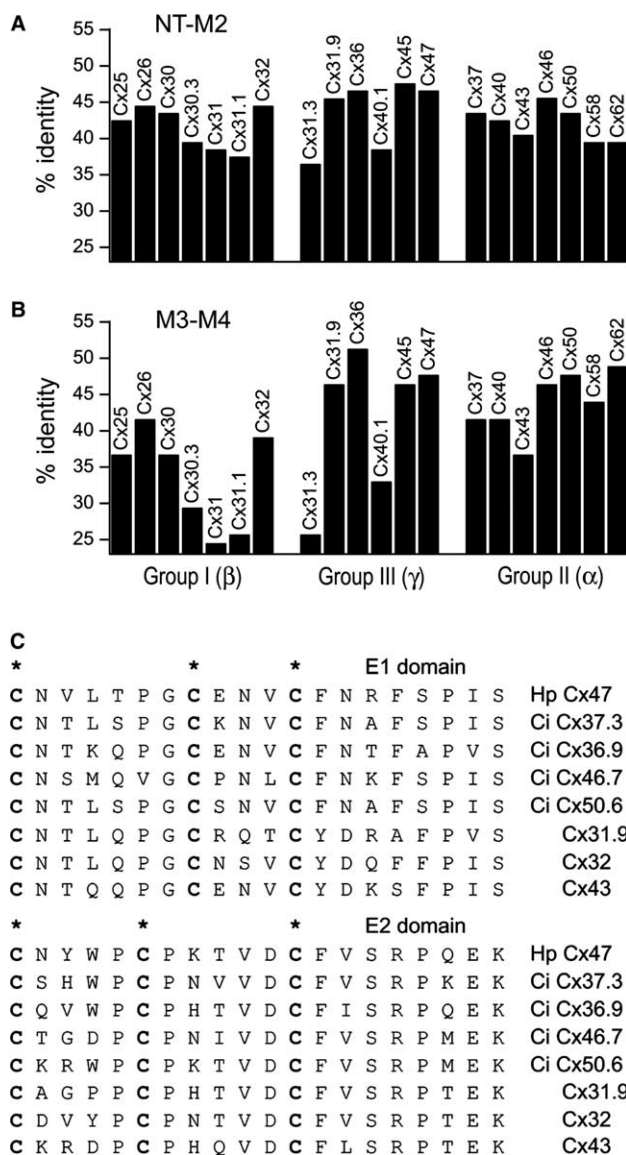


Fig. 3. Conserved domains shared by vertebrate and invertebrate connexins. (A) A plot of the pairwise amino acid identity in a ~100 amino acid conserved region (NT-M2) of Hp Cx47 with the 20 known human connexins. (B) Pairwise amino acid identity in a second ~80 amino acid conserved region (M3-M4) of Hp Cx47 with the 20 known human connexins. (C) Sequence alignment of *Halocynthia* Cx47 and four connexins from another tunicate, *C. intestinalis*, showing highly conserved extracellular domains containing three invariant cysteines (*) found in all vertebrate connexins. Partial sequences of three divergent human connexin sequences are included for comparison.

including invertebrate connexins, we performed multiple alignments of representative full-length connexin, innexin and pannexin protein sequences (Fig. 5A). As the number of pannexin genes is quite small, we limited the number of connexins and innexins to avoid over weighting these gene families in the tree. We found that the sequence divergence between connexins and pannexins was slightly greater than that between innexins and pannexins, while the divergence between innexins and connexins was more evident. Whether pannexins and innexins are truly members of a larger superfamily [6,13] will require further analysis. It is clear that connexins are

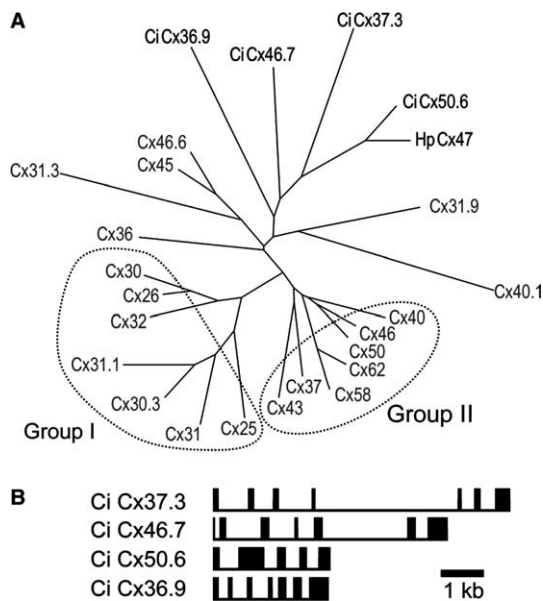


Fig. 4. Phylogeny and gene structure of invertebrate connexins. (A) Amino acid sequences containing ~180 amino acids from the conserved regions of tunicate and human connexins were aligned using Clustal and plotted as an unrooted tree. The tunicate connexins aligned outside the human groups I and II, and clustered on a distinct branch among the outlying human connexins. (B) Schematic representation of the complex gene structure of tunicate connexins. Alignment of *Ciona* connexin cDNAs with genomic sequence revealed 5–7 coding exons (thick bar) and an unbiased distribution of introns (thin bar). Vertebrate connexins have only 1–2 introns strongly biased toward the 5' end of the gene.

unrelated to innexins and these two gene families are not found within the same organism [5,29].

From the data presented here, we speculate that innexins and connexins may have arisen independently around the time when triploblasts diverged into protostomal and deuterostomal lineages (Fig. 5B). This hypothesis predicts that other deuterostomes such as echinoderms will also contain connexins rather than innexins and will be readily testable through efforts like the sea urchin genome project (<http://supg.caltech.edu>). Our finding of a complex intron-exon structure within the tunicate connexin's coding region will require that cDNA based data are also consulted in any invertebrate connexin search. Diploblast animals, like porifera, coelenterates and cnidarians, also have gap junctions and the present data do not predict whether innexins, connexins, pannexins, or an as yet unknown family of genes will encode them.

4. Discussion

Using traditional cloning methods, we have found evidence for multiple connexin genes in a second invertebrate chordate species. Both *Ciona* and *Halocynthia* are from the tunicate class *Ascidacea*. Ascidians are thought to have diverged the least from the common ancestor of all chordates [18], thus our results suggest that connexins may have arisen very early in deuterostomal evolution. Inclusion of the tunicate sequences within the greater vertebrate connexin family is supported by their ability to form voltage gated gap junction channels and

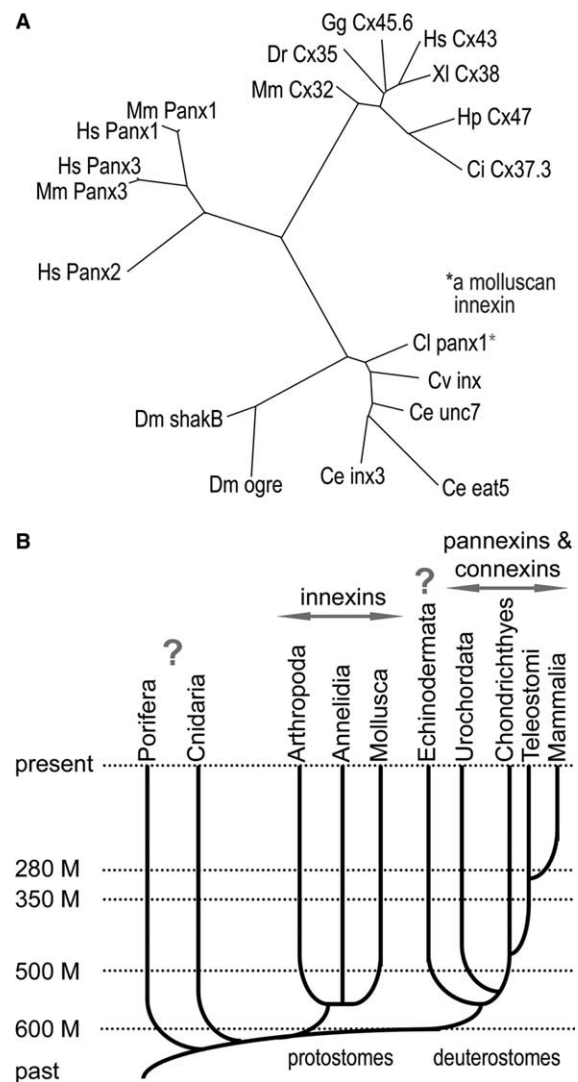


Fig. 5. Relationships between connexins, innexins and pannexins. (A) Complete amino acid sequences of gap junction genes from representative members of the three families were aligned using Clustal. Species included in the unrooted tree: Hs, *Homo sapiens*; Mm, *Mus musculus*; Dr, *Dario rerio*; Gg, *Gallus gallus*; Xi, *Xenopus laevis*; Hp, *H. pyriformis*; Ci, *C. intestinalis*; Cl, *Clione limacina*; Cv, *Chaetopterus variopedatus*; Ce, *Caenorhabditis elegans*, and Dm, *Drosophila melanogaster*. (B) Among triploblast organisms, deuterostomes have connexin and pannexin genes, while protostomes lack these two families and use innexin genes instead. The identity of gap junction genes in diploblast organisms is not known.

the presence of structural features such as four predicted transmembrane domains and highly conserved extracellular domains. However, two features of the tunicate connexins are consistent with their being evolutionarily distant from their vertebrate homologs. Firstly, their sequences cluster apart from the entire collection of human connexins, and secondly their gene structure is considerably different from that of all the vertebrate connexins. Whether the modern tunicate connexins have evolved significantly from the ancestral genes of the last common ancestor with vertebrates cannot be determined.

The modest voltage gating properties of Hp Cx47 most resembled those of the Cx35/Cx36 orthologous group of vertebrate connexins, which are prominently expressed in the

nervous system, and may represent the most ancient of the vertebrate genes. Cx35 was first cloned from the retina of a lower vertebrate, the cartilaginous skate, *Raja erinacea* [30] and its sequence has been highly conserved throughout vertebrates, including man [31]. In addition, unlike the vast majority of vertebrate connexins, genes in the Cx35/Cx36 group have retained an intron within their coding region and their sequences consistently group at the outer edges of dendrograms [26,32,38–41]. Additional experimentation will illuminate whether there is any relationship between these attributes of the Cx35/Cx36 group and the invertebrate connexins. Electrical coupling has been previously documented between the blastomeres of early ascidian embryos [42] and the cloning of functional ascidian connexins provides a molecular explanation for the basis of this intercellular communication. Interestingly, the blastomere coupling was reported to be largely insensitive to voltage [43,44] consistent with the in vitro properties of Hp Cx47. The vertebrate connexins have a wide range of voltage dependent gating [45] and it will be interesting to determine if the large family of invertebrate connexins also show such functional diversity.

Previous studies have reported staining of invertebrate gap junctions using antibodies raised against vertebrate connexins, including a report of Cx43 immunoreactivity in the *Ciona myocardium* [46]. Our data provide important support for this observation by demonstrating that connexin transcripts are present in the tunicates and these clones form bona fide gap junction channels, though they currently fail to identify a clear ortholog of Cx43. Other studies have even used connexin antibodies to stain gap junctions in Cnidaria like sea anemones [47] or hydra [48]. The importance of supporting such immunocytochemical localization with molecular and functional data is underscored by a recent review reporting that hydra exclusively use innexin genes rather than connexins [49], suggesting that the immunolocalization of connexins in hydra may have been an artifact. While determining the molecular identity of gap junctions in diploblast animals requires additional research, within the invertebrate deuterostomes, connexins have now been cloned, functionally expressed and immunolocalized at gap junctions.

The combined availability of the *C. intestinalis* genomic and cDNA databases has provided insights into the evolution of genes for many types of cellular junction. As more genomes become available, further clues to the enigma of gap junction evolution will emerge. One recent survey of cellular junction genes in *Ciona* found two pannexin-like sequences and failed to identify any innexin sequences [8]. Consistent with our analysis (Fig. 5A), these authors also reported very low conservation between the tunicate pannexins and the protostomal innexins. Thus, the vertebrate pattern of gap junction gene usage, many connexins with only a few pannexins, was already well established in the invertebrate chordates. Our data extend this cursory analysis and confirm that the invertebrate connexins can form functional gap junction channels.

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