

Homophilic interaction of the L1 family of cell adhesion molecules

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Abbreviations: FN, fibronectin type III; pdb, protein data bank; RPTP, receptor type protein tyrosine phosphatase

Abstract

Homophilic interaction of the L1 family of cell adhesion molecules plays a pivotal role in regulating neurite outgrowth and neural cell networking *in vivo*. Functional defects in L1 family members are associated with neurological disorders such as X-linked mental retardation, multiple sclerosis, low-IQ syndrome, developmental delay, and schizophrenia. Various human tumors with poor prognosis also implicate the role of L1, a representative member of the L1 family of cell adhesion molecules, and ectopic expression of L1 in fibroblastic cells induces metastasis-associated gene expression. Previous studies on L1 homologs indicated that four N-terminal immunoglobulin-like domains form a horseshoe-like structure that mediates homophilic interactions. Various models including the zipper, domain-swap, and symmetry-related models are proposed to be involved in structural mechanism of homophilic interaction of the L1 family members. Recently, cryo-electron tomography of L1 and crystal structure studies of neurofascin, an L1 family protein, have been performed. This review focuses on recent discoveries of different models and describes the possible structural mechanisms of homophilic interactions of L1 family members. Understanding structural mechanisms of homophilic interactions in various cell adhesion proteins should aid the development

of therapeutic strategies for L1 family cell adhesion molecule-associated diseases.

Keywords: cell adhesion; nervous system diseases; neural cell adhesion molecule L1; protein conformation; protein interaction domains and motifs

Introduction

The L1 family of cell adhesion molecules (the L1 family) includes L1, close homolog of L1 (CHL1), NgCAM-related cell adhesion molecule (NrCAM) and neurofascin. These molecules regulate neuronal development and networking (Burden-Gulley *et al.*, 1997; Hortsch, 2000; Katidou *et al.*, 2008). Defects in L1 family members are implicated in various neurological diseases including X-linked mental retardation, multiple sclerosis, low-IQ syndrome, developmental delay, and schizophrenia (Kenwrick *et al.*, 2000; Sakurai *et al.*, 2002; Frints *et al.*, 2003; Mathey *et al.*, 2007). Knock-out of L1 family members in mice led to guidance errors in corticospinal and retino-collicular axons, hippocampal CA3 mossy fibers, olfactory neurons, and retinal axons (Maness and Schachner, 2007). The mice also exhibited abnormal cerebellar development, dendritic misorientation defects, and behavioral deficits.

L1 family proteins are type I membrane proteins with heavy glycosylation; they contain an ectodomain (1,100 residues) with six Ig-like domains (Ig1-Ig6) and five fibronectin-type III domains (FN1-FN5), a single pass transmembrane region and a cytoplasmic domain (110 residues) (Hortsch, 1996). Homophilic and heterophilic interactions of L1 on the cell surface mediate functions including axonal guidance, neural system development, and cell migration (Maness and Schachner, 2007). Partners for heterophilic interactions include integrins, neurophilin-1, TAG-1, and receptor tyrosine phosphatases that potentiate cell mobility and signal transduction (Maness and Schachner, 2007). The adhesive function of L1 induces cellular signaling processes involving activation of tyrosine protein kinases (Silletti *et al.*, 2004). The short cytoplasmic tail binds to cytoskeletons including ankyrin and spectrin, thus mediating intracellular signal transduction (Bennett

and Baines, 2001).

When backcrossed with the C57BL/6 mouse strain, a mouse line lacking L1-L1 homophilic interactions due to the deletion of the sixth Ig-like domain of L1 resulted in a severe hydrocephalus and became embryonic lethal after several generations (Itoh *et al.*, 2004). In inflammatory conditions, homophilic interaction of L1 between dendritic cells (DCs) and the vascular endothelium mediates enhanced DC transmigration (Maddaluno *et al.*, 2009). CHL1 plays a pivotal role in neuronal positioning in the visual and somatosensory cortex by coordinating with β 1-integrins (Schmid and Maness, 2008). Homophilic interactions of neurofascin are critical for neurite induction (Pruss *et al.*, 2004).

L1 syndrome diseases include X-linked hydrocephalus as a result of stenosis of the aqueduct of sylvius (HSAS), mental retardation, aphasia, shuffling gait, and adducted thumbs (MASA) syndrome, and X-linked spastic paraplegia (Wong *et al.*, 1995; Hortsch, 2000). The studies on the crystal structure of Ig-like domain 1-4 in neurofascin suggested that many pathological L1 mutations affect conserved amino acid residues within these domains and interfere with homophilic interactions (Liu *et al.*, 2011). Sixteen mutations were found in the N-terminal horseshoe region that mediates homophilic interaction, indicating that disruption of the horseshoe was the likely cause of the diseases. Some of these mutations have been investigated by others (De Angelis *et al.*, 2002) and the possible effect on horseshoe conformation discussed. These mutants usually affect adhesion-dependent cell signaling by reducing L1-protein's adhesive abilities without altering their expression (Nagaraj *et al.*, 2009). L1 mutations are also associated with fetal alcohol spectrum disorders (FASD) (Ramanathan *et al.*, 1996; Kenwrick *et al.*, 2000). A series of structural modeling and mutagenesis studies indicate the presence of an alcohol binding site in the domain interface of the L1 horseshoe region, whose perturbation by alcohol could disrupt homophilic interactions (Arevalo *et al.*, 2008; Dou *et al.*, 2011).

Cell adhesion molecules such as integrins, cadherins, Ig-like CAMs, and selectins are often aberrantly expressed in human cancers, and contribute to disease progression (Kwak *et al.*, 2011; Witzel *et al.*, 2012). Various human tumors with poor prognosis express L1 (Fogel *et al.*, 2003). For example, the invasive front of colon cancers expresses L1 (Gavert *et al.*, 2005), and stable ectopic expression of L1 in fibroblastic cells induces the expression of metastasis-associated genes (Silletti *et al.*, 2004). Due to the wide expression of L1 in cancerous tissues and its cell surface

localization, L1 is regarded as a useful target for the diagnosis of advanced cancers and a potential candidate for therapeutic intervention (Knogler *et al.*, 2007; Wei *et al.*, 2011).

The crystal structures of L1 homologs, hemolin (Su *et al.*, 1998), axonin-1 (Freigang *et al.*, 2000), TAG-1 (Mortl *et al.*, 2007) and Dscam (Meijers *et al.*, 2007; Sawaya *et al.*, 2008) suggest that the four N-terminal Ig-like domains of L1 adopt a horseshoe-shaped conformation in which the first and second Ig-like domains (Ig1 and Ig2) fold back to interact with the fourth and third Ig-like domains (Ig4 and Ig3). The Arg-Gly-Asp (RGD) sequence in Ig6 is essential for integrin interactions (Thelen *et al.*, 2002). Other evidence indicates a regulatory role of carbohydrates in L1-mediated homophilic adhesion (Acheson *et al.*, 1991; Kleene *et al.*, 2001).

Several models for L1 homophilic interactions have been proposed based on L1 homolog structures, including hemolin (Su *et al.*, 1998), axonin-1 (Freigang *et al.*, 2000), TAG-1 (Mortl *et al.*, 2007), and Dscam (Meijers *et al.*, 2007; Sawaya *et al.*, 2008). The structure of neurofascin (Liu *et al.*, 2011), the first L1 family structure to be defined, showed a novel homophilic interaction mechanism that could be compared with previous models. The cryo-electron tomograms of L1 bound to microsomes also elucidated the patterns of homophilic interaction under physiologically relevant conditions (He *et al.*, 2009). In this review, we focus on the recent discoveries in the structural mechanisms of L1 homophilic interactions and compare them to other L1 homologs. Understanding the structural mechanism of L1 homophilic interactions may contribute to the development of therapeutic strategies for diseases mediated by defects in L1-related cellular functions.

Cell adhesion molecules with immunoglobulin-like domains

Ig-like cell adhesion molecules (Ig-CAM) such as L1 and NCAM are the most ancient and diverse family of cell adhesion proteins (Rougon and Hobert 2003; Aricescu and Jones, 2007a). Some of them have homophilic binding specificities, while others have heterophilic specificities, which allow them to interact with other Ig-CAMs or other types of CAMs. Ig-CAM ectodomains have a modular structure, with Ig-like domains located near the membrane-distal N-terminus and other types of domains such as fibronectin type III (FN domain) connecting them to the membrane-spanning domain (Figure 1) (Williams *et al.*, 1989; Shapiro *et al.*, 2007). The adhesive function of Ig-CAMs is usually mediated by Ig domains (Williams *et al.*, 1989). In comparison to Igs

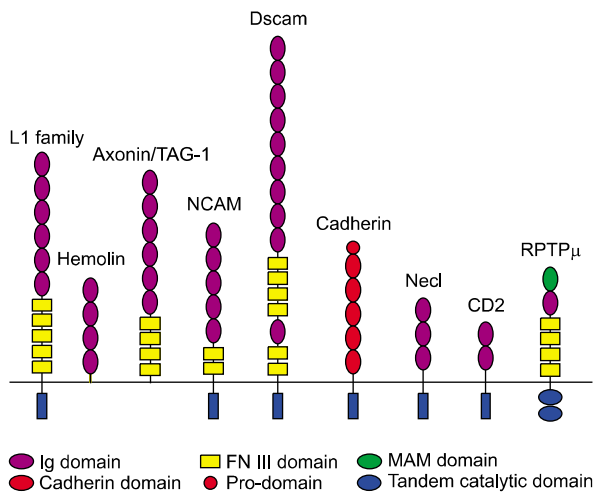


Figure 1. Domain structures of cell adhesion molecules with immunoglobulin-like domains. The L1 family includes mammalian L1, close homolog of L1 (CHL1), NgCAM-related cell adhesion molecule (NrCAM) and neurofascin. Hemolin, axonin and TAG-1 are L1 homologs with similar horseshoe domain structures (see text).

that use a defined structural mode for antigen recognition such as the complementarity-determining loops (CDRs), Ig-CAMs exhibit mechanistic diversity because of the highly diverse arrangements of domain modules.

The L1 family of proteins includes mammalian L1, CHL1, NrCAM, and neurofascin (Hortsch, 2000; Katidou *et al.*, 2008). Other homologs include the insect adhesion protein hemolin (Su *et al.*, 1998), the glycosyl phosphatidylinositol (GPI)-anchored neural cell adhesion molecule (NCAM) axonin-1 (chicken)/TAG-1 (mammalian) (Furley *et al.*, 1990; Zuellig *et al.*, 1992), and the Down syndrome protein Dscam (Figure 1) (Schmucker *et al.*, 2000). The ectodomain of the L1 family proteins has six Ig-like domains and five FN domains, whereas L1 homolog ectodomains exhibit distinct domain structures. The ectodomain of hemolin has four Ig-like domains, whereas the ectodomain of axonin-1/TAG-1 has six Ig-like domains and four FN domains (Figure 1). Different Dscam isoforms share a common domain structure, with ten Ig-like domains, six FN domains, a single transmembrane region, and a C-terminal cytoplasmic tail (Figure 1).

Other Ig-CAMs that exhibit homophilic interactions include CD2, cadherin, NCAMs and others (Figure 1). The T-cell surface protein CD2 (Selvaraj *et al.*, 1987) has two Ig-like domains that bind to its homologs CD58 (Selvaraj *et al.*, 1987) and CD48 (Kato *et al.*, 1992) on opposing cells, forming pseudo-homophilic interactions. The crystal structure of CD2 ectodomains (Jones *et al.*, 1992; Bodian *et al.*, 1994) showed a head-to-head interaction between

the GFCC'C" sheets of Ig-like domains from two interacting molecules.

Cadherins mediate various cell-cell contacts *via* homophilic interaction between the same isotypes (Steinberg and McNutt, 1999). The cadherin ectodomain comprises a tandem arrangement of cadherin domains (Figure 1). The crystal structure of a C-cadherin ectodomain containing five tandem cadherin domains showed homophilic interaction involving the first cadherin domain *in trans* (Boggon *et al.*, 2002). In addition to the *trans* interaction, the cadherin structure exhibited a *cis* interaction between the first domain of first cadherin and the interface of the first and second domains of the second cadherin, creating a zipper-like structure (Boggon *et al.*, 2002).

NCAM is one of the most extensively studied Ig-like domain-containing CAMs and play vital roles in brain development and function. Major types of NCAMs including NCAM-120, NCAM-140 and NCAM-180 contain five Ig-like domains and two FN domains in their extracellular regions (Maness and Schachner, 2007). Studies on various NCAMs suggest the zipper-like models (Walmod *et al.*, 2004). Necl molecules, which play a vital role during synapse assembly, contain three Ig-like domains in their extracellular regions. The crystal structure of the N-terminal Ig-like domain of Necl-1 showed homophilic interactions with a zipper-type arrangement (Dong *et al.*, 2006).

Type IIB receptor-type protein tyrosine phosphatases (RPTPs), such as RPTP μ have ectodomains that mediate homophilic (adhesive) interactions and intracellular phosphatase domains that dephosphorylate target proteins (Tonks, 2006). Type IIB RPTPs share a common architecture, in that their extracellular regions contain one meprin/A5/m (MAM) domain, one Ig-like domain and four FN domains. The structure of a full-length RPTP μ ectodomain (Aricescu *et al.*, 2007b) showed that the determinants required for homophilic interaction are the residues of MAM, Ig1, FN1 and FN2 domains. Homophilic interaction would generate a zipper-like structure *via cis*-interaction with adjacent molecules (Aricescu and Jones, 2007a).

Homophilic interaction of L1 homologs

The crystal structures of Ig1-4 domains from L1 homologs indicated different modes of homophilic interactions (Su *et al.*, 1998; Freigang *et al.*, 2000; Meijers *et al.*, 2007; Mortl *et al.*, 2007; Sawaya *et al.*, 2008). Hemolin, an innate immunity-related molecule in insects, contains four Ig-like domains (Ig1-4) that share 38% sequence homology with L1 Ig1-4

domains (Su *et al.*, 1998). The crystal structure determination of hemolin Ig1-4 domains revealed a unique domain arrangement among the Ig-like domain-containing cell surface proteins.

Previously described Ig-like domain proteins such as CD4 have a linearly-arranged domain structure (Wu *et al.*, 1997; Boggon *et al.*, 2002). CD4 has four Ig-like domains in a beads-on-a-string formation (Wu *et al.*, 1997). In comparison, the Ig1-4 domains of hemolin form a horseshoe structure where Ig1 and Ig2 fold back to interact with Ig4 and Ig3, respectively (Figure 2) (Su *et al.*, 1998). An extended linker between Ig2 and Ig3 creates a sharp bend of the Ig1-2 domains toward the Ig3-4 domains. The domain interactions between Ig1 and Ig4, and between Ig2 and Ig3 comprise an array of hydrophobic interactions, and residues involved in these interactions are highly conserved in the L1 family.

In the crystal of hemolin (Su *et al.*, 1998), there were no crystal contacts to suggest the mechanism of its homophilic interactions. However, based on the structure and previously described homophilic interactions in Ig-like domain-containing molecules such as CD2 and cadherin, Su *et al.*, proposed a model (Figure 2) (Su *et al.*, 1998) in which, Ig1-2 of hemolin move away from Ig3-4 to form a domain-swapped horseshoe with the Ig3'-4' of another hemolin molecule from an opposing membrane. This domain-swapping mechanism also leads to multimer formation (Figure 2A).

The chicken NCAM axonin-1 has six Ig-like domains and four FN domains (Zuellig *et al.*, 1992; Freigang *et al.*, 2000) in contrast to the five FN domains found in the L1 family proteins. The crystal structure of the Ig1-4 domains of axonin-1 also showed a horseshoe shape, confirming the generality of the Ig1-4 domain arrangement in hemolin (Figure 2B) (Freigang *et al.*, 2000). The inter-molecular interaction continues through the crystal lattice and forms a zipper-like structure. The crystal contact showed a significant burying surface, where the loop CE of Ig3 protruded to interact with the central hole in the face of the next molecule, including residues of FG loop of Ig2'. The C-termini of the dimer point in opposite directions so that facing membranes can be connected by the homophilic interaction. When residues involved in the dimeric interaction (deletion of residues 187-190 and H186A/F189A) are mutated, the myeloma cells expressing mutant axonin-1 cannot aggregate, in comparison to normal axonin-1 expressing cells, which aggregate readily (Freigang *et al.*, 2000).

Later, the Ig1-4 structure of TAG-1, the human homolog of axonin-1 was determined (Mortl *et al.*,

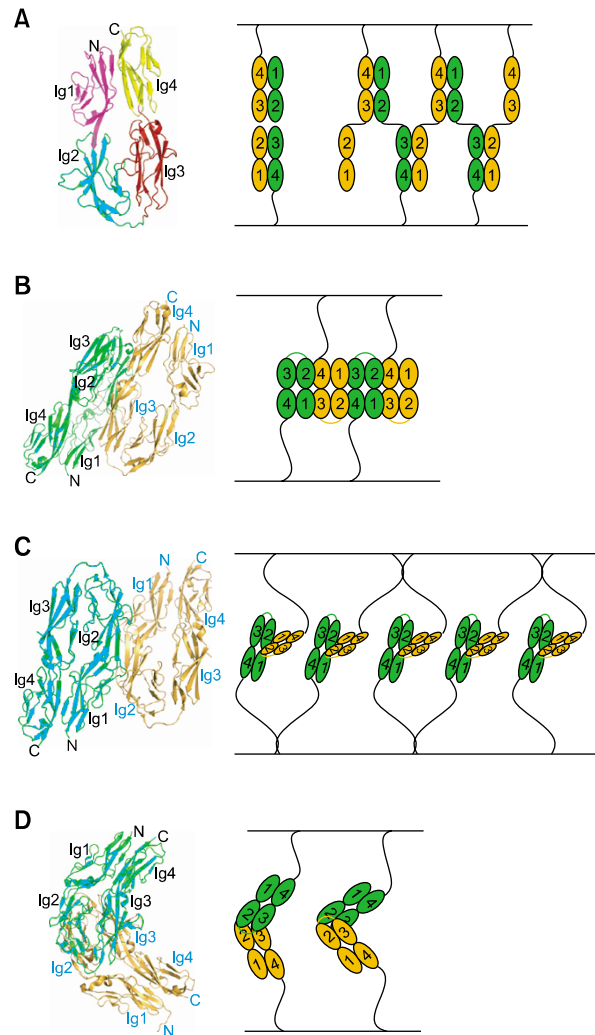


Figure 2. Different models of homophilic interactions. Proposed models (the right hand side) for homophilic interactions in the horseshoe structure are presented with their respective structures (the left hand side). Only molecules with horseshoe structures are represented. (A) Monomeric hemolin structure (PDB code: 1BIH) and the domain-swapped dimer or multimer model. (B) Dimeric axonin structure (PDB code: 1CS6) and the zipper model. (C) Dimeric TAG-1 structure (PDB code: 2OM5) and the 2-fold symmetry-related edge interaction model. The dimeric interaction occurs between edges of Ig2 domains with a perpendicular association between the two horseshoe structures. (D) Dimeric Dscam 1.34 structure (PDB code: 2V5M) and the 2-fold symmetry-related face interaction model. Interactions between large faces of Ig2-3 domains form the dimer.

2007). Although TAG-1 has a horseshoe structure similar to that axonin-1, the zipper-like dimer interaction of axonin-1 was not observed in the TAG-1 crystal. Instead, a two-fold symmetry-mediated dimer interaction is formed by side-to-side interactions between G strand of Ig2' with G strand of Ig2', generating an intermolecular β -sheet (Figure 2). The buried surface created by dimeric interaction

in the TAG-1 crystal is 2240 \AA^2 , indicating that the interaction likely has a physiological relevance. In addition, the importance of FG loop in Ig2 in myeloma cell aggregation was reported for axonin-1, the chicken homolog of TAG-1 (Freigang *et al.*, 2000). Because the same regions of two TAG-1 monomers are used in the dimeric interaction, the interaction cannot be extended to form a zipper-like structure as proposed in the case of axonin-1 (Figure 2). The possibility of two different modes of dimeric interaction in these closely related molecules must be resolved by further structural and functional analysis.

The drosophila protein Dscam, which contains ten Ig-like domains and six FN domains, mediates cell-cell interaction in brain cell networking (Schmucker *et al.*, 2000). Among the ten Ig-like domains, Ig2, Ig3 and Ig7 have 12, 47 and 33 alternative sequences, respectively, giving 18,048 potential derivatives (Schmucker *et al.*, 2000). Homophilic interactions occur only between the same isotypes; interactions between different isotypes are rare, providing necessary specificity in brain cell networking. The crystal structure of Ig1-4 domains from two different isotypes of Dscam revealed their novel dimeric interactions (Meijers *et al.*, 2007) (Figure 2D). In comparison to the dimeric interactions of axonin-1 (Freigang *et al.*, 2000) and TAG-1 (Mortl *et al.*, 2007), where interaction occurred between side regions, the dimeric interaction in Dscam involved the main surface of Ig2 and Ig3. The Ig2 surface interacts with the same surface of Ig2' and the Ig3 surface interacts with the same surface of Ig3'. A two-fold symmetry axis mediates both interactions, and the C-termini are on opposite sides, such that the opposing membranes can be associated by Dscam homophilic interaction.

Structural studies of Dscam Ig1-8 revealed the same dimeric interaction of the horseshoe region as that occurring in the the Ig1-4 domain structures (Sawaya *et al.*, 2008). In addition to the interactions mediated by Ig2 and Ig3 in the Ig1-4 region, Ig7 of Dscam interacts with Ig7' of the other monomer in a face-to-face interaction with the same two fold symmetry axis as that occurring in the Ig2 and Ig3 interactions (Sawaya *et al.*, 2008). Tight interactions of the three Ig domains in Dscam generate a double-S conformation (Figure 3). The double-S conformation of Dscam Ig1-8 indicates the critical role of the domains outside the horseshoe region in the homophilic interactions of L1 homologs. Because of the tight intermolecular interaction between two Ig1-8 molecules, alteration in non-horseshoe domains can affect the orientation of inter-horseshoe regions and direct homophilic interactions mediated by non-horseshoe domains.

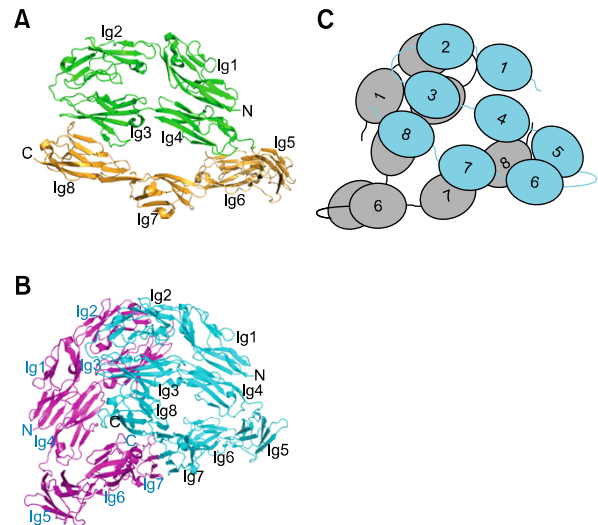


Figure 3. Dimeric structure of Dscam Ig1-8. Structure of Dscam Ig1-8 (PDB code: 3DMK) and dimeric interactions. (A) Monomeric structure. The horseshoe region (Ig1-4, green) and the remaining domains (Ig5-8, yellow) were colored differently. (B) Dimeric structure. The first monomer (Ig1-8, cyan) and the second monomer (Ig1-7, red) were represented together in their dimeric association. (C) A schematic representation of the Dscam dimer with a double-S shape.

Analysis of L1 homophilic interactions

Earlier experiments with recombinant L1 produced in *Escherichia coli* indicated that Ig2 was sufficient to induce *trans*-homophilic interaction and neurite elongation (Zhao *et al.*, 1998). The bacteria-derived FN domain spontaneously formed trimeric and higher-order complexes, suggesting that FN domains were also necessary for L1 homophilic interactions in *cis* (De Angelis *et al.*, 1999). Later, domain-mapping experiments used eukaryotic cell-derived L1 constructs and found that the homophilic interactions involved more domains. The first four Ig domains (Ig1-4) promoted homophilic cell adhesion and Ig1-6 were necessary for optimal neurite outgrowth (Haspel *et al.*, 2000; De Angelis *et al.*, 2002).

An insect cell expression system showed that a protein containing Ig1-4 domains mediated the homophilic interaction, whereas Ig2-FN5 or Ig1-3 did not, indicating that Ig2 was not sufficient for the homophilic interaction (Gouveia *et al.*, 2008). Co-immunoprecipitation studies of truncated forms of L1 and endogenous full-length L1 showed that the L1 ectodomain (L1/ECD) and L1/Ig1-4 interact homophilically in *trans* (Gouveia *et al.*, 2008). Kinetic analysis by surface plasmon resonance showed that the K_D of the whole ectodomain - whole ectodomain interaction was $116 \pm 2 \text{ nM}$, and the K_D value of the whole ectodomain - Ig1-4 interaction was $130 \pm 6 \text{ nM}$ (Gouveia *et al.*, 2008). Thus, Ig1-4 was the

minimum portion of L1 to exhibit a similar homophilic interaction activity similar to that of full length L1. Consistent with this, insect cells stably expressing L1 adhered only to L1/ECD- and L1/Ig1-4-coated surfaces or to HEK293 cells overexpressing L1 on the cell surface.

After the determination of the crystal structure of Ig1-4 domains of insect hemolin (Su *et al.*, 1998), Su *et al.*, proposed two mechanisms to explain the homophilic interaction. The first mechanism is that Ig1-4 domains exist in equilibrium between the extended and folded-back conformations and that the extended conformation mediates the homophilic interaction in *trans*. In this model, all Ig domains interact with Ig domains of the partner molecule in an antiparallel orientation (*i.e.* Ig1-Ig4', Ig2-Ig3', Ig3-Ig2', and Ig4-Ig1') (Figure 2A). The second mechanism suggests that the folded-back (horseshoe) conformation is the active form. In this model, the compact horseshoe structures of opposing L1 molecules do not open, but rather interact with each other. Although there is no clear structural evidence to favor either model, experimental data seem to rule out the extended conformation model. Deletion of Ig1 or Ig4 was sufficient to impair the homophilic interaction (Gouveia *et al.*, 2008), supporting the latter mechanism because deletion of these domains disrupted the horseshoe structure, but not the antiparallel interaction between the linearized domains.

Alcohol binding site in L1

Ethanol inhibits cell-cell adhesion mediated by L1 in neuroblastoma-glioma hybrid cells, cerebella granule cells, and human L1-transfected murine fibroblasts (Charness *et al.*, 1994; Ramanathan *et al.*, 1996; Wilkemeyer and Charness, 1998), indicating that ethanol can disrupt homophilic interaction of L1. Indeed, mutations in the L1 gene produce neuro-pathological abnormalities similar to those found in FASD caused by prenatal ethanol exposure. These abnormalities include growth deficiency, mental retardation and facial malformations (Sampson *et al.*, 1997; Hoyme *et al.*, 2005). The alcohol effect on L1 occurs for methanol through 1-butanol, but disappears for alcohols larger than 1-pentanol, indicating the presence of an alcohol binding pocket of a limited size in L1 (Ramanathan *et al.*, 1996).

To find alcohol binding sites in L1, purified L1 Ig1-4 protein was photo-labeled by photoactivatable analogs of alcohol, 3-azibutanol (1-butanol analog) and 3-azioctanol (3-octanol analog) (Arevalo *et al.*, 2008). The photoactivatable analogs labeled Tyr418 on Ig4 and several N-terminal residues including

Glu33 and Glu24-Glu27 on Ig1. In a modeled structure of the L1 Ig1-4 based on the horseshoe structure of axonin-1 (Freigang *et al.*, 2000), the photo-labeled residues were found at the interface between Ig1 and Ig4, indicating that the surface formed by these residues was likely the alcohol binding site (Arevalo *et al.*, 2008). In the model, Glu33 and Tyr418 were located closely with the distance of 2.8 Å between them, indicating that the residues formed a strong hydrogen bond. The N-terminal regions including Glu33 and Glu24-Glu27 could not be modeled because the axonin-1 structure did not contain the region. Alcohol binding to the pocket in the domain interface of Glu33 and Tyr418 likely affected the conformation of the horseshoe structure of L1, preventing of ideal interaction between the horseshoe regions.

The proximity of Glu33 and Tyr418 and their role in homophilic interaction were tested by cysteine substitution mutations (the E33C/Y418C mutant) (Dou *et al.*, 2011). Cells transfected with the cysteine mutant showed significantly increased L1 adhesion as compared to those transfected with the wild-type protein or single cysteine mutants. Moreover, reducing conditions decreased the effect of the mutation, indicating that reversible disulfide bond formation between cysteines stabilized the horseshoe conformation and the resulting homophilic interaction. The inhibitory effect of ethanol on L1-mediated adhesion decreased in cells expressing the E33C/Y418C mutant L1. Although the modeling studies and mutation analyses convincingly present the potential alcohol-binding site in L1, the shape of the binding pocket and the interactions between alcohol and L1 need to be defined by experimental structure studies. The role of the N-terminal residues including Glu33 and Glu24-Glu27 also has to be resolved.

Cryo-electron tomography of L1

The horseshoe structures found in L1 homologs (Su *et al.*, 1998; Freigang *et al.*, 2000; Meijers *et al.*, 2007; Mortl *et al.*, 2007; Sawaya *et al.*, 2008) suggest that L1 has a similar structure. However, the structures proposed different models for the homophilic interaction; therefore, a physiologically relevant analysis was necessary. Cryo-electron tomography of L1 addressed some of the questions surrounding the homophilic interaction mechanism (He *et al.*, 2009). Tomographic reconstructions of the interfaces between L1-presenting liposomes showed regularly spaced globular densities appearing as dots in a row among the overall diffuse densities (He *et al.*, 2009). The approximate size of

the dots with tomogram resolution of 4-5 nm was consistent with the size of Dscam horseshoe pairs of $60 \text{ \AA} \times 70 \text{ \AA} \times 80 \text{ \AA}$ and $60 \text{ \AA} \times 60 \text{ \AA} \times 100 \text{ \AA}$ (pdb files 2v5r and 2v5s), indicating that the dots represented the paired dimer of L1 horseshoes.

The discrete arrangement of dots in the area of membrane interaction suggested that L1 homophilic interaction did not occur as proposed in the zipper model. The interacting horseshoes in the zipper model with indefinite repeats of horseshoe domains should appear as a continuous and solid line of density in tomograms. Moreover, the domain-swapped multimerization model where domains 1 and 2 forms a horseshoe with domains 4 and 3 of the opposing membrane (Figure 2A), was not consistent with the L1 tomography observations. With the domain-swapped multimerization model, the tomograms should show a zig-zag pattern of individual horseshoe density.

Instead of the zipper or domain-swapped multimerization models, the discrete and regularly spaced dots fit the model of a dimer seen in the Dscam (Meijers *et al.*, 2007; Sawaya *et al.*, 2008) and TAG-1 structures (Mortl *et al.*, 2007). In Dscam and TAG-1 dimers (Figures 2C and 2D), one region of the first horseshoe interacts with the same region of the second horseshoe (D2-D2' and D3-D3' in Dscam; D2-D2' in TAG-1) through a two-fold symmetry axis. This dimeric interaction produces an isolated dimer, which is consistent with the discrete dots in L1 tomograms. Although the domain-swapped multimerization model does not fit the tomographic observation, one-to-one domain-swapping may result in an isolated dimer (Figure 2A), which cannot be ruled out as a possible model for the observed dots.

L1 has twenty-one glycosylations consisting of four, five and twelve sites in the horseshoe, Ig5-6 and FN domains, respectively. Various studies have shown that L1 glycosylation affects its homophilic interaction (Acheson *et al.*, 1991; Kleene *et al.*, 2001). Carbohydrate-protein or carbohydrate-carbohydrate interactions often regulate homophilic interaction of cell surface proteins (Bucior and Burger, 2004). Furthermore, the L1 ectodomain binds to sialic acid, revealing that L1 functions as a sialic acid-binding lectin (Acheson *et al.*, 1991; Kleene *et al.*, 2001).

In an experiment to analyze the role of carbohydrates in L1, He *et al.*, (He *et al.*, 2009) expressed L1 in mammalian and insect cells with attached complex N-linked and oligomannoside carbohydrates, respectively, on their membrane proteins. When the two types of L1 were reconstructed with liposomes, they produced similar dots between the two interacting liposomes; however, the dots had different inter-dot distances: 100 \AA for the mammalian cell-derived L1

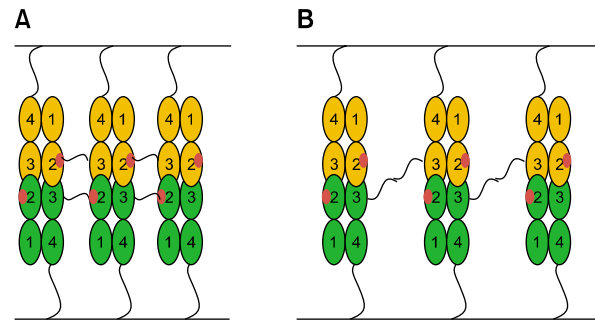


Figure 4. Models for carbohydrate interactions in L1. Models are based on the inter-membrane dots observed in cryo-electron tomograms of L1. The carbohydrate chains are represented as curved lines between the horseshoe dimers, and the sialic acid binding sites on L1 are shown as small red circles. The ends of carbohydrates interact with either the sialic acid binding site on the next L1 molecule (A) or another carbohydrate (B).

and 140 \AA for the insect cell-derived L1 (Figure 4). Thus, the regular spacing of dots in tomograms likely represents interactions with long chains of carbohydrates attached to L1 ectodomains. When the terminal sialic acids on mammalian cell-derived L1 were removed by sialidase or cell-growth in a medium containing kifunensine, the liposome interaction pattern changed (He *et al.*, 2009).

These observations indicate that the carbohydrates on L1 play a pivotal role in determining the distances between horseshoe dimers, thus modulating homophilic interaction patterns (Figure 4). The carbohydrate attachment sites of mammalian L1 are completely conserved, and there are no additional sites, further indicating the importance of carbohydrates in L1 function. However, the mechanism by which the flexible carbohydrate chain maintains the precise distance between the dots observed in the tomograms is of interest. It is unlikely that the carbohydrate alone can hold the interacting horseshoe regions in a regular fashion. The remaining domains (Ig5-6 and FN1-5 domains) are also likely to mediate the homophilic interaction and regular inter-dot spacing. The role of carbohydrates in homophilic cell adhesion interactions was shown in NCAM (Kadmon *et al.*, 1990; Acheson *et al.*, 1991) and coxsackievirus and adenovirus receptors (Excoffon *et al.*, 2007). The terminal sialic acids are also vital for NCAM homophilic interactions (Fujimoto *et al.*, 2001; Varki, 2007) as well as heterophilic interactions between L1 and CD24 (Kleene *et al.*, 2001).

Structure of the L1 family protein neurofascin

Recently, the first structure of the L1 family proteins was determined for neurofascin (Liu *et al.*, 2011). The Ig1-4 domains of neurofascin were over-expressed and purified from an insect expression system. The purified neurofascin Ig1-4 shows a 47 kDa band on SDS-PAGE, as expected from the calculation of its theoretical molecular weight. However, in a gel filtration experiment, the protein produced a peak at 100 kDa position, which was twice the monomeric size, indicating that neurofascin Ig1-4 forms a dimer in solution.

The neurofascin Ig1-4 (Liu *et al.*, 2011) has a horseshoe structure as that of L1 homologs (Su *et al.*, 1998; Freigang *et al.*, 2000; Meijers *et al.*, 2007; Mortl *et al.*, 2007; Sawaya *et al.*, 2008). The Ig1:Ig4 and Ig2:Ig3 interdomain interactions involve large surfaces with hydrophobic patches, indicating that the horseshoe structure of neurofascin Ig1-4 is likely stable. The residues involved in the domain interactions of neurofascin Ig1-4 are conserved in other members of the L1 family (Liu *et al.*, 2011).

Consistent with the dimer formation in solution, the two different crystal lattices of neurofascin Ig1-4 contain a common neurofascin Ig1-4 dimer with extensive monomer-monomer contacts (Liu *et al.*, 2011). The neurofascin Ig1-4 dimer formation is mediated by the side of Ig2 domain, resulting in the formation of a supramolecular β -sheet with a two-fold symmetry (Figure 5A). When mutations were introduced in the residues involved in the dimeric interaction, most mutant proteins showed a shift in gel filtration peak positions towards the monomeric molecular weight. Among the mutations, the substitution of Thr 222 to Ala (T222A) showed a clear movement of the peak. Thr 222 of one monomer forms a hydrogen bond with Gln 224 of the other monomer at the center of the supramolecular β -sheet, and the mutation result indicates its importance in the dimeric interaction.

Supramolecular β -sheet formation of neurofascin Ig1-4 is similar to the dimeric interaction in TAG-1 (Figure 5B) (Mortl *et al.*, 2007). The two-fold symmetry-related dimeric interaction between the side regions of neurofascin Ig2 produced an isolated dimer, consistent with the discrete inter-membrane dots observed in L1 tomograms (He *et al.*, 2009). Although the Dscam horseshoe dimeric interaction (Meijers *et al.*, 2007; Sawaya *et al.*, 2008) also generates an isolated dimer without further continuation of the interaction, the Dscam interaction occurs throughout the domain face; this differs from the edge interaction observed in neurofascin Ig1-4. Because both neurofascin Ig1-4 and TAG-1

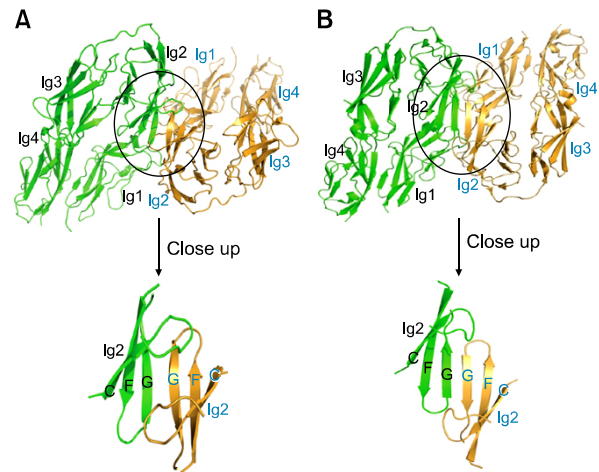


Figure 5. Common homophilic interactions. Both neurofascin (PDB code: 3P3Y) (A), an L1 family protein and TAG-1 (PDB code: 20M5) (B) have a two-fold symmetry-related edge interaction model. The conformation and dimeric interaction mode are highly similar in both molecules. In both cases, the dimeric interactions generate similar supra-molecular β -sheets.

horseshoes exhibit the same supramolecular β -sheet formation, this mode of interaction may represent homophilic interaction of L1 family molecules and their homologs.

The neurofascin Ig1-4 structure was used to map pathological mutations in neurological disorders such as HSAS, MASA syndrome, and X-linked spastic paraplegia (Wong *et al.*, 1995; Hortsch, 2000). Sixteen different mutations associated with these disorders were found in the horseshoe region. Most mutations were found in the domain interfaces (L120V, G121S, G370R, R184Q/W, and Y194C) of the horseshoe formation or in the hydrophobic core of individual domains (I179S, H210Q, P240L, C264Y, G268D, P333R, W335R, and L391P), confirming the importance of the horseshoe structure in L1 function. In contrast, one mutation (I219T) corresponding to Ile213 in neurofascin Ig1-4 produced a neurite branching defect and was located in the hydrophobic cluster that mediates dimeric interaction in neurofascin Ig1-4 crystals, supporting the model of homophilic interaction proposed by the crystal structures.

Although the neurofascin Ig1-4 structure is useful for explaining pathological mutations in the horseshoe region, it does not provide information on other domains, including Ig5-6 and FN domains (FN1-5) where various disease-inducing mutations occur (De Angelis *et al.*, 2002). The lack of homophilic interaction of L1 in a mouse line carrying a deletion of the Ig5 domain (Itoh *et al.*, 2004) also indicates that involvement of domains other than the horseshoe region can affect the homophilic

interaction of L1 family proteins. Mutations in non-horseshoe regions may change the structure of the horseshoe region or direct intermolecular interactions. Structural analysis and carefully designed mutational and functional studies of molecules containing domains outside the horseshoe region are required to answer this question.

Conclusions

Homophilic interactions between L1 family proteins are essential for neural system development and brain cell wiring. Because homophilic interaction-mediated signal transduction activates cell growth signaling, it is believed that cancer progression also involves the homophilic interaction of L1. Various models for homophilic interaction have been proposed for cell adhesion proteins (Figure 2), including zipper, domain-swapped monomer, domain-swapped multimer, two-fold symmetry related surface interaction and two-fold symmetry related edge interaction models based on the structures of axonin-1, hemolin, Dscam and TAG-1/neurofascin, respectively. The two-fold symmetry-related surface or edge interaction and domain-swapped monomer models are consistent with the discrete and equally spaced dots observed in cryo-electron tomograms of L1. Interestingly, dimeric interaction *via* the edge of Ig2 domain was observed in TAG-1 and neurofascin, suggesting that the symmetry-related edge model may represent the homophilic interaction mechanism of L1 and its homologs. Current understanding of homophilic interaction in L1 family molecules is based mainly on the crystal structures of the first four Ig-like domains of a horseshoe structure. However, for a complete understanding of homophilic interactions, structural determination of L1 including other Ig-like and FN domains is necessary. Such studies may reveal novel supra-structures such as the double-S conformation observed in the Dscam Ig1-8 dimer.

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