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THE L1CAM EXTRACELLULAR REGION: A MULTI-DOMAIN PROTEIN WITH MODULAR AND COOPERATIVE BINDING MODES

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1. ABSTRACT

L1CAM is a neural cell adhesion molecule (CAM) that is critical for proper CNS development in humans. It mediates a myriad of activities important to CNS maturation, including neurite outgrowth, adhesion, fasciculation, migration, myelination and axon guidance. L1CAM promotes these cellular activities by interacting with a diverse group of CAMs, extracellular matrix molecules and signaling receptors through interactions involving its extracellular region. This region is composed of 11 tandem immunoglobulin-like (Ig) domains. This review focuses on the L1CAM extracellular region, and how recent work has clarified important aspects of its structure and function. These studies have provided new insights into how L1CAM binds to several different extracellular molecules, how these binding activities are regulated, and how L1CAM initially folds. Furthermore, these studies suggest that the extracellular region is a dynamic, integrated structure that depends on cooperative interactions among its Ig-like domains for proper functioning.

2. INTRODUCTION

Since its discovery over 20 years ago, L1CAM has been studied intensively in attempts to understand how the nervous system develops. It is a cell adhesion molecule (CAM) belonging to the immunoglobulin (Ig) superfamily that plays several critical roles during CNS development. For example, mutations in human L1CAM, which is X-linked, leads to highly variable neurological diseases in hemizygous male children including mental retardation, gross motor defects, hydrocephalus and early mortality (1-3). L1CAM is also the prototype member of a subgroup of neural IgCAMs that includes Nr-CAM, neurofascin, and CHL1 (4-6).

In the developing CNS, L1CAM is expressed on the surface of neurons and is concentrated in growth cones and axons (7-9). It contributes to neural development by binding to a diverse set of molecules on neighboring neurons, glial cells and the extracellular matrix. This binding allows L1CAM to cluster multiple binding partners on opposing cell surfaces via its extracellular region and, at the same time, organize cytoskeletal and signaling proteins via its cytoplasmic region. L1CAM does not accomplish this alone, but rather is thought to recruit other CAMs and signaling receptors at the neuronal membrane to form a multiprotein complex (10). The end result is promotion of cellular activities important to CNS development. These activities include neuron-neuron and neuron-glial adhesion (11-13), axonal fasciculation (14, 15), neurite outgrowth (16, 17), migration (7, 18), axon guidance (19, 20), neuronal survival (21), myelination (22, 23), and memory consolidation (24, 25).

Precisely which cellular activities that L1CAM will promote in any given context cannot yet be predicted. However, it is presumed that the cellular response to L1CAM depends in part on the extracellular molecules with which it interacts. Therefore, to better understand the role of L1CAM in CNS development, we must understand how the extracellular region functions. This review focuses on recent studies that have shed light on the structure and molecular interactions of the extracellular region, and how multiple domains within this region cooperate to drive homophilic and heterophilic binding. Binding interactions of the cytoplasmic region have been reviewed elsewhere (10, 26).

3. THE L1CAM EXTRACELLULAR REGION: AN OVERVIEW

The extracellular region of L1CAM is comprised of a modular array of 11 tandem immunoglobulin-like folds (Figure 1A). It is therefore useful to review the basic structure of this fold when discussing L1CAM. The Ig fold

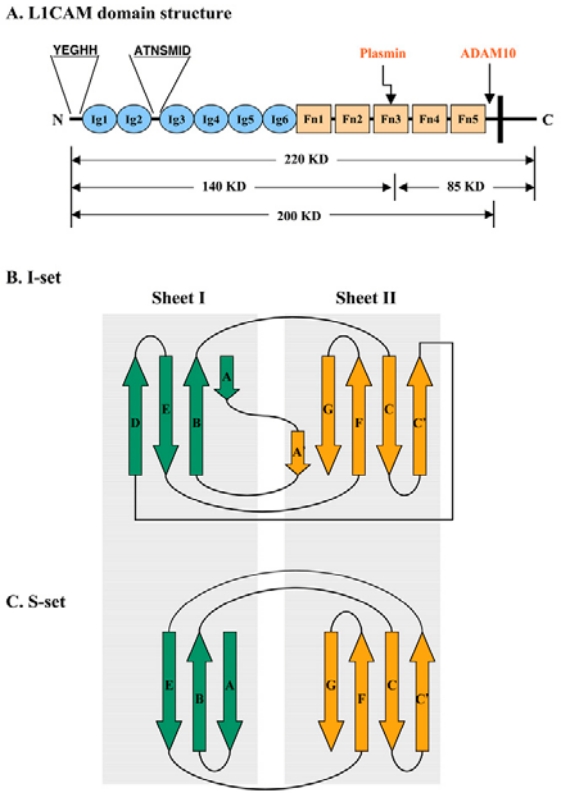


Figure 1. Domain structure of the L1CAM extracellular region A: Schematic of L1CAM. Blue ovals (Ig1-Ig6) represent I-set Ig domains, and orange rectangles represent (Fn1-Fn5) represent S-set domains. The transmembrane domain is denoted by a vertical bar. The cytoplasmic region is located at the C-terminus of the molecule following the transmembrane domain. The location of proteolytic cleavage sites recognized by plasmin and ADAM10 are denoted by arrows. The N-terminal exon 2 sequence and the 7 residue linker sequence between Ig2 and Ig3 are depicted above the cartoon. Below the schematic, the approximate molecular weights of L1CAM and its proteolytic fragments are shown. B: Ribbon diagram of an I-set Ig domain, which is representative of Ig1-Ig6. β-strands that belong to Sheet I are shaded green and those that belong to Sheet II are shaded orange. C: Ribbon diagram of an S-set Ig domain, which is representative of Fn1-Fn5.

is a highly conserved structure that is effective at driving homophilic and heterophilic protein-protein interactions. It is an all β-strand structure composed of 7-10 strands arranged into a 2 sheet "β-sandwich" (27). The strands are generally designated by letters (A, A', B-G, C' and C'' proceeding from N- to C-terminus) and the sheets by Roman numerals I and II. All Ig folds have a common "core region" composed of 2 β-strands from each sheet (E and B in Sheet I, and F and C in Sheet II (28)). Ig folds are sub-classified into "sets" based on the specific number and arrangement of their β-strands; these include the Variable (V), Constant (C1 and C2), Hybrid (H), Swapped (S), and Intermediate (I) sets (27, 29).

The first six domains of L1CAM (Ig1-Ig6) are classified as I-set Ig domains, based on sequence comparisons with other Ig folds (30). Members of this set include domains from other neural IgCAMs (such as axonin-1), FGF Receptor 2 and telokin (29). No crystallographic studies of Ig1-Ig6 have thus far been reported. However, Bateman *et al.* (30), used the crystal structure of telokin to extrapolate tertiary structures for these domains. In their model, Ig1-Ig6 each have 9 β-strands, 4 in Sheet I and 5 in Sheet II (Figure 1B), and are 92-104 residues in length. A single disulfide bridge connects the B strand in Sheet I to the F strand in Sheet II and increases the compactness of the domain (28, 29, 31). In overall tertiary structure, Ig1-Ig6 are thought to resemble Variable region Ig domains (such as the V_H domain of IgG), except that they lack a 10^{th} β-strand (labeled C'') in Sheet II (29).

The latter 5 domains in the L1CAM extracellular region (Fn1-Fn5) are S-set Ig folds (30). Because the fibronectin type III (FnIII) domain is a prototype member of the S-set (27), these domains of L1CAM are commonly referred to as "FnIII-like repeats". They each contain 7 β-strands, 3 in Sheet I and 4 in Sheet II (Figure 1C), but are approximately the same length as Ig1-Ig6 (98-106 residues). In vertebrate homologues of L1CAM, Fn1-Fn5 do not contain a disulfide bridge- although Fn1 in the *Drosophila* homologue, neuroglian, contains a disulfide bond that connects the A and G strands (32).

There are three notable peptide sequences in the extracellular region that are not included within an Ig-like domain. The first is a leader sequence at the extreme N-terminus of L1CAM (Fig. 1A). This sequence contains a five-residue stretch (YEGHH in human L1CAM) that is encoded by a small alternative exon (exon 2). L1CAM isoforms expressed by neurons include this exon 2-encoded sequence, while non-neuronal isoforms (such as expressed by B-cells) exclude this sequence (33). The second sequence is a seven-residue peptide located in between Ig2 and Ig3 (Figure 1A). This sequence is hydrophilic and is thought to be flexible, allowing Ig1-Ig2 to pivot independently of the rest of L1CAM (30). The third non-domain sequence resides between Fn5 and the transmembrane domain.

The extracellular region also contains two types of covalent modifications. The first is extensive Asn-linked glycosylation that accounts for 25% of the mass of L1CAM (34, 35). The makeup of these carbohydrate moieties has not been fully determined, but is known to include several HNK-1 chains (8, 36). The second type of covalent modification is proteolytic cleavage, which can occur at a dibasic motif within Fn3, and at another site between Fn5 and the transmembrane domain (37). Cleavage at the Fn3 site is mediated by plasmin or other similar serine proteases, and results in cleavage products of 140KD and 85KD (These weights vary somewhat between species homologues) (38). It is thought that the two fragments can remain associated through non-covalent interactions (8, 37, 39). However the 140KD fragment (containing Ig1-Fn3) may also be shed from the cell surface

and can be recovered from human CSF (40). Cleavage at the site distal to FN5 is mediated by the metalloprotease ADAM10, and results in ~200KD and 32KD L1CAM fragments (41). The 200KD fragment contains essentially the entire extracellular region and can be deposited into the extracellular matrix (42); it can also be recovered from the CSF (40).

4. THE EXTRACELLULAR REGION OF L1CAM ENGAGES IN MULTIPLE PROTEIN-PROTEIN INTERACTIONS

The L1CAM extracellular region has been shown to bind a range of cell surface and extracellular matrix molecules (summarized in Table 1). In some cases, the interactions were proposed purely on functional studies. However, even when only considering interactions where direct *in vitro* binding has been reported, it is clear the extracellular region can bind to at least 8 distinct molecules. These ligands can be loosely grouped as: 1) neural IgCAMs (such as L1CAM itself, Nr-CAM and axonin-1), 2) non-Ig family CAMs (e.g. Integrins), 3) extracellular matrix constituents (laminin, phosphacan, and neurocan) and 4) signaling receptors (NP-1). The orientation of binding between L1CAM and any particular partner is described as either *trans* (between molecules on opposing membranes) or *cis* (between molecules in the same cell membrane). In some cases the extracellular region is thought to bind the same protein in *cis* and in *trans*. Whether or not this implies two distinct binding surfaces for these targets is unclear.

5. STRUCTURE-FUNCTION ANALYSES SUGGEST MULTIPLE L1CAM DOMAINS COOPERATE DURING BINDING

One fundamental question asked about the L1CAM extracellular region is how does it accommodate so many different binding activities? Analysis of the L1CAM gene from an evolutionary perspective suggested that an ancestral L1 gene may have given rise to multiple L1 family genes by duplication (6, 43). It was also theorized that the addition of new domains to the extracellular region sequentially added new functionality, which perhaps aided the evolution of complex nervous systems. Each Ig-like domain may thus contribute unique binding activities, creating a multifunctional extracellular region.

This "modular model" gained support from studies showing that certain binding activities of L1CAM segregated to individual domains (Figure 2). For example, the binding sites for neurocan and NP-1 were localized to Ig1 (44, 45), while the binding site for several integrins was found to be an RGD containing motif in Ig6 (44, 46, 47). Another sequence in Fn3 was shown to bind independently to integrins as well (48). Zhao and Siu (49) proposed that Ig2 was necessary and sufficient for L1CAM homophilic binding in *trans*. Their conclusion was based on purified, recombinant Ig2 proteins produced in bacteria that engaged in L1CAM homophilic binding. More recently, Silletti *et al.* (48) proposed that L1CAM homophilic binding in *cis* could be mediated by Fn3. Like the previously mentioned work on Ig2, their conclusions were based on recombinant Fn3 proteins made in bacteria that were observed to spontaneously cluster *in vitro*.

However, subsequent investigations of homophilic binding suggested a different view. In an attempt to understand the molecular basis of human L1 disease, De Angelis *et al.* (50, 51), synthesized L1-Fc fusion proteins with missense mutations that are associated with disease. They then characterized these mutants for homophilic binding and heterophilic binding activity (to axonin-1 and contactin). They found that missense mutations located throughout Ig1-Ig6 and Fn2 interfered with both types of interactions. Interestingly, most of the missense mutations affected homophilic and heterophilic binding to similar extents; relatively few mutations selectively interfered with one type of interaction (51). The authors surmised that multiple domains must cooperate during L1CAM homophilic binding as well as during binding to other neural IgCAMs. An analogous conclusion was reached in a study by Kunz *et al.* (52), who conducted a deletion analysis of Ng-CAM, the chick homologue of L1CAM (Table 2). They found that deletion of any one domain from Ig1-Ig4 or Fn2 abrogated homophilic binding and binding to axonin-1. Any domain deletions within Ig1-Ig4 reduced binding. On the other hand, while deletion of Fn2 alone inhibited binding, removal of a larger segment within the FnIII repeats (Fn1-Fn2) was not deleterious (52). Similar results were obtained for human L1CAM (50). Kunz and colleagues suggested the N-terminal Ig1-Ig4 segment may directly participate in binding, while Fn2 may promote structural stability in the extracellular region (52).

Our group performed a deletion analysis of human L1CAM that sought to define a minimal contiguous segment in the extracellular region that could mediate homophilic binding (53). Like De Angelis *et al.* (51), we based our analysis on recombinant L1-Fc proteins manufactured in mammalian cells. In our study, Ig1-Ig4 was the minimal contiguous segment of L1CAM that could mediate *trans* homophilic binding; smaller segments, such as Ig1-Ig3, were inactive (53). Although Ig1-Ig4 could reproduce certain biological activities of L1CAM that are dependent on homophilic binding, such as adhesion and neurite outgrowth, it was not as potent as the native molecule. A larger segment of the protein, encompassing Ig1-Ig6, was needed to reproduce the biological potency of native L1CAM in our assays (53).

Overall, studies of L1CAM that employed eukaryotically produced proteins for analysis suggested a very different model for how the extracellular region interacts with other molecules. In terms of homophilic binding, the data suggested a "cooperative model" in which Ig1-Ig4 work together to mediate binding, while Ig5-Ig6 and Fn2 enhance the interaction, perhaps by providing structural stability. Moreover, the data also suggested that a similar cooperative mechanism underlies how L1CAM interacts with other neural IgCAMs. Interestingly, structure-function analyses of the neural IgCAMs Nr-CAM

Table 1. Binding partners of the L1CAM extracellular region

Molecule	Type of interaction	References	Direct biochemical evidence for protein-protein interaction?	Comments
L1CAM/Ng-CAM (homophilic binding)	Trans	13	Yes	Mediates neurite outgrowth and adhesion in most primary neurons (12, 17).
	Cis	48	Yes	Inferred from clustering of recombinant Fn3 in vitro (48). Thought to potentiate neurite outgrowth activity (86).
Nr-CAM	Unclear	87	Yes	May potentiate L1CAM mediated neurite outgrowth (88).
Axonin-1/TAG-1/TAX-1	Trans	89	Yes	Axonin-1 can serve as a substrate for neurite outgrowth in vitro, by binding to L1CAM on the neural cell membrane. However, whether this trans interaction occurs in vivo has been called into question (90).
	Unclear	50, 51		
	Cis	90	No	Axonin-1 was proposed to be a co-receptor for L1CAM mediated neurite outgrowth by clustering with it in the plane of the plasma membrane (90).
Contactin/F11	Unclear	51, 91	No	A member of the axonin-1 subfamily of IgCAMs.
FAR-2	Unclear	92	No	A member of the axonin-1 subfamily of IgCAMs.
N-CAM	Cis	93	No	May synergize with L1CAM to promote neurite outgrowth (94). Carbohydrate moieties on L1CAM are thought to be critical to this interaction (93).
SC1/DM-Grasp	Cis	95	No	Implicated as a co-receptor for L1CAM mediated neurite outgrowth (95).
Phosphacan	Trans	96, 97	Yes	Interaction leads to growth cone collapse (65)
Neurocan	Trans	64	Yes	Interaction leads to growth cone collapse (64)
Laminin	Trans	98	Yes	Interaction possibly mediated through HNK-1 moieties on L1CAM (99).
Integrins: $\alpha_v\beta_3$ $\alpha_v\beta_1$ $\alpha_v\beta_5$ $\alpha_5\beta_1$ $\alpha_{IIB}\beta_3$ $\alpha_9\beta_1$	Trans	44, 46, 48, 71	Yes	Cleaved L1CAM extracellular region is thought to be secreted into the extracellular matrix, where it would bind cell surface integrin receptors (46). The interaction with $\alpha_v\beta_3$ can mediate neurite outgrowth in PC12 cells, and chick DRG neurons (74). The interaction with $\alpha_5\beta_1$ potentiates epithelial cell migration on fibronectin and laminin (41).
CD9	Cis	100	No	May simultaneously interact with L1CAM and the integrin, $\alpha_6\beta_1$ (100).
Nectadrin (CD24)	Cis	101	Yes	Sialic acid moieties on CD24 are critical for its interaction with L1CAM (102). CD24 interferes with L1CAM mediated neurite outgrowth in vitro (102)
Neuropillin-1 (NP-1)	Cis and Trans	45	No	L1CAM interactions with NP-1are thought to modulate the neuronal response to Sema3A. In L1CAM knockout mice, commissural neurons are not repelled by Sema3A in vitro (19). Soluble L1-Fc added to spinal cord explant cultures converts the neural response to Sem3A from repulsion to attraction (19).
Basic FGFR	Cis	103	No	Inhibitors of FGF receptor signaling interfere with L1CAM mediated neurite outgrowth in contexts when this biological activity is subserved by homophilic binding (104). Genetic evidence for an interaction between FGFR and Neuroglian has been advanced in Drosophila (105).

Names of species homologues for certain proteins are separated by a backslash. The orientation of binding between the L1CAM extracellular region and its ligands can be described as either *cis* (within the plane of the cell membrane) or *trans* (where the binding partners are situated on opposing cells). Note that in some cases L1CAM has been proposed to interact with particular molecules in *cis* and in *trans*. In this table, evidence of a protein-protein interaction is defined strictly when purified L1CAM has been shown to interact with a purified preparation of the protein of interest. The molecules bound by the L1CAM extracellular region are organized into groups. These include neural IgCAMs (L1CAM-SC1), extracellular matrix constituents (phosphacan-Laminin), non-Ig family CAMs (integrins-nectadrin) and signaling molecules (Basic FGFR).

Table 2. Species homologues of L1CAM

Name	Species	Amino acid identity	References
L1CAM	Human	-	81
L1	Mouse	85%	13
NILE	Rat	86%	82
Ng-CAM/G4/8D9	Chick	47%	83
L1.2	Zebrafish	47%	84
L1	Pufferfish (Fugu)	40%	67
Neuroglian	Drosophila	28%	85

Selected species homologues of L1CAM are listed along with their overall amino acid identity to the human homologue.

and axonin-1 demonstrated that Ig1-Ig4 in these proteins are similarly critical for their respective protein-protein interactions (54, 55). Therefore the Ig1-Ig4 segment may represent a conserved functional unit that drives protein-protein interactions among members of the neural IgCAM family.

6. A STRUCTURAL BASIS FOR COOPERATION AMONG L1CAM DOMAINS: LESSONS FROM HEMOLIN

A structural model for how Ig1-Ig4 of L1CAM might cooperate was first proposed by Su *et al.* (56), who reported the crystal structure of hemolin. Hemolin is an immune system CAM expressed by insects, which contains four I-set Ig domains in its extracellular region. These domains assume a horseshoe-shaped quaternary structure, in which Ig1 abuts Ig4 and Ig2 contacts Ig3 (56, 57). The horseshoe structure is stabilized by hydrogen bonds between β-strands of Sheet II of Ig1 and Ig4, and Sheet I of Ig2 and Ig3 (reminiscent of the interactions that stabilize heavy and light chains in IgG Fab fragments). At the apex of the horseshoe is a 7-residue linker peptide that allows Ig1-Ig2 to pivot downward and align with Ig3-Ig4 in an anti-parallel orientation (Figure 3A). Su and colleagues noted that most of the features that enable the horseshoe structure in hemolin to form are also present in Ig1-Ig4 of L1CAM, particularly the linker sequence between Ig2 and Ig3 (56). On the basis of this, they predicted that these L1CAM domains assume a horseshoe-shaped conformation similar to hemolin (56).

Their prediction was buttressed by the work of Freigang *et al.* (58), who solved the crystal structure of Ig1-Ig4 from chick axonin-1. Axonin-1 is a neural IgCAM known to interact with L1CAM and to engage similarly in homophilic and heterophilic adhesive interactions (4). It is a GPI-linked extracellular protein containing 6 I-set Ig domains and 4 FnIII domains (59). Like hemolin, Ig1-Ig4 of axonin-1 adopts a horseshoe shaped fold of similar overall configuration (58). Again, a short linker peptide (6 residues long) is located at the apex and serves as a pivot for Ig1-Ig2. Importantly, structure-function analyses of axonin-1 localized its homophilic binding site to Ig1-Ig4, similar to L1CAM (54). Therefore, the fact that this region of axonin-1 assumes a horseshoe structure suggested that a similar arrangement might occur in Ig1-Ig4 of L1CAM as well.

Direct evidence that Ig1-Ig4 of L1CAM forms a horseshoe-shaped structure was recently advanced by electron microscopic (EM) studies. Hall *et al.* (60) imaged a trimeric L1 fusion protein via rotary-shadowing EM, and noted a globular structure located at the N-terminus of each L1CAM chain. They inferred that this globular area may represent a horseshoe-like fold (60). In collaborative studies (57), we visualized L1-Fc fusion proteins and an untagged, monomeric Ig1-Ig6 fragment of human L1CAM (61). Under rotary shadowing EM, Ig1-Ig6 appeared as an elongated, rod-like molecule. In contrast, under negative staining EM, Ig1-Ig6 appeared as a globular structure with a short, linear tail protruding from one side (like a balloon on a string). Sedimentation analysis of Ig1-Ig6 was consistent with a molecule that was at least partly globular, suggesting the folded structure is the predominant conformation of Ig1-Ig6 in solution. The results suggested that the globular structure observed on EM corresponds to Ig1-Ig4 engaged in a horseshoe fold, while the short linear projection represents Ig5 and Ig6 (57). Since these domains assumed extended or horseshoe shaped structures, depending on the imaging conditions, we theorized that Ig1-Ig4 naturally interconverts between folded and extended conformations (Figure 3A). Such a possibility was also predicted by Su *et al.* (56).

If Ig1-Ig4 can interconvert between folded (horseshoe) and extended conformations, then which conformation corresponds to the "active" form of L1CAM that mediates homophilic binding? Su *et al.* (56) noted that the same non-covalent interactions that hold the horseshoe fold together could also be used to stabilize homophilic binding in *trans*. In their proposed mechanism, the Ig1 and Ig2 domains within the horseshoe would be replaced by Ig1 and Ig2 domains from an L1CAM molecule on an opposing cell surface (Figure 3B, pathway 2). This would preserve the basic domain orientations found in the horseshoe. However, to initiate such a mechanism at least one L1CAM molecule must be in the extended conformation, which would therefore be the "active" conformation for homophilic binding. The existence of an extended conformation for L1CAM is supported by EM data (57).

On the other hand, other studies suggest that the horseshoe conformation is the active form of Ig1-Ig4 (Figure 2B, pathway 1). In the crystal structure of axonin-1, adjacent proteins associated in an anti-parallel orientation that might resemble *trans* homophilic binding *in vivo* (58). The interaction is mediated by horseshoe structures (composed of Ig1-Ig4) on adjacent axonin-1 molecules (58). Specifically, residues between the C and E strands of Ig3 formed a loop (called the "C-E" loop), which contacts a donut hole-shaped binding pocket within the

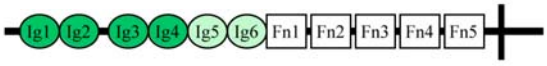
Protein	Domains Involved	References
L1CAM *trans*		50, 51
		53
		49
Ng-CAM *trans*		52
L1CAM *cis*		48
Axonin-1		52
		51
TAX-1		50
Contactin		51
Integrins: $\alpha_v\beta_3$, $\alpha_V\beta_1$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, $\alpha_{IIB}\beta_3$		46, 47, 71
$\alpha_V\beta_3$, $\alpha_5\beta_1$, $\alpha_9\beta_1$		48
Neurocan		44
Neuropilin-1		45

Figure 2. Map of binding activities in the L1CAM extracellular region. Summary of structure-function studies that identified critical L1CAM extracellular domains required for particular molecular interactions. The left hand column lists names of L1CAM binding partners (Protein). Domains shown to be critical for binding are shaded. Domains that are not critical but nonetheless may enhance binding affinity are shaded lightly. In some cases, different species homologues of a given protein were utilized in different studies (for example human TAX-1 and chick axonin-1). Results obtained using different species homologues are grouped together. Note that for L1CAM homophilic binding in *trans*, several studies arrived at divergent conclusions about which domains are sufficient for binding. It should also be noted that all proteins were generated in eukaryotic systems except for the work by Zhao and Siu (49) and the work with integrins (46-48,71), which were generated in bacteria.

horseshoe fold of an adjacent molecule. This arrangement allows for stacking and thus linear ribbons of axonin-1 proteins are observed in the crystal (58). Assuming that L1CAM adopts a similar conformation, this data would suggest that the horseshoe structure, not the extended form, is the active conformation of Ig1-Ig4. In this model, the horseshoe structure is critical for homophilic binding, as it creates the binding pocket needed to contact the C-E loop of an opposing molecule (Fig. 2B). Accordingly, the extended conformation of Ig1-Ig4 observed for L1CAM

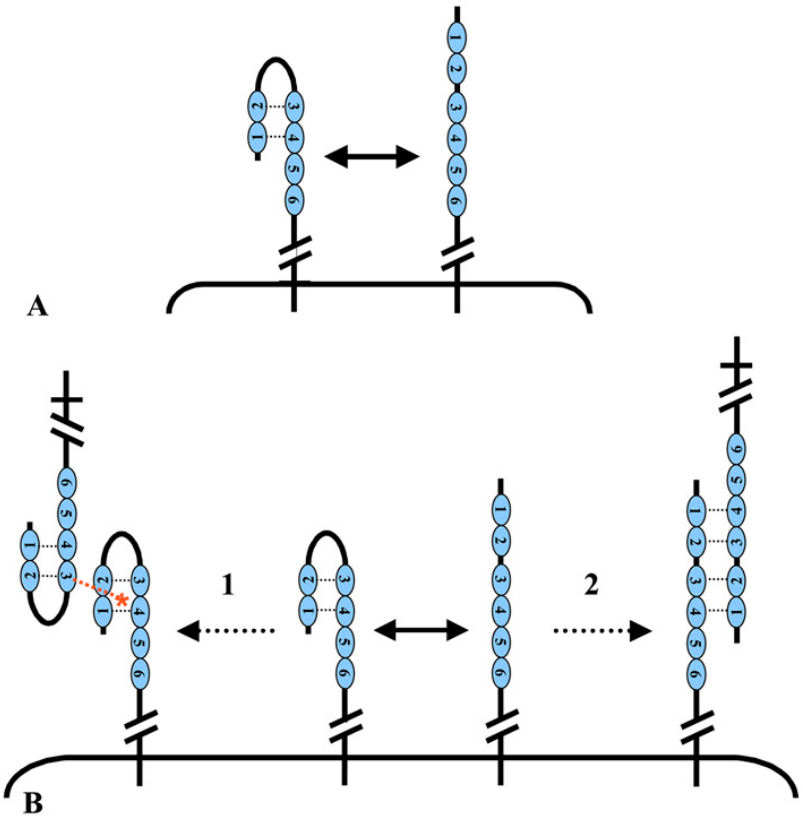


Figure 3. The L1CAM horseshoe structure and its proposed impact on activity A: The L1CAM extracellular region in folded (horseshoe) and extended conformations. The horseshoe structure is depicted schematically on the left side of the panel, and the extended form of the extracellular region is shown on the right. Note that the cartoon of L1CAM is shortened to highlight Ig1-Ig6. Dashed lines within the horseshoe represent hydrogen bonds that are proposed to link Ig1 to Ig4 and Ig2 to Ig3, and thereby stabilize the horseshoe structure. The horseshoe structure of L1CAM may reversibly open into its extended conformation, in which Ig1-Ig6 assume a linear, rod-like shape. B: Two proposed mechanisms of L1CAM homophilic binding in *trans*. Both theories are compatible with the idea that Ig1-Ig4 exist in a dynamic equilibrium between folded (horseshoe) and extended conformations (shown in the center of the panel). The first mechanism (pathway 1 on the left side of the panel), is suggested by the crystal structure of axonin-1 (58). In this model, horseshoe structures on opposing L1CAM do not open up, but rather interact with one another in *trans*. The interaction is mediated by non-covalent bonds between Ig3 of one protein with the horseshoe fold of the other L1CAM molecule (represented by a red dashed line). Specifically, a peptide loop from Ig3 is thought to contact a donut hole-shaped binding pocket (red star) within the opposing horseshoe fold. The second proposed mechanism (shown as pathway 2 on the right side of the panel), is suggested by the crystal structure of hemolin (56). In this proposed mechanism, Ig1-Ig4 first converts into its extended conformation, and then binds to Ig1-Ig4 of an opposing L1CAM protein. Non-covalent interactions that stabilize the horseshoe within individual L1CAM molecules (dashed lines) also mediate the intermolecular interaction between two L1CAM proteins.

should be unable to engage in homophilic binding (if it occurs naturally at all). De Angelis *et al.* (50) attempted to test this by producing a L1-Fc protein in which 5 of 7 residues were deleted from the Ig2-Ig3 linker. They reasoned that this mutant would lack the flexibility to form a horseshoe structure, but should fold appropriately- as the integrity of individual Ig domains is preserved. In fact, this mutation significantly reduced L1CAM homophilic binding and heterophilic binding to TAX-1 (the human homologue of axonin-1 (50)).

While this data might corroborate that the horseshoe conformation is the biologically active form of L1CAM, it is difficult to predict the structural effects of deleting the Ig2-Ig3 linker. It is believed that alternating Ig domains in L1CAM are rotated roughly 180^{o} relative to one another about the long axis of the molecule (30). For example, Sheet I in Ig1 should face in the opposite direction as Sheet I in Ig2. An exception to this is Ig2 and Ig3, which must face the same direction if they are to form the interactions needed to stabilize the horseshoe fold. Deleting residues in the linker region might rotate Ig2 about the long axis relative to Ig3, in addition to interfering with the horseshoe fold, and this could have an independent effect on homophilic binding. It is likely that a crystallographic analysis of the L1CAM extracellular

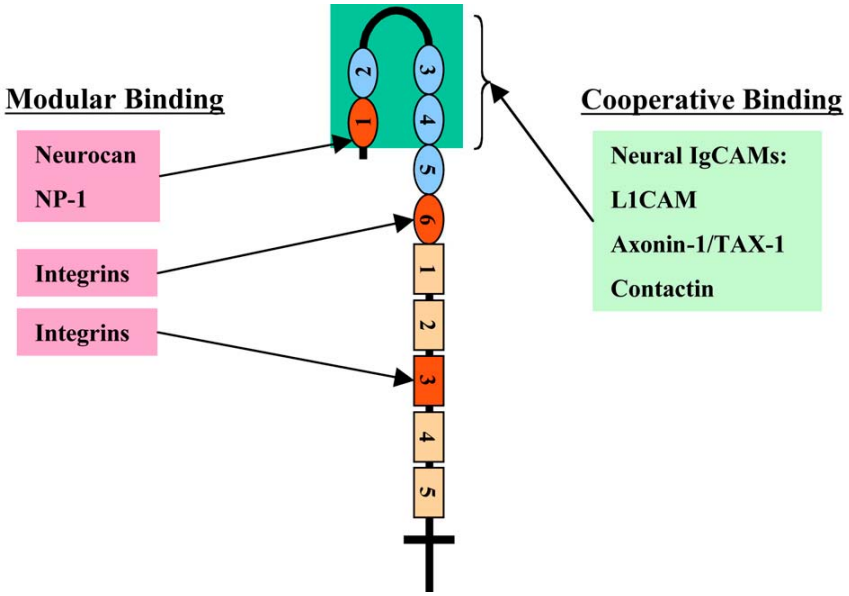


Figure 4. The L1CAM extracellular region utilizes two distinct strategies for protein-protein interactions Ligands that L1CAM interacts with via the "modular mode" are listed to the left of the cartoon. Modular mode interactions are defined as interactions mediated by single L1CAM domains that do not require contributions from neighboring domains to mediate binding. Extracellular domains of L1CAM that support modular mode binding are colored red. To the right of the cartoon is a list of ligands that interact with L1CAM via the "cooperative mode" (i.e. requiring the contribution of multiple L1CAM domains for binding). Cooperative mode binding requires contributions from Ig1-Ig4, which make up the horseshoe structure (highlighted in green).

region will be needed to resolve which conformation of Ig1-Ig4 is directly responsible for binding.

In summary, an elegant model is emerging from structural and functional studies of L1CAM that clarifies how its extracellular region can interact with so many different proteins (Figure 4). With some ligands, the extracellular region operates in a modular mode, where binding is mediated by short segments nested within individual Ig-like domains. With other ligands, the extracellular region operates in a cooperative mode, where several Ig domains form a unique quaternary "horseshoe" structure that is critical for binding. This structural and functional unit must in turn cooperate with other extracellular domains in L1CAM to optimize its biological activity. Moreover, this cooperativity is dynamic and possibly can be regulated by stabilizing or reversibly opening the horseshoe structure.

Based on the data accumulated thus far, it appears that different kinds of L1CAM ligands are segregated into different modes of binding (Figure 4). L1CAM interacts with neural IgCAMs, including axonin-1, contactin and L1CAM itself, via the "cooperative mode" (involving the horseshoe). In contrast, it interacts with extracellular matrix molecules like neurocan and non-Ig family CAMs like integrins through the modular mode of binding. It is exciting to speculate that these binding modes may at times compete with one another, and thereby modulate the biological properties of L1CAM. For example neurocan, a potent inhibitor of neurite outgrowth in the CNS (62), binds to L1CAM via a modular interaction with Ig1 (44). Interestingly, the neurocan binding site in L1CAM was recently mapped to the G and C strands of sheet II of Ig1, which are buried within the putative horseshoe fold (63). Therefore, neurocan binding may trap L1CAM in its extended form and thereby inhibit cooperative mode interactions. This may explain why neurocan directly inhibits L1CAM homophilic binding *in trans* (a cooperative mode interaction), neurite outgrowth, and cell adhesion (64, 65).

7. THE L1CAM EXTRACELLULAR REGION CONTAINS REGULATORY SEQUENCES THAT MODULATE BINDING

Typically, the L1CAM extracellular region has multiple binding opportunities at any given time. For example, it may interact with axonin-1 within the same neuronal membrane, L1CAM molecules and integrins on an adjacent cell, and neurocan in the extracellular space. What determines the choice of binding partner? Recent work suggests that binding affinities to some of these ligands can be independently regulated, thereby encouraging the extracellular region to "prefer" certain interactions over others. De Angelis *et al.* (66) showed that a short sequence N-terminal to Ig1 is important for homophilic and heterophilic binding. The sequence (YEGHH), is encoded by a miniexon (exon 2) that is included in L1CAM expressed by neurons and glia, but is excluded from L1CAM expressed by cells outside the nervous system (33, 67, 68). L1-Fc fusion proteins lacking the exon 2 sequence (exon 2^-) had a significantly reduced tendency to engage in homophilic binding and binding to

axonin-1 (66). Independently, our group found that exon 2^- L1CAM had a gradation of activity in homophilic binding assays (69). It bound with moderate efficiency to an L1-Fc containing the exon 2 sequence (exon 2^+), as compared to homophilic binding between two exon 2^+ molecules. This reduction in binding is functionally significant, as L1-Fcs lacking exon 2 showed a reduced neurite outgrowth activity (neurons express the exon 2^+ isoform). Homophilic binding between L1-Fc proteins that are both exon 2^- was very poor (69).

Interestingly, alanine substitution analysis did not identify any one residue within the exon 2-encoded sequence that was critical for supporting homophilic binding (66). This may suggest that properties other than sequence identity, such as sequence length, are important for the effects of exon 2. Despite the loss of homophilic binding for the exon 2^- isoform of L1CAM, it has been shown to bind integrins efficiently, indicating that the effect of the exon 2 sequence is selective (70). It is notable that both homophilic binding and binding to axonin-1 utilize the "cooperative mode" of L1CAM, while integrin binding employs the "modular mode". Thus, exon 2 may act as a molecular switch that regulates which binding mode will predominate.

Integrins represent the largest group of heterophilic ligands that L1CAM binds to in the modular mode. Currently, six integrins have been shown to bind L1CAM: $\alpha_V\beta_3$, $\alpha_V\beta_1$, $\alpha_V\beta_5$, $\alpha_{IIB}\beta_3$, $\alpha_5\beta_1$ and $\alpha_9\beta_1$ (41, 46-48). The best characterized site for integrin binding is an RGD-containing motif in Ig6 (47, 71). Recently Silletti *et al.* (48) identified another integrin binding site within Fn3 of human L1CAM. When produced as a recombinant protein in bacteria, Fn3 mediated binding to $\alpha_V\beta_3$, $\alpha_5\beta_1$ and $\alpha_9\beta_1$ via a non-RGD dependent mechanism (48). This protein also spontaneously aggregated in solution, predominately forming trimers. Interestingly, the trimeric form of Fn3 appeared to be the species responsible for integrin binding. When trimerization was abolished by cleaving Fn3 with plasmin, integrin binding was lost (48). Silletti and colleagues (48) predicted that, *in vivo*, Fn3 clusters L1CAM molecules in *cis* into trimeric complexes. This trimerization unmasks unique integrin binding activities, such as to $\alpha_9\beta_1$. Whether trimerization alters the overall binding preferences of L1CAM when the molecule is presented with multiple options has not yet been determined. It is interesting that Fn3 domains from neighboring L1CAM molecules may cooperate to generate new integrin binding activities. Apparently, even "modular" binding sites in the extracellular region can require cooperative interactions between Ig-like domains.

In summary, data is emerging about how the binding affinity of the L1CAM extracellular region is regulated for different molecular targets. One mechanism employs a short N-terminal sequence whose presence or absence determines broad preferences for binding partners. Another proposed mechanism is based on clustering of extracellular regions on the cell surface, which may reorganize binding preferences, as well as unmask new binding activities. Clustering of L1CAM on the surface can also result from inside-out signaling via interactions with ankyrin and associated cytoskeletal proteins that can thereby modulate cell adhesion (10). Again, cooperation among extracellular domains appears to be at the root of how L1CAM, or a pool of L1CAM molecules, selects one kind of binding partner over another. Further research will hopefully clarify the details of these mechanisms.

8. L1CAM STRUCTURAL REQUIREMENTS DURING INITIAL FOLDING MIRROR IG DOMAIN COOPERATION IN THE MATURE MOLECULE

Generally, structure-function analyses of the L1CAM extracellular region have yielded a consistent view of how it interacts with molecular targets. However, there is an interesting discrepancy among studies that investigated homophilic binding (in *trans*). Several studies concluded that Ig2 alone was sufficient for homophilic interactions, as demonstrated by *in vitro* binding and neurite outgrowth assays (49, 72-74). In contrast, other studies argued that Ig2 alone was not sufficient, but rather the minimal functional unit for L1CAM homophilic binding was Ig1-Ig4 (50-53). Both sets of studies utilized purified L1CAM fragments that were tested in similar assays and yielded reproducible results. How then can both sets of results be reconciled?

The studies that concluded that Ig2 was sufficient for homophilic binding primarily analyzed purified L1CAM proteins that were synthesized in bacteria. In contrast, studies that argued Ig1-Ig4 was necessary for binding used either L1-Fc fusion proteins that were produced in mammalian cell lines, or L1CAM mutants that were expressed on the surface of eukaryotic tissue culture cells. Recently, our group analyzed a recombinant L1CAM fragment containing Ig1-Ig3 that was produced in eukaryotic cells (31). This fragment was shown not to support homophilic binding (53, 61), although an analogous protein produced in bacteria was reported to be active (49). To our surprise, we found that Ig1-Ig3 did not fold properly when expressed in a eukaryotic context. This misfolding was reflected by intermolecular disulfide bonds that formed between adjacent Ig1-Ig3 proteins, forming dimers and higher order complexes (31). The shortest contiguous segment of the extracellular region that folded properly appeared to be Ig1-Ig4; this is also the shortest L1CAM fragment to demonstrate homophilic binding activity (53). Importantly, disulfide mediated dimerization was not reported for Ig1-Ig3 expressed in bacteria. Evidently, more structural information (at least Ig1-Ig4), is needed to properly fold L1CAM domains in the eukaryotic folding environment. However, since L1CAM is a eukaryotic protein, the need for Ig1-Ig4 to be intact for proper folding is likely to be a physiological constraint that governs its assembly *in vivo*.

Given the evidence that Ig1-Ig4 of L1CAM participate in a horseshoe fold, it is significant that shorter fragments derived from this region do not fold appropriately. The defect in folding is not limited to the horseshoe fold (a quaternary structure), but extends down to the tertiary structure of the individual Ig domains. Each

of these domains contains 2 cysteine residues that normally form an intra-domain disulfide bridge (13). The fact that the Ig1-Ig3 fragment forms intermolecular disulfide bonds indicates the conformation of at least one of these domains is sufficiently perturbed to inhibit native disulfide bridges from forming. This is important because it suggests that the folding pathway of Ig1-Ig4 proceeds contrary to the popular notion of how proteins fold. Popularly, it is believed that secondary structures arise initially, which then condense into tertiary structures (either spontaneously or with the assistance of folding cofactors). Finally, mature tertiary structures interact with one another to form quaternary structures. Implicit in this model is that tertiary structure formation is not dependent on quaternary structure relationships. However, in the case of Ig1-Ig4 of L1CAM, quaternary relationships between domains may be critical for these tertiary structures to properly assemble. In other words, just as these Ig domains cooperate with one another in mature L1CAM molecules, they also cooperate during protein folding.

Moreover, it is likely that the interdependence of L1CAM domains during protein folding is not limited to Ig1-Ig4. Many pathological missense mutations of human L1CAM result in retention of the protein in the ER, suggesting they are misfolded (50, 75). Interestingly, these mutations are spread throughout the extracellular region, including Fn4 and Fn5 that have not been directly implicated in binding to extracellular ligands (50). Clearly, the L1CAM extracellular region must meet a high standard of quality control since perturbations in any of its domains can lead to retention. It will be interesting to investigate whether molecular chaperones (such as BiP) play a role in the folding pathway.

Regarding the differing homophilic binding activities of bacterially and eukaryotically produced L1CAM proteins, we suspect the markedly different folding environments of these cells could lead to the observed discrepancies. The folding environment of bacteria may allow Ig1-Ig3 (or Ig2 alone) to fold via a non-native pathway. This possibility is reasonable, given that different Ig domains within the Ig superfamily are thought to utilize a variety of folding pathways to achieve their common, conserved structure (28). It has been observed that when isolated VH domains are expressed in bacteria, they tend to form homotypic aggregates, even though this multimerization does not occur in native IgG molecules (76, 77). A similar phenomenon occurs when domain 5 of Trk A, B or C (which is an I-set Ig domain) is expressed in isolation in bacteria (78). Therefore, non-authentic homophilic interactions can be generated in isolated Ig domains when they are expressed in bacterial systems. Likewise, it is possible that the homophilic binding activity observed for Ig1-Ig3 arose as an artifact of the bacterial expression system. If so, caution should be used in interpreting results obtained from bacterially produced L1CAM fragments.

9. PERSPECTIVE, MODEL AND FUTURE DIRECTIONS

Recent investigations of the L1CAM extracellular region have challenged our view of this important molecule (Figure 5). Previously thought of as an assembly of independent modules, the extracellular region instead appears to be a highly integrated, dynamic structure that can engage extracellular proteins via two major strategies (Figs. 4,5A). The first strategy, "cooperative mode", involves the cooperation of Ig domains 1-4 to form a horseshoe-shaped quaternary structure that interacts homophilically with like horseshoe structures in other L1CAM molecules and neural IgCAMs. The cooperative mode can be regulated by domains and sequences that abut the horseshoe structure on either side. On the C-terminal side Ig5, Ig6 and possibly additional domains enhance cooperative mode binding, perhaps by stabilizing the horseshoe structure. At the N-terminus, the short exon-2 encoded sequence can also regulate cooperative mode binding. It remains to be seen from further structural studies whether Ig5-Ig6 and the exon 2 sequence interact, perhaps forming a "linchpin" that enhances the closed (horseshoe) conformation.

The second strategy, "modular mode", involves discrete binding sites located within individual domains that mediate heterophilic interactions with integrins and certain extracellular matrix molecules. This mode of binding is indirectly enhanced when exon 2 is spliced out, such as in non-neuronal L1CAM isoforms. In our model, the absence of exon 2 promotes the conversion of Ig1-Ig4 from a folded to an extended conformation, inhibiting cooperative mode binding but leaving modular mode binding unaffected. Modular mode binding to integrins may be further enhanced by clustering of L1CAM molecules in *cis*, perhaps through the action of Fn3 (48). In our model, clustering of L1CAM in the extended conformation might also facilitate recycling. In the neuronal growth cone, L1CAM molecules are internalized at the proximal end of the growth cone via a clathrin-mediated mechanism, and transported intracellularly to the leading edge of this compartment for reinsertion into the plasma membrane (79). Internalization of L1CAM must require that the protein detaches from its extracellular ligands. Therefore L1CAM in the extended conformation should be easier to internalize, since cooperative mode binding is turned off.

Whether or not proteolytic cleavage modifies the binding preferences of the extracellular region is not yet clear. However, cleaved L1CAM can be shed into the extracellular matrix where it can play a role in epithelial cell migration (41). It is possible that extracellular matrix molecules encountered by shed L1CAM may influence its affinity for cell surface receptors and thereby modulate its ability to stimulate migration. For example, L1CAM binding to neurocan abrogates its homophilic binding activity (65), and this may indirectly produce a preference for heterophilic targets (such as integrins).

Interestingly, there are preliminary indications that the behavior of the extracellular region during initial folding mirrors the behavior of the mature molecule. Individual extracellular domains can make separate contributions to protein folding, in that derangement of any single domain can result in retention of L1CAM in the ER (50, 75). However, L1CAM folding is also a cooperative

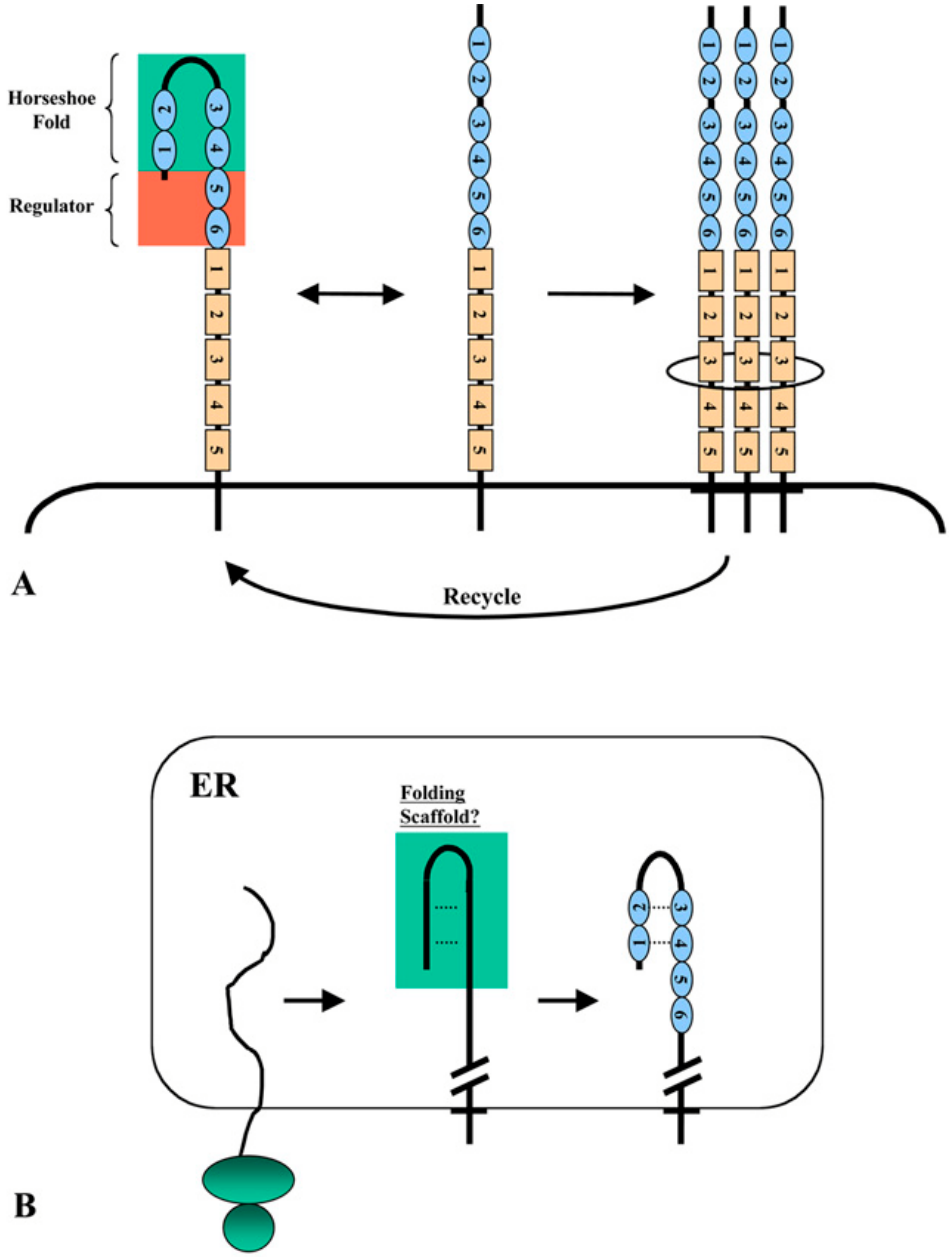


Figure 5. A model for L1CAM molecular interactions and protein folding A: At the cell surface, Ig1-Ig4 adopt a horseshoe-shaped conformation (highlighted in green) that can convert reversibly into an extended conformation. In this model, the horseshoe structure represents the critical functional unit for cooperative mode interactions (such as *trans* homophilic binding). The activity of the horseshoe is regulated by a "regulator" region (highlighted in red) that abuts the horseshoe from either side. The regulator region includes sequences from Ig5, Ig6 and exon 2, and acts by stabilizing the horseshoe structure. When Ig1-Ig4 adopt the extended conformation, cooperative mode binding is disabled but L1CAM can still engage in modular mode binding (such as binding to neurocan or integrins). In this model, L1CAM molecules may be clustered more easily in the extended form (perhaps through the action of Fn3), and can then be internalized by clathrin-mediated endocytosis (79). B: Nascent L1CAM proteins are synthesized on ribosomes (green ovals) and co-translationally inserted into the ER membrane. In this model, interactions among domains early in the folding pathway lead to a horseshoe-shaped scaffold (center, highlighted in green), which precedes the establishment of mature tertiary structures in Ig1-Ig4. Interference with this process (for example by pathological mutations) leads to misfolded Ig domains. Misfolding results in aberrant disulfide-linked dimers formed by mutant molecules that do not form stable horseshoes (right). L1CAM extracellular domains also make individual contributions to folding, in that misfolding in any domain can lead to retention in the ER (50, 75).

process, at least for Ig domains 1-4 (31). Perhaps these domains establish a horseshoe shaped "folding scaffold" (reminiscent of the horseshoe structure in the mature molecule) that mediates the proper folding of tertiary structures in this region (Fig. 4B).

Many aspects of L1CAM function await clarification by future studies. Of first importance are crystallographic studies of L1CAM to clarify its conformational structure. No such studies of Ig1-Ig6 have yet been reported, but we expect they will identify unique details of its horseshoe structure and its homophilic binding mechanism. Previously, a crystal structure of Fn1-Fn2 from neuroglian suggested that the FnIII-like domains of L1CAM form an extended, rod-like structure (32). However, antibody binding studies conducted by Silletti *et al.* (48) suggest that FnIII-like domains may assume a more globular conformation. It is possible that such a structure could have been missed by Huber *et al.* (32), because more Fn domains are needed to stabilize the putative quaternary structure. Therefore new crystallographic studies that include Fn1-Fn5 would be helpful. Proteins used for X-ray studies are commonly produced in bacteria, since this system is simple and produces deglycosylated protein. While bacterially produced protein was adequate for visualizing the horseshoe fold of axonin-1, there is evidence that L1CAM domains may utilize unpredictable folding pathways in bacteria. Therefore, L1CAM proteins produced in eukaryotic cells may be required to provide more reliable structural information.

An important lesson learned over the past few years is that seemingly modular extracellular proteins may function in a manner that is anything but modular. While L1CAM and other neural IgCAMs epitomize this new understanding, cooperative function is increasingly being noted in other classes of modularly structured proteins. In a recent article, Kirkitadze and Barlow (80) reviewed the biochemistry of Regulators of Complement Activation (RCA), which are modular proteins composed of tandem CCP domains. CCP domains are β-sandwich structures, roughly similar to Ig domains. The authors noted that neighboring CCP domains within RCA proteins cooperate with one another to promote domain stability, conformational structure and, hence, biochemical activity (80). In this family of proteins, too, modular assemblies of domains proved to be highly interdependent. We suspect that domain cooperativity represents a general principal that governs how modularly structured extracellular proteins function.

10. ACKNOWLEDGEMENTS

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