Several Extracellular Domains of the Neural Cell Adhesion Molecule L1 Are Involved in Neurite Outgrowth and Cell Body Adhesion

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The neural cell adhesion molecule L1 is a multidomain protein that plays important roles in cell adhesion, migration, and neurite outgrowth. To analyze structure–function relationships of L1 in neurite outgrowth and cell body adhesion, we have expressed and purified a set of different fragments of the extracellular part of this glycoprotein in CHO cells and in *Escherichia coli*. When neurite outgrowth from small cerebellar neurons was measured on substrate-coated L1 or L1 fragments, neurite outgrowth was promoted by the immunoglobulin-like domains I–II, III–IV, and V–VI, and by the fibronectin type III homologous repeats 1–2, while the fibronectin type III homologous repeats 3–5 were ineffective. In contrast, cell bodies of small cerebellar neurons adhered mostly to the immunoglobulin-like domains I–II and V–VI, and to the fibronectin type III homologous repeats 3–5, but less to the immunoglobulin-like domains III–IV and fibronectin type III homologous repeats 1–2. In both assays, the neuronal cell surface receptor for all active protein fragments was identified as L1. No significant differences in functional activities were found between fragments with and without carbohydrate structures. These findings indicate that L1 uses several domains for homophilic interactions overlapping for the two functions analyzed here, but also showing some regional specialization. Furthermore, we show that a homophilic molecule uses several domains in one function, with neurite outgrowth requiring more domains than adhesion for maximal activity.

[Key words: cell adhesion, fibronectin type III homologous repeats, homophilic binding, immunoglobulin superfamily, L1, neurite outgrowth]

Cell-to-cell interactions have been developed to a high degree of precision in the nervous system. Various cell surface and extracellular matrix molecules have been identified to be involved in neuron–neuron and neuron–glia recognition (for reviews, see Dodd and Jessell, 1988; Edelman et al., 1990; Edelman and Crossin, 1991; Schachner, 1991). In search for their particular functions, their structure was determined with the aim of relating specific functional traits to distinct structural

features. Several neural cell adhesion molecules were shown to belong to the immunoglobulin superfamily (Williams and Barclay, 1988) and to contain structural motifs characteristic of extracellular matrix molecules, the fibronectin type III homologous repeats. These include N-CAM (Barthels et al., 1987; Cunningham et al., 1987), L1 (Moos et al., 1988), Ng-CAM (Burgoon et al., 1991; Grumet, 1992), Nr-CAM (Grumet et al., 1991), TAG-1 (axonin-1) (Furley et al., 1990; Zuellig et al., 1992), and F3/F11 (Brümmendorf et al., 1989; Gennarini et al., 1989) in vertebrates, and their homologs in invertebrates (Harrelson and Goodman, 1988; Mayford et al., 1992).

The neural cell adhesion molecule L1, and its homologs in other species, such as human L1CAM and rat NILE, is one of these molecules (for reviews, see Schachner, 1990, 1991). The extracellular part of L1 consists of six immunoglobulin-like domains of the C2 type (Williams and Barclay, 1988) and five fibronectin type III homologous repeats. L1 contains a membrane-spanning region and a short, highly conserved cytoplasmic tail of approximately 120 amino acids (Moos et al., 1988). L1 increases neurite outgrowth on L1-positive partner cells (Bixby et al., 1987; Chang et al., 1987; Seilheimer and Schachner, 1988) or on purified L1 (Lagenaur and Lemmon, 1987; Chang et al., 1990) and is a substrate for cell body adhesion (Kadmon et al., 1990a; Miura et al., 1992). It has been suggested that neurite outgrowth on substrate-coated L1 and cell adhesion to L1 are due to a homophilic binding mechanism (Lemmon et al., 1989; Miura et al., 1992). In addition to this homophilic interaction, evidence for assisted homophilic (Kadmon et al., 1990a,b) and heterophilic binding mechanisms has been provided (Grumet and Edelman, 1988; Werz and Schachner, 1988; Drazba and Lemmon, 1990; Pollerberg et al., 1990; Smith et al., 1990; Kuhn et al., 1991). Furthermore, L1 is involved in signal transduction from the cell surface to the interior, leading to changes in the levels of intracellular second messengers, such as inositol phosphates, Ca^{2+} , pH, and cyclic nucleotides (Schuch et al., 1989; von Bohlen und Halbach et al., 1992; Williams et al., 1992), and in kinase activity (Sadoul et al., 1990; Atashi et al., 1992).

In contrast to cell surface molecules of the immune system, structure–function relationships of the neural cell surface molecules belonging to the immunoglobulin superfamily remain largely unresolved. Apart from studies on N-CAM (Frelinger and Rutishauser, 1986; Frei et al., 1992; Rao et al., 1992), little attention has been given to the involvement of the immunoglobulin-like domains and fibronectin type III homologous repeats of different neural cell adhesion molecules in such functions as adhesion of neuronal cell bodies and neurite outgrowth. To investigate such structure–function relationships in L1 and to compare them to those of N-CAM, we produced recombinant

Received Jan. 14, 1993; revised May 11, 1993; May 17, 1993.

We are grateful to Dr. Sergio Gloor, Department of Neurobiology, ETH Zürich, and Dr. Johannes Buchner, University of Regensburg, for helpful suggestions; Dr. Thomas Jessell, Columbia University, New York, for TAG-1 antibodies; Dr. James Cohen, Guy's Hospital, London, and Dr. Joanne Taylor, Department of Neurobiology, ETH Zürich, for critical reading of the manuscript; Jürg Sigrist for technical assistance; Anja Marxer for the preparation of brain L1 and brain MAG; and Studienstiftung des Deutschen Volkes for a predoctoral fellowship (F.A.). This work was supported by the Schweizerischer Nationalfonds.

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glycoprotein and protein fragments in Chinese hamster ovary (CHO) cells and *Escherichia coli* and used these as substrates for neuronal cell body adhesion and neurite outgrowth. Here we report that immunoglobulin-like domains, as well as fibronectin type III homologous repeats, are involved in L1-mediated adhesion and neurite outgrowth. We could show that at least four domains promote neurite outgrowth and at least five domains participate in short-term adhesion. As demonstrated by antibody inhibition experiments, L1 is a part of the cellular receptor for all active L1 protein fragments mediating neuronal adhesion and neurite outgrowth.

Materials and Methods

Materials

L1 (brain L1) and the myelin-associated glycoprotein (brain MAG) were purified by immunoaffinity chromatography from detergent-containing extracts of crude membrane fractions of adult mouse brain using monoclonal antibody columns (Rathjen and Schachner, 1984; Poltorak et al., 1987). Laminin from the murine EHS sarcoma was obtained from Boehringer Mannheim (Mannheim, Germany). Poly-L-lysine and fatty acid-free bovine serum albumin were from Sigma (München, Germany).

The rat monoclonal antibodies against L1 (324, 555, and 557.B6) were used as ammonium sulfate precipitates of hybridoma culture supernatants containing 1% fetal bovine serum, and were dissolved in and dialyzed against phosphate-buffered saline, pH 7.3 (PBS), before use. They will be described in a forthcoming report (F. Appel, J.-F. Conscience, J. Holm, F. von Bohlen und Halbach, A. Faissner, and M. Schachner, unpublished observations). Monoclonal antibody 324 reacts, according to ELISA, with the recombinant protein containing the immunoglobulin-like domains I–VI. Monoclonal antibodies 555 and 557.B6 react, in ELISA, with an epitope at the border between the fibronectin type III homologous repeats 2 and 3. Polyclonal antibodies against L1 were obtained from rabbits immunized with brain L1 (Rathjen and Schachner, 1984). The IgG fractions of these antibodies were purified by chromatography using a protein A Sepharose 4B fast-flow column (Sigma, München, Germany) (Ey et al., 1978). For immunoaffinity purification of polyclonal L1 antibodies, the ammonium sulfate-precipitated fraction was bound to an L1-coupled Sepharose CL4B column and eluted as described previously (Martini and Schachner, 1986). All secondary antibodies were obtained from Dianova (Hamburg, Germany). Monoclonal antibody columns containing Sepharose CL4B beads conjugated with antibodies 324 or 555 were prepared according to standard procedures (Harlow and Lane, 1988).

Analytical procedures

Protein determinations were performed as described by Bradford (1976). Proteins were separated by SDS-PAGE on 8%, 10%, or 12% slab gels (Laemmli, 1970), under reducing or nonreducing conditions. Protein bands were stained with Coomassie blue (0.05% Coomassie brilliant blue R250, 50% methanol, 10% acetic acid) or transferred to nitrocellulose filters (0.45 μ m, BA 85; Schleicher and Schuell, Dassel, Germany) for Western blot analysis according to Faissner et al. (1985), using alkaline phosphatase-coupled secondary anti-rabbit or anti-rat IgG. Polyclonal and monoclonal antibodies to L1 were used at concentrations of 0.5 μ g/ml and 50 μ g/ml, respectively.

The ELISAs were performed as described by Husmann et al. (1992) using polyclonal and monoclonal antibodies at concentrations of 0.5 μ g/ml and 100 μ g/ml, respectively. Secondary antibodies were horseradish peroxidase-coupled anti-rabbit or anti-rat IgG and developed with 1 mg/ml 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)] (Boehringer Mannheim, Mannheim, Germany) dissolved in 100 mM Na-acetate, 50 mM Na-phosphate (pH 4.2), and 0.01% H_2O_2 . The optical density was measured at 405 nm with an ELISA reader (Titertek Multiskan MKII, Flow, Basel, Switzerland).

To estimate the coating efficiency of proteins on bacteriological plasticware, 96-well plates (2-69620A, Nunc, Denmark) were coated with proteins or glycoproteins at concentrations from 0.5 to 30 μ g/ml for 3 hr at 37°C in PBS. Then, the plates were washed once with PBS, incubated with a Coomassie brilliant blue solution (0.05% Coomassie brilliant blue R250, 50% methanol, 10% acetic acid) for 30 min at room temperature and washed thoroughly with distilled water. Bound Coom-

assie blue was solubilized first with 20 μ l of 10% SDS in H_2O (10 min) and then with 50 μ l of 70% ethanol (30 min), all at room temperature. The optical density of the Coomassie blue solution was determined at 595 nm using an ELISA reader. To estimate the coating efficiency of proteins on poly-L-lysine-coated glass coverslips, the same procedure was used, but the bound Coomassie blue was solubilized with 150 μ l of 10% SDS in H_2O and 50 μ l of 70% ethanol.

Recombinant protein expression in CHO cells

To generate plasmids containing secretion signal sequences and stop codons in the right frame, different strategies were used. For the sequences coding for the complete extracellular part of L1 (sL1CHO, amino acids 1–1093 according to Moos et al., 1988) and for the six immunoglobulin-like domains (IgI-VICHO, amino acids 1–631), an oligonucleotide coding for three stop codons in every reading frame (CTAGCTAGCTAG) was ligated into the blunt-ended EcoNI [base pair (bp) 3274, sL1CHO] or ApaLI (bp 1888, IgI-VICHO) restriction site (Fig. 1A). For the fibronectin type III homologous repeats, a plasmid was constructed that encoded the signal peptide of L1 (amino acids 1–20), the first 10 amino acids of L1 (amino acids 21–30), three extra amino acids resulting from the addition of a BglII linker, the coding sequence of amino acids 631–1093 flanked by a BamHI linker (Boehringer Mannheim, Mannheim, Germany), and stop codons in all three reading frames (F. Appel, J. Holm, and S. Gloor, unpublished observations). The correct sequence of the three plasmids was confirmed by sequencing both ends. The coding sequences of the three constructs were isolated and inserted into the pEE.14 eukaryotic expression vector, which carries the human cytomegalovirus promoter and a glutamine synthetase gene as selection marker (Crockett et al., 1990; Bebbington, 1991).

Transfection with these constructs and selection of transfected CHO cells were carried out according to Bebbington (1991). In brief, semi-confluent CHO cells were transfected using the calcium phosphate precipitation method and transfected cells were selected in glutamine-free medium (GIBCO/Bethesda Research Labs, Basel, Switzerland) containing 10% dialyzed fetal calf serum and 25 μ M L-methionine sulfoximine. After 2 weeks, colonies were isolated and expanded in the same medium. Supernatants of confluent cultures were collected and subjected to ELISA and Western blot analysis. Typically, 5 out of 30 colonies secreted the protein in detectable amounts and the best producers were cloned. The conditioned medium of these clones was used to isolate the recombinant proteins.

Recombinant protein expression in *E. coli*

DNA fragments encoding seven different portions of L1 were produced using appropriate restriction enzymes (Fig. 1B), namely, fragments coding for the immunoglobulin-like domains I–VI (IgI-VIEco, amino acids 44–661 of L1 according to Moos et al., 1988), I–II (IgI-IIeco, amino acids 22–240), III–IV (IgIII-IVeco, amino acids 239–427), and V–VI (IgV-VIEco, amino acids 426–661), and the fibronectin type III homologous repeats 1–5 (FN1-5Eco, amino acids 631–1093), 1–2 (FN1-2Eco, amino acids 631–826), and 3–5 (FN3-5Eco, amino acids 828–1093). Each fragment was subcloned in the unique BamHI restriction site of the pET vector (Studier and Moffatt, 1986; Rosenberg et al., 1987; Studier et al., 1990) or modified pET vector (J. Holm, F. Appel, and S. Gloor, unpublished observations) by treating all fragments with T4 DNA polymerase and adding BamHI linkers at both ends according to standard methods (Sambrook et al., 1989). The correct sequence of the plasmids was confirmed by sequencing with a T7 polymerase sequencing kit (Pharmacia, Uppsala, Sweden). Finally, the *E. coli* strain BL21 (DE3) was transformed with the plasmids (Studier and Moffatt, 1986).

For production of recombinant protein, transformed *E. coli* cells were grown at 37°C in 1000 ml Erlenmeyer flasks containing 250 ml of LB medium with 200 μ g/ml ampicillin. Protein synthesis was induced by adding 0.4 mM isopropyl- β -D-1-thiogalactopyranoside to cultures in exponential phase (OD₆₀₀ between 0.6 and 0.8). The induced proteins were isolated as inclusion bodies by sonifying the harvested cells for 3 min in 10 ml of PBS on ice. Inclusion bodies collected by centrifugation for 10 min at 10,000 $\times$ g and 4°C were kept frozen in PBS until use.

Purification of recombinant glycoproteins from CHO cells

The glycoprotein fragments sL1CHO, IgI-VICHO, and FN1-5CHO were purified from conditioned culture medium by immunoaffinity chromatography using columns containing immobilized monoclonal anti-

body 324 or 555. The columns were washed with 0.02 M Tris·HCl, 0.15 M NaCl, 1 mM EGTA, and 1 mM EDTA, pH 7.2. The bound proteins were eluted with 0.1 M diethylamine·HCl, 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, pH 11.5, and the eluted fractions were immediately neutralized with 1 M Tris·HCl, pH 6.8. Protein-containing fractions were concentrated on Amicon YM30 membranes (Wallisellen, Switzerland) and dialyzed against PBS (Fig. 2A). The purity of IgI-VICHO was determined by visual inspection of silver-stained gels after SDS-PAGE. The yields of sgL1CHO and FN1-5CHO were so small that the glycoproteins could only be detected by Western blot analysis using polyclonal antibodies, but not by silver staining.

Purification of recombinant proteins from *E. coli* cells

Recombinant proteins obtained as inclusion bodies from *E. coli* were solubilized in 8 M urea solutions containing 1 mM dithioerythritol, in different buffers according to the calculated isoelectric point (IEP) of the recombinant protein. For the recombinant proteins IgI-IIIEco (IEP 6.7), IgIII-IVEco (IEP 6.8), and FN1-5Eco (IEP 6.7), the urea solution was buffered with 20 mM Tris·HCl, pH 8.0; for IgI-VIEco (IEP 5.1), IgV-VIEco (IEP 4.8), and FN1-2Eco (IEP 5.0), with 20 mM histidine·HCl, pH 5.5; and for FN3-5Eco (IEP 8.4), with 20 mM HEPES, pH 7.5. The recombinant proteins, with the exception of FN3-5Eco, were then purified by fast protein liquid chromatography (FPLC) on a Mono Q column (Pharmacia, LKB, Uppsala, Sweden) by applying a NaCl gradient from 0 to 750 mM. FN3-5Eco was purified by FPLC on a Mono S column (Pharmacia, LKB, Uppsala, Sweden), also by applying a NaCl gradient from 0 to 750 mM. Eluted fractions were analyzed by SDS-PAGE (12%), and the fractions with the highest purity were combined and dialyzed for at least 36 hr at 4°C against PBS, in the case of recombinant proteins IgI-IIIEco, IgIII-IVEco, and FN1-2Eco; against 20 mM Tris·HCl, pH 8.5, in the case of IgI-VIEco, IgV-VIEco, FN1-5Eco; and against 20 mM acetic acid, pH 5.5, in the case of FN3-5Eco. This dialysis against different buffers ensured that the protein fragments remained in solution when they were later diluted in PBS to the final concentration used in the test systems. All proteins exhibited a purity of more than 90% as estimated by SDS-PAGE (Fig. 2B). In the case of IgI-VIEco, the protein was also used unpurified, after solubilization in urea and dialysis against 20 mM Tris·HCl, pH 8.5. This fraction, named IgI-VIEco n.p. (n.p. = not purified), contained approximately 30% recombinant protein, as estimated by SDS-PAGE.

Determination of neurite length

Diethylether-cleaned glass coverslips (1.4 cm in diameter) were coated overnight at 4°C with poly-L-lysine (200 µg/ml in distilled water) and washed three times with distilled water. Stock solutions of proteins or glycoproteins were diluted in PBS and incubated on the coverslips overnight at 37°C. The coating efficiency for the different glycoprotein and protein fragments, estimated as described in Analytical procedures, was similar for the protein fragments, since equal concentrations of the coating solutions, measured by the method of Bradford (1976) using Coomassie blue as a detection reagent, resulted in equal OD₅₉₅ of adsorbed material. After washing the coverslips 10 times with basal medium Eagle's and twice with serum-free neuron culture medium (Fischer, 1982), purified small neurons from 6–7-d-old ICR mouse cerebella were seeded onto the coverslips (10⁵ cells in 100 µl of neuron culture medium per coverslip). The small cerebellar neurons were obtained by sedimenting trypsinized cerebellar cells through a Percoll gradient (Keilhauer et al., 1985). The resulting neurons reexpressed L1 at their surface already after 1 hr of incubation at 37°C, as determined by indirect immunofluorescence with a polyclonal antibody directed against the recombinant protein fragment IgI-VICHO (not shown). After incubation at 37°C for 3 hr, the coverslips were immersed in 2 ml of fresh medium in petri dishes (35 mm in diameter) and maintained at 37°C for additional 21 hr. The cells were then fixed for 1 hr by adding 100 µl of 25% glutaraldehyde solution and stained with crystal violet (0.5% crystal violet in 10% ethanol, 3% formaldehyde, 0.15 M NaCl) for 10 min at room temperature. Antibody inhibition experiments were carried out by including antibodies at concentrations of 61 µg/ml (324) or 360 µg/ml (557.B6) in neuron culture medium at the time of flooding the coverslips.

Crystal violet-stained cultures were analyzed by evaluating the lengths of individual neurites, using an Ai Tectron image analysis system (vms iv software, Bestobell Mobrey, Düsseldorf, Germany). Only neurites that did not contact other cells or neurites and had a length of at least one cell diameter were measured (Lemmon et al., 1989). For each experimental value, at least two independent experiments were performed in duplicate and more than 120 neurites (30 neurites/coverslip) were measured for each experimental value. For each substrate, more than two different preparations were used with similar results. Values for neurite outgrowth on different substrates are given as absolute increase in neurite length over the length obtained on poly-L-lysine alone (29 ±

Figure 2. Biochemical characterization of recombinant L1 glycoprotein and protein fragments. *A*, SDS-PAGE and subsequent Western blot of protein fragments expressed in CHO cells (for abbreviations, see Fig. 1 caption). After electrophoresis, proteins were blotted onto nitrocellulose and incubated with immunoaffinity-purified polyclonal antibody against L1 (50 μ g/ml). The antibody binding was visualized by the enzymatic activity of alkaline phosphatase conjugated to an anti-rabbit IgG. The upper band of sL1CHO is a double band of 160 and 180 kDa. *B*, Characterization of purified protein fragments from *E. coli* by SDS-PAGE under reducing conditions (for abbreviations, see Fig. 1 caption). Proteins were visualized by staining with Coomassie blue. Molecular weight markers are indicated in kilodaltons at the left margins.

3 μ m per cell). For independent experimental values, first the Kruskal-Wallis and then the Mann-Whitney *U* rank test, and for dependent values (antibody inhibition experiments) first the Friedman and then the Wilcoxon test, were used to determine the significance of differences in values, with $P < 0.05$ and $P < 0.01$ being considered significant and highly significant, respectively.

Cell-to-substratum adhesion test

On nitrocellulose. Bacteriological petri dishes (35 mm in diameter) were coated with nitrocellulose (BA85, Schleicher and Schuell, Dassel, Germany) dissolved in methanol according to Lagenaur and Lemmon (1987). Glycoprotein and protein fragments were diluted in PBS and spotted onto the nitrocellulose. The spots were not allowed to dry and, after incubating for 15 min, excess solution was removed and the dishes were blocked for 30–60 min with a solution containing 5% BSA in PBS, all at room temperature. Single cell suspensions of small cerebellar neurons were prepared (see Determinations of neurite length, above) and seeded into the petri dishes (10⁶ cells in 2 ml of neuron culture medium per dish). After incubation at 37°C for 3 hr, the dishes were shaken at 150 rpm on a rotary minishaker (Alfred Kühner, Basel, Switzerland) for 2 min at room temperature. The cells were then fixed by adding 100 μ l of 25% of glutaraldehyde for 30 min. The dishes were washed three times with distilled water to remove nonadherent cells and examined by phase-contrast microscopy. Numbers of adherent cells from four randomly chosen areas were determined with the help of an image processing system (IBAS, Kontron, Zürich, Switzerland). For any substrate, more than two different preparations were tested, with essentially identical results. In all figures, values for adhesion on different substrates are given as the percentage of cells bound to poly-L-lysine minus the unspecifically bound cells on blocked nitrocellulose. Raw data from at least four independent experiments were analyzed for statistical significance using hierarchical ANOVA and then the Newman-Keuls test, with $P < 0.05$ and $P < 0.01$ being considered significant or highly significant, respectively.

On bacteriological plastic. Ninety-six well bacteriological plates (2-69620A, Nunc, Denmark) were coated with glycoprotein and protein fragments at different concentrations from 0.5 to 30 μ g/ml for 3 hr at 37°C in PBS. The coating efficiency for the different glycoprotein and protein fragments, estimated as described in Analytical procedures, was similar for the protein fragments, since equal concentrations of coating solution, measured by the method of Bradford (1976) using Coomassie blue as a detection reagent, resulted in equal OD₅₉₅ of adsorbed material. After blocking unsaturated binding sites with 5% BSA in PBS for 30 min at room temperature, single cell suspensions of small cerebellar neurons were seeded in 100 μ l of neuron culture medium at a density of 10⁵ neurons per well. After 3 hr at 37°C in a CO₂ incubator, the cells were fixed by adding 5 μ l of 25% glutaraldehyde solution in H₂O and incubated for at least 30 min at room temperature. Plates were then washed once with PBS and the cells were stained with crystal violet (see Determination of neurite length, above) for 10 min at room temperature.

After washing the plates with distilled water, bound crystal violet was solubilized by incubating first for 10 min with 20 μ l of 10% SDS in H₂O and adding afterward 50 μ l of 70% ethanol for 30 min at room temperature. The optical density of the crystal violet solution was determined at 595 nm (OD₅₉₅) using an ELISA reader. Cell adhesion was expressed as the OD₅₉₅ value obtained on a given substrate, minus the background value obtained on blocked plastic. For comparison, the values observed on different substrates were expressed as the percentage of the value observed on poly-L-lysine. The data from at least three independent experiments carried out in triplicates were analyzed for statistical significance as described in the previous paragraph.

Results

To study the involvement of different extracellular domains of the neural cell adhesion molecule L1 of the mouse in neurite outgrowth and cell body adhesion, fragments of the molecule corresponding to the different structural domains were produced as recombinant proteins and glycoproteins in a prokaryotic and a eukaryotic expression system, respectively. In the prokaryotic expression system, *E. coli* cells, transformed with the pET expression vector (Studier et al., 1990), express nonglycosylated peptides whose disulfide bridges might not be correctly located. In the eukaryotic expression system (Bebbington, 1991), secreted soluble molecules can be expected to have correctly folded disulfide bridges and should be glycosylated, but the glycosylation pattern obtained in CHO cells may be different from that in the nervous system.

Recombinant glycoprotein fragments of L1 expressed in CHO cells

Three glycoprotein fragments of L1 (sL1CHO, IgI-V1CHO, FN1-5CHO; see Fig. 1*A*) secreted by stably transfected CHO cell clones were purified by immunoaffinity chromatography from conditioned culture medium using monoclonal antibodies reactive with epitopes expressed in the individual fragments. Preparations of IgI-V1CHO were at least 98% pure (not shown). The purity of sL1CHO and FN1-5CHO could not be ascertained, since these preparations gave no bands in silver-stained gels. In Western blots using polyclonal L1 antibodies, the purified molecules showed, for sL1CHO, three bands of 180, 160, and 120 kDa (calculated 120.8 kDa); for IgI-V1CHO, one band at 106 kDa (calculated 68.4 kDa); and for FN1-5CHO, one band at

Figure 3. Neurite outgrowth on substrate-coated laminin, brain L1, sL1CHO, IgI-VICHO, IgI-VIEco, FN1-5CHO, and FN1-5Eco. Small cerebellar neurons were plated onto poly-L-lysine-treated glass coverslips (B) coated overnight with laminin (A), L1 (C), sL1CHO (D), IgI-VICHO (E), IgI-VIEco (F), FN1-5CHO (G), and FN1-5Eco (H). Photomicrographs of cells after 24 hr in culture are shown. Scale bar, 15 μ m (for A–H).

100 kDa (calculated 54.8 kDa) (Fig. 2A). The two lower bands of sL1CHO may be cleavage products, similar to those described for brain L1 (Sadoul et al., 1988). The differences between the calculated molecular weights of the protein backbone and the observed molecular weights of the glycoproteins are most likely due to glycosylation, since 22 putative glycosylation sites have been postulated for L1 (Moos et al., 1988). Typical yields of immunoaffinity-purified glycoprotein fragment IgI-VICHO was 6 mg per liter of conditioned medium. For sL1CHO and FN1-5CHO, yields were too small to measure protein concentrations.

Recombinant protein fragments of L1 expressed in *E. coli*

All protein fragments expressed in *E. coli* (Fig. 1B) were collected as inclusion bodies that were solubilized in 8 M urea under reducing conditions and purified by ion-exchange chromatography using FPLC. In a single step, each protein fragment could be purified to at least 90% (Fig. 2B). The estimated molecular weights of purified protein fragments (Fig. 2B) are, for IgI-IIIEco, 29 kDa (calculated 26.6 kDa); for IgIII-IVEco, 25 kDa (calculated 25 kDa); for IgV-VIEco, 33 kDa (calculated 30 kDa); for IgI-VIEco, 75 kDa (calculated 73 kDa); for FN1-2Eco, 30 kDa (calculated 23.4 kDa); for FN3-5Eco, 34 kDa (calculated 30 kDa); and for FN1-5Eco, 58 kDa (calculated 53.7 kDa), as estimated by SDS-PAGE under reducing conditions. The reason for the slight discrepancy between observed and calculated molecular weights is not known, since all expression constructs were confirmed to contain the correct nucleotide sequences. After

chromatographic purification, protein fragments were refolded by extensive dialysis against appropriate buffers, the compositions of which were chosen according to the calculated IEP of each fragment. After dialysis, the protein fragments containing two immunoglobulin-like domains gave up to three bands by SDS-PAGE under nonreducing conditions and the protein fragment containing all six immunoglobulin-like domains (IgI-VIEco) gave more than 10 bands (not shown), as would be expected if several conformations were present due to different formation of disulfide bridges. Protein fragments containing fibronectin type III homologous repeats showed only one band by SDS-PAGE under nonreducing conditions (not shown). Refolding of protein fragments by a more sophisticated protocol that had previously been demonstrated to lead to antigen binding activity of F_{ab} fragments expressed in *E. coli* (Buchner and Rudolph, 1991) exhibited the same migratory properties by SDS-PAGE under nonreducing conditions and the same specific activity in the two functional test systems (not shown) as those renatured as described above. Typical yields of purified recombinant protein fragments were between 3 and 5 mg per liter of bacterial culture.

Functional tests

Two assays were performed to investigate the functional roles of the different protein fragments of L1: neurite outgrowth and short-term adhesion. Since adhesion of cell bodies is a prerequisite for neurite outgrowth, we used an assay system in which neurite outgrowth could be distinguished from and measured independently of adhesion.

Neurite outgrowth of cerebellar neurons. To determine neurite outgrowth, glycoprotein or protein fragments were adsorbed onto poly-L-lysine-coated glass coverslips. Poly-L-lysine was used to ensure good adhesion of cell bodies, and the number of adherent neurons was similar for each fragment regardless of their neurite outgrowth promoting activity. For all fragments, the effects described in the following section were titrated using 5–100 μ g/ml protein in the coating solution. Maximal effects were observed at 0.08 μ M (10 μ g/ml) for brain L1 and IgI-VICHO, 0.8 μ M for IgI-VIEco (58.4 μ g/ml) and FN1-5Eco (43 μ g/ml), and 1.6 μ M for IgI-IIIEco (42.7 μ g/ml), IgIII-IVEco (40.1 μ g/ml), IgV-VIEco (48 μ g/ml), FN1-2Eco (37.6 μ g/ml), and FN3-5Eco (48 μ g/ml). sL1CHO and FN1-5CHO could not be fully titrated, because of the low yield of the glycoproteins. However, the fragments were as active as the stock solution at a 1:2 dilution and less active at 1:4 and 1:8 dilutions.

In a first step, the neurite outgrowth-promoting ability of the extracellular part of L1 expressed in CHO cells (sL1CHO) and the protein fragments consisting of all six immunoglobulin-like domains and all fibronectin type III homologous repeats expressed both in CHO cells (IgI-VICHO and FN1-5CHO) and in *E. coli* (IgI-VIEco and FN1-5Eco) were compared to L1 isolated from adult mouse brain (brain L1) and laminin (Figs. 3, 4A). On laminin, a very good substrate for neurite outgrowth (Baron-van Evercooren et al., 1982; Manthorpe et al., 1983; Edgar et al., 1984), neurites reached an average length of 112 ± 4 μ m after 24 hr in culture, which corresponds to an increase of 83 μ m, or 285%, compared to neurites grown on poly-L-lysine alone. Brain L1 and sL1CHO gave similar increases of 71 μ m (245%) and 65 μ m (225%), respectively. IgI-VICHO and FN1-5CHO each gave an increase in mean neurite length of 42 μ m (145%) and 30 μ m (100%). Interestingly, however, a mixture of the fragments IgI-VICHO and FN1-5CHO, at the same final

Figure 4. Determination of neurite length of small cerebellar neurons maintained on different substrates coated at protein concentrations displaying maximal effects: *A*, laminin (0.02 μ M), brain L1 (0.08 μ M), sL1CHO (undiluted stock solution), IgI-VICHO (0.08 μ M), IgI-VIEco (0.8 μ M), IgI-VIEco n.p. (n.p., not purified; 0.8 μ M), FN1-5CHO (1:2 diluted stock solution), FN1-5Eco (0.8 μ M), IgI-VICHO + FN1-5CHO (0.08 μ M + 1:2 diluted stock solution), IgI-VIEco + FN1-5Eco (0.8 μ M each), brain MAG (0.08 μ M), and BSA (1.6 μ M); *B*, IgI-VIEco (0.8 μ M), IgI-IIIEco (1.6 μ M), IgIII-IVEco (1.6 μ M), IgV-VIEco (1.6 μ M), FN1-5Eco (0.8 μ M), FN1-2Eco (1.6 μ M), and FN3-5Eco (1.6 μ M) adsorbed to poly-L-lysine-coated glass coverslips. Cells were incubated for 24 hr, fixed, and stained with crystal violet. Bars represent the absolute increase in mean neurite length per cell over the mean neurite length on poly-L-lysine alone (29 $\pm$ 3 μ m per cell) $\pm$ SEM from at least two independent experiments carried out in duplicate. Bars marked by **, *, or n.s. are highly significantly ($P < 0.01$), significantly ($P < 0.05$), or not significantly different from the control (poly-L-lysine alone as a substrate), respectively.

concentration as the individual fragments, was not more active than the individual fragments.

Both protein fragments expressed in *E. coli*, IgI-VIEco and FN1-5Eco, increased neurite outgrowth by approximately 29 μ m (100%) and 26 μ m (90%) (Figs. 3, 4*A*), and the mixture of IgI-VIEco and FN1-5Eco showed the same absolute increase in neurite length as each fragment alone. The specific activities of IgI-VIEco in unpurified and purified states showed no significant difference at the same relative protein concentrations, suggesting that the maximal activity of IgI-VIEco was not influenced by inhibitory contaminants. The observed effects were similar to those observed for glycoprotein fragments expressed in CHO cells, but the specific activity of the protein fragments expressed in *E. coli* was lower, since maximal effects required approximately 10 times higher concentrations of the bacterial proteins. The reasons for the lower specific activity of the *E. coli* fragments are not clear, but it is possible that only a fraction of the protein molecules are properly folded.

Neither BSA nor MAG, also a member of the immunoglobulin superfamily, increased neurite outgrowth significantly when compared to poly-L-lysine alone, indicating that stimulation of neurite outgrowth is not a consequence of merely coating any protein or glycoprotein onto poly-L-lysine.

These results show that the two main structural extracellular domains of L1, the immunoglobulin-like domains and the fibronectin type III homologous repeats, are involved in and important for neurite outgrowth and contribute approximately equally to the functional activity of the whole molecule. However, full activity is only seen when the whole molecule is offered as a continuous protein backbone.

To determine the domains within the immunoglobulin-like domains or the fibronectin type III homologous repeats that are responsible for the neurite outgrowth promoting activity, we dissected the major domains into smaller protein fragments. These were expressed in *E. coli* only, since protein fragments

expressed in *E. coli* coated at saturating concentrations are about as active as those expressed in CHO cells, and since expression in *E. coli* is less cumbersome. When the purified immunoglobulin-like domains I-II, III-IV, or V-VI were substrate coated onto poly-L-lysine, all fragments gave a weak, but statistically significant, increase in neurite outgrowth of 11–22 μ m (38–76%), when compared to poly-L-lysine alone (Fig. 4*B*). Each pair of immunoglobulin-like domains thus exhibited a smaller neurite outgrowth-promoting activity than IgI-VIEco. However, only the activity of IgI-IIIEco or IgV-VIEco was significantly ($P < 0.05$ for each) smaller when compared to IgI-VIEco. Of the fibronectin type III homologous repeats, only fibronectin type III homologous repeats 1–2 promoted neurite outgrowth as effectively as the fragment containing all fibronectin type III homologous repeats. Fibronectin type III homologous repeats 3–5 were ineffective.

These results show that there are at least four distinct domains in the L1 molecule that promote neurite outgrowth. Two of these, IgIII-IV and FN1-2, are almost as active as the unfragmented immunoglobulin-like domains or fibronectin type III homologous repeats.

To verify further the involvement of substrate-coated L1 in the promotion of neurite outgrowth, neurite outgrowth was assayed in the presence of monoclonal L1 antibodies reacting with different domains of the molecule. Two monoclonal L1 antibodies, 324 and 557.B6, reacting with the immunoglobulin-like domains and the fibronectin type III homologous repeats, respectively, were tested for their ability to inhibit neurite outgrowth on brain L1 or protein fragments of L1. Both antibodies were able to inhibit neurite outgrowth on brain L1 by at least 80–90%, when added 3 hr after plating the cells on coverslips (Fig. 5*A*). Since the antibodies did not interfere with neurite outgrowth on laminin, cytotoxic effects can be excluded. When cells were maintained on substrate-coated sL1CHO, IgI-VICHO, or FN1-5CHO, both antibodies inhibited neurite out-

Figure 5. Effects of antibodies on neurite outgrowth from small cerebellar neurons maintained on different substrates coated at protein concentrations displaying maximal effects in the presence or absence of antibodies: *A*, laminin (0.02 μ M), brain L1 (0.08 μ M), sL1CHO (undiluted stock solution), IgI-VICHO (0.08 μ M), FN1-5CHO (undiluted stock solution); *B*, IgI-II Eco (1.6 μ M), IgIII-IV Eco (1.6 μ M), IgV-VI Eco (1.6 μ M), FN1-2 Eco (1.6 μ M), and FN3-5 Eco (1.6 μ M) adsorbed to poly-L-lysine-coated glass coverslips. Cells were incubated for 24 hr, fixed, and stained with crystal violet. Three to four hours after seeding the cells, the coverslips were flooded in culture medium with or without antibodies 324 or 557.B6 (61 μ g/ml or 360 μ g/ml for 324 or 557.B6, respectively). Bars represent the absolute increase in mean neurite length per cell over the mean neurite length on poly-L-lysine alone (29 ± 3 μ m per cell) $\pm$ SEM from at least two independent experiments carried out in duplicate. Bars marked by **, *, or n.s. are highly significantly ($P < 0.01$), significantly ($P < 0.05$), or not significantly different, respectively, from the control situation, in which the cells were maintained on the same substrate without antibodies.

growth by 60–80%. These results show that neurite outgrowth on brain L1 and L1 fragments are not caused by contaminants in the glycoprotein preparations. Furthermore, the reduction in neurite outgrowth on substrate-coated IgI-VICHO, in the presence of antibody 557.B6 (reacting with the fibronectin type III homologous repeats), can only be explained if L1 is blocked by the antibody at the cell surface. A similar situation is observed on substrate-coated FN1-5CHO in the presence of antibody 324. These observations suggest that L1 is a cell surface receptor for both protein fragments, although the inhibition of neurite outgrowth by the antibodies was not complete. Antibodies to TAG-1, a possible heterophilic ligand of L1 (Kuhn et al., 1991), did not inhibit neurite outgrowth on substrate-coated L1 (not shown).

We then tested whether the two antibodies could inhibit neurite outgrowth on the smaller protein fragments expressed in *E. coli*. Indeed, both antibodies were able to inhibit neurite outgrowth on all active L1 fragments (Fig. 5*B*). Preincubation of the cells with antibodies and removal of unbound antibody before the test was done also led to reduced neurite outgrowth, although the effect was slightly smaller than in the continued presence of the antibody (not shown).

These results confirm that the immunoglobulin-like domains and the fibronectin type III homologous repeats of L1 increase neurite outgrowth when offered as substrate and show that L1 functions as a specific cell surface receptor on cerebellar neurons for all fragments.

Cell-to-substratum adhesion test of cerebellar neurons. To determine short-term adhesion of cell bodies, glycoprotein or protein fragments were adsorbed onto nitrocellulose-coated plastic (Lagenaur and Lemmon, 1987). Unsaturated binding sites on the nitrocellulose were blocked with 5% BSA in PBS, and cerebellar neurons were incubated on these substrates for 3 hr. As for the neurite outgrowth assay, titration curves were determined from 5 to 150 μ g/ml protein in the coating solution, and

maximal effects were observed at 0.8 μ M (100 μ g/ml) for brain L1, IgI-VIEco (58.4 μ g/ml), and FN1-5Eco (43 μ g/ml), and at 1.6 μ M for IgI-II Eco (42.7 μ g/ml), IgIII-IV Eco (40.1 μ g/ml), IgV-VIEco (48 μ g/ml), FN1-2Eco (37.8 μ g/ml), and FN3-5Eco (48 μ g/ml) (Fig. 6*A,B*).

Under these conditions, only 26% of the neurons that attached to poly-L-lysine adhered to brain L1, while 83% adhered to laminin (Fig. 7*A*, solid bars). When protein fragments containing either all immunoglobulin-like domains (IgI-VIEco) or fibronectin type III homologous repeats (FN1-5Eco) were used as substrates, 67% and 63%, respectively, of the cells adhering to poly-L-lysine were attached. Unfortunately, the three glycoprotein fragments expressed in CHO cells could not be tested, because their concentrations in the stock solutions were too low to reach saturation.

MAG did not increase adhesion of cerebellar neurons, compared to blocked nitrocellulose (see also Kadmon et al., 1990a).

These results show that the two main structural domains of L1 are involved not only in neurite outgrowth, but also in adhesion, and that these domains alone are significantly more active ($P < 0.05$) in adhesion than the whole molecule.

To determine the domains within the immunoglobulin-like domains and the fibronectin type III homologous repeats that are responsible for adhesion, we also tested the smaller protein fragments. Sixty percent of the cells adhered to the immunoglobulin-like domains I–II and V–VI, a value similar to all six immunoglobulin-like domains (Fig. 7*B*, solid bars), whereas only 33% adhered to immunoglobulin-like domains III–IV, a value comparable to brain L1. Cells adhered more strongly to the fibronectin type III homologous repeats 3–5 (86%) and more weakly to fibronectin type III homologous repeats 1–2 (37%) than to the protein fragment containing all five fibronectin type III homologous repeats (63%).

These results show that there are at least five distinct L1 domains that are able to support short-term adhesion. Three of

Figure 6. Determination of the adhesion of small cerebellar neurons to different substrates adsorbed to nitrocellulose-coated plastic as a function of protein concentration: *A*, brain L1, IgI-VIEco, and FN1-5Eco; *B*, IgI-IIIEco, IgIII-IVEco, IgV-VIEco, FN1-2Eco, and FN3-5Eco. Cells were maintained for 3 hr, fixed, and counted. Values are given as percentages of cells bound to poly-L-lysine (=100%). Bars represent mean values $\pm$ SD of one representative experiment. Another independent experiment gave similar results.

these, IgI-II, IgV-VI, and FN3-5, are better substrates than the whole L1 molecule.

To evaluate the influence of the substrate carrier in this assay, we compared nitrocellulose-coated plastic with uncoated plastic (Fig. 7*A,B*, open bars). No difference in the adhesion of cerebellar neurons to the L1 fragments was observed for the two carriers (Fig. 7*A,B*, solid and open bars), even though 10 times higher protein concentrations were necessary to obtain saturation for adhesion on nitrocellulose than on plastic. These results show that differences in adhesion to the different L1 fragments are not due to differences in adsorption to the carrier, since it is unlikely that the fragments adsorb in the same manner on different nonspecific substrate carrier.

To verify further the involvement of substrate-coated L1 in adhesion, assays were performed in the presence of antibodies 324 and 557.B6 (Fig. 8*A,B*). Both antibodies inhibited adhesion to brain L1 almost completely. Since the antibodies did not interfere with adhesion to laminin, cytotoxic effects of the antibodies could be excluded. When cells were allowed to adhere to the six immunoglobulin-like domains or five fibronectin type III homologous repeats, both antibodies also inhibited adhesion (Fig. 8*A*), as they did when adhesion to the smaller L1 fragments was measured (Fig. 8*B*). Since in each case at least one monoclonal antibody did not react with the substrate but with L1 at

the cell surface of neurons, L1 has to be at least a part of the cell surface receptor for all active L1 domains.

The results of both functional assays are summarized in Table 1.

Discussion

In an attempt to characterize the functionally important domains of the neural cell adhesion molecule L1, we analyzed the capacity of different fragments of L1 to promote neurite outgrowth and to mediate short-term adhesion of neuronal cell bodies. The use of individual L1 fragments in cell-to-substratum interactions was advantageous in isolating L1-mediated adhesion and neurite outgrowth from other recognition systems present on L1-expressing neural or transfected non-neural cells (see Lemmon et al., 1989; Kadmon et al., 1990a), since cell-to-cell binding studies have demonstrated that multiple adhesion systems contribute in an apparently redundant fashion to the cell surface interactions between cell types (Bixby et al., 1987; Neugebauer et al., 1988). To study neurite outgrowth and adhesion on substrate-coated proteins and protein fragments, we adopted the following strategy. First, we purified the recombinant proteins to a purity of at least 90% to minimize possible effects of contaminants. Second, in the neurite outgrowth assay, we used protein fragments derived from both prokaryotic and eukaryotic

Figure 7. Determination of the adhesion of small cerebellar neurons to different substrates adsorbed to nitrocellulose-coated plastic (solid bars) or plastic alone (open bars) at protein concentrations displaying maximal effects: *A*, laminin (0.08 μ M), brain L1 (0.8 μ M), IgI-VIEco (1.6 μ M), FN1-5Eco (1.6 μ M), and brain MAG (2 μ M) adsorbed to nitrocellulose-coated plastic or laminin (0.02 μ M); brain L1 (0.08 μ M), IgI-VIEco (0.16 μ M), and FN1-5Eco (0.16 μ M) adsorbed to plastic; *B*, IgI-VIEco (1.6 μ M), IgI-IIIEco (3.2 μ M), IgIII-IVEco (3.2 μ M), IgV-VIEco (3.2 μ M), FN1-5Eco (1.6 μ M), FN1-2Eco (3.2 μ M), and FN3-5Eco (3.2 μ M) adsorbed to nitrocellulose-coated plastic or IgI-VIEco (0.16 μ M); IgI-IIIEco (0.32 μ M), IgIII-IVEco (0.32 μ M), IgV-VIEco (0.32 μ M), FN1-5Eco (0.16 μ M), FN1-2Eco (0.32 μ M), and FN3-5Eco (0.32 μ M) adsorbed to plastic. Cells were incubated for 3 hr, fixed, and counted. Values are given as percentages of cells bound to poly-L-lysine (=100%). Bars represent mean values $\pm$ SEM from five (nitrocellulose) or three (plastic) independent experiments. Bars marked by **, *, or n.s. are highly significantly ($P < 0.01$), significantly ($P < 0.05$), or not significantly different from the control (BSA alone as substrate), respectively.

Figure 8. Determination of the adhesion of small cerebellar neurons to different substrates coated at protein concentrations displaying maximal effects in the presence or absence of antibodies: *A*, laminin (0.02 μ M), brain L1 (0.8 μ M), IgI-VIEco (1.6 μ M), FN1-5Eco (1.6 μ M); *B*) IgI-IIIEco (3.2 μ M), IgIII-IVEco (3.2 μ M), IgV-VIEco (3.2 μ M), FN1-2Eco (3.2 μ M), and FN3-5Eco (3.2 μ M) adsorbed to nitrocellulose-coated plastic. Cells were incubated for 3 hr with or without antibodies 324 or 557.B6 (0.3 mg/ml or 1.8 mg/ml for 324 or 557, respectively), fixed, and counted. Values are given as percentages of cells bound to poly-L-lysine (=100%). Bars represent mean values $\pm$ SD of one representative experiment out of three experiments. The percentage of cells adhering to laminin was lower here than in Figure 7*A*, since lower amounts of laminin were used for these experiments. Bars marked by **, *, or n.s. are highly significantly ($P < 0.01$), significantly ($P < 0.05$), or not significantly different, respectively, from the control situation, in which the cells were maintained on the same substrate without antibodies.

expression systems to see possible difficulties arising from improper folding of bacterially expressed proteins. Third, inhibition tests with monoclonal L1 antibodies were performed to strengthen the L1 specificity of the observed effects. Fourth, all experiments were performed at the maximal activity level in saturating concentrations of substrate-coated L1 fragments with comparable molarity. Finally, experiments were carried out using at least two different preparations of all protein fragments, with similar results.

To distinguish adhesion of cell bodies from neurite outgrowth, we chose two different assay systems. Adhesion was tested by coating L1 fragments onto two substrates, nitrocellulose and plastic, since both have a broad capacity to adsorb proteins. For neurite outgrowth, we used an assay system in which neurite outgrowth could be analyzed independently of adhesion, in that proteins were adsorbed onto poly-L-lysine, since the latter was found to be the most potent substrate for adhesion. In these assay systems, we found that both immunoglobulin-like do-

main and fibronectin type III homologous repeats are involved in neurite outgrowth and adhesion. Interestingly, we found that all fragments of L1 are potent substrates for adhesion and exhibited similar or greater specific activity than L1 from mouse brain. For neurite outgrowth, none of the tested protein fragments was as potent as L1, but immunoglobulin-like domains III-IV and fibronectin type III homologous repeats 1-2 were quite active, more than immunoglobulin-like domains I-II and V-VI, whereas only fibronectin type III homologous repeats 3-5 were inactive. With respect to the maximal functional activity of different fragments, there appears to be some specialization of domains for either neurite outgrowth or adhesion, since fragments that are most active in adhesion show only weak activity in neurite outgrowth and vice versa. Our observations also indicate that major functions of L1 for adhesion and neurite outgrowth can be attributed to the protein backbone, since unglycosylated protein fragments produced in *E. coli* were active.

We could also show that L1 is always a receptor at the neuronal cell surface for the substrate-coated protein fragments, since monoclonal antibodies that recognize only the cellular receptor and not the substrate-coated fragment inhibited neurite outgrowth and adhesion on L1, but not on laminin. It can be ruled out that the L1 antibodies influence the potential of cells to adhere or extend neurites by unspecific steric hindrance of unrelated receptors or by triggering second messenger systems (see von Bohlen und Halbach et al., 1992), since adhesion and neurite outgrowth on laminin were not affected by the antibodies. However, other, possibly heterophilic receptors in adhesion or neurite outgrowth cannot be excluded, since the L1 antibodies were not always completely inhibitory. Heterophilic, yet unidentified, ligands for L1 have previously been described (Werz and Schachner, 1988; Pollerberg et al., 1990). It has recently been demonstrated that axonin-1 is a heterophilic receptor for Ng-CAM (Kuhn et al., 1991). Therefore, polyclonal antibodies against TAG-1, the rat homolog of axonin-1, were tested for their capacity to inhibit neurite outgrowth on substrate-coated L1. Interestingly, these antibodies did not inhibit neurite outgrowth, in agreement with the finding of Kuhn and colleagues,

Table 1. Summary of the effects of brain L1 and L1 glycoprotein or protein fragments on neuronal adhesion and neurite outgrowth

Substrate	Adhesion	Neurite outgrowth
Brain L1	+	+++
IgI-VI	++	++
FN1-5	++	++
IgI-II	++	+
IgIII-IV	+	++
IgV-VI	++	+
FN1-2	+	++
FN3-5	+++	-

The functional activities of brain L1 and the eukaryotically and prokaryotically expressed recombinant glycoprotein or protein fragments as substrates for adhesion and neurite outgrowth are represented as -, no effect; +, small effect; ++, intermediate effect; +++, strong effect. Strength of adhesion is rated in comparison to substrate-coated poly-L-lysine (+++) and neurite outgrowth in comparison to laminin (+++).

who found no inhibition of neurite outgrowth by antibodies to axonin-1 on substrate-coated Ng-CAM, the putative chicken homolog of L1. Nevertheless, our findings clearly show that L1 is always at least a part of the cellular receptor or receptor complex for L1, and add evidence to the model that L1 is a molecule interacting in a homophilic manner.

The observation that the protein fragments are better substrates for adhesion than the whole native molecule, but that for neurite outgrowth the whole molecule is better than the fragments, is surprising. Reasons for such a finding could be manifold. Since all protein fragments acted at least in part through cellular L1, it is unlikely that adhesion results from nonspecific cell binding to misfolded recombinant proteins. In such a case one would not expect L1 to be the receptor on the cell surface. Another explanation could be that brain L1 is partially denatured during the purification procedure and adhesion is therefore impaired. However, it is difficult to believe that the domains active in neurite outgrowth would be less affected. A more likely explanation that we favor is the possibility that cell attachment sites are more exposed in the fragments than in the whole molecule.

Given that many extracellular domains are involved in adhesion and neurite outgrowth *in vitro*, it is pertinent to ask now whether fragments of L1 could also have a functional relevance *in vivo*. Different protein fragments of 200, 180, 140, 80, and 50 kDa are described in L1 preparations from adult brain (Faisner et al., 1985). As shown for L1 (Sadoul et al., 1988) and Ng-CAM (Burgoon et al., 1991), such fragments derive from the same protein molecule and do not result from alternative splicing. Expression of sL1CHO in CHO cells yielded three fragments following purification, also suggesting that secreted L1 is cleaved at specific sites. It is not known whether these different forms of L1 are artifacts of the isolation procedure, or if they are generated naturally to subserve a particular functional role *in vivo*. Interestingly, our results show that only those domains located distally from the proteolytic site in the third fibronectin type III homologous repeat, which leads to the 140 kDa and 80 kDa fragments (Moos et al., 1988), promoted neurite outgrowth. Domains lying C-terminally from this site (FN3-5) were not active in neurite outgrowth, but were best for adhesion. It is thus tempting to speculate that the proteolytic cleavage of L1 at this particular site may subserve a functional role *in vivo*, in which the 140 kDa fragment plays a role in neurite outgrowth and the complementary 80 kDa fragment functions as a receptor for adhesion. It is interesting in this context that Durbec et al. (1992) showed that the soluble form of the L1-like adhesion molecule F3 stimulated neurite outgrowth.

It appears worthwhile to compare the present results with a similar study on N-CAM (Frei et al., 1992). For N-CAM, the immunoglobulin-like domains I and II and the fibronectin type III homologous repeats 1 and 2 are the best substrates for cell body adhesion, although other domains also show a significant adhesive potential. All five immunoglobulin-like domains together and both fibronectin type III homologous repeats were most active in neurite outgrowth, while immunoglobulin-like domain I and fibronectin type III homologous repeat 1 were less active. In summary, protein fragments of N-CAM are sufficient for adhesion, but only longer stretches of covalently linked domains are as active as the whole molecule for neurite outgrowth. Furthermore, both types of domains, immunoglobulin-like and fibronectin type III homologous ones are involved in the two functions characterized. The study did not identify the

cellular receptors for the different N-CAM fragments, so it is uncertain if the fragments are active in homophilic or heterophilic mechanisms. Thus, in both L1 and N-CAM, the fibronectin type III homologous repeats are more than an inert spacer that merely changes the distance from the cell surface of the immunoglobulin-like domains. Since our observations suggest that multiple domains are involved in homophilic interaction of L1, they are in contrast to results recently published on N-CAM (Rao et al., 1992) in which N-CAM was suggested to center its homophilic activity in one domain. Therefore, it seems likely that homophilic binding is mediated by different mechanisms.

Since N-CAM and L1 are members of a large family of molecules sharing immunoglobulin-like domains and fibronectin type III homologous repeats, it is tempting to consider whether the findings for N-CAM and L1 are also valid for other members. Is there a general rule that molecules that combine the feature of extracellular molecules (fibronectin type III homologous repeats) with that of cell surface receptors (immunoglobulin-like domains) engage more than one of these structural motifs in the same function? Studies on related adhesion molecules in the immune system, which belong to the immunoglobulin superfamily but have no fibronectin type III homologous repeats, have shown a high degree of specialization of different immunoglobulin-like domains for heterophilic receptors. Examples are the antigen binding sites of antibodies, the CD4 binding to gp120 of HIV and MHC II molecules (Fleury et al., 1991) or the binding of the different ligands to ICAM-1 (Staunton et al., 1990; Diamond et al., 1991; Berendt et al., 1992; Ockenhouse et al., 1992). In contrast to L1 and N-CAM, there are always only one or two closely located domains involved in specific recognition. Future experiments on N-CAM, L1, and related molecules should reveal whether these phylogenetically older homophilically acting recognition molecules represent a particular principle of ligand-receptor binding in that multiple domains subserve the same or similar functions. It also remains to be seen whether these multiple interactions are essential for one and redundant for another function.

With the data presented in this study, it seems possible to postulate different models for the homophilic interaction of L1 at the molecular level. Such models have to take into account that many domains of L1 are involved in adhesion and neurite outgrowth in the context of a homophilic interaction. Possible models are shown in Figure 9. The first model suggests that the interacting molecules recognize each other at different sites by a "heterophilic" mechanism (Fig. 9A,B). Two possible examples for this model are presented in which one domain interacts with one (Fig. 9A) or more than one (Fig. 9B) domain in a "heterophilic" manner. Alternatively, domains could bind to each other in a "homophilic" manner (Fig. 9C); that is, each domain interacts with the same domain on the partner molecule. These models would not only explain the observations that many protein fragments are responsible for adhesion and neurite outgrowth, but also account for the fact that all antibodies used to perturb adhesion and neurite outgrowth inhibited these functions. It is therefore tempting to speculate that perturbation of one site of the L1 molecule is sufficient to prevent recognition completely. The finding that FN3-5 shows potent adhesive properties is compatible with the proposed models, if one assumes that FN3-5 only becomes active after proteolytic cleavage. Indeed, preliminary experiments probing the binding of different L1 fragments to each other showed that FN3-5 binds more strongly than FN1-5 to other domains of L1 (J. Holm, F.

Figure 9. Different models for the homophilic interaction between L1 molecules on neighboring cells. The models presented could explain the observations in this study (for explanation of the symbols, see Fig. 1). The overall homophilic binding is mediated at the submolecular level by “heterophilic” (i.e., between different domains) (*A*, *B*) or “homophilic” recognition (i.e., between identical domains) (*C*).

Appel, and M. Schachner, unpublished observations). Further experiments will be necessary to determine which of these models is the most likely one.

References

- Atashi JR, Klinz SG, Ingraham CA, Matten WT, Schachner M, Maness PF (1992) Neural cell adhesion molecules modulate tyrosine phosphorylation of tubulin in nerve growth cone membranes. *Neuron* 8:831–842.
- Baron-Van Evercooren A, Kleinman HK, Ohno S, Marangos P, Schwartz JP, Dubois-Dalcq M (1982) Nerve growth factor, laminin, and fibronectin promote neurite outgrowth in human fetal sensory ganglia cultures. *J Neurosci Res* 8:179–193.
- Barthels D, Santoni M-J, Wille W, Ruppert C, Chaix J-C, Hirsch M-R, Fontecilla-Camps JC, Goridis C (1987) Isolation and nucleotide sequence of mouse NCAM cDNA that codes for a M, 79000 polypeptide without a membrane-spanning region. *EMBO J* 6:907–914.
- Bebbington CR (1991) Expression of antibody genes in nonlymphoid mammalian cells. *Methods* 2:136–145.
- Berendt AR, McDowall A, Craig AG, Bates PA, Sternberg MJE, Marsh K, Newbold CI, Hogg N (1992) The binding site on ICAM-1 for plasmodium falciparum-infected erythrocytes overlaps, but is distinct from, the LFA-1-binding site. *Cell* 68:71–81.
- Bixby JL, Pratt RS, Lilien J, Reichardt LF (1987) Neurite outgrowth on muscle cell surfaces involves extracellular matrix receptors as well as Ca^{2+} -dependent and -independent cell adhesion molecules. *Proc Natl Acad Sci USA* 84:2555–2559.
- Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.
- Brümmendorf T, Wolff JM, Frank R, Rathjen FG (1989) Neural cell recognition molecule F11: homology with fibronectin type III and immunoglobulin type C domains. *Neuron* 2:1351–1361.
- Buchner J, Rudolph R (1991) Renaturation, purification and characterization of recombinant F_{ab} -fragments produced in *Escherichia coli*. *Bio/Technology* 9:157–162.
- Burgoon MP, Grumet M, Mauro V, Edelman GM, Cunningham BA (1991) Structure of the chicken neuron-glia cell adhesion molecule, Ng-CAM: origin of the polypeptides and relation to the Ig superfamily. *J Cell Biol* 112:1017–1029.
- Chang S, Rathjen FG, Raper JA (1987) Extension of neurites on axons is impaired by antibodies against specific cell surface glycoproteins. *J Cell Biol* 104:355–362.
- Chang S, Rathjen FG, Raper JA (1990) Neurite outgrowth promoting activity of G4 and its inhibition by monoclonal antibodies. *J Neurosci Res* 25:180–186.
- Crockett MI, Bebbington CR, Yarranton GT (1990) High level expression of tissue inhibitor of metalloproteinases in chinese hamster ovary cells using glutamine synthetase gene amplification. *Bio/Technology* 8:662–667.
- Cunningham BA, Hemperly JJ, Murray BA, Prediger EA, Brackenbury R, Edelman GM (1987) Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science* 236:799–806.
- Diamond MS, Staunton DE, Marlin SD, Springer TA (1991) Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. *Cell* 65:961–971.
- Dodd J, Jessell TM (1988) Axon guidance and the patterning of neuronal projections in vertebrates. *Science* 242:692–699.
- Drazba J, Lemmon V (1990) The role of cell adhesion molecules in neurite outgrowth on Müller cells. *Dev Biol* 138:82–93.
- Durbec P, Gennarini G, Goridis C, Rougon G (1992) A soluble form of the F3 neuronal cell adhesion molecule promotes neurite outgrowth. *J Cell Biol* 117:877–887.
- Edelman GM, Crossin KL (1991) Cell adhesion molecules: implications for a molecular histology. *Annu Rev Biochem* 60:155–190.
- Edelman GM, Cunningham BA, Thiery JP (1990) Morphoregulatory molecules. Neurosciences Institute. New York: Wiley.
- Edgar D, Timpl R, Thoenen H (1984) The heparin-binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. *EMBO J* 3:1463–1468.
- Ey PL, Prowse SJ, Jenkin CR (1978) Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-Sepharose. *Biochemistry* 15:429–436.
- Faissner A, Teplow DB, Kübler D, Keilhauer G, Kinzel V, Schachner M (1985) Biosynthesis and membrane topography of the neural cell adhesion molecule L1. *EMBO J* 4:3105–3113.
- Fischer G (1982) Cultivation of mouse cerebellar cells in serum free, hormonally defined media: survival of neurons. *Neurosci Lett* 28:325–329.
- Fleury S, Lamarre D, Meloche S, Ryu S-E, Cantin C, Hendrickson WA, Sekaly R-P (1991) Mutational analysis of the interaction between CD4 and class II MHC: class II antigens contact CD4 on a surface opposite the gp120-binding site. *Cell* 66:1037–1049.
- Frei T, von Bohlen und Halbach F, Wille W, Schachner M (1992) Different extracellular domains of the neural cell adhesion molecule (NCAM) are involved in different functions. *J Cell Biol* 118:177–194.
- Frelinger AL III, Rutishauser U (1986) Topography of NCAM structural and functional determinants. II. Placement of monoclonal antibody epitopes. *J Cell Biol* 103:1729–1737.
- Furley AJ, Morton SB, Manalo D, Karageorgos D, Dodd J, Jessell TM (1990) The axonal glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite outgrowth-promoting activity. *Cell* 61:157–170.
- Gennarini G, Cibelli G, Rougon G, Mattei M-G, Goridis C (1989) The mouse neuronal cell surface protein F3: a phosphatidyl inositol-anchored member of the immunoglobulin superfamily related to chicken contactin. *J Cell Biol* 109:775–788.
- Grumet M (1992) Structure, expression, and function of Ng-CAM, a member of the immunoglobulin superfamily involved in neuron-neuron and neuron-glia adhesion. *J Neurosci Res* 31:1–13.
- Grumet M, Edelman GM (1988) Neuron-glia cell adhesion molecule interacts with neurons and astroglia via different binding mechanisms. *J Cell Biol* 106:487–503.
- Grumet M, Mauro V, Burgoon MP, Edelman GM, Cunningham BA (1991) Structure of a new nervous system glycoprotein, Nr-CAM, and its relationship to subgroups of neural cell adhesion molecules. *J Cell Biol* 113:1399–1412.
- Harlow E, Lane D (1988) Antibodies: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Harrelson AL, Goodman CS (1988) Growth cone guidance in insects: fasciclin II is a member of the immunoglobulin superfamily. *Science* 242:700–708.
- Husmann K, Faissner A, Schachner M (1992) Tenascin promotes cerebellar granule cell migration and neurite outgrowth by different domains in the fibronectin type III repeats. *J Cell Biol* 116:1475–1486.
- Kadmon G, Kowitz A, Altevogt P, Schachner M (1990a) The neural cell adhesion molecule N-CAM enhances L1-dependent cell-cell interactions. *J Cell Biol* 110:193–208.

- Kadmon G, Kowitz A, Altevogt P, Schachner M (1990b) Functional cooperation between the neural adhesion molecules L1 and N-CAM is carbohydrate dependent. *J Cell Biol* 110:209–218.
- Keilhauer G, Faissner A, Schachner M (1985) Differential inhibition of neurone-neurone, neurone-astrocyte and astrocyte-astrocyte adhesion by L1, L2 and N-CAM antibodies. *Nature* 316:728–730.
- Kuhn TB, Stoeckli ET, Condreau MA, Rathjen FG, Sonderegger P (1991) Neurite outgrowth on immobilized axonin-1 is mediated by a heterophilic interaction with L1(G4). *J Cell Biol* 115:1113–1126.
- Lacmml UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Lagenaur C, Lemmon V (1987) An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. *Proc Natl Acad Sci USA* 84:7753–7757.
- Lemmon V, Farr KL, Lagenaur C (1989) L1-mediated axon growth occurs via a homophilic binding mechanism. *Neuron* 2:1597–1603.
- Manthorpe M, Engvall E, Ruoslahti E, Longo FM, Davis GE, Varon S (1983) Laminin promotes neuritic regeneration from cultured peripheral and central neurons. *J Cell Biol* 97:1882–1890.
- Martini R, Schachner M (1986) Immunoelectron microscopic localization of neural cell adhesion molecules (L1, N-CAM, and MAG) and their shared carbohydrate epitope and myelin basic protein in developing sciatic nerve. *J Cell Biol* 103:2439–2448.
- Mayford M, Barzilai A, Keller F, Schacher S, Kandel ER (1992) Modulation of an N-CAM-related adhesion molecule with long-term synaptic plasticity in *Aplysia*. *Science* 256:638–644.
- Miura M, Asou H, Kobayashi M, Uyemura K (1992) Functional expression of a full-length cDNA coding for rat neural cell adhesion molecule L1 mediates homophilic intercellular adhesion and migration of cerebellar neurons. *J Biol Chem* 267:10752–10758.
- Moos M, Tacke R, Scherer H, Teplow D, Früh K, Schachner M (1988) Neural cell adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. *Nature* 334:701–703.
- Neugebauer KM, Tomaselli KJ, Lilien J, Reichardt LF (1988) N-cadherin, NCAM, and integrins promote retinal neurite outgrowth on astrocytes *in vitro*. *J Cell Biol* 107:1177–1187.
- Ockenhouse CF, Betageri R, Springer TA, Staunton DE (1992) Plasmodium falciparum-infected erythrocytes bind ICAM-1 at a site distinct from LFA-1, Mac-1, and human rhinovirus. *Cell* 68:63–69.
- Pollerberg GE, Davoust J, Schachner M (1990) Lateral mobility of the cell adhesion molecule L1 within the surface membrane of morphologically undifferentiated and differentiated neuroblastoma cells. *Eur J Neurosci* 2:712–717.
- Poltorak M, Sadoul R, Keilhauer G, Landa C, Fahrigh T, Schachner M (1987) Myelin-associated glycoprotein, a member of the L2/HNK-1 family of neural cell adhesion molecules, is involved in neuron-oligodendrocyte and oligodendrocyte-oligodendrocyte interaction. *J Cell Biol* 105:1893–1899.
- Rao Y, Wu X-F, Garipey J, Rutishauser U, Siu C-H (1992) Identification of a peptide sequence involved in homophilic binding in the neural cell adhesion molecule NCAM. *J Cell Biol* 118:937–949.
- Rathjen FG, Schachner M (1984) Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. *EMBO J* 3:1–10.
- Rosenberg AH, Lade BN, Chui D-S, Lin S-W, Dunn JJ, Studier FW (1987) Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* 56:125–135.
- Sadoul K, Sadoul R, Faissner A, Schachner M (1988) Biochemical characterization of different molecular forms of the neural cell adhesion molecule L1. *J Neurochem* 50:510–521.
- Sadoul R, Kirchhoff F, Schachner M (1989) A protein kinase activity is associated with and specifically phosphorylates the neural cell adhesion molecule L1. *J Neurochem* 53:1471–1478.
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schachner M (1990) Functional implications of glial cell recognition molecules. In: *Seminars in the neurosciences*, Vol 2, Neurobiology of glia (Jessen KR, Mirsky R, eds), pp 497–507. New York: Saunders.
- Schachner M (1991) Neural recognition molecules and their influence on cellular functions. In: *The nerve growth cone* (Letourneau PC, Kater SB, Macagno ER, eds), pp 237–254. New York: Raven.
- Schuch U, Lohse MJ, Schachner M (1989) Neural cell adhesion molecules influence second messenger systems. *Neuron* 3:13–20.
- Seilheimer B, Schachner M (1988) Studies of adhesion molecules mediating interactions between cells of peripheral nervous system indicate a major role for L1 in mediating sensory neuron growth on Schwann cells in culture. *J Cell Biol* 107:341–351.
- Smith GM, Rutishauser U, Silver J, Miller RH (1990) Maturation of astrocytes *in vitro* alters the extent and molecular basis of neurite outgrowth. *Dev Biol* 138:377–390.
- Staunton DE, Dustin ML, Erickson HP, Springer TA (1990) The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. *Cell* 61:243–254.
- Studier FW, Moffatt BA (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* 189:113–130.
- Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1990) Use of T7 polymerase to direct expression of cloned genes. *Methods Enzymol* 185:60–94.
- von Bohlen und Halbach F, Taylor J, Schachner M (1992) The adhesion molecules L1 and N-CAM influence different second messenger systems in different neural cell types. *Eur J Neurosci* 4:896–909.
- Wertz W, Schachner M (1988) Adhesion of neural cells to extracellular matrix constituents. Involvement of glycosaminoglycans and cell adhesion molecules. *Dev Brain Res* 43:225–234.
- Williams AF, Barclay AN (1988) The immunoglobulin superfamily-domains for cell surface recognition. *Annu Rev Immunol* 6:381–405.
- Williams EJ, Doherty P, Turner G, Reid RA, Hemperly JJ, Walsh FS (1992) Calcium influx into neurons can solely account for cell contact-dependent neurite outgrowth stimulated by transfected L1. *J Cell Biol* 119:883–892.
- Zuellig RA, Rader C, Schroeder A, Kalousek MB, von Bohlen und Halbach F, Osterwalder T, Inan C, Stoeckli ET, Affolter H-U, Fritz A, Hafen E, Sonderegger P (1992) The axonally secreted cell adhesion molecule, axonin-1. *Eur J Biochem* 204:453–463.