

## Alternative Use of a Mini Exon of the *L1* Gene Affects L1 Binding to Neural Ligands\*

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Elena De Angelis‡§, Thomas Brümmendorf¶, Ling Cheng||, Vance Lemmon||, and Sue Kenwrick‡

From the ‡Wellcome Trust Centre for Molecular Mechanisms in Disease and Cambridge University Department of Medicine, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2XY, United Kingdom, the ¶Max Delbrück Center for Molecular Medicine, Robert-Rössle-Strasse 10, 13092 Berlin, Germany, and the ||Department of Neurosciences, Case Western Reserve University, Cleveland, Ohio 44106-4975

**Neural cell adhesion molecule L1 is a cell surface glycoprotein required for the correct development of the nervous system. L1 exists as two isoforms encoded by mRNA species that either collectively incorporate or exclude exons 2 and 27. Neurons utilize only the full-length isoform, whereas Schwann cells, kidney cells, and blood lymphocytes only express the short form of L1. Still other cells, oligodendrocytes, regulate L1 isoform expression in a maturation-dependent manner. The RSLE motif encoded by exon 27 is known to have a role in clathrin-mediated endocytosis of L1, but the function of the exon 2-encoded motif (YEGHHV) is unknown. Here we show that this motif is required for the optimal binding of L1 to several neural ligands and is likely to be important for nervous system development. Thus, alternative use of exon 2 is a mechanism for regulating ligand interactions with L1.**

Neural cell adhesion molecule L1 is one member of a subfamily of immunoglobulin (Ig) superfamily proteins that acts as a cellular and axonal guidance cue and receptor during nervous system development. L1 is expressed primarily on the surface of axons and growth cones of developing neurons and is thought to have a role in neuronal cell migration, fasciculation, axon growth, and guidance (1).

Mutations in the *L1* gene are responsible for a congenital neurological disorder in humans (X-linked hydrocephalus, mental retardation, aphasia, shuffling gait, and adducted thumbs (MASA) syndrome, or spastic paraplegia type I). Clinical signs of this disease include hydrocephalus, mental retardation, adducted thumbs, and lower limb spasticity. Abnormalities of corpus callosum and corticospinal tract development in patients and L1 knockout mice indicate a pivotal role for L1 in the growth or guidance of these particular axon tracts. L1-deficient mice also show reduced interaction between Schwann cells and the axons of unmyelinated sensory nerves, resulting in degeneration of axons and impaired sensory function. Thus L1 may also be a neuronal receptor for survival cues provided by glial cells.

Outside the nervous system L1 is expressed on subsets of cells including B-lymphocytes, cells of the intestinal crypt, and kidney epithelial cells. L1 is clearly required for normal kidney

morphogenesis, but specific roles in the intestine and immune system have not yet been defined (reviewed in Ref. 2).

The L1 glycoprotein comprises six Ig-like domains and five fibronectin type III repeats in the extracellular region, a single pass transmembrane domain, and a cytoplasmic tail. Through its extracellular domains L1 can interact with a variety of ligands. Determining which interactions are important for the development or function of the nervous system or other tissues is fundamental to understanding the complex biology of L1. Homophilic interaction is likely to be an important mediator of neurite outgrowth in nervous system development, because explanted neurons will extend neurites on an L1 substrate only if L1 is present as a receptor. However, interactions with other proteins on the same membrane or in *trans* are likely to be involved in L1 function. For example, when dorsal root ganglion cells extend neurites on an NgCAM substrate (the chick homologue of L1), cell surface NgCAM recruits axonin-1, another Ig superfamily member, in a receptor complex (3). Thus L1 is likely to be a dynamic receptor with multiple binding mechanisms and functions. The factors that regulate L1 interactions in different cell types are not understood.

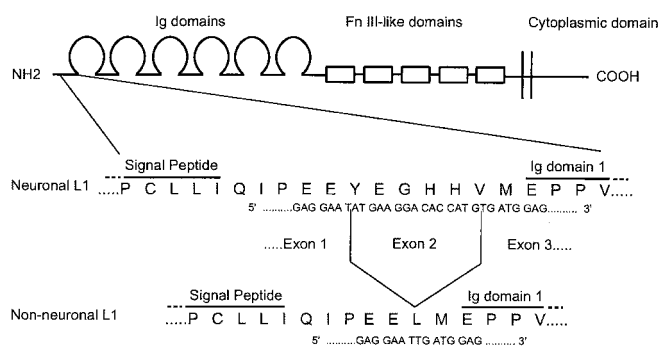
A major difference between L1 present in neurons and in most other cell types is dictated by alternative splicing of L1 pre-mRNA (4, 5). L1 has 29 exons, 28 of which are coding (here designated 1b-28) and one that contains an 5'-untranslated sequence (exon 1a). Neurons utilize the entire 28-exon coding sequence (6). In contrast, mRNA that lacks exons 2 and 27 is exclusive to nonneuronal cells, although oligodendrocytes have been found to express both isoforms in a maturation-dependent manner (7). Exon 2 nonsense or frameshift mutations have been found in patients with X-linked hydrocephalus, in keeping with the observation that all neuronal transcripts are of this form. Differential use of exons 2 and 27 in neuronal *versus* nonneuronal cells is conserved for L1 orthologues in rodents (5, 8) and the teleost fish, fugu (9), suggesting that it has functional importance.

Exon 27 encodes a 4-amino acid peptide, RSLE, that is part of a cytoplasmic tyrosine-based sorting motif, YRSL. Studies by Kamiguchi *et al.* (10, 11) have shown that indeed this motif is required for the recycling of L1 by clathrin-mediated endocytosis.

The inclusion of exon 2 in L1 mRNA provides a 6-amino acid motif (YEGHHV) in place of a single leucine residue prior to the first Ig domain (Fig. 1). The function of this motif is, however, largely unexplored. We have examined the influence of the exon 2 motif on L1 trafficking in neuronal cells and on the ability of L1 to bind to itself and two other Ig superfamily ligands found primarily in the nervous system, TAX-1 (rodent TAG-1/chick axonin-1) and contactin (rodent F3/chick F11). We find that inclusion of the exon 2-encoded motif is not required

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§ To whom correspondence should be addressed: Tel.: 44-1223-331756; Fax: 44-1223-331206; E-mail: ed214@mole.bio.cam.ac.uk.



**Fig. 1. Schematic representation of the neuronal and nonneuronal isoforms of L1.** The exon 2-encoded region prior to Ig domain 1 is highlighted in relation to the full-length L1 protein. The mRNA sequences of wild type and exon 2-minus isoforms of L1 are shown below the protein translation. In the nonneuronal isoform, exon 2-minus L1, the YEGHHV peptide is replaced by a single leucine residue. *Fn III*, fibronectin type III.

for the correct trafficking of L1 in neuronal cells but is required for efficient binding to all three ligands. Similarly reduced binding has been found for patient mutations that cause X-linked hydrocephalus (12), indicating that exon 2-encoded residues are required for the neuronal function of L1. Furthermore, this suggests that distinct functions of L1 in neuronal and nonneuronal tissues could be mediated by differences in binding preferences.

#### EXPERIMENTAL PROCEDURES

**Mutagenesis**—L1 cDNA without the 15-bp<sup>1</sup> sequence encoded by exon 2 was created by polymerase chain reaction-directed mutagenesis (12) and BIO-X-ACT™ (Bioline) proofreading polymerase. In a first-round reaction, an antisense primer containing the junction sequence of exon 2-minus cDNA (5'-TGACAGGTGGCTCCATCA/ATTCCTCGGGG-ATCTGGA-3') was used with a primer designed from pBluescript (V1, 5'-CATCAAAGGGAACAAAAGCTGGAG-3') to amplify L1 sequences from a pBluescript clone containing the full 3.9-kilobase L1 cDNA (pBS-L1, a gift from John Hemperly). The 189-bp product was used as a megaprimer in a second-round reaction with a downstream antisense primer (5'-ATGGCTCTGGGGCTTGTGCAG-3'). The 1111-bp product was digested with *EcoRI* and *SphI*, and the resultant 980-bp fragment was substituted for the wild-type *EcoRI/SphI* section of L1 cDNA. The mutated cassette was sequenced using the Prism™ dye terminator kit (PerkinElmer Life Sciences) and an ABI 373 semi-automated DNA sequencer.

The individual amino acid residues of exon 2 were sequentially substituted with an alanine residue using a similar method. First-round reactions utilized a sense mutagenic primer containing the altered bases with antisense primer L9 (5'-CCGTTCTGCCCCATCGTCA-C-3') to produce a 490–505-bp megaprimer from wild-type pBS-L1 template. An aliquot of megaprimer was then used with a sense-flanking primer ER1 (5'-CCGAATTCGCGCGCCGGAAAGAT-3') in a second-round polymerase chain reaction producing a 596-bp L1 fragment containing the mutation. This was digested with *EcoRI* and *BglII* restriction enzymes and substituted for the equivalent region in pBS-L1. The clones were sequenced across the mutated cassettes.

The mutagenic primers (with the mutations highlighted in bold) were: Y26Amp (CCCAGGAAGCCGAAGGACAC), E27Amp (GAGGAATATGCCGACACCAT), G28Amp (GAATATGAAGCCACCATGTG), H29Amp (TATGAAGGAGCCCATGTGATG), H30Amp (GAAGGACACGCCGTGATGGAG), and V31Amp (GGACACCATGCCATGGAG-CCA).

**Proteins**—Constructs containing sequences encoding the extracellular portions of human L1, mouse F3 (the mouse orthologue of chick F11 and human contactin), or human TAX-1 (the orthologue of chick axonin-1 and mouse TAG-1) in conjunction with sequences encoding human IgG Fc domains were used to produce soluble chimeric proteins. The L1-Fc construct was described by De Angelis *et al.* (12). The F3-Fc construct was a gift from C. Faivre-Sarrailh and G. Rougon (CNRS,

France). The TAX-Fc clone was constructed by engineering the extracellular portion of human TAX-1 cDNA into pIgplus (Invitrogen). The primers TH3F (5'-CTGGACTTTCTCAAGCTCTAGTC-3') and THXE (5'-GCCATGATATCCACCATCATTCTAGAGCCTCCATTCTCT-3') were used to amplify a 252-bp polymerase chain reaction product from a human full-length TAX-1 clone (a gift from P. Sonderegger, Switzerland). This introduced an *XbaI* restriction enzyme site immediately prior to the glycosylphosphatidylinositol-linkage sequence. This fragment was cut with *BstBI* and *XbaI* and used in a three-way ligation with a 2.8-kilobase *HindIII/BstBI* fragment of TAX-1 cDNA corresponding to the upstream coding sequence and *HindIII/XbaI*-cut pIgplus. The *BstBI/XbaI* cassette and cloning sites were sequenced.

Soluble L1-Fc (mutated and wild type), TAX-Fc, and F3-Fc chimeric proteins were produced by transiently transfecting COS-7 cells with 10  $\mu$ g of DNA/150-mm culture dish. The soluble Fc chimeric protein was allowed to accumulate in the medium for 6 days and was recovered and purified by Protein A-Sepharose affinity chromatography as described by De Angelis *et al.* (12).

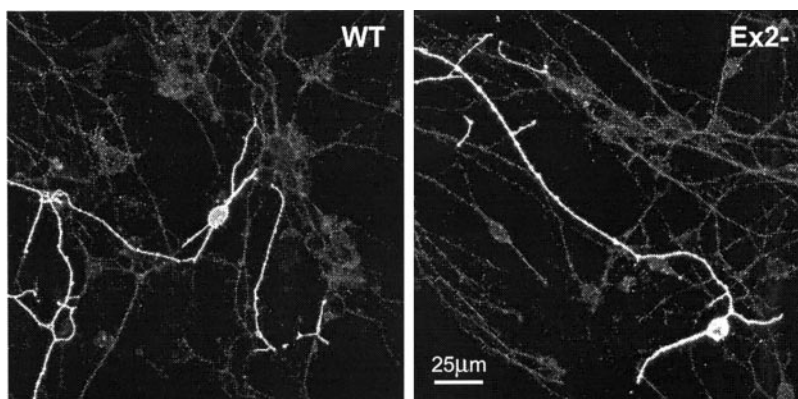
**Homophilic Binding**—The homophilic binding assay was conducted according to the method described by De Angelis *et al.* (12). Bioclean fluorescent microsphere beads (Red, 0.6  $\mu$ m, Duke Scientific Corp.) were precoated with anti-human IgG antibody (Fc-specific, Sigma, I-2136). Briefly, 250  $\mu$ l of fluorescent beads were incubated for 1 h at 37  $^{\circ}$ C with 50  $\mu$ l of antibody and 700  $\mu$ l of PBS. The beads were then washed with PBS/5% fetal calf serum, incubated for 30 min at room temperature, and stored in 250  $\mu$ l of PBS/0.02% azide. The same batch of antibody-coated beads was used throughout the study. 2.5  $\mu$ g of L1 mutant or wild-type Fc protein was conjugated to 10  $\mu$ l of antibody-coated beads by incubating for 2 h at 37  $^{\circ}$ C. Excess unbound protein was removed by washing with PBS/5% fetal calf serum, and the beads were resuspended in 60  $\mu$ l of PBS/5% fetal calf serum. For the homophilic binding assay, L1-Fc-coated beads were disaggregated to a single bead suspension by trituration followed by a 30-min incubation in an iced water bath sonicator. Homophilic aggregation was allowed to occur by incubating the single bead suspension at 37  $^{\circ}$ C with samples removed in duplicate over a 30-min time course. Samples were diluted 1:5000 times in ice-cold PBS and analyzed using a Becton Dickinson FACSort. 10,000 particles were sampled for each time point, and the number of particles with single bead fluorescence levels was measured in comparison to the number of particles with multiple bead fluorescence levels (clustered particles). Each Fc protein was assayed at least three times in parallel with WT to control for variation between experiments and standardized to a WT binding curve. As a control to ensure that equivalent concentrations of the Fc chimeric proteins were captured by the antibody-coated beads, 30- $\mu$ l samples of coated beads were denatured at 95  $^{\circ}$ C in sample buffer, and the released Fc chimeric proteins were subjected to SDS-polyacrylamide gel electrophoresis and shown to be present in equal proportions (12). No unbound Fc protein was detected under assay conditions as assessed by SDS-polyacrylamide gel electrophoresis.

A two-color aggregation assay was developed using red and green fluorescent microsphere beads. Microspheres were coated with anti-human IgG antibody and conjugated with either L1 or exon 2-minus L1 as described above. The protein-conjugated microspheres were disaggregated to produce single bead suspensions by trituration. A 1:1 mixture of red (WT-L1) and green (exon 2-minus L1) protein-conjugated beads was then prepared and further disaggregated by sonicating in an iced bath for 30 min. To allow mixed bead aggregation to occur, the beads were incubated at 37  $^{\circ}$ C with samples removed over a 30-min time course and diluted 1:5000 times in ice-cold PBS. 10,000 particles/sample were analyzed by FACS analysis. The number of two-color aggregates was quantified and compared with the number of mixed aggregates obtained when wild-type L1 was coated onto both red and green beads or when exon 2-minus L1 was coated onto both red and green beads.

**Heterophilic Binding**—Bioclean fluorescent microspheres were coated with anti-human IgG as described above and saturated with TAX-Fc or F3-Fc fusion proteins. Binding of the protein-conjugated beads to L1-transfected COS cells was conducted as described by Brümendorf *et al.* (13) with the exception that cells were maintained in Dulbecco's modified Eagle's medium/1% fetal calf serum before bead incubation and fixed with 1% formaldehyde for 15 min at room temperature before staining with polyclonal antibodies. COS cells are heterogeneous with respect to size, form, surface properties, and expression levels of heterologously expressed proteins; therefore, large numbers of cells have to be evaluated to quantify bead binding. An image analysis system described in detail previously (14) was adapted to quantify bead binding. Cells forming uninterrupted confluent monolayers were se-

<sup>1</sup> The abbreviations used are: bp, base pair(s); WT, wild type; FACS, fluorescence-activated cell sorter.

**FIG. 2. Deletion of the exon 2 motif does not affect L1 trafficking in cerebellar granule cells.** Cerebellar granule cells were transiently transfected with expression plasmids encoding wild-type L1 or L1 lacking exon 2 (*Ex2-*). Cell surface expression was detected by immunofluorescent labeling.



lected, and the integrity of the cells was monitored with monoclonal antibody 412, which is specific for the intracellular large T antigen, expressed by COS cells (15). After bead incubation, the cell monolayer was washed and fixed; L1-expressing cells (representing 10–50% of cells in the confluent monolayer) were identified by immunofluorescence analysis using L1-specific polyclonal antibodies (16). Images were captured separately for L1-expressing cells (Cy3-fluorochrome, red fluorescence) and beads (yellow fluorescence). L1-expressing cells were identified automatically and distinguished from untransfected cells by a fluorescence intensity threshold. Fluorescent beads were identified using size-exclusion criteria and intensity threshold. An automated comparison of each cell image with its corresponding bead image allowed the calculation of bead density on L1-expressing cells and background density on untransfected cells. Background binding, which was below 5% of WT L1 binding, was subtracted. Five independent analyses were performed for each combination of ligand and L1 variant, and in each analysis at least 200 transfected cells were evaluated. The data sets were compared with the Mann-Whitney *U* test implemented in the Statview program (Abacus Concepts, Inc., Berkeley, CA).

**Immunofluorescence Studies**—For transfection of cerebellar granule cells, 10  $\mu$ g of DNA diluted in 1 ml of Dulbecco's modified Eagle's medium was added to 30  $\mu$ l of TransFast transfection reagent (1 mg/ml) (Promega) and incubated at 37 °C for 45 min. Meanwhile, the cerebellum was dissected from P8 mice, dissociated in trypsin, and triturated in DNase solution. Dissociated cells were centrifuged and resuspended in Dulbecco's modified Eagle's medium to a concentration of  $2 \times 10^6$  cells/ml. 1 ml of cell suspension was added to the TransFast/DNA mix, plated onto poly-L-lysine and laminin-coated coverslips, and incubated at 37 °C for 90 min. They were washed and incubated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 5 mM KCl at 37 °C for 3 days prior to immunofluorescent labeling. The cells were fixed with 4% paraformaldehyde and labeled with rabbit anti-human L1 polyclonal antibody (1:1000) for 1 h and goat anti-rabbit IgG (Molecular Probes) Alexa 488-conjugated secondary antibody (1:300) for 1 h. The coverslips were mounted onto slides with the Slowfade Antifade kit (Molecular Probes).

## RESULTS

Because the exon 2-minus L1 mRNA is not found in neurons, we investigated the possibility that inclusion of the exon 2-encoded motif is required to ensure correct trafficking of L1 along neuronal processes. Exon 2-minus L1 was transfected into mouse cerebellar granule cells, and the expression pattern was compared with that of full-length protein (Fig. 2). Exon 2-minus L1 was expressed on the cell body and along neurites of the cerebellar granule cells in an identical pattern to full-length L1. The transfection efficiency of cerebellar granule cells was ~5%, and the experiment was repeated three times with similar results obtained. Similar results were also obtained using chick dorsal root ganglion cells.<sup>2</sup>

To investigate whether the alternative splicing of exon 2 has an influence on cell surface interactions, binding assays were conducted for three ligands, L1 and glycoposphatidylinositol-linked Ig superfamily members TAX-1 and F3, for the following

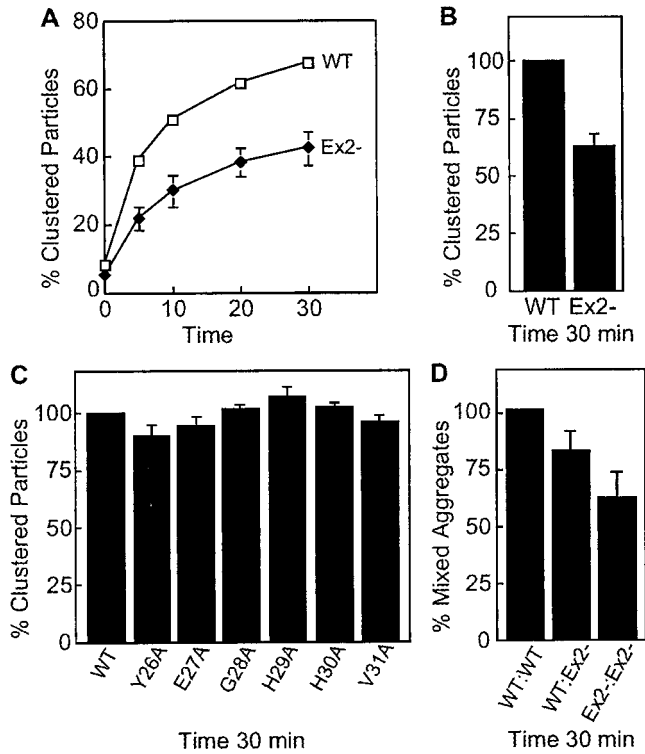
reasons. Homophilic binding by L1 is essential for promoting neurite outgrowth in response to immobilized or soluble L1 for several different types of neuron in *in vitro* assays (reviewed in Ref. 17) and is therefore likely to be an important mediator of axon growth *in vivo*. Studies using chick neurons indicate that this may depend on cooperation between L1 and TAX-1 on the neuronal surface (3). F3 was included, because it is a paralog of TAX-1 and may therefore function in a similar fashion. Moreover, several pathogenic missense mutations reduce binding to one or more of these ligands (12).

To compare homophilic binding of the peripheral (exon 2-minus) and neuronal (WT)-encoded forms of L1, fluorescent bead aggregation assays were used. WT-L1 or exon 2-minus L1-Fc was coated onto antibody-conjugated fluorescent beads and allowed to aggregate with samples removed between 0 and 30 min. The level of fluorescence associated with single particles compared with clustered particles was quantified by FACS analysis, in which the percentage of clustering reflects the percentage of homophilic binding. A time course of aggregation is shown in Fig. 3A. The exon 2-minus isoform of L1 has significantly reduced binding (63% of WT after 30 min), suggesting that the YEGHHV motif may be involved directly or indirectly in homophilic adhesion (Fig. 3, A and B). Binding of anti-Fc-coated beads only is ~4% of WT; therefore the antibody does not contribute to binding (12). A two-color fluorescent bead aggregation assay was used to investigate the interaction of WT-L1 with exon 2-minus L1. To examine this interaction, a two-channel FACS detection system was used to quantify the proportion of beads that formed mixed aggregates of L1 and exon 2-minus L1. The binding of WT-L1 to exon 2-minus L1 (81% of WT after 30 min) was greater than the binding of exon 2-minus L1 only (60% of WT after 30 min), suggesting WT-L1 partially rescued the loss of homophilic binding shown by exon 2-minus L1 (Fig. 3D).

To investigate whether the YEGHHV motif as a whole or individual amino acids are required for homophilic binding, single residues of this segment were replaced by alanine (Y26A, E27A, G28A, H29A, H30A, and V31A). Chimeric L1-Fc proteins were produced for each individual alanine substitution, and homophilic binding was assessed. All six mutated proteins exhibited wild-type levels of binding. Therefore, it is unlikely that an individual residue within the motif is required as part of a homophilic binding site (Fig. 3C).

For analysis of heterophilic interactions, we compared the binding of TAX-Fc and F3-Fc to full-length (WT) L1 with binding to L1 lacking the exon 2-encoded protein sequence. L1 protein was expressed at the surface of transiently transfected COS cells, and the binding of fluorescent microspheres coated with TAX-Fc or F3-Fc was quantified. To achieve this, digital image analysis procedures were used to evaluate more than 1000 transfected cells for each experimental condition. These

<sup>2</sup> L. Cheng, unpublished data.



**FIG. 3. Deletion of the exon 2 motif but not alanine substitution reduces homophilic binding.** Exon 2-minus L1-Fc constructs (*Ex2-*) or L1-Fc constructs with single amino acids of exon 2 replaced by alanine were assessed for their ability to bind homophilically (see "Experimental Procedures"). Homophilic binding is measured as the percentage of clustered particles formed over time by exon 2-minus L1 or alanine scan chimera compared with wild-type L1, in which clustered particles = >1 bead. The binding of exon 2-minus L1 compared with wild-type L1 is expressed as a time course (A), and the 30-min time point is shown in a histogram (B). Results for the alanine scan chimeric proteins are shown in a histogram of the 30-min time point (C). The values are expressed as a percentage of wild-type binding and are the mean and S.E. of at least four independent assessments. The binding of exon 2-minus L1 to wild-type L1 was assessed by FACS analysis using a two-color bead aggregation assay (D). The histogram shows the percentage of WT-L1 and exon 2-minus L1 mixed aggregates at the 30-min time point compared with the percentage of mixed aggregates formed when wild-type L1 or exon 2-minus L1 was coated onto both red and green beads. The values are the mean and S.E. of at least four independent assessments.

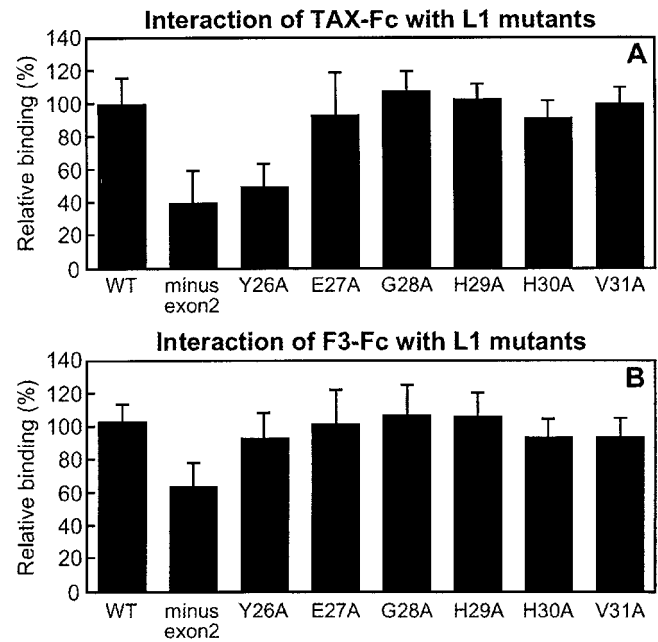
analyses demonstrate that the absence of exon 2 compromises binding of heterophilic ligands by 40 or 60% for F3-Fc ( $p < 0.05$ ) and TAX-1-Fc ( $p < 0.05$ ), respectively. Thus, the YEGHHV motif may also be directly or indirectly involved in heterophilic interactions (Fig. 4, A and B).

Quantification of bead binding to L1 mutant proteins revealed no significant effects of the alanine substitutions E27A, G28A, H29A, H30A, and V31A on both heterophilic interactions. However, substitution Y26A led to a reduced binding of TAX-Fc ( $p < 0.05$ ) but did not interfere with F3-Fc binding. This suggests that Y26A may have a more direct role in the binding of TAX-1 by L1 and argues for a difference in binding requirements for the two closely related ligands.

#### DISCUSSION

Neural cell adhesion molecule L1 exists as two isoforms that have distinct tissue distributions. Neuronal L1 is full-length, whereas other cell types express L1 that lacks residues encoded by alternatively spliced exons 2 and 27. Here we show that exon 2-encoded sequences influence the ability of L1 to bind biologically important ligands *in vitro*.

Exon 2-encoded sequences are not required for the move-



**FIG. 4. Deletion of the exon 2 motif interferes with heterophilic interactions of L1.** COS cells were transiently transfected with expression plasmids encoding wild-type L1, exon 2-minus L1, or L1 with single amino acids of exon 2 replaced by alanine. Shown are the binding of TAX-Fc-coated beads (A) and F3-Fc-coated beads (B) to the transfectants. The values are expressed as a percentage of wild-type binding with the mean and S.E. calculated from at least five independent analyses. The bar on each histogram represents data collected from more than 1000 transfected cells (>5 experiments with >200 cells).

ment of L1 along neuronal processes in culture. This is not surprising, because apart from the signal peptide, sorting signals usually reside in cytoplasmic regions. However, YEGHHV exclusion significantly reduces binding of L1 to itself and two heterophilic ligands, namely TAX-1/TAG-1/axonin-1 and contactin/F3/F11. The involvement of individual L1 (or NgCAM) domains in homophilic binding or interaction with orthologues of TAX-1 and F3 has been examined using domain-deletion studies and by examining the effects of patient mutations (12, 18, 19). These studies indicate that at least the first four Ig domains are required for all three types of interaction. In light of our results, a model for L1 interaction with these ligands must now incorporate the sequences encoded by exon 2, immediately upstream of Ig domain 1. There are two possibilities: either the YEGHHV sequence is part of a contact site between L1 and all three interacting proteins or the sequence provides an indirect structural function. If the region is part of an intermolecular contact site, we would expect that mutation of one or more individual residues would also affect binding. Sequential mutation of each residue to an alanine, however, had no effect on binding of L1 to either itself or F3. It is more likely therefore that the YEGHHV motif has an indirect effect on binding to these two ligands. Comparison of the first four domains of L1 with the resolved structures of those of axonin-1 and the insect protein hemolin strongly suggests that they adopt a horseshoe structure dependent on a short-hinge sequence located between Ig domains 2 and 3 (20, 21). The region prior to Ig domain 1 containing the YEGHHV motif would lie in the vicinity of the junction between Ig domains 4 and 5. A function of the motif could be to stabilize the horseshoe by direct interaction with these domains, or it may provide a 'spacer,' allowing NH<sub>2</sub>-terminal residues to undertake this role.

Oleszewski *et al.* (22) have shown that binding of L1 to neurocan, an extracellular matrix proteoglycan expressed primarily in the nervous system, depends on the presence of the Ig

domain 1 of L1. They also demonstrated that YEGHHV is not required, indicating that the exon 2 motif does not affect the geometry of neighboring Ig domain 1. This is in keeping with an effect on tertiary structure or domain presentation rather than secondary structure.

Alanine substitution revealed a difference in the binding characteristics of TAX-1 compared with F3. Mutation Y26A significantly reduced binding to TAX-1, suggesting that binding may directly involve this residue. Interestingly, this tyrosine is the only residue within the motif that is identical in L1 orthologues from human (GenBank<sup>TM</sup> accession number M77640), rodent (GenBank<sup>TM</sup> accession numbers X12875 and X59149), chick (GenBank<sup>TM</sup> accession number Z75013), and fugu (GenBank<sup>TM</sup> accession number Z71926). This is in contrast to the cytoplasmic RSLE motif that is absolutely conserved between L1-like molecules from these species as well as in other members of the L1 subfamily such as neurofascin (reviewed in Ref. 1).

The result that binding activities for L1 can be modulated at the RNA splicing level is paralleled by studies on isoforms of neurofascin. This protein has the same overall domain structure as L1 but many different isoforms produced through alternative splicing of exons. Interaction of neurofascin with four ligands including F11 and axonin-1 is modulated in a complex fashion by the combinatorial inclusion of different stretches of the protein (23). The most NH<sub>2</sub>-terminal exon that is subject to alternative splicing encodes a peptide immediately upstream of Ig domain 1, *i.e.* in an equivalent position to YEGHHV in L1. The differential use of this exon modulates binding to several ligands in the context of some domain combinations but not others. Thus, for neurofascin, this region is also more likely to influence the tertiary structure of the protein than belong to a binding domain.

Our results show for the first time that the choice or strength of L1 interactions could be in part determined by differential exon usage. It follows that L1 on neurons has a different interactive potential compared with L1 on most peripheral cells. This has implications for understanding the function of L1 in different cell types. Oligodendrocytes, for example, express different levels of the two potential isoforms depending on their state of maturation (7). As we have shown that binding of wild-type L1 to exon 2-minus L1 is also reduced *in vitro*, developmental regulation of isoform expression may be accompanied by an *in vivo* change in affinity for L1 as well as TAX-1 and F3 on neighboring neurons.

Do these data suggest that exon 2-encoded sequences are important for normal development of the nervous system? With respect to this question, we have reported previously that sim-

ilar levels of reduction in homophilic or heterophilic binding to F3 and/or TAX-1 were observed for mutations in L1 that are pathogenic in man (12). This would suggest that wild-type levels of binding are required for correct nervous system development.

In summary we have shown that the omission of sequences encoded by exon 2 reduced binding *in vitro* to several L1 ligands that are important to the development of the nervous system. Differential utilization of this sequence may therefore be a way of spatially and temporally regulating the type of interaction L1 undertakes.

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#### REFERENCES

- Brümmendorf, T., Kenrick, S., and Rathjen, F. G. (1998) *Curr. Opin. Neurobiol.* **8**, 87–97
- Kenrick, S., Watkins, A., and De Angelis, E. (2000) *Hum. Mol. Genet.* **9**, 879–886
- Buchstaller, A., Kunz, S., Berger, P., Kunz, B., Ziegler, U., Rader, C., and Sonderegger, P. (1996) *J. Cell Biol.* **135**, 1593–1607
- Reid, R. A., and Hemperly, J. J. (1992) *J. Mol. Neurosci.* **3**, 127–135
- Jouet, M., Rosenthal, A., and Kenrick, S. (1995) *Brain Res. Mol. Brain Res.* **30**, 378–380
- Takeda, Y., Asou, H., Murakami, Y., Miura, M., Kobayashi, M., and Uyemura, K. (1996) *J. Neurochem.* **66**, 2338–2349
- Itoh, K., Sakurai, Y., Asou, H., and Umeda, M. (2000) *J. Neurosci. Res.* **60**, 579–586
- Miura, M., Kobayashi, M., Asou, H., and Uyemura, K. (1991) *FEBS Lett.* **289**, 91–95
- Coutelle, O., Nyakatura, G., Taudien, S., Elgar, G., Brenner, S., Platzer, M., Drescher, F., Jouet, M., Kenrick, S., and Rosenthal, A. (1998) *Gene (Amst.)* **208**, 7–15
- Kamiguchi, H., Long, K. E., Pendergast, M., Schaefer, A. W., Rapoport, I., Kirchhausen, T., and Lemmon, V. (1998) *J. Neurosci.* **18**, 5311–5321
- Kamiguchi, H., and Lemmon, V. (1998) *J. Neurosci.* **18**, 3749–3756
- De Angelis, E., MacFarlane, J., Du, J. S., Yeo, G., Hicks, R., Rathjen, F. G., Kenrick, S., and Brümmendorf, T. (1999) *EMBO J.* **18**, 4744–4753
- Brümmendorf, T., Hubert, M., Treubert, U., Leuschner, R., Tarnok, A., and Rathjen, F. G. (1993) *Neuron* **10**, 711–727
- Treubert, U., and Brümmendorf, T. (1998) *J. Neurosci.* **18**, 1795–1805
- Deppert, W., Gurney, E. G., and Harrison, R. O. (1981) *J. Virol.* **37**, 478–482
- Wolff, J. M., Frank, R., Mujoo, K., Spiro, R. C., Reisfeld, R. A., and Rathjen, F. G. (1988) *J. Biol. Chem.* **263**, 11943–11947
- Kamiguchi, H., and Lemmon, V. (1997) *J. Neurosci. Res.* **49**, 1–8
- Kunz, S., Spirig, M., Ginsburg, C., Buchstaller, A., Berger, P., Lanz, R., Rader, C., Vogt, L., Kunz, B., and Sonderegger, P. (1998) *J. Cell Biol.* **143**, 1673–1690
- Haspel, J., Friedlander, D. R., Ivgi-May, N., Chickramane, S., Roonprapunt, C., Chen, S., Schachner, M., and Grumet, M. (2000) *J. Neurobiol.* **42**, 287–302
- Su, X. D., Gastinel, L. N., Vaughn, D. E., Faye, I., Poon, P., and Bjorkman, P. J. (1998) *Science* **281**, 991–995
- Freigang, J., Proba, K., Leder, L., Diederichs, K., Sonderegger, P., and Welte, W. (2000) *Cell* **101**, 425–433
- Oleszewski, M., Gutwein, P., von Der Lieth, W., Rauch, U., and Altevogt, P. (2000) *J. Biol. Chem.* **275**, 34478–34485
- Volkmer, H., Zacharias, U., Norenberg, U., and Rathjen, F. G. (1998) *J. Cell Biol.* **142**, 1083–1093