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EVOLUTION OF CLAMS (CHOLINESTERASE-LIKE ADHESION MOLECULES): STRUCTURE AND FUNCTION DURING DEVELOPMENT

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1. ABSTRACT

The protein family known as CLAMS (cholinesterase-like adhesion molecules) forms a novel class of heterophilic cell adhesion proteins. Family members are found through a wide range of metazoans and play a role during the development of multiple tissues. The majority of members of this family are transmembrane proteins with an extracellular domain that is conserved with cholinesterases including acetylcholinesterase. Yet all family members lack one or more of the residues that make up the catalytic triad necessary for enzymatic function. Therefore the conserved cholinesterase-like domain is not necessary for enzymatic function but does appear to play a role in heterophilic binding. CLAMS are expressed in a wide array of tissues and most family members appear to play a role in cell adhesion and junction formation. The development of junctions including septate junctions and synaptic junctions require CLAM family members such as Gliotactin and Neuroligins respectively. Modeling of the cholinesterase-like domain reveals that evolutionary changes to the binding pocket of the cholinesterase domain may produce a range of different ligand binding partners for CLAM family members. In this vein, previous chimera experiments and recent work has identified mutations in CLAM family members that affect the structure of the cholinesterase-like domain. These mutant forms affect protein function during the development of specialized junctions and confirm the role of the cholinesterase domain in mediating heterophilic binding.

2. THE CHOLINESTERASE-LIKE ADHESION MOLECULES

Members of the cholinesterase-like adhesion molecule (CLAM) class of proteins have the ability to form heterophilic adhesive complexes between cells via their cholinesterase-like domains (CLD) (1-5). This function appears to be conserved, as family members have been isolated from a wide range of tissues and developmental

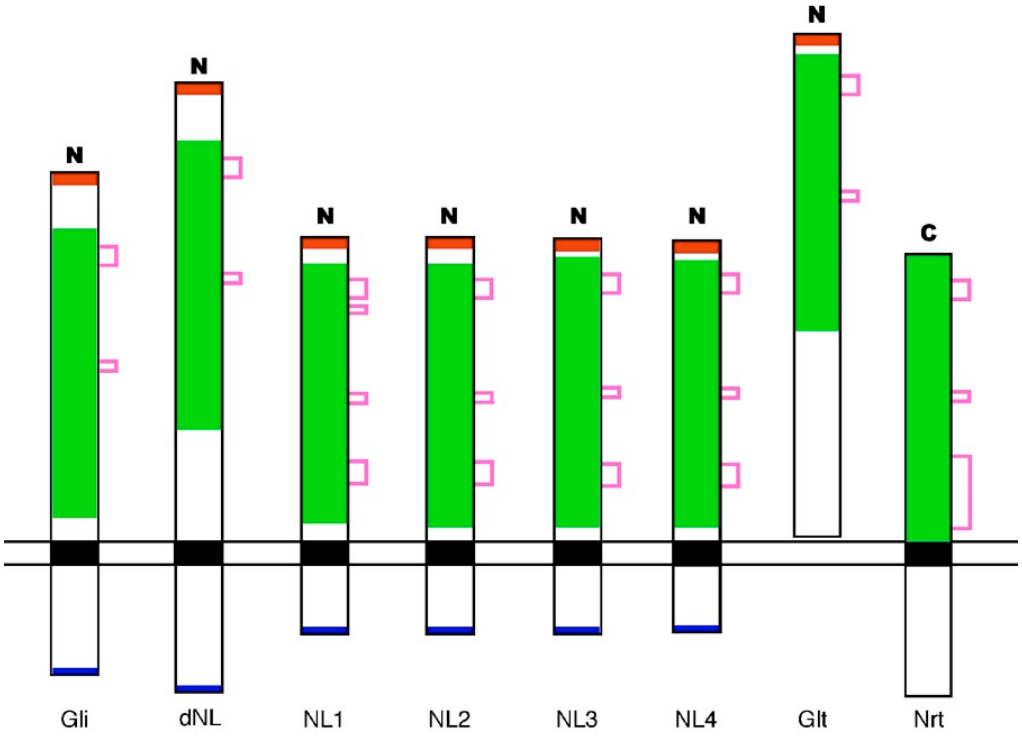

Figure 1. The general structure of the CLAM family members. The names of each protein are abbreviated below: Gli (Gliotactin), dNL (*Drosophila* Neuroligin), NL1, NL2, NL3 and NL4 (vertebrate Neuroligins 1-4), Glt (Glutactin) and Nrt (Neurotactin). Black rectangles indicate the transmembrane domain, blue indicates the PDZ-binding domain, green indicates the cholinesterase-like domain, red the signal sequence and magenta brackets indicate the relative size and position of the disulfide-bonded loops. Glutactin is the only secreted family member. Neurotactin is the only type II transmembrane protein i.e. oriented with the carboxyl portion of the protein on the extracellular face of the membrane (indicated by C, all others are N).

stages in many divergent metazoans. The following review will introduce the most characterized family members and their roles during development. In addition significant alterations or conservation in the cholinesterase-like domains with respect to the modeled structure of the domain and potential changes to ligand binding will be discussed. Finally a series of naturally occurring or induced mutations in the cholinesterase-like domain have recently been analyzed and their affect on protein function determined.

2.1. CLAM family characteristics

All members of cholinesterase like adhesion molecule (CLAM) family are comprised of an extracellular cholinesterase-like domain (CLD), which is conserved across a wide range of phylogeny (Figure 1). The cholinesterase-like domain is conserved with the carboxyl/cholinesterase enzymes, which constitute a subset of the alpha/beta-hydrolase fold superfamily of enzymes. (For additional information on this superfamily visit the website http://bioweb.ensam.inra.fr/ESTHER/general?what=index). The alpha/beta-hydrolase fold is made up of a parallel sheet of eight to ten beta-strands and forms the core structure of this domain. Even with a high degree of sequence divergence across this family of proteins, the tertiary structure of the core is conserved amongst all family members (6). While the enzymes all contain a Nucleophile-His-Acid catalytic triad they have also evolved to operate on substrates with different chemical composition and properties (7). It is the associated loops and helices interspersed among the beta-strands that provide the substrate specificity for the various enzymes and ligand specificity in the case of the CLAM family members. For instance, the cholinesterase family is characterized by a catalytic triad of Ser-His-Glu, which lies at the base of a gorge. In the cholinesterase family there is a wide range in the volume of the gorge and in the charge, which is reflected in the diversity of substrate specificities and reaction kinetics (6). The CLAM family of proteins is distinct from the cholinesterases in that one or more of the residues that form the catalytic triad are absent. Thus CLAM family members have no enzymatic function yet have maintained a high degree of conservation within the rest of the cholinesterase-like domain.

In addition to the extracellular CLD, the majority of family members are also single-pass transmembrane proteins and a subset of CLAM proteins have a highly conserved PDZ recognition peptide at the C-terminal end of the intracellular domain. Excluding the PDZ recognition peptide, there does not appear to be any significant sequence conservation in the intracellular domains of CLAM family members. Presumably, these divergent intracellular sequences have functions *in vivo* that have evolved to match the specific cellular role for each protein. In support of this recent studies have highlighted the importance of both the PDZ recognition peptide and the intracellular domain to protein function *in vivo*.

The best-characterized CLAM proteins have a clear role in cell adhesion and junction formation suggesting that the non-catalytic cholinesterase domain is responsible for mediating binding. As will be discussed in the following sections, a broad range of tissues express and utilize CLAM proteins during development and it is thought that this heterogeneity of expression reflects a wide range of potential ligand binding partners for CLAM family members.

2.2. Glutactin and Neurotactin

The first two CLAM family members to be identified, Glutactin (Glt) and Neurotactin (Nrt), are also the most divergent members of the CLAM family. Both proteins appear to be limited to insects, as no homologues have been identified outside of *Drosophila melanogaster, Apis mellifera* (honey bee) and *Anopheles gambiae* (mosquito).

Glutactin encodes an acidic sulfated glycoprotein that is secreted and localizes to basement membranes in *Drosophila* embryos (8). It is divided into two domains by a string of Threonine residues: the amino portion, which contains the cholinesterase-like domain (CLD) and the carboxy portion, which has been shown to bind Ca^{2+}. The C-terminal domain contains a high proportion of acidic residues some of which are O-sulfated. Its function is unknown though it is highly expressed in the CNS and muscle apodemes suggesting a role in cell adhesion.

Neurotactin is another CLAM that appears to be involved in cell adhesion during *Drosophila* development (9, 11). Neurotactin is a type II transmembrane protein and like Glutactin only the CLD is conserved with other CLAM family members. Over the course of embryogenesis Neurotactin is expressed in proliferating and differentiating cells. Neurotactin is first expressed in early embryogenesis at the onset of cellularization. Initially Neurotactin is first detected in Golgi peripheral membranes basal to the nuclei (12) and then is inserted in the apical plasma membrane prior to membrane invagination. As cellularization proceeds Neurotactin is found progressively in the growing lateral membrane (12). As development continues, expression becomes restricted to cells of the CNS and PNS, particularly at points of cell-to-cell contact (11, 13). Later Neurotactin is also expressed in imaginal discs and mesoderm (13).

Both gain-of-function and loss-of-function mutants in *Neurotactin* have similar mutant phenotypes including defasciculation and commissural breaks in the CNS, suggesting a role in axon guidance during embryonic and postembryonic development (14). Double mutant combinations of *Neurotactin* and mutations in other genes encoding adhesion/signaling molecules, including *Neuroglian*, *derailed*, and *kekkon-1*, enhance the CNS defects. This result provides evidence for cooperation *in vivo* between Neurotactin mediated adhesion and other adhesion/signaling pathways in axon outgrowth, fasiculation and guidance (14).

A ligand for Neurotactin was identified as Amalgam, a secreted protein of the immunoglobulin superfamily (15). Mutations in both *Amalgam* and *Neurotactin* were shown to act as modifiers of Abelson tyrosine kinase (*Abl*) mutants resulting in disrupted axon pathfinding. These results suggest that Amalgam/Neurotactin mediated adhesion may play a role in triggering Abl during growth cone guidance (16).

2.3. Gliotactin

Another invertebrate CLAM whose role in development has been extensively studied in *Drosophila* is Gliotactin (17, 19). Gliotactin is a single pass transmembrane protein that is expressed in a wide range of epithelial-like tissues including glia, trachea, epidermis, hindgut, and the eye, wing, and leg imaginal discs. Gliotactin is localized to pleated septate junctions, which are the physiological equivalent of vertebrate tight junctions. However Gliotactin is distinct from other septate junction proteins in polarized epithelia in that it is uniquely localized to the tricellular junction, a specialized junction that meets at the convergence of septate junctions in three neighbouring cells (18). Septate junctions are basal lateral to adherens junctions and form a permeability barrier. In transmission electron micrographs, septate junctions are characterized by a ladder-like array with septa spanning the 15-20 nm space between adjacent plasma membranes (20). The highest expression of Gliotactin during embryonic development is in the peripheral glia starting at stage 13, peaking at stage 17 during the formation of the septate junctions that form the blood-nerve permeability barrier (17). *Gliotactin* mutants result in paralysis due to the break down of the blood-nerve barrier (17). *Gliotactin* mutants also have septate junction and permeability barrier defects in salivary glands, gut, and trachea (18).

Gliotactin is necessary for the correct localization of other septate junction components such as Neurexin IV and Coracle in polarized epithelial cells including epidermis and salivary glands. Conversely, the localization of Gliotactin is dependent on Neurexin IV and remains uniformly distributed around epithelial cells and extending basally in *Neurexin IV* mutant embryos (18). Furthermore, in *Gliotactin* mutants other septate junction proteins are mislocalized (18). Loss of Gliotactin function appears to retard maturation and compaction of septate junctions and result in compromised permeability barriers and embryonic lethality. In addition, Gliotactin mutant phenotypes are enhanced by mutations in other septate junction genes, including *discs-large*, *coracle*, and *Neurexin IV* (19). Like all CLAM family members Gliotactin appears to be functioning as a heterophilic adhesion molecule whose ligand is unknown at this time. Gliotactin is highly conserved in honeybee, mosquito and *C. elegans* as well as with the Neuroligins discussed below.

2.4. Neuroligins

Neuroligins are type 1 membrane proteins with an extracellular CLD, a linker domain containing an O-glycosylation cassette, a single transmembrane region, and a cytoplasmic C-terminal tail (2). Three Neuroligin genes have been identified in mice and rats while five Neuroligin genes have been identified in humans. In addition there has been at least one homologue of Neuroligin identified in

Drosophila and *C.elegans* respectively. The Neuroligins were initially identified as CNS specific binding partners for the beta-Neurexin family of transmembrane proteins. As in other CLAMS, the extracellular CLD of Neuroligins mediates the binding to the ligand. Rat Neuroligins 1, 2 and 3 in the presence of Ca^{2+} bind a beta-Neurexin isoform that lacks an insert in the G domain (1, 21). The Ca^{2+} dependency is a function of Neuroligins as Neuroligin 1 binds $^{45}Ca^{2+}$, whereas beta-Neurexin 1 does not(1). It is clear that the different forms of Neuroligins have very similar binding properties and can physically substitute for each other (21). However, Neuroligins have also been found in a wide range of tissues outside of the CNS and over a wide range of developmental times. Since beta-Neurexins are not known to be expressed outside of the CNS, this suggests that Neuroligins have non-Neurexin ligands as well.

2.4.1. Neuroligin 1

Of all family members, Neuroligin 1 appears to be specifically expressed in neurons of the CNS. Rat and mouse Neuroligin 1 for instance is concentrated in synaptic junctions in the CNS and is found associated with NMDA-R, PSD-95 and S-SCAM at the synaptic cleft and postsynaptic densities (4, 22, 25). Neuroligin 1 binds to a specific splice isoform of beta-Neurexin proteins (1, 2, 21), which are also concentrated at CNS synapses (5). Neuroligin 1 (as well as Neuroligin 2) has been demonstrated to induce presynaptic differentiation. For instance, mouse Neuroligin 1 and Neuroligin 2, when expressed heterologously in cells, are capable of inducing synaptic vesicle accumulation in pontine axons or granule cells (3, 5). Furthermore, heterologous expression of mouse Neuroligin 1 and 2 induced clustering and vesicle turnover in a depolarization-dependent manner in pontine explants (3).

The binding to beta-Neurexins is a key component of Neuroligin function during synapse formation. Purified Neuroligin 1 will cluster Neurexin on the presynaptic membrane and trigger recruitment of synaptic vesicles to the cytoplasmic domain of Neurexin (5). The heterophilic association of Neuroligin 1 and beta-Neurexins may facilitate the aggregation of synaptic vesicles through binding of the intracellular PDZ recognition peptide of beta-Neurexins with CASK (26) on the presynaptic side. Subsequently, a tripartite complex formed between CASK, Mint 1 and Velis will create the scaffold on which the synaptic vesicle fusion machinery is based (26, 28). Finally, Neurexins can couple to synaptotagmins, part of the synaptic vesicle machinery that regulate vesicle exocytosis, suggesting that this complex is a trigger for the formation of the presynaptic structure (29).

Neuroligin 1 has also been shown to play a role in the formation of inhibitory synapses. Neuroligin 1 expression in cultured neurons increased both excitatory and inhibitory presynaptic contacts and the frequency of miniature excitatory and inhibitory synaptic currents (30). This and other work has lead to the hypothesis that Neuroligins are involved in establishing initial synaptic contacts in multiple neuronal types perhaps in the recruitment of beta-Neurexins as outlined above. Other mechanisms such as Neuroligin interaction with PSD-95 may dictate the phenotype of the postsynaptic density.

All rat Neuroligin proteins have a conserved PDZ recognition peptide and have been shown to bind to the MAGUK protein PSD-95. The PSD-95 family of proteins contains three amino-terminal PDZ domains followed by an src homology 3 (SH3) domain and a guanylate kinase (GuK) domain. The first two PDZ domains of PSD-95 bind to NMDA receptor subunits and K^+ channels whereas the third PDZ domain interacts with the C-terminus of Neuroligins (24). The localization, subcellular distribution, and developmental expression of Neuroligin 1 match that of PSD-95 and NMDA receptor subunits (4). Neuroligin 1 is responsible for recruitment of PSD-95 to the postsynaptic density. Overexpression of Neuroligin 1 in cultured primary neurons blocks synaptic accumulation of PSD-95 (31). As well, a PSD-95 mutant that lacks the PDZ domain necessary for Neuroligin binding region does not affect the localization of Neuroligin 1 (31). In addition, mutant Neuroligin 1 that lacks the PDZ recognition peptide is correctly localized in primary neurons (32). Therefore the localization of Neuroligin in postsynaptic densities determines the localization of PSD-95 but not vice-versa. If not PSD-95, then what is necessary for the proper localization of Neuroligin 1? Recent work suggests that S-SCAM is responsible. S-SCAM is another scaffolding protein with PDZ domains found at the postsynaptic density in neurons (33). S-SCAM appears to be one of the first proteins to be localized to the synapses through an interaction with beta-catenin. Neuroligin 1 binds to the WW domain of S-SCAM and over-expression of Neuroligin 1 binding region of S-SCAM blocks the synaptic localization of Neuroligin in cultured neurons (31). S-SCAM, PSD-95, and Neuroligin 1 form a complex *in vivo*, which leads to the possibility that Neuroligin 1 binds to S-SCAM, which in turn recruits PSD-95 (31).

Beyond recruiting PSD-95 to the postsynaptic membrane, it appears that the ratio of PSD-95 and Neuroligin 1 is also an important factor in determining the phenotype of the postsynaptic membrane. Changing the ratio of Neuroligin 1 with respect to PSD-95 can determine the ratio of excitatory-to-inhibitory synaptic contacts. Overexpression of PSD-95 alone in cultured neurons enhances excitatory synapse size and miniature current frequency while reducing the number of inhibitory synapses. Furthermore, overexpression of Neuroligin 1 alone increased both the number of both excitatory and inhibitory synapses and miniature current frequencies. In contrast, coexpression of PSD-95 and Neuroligin 1 in cultured neurons induces maturation of excitatory presynaptic and postsynaptic elements and negates Neuroligin 1 enhancement of inhibitory synapses (30). These properties of Neuroligin 1 suggest a model that allows Neuroligin to nucleate the assembly of postsynaptic components and, through its interactions with beta-neurexin, pre-synaptic components at the proper subcellular location.

2.4.2. Neuroligin 2

Neuroligin 2 has also been found to localize

to rat CNS synapses but appears to be localized to inhibitory synapses *in vivo*. In immature neurons, Neuroligin 2 is also associated with aggregates of the GABA-A receptor (34), which suggests a role of Neuroligin 2 in the formation of inhibitory synapses. Much less is known about Neuroligin 2 expression beyond the CNS. However ESTs from both mice and humans corresponding to Neuroligin 2 have been found expressed in a much broader range of tissues compared to other Neuroligins including pancreas, lung, endothelia, uterus, and colon (Unigene set at NCBI).

2.4.3. Neuroligin 3

Neuroligin 3 in mice and rats is expressed in a wide range of glia during CNS and PNS development including immature astrocytes, Schwann cells, satellite glia and olfactory ensheathing glia (35). Furthermore, Neuroligin 3 is the only Neuroligin expressed in the olfactory ensheathing glia whereas Schwann cells expressed both Neuroligin 2 and 3 (35). Human Neuroligin 3 appears to have three isoforms that are expressed differentially in skeletal and heart muscle, brain and pancreas (36).

2.4.4. Neuroligin 4/5

Human Neuroligin 4 is expressed in heart at relatively high levels, and at lower levels in skeletal muscle, pancreas and liver, and at very low levels in brain, placenta, lung and kidney (37). Another gene very closely related to human Neuroligin 4 has been placed on the Y chromosome by the human genome project. This gene has been dubbed NL4y or Neuroligin 5 (37, 38). While NL4y differs by only 19 amino acids from NL4, it has diverged significantly in sequence within its introns (between 70-80% identity (37)) suggesting that this is a gene duplication event, which has undergone divergence of function.

2.4.5. *in vivo* function of Neuroligins

While Neuroligin function has been tested *in vitro* in primary cultured neurons, the roles of Neuroligins *in vivo* have not been examined. Knock out mutants in the mouse Neuroligin 1, 2 and 3 genes have been generated and in each case found to be viable and fertile (4). Given the similarity of the different Neuroligins and their ability to physically substitute for each other, the lack of phenotypes in the knock out mice may reflect the redundancy of Neuroligin function. However, it is clear that the Neuroligins are expressed in a variety of tissues and have functions beyond synaptogenesis in the CNS. Therefore, it is possible that while the animals are viable and fertile they may have less detrimental, but significant behavioral and structural defects that have yet to be characterized. As will be discussed later, this is a strong possibility given the discovery of a series of mutations in human Neuroligin 3 and 4 that have been found associated with a small subset of autism and related syndromes.

3. CONSERVATION AND EVOLUTION OF THE CHOLINESTERASE PROTEIN DOMAINS

Phylogenetic analysis has been used to evaluate the relationship between CLAM family members. The purpose was to determine which regions might be important for CLAM function and thus more likely to be conserved in all family members. In addition, phylogenetic analysis is able to highlight those regions within orthologous CLAMs that are conserved for functions unique to that CLAM type. Previously the relationship between the closest family members Neuroligins and Gliotactins from human, rat, mouse, *D. melanogaster* and *C. elegans* were determined (35). Since then, several new potential members of the CLAM family have been identified, most notably in *Fugu rubripes*, and the phylogeny of the CLAM family extended. Sequences were initially aligned using Clustal X (39) and refined manually in conjunction with secondary structural predictions to optimize the alignment (Figure 2). The alignment and subsequent modeling of CLD structure revealed several interesting features with regards to CLD conservation and divergence.

3.1. Conservation of primary sequence

The primary hallmark of the group has been defined as an extracellular domain with strong sequence similarity to acetylcholinesterase (AChE) but lacking the essential serine residue necessary for enzymatic function. However, this serine is only one of three residues that are essential for enzymatic function in cholinesterases, the other two being a glutamic acid and a histidine in close proximity to the third disulfide-bonded loop (7) (Figure 2). In all family members, with the exception of Glutactin and *C.elegans* Neuroligin, the glutamic acid residue is conserved whereas the histidine residue is conserved in twelve of the eighteen sequences. Notably, the histidine is changed to methionine in Neurotactin, the one CLAM family member that has retained the serine residue. Thus loss of function of the catalytic triad is a defining hallmark of this group rather than simply the absence of the serine.

A second hallmark of the CLAMs family is the conservation of two of the three disulfide-bonded loops in acetylcholinesterases (Figure 2) (40, 41). All vertebrate forms of Neuroligin 1 are unusual in that they contain two additional cysteines in alternatively spliced domain A, which form an additional disulfide loop (42) for which there is no known function (Figure 2**)**. The third pair of cysteines is shifted and significantly shorter in the vertebrate Neuroligins compared to functional serine esterases, and is completely absent in the invertebrate Neuroligins, Gliotactins and Glutactin. Only Neurotactin retains this third pair of cysteines in the same region as AChE. The C-terminal third of the CLD domain is the most divergent in sequence, both between family members and in comparison with acetylcholinesterases. For instance, the *Drosophila* Neuroligin contains an 80 amino acid sequence not shared with *C. elegans* Neuroligin or any other member. Likewise, the vertebrate Neuroligins contain a twelve amino acid sequence not shared with the invertebrate family members. Furthermore, Glutactin and Neurotactin share very few amino acids with the other CLAMs in this domain. The third domain is thus most likely to be the site of specialization for each of the CLAM family members for their unique ligands.

Unlike AChE, CLAM family members lack the unpaired cysteine residue following the CLD through

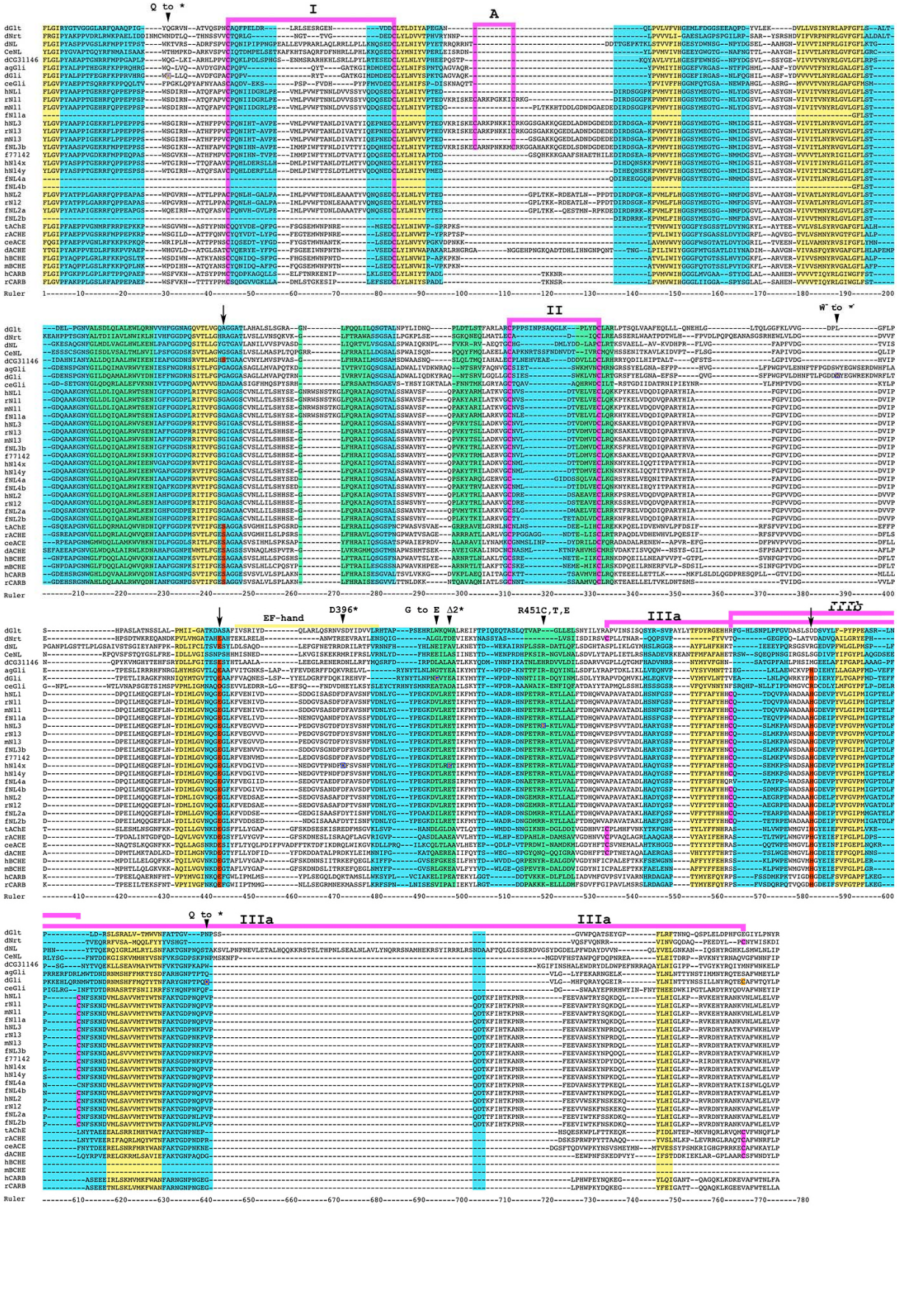
Q to *
I
A
II
W to *
EF-hand
D396*
G to E
Δ2*
R451C,T,E
IIIa
IIIb
Q to *
IIIa
IIIa

Figure 2. Alignment of CLD of CLAM family members and active cholinesterases. The alignment of CLAM family members and selected members of the functional cholinesterases across the cholinesterase-like domain. Conserved secondary structures are highlighted (random coils, blue; alpha-helices, green; beta-sheets, yellow). Additional key features shown include: disulfide-bonded loops (magenta), catalytic triad amino acids (red and arrows), potential EF-hand (horizontal yellow bar), characterized mutations (arrowheads and blue boxes), and an unpaired cysteine in dGli (orange box). The alignment was initially generated using Clustal X and then optimized manually. Conserved secondary structures were identified using the PELE suite of algorithms available on Biology Workbench 3.2, San Diego Supercomputer Center, UCSD. Organismal abbreviations are *Drosophila melanogaster* (d), *Anopheles gambiae* (Ag), *Caenorhabditis elegans* (Ce), *Homo sapiens* (h), *Rattus norvegicus* (r), *Mus musculus* (m), *Fugu rubripes* (f), *Torpedo californicus* (t). The accession numbers for each of the proteins shown are as follows: dGlt (462182), dNrt (128570), dNL (NM_078772), CeNL (CAA94208), dCG31146 (NP_731172), agGli (EAA12448), dGli (L39083), CeGli (U40948), hNL1 (AI056353), rNL1 (U22952), mNL1 (NM_138666), fNL1a (SINFRUP00000068675), hNL3 (NP061850), rNL3 (U41663), mNL3 (NM_172932),fNL3b (SINFRUP00000087769), f77142 (SINFRUP00000077142), hNL4x (AF376803), hNL4y (NM_014893), fNL4a (SINFRUP00000081112), fNL4b (SINFRUP00000085457), hNL2 (NM_020795), rNL2 (U41662),fNL2a (SINFRUP00000078547), fNL2b (SINFRUP00000076572), tACHE (X03439), rACHE (584716), CeACE (NP_510660), dACHE (P07140), hBCHE (M16541), mBCHE (NM_009738), hCARB (I61085) and rCARB (CAA55241).

which dimerization occurs in AChE (43). Yet evidence from genetic studies and biochemical studies indicate that *Drosophila* Gliotactin and mammalian Neuroligin 1 do dimerize via their extracellular domains (19, 44). The alpha helices thought to mediate AChE dimerization (45, 47) are highly conserved in all CLAM family members suggesting that CLAM family members can form dimers. Indeed as will be discussed below, dimerization has been proven both biochemically and genetically for two family members.

Two putative EF-hand metal binding domains have been identified in AChE and one in Neuroligin based on modeling algorithms (48). There is clear evidence that at least one CLAM family member, Neuroligin 1, is dependent on Ca^{2+} for binding to its ligand beta-Neurexin (1). However as the critical residues for an EF-hand region are poorly conserved (Figure 2) this suggests that if the predicted EF hand in Neuroligin is the site of Ca^{2+} binding that other CLAM members either do not bind Ca^{2+} or do so at another site in the protein which has yet to be identified.

Other regions of the CLD have gene-specific changes, which may reflect a change in function or binding specificity. For instance, the vertebrate Neuroligin 1 sequences all contain a unique nonapeptide (GNRWSNSTK) just downstream of the usual site of the catalytic site serine. *Drosophila* and mosquito, but not *C. elegans*, Gliotactins contain a unique sequence (NFTLPGDDWYEGWREKDW) just downstream of the second disulfide-bonded loop. *Drosophila* Neuroligin contains two unique insertions, one of 16 amino acids midway between the second loop and the glutamic acid of the enzymatic triad, and an 80 amino acid insert within the last third of the CLD.

3.2. Conservation of secondary and tertiary structure

Previous work on the crystal structure of *Torpedo*, *Drosophila* and mouse AChE (45, 46, 49, 50) has greatly facilitated the modeling of the CLD of CLAM family members. Given the high degree of amino acid conservation to AChE many models of the cholinesterase domain of CLAM family members have been generated (5, 10, 51).

With the inclusion of new family members from different species, the CLD domains for different CLAM members were remodeled providing new insight into regions that are structurally similar or distinct. The predicted two dimensional structures for each of the CLAM family members and several other members of the serine esterase superfamily were determined using the PELE suite of algorithms available on Biology Workbench 3.2, San Diego Supercomputer Center, UCSD (http://workbench.sdsc.edu/). The results from each algorithm were compared and the consensus structures superimposed on the sequence alignment for these proteins (Figure 2, Figure 3). The results of the two-dimensional analysis suggested that CLAM family members would be capable of forming three-dimensional structures very similar to that of acetylcholinesterase. In spite of the increased divergence of the sequences of each of the family members, approximately 65% of the CLD for all family members is predicted to share the same two-dimensional structure as the members of the cholinesterase superfamily (Figure 3). This conservation of structure is greatest in the first half of the CLD between and including the first and second disulfide-bonded loops to position 335 (Figure 2). For the second half of the CLD the most notable conservation of structure is from position 430-640 (Figure 2) in the vicinity of the cysteine residue.

The portions of the CLAM proteins sharing the most conservation of structure are those required to form the alpha beta -hydrolase fold and position the first member of the active-site triad in the gorge. The second block of conserved structure positions the remaining two members of the catalytic triad and forms a large portion of the mouth of the gorge. The loss of the third disulfide-bonded loop, or the repositioning and reduction of its size, in the CLAM proteins would significantly alter the conformation of the gorge mouth (Figure 3). Taken together with the concentration of sequence divergence among family members in this region, these results suggest that this is a likely target for the various ligands to associate with each member of the CLAM family.

The other portions where alternative exons or insertions unique to particular family members all appear to map to regions on the outer surface of the three-dimensional structure away from the gorge mouth, and do not appear to impinge on ligand binding. This is supported by the *in vitro* studies that show that all splice isoforms of

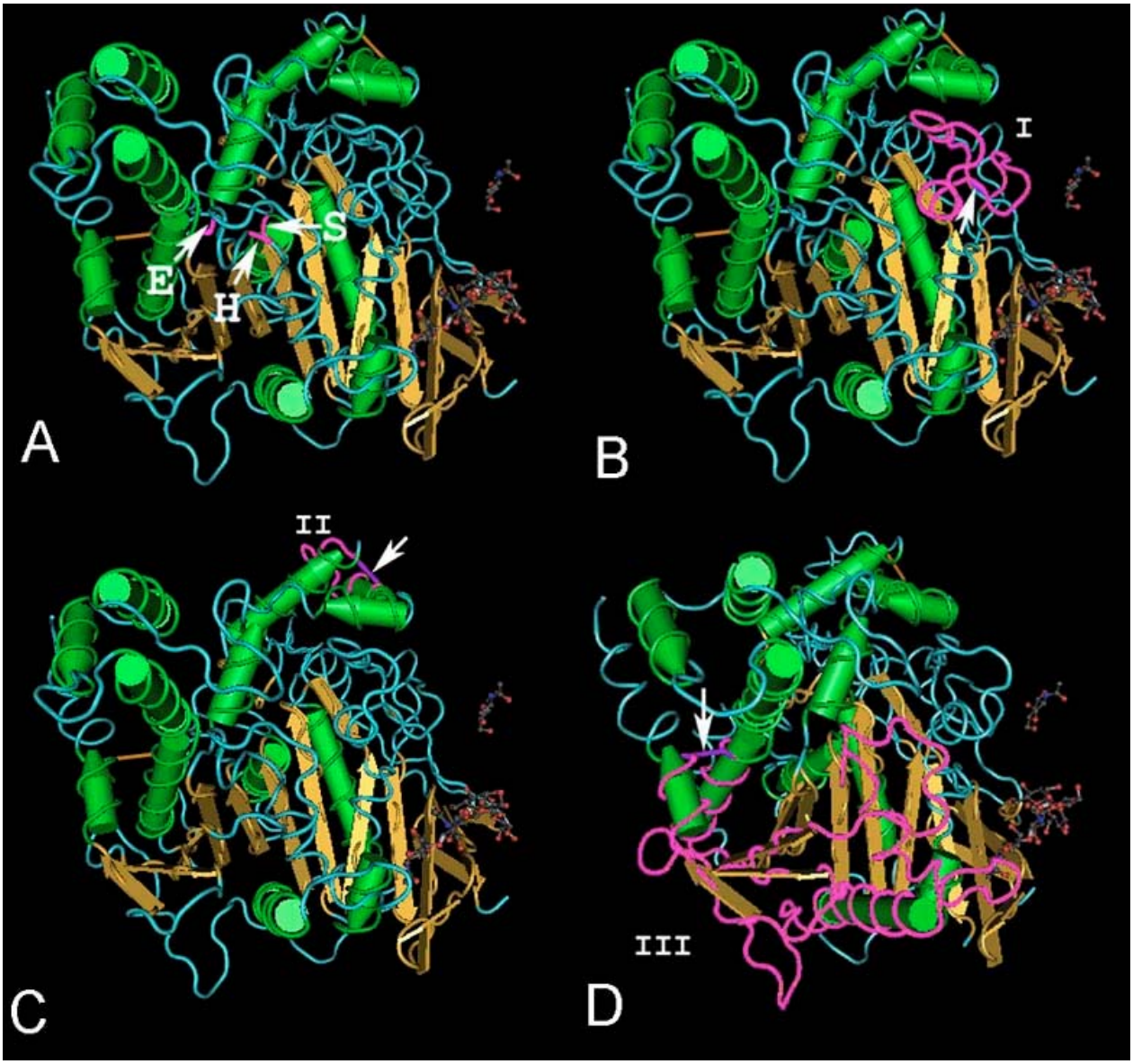

Figure 3. The CLD model for CLAM family members. Three dimensional models of *Drosophila* acetylcholinesterase showing the position of A) the catalytic triad, B) the first disulfide-bonded loop, C) the second disulfide-bonded loop and D) the third disulfide-bonded loop. In each of the CLAM family members, this last domain is most diverged in sequence. For most of the vertebrate Neuroligins (except fNL4a) this loop is shifted and diminished in size. Most invertebrate family members (except Neurotactin) have not acquired or have lost, either one or both cysteines and do not form this third loop. The structure is oriented looking down the catalytic gorge and each of the featured portions are highlighted in magenta. The amino acids of the catalytic triad further highlighted by arrows. The image was extracted from Cn3d 4.1 projection of 1Q09 from the NCBI 3D structure database and (49), and modified using Photoshop 7.0.

Neuroligin 1 are capable of inducing synaptic formation (3) suggesting that these the different isoforms have no impact on ligand interactions.

AChE is known to form tetramers by parallel association of two primary dimers with each other. While the cysteine residue necessary for dimerization in *torpedo* AChE appears to be absent in CLAM family members, the alpha helix pair in AChE responsible for dimerization (45, 46, 50) (Figure 4), is retained in Neuroligin 1 (5). Previous models of Neuroligin 1 structure have shown that this region is perfectly positioned to mediate lateral interactions with other Neuroligin molecules through a second alpha helix in the protein (5) (Figure 4). This model was confirmed by showing that mutants in the second helix also lead to a loss of Neuroligin 1 synaptogenic inducing activity. In other CLAM family members the two alpha helical regions are conserved in both the primary and

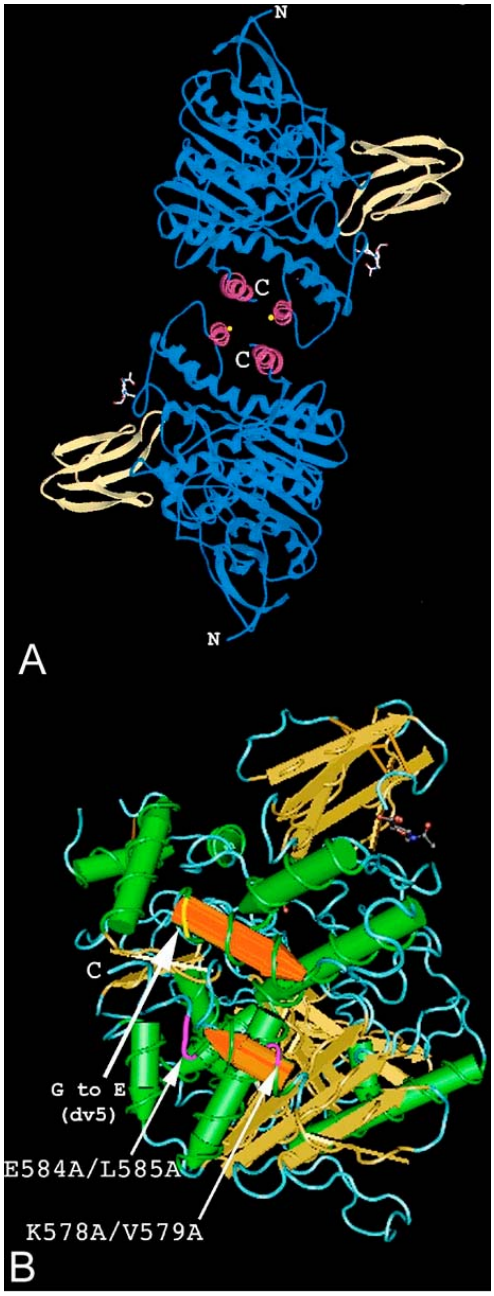

Figure 4. Dimerization domain of the CLD. The dimerization of AChE and Neuroligins occurs between two alpha helices (alpha$3_{7,8}$ and alpha$_{10}$ in mAChE). A) A top-down view of dimerized mAChE showing the alignment of the two pairs of alpha-helices (magenta) and the relative position of dv5 (yellow dot) in the first alpha helix and the Neuroligin 1 mutants in the second alpha helix (the N and C termini of the protein are marked N and C). This panel is modified from (46). B) The location of mutations in Neuroligin and Gliotactin with respect to the dimerization domain are shown. The position of the Neuroligin mutants E584A/L585A and K578A/V579A map to the last alpha helix (magenta). The position of the Gli^{dv5} (G to E hypomorphic mutation; yellow and arrow) maps to the outer face of one of two alpha-helices (in red) necessary for dimerization of mouse acetylcholinesterase (PDB# 1KU6).

secondary structures (Figure 2, 4), which suggests that dimerization could be a common property of CLAM proteins.

AChE proteins also contain a region known as the peripheral anionic site (PAS), which is known play a role in regulation of AChE-catalyzed hydrolysis and contain binding sites for allosteric activators and inhibitors. The PAS is composed of largely aromatic residues clustered around the rim of the active site gorge. The peripheral site has also been proposed to mediate non-catalytic functions of AChE such as heterophilic binding. Examples of non-catalytic functions of AChE include: neurotrophic support (52); neurite outgrowth and synaptogenesis (53, 57); binding to basement membrane components such as laminin and collagen (58); the nucleation of amyloid peptides during the onset of Alzheimer's disease (59, 60). This adhesive role is non-enzymatic, as AChE active site inhibitors do not affect these proposed functions (53, 54, 61) and is localized to the peripheral anionic site (PAS) (52, 62). Structural and chemical data suggests that residues W84 and F330 belong to the anionic subsite located in the active site gorge and W279, Y121, and Y70 to the peripheral anionic site located at the entrance of the gorge (45, 63). While these sites and the PAS regions in general are highly conserved in AChE proteins they are not conserved with other CLAM family members. For instance the equivalent residue to W279 in *torpedo* AChE (position 345 Figure 2) or other residues known to interact with AChE inhibitors in this region (50) are poorly conserved in CLAM family members. However previous models of CLAM family proteins have shown that these proteins share an "annular" electrostatic motif of negative potential around the "active-site" gorge encompassing the equivalent to the PAS region in AChE (51). Even in the absence of conserved residues three diverse CLAM family members Neurotactin, Gliotactin and Neuroligin share this motif suggesting that this domain may facilitate ligand regulation prior to the generation of a more specific and higher affinity ligand pairing.

3.3. Evolutionary relationship between CLAMs

A question remains regarding the evolution of the CLAM family members. What is the relationship between CLAMs and AChE or other cholinesterases? This is significant because of the body of research that suggests acetylcholinesterases have adhesive properties in addition to their enzymatic role indicating a potential conservation of an adhesion rather than enzymatic function (55, 57).

The evolutionary tree generated from the alignment of CLAM CLD domains (Figure 5) was accomplished using the heuristic maximum parsimony algorithm with bootstrapping from PAUP 4beta (64). We have used Glutactin and Neurotactin as outgroups to reveal the evolutionary divergence of the CLAMs from the functional serine esterases, particularly acetylcholinesterases. Glutactin and Neurotactin are distinct from the Neuroligins and Gliotactin in both sequence similarity and structural organization (35). The resulting evolutionary tree suggests that Gliotactins (*C. elegans*, *Drosophila* and mosquito) are more closely related to the vertebrate Neuroligins than the invertebrate Neuroligins,

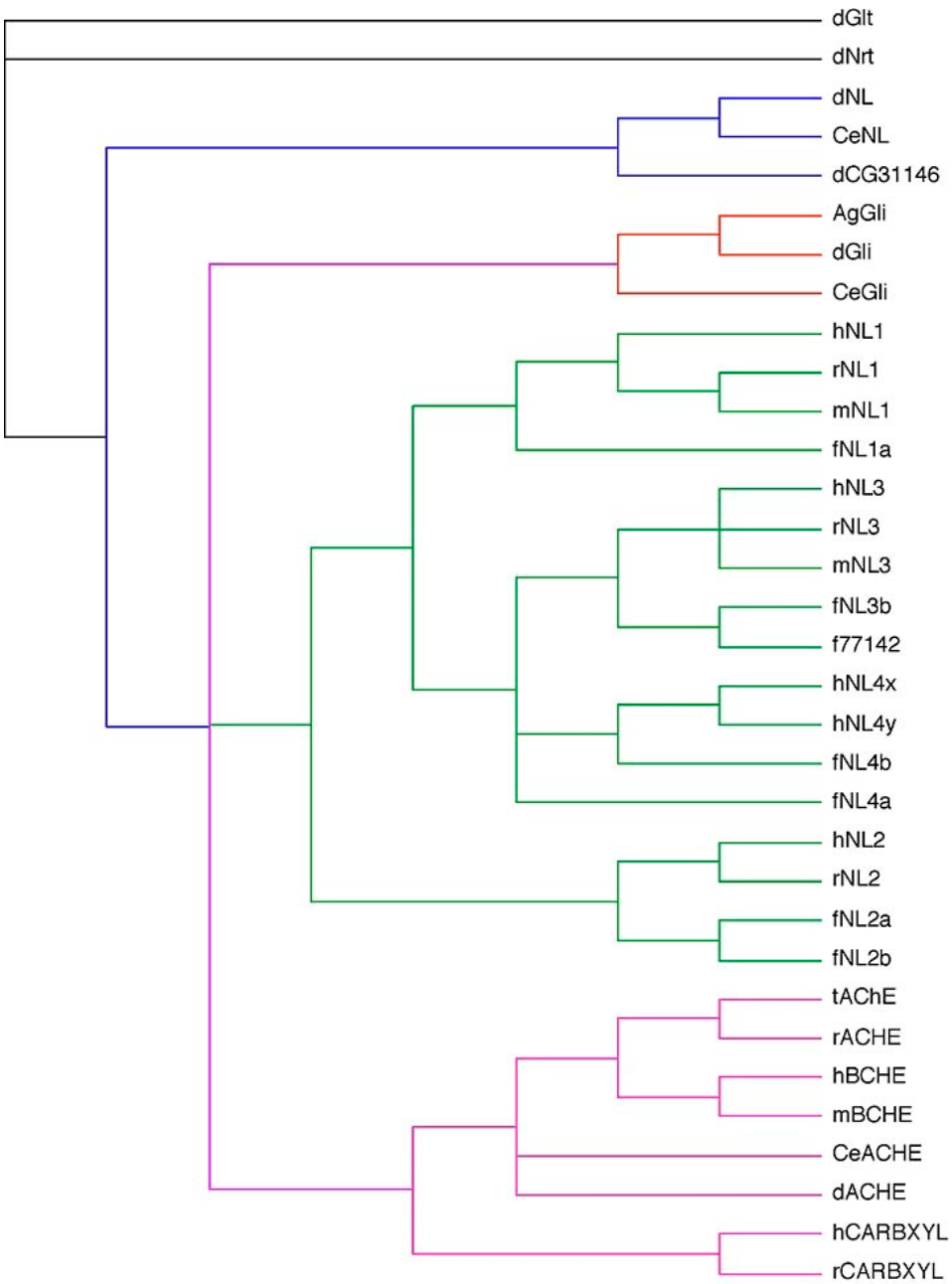

Figure 5. The evolutionary analysis of 33 cholinesterase-like proteins. Generation of the evolutionary tree of relationships was obtained using a heuristic maximum parsimony algorithm with bootstrapping from PAUP 4.0beta 10 (64). Maximum parsimony was used in order to obtain a tree with the fewest number of steps to explain the amino acid differences in the alignment. The heuristic algorithm, tree bisection-reconnection, was used to speed the analysis instead of exhaustively building and evaluating every single configuration of the data set. Bootstrapping gives a measure of confidence that the tree generated is accurate. Glutactin and Neurotactin (black lines) were used as outgroups to reveal the evolutionary divergence of the CLAMs {{the invertebrate Neuroligins (blue), the vertebrate Neuroligin variants (green) and Gliotactins (red)}} from the functional cholinesterases (magenta).

Glutactin or Neurotactin and that the functional serine esterases belong to distinct branches (Figure 5). The invertebrate Neuroligins in this analysis appear to be ancestral to the other CLAM members. The *Fugu* Neuroligins indicate that the gene duplication events that gave rise to the four vertebrate Neuroligins occurred relatively early in the evolution of vertebrates, before the branches for bony fish and mammals separated but after the branches for chordates, nematodes and arthropods diverged. Similarly, the Gliotactins arose before the nematodes and arthropods diverged. What cannot be determined at this time is the relationship between the Gliotactins and the vertebrate Neuroligins as there is no intermediate organism, such as a mollusk or echinoderm,

that has been demonstrated to possess CLAM proteins that are closely related to both branches. Consequently, developmental and subcellular expression and functional studies will have to be employed to resolve this issue.

From our analysis it appears that the vertebrate Neuroligins diverged from the invertebrate Gliotactins and Neuroligins at the same point that the cholinesterases and carboxylesterases diverged. We do not think this is the case given the existence of cholinesterases and carboxylesterases in more ancestral species such as bacteria, yeast and *Dictyostelium* (6). In addition our results with respect to the relationship between Glutactin/Neurotactin and rat Neuroligin-1 are validated by earlier phylogenetic analyses that investigated the evolutionary relationship of the carboxyl/cholinesterase family (6). Instead our results appear to be a case of long branch attraction, a phenomenon that commonly arises when there is mutational saturation and unequal rates of evolution between homologous sequences (65). This can be overcome by choosing outgroup molecules that are more closely related to those being studied, and from sister groups at the base of the branch being examined. Unfortunately, the CLAM family is still quite small which makes choosing a molecule more closely related to the Neuroligins and Gliotactins than Neurotactin and Glutactin impossible at this time.

4. MUTATIONAL ANALYSIS OF THE CLD STRUCTURE AND FUNCTION

It is clear that the cholinesterase-like domain has been conserved to maintain a function in heterophillic binding. The modeling of the CLD suggests that specific regions of the domain may play a role in mediating dimerization or ligand binding. A series of mutants in CLAM family members has begun to address the functional importance of different subdomains in the CLD.

4.1. Model organism studies

4.1.1.CLD domain studies

Previous chimera studies using domain swapping highlighted the importance of the CLD for ligand interactions. For instance, chimera experiments between Glutactin and Neurotactin were carried out to determine the site of ligand binding. Fusions created between *Drosophila* or *Torpedo* acetylcholinesterase (AChE) and the CLD domain of Glutactin to the Neurotactin cytoplasmic domain were able to mediate aggregation of cells. Using truncated forms of Neurotactin it was determined that the binding site for Amalgam (its ligand) was localized within the CLD in a region that encompasses the C-terminal third of the CLD (His347–His482 domain) (15).

In a similar vein, chimeras between Neuroligin 1 and AChE were used to identify the region necessary to induce synaptic vesicle clustering in pontine neurons. NL1-AChE chimeric proteins containing either the C-terminal third of the acetylcholinesterase domain or the entire acetylcholinesterase domain were unable to induce vesicle clustering. This suggests that the vesicle-clustering activity of Neuroligin 1 is located in the last third of the CLD (3). Furthermore, this region was shown to mediate binding to the ligand beta-Neurexin 1 (3). Therefore the last region of the CLD, which corresponds to the most divergent region between CLAM family members, mediates ligand-binding interactions.

In an additional study of Neuroligin-AChE chimeras, a small region at the carboxy-terminal of the CLD was found to be essential for the synaptic vesicle recruitment function of Neuroligin but not for Neurexin binding. This region was subsequently modeled to be a potential dimerization domain. By testing mutants with specific amino acid changes in this region, it was shown that this region is necessary for Neuroligin dimerization and function (Figure 4)(5).

A similar experimental approach was used to study Gliotactin function *in vivo*. A series of hypomorphic mutations were generated by chemical mutagenesis in Gliotactin and the effects on protein localization and function assessed (19). Gli^{dv5} as a homozygote is a weak mutation and a small percentage of flies survive to adulthood. The mutated protein is correctly localized to the tricellular junction but at much reduced levels compared to wild type suggesting a defect in protein processing. However, the Gli^{dv5} mutation can almost be completely corrected when placed in trans to a second embryonic lethal mutation, Gli^{RAR77}, a nonsense mutation that changes Ser820 to a stop in the intracellular domain of Gliotactin. Given that both mutant proteins have one normal domain, either intracellular or extracellular, they are capable of forming a dimer with partial function on each face of the cell membrane. Both the Gli^{dv5} allele and the Neuroligin 1 mutants that block dimerization map to the alpha helices known to mediate AChE protein dimerization (Figure 4). Another Gliotactin mutant, Gli^{cq1} is a Gln to a stop resulting in a truncation just prior to the C-terminal end of the CLD (Figure 2, 5). Gli^{cq1} cannot be rescued *in trans* with Gli^{dv5}, perhaps because it truncates Gliotactin monomers prior to the last alpha helix associated with dimerization and thus lacks that ability to from functional dimers.

A second example of complementation between different alleles of Gliotactin occurs between Gli^{dv5} and Gli^{dv1}, a nonsense mutation that changes Trp454 to a stop truncating the protein between the second disulfide loop and the predicted EF-hand domain (Figure 2, Figure 6). This result suggests that the increased survival of Gli^{dv1} mutants occurs presumably through the ability of Gli^{dv1} to form a complex with Gli^{dv5} via the first half of the CLD including the conserved first and second disulfide loops prior to targeting to the membrane. In addition these results also point to the possibility that the multimerization of CLAM family proteins extends beyond the known alpha-helix interactions to include other regions of the protein. This is supported by work on tetrameric forms of mAChE where flanking alpha-helices from one half of an AChE dimer can make tight associations with the peripheral site region (PAS) of the gorge entrance of the facing subunit of the second dimer (47).

4.1.2 Intracellular domain studies

While the intracellular domains of CLAM family members are quite divergent, mutant analysis has indicated

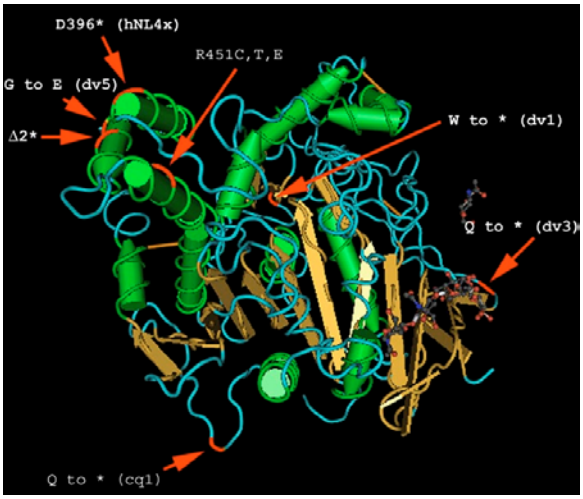

Figure 6. Position of CLAM mutations in the CLD structure. The position and type of mutations characterized in the CLAMs were mapped to the three-dimensional structure of *Drosophila* acetylcholinesterase. The orientation is looking down into the active site gorge. Gliotactin: Three of the four *Drosophila* Gliotactin mutations result in truncated proteins (cq1, dv1 and dv3) whereas the fourth (dv5) changes a glycine to glutamic acid in an alpha-helix adjacent to the predicted EF-hand domain. Neuroligin: Similarly, a two base pair deletion that results in a truncated protein in hNL4x (delta2*) is three amino acids away from the Gli^{dv5} mutation and like it, accumulates in the endoplasmic reticulum with very little protein reaching the plasma membrane. Another mutation in hNL4x that is proximal to dv5 and delta2* within the predicted EF-hand domain (D396*) also accumulates in the endoplasmic reticulum and like delta2*, is associated with mental retardation and autism-spectrum disorders. The R451C,T,E mutations originally identified in hNL3 have been found to have a greatly reduced ability to induce synapse maturation of cultured neurons, accumulate in the endoplasmic reticulum and R451C has been associated with autism-spectrum disorders.

that the intracellular domain of the Gliotactin/Neuroligin family members are necessary for protein function. In addition these family members all contain a PDZ recognition peptide that was initially hypothesized to be important for protein function. The surprising result from a number of recent studies is that the PDZ recognition peptide is not necessary for protein localization to the correct subcellular domain. For instance removal of the PDZ recognition peptide from Neuroligin 1 does not affect targeting to the postsynaptic density in primary cultured neurons (31, 32). In addition mutant forms of Gliotactin that lack this same conserved region when expressed in Drosophila are correctly localized to the tricellular septate junction region and are able to rescue null Gliotactin mutants (J. Schulte and V. Auld; unpublished results). However it is clear that other regions in the intracellular domain are necessary for protein localization and function. For instance, a truncation mutation in Gliotactin that removes two thirds of the intracellular domain is a strong loss-of-function mutation resulting in the embryos dying at the same stage as Gliotactin null alleles (19). Similarly, truncation mutants of Neuroligin 1 that lack the last two thirds of the intracellular domain are not correctly localized (31). When further investigated it appears that the middle of the cytoplasmic domain of Neuroligin 1 binds to the WW domain of S-SCAM and this association maybe the key to correct localization of Neuroligin 1 in cultured neurons (31). Similar results from another study show that a truncation mutant of Neuroligin 1 that lacks the entire intracellular domain is not correctly localized to the postsynaptic membrane but that a mutant with the first 30 residues of the intracellular domain is correctly localized (32). Of interest is that the intracellular domains of these two independent studies on Neuroligin 1 do not overlap suggesting that there maybe multiple interactions that mediate the correct localization of Neuroligins.

4.2 Inherited human mutations and disease

A series of mutations associated with forms of autism and Asperger syndrome have also pinpointed important regions in the CLD of human Neuroligins 3 and 4 (36-38). A mutation in human Neuroligin 3, in which Arg451 is changed to a Cys (R451C: Figure 2, 5), was found in autistic or Aspberger syndrome-affected members of a Swedish family (38). However subsequent mutation analysis indicated that it was the loss of an Arg at this site, not the change to a Cys, that caused the functional deficit (66). The mutant Neuroligin 3 protein is largely retained in the endoplasmic reticulum when expressed in COS cells suggesting a problem in protein folding. Furthermore, what little protein gets to the cell surface has a decreased ability to promote synaptic vesicle clustering in contacting hippocampal axons in co-culture in contrast to wild-type protein (66). These results show that Neuroligin 3, like Neuroligin 1 and 2, can trigger synaptic formation *in vitro* and thus may be involved in neural or synaptic development *in vivo*. However given the broad expression of Neuroligin 3 in developing glia (35), it is also possible that these mutations point to an as yet unknown function of glia in autism and related syndromes.

A novel insertion in human Neuroligin 4 resulting in a frame-shift and premature termination of the transcript at Asp396 (D396*: Figure 2, 5) was identified in one Swedish family with two affected brothers, one with typical autism and the other with Asperger syndrome (38). This mutation terminates the gene product upstream of the two members of the catalytic triad proximal to the C-terminal third of the CLD (Figure 5). This would generate a protein only capable of forming the alpha beta-hydrolase fold but lacking a proper gorge, ligand binding domain, dimerization domain and transmembrane domain. When expressed in COS cells the D396X mutant protein was either secreted or concentrated in the endoplasmic reticulum (66). Furthermore, COS cells expressing the D396X mutant protein were unable to promote synaptic vesicle clustering in contacting axons in a co-culture assay with rat hippocampal neurons compared to wild-type Neuroligin 4 (66).

A second deletion mutation resulting in a premature termination of the protein was found in an

extended family with members affected by X-linked mental retardation with or without autism (67). This mutation is 22 amino acids downstream of D396 (Delta2*: Figure 2) and would also result in a protein solely encoding an alpha beta-hydrolase fold (Figure 5) and that is most likely secreted.

As expected given the broad diversity of autism and related disorders, changes in Neuroligin proteins represent only one aspect of this disease. Recent studies have shown that mutations in Neuroligin 3 and 4 occur infrequently in autism and represent only a small fraction of autism cases (68, 69). Regardless, these results are informative about Neuroligin function for a number of reasons. First these results suggest that the Y chromosome associated Neuroligin 4y/Neuroligin 5 cannot functionally replace the X chromosome associated Neuroligin 3 or 4 proteins in males. Additionally these results point to a role for Neuroligins in nervous system function and suggest that behavioral tests will be needed to test for Neuroligin function in mouse knock out models of Neuroligins 1, 2 and 3.

5. PERSPECTIVE

Evolutionary and structural analyses of the cholinesterase-like domain of CLAM proteins has given us insights into the primary, secondary and tertiary elements that are crucial to the common and unique functions of this protein family. Evolutionary analysis indicates that while closely related to acetylcholinesterases and butylcholinesterases, the CLAMs form a distinct family. The CLAM family arose earlier than the insects, nematodes and vertebrates diverged and it is very likely that additional CLAM family members will also be found in sponges, coral and echinoderms. This analysis further indicates that from invertebrates to vertebrates the cholinesterase-like domain is the most highly conserved portion of CLAM proteins. In particular the portions of the CLD that are required to form the alpha-beta hydrolase fold in the first half of the CLD, the portions forming the gorge and the two alpha helices for dimerization are most conserved. While the tertiary structure of the gorge has been conserved, one or more members of the catalytic triad have been inactivated by mutation, indicating that the structure is required for recognition of the various ligands but not for enzymatic function. The sequences downstream of the last member of the inactivated catalytic triad which in AChE forms the third disulphide loop have diverged significantly across species and between paralogs within a species. The vertebrate Neuroligins have demonstrated ability to bind beta-neurexins lacking an insertion in the G-domain. In contrast, Neurotactin has very little conservation of sequence in the C-terminal third of the CLD and has the ability to bind Amalgam, a secreted protein of the immunoglobulin superfamily. Our analyses indicate that the diversity seen in this region reflects the broad range of ligands that bind to CLAM family members.

This type of structural analysis of the CLD suggests a model tertiary structure very similar to acetylcholinesterases in ligand binding and dimerization. This model is supported by genetic analysis of Gliotactin and *in vitro* studies of spontaneous mutations in human Neuroligins 3 and 4, as well as biochemical studies of vertebrate Neuroligins. These mutations all appear to affect nervous system and epithelia derived tissues during development. However, based on tissue distribution analyses, the CLAM members may be of more wide spread importance in development perhaps mediating the formation of junctions in many tissues throughout development in addition to axon guidance, development of synapses, and blood-brain barrier and epithelial sheet formation. Correlations of the positions of various mutations on the tertiary structure with the phenotypic outcomes in both *in vivo* and *in vitro* systems will now provide a context for future functional analyses of cell-to-cell interactions during development. Furthermore, extension of the structural and mutational analyses to the intracellular domains of the membrane-bound CLAMs will be essential to understanding the interactions with cytoskeletal structures and signaling pathways and how these interactions change within a cell during development.

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