

Molecular Structure and Functional Testing of Human L1CAM: An Interspecies Comparison

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The rodent, avian, and insect L1-like cell adhesion molecules are members of the immunoglobulin superfamily that have been implicated in axon growth. We have isolated an L1-like molecule from human brain and found that it also supports neurite growth *in vitro*. We have also cloned and sequenced the entire coding region of human L1CAM and found that it shows a very high degree of homology to mouse L1cam, with 92% identity at the amino acid level. This similarity suggests that L1CAM is an important molecule in normal human nervous system development and nerve regeneration. Overall, there is substantially less homology to chick Ng-CAM; they are 40% identical at the amino acid level but many regions are highly conserved. Comparison of the sequences from human, mouse, chick, and *Drosophila* indicates that the L1 immunoglobulin domain 2 and fibronectin type III domain 2 are strongly conserved and thus are likely functionally important. © 1991

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INTRODUCTION

The L1¹-related molecules are integral membrane glycoproteins that have been described in the nervous system of several species. Representative examples include L1cam in mouse (Rathjen and Schachner, 1984), NILE² in rat (McGuire *et al.*, 1978), Ng-CAM/8D9/G4 in chick (Grumet *et al.*, 1984; Lemmon and McLoon, 1986; Rathjen *et al.*, 1987), and neuroglian

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. M64296.

¹ To conform with the HGMW-approved nomenclature we refer to human L1 as L1CAM and mouse L1 as L1cam.

² Abbreviations used: L1CAM, human L1 cell adhesion molecule; L1cam, mouse L1 cell adhesion molecule; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NILE, NGF inducible large external glycoprotein; Ng-CAM, neuron-glial cell adhesion molecule; Ig, immunoglobulin C2 type domain; Fn, fibronectin type III domain; TM, transmembrane region; CP, cytoplasmic tail.

in *Drosophila* (Bieber *et al.*, 1989). These molecules share similar biochemical properties, immunological cross-reactivity, localization predominantly on axons of projection neurons, homology in nucleotide sequence, and functional similarity. Several lines of evidence suggest that L1 plays an important role in neuronal growth and fasciculation. First, it is expressed early on developing (Martini and Schachner, 1986) and regenerating axons (Daniloff *et al.*, 1986; Martini and Schachner, 1988). Second, antibodies to L1 disrupt fascicle formation *in vitro* (Stallcup and Beasley, 1985) and *in vivo* (Landmesser *et al.*, 1988). Finally, purified L1 is a potent substrate for neurite growth (Lagenaur and Lemmon, 1987).

A number of observations are consistent with the existence of an L1 human homologue. Human tumors, especially neuroblastoma, demonstrate immunoreactivity to L1 antibodies (Mujoo *et al.*, 1986; Figarella-Branger *et al.*, 1990). Biochemical analysis of a glycoprotein isolated from human brain using an anti-neuroblastoma monoclonal antibody revealed that the antigen was very similar to mouse L1cam (Wolff *et al.*, 1988). Recently, partial sequences obtained for a human genomic clone (Djabali *et al.*, 1990) and a human melanoma cDNA clone (Harper *et al.*, 1991) confirmed that a human L1-like molecule exists. Further studies localized the gene for human L1 to the q28 band on the X chromosome (Djabali *et al.*, 1990), the homologous region to the A6-B region of the mouse X chromosome where the L1cam gene is located.

The knowledge that an L1-like molecule exists in humans leads to the idea that L1CAM may be important in promoting axon regeneration in trauma or disease states of the human nervous system. Therefore, we purified L1 from human brain and conducted *in vitro* experiments on the natural substance that demonstrate that human L1CAM, like chick and mouse L1cam, can support neuron attachment and neurite growth. We have also cloned and sequenced cDNAs encompassing the entire coding region of L1CAM.

This information will allow future studies on the structure and function of L1CAM and permit the construction of cell lines expressing L1CAM for *in vitro* and *in vivo* experiments on nerve growth and regeneration.

MATERIALS AND METHODS

L1CAM Purification

L1CAM was purified by immunoaffinity chromatography using methods previously described (Lemmon *et al.*, 1989). Briefly, neural membranes from 12-day full-term neonatal human brain (the infant succumbed from complications of trisomy 18) were isolated on sucrose gradients and then extracted with 1% deoxycholate. The extract was then run over a 74-5H7 IgG monoclonal antibody to L1cam (Lemmon *et al.*, 1989) affinity column. Antigens were eluted with 0.1 M diethylamine (pH 11.5) and the solution was rapidly neutralized with Tris-HCl. Fractions were then dialyzed against PBS overnight. Gel electrophoresis of the purified product was performed on a 5% SDS-polyacrylamide gel.

Cell Culture

Functional assays of the L1CAM purified from human brain were performed by two neuronal culture methods. Dissociated P1 rat cerebellar cells were plated on L1CAM-nitrocellulose-coated plates as described by Lemmon *et al.* (1989). E7 chick retinal strips were grown on L1CAM-nitrocellulose-coated plates based on a system developed by Halfter *et al.* (1981). Plain nitrocellulose coated with bovine serum albumin was used as a negative control substrate.

Molecular Cloning

A human fetal brain cDNA library in λ ZAP II (Stratagene) was amplified in the *Escherichia coli* strain XL1-Blue (Stratagene). The library was probed with a mix of ^{32}P 5'-end-labeled synthetic degenerate oligonucleotides, 50 nucleotides in length, corresponding to a region that is highly conserved between L1cam and the rat homologue NILE glycoprotein (Prince *et al.*, 1989). The screening was carried out overnight at 30°C in a solution containing 6× SSC, 5× Denhardt's, 0.5% SDS, 20% formamide, and 100 $\mu\text{g}/\text{ml}$ salmon sperm DNA. Phage clones isolated from the library were plaque-purified and converted to plasmid form, and the insert sizes were determined. A second separate screening of the library was performed using a 40-base oligonucleotide probe, the sequence of which was derived from the furthest 5' sequence of an earlier obtained clone. The oligonucleotides used for screening are indicated in Fig. 2.

DNA Sequencing

Double-stranded DNA sequencing was carried out by the dideoxynucleotide method (Sanger *et al.*, 1977) using a Sequenase Kit (USBC) and [^{35}S]-deoxyadenosine 5'-(thio)triphosphate from Amersham. The primers for the reactions were custom synthesized at this institution. Sequencing primers to the T7 and T3 promoters were used for the initial sequencing, and subsequent sequencing was performed using additional synthetic oligonucleotide primers generated from the newly acquired L1CAM sequence. The complete sequence was obtained from both DNA strands. Sequence analysis was carried out using the MacVector (IBI) sequence analysis program.

RESULTS AND DISCUSSION

A silver stained SDS-polyacrylamide gel of purified L1CAM is shown in Fig. 1A. Notable is the characteristic doublet of bands at 190–180 kDa, a major band at 130 kDa, and minor bands at 105, 80, and 62 kDa. This is similar to the pattern reported by others for L1CAM (Mujoo *et al.*, 1986; Wolff *et al.*, 1988), chick Ng-CAM (Burgoon *et al.*, 1991), and L1cam (Sadoul *et al.*, 1988).

Purified L1CAM was extremely potent in supporting neurite outgrowth. Chick retinal explants produced extended defasciculated neurites on L1CAM (Fig. 1B) and dissociated rat cerebellar neurons grew long neurites (data not shown). Controls grown on nitrocellulose without L1CAM showed poor attachment and no neurite extension (data not shown).

A total of 3×10^6 plaques were screened, representing a threefold screening of the library. Twenty plaques were initially positive; however, only three of the clones initially isolated from the cDNA library remained positive after successive rounds of screening and proved subsequently to correspond to L1CAM cDNA. After excision of the inserts from the phage vector, these clones were 3.4, 2.6, and 1.4 kb in length and were designated 3.1, 4, and 17, respectively. Because none of these contained a start methionine and initial signal sequence, a second screening of the library was performed using a 40-base oligonucleotide deduced from sequencing the 5' end of clone 3.1. A positive clone from this screening, C2, contained an initiation codon preceded by a stop codon and followed by a sequence that corresponded to a hydrophobic stretch of amino acids that is presumed to be a signal sequence, as well as a sequence that overlapped with the previously obtained clones. This clone was 1.2 kb in length.

The nucleotide and deduced amino acid sequences for the L1CAM cDNA coding region are shown in Fig. 2. The open reading frame encodes a protein of 1256

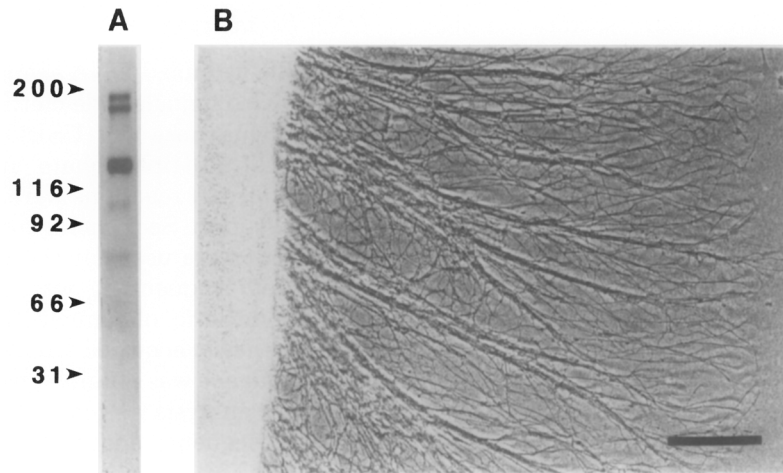


FIG. 1. (A) Silver stained SDS-PAGE gel of immunopurified L1CAM. Molecular weight standards are indicated at the left. (B) E7 chick retinal explants grown on L1CAM-coated dishes. Control dishes coated with BSA had no neurite outgrowth. Scale bar = 100 μ m.

amino acids and 142,698 Da. The nucleotide sequences of the human L1CAM and mouse L1cam cDNAs were compared and found to be 85% identical. At the amino acid level, this rose to 92% overall identity (Fig. 3).

Structurally, L1-related molecules are similar to other immunoglobulin superfamily cell adhesion molecules having a motif of repeating immunoglobulin domains followed by fibronectin type III domains. The L1-like molecules in particular have six repeating immunoglobulin C2 (Ig) domains followed by five repeating fibronectin type III (Fn) domains (Moos *et al.*, 1988; Burgoon *et al.*, 1991). These are linked to a cytoplasmic portion of the molecule by a transmembrane domain. A domain by domain comparison of the protein sequence of human L1CAM to mouse L1cam, chick Ng-CAM, and *Drosophila* neuroglian was performed and is summarized in Table 1.

Two short stretches of L1CAM nucleotide sequence have been published previously. A comparison of the genomic sequence obtained by Djabali *et al.* (1990) with ours is an identical match from nucleotides 991 to 1091 (see Fig. 2). The first 23 nucleotides of the Djabali sequence, however, did not match our sequence or the L1cam sequence and likely represents an intron. The sequence obtained by Harper *et al.* (1991) from human melanoma cDNA differs from ours at L1CAM nucleotides 3528 to 3540, where they show a 12-nucleotide deletion. We obtained identical sequence for this region from three independent clones and the sequence matches the corresponding mouse nucleotide sequence perfectly. This discrepancy could represent a mutation in the tumor line or a splicing variant. The sequence also varies between nucleotides 3344 and 3349, where Harper *et al.* have an extra 3 nucleotides introduced non-sequentially.

Interestingly, we found a one-amino-acid deletion here compared to the mouse L1cam sequence, with matching of the flanking amino acids on either side. This region was particularly difficult to sequence on the antisense strand, requiring dITP reactions to resolve compressions. The coding strand, however, had unambiguous sequence. We found a final difference at base 3086, where we have an A.

A comparison of the sequences of human L1CAM and mouse L1cam with Ng-CAM raises an interesting question about the relationship between mammalian L1 and Ng-CAM: Are they homologous? Burgoon *et al.* have suggested that Ng-CAM may not be "equivalent" to L1 due to the relatively low sequence (40%) identity between the two molecules (Burgoon *et al.*, 1991). In contrast, mouse NCAM is about 80% identical with chick NCAM and rat fibronectin is about 80% identical with chick fibronectin. They also state that "experiments to identify an L1 homologue in chickens and an Ng-CAM homologue in mice have not yet revealed such molecules."

Despite the poor sequence homology between Ng-CAM and mammalian L1, there are many similarities among the molecules. If conservative amino acid substitutions in Ng-CAM are permitted, the overall similarity between L1CAM and Ng-CAM rises to 66%. The Ig domains show relatively higher degrees of similarity, up to 83%, with 75% conservation of the cytoplasmic domain. Furthermore, the overall structure of the molecule is preserved from species to species; there are six Ig domains and five Fn domains in each molecule and each structural domain in the chick molecule is most closely related to the same domain in the human. The domains that show the highest degree of similarity between human and mouse (Ig 2, Fn 2, the transmembrane and cytoplasmic domain) also demon-

L1	17	Mouse L1	-----R-R-Q-E-----I-----V-HEA-Y-----E-----Y-----Q-V
	17	Human L1	VITEGSPRLVVFPTDILSKCEASGKVEQFRTWDRGVHFKKELGVTVYQSPHSGFRTITGNMNSFAQRFGQIYRGSFNKLGCTAMSHSIRLM
	17	Ng-CAM	EL--EP-EQ---S---V--V-T-N-P--Y--S--EISPSS-RSTG--RM-PDRHLVI--ATLA-Q-L--RF---T-A---V-P--ANVI
L2	112	Mouse L1	-----A-P-----D-----S-----N-----P-----
	113	Human L1	AEGARFKFKETVPEVEGESVVLPCNPPSAPFLRIYWNNSKILHIKQDERVTWQNGNLIFANVLISDNHSDYICHANHPGTTRIIQKPEIDLVRKATNSMD
	107	Ng-CAM	--NT-Q---KK-T---DP---D--E--V-PK---L--D-V--A---S--AMVG--S--D---L--P---AFS-AVRS
L3	218	Mouse L1	-----H--D---T---I-----N-----
	219	Human L1	RKPRLLFTNSSHLVALQOPLVLECIAEGFPTIKMLRPSGMPADRVTYQNHKTLQLLKGVEEDDGEYCLAENSLGSRARHAYVTVAAAP
	213	Ng-CAM	-----L--RDEQTTI--R-GSV-----L--NVR-R-LN--LLPGG--F--R-WG-T-S--E-V--GR-T--GTHS
L4	314	Mouse L1	-----I-----MSM-TYN-----EQ-S-----Q-----
	315	Human L1	YWLHKPQSHLYGFGETARLDQCQVGRPQPEVTWRIKIPVPEELAKOKQYRIGRALLILSNVQFSDTWVTCQEARHGLLLANAYIYVOLLP
	307	Ng-CAM	-----E-G-K-R-OIQ-S---V-I- A-GAERRMLRG---V-PELR-N-SA-L---P---FLH--E--
L5	406	Mouse L1	-----E-----R-----K--Q-----E-----
	407	Human L1	AKILTADNOTYMAVGSTAYLLCKAFKAFVPSQMLDEGTVLQDERFFPYANGTLGIRDLOANDTGRYFCLAANDONNVTIMANIKVKDAT
	398	Ng-CAM	LRN---B-R-EV-ENQTFE-H-RT---A-N-E---TPLEPA---D-S-VF-T--S-RVSAVRGG-G-V-T-K-O-AHS-GSLA-L-E-RAE-
L6	499	Mouse L1	-----A-----A-----R-----K--Q-----E-----
	500	Human L1	QITGPRSTIEKGSVFTCOASFPDSIQESIT WRGDGRDQELGSDKYFIEDGRLVHLSLDYSDQNSYCVASTELDYVESRRAQLLVGSGP
	491	Ng-CAM	R--SAP---ATA---ET---H-G-T---AVT-GEUR-LRG-OP-P --DPR-SVAAMET-S NV--G-E-TIO-R---P--SA-AE--R--R--
Fn.1	595	Mouse L1	-----H--E---R--K--HL-----S-----S-----
	596	Human L1	VPLRVLSDILLTQSQVRSVSPAEHDHAPTEKVDIEFEDKEMAPKWKVSLGKVPGNQSTLKLSPYHYTFVTAINKYGPGEPSYSEVAVTPEAPE
	583	Ng-CAM	SRD-- QVMEVDEHR--J--T-GD---S---FVV-E-EBERDQRGFGAAND--QPTFP-P---GRFP---V-V-A-R--HHAP-AFIE--P---S--
Fn.2	696	Mouse L1	-----N-----I-----KQE-----R-K-T-----N-----
	697	Human L1	KNFVDYKGEGETITNMTWPKFLRWDMNAPQVQYVQWRPQSTRG PWOEQIVSDPFLVVSNTSTFVPEIKVQAVNSQCKGPFQVITIGYSGEDY
	681	Ng-CAM	R--GG-H---G-L---K---PQA---WAR---LEEP---GGGF-SGGF--A-ST-DA-PV--GGLFP--S-PQ-R---GA---ATPGV-H---L
Fn.3	792	Mouse L1	-----V-S---D-T-F---T---R---W-K---S-I---A-----V-----L-----M-----
	793	Human L1	POAIPLEGIEILNLSAVLVKWR FVDLQAVKHLRGVYVWREGSQRKSHKHSHKDVVPANTTSVILSLRPSYSYHLEVOAFNGRGGSPASEFT
	785	Ng-CAM	-LVT--NV-V-L---T-R-R-TLGGGPEUR-N--FR-L---L-WVGER-R-OAPP-FPOI-QSPA EDDP-FPPVA-T-GG DA--ALLGGLRPSRYQIRVLFNFRGDDGPPSEPIA-E-----FSTPEGV
Fn.4	898	Mouse L1	-----D---H---H---VEGES-E-F-S-----N-N-DIQ-----QQG-----
	899	Human L1	PGHPEALHLECOQNTLLLRWOPPLSHNGVLTGVLSYHPLDGEGKGLSEFLRDLDELPHLTHLTDLSPLRVRFOQAIT KEGPGEAIVREGGTMAL
	911	Ng-CAM	--P--E-RV-RLDD-A-SWVERTEKRSI ---R-QVEEP-SALPGGSV--- QCD-RG-NARS---LA-PS -PR-R-ALOT--GSTKEPEPSPWMSR
Fn.5	996	Mouse L1	F-RP-----K-----P-G-VSPDHPQ-----N-----K-----I--KULL-HLD-----P--VS
	996	Human L1	SGLSDFGMSATAGENYSVWSM VPKECOGNFRPHLFLKALGEGKGCASLS PQVYSYNSQSYTQMDLQDPTDYEHLFKERMPRHQHAVKTINGTGRVRLP
	1009	Ng-CAM	F-VGCR-GFHGA-V-FGAQOEDD-EF-V-FNNKSTDEPWRTSGRANS --RRYYLEGLRPGTA-RVQFVGRNRS-G-NVA-W-SE -Q----- - -Q
Transmembrane			
	1097	Mouse L1	TT--S--S---A-----I-----
	1095	Human L1	PAG FATEGWFI GEVSAIILLVLLILCFI KRSGKGVSKVDKEDTQVDSEARPKDETFGEYRSLESDNEEK AEGSSQPSLNGDIK PLGSDDSLADYGGSDYQFVEDGSGFICQYSKKEKEAAGNDSSGATSPINPAVALE
	1102	Ng-CAM	-G-GVC-K---S---SVV---I-----EA-KGS-S--GAG-GV-SFGRGFCAM--E--G-----R-PGAGFGSS-FA-SG-GF-LD

FIG. 3. Comparison at an amino acid level of L1CAM domains with corresponding domains in L1cam and chick Ng-CAM. Gaps were introduced to maximize identities between the sequences. Identical residues are represented by a hash mark. The amino acids shown as being the transmembrane region include the link between the Fn-5 domain and the presumed membrane spanning region that begins with GWFI.

TABLE 1

Percentage Identity of Amino Acids in Different Domains Compared to Human L1

Domain	Ig 1	Ig 2	Ig 3	Ig 4	Ig 5	Ig 6	Fn 1	Fn 2	Fn 3	Fn 4	Fn 5	TM	CP
M L1cam	83	93	91	87	90	93	89	89	86	82	75	94	100
NGCAM	44	66	55	57	39	43	38	56	33	30	18	78	63
NGCAM*	62	83	71	79	66	56	67	73	58	49	32	100	75
Neuroglian	26	32	27	24	29	32	32	36	31	29	18	22	26
Neuroglian*	41	54	47	49	49	48	51	68	50	52	40	67	40

Note. Asterisk shows analysis with conservative amino acid substitutions allowed (Ref. (6)). The following amino acid groupings were considered conserved: (S,T,P,A,G), (N,D,E,Q), (H,K,R), (M,I,L,V), (F,Y,W), (C).

projection axons in the CNS and PNS express L1 or Ng-CAM in the corresponding species.

Functional experiments have shown that anti-L1 and anti-Ng-CAM antibodies disrupt axon fasciculation in both chicks and mammals (Stallcup and Beasley, 1985; Rathjen *et al.*, 1987) and inhibit neuron-neuron adhesion (Keilhauer *et al.*, 1985; Grumet and Edelman, 1988). They also perturb migration of granule cells from the external granule cell layer to the internal granule cell layer in the cerebellum (Lindner *et al.*, 1983; Hoffman *et al.*, 1986). Finally, studies with purified L1 and with Ng-CAM show that mammalian cells can bind to chick Ng-CAM and that chick neurons can bind to mammalian L1 in a homophilic binding interaction between the L1cam and the chick Ng-CAM (Lemmon *et al.*, 1989). Therefore, while there are clearly structural differences between Ng-CAM and L1, it seems most likely that they represent homologous and not merely analogous molecules.

Comparison of L1CAM, L1cam, rat NILE, and chick Ng-CAM confirms previous reports that the cytoplasmic portion of this molecule is highly conserved, suggesting an important functional role for this region of the molecule (Prince *et al.*, 1989; Harper *et al.*, 1991). Evidence points to an interaction of L1 with the cytoskeleton, directly or indirectly, because in differentiated neuroblastomas L1 is relatively immobile (Pollerberg *et al.*, 1990). On axons and growth cones of chick retinal ganglion cells, however, L1 is freely diffusible (Drazba and Lemmon, 1990). This suggests that attachment to the cytoskeleton is not a prerequisite for functional binding as is the case for cadherins (Nagafuchi and Takeichi, 1988). The cytoplasmic domain of L1 also may be involved in regulating cell adhesion molecule function. L1 is phosphorylated and is associated with a casein kinase (Sadoul *et al.*, 1988). Anti-L1 antibody binding to L1 on PC12 cells has been shown to alter intracellular calcium and pH (Schuch *et al.*, 1989). Agents such as TPA or okadaic acid that increase cytoplasmic phosphorylation increase fasciculation in a manner consistent with increased affinity of L1 for its ligand

(Cervello, Lemmon, Rutishauser), (unpublished results). This evidence indicates that L1 either regulates cell function or has its function regulated via its cytoplasmic region.

Interspecies comparison of the amino acid sequence of the extracellular portion of L1 suggests that the Ig domain 2 and Fn domain 2 may have some conserved function since these are the immunoglobulin and fibronectin domains with greatest homology. One possibility is that the Ig domain 2 is important in L1-L1 homophilic binding. The Ig domains 2 and 3 of NCAM are believed to be involved in heparan and cell binding (Frelinger and Rutishauser, 1986; Cole and Akeson, 1989). This demonstrates that Ig superfamily molecules do not necessarily bind amino terminus to amino terminus. It is also possible that a large region of L1 is involved in L1-L1 binding, similar to the manner in which Ig heavy chains bind to each other; according to this model, the L1 Ig domains would bind in a long parallel or antiparallel interaction. This possibility is consistent with the report that all monoclonal antibodies to G4 (thought to be identical with chick Ng-CAM) that were tested were able to inhibit G4-G4 binding (Chang *et al.*, 1990). No highly positively charged regions were observed in any of the Ig domains that would be analogous to the heparan binding domain in the second Ig domain of NCAM (Cole and Akeson, 1989). However, the second Ig domain of L1 and Ng-CAM does have a highly negatively charged region with 4 of 6 amino acids being aspartic acid or glutamic acid. The third Fn domain of L1 has one region with 9 of 12 positively charged amino acids. A similar region is present in L1cam but is absent from chick. Conclusions about structure-function relations must await more detailed experiments using well-defined antibodies and mutated forms of L1.

The information provided in this paper extends previous work by providing the entire coding sequence of human L1CAM and demonstrating that like the related molecules in mouse, rat, and chick, L1CAM purified from human brain can support neurite growth.

The structural knowledge gained allows comparisons with nearby and more distant species, mice, chick, and *Drosophila* and speculation about structurally important areas of the molecule. The results of *in vitro* testing of natural L1CAM support the idea that L1CAM is likely to be an important molecule in the development of the human nervous system by providing evidence that L1CAM can mediate neurite growth. This complements reports that the human L1 gene is on the X chromosome in a region where disease of CNS axonal tract development has been mapped (Djabali *et al.*, 1990), and strengthens the need for further understanding of the molecule. Use of the cDNA will enable comparison of recombinant L1CAM with the product purified from human brain and permit functional studies on the recombinant molecule *in vitro*. Finally, using the cDNA, cell lines expressing normal and altered L1CAM can be constructed for use in transplantation or regeneration studies.

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