Colocalization of the Homophilic Binding Site and the Neuritogenic Activity of the Cell Adhesion Molecule L1 to Its Second Ig-like Domain*

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The cell adhesion molecule L1 has been implicated in mediating cell-cell adhesion and in promoting neurite outgrowth. The extracellular region of L1 contains six immunoglobulin (Ig)-like domains in the amino-terminal region, followed by five fibronectin type III-like repeats. L1 is capable of undergoing homophilic binding as well as heterophilic interactions. To map the homophilic binding domain in L1, three glutathione S-transferase (GST) fusion proteins (GST-Ig1-2-3, GST-Ig4-5-6, and GST-Fn) were prepared and coupled to Covaspheres and their homophilic binding activity was determined using a substrate-coated Covasphere binding assay. Only GST-Ig1-2-3 was capable of homophilic binding. Next, His-tagged recombinant Ig-domain proteins (His-Ig1-2, His-Ig1, and His-Ig2) were expressed and subjected to similar assays. Only His-Ig1-2 and His-Ig2 were capable of homophilic interactions. Binding of His-Ig2-conjugated Covaspheres to substrate-coated His-Ig2 was inhibited by anti-Ig1-2 Fab and soluble His-Ig2. These results indicate that the L1 homophilic binding site resides within Ig2. To examine effects of these L1 recombinant proteins on neurite outgrowth, neural retinal cells were cultured on different substrate-coated fusion proteins. Both GST-Ig1-2-3 and His-Ig2 were potent inducers of neurite extension. These results thus indicate that the L1 Ig-like domain 2 alone is sufficient to mediate L1-L1 interaction and promote neurite outgrowth from retinal cells.

Intercellular adhesion plays an important role during neural development, when specific synaptic connections are established primarily by extension of axons along restricted pathways (1, 2). The molecular basis of these processes involves cell adhesion molecules and diffusible factors. In recent years, an increasing number of cell adhesion molecules have been found to be associated with neuronal cells. These cell adhesion molecules have been categorized according to their structure into three major groups: the cadherins (3), the integrins (4), and proteins of the immunoglobulin (Ig)1 superfamily (5, 6).

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1 The abbreviations used are: Ig, immunoglobulin; NCAM, neural cell adhesion molecule; PCR, polymerase chain reaction; GST, glutathione S-transferase; PBS, phosphate-buffered saline; DTT, dithiothreitol; L1 was first described as a 200-kDa transmembrane glycoprotein in the central nervous system, and it belongs to the Ig superfamily (7, 8). L1 consists of six C2-type Ig-like domains in the amino-terminal region, followed by five fibronectin type III-like repeats, a transmembrane domain, and a cytoplasmic domain (8). Nile in rat, NgCAM, G4, and 8D9 in chicken are the species homologues of mouse L1 (9-11). L1 cDNAs have been cloned from mouse (8), rat (12, 13), and human (14, 15). The L1 gene in human has been mapped to chromosome Xq28 (16, 17). An association has been made between mutations in the L1 gene and the X-linked hydrocephalus phenotype (18-20).

L1 can undergo homophilic binding as well as heterophilic interactions with several other cell adhesion molecules, such as NCAM (21, 22), TAG-1/axonin-1 (23, 24), F3/F11 (25), glia (26, 27), and the extracellular matrix protein laminin (28). Some of these heterophilic interactions are known to modulate L1 functions. For instance, NCAM has been shown to undergo disinteractions with L1, which in turn facilitates L1-L1 homophilic binding (21). Neurocan, in contrast, binds to L1 and inhibit neuronal adhesion and neurite extension promoted by the L1 substrate (29).

L1 has been implicated in a wide range of neuronal cell differentiation. Substrate-coated L1 is a potent inducer of neurite outgrowth from a number of primary neurons (14, 30, 31). Axonal growth involves both adhesion and the transmission of extracellular signals into the interior of a growth cone to activate intracellular events (32, 33). L1 appears to play an important role in this signal transduction process (34, 35). A L1-Fc chimeric protein has been reported to induce protein tyrosine phosphorylation in neuronal cells (36) as well as promote neurite outgrowth (37), suggesting that the clustering of L1 molecules may trigger the signaling pathway leading to neurite extension.

It is evident that the formation of adhesion complexes via L1 homophilic binding may serve as an initiation point for many important signaling events. However, very little is known about the homophilic binding site of L1 and the mechanism of its interaction. In this report, experiments were carried out to investigate the relationship between L1 homophilic binding and its neuritogenic activity. We found that the second Ig-like domain of L1 was capable of binding to cell membrane-associated L1, as well as undergoing homophilic binding by itself. In addition, the Ig-like domain 2 of L1 was capable of promoting neurite outgrowth from retinal neurons, suggesting an intimate relationship between L1 homophilic binding and L1-mediated neurite outgrowth.

BSA, bovine serum albumin; HBSS, Hank’s balanced salt solution; CEA, carcinoembryonic antigen; FGF, fibroblast growth factor; NgCAM, neuron-glia cell adhesion molecule.
EXPERIMENTAL PROCEDURES

Materials—The pGEX-3X plasmid and glutathione-Sepharose 4B were purchased from Pharmacia Biotech Inc. (Tokyo, ON). The pQE-8 plasmid and nickel-nitrilotriacetic acid resin were purchased from Qiagen (Chatsworth, CA). Covaspheres were obtained from Duke Scientific Corp. (Palo Alto, CA). Freund’s adjuvant, trypsin, α-minimal essential medium, and N2 supplement were purchased from Life Technologies, Inc. (Toronto, ON). Poly-L-lysine was purchased from Sigma, Dial from Molecular Probes (Eugene, OR). The BCA (bicinchoninic acid) protein assay kit from Pierce (Rockford, IL).

Construction of Expression Vectors—Standard recombinant DNA methods were followed in the construction of expression vectors (38). DNA fragments encoding different portions of L1 were obtained using appropriate restriction enzymes or PCR amplification. The cDNA fragment coding for the Ig-like domains 1, 2, and 3 (Ig1-2-3) between amino acid positions 24 and 351 (amino acid and nucleotide numbering according to Hlavin et al. (14)) was released by NarI at nucleotide position 113 and BamHI at nucleotide position 1114, followed by treatment with Klenow enzyme and addition of EcoRI linkers at both ends. This fragment was subcloned into the unique EcoRI site of the pGEX-3X vector for expression of the fusion protein glutathione S-transferase (GST)-Ig1-2-3. The cDNA fragment coding for all three fibronectin type III repeats (Fn) between amino acid positions 596 and 1094 was amplified by PCR using the forward primer 5′-GGGATCCGTCATCACGGAACAGT-3′ and the reverse primer 5′-GGGAATTCCATGAGCCGGATCTCA-3′. This fragment was subcloned into the unique BamHI site of the pGEX-3X vector for expression of the fusion protein GST-Ig1-2-3Fn. DNA fragments coding for the Ig-like domains 4, 5, and 6 (Ig4-5-6) between amino acid positions 352 and 595 was obtained by BamHI digestion of a PCR product, which was generated using the forward primer 5′-GCCCCTGGGACCATTGCTCATCGGAAGACTGCT-3′ and the reverse primer 5′-CGGAAATTCGGATCCGGCGGCCAGGGCTTCACCACG-3′. This fragment was subcloned into the unique BamHI site of the pGEX-3X vector for expression of the fusion protein GST-Ig4-5-6.

Expression and Refolding of Recombinant L1 Fusion Proteins—To produce GST-L1 fusion proteins, transformed E. coli cells were grown at 37°C in 250 ml of LB medium with 100 μg/ml ampicillin. Protein synthesis was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside when A600 reached 0.6–0.8. Cells were collected 3 h after induction by centrifugation at 4,000 × g for 10 min. The pellet was resuspended in 10 ml lysis buffer (50 mM Hepes buffer, pH 7.9, 5% glycerol, 2 mM EDTA) and the fusion proteins were solubilized as inclusion bodies by sonication of the cell suspension on ice, followed by centrifugation for 10 min at 10,000 × g at 4°C. Inclusion bodies were washed two times using washing buffer (50 mM Hepes, pH 7.9, 5% glycerol, 2 mM EDTA, 0.1 M DTT, 0.05% deoxycholate, and 1% Triton X-100) followed by washing once with lysis buffer. Then they were solubilized by overnight incubation at 4°C in 10 ml of 6 M guanidine buffer, containing 10 mM Hepes, 50 mM DTT, and 0.2 M EDTA. Samples were centrifuged at 10,000 × g for 15 min to remove the insoluble material, the supernatant was slowly diluted by adding 40 ml of dilution buffer (10 mM Hepes, pH 7.9, 0.2 mM EDTA, 2 mM DTT) and allowed to sit at 4°C overnight. Then the denaturant and reducing reagents were slowly removed by dialyzing against the storage buffer (20 mM Hepes, 0.5 M NaCl, 0.2 M Na2EDTA, 0.1% Triton X-100, 0.2% BSA, 0.2 M NaCl, 0.2 mM EDTA). The refolded GST fusion proteins were passed through a glutathione-Sepharose 4B column according to the manufacturer’s protocol. Eluted proteins were dialyzed against PBS at 4°C. His-Ig1-2, His-Ig1, and His-Ig2 were purified and refolded as described previously (39). In brief, column fractions containing His-tagged proteins were pooled and adjusted to −2 mg/ml. They were reduced by adding β-mercaptoethanol to 0.1 M, followed by incubation at 37°C for 90 min. To reoxidize, His-tagged proteins were dialyzed against 8 M urea, 150 mM NaCl, 50 mM Tris·HCl, pH 8.0, at 4°C overnight. Samples were diluted 1:20 with PBS and concentrated by ultrafiltration. A single cycle of sonication and then collecting and resuspended in 2 ml of saline (0.9% NaCl). The cell/saline suspension was kept on ice for 5 min. Then 8 ml of acetone (−20°C) was added and mixed vigorously. The acetone suspension was kept on ice for 30 min, and the precipitate was collected by centrifugation at 10,000 × g for 10 min. The supernatant was discarded, and the pellet was resuspended with fresh acetone and mixed vigorously. The mixture was kept on ice for another 10 min. The pellet was collected and air-dried at room temperature. The dried acetone powder was added to the antiserum at a final concentration of 1%, and the mixture was incubated at 4°C overnight with gentle rotation. After spinning at 10,000 × g for 10 min, the supernatant was discarded and passed through an Econo-Pac serum IgG purification column (Bio-Rad) according to the manufacturer’s protocol.

Covasphere-to-Substratum Attachment Assay—Green or red MX Covaspheres (50 μl) were briefly sonicated before the addition of 10 μg of fusion protein, and the final volume was adjusted to 100 μl with PBS. After rotation at room temperature for 75 min, excess active sites on Covaspheres were blocked with 1% BSA. The Covasphere-to-substratum attachment assay was carried out as described previously (39). Recombinant proteins (5 μl of a 1 μM solution) or anti domaine IgG (5 μl at 10 μg/ml) in PBS was used to coat spots (2.5 mm in diameter) on 35-mm plastic Petri dishes at room temperature for 1 h, followed by washing once with 30 mM BSA in PBS at room temperature for 30 min. In竞争 experiments, biotin-conjugated Covaspheres were added to 100 μl of PBS and dispersed with sonication for 5 min in a chilled water bath sonicator. Samples of the diluted Covaspheres (30 μl each) were added to the coated spots and incubated at room temperature for 30 min. After five gentle washes with PBS, the binding of Covaspheres to the substratum was observed by epifluorescence microscopy and recorded images were analyzed using NIH I mage program. The relative amounts of bound Covaspheres per unit area were calculated. In competition experiments, Covaspheres were incubated with competitors at room temperature for 10 min before the binding assay.

Covasphere-to-Cell Binding Assay—Neuronal retinal cells from day 6 chick embryos were isolated and cultured previously (40) and further cultured on coverslips for 5 to 6 h at 37°C. Coverslips were incubated with 1% BSA in HBSS for 5 min and incubated with Covaspheres at room temperature. In inhibition studies, inhibitors at different final concentrations were incubated with cells for 25 min at room temperature after blocking with BSA. After the removal of excess inhibitor and one gentle wash with HBSS, 100 μl of fusion protein-conjugated Covaspheres were added to the coverslip. All coverslips were incubated for 45 min at room temperature on a platform shaker. After washing several times with PBS, cells were observed using epifluorescence microscopy. Cells with more than 5 Covaspheres attached on the surface were scored positive, and generally 100–200 cells were scored for each coverslip.

Neurite Outgrowth Assay—Neuronal retinal cells from day 5 chick embryos were isolated as described previously (41). Round glass coverslips (12-mm diameter) were coated with 0.01% (w/v) poly-L-lysine at room temperature for 3 h. After washing three times with water, 80 μl samples of recombinant proteins at 1 μM were used to coat the coverslips overnight at 4°C. To determine the efficiency of protein coating, protein adsorbed to coverslips was solubilized by 1% SDS (10 μl) at 37°C for 10 min. The relative amounts of bound proteins were determined using the BCA protein assay. Protein samples were adjusted to a final volume of 50 μl and transferred to a 96-well plate. Then 200 μl of working reagent was added to each well, followed by incubation at 37°C for 2 h, color development was stopped by mixing 0.04% (w/v) sodium dodecyl sulfate, and absorbance at 570 nm was measured using a microtitr plate reader. Approximately 60% of the input protein was found adsorbed to the substratum. Similar results were obtained for both GST fusion proteins and His-tagged proteins. The unbound protein was washed away with water, and the
Expression and Refolding of GST Fusion Proteins—To investigate the structure-function relationships of L1, cDNA fragments encoding three different extracellular segments of L1 were fused to GST for expression in E. coli (Fig. 1A). These three GST fusion proteins contained Ig-like domains 1, 2, and 3 (GST-Ig1-2-3), Ig-like domains 4, 5, and 6 (GST-Ig4-5-6) and fibronectin type III-like domains 1 to 5 (GST-Fn), respectively. Samples of purified and refolded proteins were analyzed by SDS/polyacrylamide gel electrophoresis. Under reducing conditions, fusion proteins migrated with apparent molecular sizes of 65,000, 55,000, and 85,000 for GST-Ig1-2-3, GST-Ig4-5-6, and GST-Fn, respectively (Fig. 1B). These values corresponded closely to the expected molecular size of these fusion proteins. Several minor bands of lower molecular size were observed. Since these bands were recognized by anti-GST antibodies (data not shown), they were probably partially degraded products of the recombinant proteins. The yield of fusion protein was ~5 mg of protein/liter of bacterial culture in all three cases.

To obtain L1 domain-specific antibodies, rabbits were immunized with the purified fusion proteins. The antisera were absorbed against acetone powder to remove antibodies that recognized bacterial protein and the GST moiety of these fusion proteins. The IgG fraction was isolated from each antiserum to obtain L1 domain-specific antibodies. Western blots were carried out using these purified IgG to ensure that they did not cross-react with the other two fusion proteins (data not shown).

Binding of Fusion Protein-conjugated Covaspheres to Substrate-coated Proteins—To determine which fusion protein contained the L1 homophilic binding activity, a Covasphere-to-substratum attachment assay was developed. Fusion proteins were conjugated to Covaspheres, which were tested for their ability to adhere to different substrate-coated proteins. As a positive control, IgG that recognized specific L1 segments was adsorbed on to Petri dishes for Covasphere binding (Fig. 2a). Covaspheres were also incubated on substrate-coated GST to monitor background resulting from GST-to-GST interactions (Fig. 2b). Covaspheres conjugated with GST-Ig1-2-3 attached very well to the GST-Ig1-2-3 substrate, whereas the level of binding on GST-Ig4-5-6 was close to background level (Fig. 2, c and d).

The relative percentages of Covaspheres bound per unit area were estimated by normalizing the results to the level of Covasphere binding on the IgG-coated substrate (Fig. 3). The amount of GST-Ig1-2-3-conjugated Covaspheres bound to the GST-Ig1-2-3 substrate was ~25-fold higher than that attached on the GST substrate. In contrast, binding of GST-Ig4-5-6-conjugated Covaspheres to the GST-Ig4-5-6 substrate was at the background level (Fig. 3). In the case of GST-Fn-conjugated Covaspheres, a higher background level of binding to the GST substrate was observed, but there was no significant difference between the level of binding to the GST-Fn substrate and the level of binding to GST (Fig. 3). Further, GST-Ig1-2-3-conjugated Covaspheres did not attach to substrate-coated GST-Ig4-5-6 or GST-Fn (data not shown). It was evident that...
the Ig-like domains 1, 2, and 3 did not interact with other extracellular segments of L1. These results thus demonstrate that the L1 homophilic binding site resides within the first three Ig-like domains, and that the last three Ig-like domains and the fibronectin domains may not be directly involved in L1 homophilic interactions.

The dose effect of substratum-associated GST-Ig1-2-3 on Covasphere binding was also examined. When equal amounts of Covaspheres were loaded onto substrates coated with different concentrations of GST-Ig1-2-3, Covasphere attachment was found to be dose-dependent and maximal binding was achieved when the substratum was coated with 1 μM GST-Ig1-2-3 (Fig. 4A). When binding was carried out on a GST-coated substratum, no significant binding was observed up to a concentration of 5 μM.

To demonstrate the specificity of Covasphere binding, competition experiments were carried out using either anti-Ig1-2-3 Fab or soluble GST-Ig1-2-3. Anti-Ig1-2-3 Fab blocked the binding of GST-Ig1-2-3-conjugated Covaspheres to substrate-coated GST-Ig1-2-3 in a dose-dependent manner (Fig. 4B). Fifty percent inhibition was achieved at ~35 nM anti-Ig1-2-3 Fab. However, the effects of anti-Ig4-5-6 Fab was negligible up to a concentration of 5 μM. The attachment of GST-Ig1-2-3-conjugated Covaspheres to substrate-coated GST-Ig1-2-3 was also inhibited by soluble GST-Ig1-2-3 (Fig. 4C). The inhibition was dose-dependent, and 50% inhibition was achieved at ~80 nM soluble GST-Ig1-2-3. In contrast, the attachment of GST-Ig1-2-3 Covaspheres to GST-Ig1-2-3 substrate was not affected by GST or GST-Ig4-5-6, up to a concentration of 3 μM.

Localization of the Homophilic Binding Site to Ig-like Domain 2 of L1—To identify the domain that contains the homophilic binding site, smaller fragments of the extracellular portion of L1 were expressed as fusion proteins using the pQE expression vectors (Fig. 5A). His-Ig1-2 contained the first two Ig-like domains of L1; His-Ig1 and His-Ig2 contained the first and the second Ig-like domain, respectively. The recombinant proteins were refolded proteins, and SDS/polyacrylamide gel electrophoretic analysis revealed that they were at least 90% pure (Fig. 5B).

The fusion proteins were used to coat Petri dishes and then assayed for their ability to bind GST-Ig1-2-3-conjugated Covaspheres. A large number of GST-Ig1-2-3-conjugated Covaspheres attached to the His-Ig1-2 substrate, suggesting that the third Ig-like domain of L1 is not needed for homophilic interactions. When recombinant proteins containing a single Ig-like domain were tested, Covaspheres attached to the His-Ig2 substrate, but not to the His-Ig1 substrate (Fig. 6A), suggesting that it is Ig2, and not Ig1, that is directly involved in L1-L1 binding. Consistent with this observation, His-Ig2 was able to function as a competitor to displace GST-Ig1-2-3-conjugated Covaspheres in the attachment assay. Only residual
binding (−5%) was observed when binding was carried out in 10 μM soluble His-Ig2 (Fig. 6A). In contrast, a relative level of 70% binding was retained when the same concentration of His-Ig1 was included in the assay.

To determine whether Ig2 can bind to Ig2, His-Ig2-conjugated Covaspheres were assayed for their ability to attach to substrate-coated His-Ig2. Binding of Covaspheres was observed on the His-Ig2 substrate, but not on GST (Fig. 6B). Positive results were also obtained when these Covaspheres were deposited on substratum coated with either GST-Ig1-2-3 or His-Ig1-2. However, His-Ig2-conjugated Covaspheres did not bind to substrate-coated His-Ig1. These results are consistent with the notion that L1-L1 binding is mediated by homophilic interactions between the second Ig-like domains of two apposing L1 molecules.

Whether His-Ig2 was able to interact with L1 molecules expressed by neural retinal cells was also examined. Retinal cells were isolated from day 6 chick embryos and cultured on coverslips. His-Ig2-conjugated Covaspheres were assayed for their ability to attach to substrate-coated His-Ig2. Binding of Covaspheres was observed on the His-Ig2 substrate, but not on GST (Fig. 6B). Positive results were also obtained when these Covaspheres were deposited on substratum coated with either GST-Ig1-2-3 or His-Ig1-2. However, His-Ig2-conjugated Covaspheres did not bind to substrate-coated His-Ig1. These results are consistent with the notion that L1-L1 binding is mediated by homophilic interactions between the second Ig-like domains of two apposing L1 molecules.

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Promotion of Neurite Outgrowth by L1 Fusion Proteins—L1 as a substrate has been found to be a potent neurite outgrowth promoter (14, 30, 31). To determine which fusion protein contained the stimulatory activity of L1-dependent neurite outgrowth, neural retinal cells were isolated from day 5 chick embryos and cultured on top of substrate-coated coverslips. The length of neurites extending from retinal neurons was measured after 18 h. Relatively long neurites were observed in cells cultured on the GST-Ig1-2-3 substrate (Fig. 7A). However, only short neurites were observed on substrate-coated with either GST, GST-Ig4-5-6, or GST-Fn (Fig. 7, b and c).

Quantitative analysis showed that the majority of neurites (>80%) extending from retinal cells cultured on GST-Ig4-5-6, GST-Fn, or GST were <25 μm, with mean neurite lengths ranging between 15 and 20 μm (Fig. 8). In contrast, retinal cells cultured on top of GST-Ig1-2-3 sent out much longer neurites, with a wider range of size distribution (Fig. 8A). Approximately 90% of them were ≥25 μm.
The patterns of neurite length distribution for GST-Ig1-2-3 and intact L1 were almost identical (Fig. 8). The data indicated that GST-Ig1-2-3 retained most of the neuritogenic activity of native L1. In comparison to substratum coated with GST where cells yielded a mean neurite length of 13 μm, retinal cells cultured on the GST-Ig1-2-3 substrate extended neurites with a 3-fold increase in their average length, whereas GST-Ig4-5-6 and GST-Fn did not lead to a significant increase in neurite outgrowth over the GST control.

Competition experiments were carried out using either soluble fusion protein or Fab fragments directed against Ig1–2-3 for 25 min and the excess competitor was removed by washing. Then fusion protein-conjugated Covaspheres were added, and incubation was carried out at room temperature for 45 min.

### Table I

| Protein conjugated to Covaspheres | Competitor added | Cells with bound Covaspheres | Inhibition |
|----------------------------------|------------------|------------------------------|------------|
| BSA                              |                  | 8.3 ± 0.3                    |            |
| His-Ig1                          |                  | 14.0 ± 1.3                   |            |
| His-Ig2                          |                  | 40.7 ± 1.5                   |            |
| His-Ig2 His-Ig2 (10 μg/ml)        |                  | 27.0 ± 3.6                   | 42.3       |
| His-Ig2 Fab (10 μg/ml)            |                  | 16.7 ± 1.5                   | 74.1       |
| His-Ig2 Fab (75 μg/ml)            |                  | 12.0 ± 2.0                   | 88.6       |

### Fig. 7. Epifluorescence micrographs of neurites extended by retinal cells.

(a) Neural retinal cells were isolated from E5 chick embryos and labeled with Dil. Retinal cells were cultured for 18 h on different fusion protein-coated substrates: GST-Ig1-2-3 (a), GST-Ig4-5-6 (b), and GST (c). Bar, 10 μm.

(b) The patterns of neurite length distribution for GST-Ig1-2-3 and intact L1 were almost identical (Fig. 8A), and their mean neurite lengths were 42.3 and 47.4 μm, respectively (Fig. 8B). The data indicated that GST-Ig1-2-3 retained most of the neuritogenic activity of native L1. In comparison to substratum coated with GST where cells yielded a mean neurite length of 13 μm, retinal cells cultured on the GST-Ig1-2-3 substrate extended neurites with a 3-fold increase in their average length, whereas GST-Ig4-5-6 and GST-Fn did not lead to a significant increase in neurite outgrowth over the GST control.

Competition experiments were carried out using either soluble fusion protein or anti-Ig1-2-3 Fab. When retinal cells were cultured in the presence of soluble GST-Ig1-2-3, neurite outgrowth was reduced to the background level. The pattern of neurite length distribution was similar to that of cells cultured on GST (Fig. 9). Similar inhibitory effects were observed when cells were cultured in the presence of anti-Ig1-2-3 Fab. In both cases, the active L1 sites on retinal cells and substratum were blocked by the competitor. Nevertheless, the number of cells attached to the coverslip did not decrease, suggesting that the anchorage of cells to the substratum per se was not sufficient to promote neurite outgrowth and that neurite outgrowth was dependent on the cellular response to the L1 substrate.

Localization of the Neuritogenic Activity of L1 to Ig-like Domain 2—To further narrow down the segment of L1 polypeptide that harbored the neuritogenic activity, the inhibitory effects of soluble His-Ig1-2, His-Ig1, and His-Ig2 on neurite outgrowth were examined using neural retinal cells cultured on substrate-coated GST-Ig1-2-3. His-Ig1 had relatively little effect on neurite outgrowth. However, the neurite outgrowth promotion activity of the substrate was abolished in the presence of His-Ig1-2 and His-Ig2 (Fig. 10). These results suggest that the second Ig-like domain can compete for the neuritogenic
This sequence corresponds to the C'-β-strand and the C'-E loop of the Ig fold (40, 47). The charged residues as well as the aromatic side-chains appear to play a crucial role in NCAM homophilic binding (40). The NCAM homophilic binding sequence is unique to Ig-like domain 3, and it probably interacts isologously with the same sequence on NCAM molecule present on apposing cells (39). A similar strategy of binding is used by the cell adhesion molecule gp80 in Dictyostelium discoideum (48–51). gp80 is a primitive member of the Ig superfamily of recognition molecules (51, 52), and it mediates cell-cell adhesion in a Ca\(^{2+}\)-independent manner. The homophilic binding site has been mapped to an octapeptide sequence (YKLN-VNDS), which is also predicted to adopt a β-strand conformation followed by the beginning of a β-turn structure (53). As in NCAM, both the amino-terminal Tyr residue and the two internal charged residues are vital to the homophilic binding activity of gp80. Furthermore, the homophilic binding site of gp80 is capable of undergoing isologous interaction with the same sequence in an anti-parallel manner (53).

The exact location of the homophilic binding site within Ig-like domain 2 of L1 is not yet known. However, two point mutations within this domain have been implicated in X-linked hydrocephalus and mental retardation. One of the mutations resulted in the replacement of Arg-184 with Gln, while the other mutation substitutes Gln for His-210 (20). Both mutations affect the folding of the Ig-like domain 2, resulting in the abolition or reduction in the affinity of L1 homophilic interactions. It is of interest to note that Arg-184 lies within a region corresponding to the predicted C'-β-strand of the Ig fold (14), suggesting that Arg-184 and its flanking sequences may participate in L1 homophilic binding in a manner similar to the C'-β-strand in the third Ig-like domain of NCAM.
Whereas L1 and NCAM undergo homophilic binding via interactions between two identical domains, the carcinoembryonic antigen (CEA), which is also a member of the Ig superfamily, adopts a heterologous binding mechanism. This involves the reciprocal interactions between the amino-terminal Ig-like domain of one molecule and an internal Ig-like domain of the opposing molecule (54). Since the Ig superfamily consists of a great variety of recognition molecules, it is conceivable that different mechanisms may be utilized in the adhesive processes mediated by different molecules. It remains to be determined whether the two mechanisms utilized by L1/NCAM and CEA are widely adopted by other members of the Ig superfamily.

In addition to being able to undergo homophilic binding, the Ig-like domain 2 of L1 is a potent inducer of neurite outgrowth from neural retinal cells. Our results showed that the Ig-like domains 4-6 and all five fibronectin type III repeats failed to promote neurite outgrowth from retinal cells. In contrast, Appel et al. (55) reported that L1 fusion proteins containing Ig-like domains 1-2, 3-4, 5-6, or fibronectin type III repeats 1-2 were all capable of promoting neurite outgrowth from small cerebellar neurons. Interestingly, a more recent study on NgCAM, a chicken homolog of L1, showed that only the fourth and fifth fibronectin-like domains of NgCAM were required for stimulating neurite outgrowth from dorsal root ganglia cells (56). It is possible that, depending on the relative levels of endogenous L1 and L1 receptors, different types of primary neurons may respond differently to these external peptide substrates. These apparently conflicting results may also reflect the complexities involved in neurite outgrowth.

It should be pointed out that the second Ig-like domain alone is sufficient to stimulate neurite outgrowth. Since the potency of His-Ig2 in our neurite outgrowth assay was comparable to that of intact L1, the other structural domains of L1 do not seem to be required in the initial step of activating the neurite outgrowth pathway. Our results suggest that an intimate relationship exists between L1 homophilic binding and L1-induced neurite outgrowth. Similar observations have been made when retinal cells were cultured on top of a monolayer of NCAM-expressing L1 cell transfectants. Here retinal neurons extend much longer neurites than those cultured on control cells (38, 57). However, mutations in the NCAM homophilic binding site abrogate the ability of NCAM to stimulate neurite outgrowth (38).

Lemmon et al. (27) have shown that L1 stimulated neurite outgrowth via a homophilic binding mechanism. It is therefore conceivable that the substrate-coated His-Ig2 may interact with the Ig-like domain 2 of L1 on cells, which in turn generates neurite outgrowth signals either by inducing conformational changes in the L1 molecule or by altering L1 interactions with neighboring membrane and cytoplasmic components. A direct association between the cytoplasmic domain of L1 and ankyrin has been reported (58). The cytoplasmic domain of L1 has also been found to associate with both protein kinase C and non-receptor tyrosine kinase activities and it can be phosphorylated (59, 60). Both tyrosine phosphorylation and Ca\(^{2+}\) influx have been found to be key steps in the signaling pathways initiated by cell adhesion molecules (30, 35, 57).

A recent study (37) showing that L1 clustering by a soluble bivalent L1-Fc chimeric proteins leads to an increase in neurite outgrowth is consistent with the notion that L1-L1 homophilic interaction serves as the first step in the signaling cascade that leads to neurite extension. It is of interest to note that NCAM behaves somewhat differently in this respect. We have previously found that a synthetic peptide which contains the NCAM homophilic binding site within a 21-amino acid sequence is a potent inducer of neurite outgrowth from retinal cells (38). This suggests that while NCAM homophilic binding is required, clustering of NCAM molecules may not be essential.

The signaling cascade involved in L1-dependent neurite outgrowth is a subject of considerable debate. Several recent reports implicate an essential role for the FGF receptor in neurite outgrowth induced by several cell adhesion molecules, including L1, NCAM, and N-cadherin (36, 37). A similar neurite outgrowth response can be elicited by treatment with basic FGF (61), suggesting that L1-L1 binding may lead to the activation of FGF receptor. Since both the L1-Fc chimera and basic FGF induce increases in tyrosine phosphorylation on a common set of neuronal proteins (36), an identical pathway that involves the activation of FGF receptor has been postulated for all cell adhesion molecule-dependent neurite outgrowth (36, 61). On the other hand, studies using sry or fyn knock-out mice have indicated that pp60\(^{src}\) is an essential component of the intracellular signaling pathway in L1-mediated neurite outgrowth (62). Whereas the L1 response is dependent on the non-receptor tyrosine kinase pp60\(^{src}\), NCAM-stimulated neurite outgrowth is dependent on p59\(^{yn}\) since neuronal cells derived from fyn-minus mice fail to respond to NCAM (63). These results argue for the involvement of distinctly different components in the early steps of signaling pathways induced by L1 and NCAM. However, the nature of the association between either L1 and sry or NCAM and fyn is still not known. Similarly, there is no direct evidence demonstrating physical interactions between L1 and FGF receptor. Future experiments to address this and related issues will be required to resolve the discrepancy between these models.

L1 is a multidomain molecule and is known to undergo heterophilic interactions with other molecules such as NCAM (21, 22, 64), TAG-1/axonin-1 (23, 24), F3/F11 (25), and brain proteoglycans (29, 65). Interactions with these membrane and extracellular components may have important roles at specific stages of brain development. In addition to the mutations detected in Ig-like domain 2, several other mutations in L1 have been reported to co-segregate with X-linked hydrocephalus (18, 19, 66). Some mutations have been found to affect the expression of L1; others may have deleterious effects on L1 interactions with its ligands. Further investigation of the role of L1 in brain development will depend on the identification of its homophilic and heterophilic binding sites as well as the elucidation of their mechanisms of interaction. These studies should help us better understand how the hydrocephalus-related mutations affect L1 functions and provide new insights in the cause of X-linked hydrocephalus and mental retardation.

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REFERENCES

1. Carpenter, E. M., and Holiday, M. (1992) Dev. Biol. 150, 160–170
2. Martini, R. (1994) J. Neurocytol. 23, 1–28
3. Takahashi, R. (1990) Science 245, 1455
4. Reichardt, L. F., and Tomasselli, K. J. (1991) Annu. Rev. Neurosci. 14, 531–570
5. Rathjen, F. G., and Jessel, T. M. (1991) Semin. Neurosci. 3, 297–307
6. Grumet, M., Mauro, V., Burgoon, M. P., Edelman, G. M., and Cunningham, B. A. (1991) J. Cell Biol. 113, 1399–1412
7. Lindner, J., Rathjen, F. G., and Schachner, M. (1983) Nature 305, 427–430
8. Moos, M., Tacke, R., Scherer, H., Teplow, D., Früh, K., and Schachner, M. (1988) Nature 334, 703–707
9. Grumet, M., Hoffman, S., Chuang, C. M., and Edelman, G. M. (1984) Proc. Natl. Acad. Sci. U S A 81, 7989–7993
10. Rathjen, F. G., Wolff, J., McLaughlin, R., Bonhoeffer, F., and Rutishauser, U. (1987) J. Cell Biol. 104, 343–353
11. Lemmon, V., and McDon, C. S. (1986) J. Neurosci. 6, 2987–2994
12. Miura, M., Kobayashi, H., Asou, H., and Uyemura, K. (1991) FEBS Lett. 289, 91–95
13. Prince, J. T., Alberti, L., Healy, P. A., Nauman, S. J., and Stallcup, W. B. (1991) J. Neurosci. Res. 30, 567–581
14. Hlavac, M. L., and Lemmon, V. (1991) Genomics 11, 416–423
15. Kobayashi, M., Miura, M., Asou, H., and Uyemura, K. (1991) Biochim.
Homophilic Binding Site and Neuritogenic Site of L1

16. Chapman, V. M., Keltz, B. T., Stephenson, D. A., Mullins, L. J., Moos, M., and Schachner, M. (1990) Genomics 8, 113–118
17. Djabali, M., Mattei, M. G., Nguyen, C., Roux, D., Demengeot, J., Denizot, F., Moos, M., Schachner, M., Goridis, C., and Jordan, B. R. (1990) Genomics 7, 587–593
18. Rosenthal, A., Jouet, M., and Kenwrick, S. (1992) Nature Genet. 2, 107–112
19. Camp, G. V., Vits, L., Coucke, P., Lyonnet, S., Schrander-Stumpel, C., Darby, J., Holden, J., Mun nich, A., and Willems, P. J. (1993) Nature Genet. 4, 421–425
20. Jouet, M., Rosenthal, A., Armstrong, G., MacFarlane, J., Stevenson, R., Rosenthal, A., Jouet, M., and Kenwrick, S. (1992)
21. Kadmon, G., Kowitz, A., Altevogt, P., and Schachner, M. (1990) J. Cell Biol. 110, 193–208
22. Horstkorte, R., Schachner, M., Magyar, J. P., Vorherr, T., and Schmitz, B. (1990) J. Cell Biol. 121, 1409–1421
23. Kuhn, T. B., Stoeckli, E. T., Condrau, M. A., Rathjen, F. G., and Sonderegger, Felsenfeld, D. P., Hynes, M. A., Skoler, M., Furley, A. J., and Jessel, T. M. (1993) Neuron 10, 711–727
24. Grumet, M., Friedlander, D. R., and Edelman, G. M. (1982) Proc. Natl. Acad. Sci. U. S. A. 84, 7753–7757
25. Hoffman, S., and Edelman, G. M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5762–5766
26. Pedc, D., and Walsh, F. S. (1993) J. Cell Biol. 123, 1587–1595
27. Lemmon, V., Farr, K. L., and Lagenaur, C. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 675–680
28. Grumet, M., Rutishauser, U., and Edelman, G. M. (1982) Nature 295, 693–695
29. Edelman, G. M. (1988) Biochemistry 27, 3533–3543
30. Williams, E. J., Furness, J., Walsh, F. S., and Doherty, P. (1994) J. Cell Biol. 123, 269–278
31. Doherty, P., Ashton, S. V., Moore, S., and Walsh, F. S. (1991) J. Cell Biol. 110, 269–278
32. Doherty, P., and Walsh, F. S. (1993) J. Cell Biol. 121, 1409–1421
33. Doherty, P., and Walsh, F. S. (1992) J. Cell Biol. 121, 1409–1421
34. Williams, E. J., Doherty, P., Ashton, S. V., Moore, S., and Walsh, F. S. (1991) J. Cell Biol. 110, 269–278
35. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
36. Williams, E. J., Furness, J., Walsh, F. S., and Doherty, P. (1994) Neuron 13, 583–594
37. Doherty, P., Williams, E., and Walsh, F. S. (1995) Neuron 14, 57–66
38. Sandig, M., Rao, Y., and Siu, C. H. (1994) J. Biol. Chem. 269, 27540–27548
39. Rao, Y., Zhao, X., and Siu, C. H. (1994) J. Biol. Chem. 269, 27540–27548
40. Rao, Y., Wu, X. F., Yip, P., Gariephy, J., and Siu, C. H. (1993) J. Biol. Chem. 268, 20630–20638
41. Sandig, M., Rao, Y., and Siu, C. H. (1994) J. Biol. Chem. 269, 14841–14848
42. Lagenaur, C., and Lemmon, V. (1987) J. Cell Biol. 105, 2523–2533
43. Hoffman, S., and Edelman, G. M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5762–5766
44. Grumet, M., Rutishauser, U., and Edelman, G. M. (1982) Nature 295, 693–695
45. Edelman, G. M. (1988) Biochemistry 27, 3533–3543
46. Pedc, D., and Walsh, F. S. (1993) J. Cell Biol. 123, 1587–1595
47. Rao, Y., Wu, X. F., Gariephy, J., Rutishauser, U., and Siu, C. H. (1992) J. Cell Biol. 118, 937–945
48. Siu, C. H., Lam, T. Y., and Choi, A. C. (1985) J. Biol. Chem. 260, 1620–1626
49. Siu, C. H., Cho, A., and Choi, A. H. C. (1987) J. Cell Biol. 105, 2523–2533
50. Kamboj, R. K., Wong, L. M., Lam, T. Y., and Siu, C. H. (1988) J. Cell Biol. 107, 1835–1843
51. Kamboj, R. K., and Kamboj, R. K. (1990) Dev. Genet. 11, 377–387
52. Matsunaga, T., and Mori, N. (1987) J. Cell Biol. 106, 485–495
53. Kamboj, R. K., Gariephy, J., and Siu, C. H. (1989) Cell 59, 635–639
54. Zhou, H., Fuks, A., Alcaraz, A., Bolling, T. J., and Stanners, C. P. (1993) J. Cell Biol. 122, 951–960
55. Appel, F., Holm, J., Conscience, J., and Schachner, M. (1993) J. Neurosci. 13, 4764–4775
56. Burgoon, M. P., Hazan, R. B., Phillips, G. R., Crossin, K. L., Edelman, G. M., and Cunningham, B. A. (1995) J. Cell Biol. 130, 733–744
57. Doherty, P., Ashton, S. V., Moore, S., and Walsh, F. S. (1991) J. Cell Biol. 110, 217–2176
58. Davis, J. Q., and Bennett, V. (1994) J. Biol. Chem. 269, 879–892
59. Sadoul, R., Winkelmann, P., and Schachner, M. (1989) J. Neurochem. 53, 1471–1476
60. Bixby, J. L., and Jhabvala, P. (1990) J. Cell Biol. 111, 2725–2732
61. Williams, E. J., Furness, J., Walsh, F. S., and Doherty, P. (1994) Development 120, 1685–1693
62. Ignotz, R. A., Miller, D. R., Soriano, P., and Maness, P. F. (1992) Neuron 12, 873–884
63. Beggs, H. E., Soriano, P., and Maness, P. F. (1994) J. Cell Biol. 123, 825–833
64. Kamboj, R. K., Altevogt, P., and Schachner, M. (1990) J. Cell Biol. 110, 209–218
65. Morgan, D. R., Sakurai, T., Kanno, I., and Maness, P. F. (1994) J. Cell Biol. 123, 1703–1715
66. Wong, E. V., Kenwrick, S., Willems, P., and Lemmon, V. (1995) Trends Neurosci. 18, 168–172
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