Evolutionary Diversification of Insect Innexins

Austin L. Hughes

Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA

Corresponding author, e-mail: austin@biol.sc.edu

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ABSTRACT. Phylogenetic analysis of insect innexins supported the hypothesis that six major clades of insect innexins arose by gene duplication prior to the origin of the endopterygote insects. Within one of the six clades (the Zpg Clade), two independent gene duplication events were inferred to have occurred in the lineage of Drosophila, after the most recent common ancestor of the dipteran families Culicidae and Drosophilidae. The relationships among these clades were poorly resolved, except for a sister relationship between ShakB and Ogre. Gene expression data from FlyAtlas supported the hypothesis that the latter gene duplication events gave rise to functional differentiation, with Zpg showing a high level of expression in ovary, and Inx5 and Inx6 showing a high level of expression in tests. Because unduplicated members of this clade in Bombyx mori and Anopheles gambiae showed high levels of expression in both ovary and tests, the expression patterns of the Drosophila members of this clade provide evidence of subdivision of an ancestral gene function after gene duplication.

Key Words: gap-junction channel, gene duplication, innexin, multigene family

Intercellular communication is a fundamental need of multicellular organisms (Panchin 2005). In animals, an important pathway of cell–cell communications is provided by gap junctions, constituted by the junction of two hexameric protein arrays or hemichannels (Bauer et al. 2005, Phelan 2005). Each of the two communicating cells produces one hemichannel, and their alignment creates a channel allowing the passage of ions and small molecules (Phelan and Starich 2001). In invertebrates, the hexamers are composed of proteins belonging to a family known as innexins, which have apparent distant homologs (pannexins) in vertebrates but are distinct from the widely studied vertebrate connexin family of gap-junction proteins (Yen and Saier 2007).

Genomic studies have revealed multiple innexin family genes in insect species. In the best-studied insect model, Drosophila melanogaster, there are seven genes (Bauer et al. 2005): 1) ogre (‘optic ganglion reduced’), also known as inx1, 2) inx2, 3) inx3, 4) zpg (‘zero population growth’), also known as inx4, 5) inx5, 6) inx6, and 7) inx7, and shakB (‘shaker B’). Each of these genes encodes a protein with four transmembrane domains (Bauer et al. 2005). Certain hemichannels may be heteromeric, while others are homomeric. When two homomeric semichannels of different types form a channel together, that channel is known as heterotypic, whereas a channel formed by two homomorphic semichannels of the same type is called homotypic (Phelan and Starich 2001). In Drosophila, the Ogre and Inx3 proteins both form heteromeric channels with Inx2, while Zpg can form heterotypic channels with Inx5 (Phelan 2005). By contrast, ShakB forms homotypic channels, as can Inx2 (Phelan 2005). Finally, there is evidence that at least in some animal species, innexins or their homologs can form non-junctional channels (‘hemichannels’ or innexons) of poorly understood function (Bao et al. 2007, Scemes et al. 2009).

A number of researchers have presented phylogenetic analyses of selected innexins (Phelan and Starich 2001, Phelan 2005, Hong et al. 2009). However, no study has attempted to use phylogenetic methods to estimate the time of gene duplications within the insect family relative to major events of cladogenesis within the insects. Here, I take advantage of the information available from insect genome projects to reconstruct the evolutionary history of insect innexins. In addition, making use of data from gene expression atlases of model species, I examine patterns of functional differentiation of duplicate innexin genes.

Methods

Phylogenetic Analyses. Phylogenetic analyses were based on the 79 selected innexin protein sequences from 14 insect species representing two orders of exopterygotes (insects with incomplete metamorphosis) from the infraclasse Paraneoptera; and four orders of the infraclasse Endopterygota (endopterygotes or insects with complete metamorphosis) were downloaded from the NCBI website (Table 1; Fig. 1). From the genus Drosophila, D. melanogaster was chosen along with Drosophila grimshawi, which represents the clade of Drosophila species with sequenced genomes that are phylogenetically most distant from D. melanogaster (Drosophila 12 Genomes Consortium 2007). Sequences were aligned by the CLUSTAL algorithm in MEGA 5.05 (Tamura et al. 2011); and any site at which the alignment postulated a gap in any of a set of aligned sequences was excluded from analyses involving that set of sequences. Phylogenetic trees rooted with four sequences from the nematode Ascaris sum (Fig. 1). Phylogenetic trees were reconstructed by two methods: 1) maximum likelihood (ML), based on the JTT+G+I+F model and 2) minimum evolution (ME) based on the JTT+G distance.

The model for the ML analysis was chosen in MEGA 5.05 using the Bayes Information Criterion (Tamura et al. 2011). The gamma parameter (measuring rate variation among sites) used in the ME analysis (1.9926) was estimated by the ML analysis. The reliability of branching patterns in ML trees was tested by bootstrapping; 1,000 bootstrap pseudosamples were used. Significance of internal branches in the ME tree was tested by the interior branch test, with the standard error of branch lengths estimated by bootstrapping (Nei and Kumar 2000). The ML method was used to reconstruct ancestral sequences (most probable ancestors) at major nodes within the phylogenetic tree in MEGA 5.05.

Gene Expression Data. Gene expression data were downloaded from the Gene Expression Omnibus (GEO) platform at the National Institute for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/). For D. melanogaster, gene expression data across nine tissue types (larval fat body and Malpighian tubules; and adult hindgut, midgut, accessory gland, brain, crop, ovary, and tests) were derived from the FlyAtlas database (GEO accession GSE7763; Chintapalli et al. 2007). Each tissue was represented by four biological samples,
Table 1. Insect species and major innexin groups\(^1\) from which sequences are analyzed

| Infraclass   | Order         | Family         | Species                          | Innexin group (number of sequences) |
|--------------|---------------|----------------|----------------------------------|-------------------------------------|
|              |               |                |                                  | Inx2  | Inx3 | Inx7 | Ogre | ShakB | Zpg/Inx5/Inx6 | Other |
| Paraneoptera | Hemiptera     | Aphisidae      | Acyrthosiphon pismum             | 1     | 1    | 2    | 1    | 1     | 1               | 2     |
|              | Hymenoptera   | Tenebrionidae  | Pediculus humanus               | 1     | 1    | 1    | 1    | 1     | 1               | –     |
|              | Coleoptera    | Bombbycidae    | Tribolium castaneum             | 1     | 3    | 1    | 1    | 1     | 1               | –     |
|              | Lepidoptera   | Culicidae      | Bombbyx mori                    | 1     | –    | 1    | –    | –     | 1               | –     |
|              | Diptera       | Drosophilidae  | Aedes aegypti                   | 1     | 1    | 1    | 1    | –     | –               | –     |
|              |               |                | Anopheles gambiae               | 1     | 1    | 1    | 1    | 2     | 1               | –     |
|              | Hymenoptera   | Pteromalidae   | Culex quinquefasciatus          | 1     | –    | 1    | –    | –     | –               | –     |
|              | Apidae        |                | Drosophila melanogaster         | 1     | 1    | 1    | 1    | 1     | 3               | –     |
|              |               |                | Drosophila grimshawi            | 1     | 1    | 1    | 1    | 1     | 3               | –     |
|              |               |                | Nasonia vitripennis             | –     | 1    | 1    | –    | –     | –               | –     |
|              |               |                | Apis mellifera                  | 1     | 1    | 1    | 1    | 1     | –               | –     |
|              |               |                | Bombus impatiens                | 1     | 1    | 1    | 1    | 1     | –               | –     |
|              |               |                | Camponotus floridanus           | 1     | 1    | 1    | 1    | 1     | –               | –     |
|              |               |                | Harpegnathos saltator           | 1     | 1    | 1    | 1    | 1     | –               | 1     |

\(^1\) Groups are named as in *D. melanogaster*.
insect innexins have been separated for at least 300 Mya. The relationships among the six clades were not well resolved, except for evidence of a sister relationship between ShakB and Ogre.

Within one of the six clades (the Zpg Clade), two independent gene duplication events were inferred to have occurred in the lineage of Drosophila, after the MRCA of dipteran families Culicidae and Drosophilidae, which probably occurred in the early Jurassic around 250 Mya (Grimaldi and Engel 2005). These duplications gave rise to Drosophila zpg, inx5, and inx6, which lack orthologs in Culicidae or other available insect genomes. The presence of zpg, inx5, and inx6 orthologs in both D. melanogaster and D. grimshawi indicates that these duplications occurred prior to the MRCA of these two species, which has been estimated to have occurred over 60 Mya (Tamura et al. 2004). More information about the timing of these duplications will be provided by more fully sequenced genomes of Diptera.

Gene expression data from the FlyAtlas database (Chintapalli et al. 2007) indicated marked differences in expression among tissues in the case of every D. melanogaster innexin except ShakB. The ShakB protein also differed from other insect innexins in its high level of amino acid sequence conservation, with much higher proportions of ancestral residues and of unique ancestral residues being conserved in the ShakB clade than in other clades. A high level of sequence conservation in the case of a very broadly expressed protein is consistent with data suggesting that broadly expressed proteins tend to be highly conserved (Zhang and Li 2004). In spite of the sister relationship between ShakB and Ogre, there was no evidence of a similar pattern of gene expression in Drosophila (Supp Fig S2).

The Zpg Clade of Drosophila provided a striking contrast in patterns of gene expression, with Zpg expressed at a high level in ovary and Inx6 and Inx7 expressed at a high level in testis. Because the unduplicated members of this clade from B. mori (Lepidoptera) and A. gambiae (Diptera: Culicidae) were expressed at high levels in both ovary and

Fig. 1. ME tree of insect innexins, rooted with sequences from the nematode A. sum. The tree was based on the JTT + G distance at 267 aligned amino acid positions. Numbers on the branches are the confidence levels of the interior branch test; only values ≥95% are shown.

Fig. 2. (A) The percentage of residues inferred to have been present in the common ancestor of each of six clades of insect innexins that were conserved in all clade members analyzed (out of 267 total aligned residues in each case). Fisher’s exact test of the equality of the proportion conserved with the ShakB clade: *P < 0.05; **P < 0.01 (Bonferroni-corrected). (B) The percentage of apomorphic residues inferred to have arisen in the common ancestor of each clade that were conserved in all clade members analyzed (total numbers of apomorphic residues in the ancestor are shown above each bar). Fisher’s exact test of the equality of the proportion conserved with the ShakB clade: *P < 0.05; **P < 0.01 (Bonferroni-corrected).
testis, it seems likely that this broader pattern of expression in gonads of both sexes was the ancestral pattern prior to gene duplication in the Drosophila lineage. Thus, the patterns of expression of the Drosophila members of this clade provide evidence of subdivision of an ancestral gene function after gene duplication (Hughes 1994, Lynch and Force 2000).

It is known that Drosophila Zpg can form heterotypic channels with Inx2 (Phelan 2005), and it is of interest that, in spite of a complex pattern of tissue expression, Inx2 shared with Zpg a high level of expression in ovary (Fig. 2B). Because no other Drosophila innexin showed the same high level of expression in the testis as Inx5 and Inx6, it is possible that these two proteins together form heterotypic channels in the testis. On the other hand, it is known that Inx6 forms heterotypic channels with Inx7 in the Drosophila brain and that these channels are important for memory (Wu et al. 2011).

A more detailed knowledge of expression patterns and the formation of heteromeric and heterotypic channels in different tissues will shed further light on the functional differentiation of the insect innexins.

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