

FAST TRACK

L1 Mediated Homophilic Binding and Neurite Outgrowth Are Modulated by Alternative Splicing of Exon 2

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ABSTRACT: The neural cell adhesion molecule (CAM) L1 is a member of the immunoglobulin superfamily that has been implicated in neuronal adhesion, neurite outgrowth, and axon guidance. The clinical importance of L1 is illustrated by pathological mutations that lead to hydrocephalus, mental retardation, motor defects, and early mortality. The L1 gene is composed of 28 exons, including exons 2 and 27 that are spliced alternatively, and mutations in exon 2 are associated with severe neurological abnormalities in humans. To elucidate the role of L1 exon 2, a recombinant Fc fusion protein called $\Delta 2L1$ was constructed lacking the second exon in the extracellular domain of L1. When bound to fluorescent beads, L1 exhibited homophilic binding while $\Delta 2L1$ did not. However, L1 beads coaggregated with the $\Delta 2L1$ beads. Similarly, in cell binding studies, L1 bound to L1 and $\Delta 2L1$ did not bind to $\Delta 2L1$ but it

bound moderately to L1. Given the reduced binding of $\Delta 2L1$, we tested its effect on neurons. By comparison to L1, a lower percentage of dissociated neurons extended neurites on $\Delta 2L1$, and there was a modest decrease in length of the neurites that grew. Neurite outgrowth from reaggregated neurons was much less robust on $\Delta 2L1$ than on L1. The combined results indicate that $\Delta 2L1$ does not bind homophilically but it can interact with L1 containing exon 2. The reduced binding and neurite promoting activity of $\Delta 2L1$ provides an explanation for certain pathological mutations in L1 that lead to clinically apparent disease in the absence of the normal form of L1 in the nervous system. © 2002 Wiley Periodicals, Inc. *J Neurobiol* 51: 177–189, 2002

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INTRODUCTION

L1 is a neural cell adhesion molecule of the immunoglobulin superfamily that has been implicated in axonal growth and guidance during development (Brummendorf and Rathjen, 1995; Grumet, 1992). It is expressed primarily on the surface of neurons in-

cluding their growth cones and functions via homophilic and heterophilic interactions. L1 consists of six Ig domains and five fibronectin type III repeats in its extracellular regions, a single transmembrane region and a short cytoplasmic region. The region containing the Ig domains folds into a horseshoe shape (see Materials and Methods, Fig. 1C) (Bateman et al., 1996; Su et al., 1998) and acts as a functional unit of the protein that is equipotent with the full length extracellular region in promoting neurite outgrowth *in vitro* (Haspel et al., 2000; Schurmann et al., 2001). Although the functions of the fibronectin repeats in L1 are less well characterized, there is evidence that they are involved in homomultimerization as well as in heterophilic interactions and thus may modulate neurite outgrowth (Stallcup, 2000).

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A range of neurological disorders in humans including hydrocephalus, mental retardation, and spastic paraplegia (termed CRASH syndrome) are caused by mutations in the L1 gene (reviewed in Kamiguchi et al., 1998a; Kenwrick and Doherty, 1998). Many of these are missense mutations and an initial analysis of some of them located in various Ig and FnIII domains in L1 indicate that disruption of multiple regions of the protein can interfere with homophilic and heterophilic L1 binding (De Angelis et al., 1999). Another pathological human mutation in L1 results in the loss of exon 2 (Jouet et al., 1995). Interestingly, exons 2 and 27 represent two alternatively spliced exons that are normally expressed in neurons but are absent in other L1-expressing cells (Takeda et al., 1996). Exon 27, which is in the cytoplasmic region, encodes the sequence RSLE that is involved in targeting neuronal L1 to the growth cone (Kamiguchi and Lemmon, 1998). Exon 2, which is in the extracellular domain, encodes the sequence YEGHHV and is located immediately N-terminal to the first Ig domain. This exon is included in neuronal L1 isoforms expressed in the nervous system (by neurons but not by Schwann cells), but is absent from L1 protein expressed by melanocytes, kidney epithelia, and B-cells (Debiec et al., 1998; Jouet et al., 1995; Takeda et al., 1996). While the cytoplasmic mutations of L1 involving the RSLE mini-exon have been investigated extensively (Kamiguchi and Lemmon, 1998; Kamiguchi et al., 1998b; Long et al., 2001; Takeda et al., 1996), a report on the role of exon 2 in L1 binding has only appeared recently (De Angelis et al., 2001).

Here we have examined the role of exon 2 in L1 utilizing soluble and transmembrane recombinant L1 proteins in the context of the six Ig domains that have been shown to be critical for the neurite outgrowth promoting activity of L1 (Haspel et al., 2000). The results confirm a role for exon 2 in homophilic binding (De Angelis et al., 2001) and further suggest that exon 2 critically modulates the homophilic binding mediated by the Ig domains of L1. In addition, we provide the first evidence that deletion of exon 2 from L1 reduces its neurite outgrowth promoting activity. These results also suggest a modulatory role for exon 2 of L1 that favors homotypic interactions among neurons expressing L1 with this exon versus potential heterotypic interactions with L1 on nonneuronal cells lacking this exon.

MATERIALS AND METHODS

Construction of the Exon 2 Deletion Mutant of L1

To investigate the functional properties of the second exon of L1 cell adhesion molecule (CAM), we constructed an Fc

fusion protein called $\Delta 2L1$ -Fc that contains the six Ig domains of L1 but lacks the second exon near the amino terminus of the L1 gene that consists of six amino acids (Fig. 1A). The plasmid pCR/L1-16TEV-Fc (Haspel et al., 2001a,b) was used as a template in PCR reactions. A segment containing the L1 sequence 5' to exon 2 was amplified with the oligos 5'-GGAGGTCTATATAAGCAGAGC-3' (pCR_upstr_sac) and 5'-TTTCATGAGCTCCTCGGGG-ATCTGGATAAG-3' (dwnstr_5'exon2) using *Pfu* polymerase (Stratagene, La Jolla, CA), and was digested with *EcoRI* and *SacI*. Another fragment containing sequences 3' to exon 2 was prepared using 5'-CCATGGATCCAGAGCTCATGGAGCCACCTGTCATCAC-3' (upstr_3'exon2) and 5'-TGATCTTGATCATTCGGCTTCCTGTCAATCATGC-3' (dwnstr_Ig2). This was cut with *SacI* and *BglIII*. The latter two fragments were ligated into pCR/L1-16TEV-Fc that was cut with *EcoRI* and *BglIII*, and the resultant construct called pCR/L1-16 $\Delta 2$ -Fc (encoding a protein called $\Delta 2L1$ -Fc) was confirmed via sequencing (data not shown). The exon 2-deleted isoform of L1 is distinguished by both the absence of six residues encoded by exon 2 and by conversion of Val-30 \rightarrow Leu (Jouet et al., 1995). Both of these alterations are included in Fc fusion constructs used here. As control proteins, we used L1-1212-Fc (Haspel et al., 2000) containing tandem repeats of the first and second Ig domains of L1, and neurofascin-Fc (a generous gift from Dr. Steven Lambert).

Protein Production

Fc fusion proteins were produced essentially as described (Haspel et al., 2001a). Briefly, HEK 293 cells were stably transfected and protein from the culture supernatant was precipitated and dialyzed. Ammonium sulfate dialysates were incubated with protein A-Sepharose (Pierce, Rockford, IL) and the respective proteins (L1-Fc and $\Delta 2L1$ -Fc) were eluted with 100 mM glycine, pH 2.8, as described for other Fc fusion proteins (Sakurai et al., 1998). Typical yields were 1–3 mg/L cultured supernatant.

Fluorescent Bead Aggregation

Red and green fluorescent protein-A fluorosphere beads (diameter = 1 μ m) were purchased for aggregation studies (Molecular Probes, Eugene, OR). Ten microliters of fluorescent bead slurry were mixed in 200 μ l with 25 μ g of each protein; red or green beads were used for L1-Fc and green for $\Delta 2L1$ -Fc in PBS at pH 8.0. These suspensions were vortexed briefly to ensure mixing and incubated in 1.5 ml microfuge tubes at 4°C overnight. The next day, the suspensions were sedimented by centrifugation at 10,000 rpm for 5 min and washed three times with PBS. Finally, the beads were resuspended in 100 μ l (10 times the original bead volume) in PBS containing 0.1 mg/ml BSA. SDS-PAGE analysis confirmed that equal amounts of each protein were bound to the beads (Fig. 1F). Aggregation of beads was measured using a Coulter Counter set to count superthreshold particles between 2.5 and 6 μ m as described

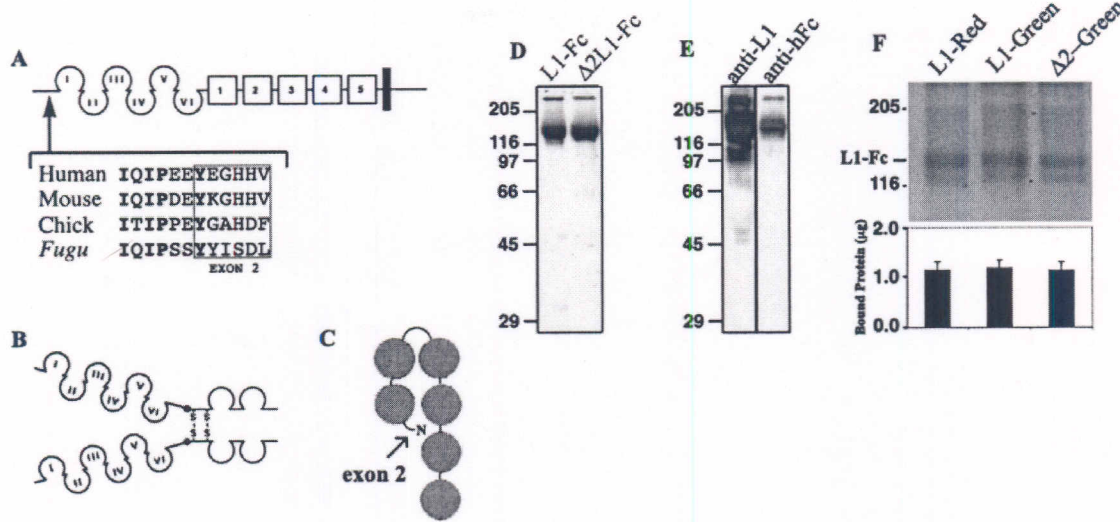


Figure 1 Model of L1 and production of exon 2-deleted proteins. (A) Schematic diagram of L1 illustrating the position of the exon 2-encoded sequence, which is N-terminal to the first Ig domain. Below the diagram, the primary sequence of the amino terminus of L1 is shown in different species. Conserved residues are indicated in bold. (B) Schematic representation of the L1-16-Fc proteins used in this study containing the 6 Ig domains of the human L1 ectodomain fused to the Fc region of human IgG. (C) Model of the L1 Ig domains showing the amino terminus (N) and the six Ig domains (represented by filled circles), with the first four Ig domains folded in a horseshoe conformation. (D) SDS-PAGE analysis of $\Delta 2$ L1-Fc. For comparison, L1-Fc was run in parallel. (E) Western blots of $\Delta 2$ L1-Fc probed with monoclonal anti-L1 Neuro 4.1 (Williams et al., 1992) and anti-human Fc antibodies. (F) Fc fusion proteins were bound to red and green fluorescent beads coupled with protein A, and bound proteins were eluted in SDS sample buffer, resolved by SDS-PAGE and visualized using the fluorescent dye SYPRO orange (top). Quantitation of SDS gels (lower panel) shows that approximately 1 μ g of each protein bound to 10 μ l of beads in each case ($n = 3$).

previously (Grumet and Edelman, 1988). For mixed bead aggregation experiments, different bead preparations were sonicated for 15 s to ensure dissociation, which was monitored by fluorescence microscopy, and 5 μ l of each were combined in a 1.5 ml microfuge tube, and incubated at 37°C for 1.5 h. Following the incubation, 1.2 μ l of each aggregation mix was spotted on a glass microscope slide (Fischer Scientific, Fair Lawn, NJ) and images were digitally captured using a Zeiss (Thornwood, NY) LSM 510 confocal microscope. LSM confocal images were quantitated for colocalization using Bitplane Advanced Imaging Software running on an SGI-octane (Silicone Graphics Inc., Mountain View, CA). Briefly, images were analyzed using the colocalization function after applying a Gaussian filter; the colocalization index was defined as the ratio of (numbers of green colocalized pixels)/(total number of green pixels).

Dissociated Cell Neurite Outgrowth

Protein coating on plastic dishes was conducted essentially as described (Haspel et al., 2000). Briefly, 1 cm radius circles were etched into 35 mm petri dishes (Falcon, Lincoln Park,

NJ), and the inscribed area was coated with 10 μ g/ml poly-L-Lysine (Sigma, St. Louis, MO) in ddH₂O for 5 min at room temperature. After three washes with 1 ml of Hanks Buffered Saline Solution (HBSS) (Bio-Whittaker, Walkerville, MD), L1 and $\Delta 2$ L1 Fc fusion proteins (diluted in HBSS) were spotted (1.2 μ l/spot). Duplicate spots of each protein were made per plate and duplicate plates were prepared in each experiment. The plates were incubated in a 37°C tissue culture incubator for 1 h. The plates were then washed with HBSS and the surfaces were blocked with 1% BSA in HBSS for 1 h at 37°C. Then, they were washed two times with HBSS and once with neurite outgrowth medium (Neurobasal, B27, 0.5 mM L-Gln, 25 μ M L-Glu, Life Technologies, Inc., Rockville, MD). Mouse cerebellar granule cells were prepared from P4-5 mouse pups as described (Haspel et al., 2000), suspended in neurite outgrowth medium at 2×10^5 cells/ml and 250 μ l of this suspension was added per plate. The plates were incubated for 18 h in a 5% CO₂ tissue culture incubator and the experiment was stopped by adding 250 μ l of 2 \times fixative solution (6% paraformaldehyde, 0.05% glutaraldehyde, 8% sucrose in PBS) for 15 min at 37°C. Randomly selected fields were digitally captured from each spot using a phase contrast microscope

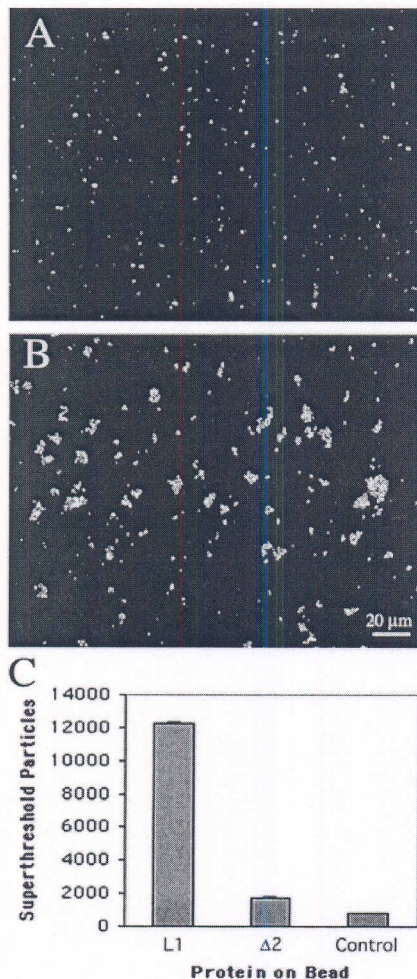


Figure 2 $\Delta 2L1$ lacks homophilic binding as compared with L1. (A) $\Delta 2L1$ coated fluorescent beads demonstrated little or no homophilic binding while (B) L1 coated fluorescent beads aggregated extensively following 2 h incubation at 37°C. Quantitation of superthreshold particles (C) confirmed little or no aggregation of $\Delta 2L1$ ($\Delta 2$) coated beads by comparison to beads coated with L1-Fc (L1) and L1-1212-Fc (control).

(Nikon Diaphot, Diagnostic Instruments, Sterling Heights, MI) using a digital Spot camera. The length of the longest neurite of all cells was measured using NIH Image software and measurements from duplicate spots were averaged. The number of cells containing neurites longer than the diameter of two cell bodies were counted and divided by the total number of cells to obtain the fraction of cells with neurites.

Reaggregate Neurite Outgrowth

This assay was procedurally similar to that of dissociated cell neurite outgrowth except that 6 cm petri dishes (Falcon)

were used and 0.5 cm radius circles were etched into the plastic. In brief, dissociated cerebellar granule cells were diluted to a density of $1-2 \times 10^7$ cells/ml in DMEM 10% FCS to produce cell aggregates in hanging drops; 10 μ l cell suspensions were spotted on the lid of 96-well microtiter plates and the lid was inverted onto the 96-well dish to create hanging drops that were incubated in a 10% CO_2 tissue culture incubator for 20 h to induce formation of aggregates. Aggregates were collected and washed by settling through a 3.5% BSA cushion and resuspended in DMEM 10% FCS. Five to 20 reagggregates were added in 30 μ l of DMEM 10% FCS to each circle and the plates were incubated in a 10% CO_2 tissue culture incubator for 18 h. The cultures were fixed by adding equal volumes of 2 \times fixative solution as described above. Digital images were captured and the length of the tenth longest neurite of each aggregate was measured as described (Haspel et al., 2000).

Construction of L1 and $\Delta 2L1$ Sindbis Virus

L1 containing viruses were constructed using the Sindbis Expression System essentially as described by the manufacturer's protocol (Invitrogen, Carlsbad, CA). Briefly, plasmid cDNA encoding full length L1 was digested with *Xba*I and *Pme*I and ligated into the Sindbis expression construct pSinRep5, which carries the nonstructural genes and the transgenes, that had been cut with *Xba*I and *Stu*I, to make pSinRep5/L1 (RSLE⁻). From this vector, we prepared pSinRep5/hL1 $\Delta 2$ (RSLE⁻) lacking exon 2 by replacing the *Eco*RI/*Bgl*II fragment with the corresponding fragment of L1 lacking exon 2 as was done for the Fc construct above. After transformation into bacteria, plasmid DNA was isolated and 50 μ g aliquots of each construct was linearized with 50 units of *Pac*I overnight under strict RNase-free conditions; the reaction was stopped with EDTA and the DNA was extracted with phenol/chloroform, chloroform, and then precipitated in potassium acetate/ethanol. The DNA pellets were then washed once in 70% ethanol to remove any residual organic solvents. This typically gave a yield of approximately 10 μ g of linearized plasmid. A helper plasmid, DH-26S, provided by the manufacturer, which contains the structural proteins for packaging the Sindbis viral genome, was linearized by overnight digestion with *Xho*I, and prepared as described for pSinRep5 above. RNA was prepared from linearized pSinRep5/L1, pSinRep5/ $\Delta 2L1$, and DH-26S via *in vitro* transcription under a Sp6 promoter using the Invitrogen Sp6 transcription kit as suggested by the manufacturer's protocol. Each reaction typically produced approximately 20 μ g of RNA. For virus production, 3×10^5 baby hamster kidney (BHK) cells were plated in 2 ml α MEM, 5% FCS, 2 mM glutamax-1, 60 μ g/ml gentamicin, in a standard tissue culture six-well plate overnight. The next day, the cells (at ~80–90% confluency) were co-lipofected with viral and helper plasmid RNAs using DMRIEC reagent (Life Technologies, Inc.). Briefly, 6 μ l of DMRIEC reagent was combined with 10 μ l of the RNA in 1 ml Optimem in 5 ml plastic test tubes (Falcon)

and vortexed immediately. Meanwhile, BHK cells were washed two times with Optimem, and the lipofection suspensions (1 ml each from L1 viral RNA and DH-26S RNA) were gently added to the BHK cell monolayer and swirled briefly to disperse the complexes. These cells were incubated in a 5% CO₂ tissue culture incubator for 30–48 h after which the supernatant medium containing the virus was collected, centrifuged to remove cellular debris and stored at –70°C. Viral titer was determined by staining the transfected BHK cells for L1 expression.

L1 Cell Binding Assay

BHK cells were plated on glass coverslips in a 24-well plate at 20,000 cells per coverslip 1 day before infection to attain optimal cell density at 37°C. Either L1 or Δ 2L1 Sindbis virus, at a ratio of 1:100 in α MEM (GIBCO) with 5% FCS, was added to the cells and incubated in a 5% CO₂ tissue culture incubator for 1 h at 37°C. The media was then replaced with α MEM containing 1% FCS and incubated overnight. The next day, the coverslips were washed three times with Leibovitz-15 (L15) medium (GIBCO) and 30 μ l of either Δ 2L1-Fc or L1-Fc protein was incubated with the coverslip for 1 h at 4°C and then the coverslips were washed three times in L15 media. Binding of Fc fusion protein was visualized following incubation with anti-human IgG conjugated to Alexa 594 (Molecular Probes) for 30 min at 37°C, washing three times with L15 media and fixation with 4% paraformaldehyde. Infection of L1 proteins was assessed post-fixation with an anti-L1 mAb 2C2 (30 min at 37°C) and visualized with a secondary anti-mouse IgG conjugated to Alexa 488 (Molecular Probes) for 30 min at 37°C. Images were captured digitally with a Zeiss LSM510 confocal microscope and the mean intensities were measured using the histogram function of version 2.8 software; data was downloaded into Excel and means were calculated.

RESULTS

L1 without Exon 2 Lacks Homophilic Binding Activity

To assess the functional properties of exon 2 in L1, we prepared fusion proteins consisting of the six Ig domains of human L1 with the constant regions of human IgG either with (L1-Fc) or without (Δ 2L1-Fc) exon 2 (Fig. 1). Given that previous studies using protein coated beads demonstrated that L1 bound by a homophilic mechanism (De Angelis et al., 1999; Grumet and Edelman, 1988), we analyzed the binding properties of the Δ 2L1-Fc and L1-Fc when bound to fluorescent protein A beads. A comparison of Δ 2L1-Fc and L1-Fc proteins on SDS gels did not reveal any difference between them, consistent with the negligible difference in their sizes [Fig. 1(D)]. Like L1-Fc (Haspel et al., 2000), Δ 2L1-Fc was rec-

ognized by the anti-L1 monoclonal antibody Neuro 4.1 [Fig. 1(E)]. Δ 2L1-Fc and L1-Fc proteins bound equally well to protein A coated beads resulting in bead preparations containing equivalent amounts of each protein [Fig. 1(F)]. To test for homophilic binding, equal volumes of protein-bound beads were dissociated by sonication and allowed to aggregate at 37°C for 2 h. Microscopic observation of aliquots immediately following sonication confirmed that the beads were dissociated. At 2 h of incubation, nearly all of the L1-Fc beads were in aggregates, in contrast to Δ 2L1-Fc beads that showed very little evidence of aggregation (Fig. 2). Most of the Δ 2L1-Fc beads were still present as individual beads, and no large aggregates were observed. Quantitation to measure the appearance of aggregates indicated much fewer super-threshold particles with Δ 2L1-Fc beads than with L1-Fc beads [Fig. 2(C)]. By comparison to L1-Fc that aggregated well and the negative control (L1-1212-Fc) that had no detectable L1 binding activity (Haspel et al., 2000), there was little or no homophilic binding activity in the Δ 2L1-Fc beads.

Δ 2L1 Can Interact with L1 on Beads

To investigate further the binding activity of Δ 2L1, we tested its interaction with L1. In these experiments, equal volumes of purified Δ 2L1-Fc bound to green fluorescent beads and purified L1-Fc bound to red fluorescent beads were dissociated and then combined and allowed to aggregate. As a positive control for coaggregation, we used mixtures of red and green beads both of which contained L1 protein [Fig. 3(A)]. The aggregates formed in this control exhibited significant intermixing of red and green beads as expected. Interestingly, we saw coaggregation of Δ 2L1 beads with L1 coated beads [Fig. 3(B)], but most of the beads in the aggregates were the red L1 beads with much fewer green Δ 2L1 beads. Moreover, the Δ 2L1 beads typically surrounded the L1 beads suggesting that Δ 2L1 bound more weakly than L1. In contrast, there was little or no binding of the Δ 2L1-Fc coated beads to aggregates formed by beads coated with a different adhesion molecule [Fig. 3(D)]. Quantitation of the coaggregation index confirmed a relatively high degree of co-localization for Δ 2L1 beads with L1 beads by comparison to the strong signal for L1 beads with L1 beads [Fig. 3(E)]. These observations suggest that Δ 2L1 can interact with L1 despite the fact that it does not interact with itself sufficiently well to support bead self aggregation (Fig. 2). As an additional control, we tested beads coated with L1-1212-Fc, a recombinant L1 fusion protein containing tandem repeats of the first and second Ig domains that was

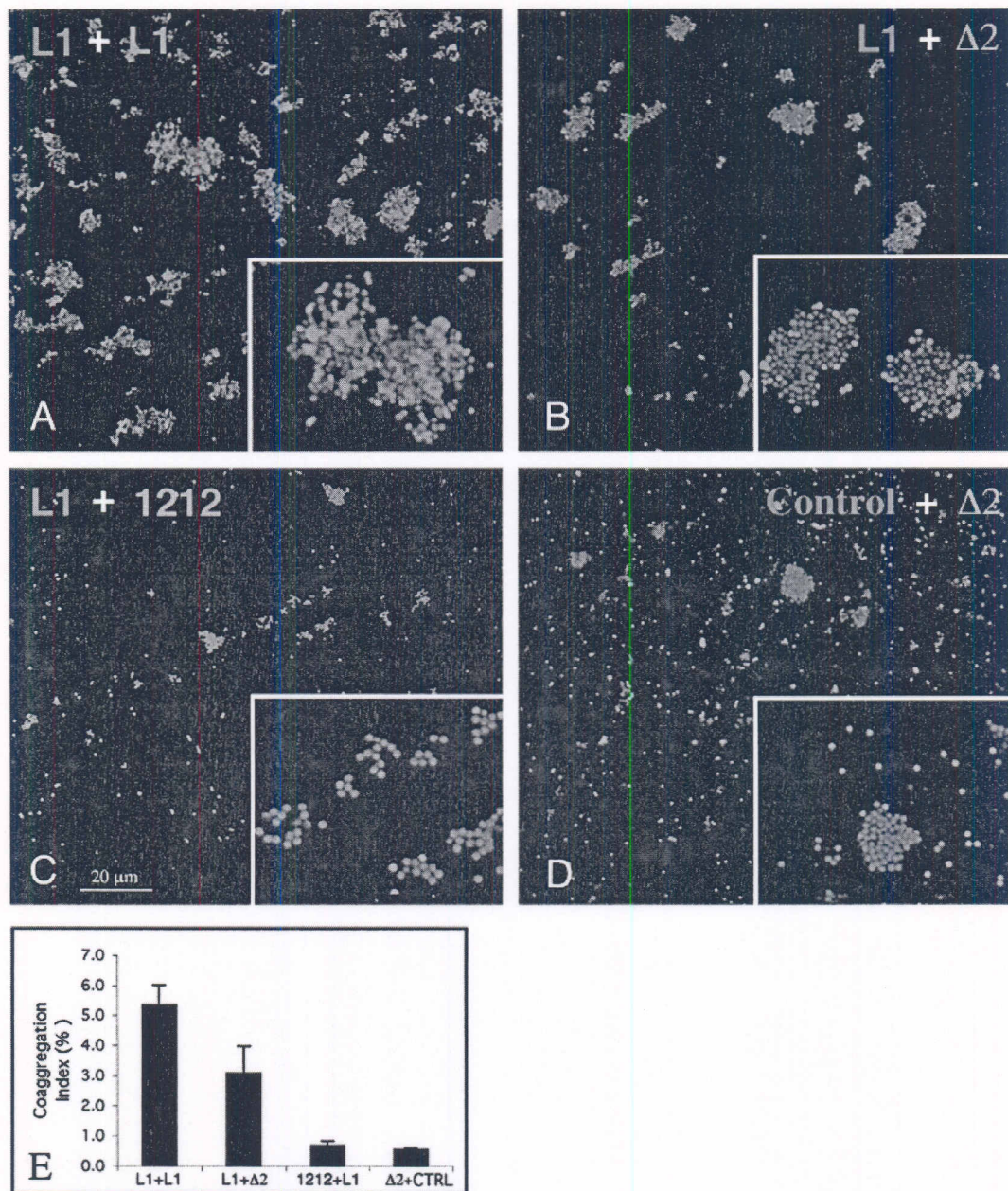


Figure 3 Mixed fluorescent bead aggregation experiments reveal interaction between $\Delta 2L1$ and L1. (A) Red and green fluorescent beads each coated with L1-Fc demonstrated robust coaggregation. (B) Red fluorescent beads coated with L1-Fc coaggregated with green fluorescent beads coated with $\Delta 2L1$ protein. (C) Green beads coated with L1-1212-Fc showed no coaggregation or trapping in aggregates of red beads coated with L1-Fc. (D) Green fluorescent beads coated with $\Delta 2L1$ did not bind to red beads coated with neurofascin-Fc that aggregated. Bar = 20 μm . (E) Random fields from two independent experiments were quantitated using Bitplane software to determine the colocalization index as described in Materials and Methods, and average values were calculated.

found previously to be inactive (Haspel et al., 2000), and we confirmed that it did not coaggregate with L1 [Fig. 3(C,E)].

Interactions of $\Delta 2L1$ with L1 when Expressed on Cells

The experiments using protein coated beads indicated that $\Delta 2L1$ did not interact with itself but it could bind to L1. To investigate this interaction further in a more biological context, we expressed full-length L1 as transmembrane proteins in BHK cells transduced with viruses encoding the protein either with (L1) or without ($\Delta 2L1$) exon 2. Following the infection, we confirmed the surface expression of L1 and $\Delta 2L1$ using monoclonal antibody Neuro 4.1 that binds to the Ig domains (data not shown). To test for L1 binding, live cells were incubated with soluble L1-Fc or $\Delta 2L1$ -Fc and the Fcs were visualized following incubation with a mouse antibody against human IgG. The cultures were then fixed and L1 expressing cells were visualized by immunofluorescence staining with monoclonal antibody 2C2, directed against the conserved cytoplasmic domain of L1 (Lustig et al., 2001) that is not present in the Fc fusion protein. Strong binding of L1-Fc was detected to BHK cells expressing L1 proteins but not to the nonexpressing cells in the same culture [Fig. 4(A)]. In contrast, relatively weaker binding of L1-Fc was detected to BHK cells expressing $\Delta 2L1$ protein (Figs. 4, 5). Moderate to strong binding was detected for $\Delta 2L1$ to cells expressing L1 but not to cells expressing the $\Delta 2L1$ form of the protein. Quantitation of the degree of co-localization of the different bound L1 proteins with the L1 proteins expressed in the cells by viral infection [Fig. 5(A)] indicated strong binding of L1 to L1, little or no binding of $\Delta 2L1$ to $\Delta 2L1$, and intermediate levels of binding between $\Delta 2L1$ and L1. The average level of expression of L1 was not found to differ significantly from that seen for $\Delta 2L1$, indicating that both proteins are efficiently expressed on the cells [Figs. 4, 5(B)]. Taken together, our results indicate that the $\Delta 2L1$ can interact with L1 on cells but the $\Delta 2L1$ does not appear to bind to itself.

$\Delta 2L1$ Supports Diminished Levels of Neurite Outgrowth from Cerebellar Neurons

Homophilic binding is believed to be an important mechanism by which L1 mediates neurite outgrowth and fasciculation of neurites (Lemmon et al., 1989), and therefore it was of interest to test $\Delta 2L1$ in neurite outgrowth assays. L1 provides an excellent substrate

for promoting neurite outgrowth, but $\Delta 2L1$ was found to be less effective in promoting neurite outgrowth from dissociated neurons [Fig. 6(A–D)]. The percentage of neurons with neurites on $\Delta 2L1$ was less than half that observed on L1 [Fig. 6(D)]. The average length of neurites that did extend on $\Delta 2L1$ was lower than on L1. The observation that neurite outgrowth on $\Delta 2L1$ was somewhat less effective than on L1 is consistent with the idea that the avidity of $\Delta 2L1$ binding to L1 is weaker than that for L1 to L1.

We also tested neurite outgrowth from reagggregates of neurons that may more closely approximate the situation *in vivo* where neurites make choices among different substrates. Again, L1 was clearly more effective than the $\Delta 2L1$ as a substrate in promoting outgrowth from neuronal reagggregates over a range of concentrations tested [Fig. 6(E–G)]. There appeared to be fewer neurites per aggregate on $\Delta 2L1$ than on L1, but this was not quantitated because the number of cells per aggregate varied and could not be measured. In any case, the neurites that did extend processes on the $\Delta 2L1$ substrate on average were shorter than those on the L1 over a range of doses. A possible explanation for the more dramatic differences between the $\Delta 2L1$ and L1 in the reaggregate assay is that neurites expressing L1 prefer to bind to other neurons rather than to the $\Delta 2L1$ substrate. This difference is less pronounced with the dissociated cells possibly because they have little or no contact with their neighbors; rather, they are exposed primarily to the substrate and there is no competition with other surfaces (e.g., cells) as can occur in the reaggregate.

DISCUSSION

This study indicates that the alternatively spliced second exon in the extracellular region of L1, which lies amino terminal to the Ig domains, can modulate L1 function. We focused here on the Ig domains of L1 insofar as they appear to represent a critical functional region of L1 that can account for all or most of the neurite outgrowth promoting activity of the molecule (Haspel et al., 2000). In the absence of exon 2 in L1, we found little or no homophilic binding between the $\Delta 2L1$ proteins. However, the $\Delta 2L1$ protein can still interact sufficiently well with the neuronal form of L1 protein to support a moderate level of neurite outgrowth when the protein is used as a substrate. Previous studies demonstrated reduced binding for mutated forms of L1 (De Angelis et al., 1999, 2001), but our study is the first to show that a pathological mutation in human L1 is associated with a deficiency

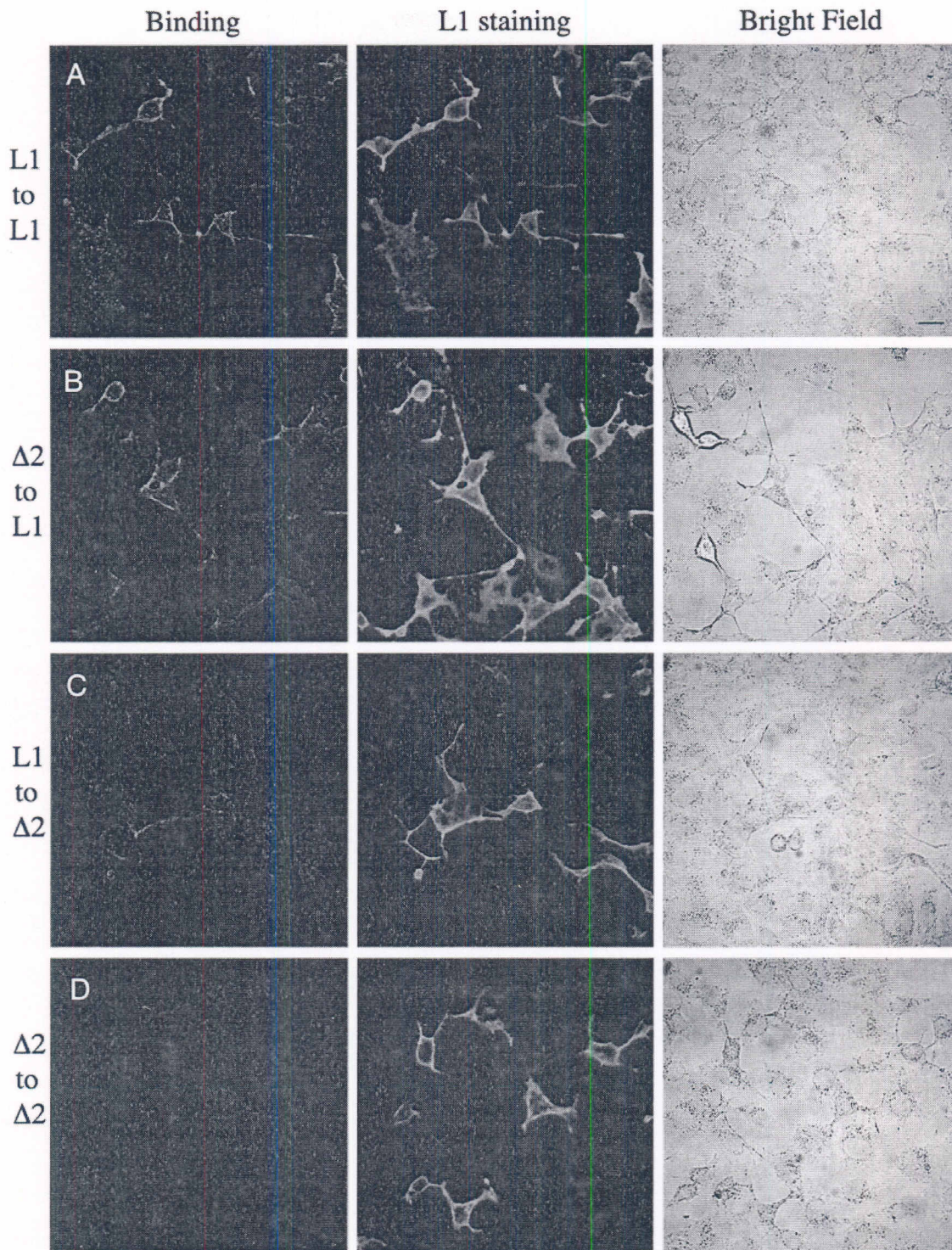


Figure 4

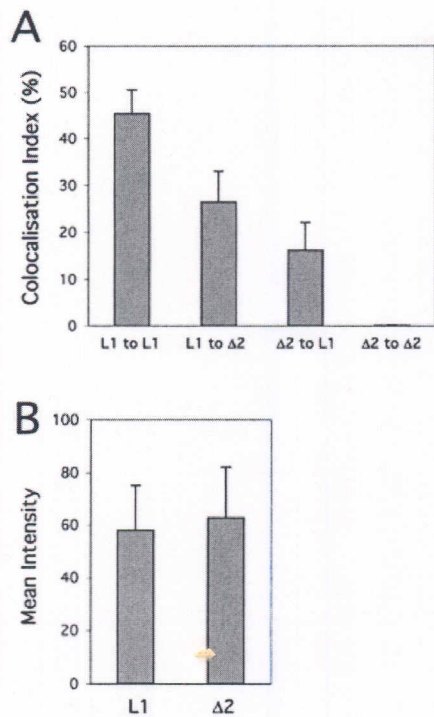


Figure 5 Quantitative analysis of binding of soluble L1 and $\Delta 2$ L1 to BHK transductants. (A) Binding of soluble L1 proteins to cells expressing transmembrane forms of L1 was performed as described in Figure 4 and quantitated using Bitplane software to determine the co-localization index as described in Materials and Methods. Labels correspond to those in Figure 4 and average values were calculated. (B) Intensity of immunofluorescence staining for L1 and $\Delta 2$ L1 expressed in cells (see Fig. 4) was quantitated using LSM software and the mean intensity values were calculated.

in neurite outgrowth activity. This was manifest both as a decrease in the percent of cells extending neurites as well as a decrease in the length of neurites that

Figure 4 Binding of L1 and $\Delta 2$ L1 to cells expressing L1 and $\Delta 2$ L1. BHK cells were infected with Sindbis virus encoding either L1 or $\Delta 2$ L1 and subsequently incubated with soluble Fc fusion proteins. Binding of L1 proteins to live cells (red) was detected by an Alexa 594-conjugated antibody against hIgG. Following fixation, L1 staining (green) was detected by immunofluorescence labeling with the L1 monoclonal antibody 2C2 and visualized with anti-mouse IgG conjugated to Alexa 488. (A) L1 infected cells show strong binding of soluble L1-Fc. (B) L1 infected cells show strong-to-weak binding of soluble $\Delta 2$ L1 protein. (C) Cells infected with $\Delta 2$ L1 show weaker binding of soluble L1-Fc. (D) Cells infected with $\Delta 2$ L1 show little to no binding of the soluble mutant $\Delta 2$ L1-Fc.

were observed. Whereas one might have predicted reduced neurite outgrowth on $\Delta 2$ L1 based on its reduced binding activity (De Angelis et al., 2001), the reduction in the percentage of neurons extending neurites suggests a role for L1 in initiating neurite growth or promoting neurite stability as well as extension.

A role for the L1 exon 2 in homophilic and heterophilic binding was recently reported (De Angelis et al., 2001) while our manuscript was in preparation. In their study, deletion of exon 2 in the context of the full extracellular region of L1 reduced, but did not eliminate, homophilic binding. In contrast, we detected little or no homophilic binding in the context of only the Ig domains. While different kinds of beads were used, it is unlikely that this can account for the differences observed insofar as both studies employed anti-human IgG to anchor the Fc fusion proteins. It is possible that the residual level of homophilic binding detected between pairs of full-length extracellular L1 molecules lacking exon 2 could be mediated via the fibronectin type III repeats given evidence for its involvement in L1 multimerization in the full length molecule (Silletti et al., 2000). Whereas the FnIII repeats appear to mediate multimerization among adjacent molecules in *cis* (i.e., in the plasma membrane), they may also modulate the conformation of L1 and thereby its interactions in *trans* between cells (Silletti et al., 2000; Stallcup, 2000). Although there is no evidence for *trans* homophilic binding involving the FnIII repeats of L1 molecules on adjacent cells, such interactions could mediate binding when L1 is present on beads where the proteins are not constrained by anchorage in the plasma membrane. In any event, our inability to detect significant levels of binding of $\Delta 2$ L1 Ig domains as Fc fusion proteins to full-length $\Delta 2$ L1 expressed in cells (Figs. 4, 5) argues against *trans* homophilic interactions of the FnIII repeats with the Ig domains. Thus, there are multiple *cis* and *trans* interactions among L1 molecules that are mediated by different regions of the molecule, and additional studies using transgenic approaches will probably be needed to determine further their contributions to cell-cell interactions in vivo.

In addition to affecting homophilic binding, deletion of exon 2 in L1 also reduced heterophilic binding of TAG-1 and contactin/F11/F3 coated beads to cells expressing L1 (De Angelis et al., 2001). Heterophilic L1 binding may also have consequences for neurite outgrowth given known interactions of L1 (Ng-CAM, its chicken homologue) with TAG-1 (axonin-1, its chicken homologue) (Buchstaller et al., 1996; Malhotra et al., 1998). However, not all heterophilic interactions are modulated by exon 2, for example, its deletion did not effect binding of L1 to neurocan

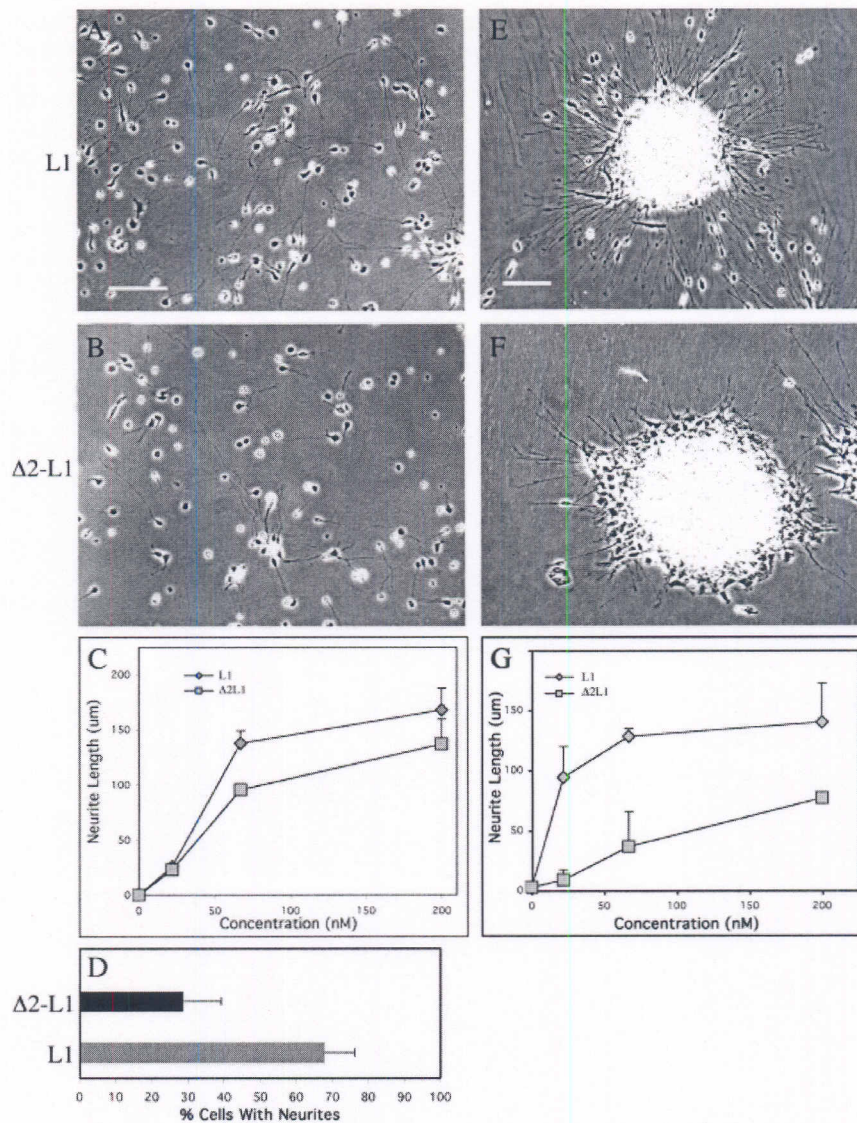


Figure 6 Δ2L1 supports reduced levels of neurite outgrowth from cerebellar neurons. Neurite outgrowth of mouse cerebellar neurons was examined using dissociated cells (A–D) and reaggregates (E–G) on L1-Fcs. P3-P4 mouse cerebellar cells or reaggregates were plated on substrates coated with 10 μg/mL poly-L-lysine, followed by 20–200 nM L1-Fc or Δ2L1-Fc. After 18 h, the cultures were fixed and randomly selected fields were captured for analysis. Representative images of neurons growing on substrates with 200 nM of the specified proteins are shown. Dissociated cerebellar granule neurons showed more robust neurite outgrowth on L1-Fc (A) than on Δ2L1 (B). Reaggregated cerebellar neurons grown on L1 substrate (E) show robust neurite outgrowth activity while reaggregates on Δ2L1 substrate (F) show fewer and shorter neurites. Quantitation of dissociated (C) and reaggregate (G) cell neurite outgrowth was performed as described (see Materials and Methods) and dose response curves are shown. The percent of cells extending neurites in dissociated cerebellar cultures was much lower on Δ2L1 than on L1 when measured (D) as described in Materials and Methods. Values in panels (C–E) represent means averaged over three experiments. Bars = 100 μm.

(Oleszewski et al., 2000). It therefore appears that exon 2 can selectively affect different functions of L1.

How exon 2 acts to modulate L1 function is still unclear. It is interesting that only the tyrosine residue present in exon 2 (YEGHHV) is conserved among L1 proteins in different species [Fig. 1(A)] and that alanine substitution of each of these residues except the tyrosine had no significant effect on homophilic binding (De Angelis et al., 2001). The horseshoe model of Ig superfamily proteins including L1 predicts that Ig domain 1 lies adjacent to Ig domain 4, and therefore exon 2, which is at the amino terminus of Ig domain 1, may lie adjacent to the border between Ig domains 4 and 5 [see Fig. 1(C)]. It has been suggested that exon 2 itself may not be an intramolecular binding site insofar as alanine substitution had no major effect on binding (De Angelis et al., 2001). However, it is possible that exon 2 could act perhaps as a spacer to modulate interactions of the six highly conserved amino terminal residues of L1 [e.g., IQIPEE; Fig. 1(A)] with the Ig domains. Deletion of exon 2 might alter the accessibility of these conserved residues such as the Proline (position 4) to interact with Ig domains 4 and/or 5, possibly to modulate the stability of the horseshoe structure in one L1 molecule or in a neighboring L1 molecule in the same plasma membrane (*in cis*). It has been suggested that Fc fusion proteins may mimic the close parallel packing of L1 at the cell surface that is believed to be important for adhesion (Doherty et al., 1995), and the functional differences between $\Delta 2L1$ -Fc and L1-Fc may reflect *cis* interactions between L1 molecules that are modulated by exon 2.

Pathological human mutations are believed to eliminate exon 2 in L1 in certain patients with X-linked hydrocephalus (Finckh et al., 2000; Jouet et al., 1995). Our observations, and those of DeAngelis and colleagues on defects in L1 homophilic binding in the absence of exon 2 (De Angelis et al., 2001) predict a significant disruption of L1-mediated function including axonal growth, guidance, and fasciculation that could account for the human phenotype (Kamiguchi et al., 1998a). Given that both the number of neurites extended and the average length of neurite outgrowth from neurons were reduced significantly on substrates coated with L1 lacking exon 2 by comparison to L1 with exon 2, it is likely that neurons expressing only $\Delta 2L1$ would be affected more severely. Interestingly, there is little evidence of brain defects in humans that are heterozygous for various mutations in L1 suggesting that few, if any, behave as dominant negatives (Wong et al., 1995). The lack of defects in heterozygotes may simply reflect selective survival of neurons expressing the native form of L1. However, the ability

of $\Delta 2L1$ to interact somewhat with neuronal L1 raises the possibility that some mutant neurons expressing $\Delta 2L1$ may be able to survive in heterozygotes by interacting with cells expressing normal neuronal L1. While $\Delta 2L1$ may retain residual binding activity, other deletions or truncations may lead to inactive forms of the protein. For example, we recently reported that a truncated form of L1 containing only Ig domains 1–3 formed *cis* dimers that were completely inactive in promoting neurite outgrowth (Haspel et al., 2001b).

Neurons normally express L1 containing both exons 2 and 27 but alternative splicing of these exons results in their absence from L1 expressed in nonneuronal cells. Alternative mRNA splicing has been found in several related proteins in the Ig superfamily and is believed to play a functional role for N-CAM (Cunningham et al., 1987) and neurofascin (Volkmer et al., 1998) where complex patterns of splicing have been observed. Both exons 2 and 27 are absent from L1 found in nonneuronal cells including Schwann cells, astrocytes, oligodendrocytes and lymphocytes (Itoh et al., 2000; Jouet et al., 1995; Takeda et al., 1996). The dramatic increase in L1 isoforms containing exons 2 and 27 during oligodendrocyte maturation suggest that the more mature oligodendrocytes may have increased affinity for axons because of changes both in the amount and form of L1 expressed (Itoh et al., 2000). For nonneuronal cells expressing L1 lacking exon 2, however, the results suggest there would be little or no contribution of the Ig domains to L1 homophilic binding and it is uncertain whether the fibronectin repeats might contribute to this interaction *in vivo*. There is no evidence suggesting that L1 mediates homotypic interactions among nonneuronal cells such as Schwann cells. However, there is evidence in the peripheral nervous system (Seiheimer et al., 1989; Wood et al., 1990) for a role of L1 on the axon in myelination, not with L1 lacking exon 2 on Schwann cells, but rather via a heterophilic mechanism (Haney et al., 1999).

Heterophilic L1 interactions may also play a role in lymphocyte activation by at least two mechanisms including L1 binding via the RGD sequence in its sixth Ig domain to integrins, and L1 binding via its FnIII repeat to integrins (Balasian et al., 2000; Montgomery et al., 1996; Silletti et al., 2000). The involvement of homophilic L1 binding has been suggested but that was based on experiments using neuronal isoforms of L1 containing exon 2 that would not normally be found in lymphocytes (Balasian et al., 2000). Thus, considering the weak interaction of $\Delta 2L1$ to $\Delta 2L1$, it is possible that homophilic $\Delta 2L1$ binding may not play a significant role in mediating

adhesion among lymphocytes and other nonneuronal cells. On a general note, it is remarkable how the immune system and nervous system express many of the same CAMs including L1, yet immune attack of neural tissue is rare, even in the peripheral nervous system. Perhaps one way of ensuring that the immune system treats neural tissue as "immune privileged," is to ensure that CAMs shared by these tissue types such as L1 do not preferentially bind to each other. More research will be necessary to explore the idea that nonneuronal L1 lacking exon 2 may not effectively mediate homophilic binding *in vivo*.

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