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Evidence for a Diverse Cys-Loop Ligand-Gated Ion Channel Superfamily in Early Bilateria

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Abstract. The genome sequences of *Caenorhabditis elegans* and *Drosophila melanogaster* reveal a diversity of cysteine-loop ligand-gated ion channels (Cys-loop LGICs) not found in vertebrates. To better understand the evolution of this gene superfamily, I compared all Cys-loop LGICs from rat, the primitive chordate *Ciona intestinalis*, *Drosophila*, and *C. elegans*. There are two clades of GABA receptor subunits that include both verterbate and invertebrate orthologues. In addition, I identified nine clades of anion channel subunits found only in invertebrates, including three that are specific to *C. elegans* and two found only in *Drosophila*. One well-defined clade of vertebrate cation channel subunits, the α7 nicotinic acetylcholine receptor subunits (nAChR), includes invertebrate orthologues. There are two clades of invertebrate nAChRs, one of α-type subunits and one of non-α subunits, that are most similar to the two clades of vertebrate neuronal and muscle α and non-α subunits. There is a large group of divergent *C. elegans* nAChR-like subunits partially resolved into clades but no orthologues of 5HT3-type serotonin receptors in the invertebrates. The topology of the trees suggests that most of the invertebrate-specific Cys-loop LGIC clades were present in the common ancestor of chordates and ecdysozoa. Many of these disappeared from the chordates. Subsequently, selected subunit genes expanded to form large subfamilies.

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Introduction

The cysteine-loop ligand-gated ion channels (Cys-loop LGICs) are neurotransmitter receptors central to neurotransmission in bilateria. These pentameric channels are encoded by a superfamily of subunit genes that share a membrane topology consisting of an amino terminal ligand-binding domain and four transmembrane domains that form the ion selective pore (Hille 1992) (Fig. 1).The subunit composition determines ion selectivity, neurotransmitter affinity, subcellular localization, gating kinetics, and pharmacology. In vertebrates, four families of LGIC subunits have been recognized based on their ligand specificity: excitatory (cation selective) nicotinic acetylcholine receptors (nAChRs), excitatory 5HT3 receptors, inhibitory (anion selective) GABA receptors, and inhibitory glycine receptors (Le Novere and Changeux 1995; Ortells and Lunt 1995). Families of subunits are broken down into subfamilies, designated α, β, γ, etc., based on sequence motifs and pharmacology. The nAChR subunits are designated α and non-α subunits, based on the presence of vicinal cysteines in the α subunits that are necessary to bind acetylcholine. Invertebrates encode, in addition to nAChRs and GABA receptors, a wide array of Cys-loop LGICs including inhibitory histamine (Gisselmann et al. 2002; Zheng et al. 2002), glutamate

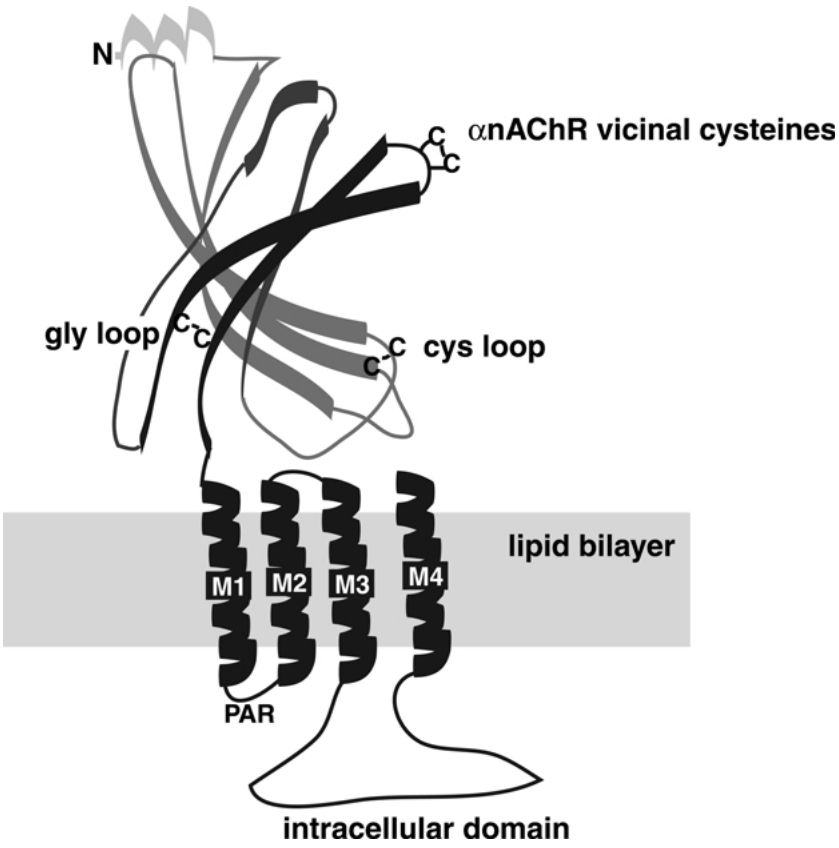

Fig. 1. The structure of a Cys-loop ligand-gated ion channel (LGIC). The extracellular amino terminal domain (''N'' indicates the amino terminus) is the ligand-binding domain. It consists primarily of ß-sheets interrupted by loops. ''Cys loop'' indicates the position of the disulfide bond that defines the Cys-loop LGICs. The vicinal cysteines that define α subunits of nAChRs are indicated, as well as the approximate position of a pair of cysteines (''gly loop'') found in glycine receptors (''double Cys-loop'' subunits; see text). Four α-helical transmembrane domains (''M1–4'') form the ion selective pore. The anion selectivity motif, ''PAR,'' is indicated between M1 and M2. A poorly conserved intracellular loop of variable length appears between M3 and M4.

(Cully et al. 1994; Dent et al. 1997; Vassilatis et al. 1997a; Horoszok et al. 2001), acetylcholine (Putrenko et al. 2005), and serotonin (Ranganathan et al. 2000)-gated chloride channels as well as excitatory GABA receptors (Beg and Jorgensen 2003; Gisselmann et al. 2004).

Given the diversity of the Cys-loop LGIC superfamily and its central role in fast neurotransmission, understanding the evolution of the Cys-loop LGICs is of considerable interest. The Cys-loop LGICs are related to a highly diverged collection of prokaryotic LGIC-like proteins that lack the Cys-loop cysteines (Tasneem et al. 2004), suggesting that the Cys-loop LGICs may have evolved from a protein that predates the prokaryote/eukaryote split. The Cys-loop LGICs have been grouped into two clades, the nAChR/5HT3-like cation channels and the GABA/glycine-like anion channels, based on trees rooted to prokaryotic the LGIC-like proteins (Tasneem et al. 2004). The anion channel/cation channel divergence, and thus the origin of the Cys-loop LGICs, has been estimated to have occurred >2 billion years ago (Cockroft et al. 1992; Ortells and Lunt 1995).

The vertebrate cation channel subunits consist of the serotonin-gated 5HT3 receptor subunits and the acetylcholine-gated nAChR subunits. Based primarily on vertebrate subunits, the nAChRs have been further split into approximately four groups: α7-like subunits, invertebrate α and non-α subunits, vertebrate α subunits, and vertebrate non-α subunits (Cockroft et al. 1992; Le Novere and Changeux 1995; Ortells and Lunt 1995; Tsunoyama and Gojobori 1998). The vertebrate subunits can also be classified based on expression in neurons or in muscles at the neuromuscular junction. It has also been proposed, in contrast to the classification above, that the muscle subunits may have diverged from the neuronal subunits and each clade subsequently evolved α and non-α subunits (Le Novere and Changeux 1995; Le Novere et al. 2002). Some invertebrate nAChR subunits appear to have diverged after the split between the α7 family and the other nAChR subfamilies, indicating that at least two nAChR subfamilies existed in the last common bilaterian (Le Novere and Changeux 1995; Ortells and Lunt 1995; Ballivet et al. 1996). However, it has subsequently become clear that the family of nAChRs in invertebrates, especially nematodes, is quite diverse including some subunit types that are likely to be quite ancient (Treinin and Chalfie 1995; Ballivet et al. 1996; Mongan et al. 1998; Mongan et al. 2002; Jones and Sattelle 2003).

The vertebrate anion channels have been divided into three groups: the glycine receptors, the α/γ/ε GABA receptors, and the β/ρ/δ/π/τ GABA receptors (Ortells and Lunt 1995; Vassilatis et al. 1997b; Xue 1998). Some analyses support the divergence of the β/ρ/δ/π/τ GABA subunits followed by a split between the glycine receptors and the α/γ/ε GABA

receptors (Ortells and Lunt 1995). Invertebrates encode both β and non-β (i.e., α/γ/ε)–type $GABA_A$ subunits as well as a distinct class of invertebrate GABA receptors (Xue 1998). The invertebrate-specific glutamate-gated chloride channels (GluCls) appear to be most closely related to the vertebrate glycine receptors (Vassilatis et al. 1997b; Xue 1998). Since no invertebrate glycine receptors have been identified (but see Pierobon et al. 2004), the glycine receptors and the GluCls could be orthologous (Vassilatis et al. 1997b). Moreover, *C. elegans* encodes a number of unusual subunits not found in vertebrates that may belong to a monophyletic family distinct from the GABA, glycine and GluCl families of subunits. These too appear to be of ancient origin (Xue 1998).

An understanding of the history of the Cys-loop LGIC neurotransmitter receptors should provide insight into the structure of primitive nervous systems and the forces that drove their evolution. With the completion of the genome sequences of the nematode *C. elegans* and the fruit fly *Drosophila*, it seemed an opportune time to re-examine the evolution of the Cys-loop LGICs. I have confirmed and extended the previous analyses showing the existence of several LGIC families unique to invertebrates. I provide evidence that these families diverged prior to the split between chordates and invertebrates and were subsequently lost from the vertebrate lineage. Thus, there appears to have been a surprisingly diverse Cys-loop LGIC superfamily in the last common bilaterian.

Materials and Methods

Vertebrate sequences with the following accession numbers were acquired from GenBank: NP_062170, NP_058774, NP_434692, NP_077330, T01378, P12389, NP_062018, NP_062171, NP_058890, NM_024485, NP_036660, NP_598281, NP_434693, NP_075219, NP_476532, NP_077370, NP_899155, NP_058987, P47742, P50573, NP_899156, AAH87714, P15431, NP_077346, NP_112291, NP_058991, NP_058761, NP_058765, NP_037089, NP_113921, NP_542154, NP_075579, NP_068613, NP_542153, NP_036700, NP_446176, NP_445748, and AAF73283. *C. elegans* sequences were taken from Wormbase (http://www.wormbase.org release 136) (Stein et al. 2001) Wormbase gene predictions are based on cDNAs in many cases but only on Genefinder predictions and annotation in other cases. The *Drosophila* sequences were taken from Flybase (http://flybase.bio.indiana.edu/) and corresponding cDNA have been characterized for all *Drosophila* Cys-loop LGICs discussed (Gelbart et al. 1997). The *Ciona intestinalis* genes were taken from the *Ciona* database (http://genome.jgi-psf.org/Ciona4/ciona4.home.html). These sequences are based entirely on gene predictions.

To identify Cys-loop LGICs I scanned the sequenced genomes of *C. elegans*, *D. melanogaster* and *C. intestinalis* for genes encoding putative channel subunits. Characterized Cys-loop ligand-gated ion channel subunits from *C. elegans* and vertebrates were used in BLAST (tblastn) searches of the genomes of these organisms. I confirmed these proteins as Cys-loop LGIC subunits by alignment with known channels. These were then used in the next round of BLAST searches until no new subunits were identified. The putative open reading frames of the LGIC subunits I identified were recovered from the respective organismal databases as annotated. Some sequences appear to have insertions or deletions that may be artifacts of annotation. However, verified sequences (e.g., UNC-38 and UNC-63) also have insertions so I did not attempt to edit the sequences on this basis. The available Ciona genome sequence encodes several Cys-loop LGIC subunit fragments, usually near the end of a contig. These were not included in the analysis. In cases where splice variants are known, the shortest variant containing all domains (Fig. 1) was generally chosen. Only proteins that contained the defining cysteine pair were used in spite of other evidence of homology.

Alignments were performed on amino acid sequences because the ancient nature of the channels and the rapid evolution of *C. elegans* suggested that the DNA sequence would be mutagenically saturated. Sequences were aligned using MUSCLE (Edgar 2004), and nonalignable, noninformative sites were removed manually (239 informative sites among cation channels, 276 among anion channels). Because the various tree-building algorithms have different biases (Kolaczkowski and Thornton 2004), I used three different algorithms. PROTPARS from the PHYLIP software package was used for maximum parsimony (Fitch 1971) analysis. Prot Test (Abascal et al. 2005) was used to determine the optimal model and parameters (anion channels—Jones-Taylor-Thornton matrix, 0.01 invariant site, $\alpha = 1.09$; cation channels—WAG model, 0.02 invariant site, $\alpha = 1.86$). Neighbor-joining trees were created using PROTDIST and NEIGHBOR (Saitou and Nei 1987). Maximum likelihood analyses were performed using PHYML (Guindon and Olivier 2003). SEQBOOT and CONSENSE were used for bootstrap analyses and the generation of consensus trees. Pilot studies indicated that the choice of model did not substantially influence tree topology. Bootstrap values are normalized to 100 for replicates of 100 for maximum likelihood, 400 for neighbor joining, and 1000 for maximum parsimony. Trees were rooted at a point between the anion and cation channel clades (Tasneem et al. 2004). Figures were created using DRAWGRAM and Adobe Illustrator 8.0 (Adobe Systems Inc., San Jose, CA).

Results

To better understand the evolution of the Cys-loop LGIC receptor superfamily, I examined channel subunits cloned from rat and identified in the genomes of the primitive chordate *C. intestinalis* (Dehal et al. 2002), the nematode *C. elegans* (Consortium 1998), and the insect *Drosophila melanogaster* (Adams et al. 2000). These four organisms represent major phyla that were among the first to diverge from the last common bilaterian and for which well annotated genome sequences were available. The Cys-loop LGICs of additional vertebrate, insect (e.g., *Anopheles*) and nematode (e.g., *Caenorhabditis briggsae*) genome sequences are largely orthologous to those of rat, *C. elegans*, and *Drosophila* superfamilies and therefore their addition would not illuminate the divergence of Cys-loop LGICs in early bilateria (Stein et al. 2003; Jones et al. 2005). The putative cation channel subunits and anion channel subunits were analyzed separately (with exceptions noted below) using maximum likelihood (ML), maximum parsimony (MP), and neighbor joining (NJ) algorithms.

The mammalian anion channel subunits consist of the $GABA_A$ receptor subunits, which fall into seven

subfamilies (6α, 3β, 3γ, ε, δ, π, τ), and glycine receptor subunits, with two subfamilies (3α, β). In *Ciona*, I identified seven GABA/glycine-like subunits. *Drosophila* encodes eight GABA/glycine-like subunits. These include rdl, Grd, and LCCH3, all of which have been shown to form GABA-responsive channels in heterologous systems (Hosie et al. 1996; Gisselmann et al. 2004), the GluCl subunit dm_GluClα (Cully et al. 1996), and the histamine receptor subunits ORT and HisCl1 (Gisselmann et al. 2002; Zheng et al. 2002). *C. elegans* encodes two characterized GABA receptor subunits UNC-49 (Bamber et al. 1999) and GAB-1 (Feng et al. 2002) as well as the predicted genes ZC482.5 and F11H8.2. *C. elegans* also encodes a family of six GluCls, a serotonin-gated chloride channel (MOD-1) (Ranganathan et al. 2000) and several acetylcholine-gated chloride channels (ACCs) (Putrenko et al. 2005). Moreover, a large number of uncharacterized subunits are likely to form anion selective channels based on the presence of a consensus pro-ala-arg motif (or a variation thereof) on the cytoplasmic side of the second transmembrane domain (Galzi et al. 1992). I included the GABA-gated cation channel subunits, EXP-1 (Beg and Jorgensen 2003) (which has a corresponding cation selectivity motif) and Grd (Gisselmann et al. 2004), in this category because previous results indicated a greater overall similarity to the anion channels (Beg and Jorgensen 2003).

I included 18 mammalian cation channel subunits: 15 nAChRs (8α, 4β, γ, δ, ε), two 5HT3 serotonin receptor subunits and the human zinc-activated cation channel (ZAC; Davies et al. 2003). I identified 12 cation channel subunits from *Ciona*. Seven nAChR-like subunits and one divergent cation channel subunit (Dβ3/CG11822) from *Drosophila* were included. The α3 and β3 subunit have been shown to form acetylcholine-responsive heteromers with vertebrate nAChR subunits (Schulz et al. 1998; Lansdell and Millar 2000) Among the 52 *C. elegans* subunits are UNC-38, UNC-29, LEV-1, UNC-63, EAT-2, ACR-16, and LEV-8, all of which have been shown to form acetylcholine receptors (Ballivet et al. 1996; Fleming et al. 1997; Culetto et al. 2004; McKay et al. 2004; Towers et al. 2005). A heteromer of DEG-3/DES-2 responds most robustly to choline but also responds to acetylcholine (Yassin et al. 2001).

The Invertebrate and Ciona *GABA/Glycine Receptor Families Are Sparse Compared to Vertebrates*

Ciona appears to encode one gene corresponding to several, but perhaps not all, of the major subunit families found in vertebrates (Fig. 2; Supplemental Data). There is strong support for grouping the subunits ci01001314, ci01001338, and ci01001485 as a monophyletic group within the rho subfamily of GABA subunits (bootstrap values: ML100, MP78, NJ95). ci01001443 groups with mammalian β subunits (ML67, MP81, NJ98), whereas ci01001379 appears orthologous to the π subunit (ML95, MP69, NJ86). ci01001456 groups with the α-type GABA subunits (ML86, MP91, NJ98) but not with any particular member of this subfamily. I identified one putative *Ciona* glycine receptor subunit ci01001376 (ML96, MP86, NJ82) that likely diverged after the α/β subunit split (ML < 50, MP90, NJ82).

As with *Ciona*, the invertebrate GABA-like subunits represent a subset of the mammalian receptors. *Drosophila* LCCH3 and *C. elegans* GAB-1 are orthologous (ML100, MP100, NJ100) and form part of the chordate β/ρ/δ/π/τ $GABA_A$ receptor subunit clade (ML100, MP59, NJ88). *C. elegans* ZC482.5 is orthologous to the *Drosophila* paralogues Grd and CG8916 (ML95, MP71, NJ66) and these, together with the chordate α/γ/ε $GABA_A$ receptor subunits, form a monophyletic group (ML100, MP92, NJ95). Finally, the *Drosophila* rdl joins with UNC-49 and its paralogue F11H8.2 to form a monophyletic group of invertebrate-specific GABA receptor subunits (ML89, MP93, NJ80). The ML(57) phylogeny is consistent with all GABA receptor subunits having a common ancestor. There are no clear invertebrate orthologues to the chordate glycine receptors.

Invertebrates Encode Several Families of Anion Channel Subunits Not Found in Vertebrates

Three clades of invertebrate channel subunits share with the vertebrate glycine receptor subunits a pair of cysteines in addition to the Cys-loop cysteines (Fig. 3). These clades include the *Drosophila* histamine receptors, ORT and HisCl1 (ML100, MP100, NJ100), which, together with CG12344, appear to form a monophyletic group (all bootstraps < 50), the invertebrate GluCl subunits, which include six *C. elegans* subunits and one *Drosophila* subunit (ML100, MP90, NJ87), and a group of six subunits from *C. elegans* here referred to as "ce_Group I" (ML97, MP95, NJ98) (Fig. 1; supplemental data). In my analysis, these clades do not form a monophyletic group although the HisCl subunits consistently group with ce_Group I (bootstraps < 50).

In addition to the GABA/glycine-like channels, *C. elegans* encodes three clades of Cys-loop LGICs that are not found in vertebrates, *Ciona*, or *Drosophila*. One clade consists only of EXP-1 and an uncharacterized paralogue, Y46G5A.26 (ML100, MP100, NJ100). A second clade consists of 16 subunit genes and includes the ACC acetylcholine-gated chloride channel subunits and the MOD-1 serotonin-gated chloride channel (ML100, MP100, NJ100). This clade can be further broken down into three subgroups: MOD-1 and T20B12.9 (ML94, MP70, NJ86), a group of eight su-

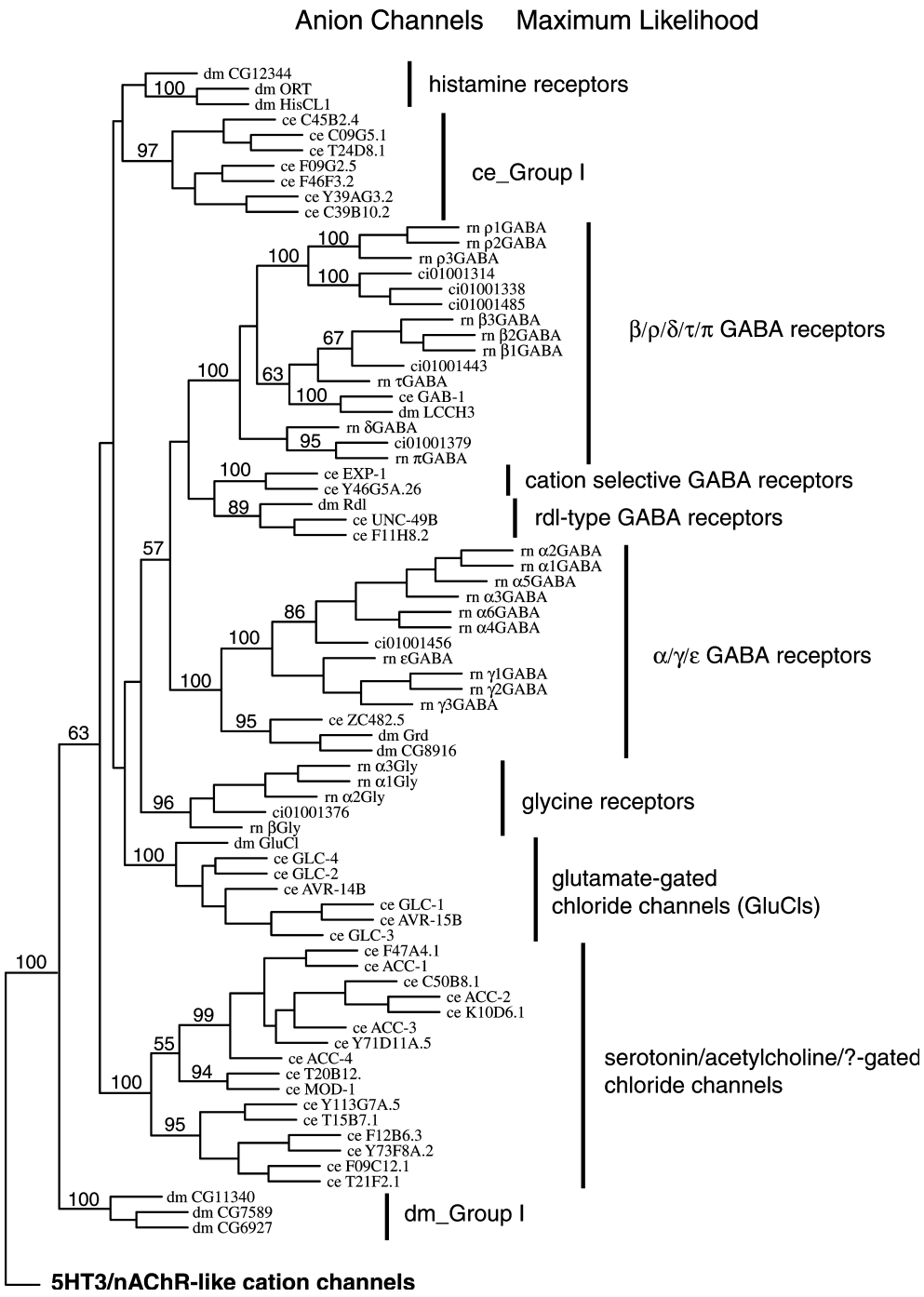

Fig. 2. Dendrogram of anion channel subunits. Maximum likelihood tree derived from amino acid sequences of all putative anion channel-like subunits (including the cation channel subunits EXP-1 and Grd) from rat (rn), *Ciona* (ci), *Drosophila* (dm), and *C. elegans* (ce). Bootstrap values (of 100) indicated on selected branches. Tree rooted to nAChR and 5HT3 cation channel subunits.

bunits including the four ACC subunits (ML99, MP84, NJ100), and six uncharacterized subunits (ML95, MP82, NJ87). Finally, *Drosophila* also encodes a family of three LGIC subunits, CG7589, CG6927, and CG11340 (ML100, MP100, NJ100), not found in the chordates or *C. elegans*. Orthologues of at least two of these subunits are also found in *Anopheles* (data not shown), indicating that this family is conserved among the diptera.

Ciona and Mammals Show Different Patterns of Expansion of Cation Channel Subunit Families

Similar to the anion channels, several mammalian cation channel subunit subfamilies are represented by a single subunit in *Ciona* with independent expansion of selected *Ciona* subgroups (Fig. 4). Three *Ciona* subunits, ci01001543, ci01001441, and ci01001389, consistently group with the mammalian 5HT3 receptor

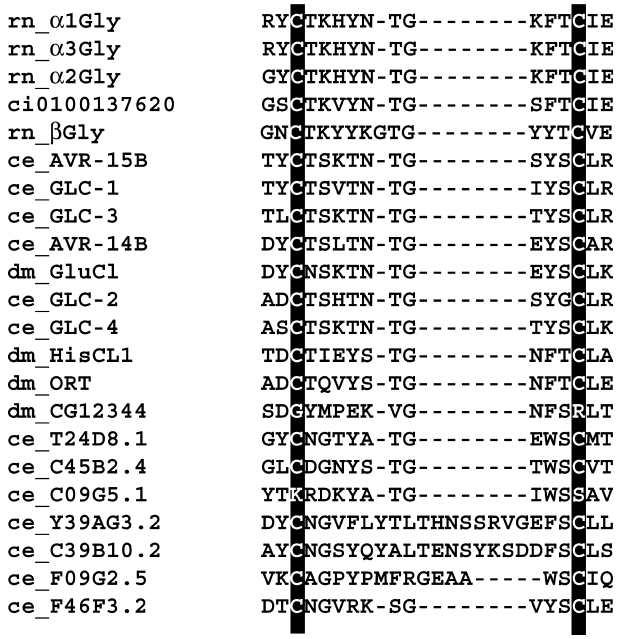

Fig. 3. Lineup of the putative second Cys-loop domain. A lineup manually edited to highlight the conserved pair of cysteines found in glycine receptors, GluCls, HisCls, and ce_Group I. These cysteines may form a second Cys-loop defining the proposed "double Cys-loop" family of subunits. C09G5.1 and dm_CG12344 do not have these cysteines but clearly fall into the ce_GroupI and HisCl clades, respectively.

(ML73, MP71, NJ < 50). ci01001505/01001547 belong to a clade of α7-like subunits (ML72, MP50, NJ69) but do not appear to be orthologous to the mammalian α7 subunit. There is strong support for orthology of ci01001490 and neuronal β2/4 subunits (ML99, MP99, NJ100), of ci01001451 and neuronal α3/6 subunits (ML94, MP94, NJ99), and of ci01001422 and the muscle α1 subunit (ML97, MP92, NJ99). There is support for ci1001324, ci01001322, and ci01001376 forming a monophyletic (ML75, MP89, NJ59) sister group (ML97, MP89, NJ97) to a monophyletic group of mammalian β1/δ/γ/ε non-α muscle subunits (ML80, MP83, NJ50).

Invertebrates Encode Several Distinct Classes of nAChR-Type Receptors

C. elegans and *Drosophila* encode at least five major clades of nAChR-like subunits, two of which are unique to *C. elegans*. Among the shared clades, there is a monophyletic group of 11 subunits (ML96, MP57, NJ93), referred to here as the UNC-38-like clade after the best-characterized member. All but one member of this group (Dβ2/CG6798) has the vicinal cysteines that define them as α subunits. The clade divides roughly into a *Drosophila* (ML90, MP85, NJ99) and a nematode (ML < 50, MP < 50, NJ polyphyletic) subgroup, indicating much of the expansion of this family occurred after the nematode insect split and that the non-α subunit Dβ2 evolved from α subunits. LEV-8, ACR-8, and ACR-12 appear to derive from a common ancestor (ML100, MP100, NJ100) that may have branched early within the UNC-38-like clade (MP and NJ). A second clade consists of five non-α 5 subunits (the UNC-29-like family): one insect and four nematode subunits (ML100, MP99, NJ100). These two invertebrate clades group with the chordate neuronal/muscle α and non-α clades (ML100, MP88, NJ94). The UNC-29-like clade consistently diverges before the other clades but without strong bootstrap support (ML62). The relationship of the remaining three clades is ambiguous, with NJ (66) supporting a grouping of the invertebrate and vertebrate α-subunit clades and ML (66) and MP (53) supporting the grouping of the vertebrate α and non-α clades.

There is support for a large and diverse clade of vertebrate and invertebrate α7-like subunits including three *Drosophila* subunits and nine *C. elegans* subunits (ML72, MP50, NJ69). ce_ACR-19 shows the highest degree of similarity to mammalian α7, whereas the other eight *C. elegans* subunits form a monophyletic subgroup (ML99, MP91, NJ95) that includes both α and non-α (ACR-9, ACR-14, and EAT-2) subunits. The three *Drosophila* subunits also form a monophyletic group (ML100, MP99, NJ100) closely related to mammalian α7. Interestingly, all analyses are consistent with the divergence of the *Ciona* α7-like subunits prior to divergence of mammalian α7, ce_ACR-19 (except MP), and the *Drosophila* α7-like subunits. There is no clear invertebrate orthologue of the mammalian α9nAChR but it is most similar to the nematode paralogues ACR-21 and F18G5.4.

Of the two remaining large families of *C. elegans*-specific nAChR-like subunits, one group of six α subunits is defined by the characterized genes DEG-3 and DES-2 (ML100, MP99, NJ100). The DEG-3 clade consistently formed a monophyletic group with ACR-17, ACR-18, F21A3.7, and F58H7.3, but with weak bootstrap support (< 50). There is evidence for a related 15-member group of non-α subunits, represented by ACR-22, which includes a group of seven genes of the T01H10 type (ML85, MP < 50, NJ73). Six of these (T01H10.1, −.2, −.3, −.5, −.6, −.7) cluster within a 20-kb region of the X chromosome (Consortium 1998; Stein et al. 2001). This clade consistently includes two additional subunits, F11C7.1 and Y44A6E.1 (ML62). Together the DEG-3 and ACR-22 clades consistently form a monophyletic group but with weak bootstrap support (ML58, MP < 50, NJ57).

Finally, there are four non-α *C. elegans* subunits, defined by CUP-4, that consistently group together but with inconsistent bootstrap support (ML71, MP polyphyletic; NJ < 50). These, together with the *Drosophila* subunit Dβ3/CG11822 and ZAC, repre-

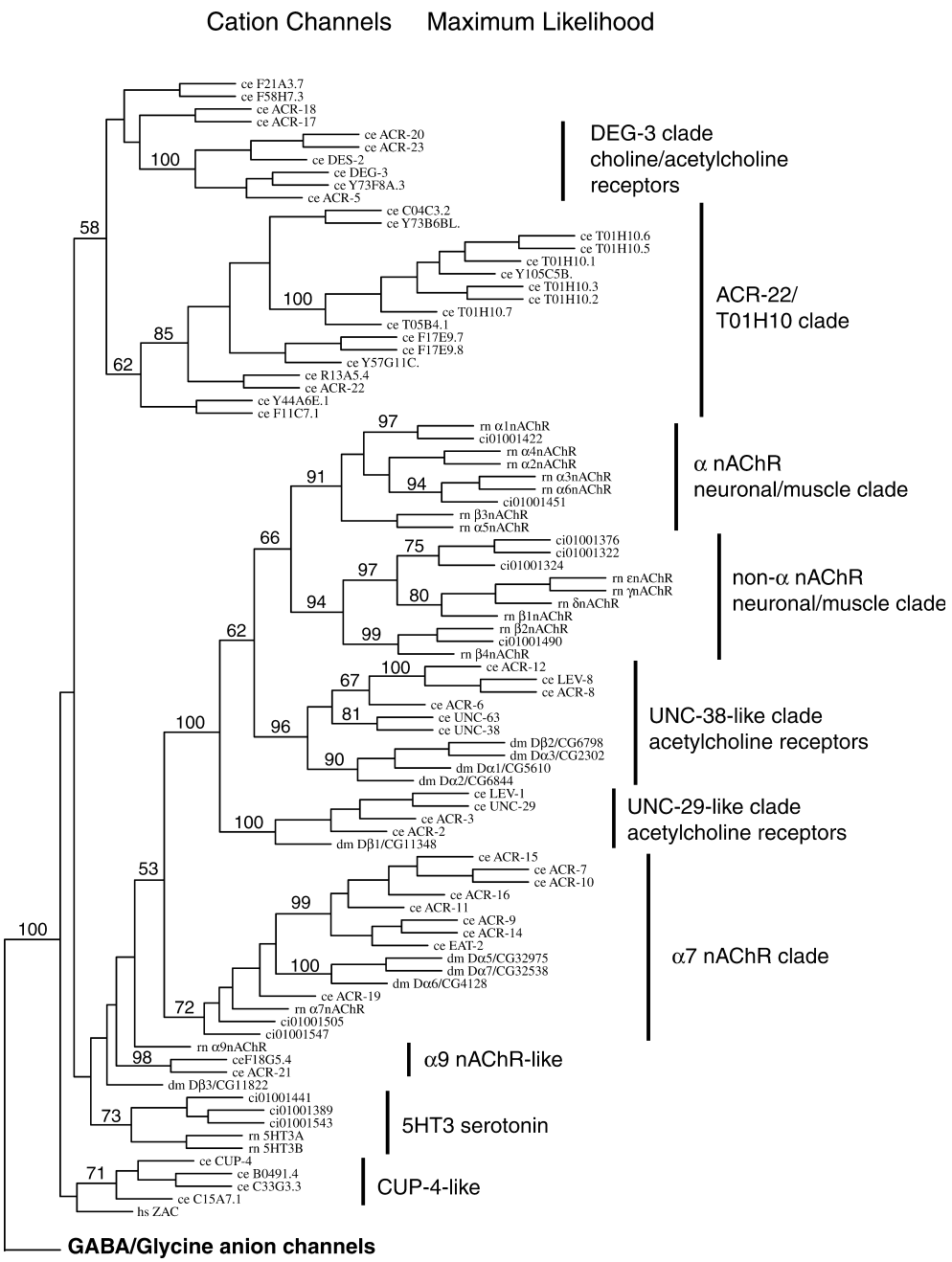

Fig. 4. Dendrogram of cation channel subunits. Maximum likelihood tree derived from amino acid sequences of all putative cation channel-like subunits from rat (rn), human (hs), *Ciona* (ci), *Drosophila* (dm), and *C. elegans* (ce). Bootstrap values (of 100) indicated on selected branches. Tree rooted to vertebrate GABA and glycine anion channel subunits.

sent highly divergent nAChR-like subunits that do not clearly fall into any family.

Discussion

Using a larger and more complete data set, my results confirm and extend previous analyses of Cys-loop LGIC phylogeny. The results are consistent with a fundamental grouping into anion and cation channels (with the exception of EXP-1 and Grd). Among the anion channels, I see the GABA receptors divided into two groups, with the invertebrate-specific rdl-type receptors forming a third group. The glycine receptors form an additional family, possibly a sister group to the families of GluCls, HisCls, and *C. elegans* Group I. Among the cation channels, my results are consistent with a clade of 5HT3-like subunits, a clade of α7-like subunits, two clades of invertebrate subunits, and two clades, α and non-α, of vertebrate subunits. However, my results also differ from previous studies in a number of aspects.

The Relationship of the GABA and Glycine-like (Double Cys-Loop) Subunits

Ortells and Lunt (1995; see also Vassilatis et al. 1997b) proposed that β GABA subunits were primitive and, along with ρ/δ/π/τ subunits, diverged from the other anion channel subunits before the glycine receptors branched from the α/γ/ε GABA subunits. They therefore concluded that the glycine receptors evolved from the GABA receptors. I see no evidence of this. Although the glycine receptors sometimes group with a GABA receptor clade, the bootstrap support for this grouping is very weak. Vassilatis et al. (1997) noted the similarity of the vertebrate glycine receptors and invertebrate GluCls and proposed they could represent orthologous groups. My results do not strongly support an orthologous relationship between these families. Nevertheless, there are compelling reasons for grouping the GluCls glycine receptors, the histamine receptors, and the ce-Group I subunits. All but two members of these clades share a second pair of cysteines (in addition to the Cys-loop cysteines) in the extracellular ligand-binding domain. In lineups with acetylcholine binding protein (AChBP) these cysteines flank the ligand-binding C-loop and are juxtaposed (data not shown) (Brejc et al. 2001; Karlin 2002). Evidence from mutagenesis studies of glycine receptors indicate the cysteines form a disulfide bond important for ligand binding (Rajendra et al. 1995). I therefore refer to the glycine GluCl, HisCl, and Group I channels as "double Cys-loop channels," a group I propose is monophyletic. Because tree-building algorithms do not clearly resolve the topology within the double Cys-loop group, or even identify them as a clade, it is not clear whether the double Cys-loop subunits diverged into its member clades before or after the chordate/invertebrate split.

Evolutionary Relationships Among the Cation Channel Subunits

My results are consistent with the previous analyses showing that the vertebrate cation channels fall into 5HT3, α7, α9, and neuronal/muscle α and non-α clades (Cockroft et al. 1992; Ortells and Lunt 1995). I found the muscle and neuronal α subunits form a monophyletic group rather than the muscle α1 and muscle non-α subunits (β1, γ, δ ε) forming a clade, suggesting that structure, not expression pattern, is ancestral. Early analyses with limited data indicated that invertebrate subunits form a monophyletic or paraphyletic group (Le Novere and Changeux 1995; Ortells and Lunt 1995). My results indicate the presence of a large monophyletic group of α7-like channels subunits that includes nine nematode subunits and three *Drosophila* subunits. I find two monophyletic groups of invertebrate subunits, containing both nematode and insect subunits that are most similar to the vertebrate muscle and neuronal α and non-α subunits. It is interesting that the two vertebrate and invertebrate clades divide into α (with the exception of the rn_β3 in the vertebrate case and dm_Dβ2 in the case of invertebrates) and non-α subunits. This is consistent with a model wherein the α and non-α subunits diverged before the chordate/invertebrate split. However, non-α subunits have evolved from α subunits several times (Ortells and Lunt 1995; Jones et al. 2005), so in the absence of less ambiguous evidence the orthology of these vertebrate and invertebrate α-subunit nAChR clades must remain an open question. On the other hand, it is clear that *C. elegans* encodes a vast superfamily of nAChR-like subunits that are not orthologous to any Chordate or *Drosophila* proteins. These include a clade characterized by DEG-3/DES-2 and a less well-defined clade characterized by ACR-22 and the T01H10 group of subunits.

Although the nAChR-like cation channels (including 5HT3 receptors) form a group based on sequence, the ligand sensitivity of the more divergent members is unknown. There is clear evidence of acetylcholine sensitivity among members of the UNC-38-like, UNC-29-like, and α7-like clades (Fleming et al. 1997; Culetto et al. 2004), including some of the more divergent members like EAT-2 (McKay et al. 2004) and LEV-8 (Towers et al. 2005). There is evidence for DEG-3 sensitivity to choline in vivo (Yassin et al. 2001), but no experimental evidence identifying the ligand-sensitivity of members of the ACR-22/T10H10 or CUP-4 clades.

Evidence of a Diverse Family of Cys-Loop LGICs in the Last Common Bilaterian

The presence of invertebrate orthologues of vertebrate channels among several of the major Cys-loop LGIC clades implies that these major families of subunits evolved before the chordate-invertebrate split and may have been present in the last common bilaterian. This is most clear in the orthology of LCCH3/GAB-1 to the α/γ/ε $GABA_A$ receptor subunits, the orthology between ZC482.5/Grd and the β/ρ/δ/π $GABA_A$ subunits, and the orthology of ACR-19 and α7. Less well supported is the orthology of the double Cys-loop channel clades, the orthology of α9 and ACR-21, and the possible orthology of invertebrate α and non-α subunits to the vertebrate muscle and neuronal α and non-α clades. If the invertebrate subunits diverged after the major families diverged, then at least one original member of each family must have been present in the last common ancestor.

There are caveats to this conclusion. First, the putative ancient invertebrate-specific clades could have been formed more recently by domain shuffling to produce chimeras. However, I found that a phylogeny based on just the ligand-binding domains or just the transmembrane domains does not change the overall topology of the tree (data not shown). Second, horizontal transfer of subunit genes occurring after the chordate-invertebrate split would make the origin of vertically transmitted genes appear to have occurred before the split. However, horizontal gene transfer among metazoans is probably very rare and may be restricted to transposable elements (Daniels et al. 1990; Jordan et al. 1999). Moreover, assuming nematodes and insects group into ecdysozoa (Aguinaldo et al. 1997), horizontal transfer would have had to occur at least three times (two GABA subunit genes and an α7-like subunit gene; Fig. 5A). The greater similarity of the vertebrate α7 to nematode ACR-19 gene or to the insect α7-like genes is consistent with horizontal transfer between an ecdysozoan and a chordate. However, this association is weakly supported and could reflect the recent loss of orthologous α7-like genes from the *Ciona* lineages. Finally, significant variations in the rates of Cys-loop LGIC evolution could make some clades appear more ancient than they truly are (Aguinaldo et al. 1997). Although one cannot rule out this possibility, the existence of conserved orthologues in nematodes and insects (GAB-1/LCCH3, Grd/ZC482.5, and the invertebrate UNC-38 and UNC-29 nAChR clades) suggests that a faster rate of evolution of invertebrate Cys-loop LGICs is not a general phenomenon. Similarly, the sheer diversity of the Cys-loop LGIC superfamily, including independently evolved ACh binding subunits (nAChRs and ACCs), argues against constraints to LGIC structure strongly influencing tree topology through convergent evolution.

Assuming that the phylogenetic trees presented here reflect the true order of divergence, there were at least five types of Cys-loop LGICs (two GABA receptor types, α7-type nAChRs, another cation channel, and another anion channel) in the last common ancestor of chordates and invertebrates (Fig. 5B). Probably there were many more. Based on the distance between the identified chordate/invertebrate orthologues and assuming a constant rate of evolution, each of the major clades likely diverged before the chordate/ecdysozoa split. This would imply that the common ancestor encoded seven GABA/anion channel subunit types: a α/γ/ε-type GABA receptor subunit, a β/ρ/δ/τ GABA receptor subunit, an rdl-type GABA receptor subunit, a double Cys-loop subunit, an ACC/MOD type subunit, a dm_Group I-type subunit, and an EXP-1-type subunit. If some members of the double Cys-loop clade diverged before the chordate/ecdysozoa split, which is not inconsistent with the topology and branch lengths, that would add a HisCl-type subunit, a GluCl-type subunit, a glycine receptor subunit, and/or a ce_Group I-type subunit. The cation channel subunits in the last common ancestor would have consisted of: a 5HT3-type subunit, an α7 subunit (possibly several; see above), an α9 subunit, the muscle/neuronal-type subunits, DEG-3-type subunits (possibly also ACR-22-type), and some subset of

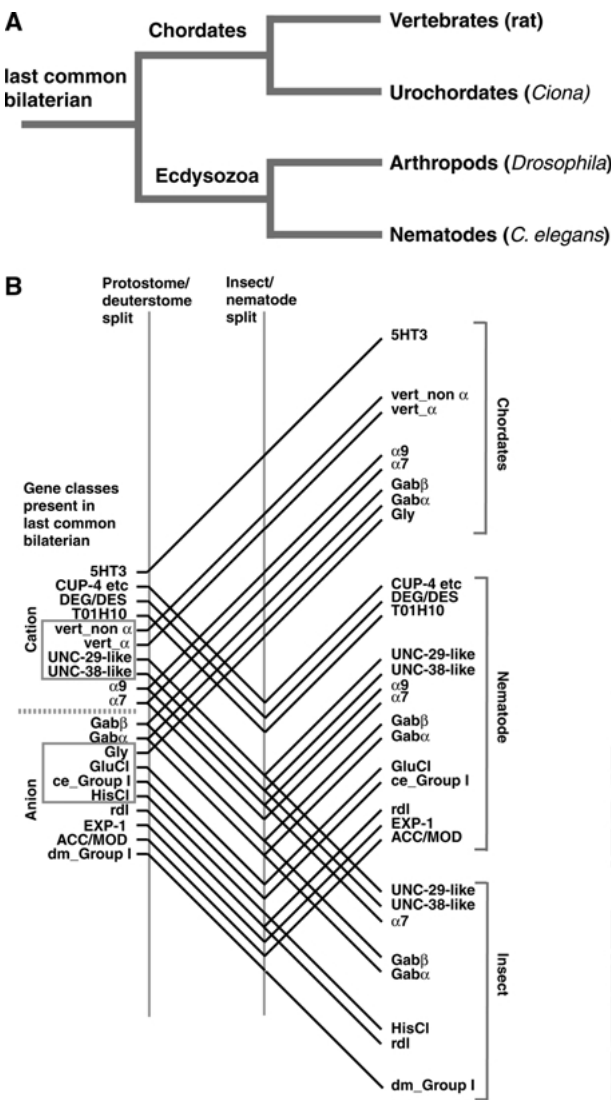

Fig. 5. Proposed pattern of Cys-loop LGIC evolution from early bilateria to the divergence of chordates, nematodes, and insects. **A** Phylogenic relationship of the chordates (rat and Ciona) and the ecdysozoa (*C. elegans* and *Drosophila*) according to Aguinaldo et al. (1997). **B** Proposed pattern of Cys-loop LGIC divergence. To the left are subunit types proposed to have been present in the common ancestor of chordates and invertebrates. The gray boxes enclose subunit types that may not have diverged before the protostome/deuterostome split (see text). The divergence of deuterostomes and protostomes resulted in the eventually loss of several subunit types from chordates but the family remained stable with the divergence of *Ciona* and vertebrates. The divergence of insects and nematodes resulted in further losses of subunit types as well as the retention of shared types such as the rdl-type GABA receptors and the GluCls. The subsequent expansion of the retained subunits into subfamilies is not shown.

CUP-4-like subunits. If the chordate muscle/neuronal nAChR subunits diverged early, the last common ancestor could already have encoded ancestors of each of the two vertebrate and invertebrate muscle/neuronal-type nAChR clades.

Most of the bilaterian phyla, including the ecdysozoa (nematodes and insects) and the deuterostomes (*Ciona* and mammals), appear to have diverged at approximately the same time, before the Cambrian explosion (~540 million years ago), when body plans of major phyla suddenly appear in the fossil record (Valentine et al. 1999). Molecular phylogenies place the split from 600 million to >1 billion years ago (Wray et al. 1996; Peterson et al. 2004). Thus, many if not most of the subunit classes I have outlined may have been present in the last common bilaterian (but see Ruiz-Trillo et al. 1999). If so, a sister species to the bilateria should share many of the invertebrate- and vertebrate-specific subunit families.

Patterns of Contraction and Expansion of Cys-Loop LGIC Families

There is a striking pattern of expansion and contraction of the LGIC superfamily at least from the time of the chordate-invertebrate split. This split appears to have been followed by the eventual loss from the chordate lineage of the subunit types that are currently found only in invertebrates. The invertebrates in turn lost the 5HT3 receptor subunits and possibly the glycine receptors. When the nematodes split from insects, nematodes lost dm_Group I and the HisCls. Insects lost the MOD/ACC subunits, EXP-1-type GABA-gated cation channels and possibly ce_Group I. With the divergence of the *Ciona* lineage from the vertebrates, the α9 nAChR subunit, the neuronal nAChR subunits α2, 4, 5, and β3, the GABA γ/ε subunits may have been lost as well. Why chordates might undergo a Cys-loop LGIC bottleneck is a puzzle I address below.

Following these losses, specific subunit lineages have expanded. All of the invertebrate-specific lineages expanded by at least one duplication event, and quite dramatically in several cases. In contrast, the invertebrate orthologues of the vertebrate GABA subunits were restricted to a single subunit per species in the case of LCCH3/GAB-1 or one duplication event in the case of *Drosophila* Grd and CG8916. There appears to have been a modest expansion of chordate GABA receptor subunits prior to the *Ciona*/vertebrate split and a dramatic expansion in the vertebrate lineage afterward.

It is interesting to note examples of what appear to be parallel expansion of subunit subfamilies. Both *Ciona* and vertebrate ρGABA subfamilies independently expanded to at least three subunits. It appears the muscle non-α (β1, γ, δ, ε) nAChR families of *Ciona* and vertebrates expanded independently after diverging from the neuronal βnAChR subunits. Finally, the data are consistent with an expansion of the 5HT3 family of subunits to three members in *Ciona*, while two subunit genes serve in mammals. Similar evolutionary pressures may have been independently exerted on Ciona and vertebrates to cause the expansion of these particular subfamilies.

Cys-Loop LGICs Readily Assume New Functions

What we know of the function of the vertebrate and invertebrate Cys-loop LGICs indicates that there is very little functional homology. Most obviously, nAChRs mediate neurotransmission at all neuromuscular junctions in vertebrates, whereas insects use excitatory glutamate receptors (Littleton and Ganetzky 2000). In *C. elegans*, acetylcholine-gated channels formed by the subunits UNC-29, UNC-38, LEV-1, and UNC-63 mediate neuromuscular transmission of the somatic body wall muscle (Fleming et al. 1997; Richmond and Jorgensen 1999; Culetto et al. 2004). However, the subunits most similar to these are the insect, not the vertebrate muscle nAChR subunits (as predicted by Le Novere and Changeux 1995). The *C. elegans* subunits also act in neurons (Fleming et al. 1997). Mammalian α7 acts in the central nervous system. The function of ACR-19 is not known but the divergent α7-like subunit EAT-2 mediates cholinergic neuromuscular transmission in the pharyngeal muscle (McKay et al. 2004).

Vertebrate GABA receptors act in the central nervous system. The function of the invertebrate orthologues are not known but the UNC-49 GABA receptor acts in somatic muscle to mediate inhibition necessary for locomotion (Bamber et al. 1999; Richmond and Jorgensen 1999). The vertebrate glycine receptors act in inhibitory interneurons in the spinal chord, whereas the HisCls mediate neurotransmission in eye (Gengs et al. 2002) and the GluCl AVR-15 is a postsynaptic receptor in the pharyngeal muscle (Dent et al. 1997). On the other hand, there is evidence for a role for both GABA receptors and nAChRs in the *Drosophila* central nervous system (Aronstein et al. 1996; Brotz et al. 2001). Unfortunately, the apparent lack of correlation between a subunit's origin and its current role in nervous system function may mean that it will not be possible to deduce the functional properties of ancient nervous systems from the channels that were expressed.

The Origin of Cys-Loop LGIC Superfamily

If the Cys-loop LGICs diversified before the evolution of sophisticated bilaterian body plans and therefore, presumably, before the evolution of complex nervous

systems, what drove ion channel expansion and diversification? Littleton and Ganetsky (2000) hypothesized that a large Cys-loop LGIC superfamily increases the computational sophistication of small nervous systems. This could have been important in early, simple bilateria and may continue to drive the retention of a large Cys-loop LGIC superfamily in nematodes today. Conversely, the expansion of the nervous systems in chordates and insects may have obviated the need, at least temporarily, for a larger Cys-loop LGIC superfamily.

Alternatively the Cys-loop LGICs may originally have diversified in early bilateria to serve as chemoreceptors. LGIC-like proteins found in prokaryotes, which could represent primitive Cys-loop LGICs, may play a role in chemotaxis (Tasneem et al. 2004). A chemosensory role for Cys-loop LGICs has been proposed to explain their presumed origin in unicellular eukaryotes (Cockroft et al. 1992). Today, both vertebrates and invertebrates encode a large family of chemoreceptors that belong to the family of G-protein-coupled, seven transmembrane receptors, and so too, therefore, did the last common ancestor (Buck and Axel 1991; Troemel et al. 1995; Scott et al. 2001). These may be ancestors of the related class of G-protein-coupled (metabotropic) neurotransmitter receptors found in both protostomes and deuterostomes (Hille 1992; Perovic et al. 1999). However, there is evidence that at least one Cys-loop LGIC still acts as a chemoreceptor. The *C. elegans* DEG-3/DES-2 heteromeric channel responds to choline and is expressed in chemosensory neurons. Mutating DEG-3 reduces the efficiency of chemotaxis to choline (Yassin et al. 2001). Thus, the last common bilaterian (or an earlier metazoan ancestor) may have evolved a large and diverse family of both metabotropic and Cys-loop LGIC chemoreceptors. Some receptors of each superfamily were co-opted for neurotransmission, with different neurotransmitter receptors being retained in different phyla as they diverged. Eventually the Cys-loop LGICs ceased functioning as chemoreceptors in chordates but some Cys-loop LGICs may have retained that role in *C. elegans*. The Cys-loop LGICs that were retained in a given phylum may represent those that were fixed in the genome by conversion from chemoreceptor to neurotransmitter receptor.

Note Added at Proof. Schnizler et al., 2005 (J. Biol. Chem. 280(16): 16254–16262) reported that the Drosophila gene CG6112, which was omitted from our phylogeny, encodes a Cys-loop LGIC from Drosophila that is pH sensitive. It appears to define a new anion channel subunit clade.

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