Correspondence

Evolution of gap junctions: the missing link?

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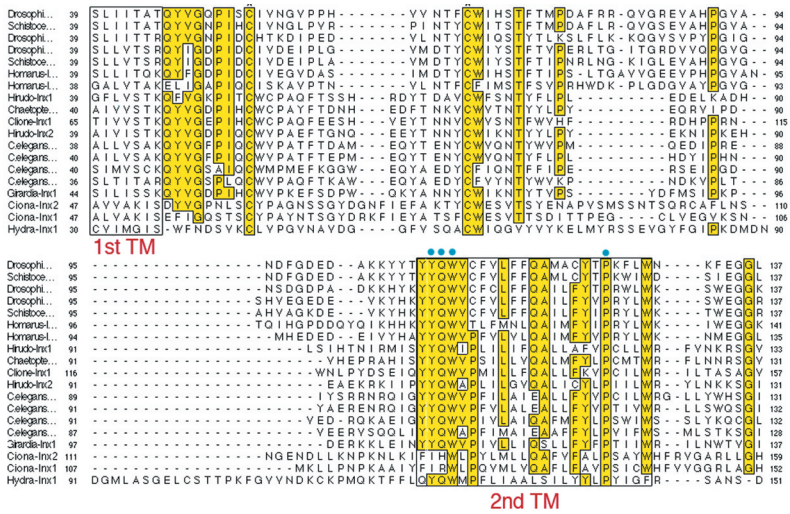

Figure 1. Multiple sequence alignment of innexins, from a variety of taxa.
The boxes highlight part of the predicted first and the entire second transmembrane (TM) domains of innexins. Identical amino acids in all the sequences in a percentage higher than 65% are highlighted in yellow while the black stars mark the invariant cysteines of the 1st extracellular loop. The blue dots mark the signature amino acids Y, Q, W and P of the second TM domain. The alignment was made using the ClustalW algorithm and the decorations of the identical amino acids using the SeqVue software (University of Washington). The proteins aligned in order are: *Drosophila melanogaster* ogre, *Schistocerca* Inx1, *D. melanogaster* Shaking-B and Inx2, *Schistocerca* Inx2, *Homarus* Inx2 and Inx1, *Hirudo* Inx1, *Chaetopterus* Inx1, *Clione* Inx1, *Hirudo* Inx2, *C. elegans* Unc7, Unc 9, Inx3 and Eat5, *Girardia* Inx1, *Ciona* Inx2 and Inx1 and *Hydra* Inx1.

Connexin molecules form gap-junction channels in vertebrates and there are at least 20 of them in humans [1]. Intuitively, one would imagine that cardinal features of the cellular machinery, such as gap-junctions, would be highly conserved. Paradoxically, however, *Drosophila* and *Caenorhabditis elegans* do not have connexin genes, but instead use innexins for gap-junctional communication, a protein family with the same 4-transmembrane topology but no sequence similarity to the connexins [2,3]. In this paper we show that the simple diploblastic organism *Hydra* appears to possess only innexins. We conclude that innexins are the primordial gap-junction molecules, while connexins evolved more recently in the deuterostomes.

The major question is whether the connexin–innexin dichotomy is an extreme case of sequence divergence from a common ancestor or a convergent solution to the problem of intercellular communication. A critical experiment was to identify gap-junction proteins in diploblastic organisms, e.g cnidaria. These organisms have functional gap junctions [4] and represent an evolutionary grade before the deuterostome–protostome divergence. We focused on the hydrozoan *Hydra* because the cells that comprise its body have large gap-junction plaques and are electrically and dye coupled [4,5].

During a signal peptide screen, we recovered a fragment, which matched a set of 14 overlapping ESTs (contig number C_CD267995, available at http://mpc.uci.edu/hampson/public_html/blast/jf), which we identified as a true innexin. The original EST collection had 3500 distinct sequences derived from 13,000 ESTs. The novel sequence has 396 amino acids and a predicted molecular weight of 44.9 kDa. A structural prediction using the Kyte-Doolittle algorithm showed that this molecule, named *Hydra* innexin-1 (Hv-inx1), has a typical innexin topology with 4-transmembrane (TM) domains and amino- and carboxy-terminal domains on the cytoplasmic face of the membrane. The extracellular loops contain pairs of invariant cysteine residues and the transmembrane domains (TM) contain signature residues Y, Q, W, P (second TM) and W, F (fourth TM) at conserved positions ([6]; Figure 1). This strongly supports the identification of Hv-inx1 as a true innexin despite low overall sequence identity. Expression of a Hv-inx1-GFP fusion protein in *Hydra* revealed a punctate pattern of GFP fluorescence along the basal lateral membrane of epithelial cells (Figure 2), corresponding to known sites of gap junctions [5].

A search of an enlarged Hydra EST collection, containing 93,000 entries (H.R. Bode and R. Steele, UC Irvine; http://www.hydrabase.org) revealed four more innexin homologs in addition to innexin-1. The enlarged EST collection was also searched for connexins using the BLAST algorithm but no statistically significant hits (*e*-value < 1) were recorded. This suggests that an earlier report of connexin 32 immunoreactivity in *Hydra* [4] is due to cross-reaction with a conserved epitope. In support of this, we note that punctate patterns of connexin 32 immunoreactivity have also been observed in *Drosophila* and *C. elegans*, which do not have connexin genes (D. Becker, C. Green, P. Phelan, unpublished data). Thus, we conclude that *Hydra* contains innexins but not connexins.

The most parsimonious explanation of our results is that innexins are the primeval gap-junction proteins. They evolved in diploblasts and persisted in the

protostome lineage. To resolve the provenance of connexins, we turned to protochordates, which represent an intermediate point of evolution between invertebrates and vertebrates. Recently, 17 connexin homologs and two presumptive innexin-like homologs were identified in the protochordate *Ciona intestinalis* [7,8]. We also searched genomic and EST data from the echinorderm *Strongulocentrotus purpuratus* (www.ncbi.nlm.nih.gov/genome/seq/SpuBlast.html; ~50,000 ESTs) and the cephalochordate *Branchiostoma floridae* (goblet.molgen.mpg.de/cgi-bin/Blast-amphioxus.cgi; ~7,000 ESTs) but no positive hits were recorded for innexins or connexins. Presumptive innexin homologs have also been reported for humans and mice [9]. These proteins, named pannexins, are expressed in the CNS and form functional gap junctions when expressed in *Xenopus* oocytes [10]. So far, only three such proteins appear in the human genome, and these proteins are completely unrelated in sequence to the connexins.

It is now possible to propose a solution to the conundrum of gap-junction evolution. Diploblasts, such as *Hydra,* evolved innexins for gap-junctional communication. These were inherited by protostomes and deuterostomes, while an additional class of 4-transmembrane molecules, the connexins, arose *de novo* in deuterostomes. Gene duplications in the early protochordate lineage [11] may have allowed the connexins to assume a pivotal role in forming gap-junctions, allowing the innexin family to diverge and form a subfamily, the pannexins. We conclude that the innexin versus connexin dichotomy is a convergent solution to the problem of intercellular communication.

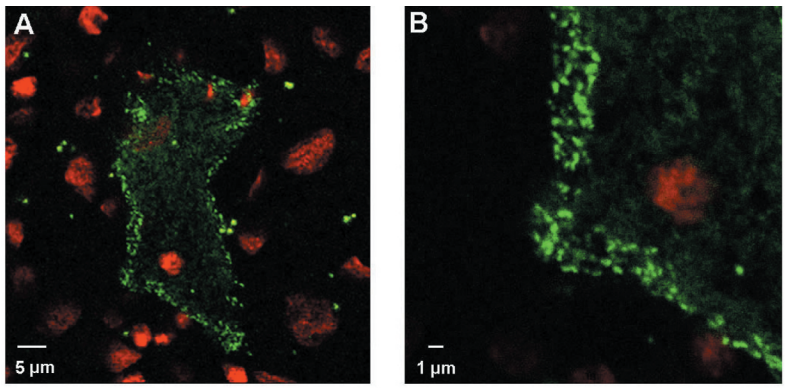

Figure 2. Expression of Hv-inx1-GFP in a *Hydra* epithelial cell.
Full length Hv-inx1 was amplified by PCR from *Hydra* cDNA, cloned into the HyEGFP expression vector and transfected into *Hydra* with a particle gun [12]. After 1–2 days epithelial cells expressing the Hv-inx1-GFP fusion protein were clearly visible. (A) The epithelial cell is surrounded by numerous smaller interstitial cells (nuclei stained red with TOPRO). The Hv-inx1-GFP signal (green) is localized to small spots (100–500 nm) in the basal lateral membrane, which is the site of gap junctions in Hydra [5]. Some areas of the membrane are shown *en face* facilitating imaging of the gap junction plaques. Control transfections of GFP alone result in GFP localising to the cytoplasm and nucleus [12]; transfections of GFP fusion proteins with nuclear localisation signals (NLS) localize exclusively to the nucleus [13]. (B) Enlargement of the Hv-inx1-GFP expressing epithelial cell in the left panel.

Supplemental data

Supplemental data are available at http://www.current-biology.com/cgi/content/full/14/20/R879/DC1/

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