The Neural Recognition Molecule L1 Is a Sialic Acid-binding Lectin for CD24, Which Induces Promotion and Inhibition of Neurite Outgrowth*

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Among the recognition molecules that determine a neuron’s interaction with other cells, L1 and CD24 have been suggested to cooperate with each other in neurite outgrowth and signal transduction. Here we report that binding of CD24 to L1 depends on α2,3-sialic acid on CD24, which determines the CD24 induced and cell type-specific promotion or inhibition of neurite outgrowth. Using knockout mutants, we could show that the CD24-induced effects on neurite outgrowth are mediated via L1, and not GPI-linked CD24, by trans-interaction of L1 with sialylated CD24. This glycoform is excluded together with L1 from raft microdomains, suggesting that molecular compartmentation in the surface membrane could play a role in signal transduction.

Path-finding of growth cones and neurite outgrowth toward targets are important events in the developing and regenerating nervous system and in synaptic remodeling during learning and memory. Axonal guidance depends on molecules at the cell surface and in the extracellular matrix. The different and often changing combinations of molecularly associated recognition molecules at the cell surface are important determinants of the ways by which the cell surface communicates with the cell interior, where cell surface signals are integrated to influence cell behavior.

Two recognition molecules, L1 of the immunoglobulin superfamily and CD24, a highly glycosylated mucin type glycoprotein, interact with each other functionally (1, 2). L1 is a 200-kDa homophilic and heterophilic adhesion molecule expressed by many postmitotic neurons in the central nervous system (for reviews, see Refs. 3 and 4). It is one of the most potent promoters of neurite outgrowth in vitro known so far. Mutants of L1 in mice and men strongly underscore its importance during embryonic development in vivo (3, 5, 6).

CD24 is linked to the surface membrane by a glycosyl phosphatidylinositol anchor and is, therefore, unable to directly interact with cytoplasmic proteins. It is also known as heat-stable antigen or nectadrin with a peptide core of only 30 amino acids (for references, see Ref. 1). Similar to L1, it is highly expressed by neurons (7, 8). The apparent molecular weight of CD24 varies considerably among cell types and also within each cell type, depending on its developmental stage due to differences in glycosylation pattern (for references, see Refs. 1 and 7). These observations suggest that post-translational modifications of CD24 play an important functional role. CD24 acts as a co-stimulator for various physiological functions. In the nervous system, CD24 has been reported to interact with L1 to stimulate cell adhesion and to increase intracellular Ca2+ levels (1, 2). Interestingly, CD24 has been shown to inhibit neurite outgrowth of neonatal retinal ganglion cells and dorsal root ganglion neurons in culture (8) by yet unknown signal transduction mechanisms.

Based on these observations on the functional interplay and molecular association between L1 and CD24, we decided to further study their functional interdependence. Here we report that L1 is a sialic acid-binding lectin for CD24 and that CD24 inhibits neurite outgrowth of dorsal root ganglion neurons and promotes neurite outgrowth of cerebellar neurons via interaction in trans-position with L1 at the cell surface of the neurite outgrowth-competent cell. Our experiments appear to underscore four important observations in neural cell interactions: 1) the pivotal function of glycans as mediators of interactions between neural recognition molecules in trans-association, 2) the characterization of L1 as a sialic acid-binding lectin and identification of the siglec domain (9) in the first fibronectin type III homologous repeat of the L1 family, 3) the implication of L1 as a signal-transducing cell surface receptor for CD24-induced cell type-specific effects on neurite outgrowth, and 4) the importance of the intracellular signal transduction machinery of a particular neuronal cell type that determines whether neurite outgrowth enhancement or inhibition result from cell surface interactions.

EXPERIMENTAL PROCEDURES

Animals—Outbred ICR wild type mice were used for purification of L1 and CD24, and C57BL/6J mice were used for subfractionation. ICR wild type, CD24-deficient (CD24−/−) (10), and L1-deficient (L1−/−) (5) mice were used for cell culture.

Antibodies—Polyclonal antibodies against recombinant L1-Fc fusion protein comprising the whole extracellular part of mouse L1,1 rat monoclonal antibody 555 against mouse L1, or monoclonal rat antibody 79 reacting with mouse CD24 were used (11).

Purification of L1 and CD24—L1 was purified by immunoaffinity chromatography from 2-3-day-old ICR mouse brains as described (11). For purification of CD24, frozen brain tissue was thawed on ice and homogenized with 10 volumes of cold acetonitrile (~20 °C) using a Potter homogenizer. The residue was recovered by filtration on a G3 glass filter under reduced pressure, and the collected tissue pieces and powder were again homogenized in cold acetone and filtered. All of the following steps were carried out at 4 °C. The freshly prepared acetone powder was washed twice in 25 ml of Hepes-buffered saline (10 mM Hepes, 150 mM NaCl, pH 7.4) by incubation for 15 min in a head-over-head mixer and subsequent centrifugation at 20,000 × g for 15 min. The washed residue was homogenized and agitated for 2 h in 50 ml of

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Hepes-buffered saline containing 1% (w/v) deoxycholate, 0.1 mM phenylmethylsulfonyl fluoride, and 0.02% (w/v) NaN3. After centrifugation at 100,000 × g for 60 min, the supernatant was collected and applied to an affinity column prepared by coupling monoclonal antibody 79 to CNBr-activated Sepharose B (Amersham Pharmacia Biotech). After loading the sample, affinity chromatography was performed with 1 ml of saline in 5 mM Hepes, pH 7.4, 150 mM NaCl, 2% Triton X-100, 0.02% NaN3, followed by washing with buffer B (10 mM Hepes, pH 7.4, 500 mM NaCl, 0.1% Triton X-100, 0.02% NaN3) and with 50 ml of Hepes-buffered saline. Bound CD24 was eluted with buffer C (50 mM ethanamine, pH 11.5, 150 mM NaCl, 0.2% (w/v) CHAPS). The eluate was immediately neutralized by adding HCl (1 M) to pH 7.4. The mixture was then centrifuged for 2 h at 37 °C in a head-over-head mixer for 2 h. After centrifugation, the material at the 0.32/1.1 M sucrose interface contained both L1 and CD24, whereas membranes pelleted by centrifugation for 30 min at 120,000 × g contained CD24 but no L1. Western blot analysis of the different proteins diluted in buffer H and overlaid with 1.1 and 0.32 M sucrose in buffer H. After centrifugation for 10 min at 700 × g, the resulting membrane fraction was first incubated with the polyclonal L1 antibody for 2 h at room temperature. WTOX1-100 was added to 1 mg of protein in 1 ml of PBS. After an incubation time of 4 h at room temperature, reaction mixtures were desalted on PD10 columns and stored at 5 °C.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis**—SDS-polyacrylamide gel electrophoresis was performed on 10 or 12.5% gels. Proteins were visualized by Coomassie or silver staining. Proteins separated by SDS-polyacrylamide gel electrophoresis were transferred electrophoretically onto nitrocellulose membranes (BA 85; Schleicher & Schuell). For antibody detection assays, nitrocellulose membranes were incubated for 1 h at room temperature in blocking buffer (TBS or PBS containing 5% skim milk powder), washed three times with TBS or PBS, and incubated at room temperature for 1 h with monoclonal antibody 79 or 555 (1 μg/ml) in blocking buffer. Filters were then washed five times with TBS, the plates were incubated with biotinylated affinity-purified antibodies against L1 and CD24 as indicated for 1 h at room temperature. WTOX1-100 was added to 1 mg of protein in 1 ml of PBS. After an incubation time of 4 h at room temperature, reaction mixtures were desalted on PD10 columns and stored at 5 °C.

**RESULTS**

**Purification of L1 and CD24 from Mouse Brain**—Brains were removed from 7-day-old C57BL/6J mice and homogenized in homogenization buffer (0.32 M sucrose in buffer H: 50 mM Tris/HCl, pH 7.4, 1 mM CaCl2, 1 mM MgCl2, and 1 mM NaHCO3) using a Potter homogenizer and applying 10 strokes. All steps were carried out at 4 °C. The homogenate was centrifuged for 10 min at 700 × g, the resulting supernatant was further centrifuged at 17,000 × g. The pellet was resuspended in homogenization buffer and applied on top of a discontinuous sucrose gradient consisting of 1.2, 1.0, 0.8, and 0.65 M sucrose in buffer H. After centrifugation for 2 h at 100,000 × g, the turbid material from the different interfaces was collected, diluted with buffer H, and pelleted by centrifugation for 30 min at 100,000 × g. Western blot analysis of the sample fraction collected from the 1.0/1.2 M sucrose interface contained both L1 and CD24, whereas membranes collected from the other interfaces did not contain L1 together with CD24. Myelin was prepared according to Norton and Poduslo (14). The pelleted membrane fraction from the 1.0/1.2 M interface was resuspended in buffer H. To disrupt noncovalent protein interactions, one volume of the resuspended membrane fraction was supplemented with one volume of either 10 mM EDTA or 300 mM NaHCO3, pH 10, and incubated for 1 h on ice. For the selective solubilization of proteins, cold Triton X-100 was added to the membrane suspension to a final concentration of 1% and incubated for 1 h on ice. The samples were then centrifuged for 30 min at 120,000 × g. To isolate detergent insoluble lipid microdomains of low density (rafts) the Triton X-100-insoluble membrane fraction was adjusted to 1.2 M sucrose using 2.34 M sucrose in buffer H and overlaid with 1.1 and 0.32 M sucrose in buffer H. After centrifugation for 2 h at 120,000 × g, the material at the 0.32/1.1 M sucrose interface was collected, diluted with buffer H, and pelleted by centrifugation for 30 min at 120,000 × g.

**For immunoprecipitation, the Triton X-100-insoluble membrane fraction was first incubated with the polyclonal L1 antibody for 2 h at 4 °C and then with protein A/G beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4 °C. The beads were pelleted and washed five times with buffer H. Proteins bound to the beads were eluted by boiling the beads at 100 °C in SDS-sample buffer.

**RESULTS**

**Purification of L1 and CD24 from Mouse Brain**—Since L1 and CD24 tend to bind to each other, particular attention was paid to the isolation procedure to assure a maximal degree of purity. L1 was directly isolated from homogenates of early postnatal mouse brain, while CD24 was purified after extracting lipids from the brain tissue homogenate by acetone. The purity of the L1 and CD24 preparations was checked by Coo-

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*The abbreviations used are: CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; TBS, Tris-buffered saline; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; PLL, poly-l-lysine.*
molecular masses of glycosylated proteins in which three bands with apparent masses from experiments carried out with different batches of purified L1 and CD24 are shown.

The CD24 preparation yielded a smear characteristic of highly sialylated in α2,3- but not in α2,6-linkage, whereas the 27-kDa glycoform does not appear to be sialylated. Peanut agglutinin, which recognizes the unsubstituted terminal Galβ1,3-GalNAc core unit of O-linked mucin type glycan chains, binds to the 30- and 33-kDa glycoforms of CD24 only upon the removal of sialic acid residues by neuraminidase and not without removal. These observations indicate that only the 30- and 33-kDa glycoforms of CD24 carry α2,3-linked sialic acid on the O-glycan core unit Galβ1,3-GalNAc and not the 27-kDa glycoform. However, it could not be determined whether the 30- and 33-kDa glycoforms also carry α2,3-linked sialic acid residues on complex type N-glycans.

Fig. 1. Analysis of L1 and CD24 purified from mouse brain. L1 and CD24 were separated on 10% SDS-polyacrylamide gels and visualized by Coomassie (lanes 1 and 3) and silver staining (lanes 2 and 6) or transferred to nitrocellulose membranes. For immunodetection of L1 or CD24, monoclonal antibodies 555 against L1 (lanes 8 and 9) and 79 against CD24 (lanes 4 and 7) were used, while for lectin affinity blotting of purified CD24 (lanes 9–13) or asialo-CD24 (lane 14; +SAase), digoxigenin-labeled agglutinins from D. stramonium (DSA; lane 9), G. nivalis (GNA; lane 10), M. amurensis (MAA; lane 11), S. nigra (SNA; lane 12), and peanut (PNA; lanes 13 and 14) were applied.

Fig. 2. Binding of CD24 to immobilized L1. L1 was coated to 96-well microtiter plates and incubated with different amounts of CD24, asialo-CD24, or CD24 peptide. Binding was evaluated through enzyme-linked immunosorbent assay using biotinylated monoclonal antibody 79 against CD24 and streptavidin-horseradish peroxidase. Values from experiments carried out with different batches of purified L1 and CD24 are shown.

Immunoaffinity-purified L1 yielded two bands with apparent molecular masses of 200 and 180 kDa as detected by Coomassie staining (Fig. 1, lane 1), silver staining (Fig. 1, lane 2), and immunostaining with monoclonal L1 antibodies (Fig. 1, lane 3). The CD24 preparation yielded a smear characteristic of highly glycosylated proteins in which three bands with apparent molecular masses of ~33, ~30, and ~27 kDa could be detected, although not very distinctly. When characterizing the three bands as three glycoforms of CD24, we refer to the upper, middle, and lower parts of the smear. The smear containing the three faint bands could be visualized only by silver staining but not by staining with Coomassie (Fig. 1, lanes 5 and 6). Three bands could usually be recognized by monoclonal antibody 79 (Fig. 1, lane 7). The L1 preparation did not contain any immunoactivity for CD24 (Fig. 1, lane 4); nor did the CD24 preparation contain any L1 immunoreactivity (Fig. 1, lane 8).

Different Glycoforms of CD24—Glycans of purified CD24 and L1 were characterized by lectin affinity blotting. The three glycoforms of CD24 showed a different extent of reactivity with Datura stramonium agglutinin (DSA; Fig. 1, lane 9), which recognizes Galβ1,4-GlcNAc, whereas no reactivity was observed for Galanthus nivalis agglutinin (GNA; Fig. 1, lane 10), which is specific for terminal mannose residues. These results indicate the absence of hybrid or oligomannosidic type oligosaccharides and the presence of complex type oligosaccharides on CD24. The 30- and 33-kDa glycoforms of CD24 were recognized by Maackia amurensis agglutinin (MAA; Fig. 1, lane 11) but not by Sambucus nigra agglutinin (SNA; Fig. 1, lane 12), showing that these glycoforms are solely sialylated in α2,3- but not in α2,6-linkage, whereas the 27-kDa glycoform does not appear to be sialylated. Peanut agglutinin, which recognizes the unsubstituted terminal Galβ1,3-GalNAc core unit of O-linked mucin type glycan chains, binds to the 30- and 33-kDa glycoforms of CD24 only upon the removal of sialic acid residues by neuraminidase and not without removal. These observations indicate that only the 30- and 33-kDa glycoforms of CD24 carry α2,3-linked sialic acid on the O-glycan core unit Galβ1,3-GalNAc and not the 27-kDa glycoform. However, it could not be determined whether the 30- and 33-kDa glycoforms also carry α2,3-linked sialic acid residues on complex type N-glycans.

Glycosylation-dependent Binding of CD24 to L1—It has been shown that CD24 binds to L1 (1, 2). To assess the importance of glycan chains on the CD24 molecule for its binding to L1, we investigated in a solid phase immunoassay whether the two molecules interact with each other via their glycan chains. Binding of CD24 to immobilized L1 was dose-dependent and saturable, whereas a synthetic full-length CD24 polypeptide devoid of glycans did not bind to immobilized L1 (Fig. 2). We also analyzed whether the binding of CD24 to L1 depends on α2,3-linked sialic acid residues. Therefore, sialic acid-free CD24 was generated by treatment with neuraminidase from V. cholerae, which removes terminal α2,3-linked sialic acid residues. In its asialo form, CD24 no longer bound to L1 (Fig. 2). These observations indicate that the binding of CD24 to L1 depends on its glycan chains and that sialic acid residues on CD24 are essential for the binding to L1.

Inhibitory Effect of CD24 on Neurite Outgrowth from Dorsal Root Ganglion Neurons—To assess the functional importance of the glycan chains on CD24 for neurite outgrowth, dorsal root ganglion neurons from neonatal mice were maintained on different substrate-coated proteins. Total neurite length per cell was significantly reduced on the CD24 substrate in comparison with the poly-L-lysine control (Fig. 3A, compare CD24 with PLL). Neurons plated either onto the CD24 peptide or asialo-CD24 were not inhibited in their neurite outgrowth when compared with the poly-L-lysine control (Fig. 3A, compare CD24 peptide and asialo-CD24 with PLL). A monoclonal antibody against CD24 and polyclonal antibodies against L1 were able to reduce or inhibit the negative influences on neurite outgrowth elicited by substrate-coated CD24 but not on poly-L-lysine (Fig. 3A, compare CD24 + anti-CD24 and CD24 + anti-L1 with CD24 and PLL), whereas nonimmune IgG control antibodies had no effect (Fig. 3A, compare CD24 + IgG with CD24).

CD24 preparation

- Protein (µg/ml)
- CD42
- asialoCD24
- CD24 peptide

| Protein (µg/ml) | CD24 | asialoCD24 | CD24 peptide |
|----------------|------|------------|--------------|
| 0              | 0.5  | 0.3        | 0.2          |
| 6              | 0.4  | 0.2        | 0.1          |
| 12             | 0.3  | 0.1        | 0.05         |

CD24 preparation

- Poly-L-lysine control
- Poly-L-lysine control
- Poly-L-lysine control

CD24 preparation

- Poly-L-lysine control
- Poly-L-lysine control
- Poly-L-lysine control
indicating that the L1 antibody binds to L1 at the cell surface of dorsal root ganglion neurons and masks it for binding to CD24 in trans-interaction.

When neurite outgrowth was assayed on L1-coated substrates, neurite outgrowth was enhanced over the poly-L-lysine control (Fig. 3, compare L1 with PLL). In the presence of L1 antibodies, neurite outgrowth was reduced to control levels (Fig. 3B, compare L1 anti-L1 with PLL and L1). Antibodies to CD24, which were able to neutralize the inhibitory effect of substrate-coated CD24, did not affect the enhanced neurite outgrowth on L1 (Fig. 3B, compare L1 anti-CD24 with L1). As control, nonimmune IgG did not reduce the enhanced neurite outgrowth on substrate-coated L1 (Fig. 3B, compare L1 IgG with L1).

Neurite outgrowth was considerably reduced on the mixture of L1 with CD24 in comparison with that seen on the L1 substrate and was rather similar to that observed on the CD24 substrate alone (Fig. 3B, compare L1 + CD24 with PLL, L1, and CD24). The CD24 peptide and asialo-CD24 had no effect on L1-enhanced neurite outgrowth (Fig. 3B, compare L1 + CD24 peptide and L1 + asialo-CD24 with PLL and L1).

These experiments show that in mixture with L1, CD24 completely abolishes the enhancing effect of L1 on neurite outgrowth and thus predominates over L1. That the inhibitory effect of CD24 on L1-enhanced neurite outgrowth is abolished when sialic acid is removed or glycans on CD24 are absent indicates that this interaction depends on glycans and, in particular, on sialic acid residues present on CD24.

**CD24 Inhibits L1- but Not CD24-mediated Neurite Outgrowth of Dorsal Root Ganglion Neurons**—To investigate the functional consequences of the interaction between CD24 and L1 on neurite outgrowth, neurite outgrowth from dorsal root ganglion neurons from neonatal wild type mice was compared with neurite outgrowth from CD24- or L1-deficient mice. In these experiments, dorsal root ganglion neurons from knockout mutant mice were maintained on different substrates, and total lengths of neurites per cell were measured. Neurons from CD24-deficient animals responded in the same manner as neurons from the wild type animals on the different substrates; neurite outgrowth of CD24-deficient neurons was substantially reduced on the CD24-coated substrate (Fig. 3C, compare CD24 with PLL), was not reduced on asialo-CD24 (Fig. 3C, compare asialo-CD24 with PLL and CD24), and was significantly better on the L1-coated substrate when compared with the poly-L-lysine control (Fig. 3C, compare L1 with PLL and CD24). When neurons were cultured on the mixture of L1 with CD24, neurite outgrowth was considerably reduced when compared with that on the L1-coated substrate and was similar to neurite outgrowth on the CD24 substrate alone (Fig. 3C, compare L1 + CD24 with L1 and CD24). Asialo-CD24, when coated in a mixture with L1, had no influence on the L1-induced enhancement of neurite outgrowth (Fig. 3C, compare L1 + asialo-CD24 with CD24 and L1).

When neurons from L1 knockout mice were used, substrate-coated L1 no more enhanced neurite outgrowth, and CD24 no more inhibited neurite outgrowth (Fig. 3D, compare L1 and CD24 with PLL). Similarly, the mixture of L1 and CD24 did not show any effects different from those on the control substrate.
tissue culture plastic coated without (PLL) or with CD24, L1, and their mixture (PLL1). Bars indicate S.D. from at least three independent experiments. Error bars indicate S.D. from at least three independent experiments. Bars marked by asterisks and double asterisks are significantly (p < 0.01) different from the control (PLL) and L1, respectively.

CD24 Promotes Neurite Outgrowth of Cerebellar Neurons—To investigate whether the inhibitory effect of CD24 was due to unique functions of CD24 or whether CD24 exerts different effects on different neuronal cell types, cerebellar neurons from 5-day-old mice were maintained on different substrates. Cells cultured on the CD24-coated substrate extended longer neurites than on the poly-l-lysine control (Fig. 4A, compare CD24 with PLL). Asialo-CD24 did not enhance neurite outgrowth (Fig. 4A, compare asialo-CD24 with PLL). On L1-coated substrates, cells had even longer neurites than on CD24 or poly-l-lysine (Fig. 4A, compare L1 with CD24 and PLL). On the mixture of CD24 with L1, neurons extended neurites longer than on the poly-l-lysine control, but neurite lengths were significantly reduced when compared with L1 alone and comparable with CD24 alone (Fig. 4A, compare L1 + CD24 with PLL, L1, and CD24). Asialo-CD24 was not neurite outgrowth-promoting or -inhibiting in substrate mixture with L1 (Fig. 4A, compare L1 + asialo-CD24 with L1). These results show that CD24 predominates in its effect over that of L1 and that the different responses to neurite outgrowth depend on the neuronal cell type.

CD24 Enhances L1-mediated but Not CD24-mediated Neurite Outgrowth of Cerebellar Neurons—Next, the interaction between CD24 and L1 and the response on the neurite outgrowth of cerebellar neurons was further investigated using neurons from CD24- or L1-deficient mice. In these experiments, neurite lengths of cerebellar neurons from wild type and knockout mutant mice maintained on different substrates were determined. Neurons from CD24-deficient animals and from wild type animals showed comparable neurite outgrowth on the different substrates; neurite outgrowth was substantially enhanced on the CD24-coated substrate (Fig. 4B, compare CD24 with PLL) but less extensive than on the L1 substrate (Fig. 4B, compare L1 with PLL and CD24), while the mixture of L1 and CD24 enhanced neurite outgrowth to the level of the CD24 substrate only (Fig. 4B, compare L1 + CD24 with L1 and CD24). In contrast, cerebellar neurons from L1 knockout mice showed no enhanced neurite outgrowth on L1, CD24, or a mixture of L1 and CD24 when compared with the poly-l-lysine control (Fig. 4C, compare L1, CD24, and L1 + CD24 with PLL). These experiments indicate that L1, but not CD24, is involved in L1 and CD24 substrate-dependent enhancement of neurite outgrowth of cerebellar neurons.

Distinct Glycoforms of CD24 Interact with Neuronal L1—The interaction of L1 and CD24 was investigated biochemically by analyzing the distribution of L1 and CD24 in membrane subfractions from 7-day-old mouse brain obtained by sucrose gradient centrifugation. Western blot analysis of the different membrane fractions showed that L1 was exclusively found in a membrane fraction obtained from the 1.0/1.2 m sucrose interface (data not shown), which has been reported to contain synaptosomal membranes. In this membrane fraction, the three glycoforms of CD24 (27, 30, and 33 kDa) were detected by the CD24 antibody (Fig. 5A). The astrocyte marker glial fibrillary acidic protein was also present in this fraction, whereas the oligodendrocyte marker MAG was not detectable (Fig. 5A). In contrast, analysis of a crude myelin fraction as a control showed that the preparation contained no L1 and negligible amounts of CD24 and glial fibrillary acidic protein but large amounts of MAG (Fig. 5A).

The co-distribution of the three glycoforms of CD24 with L1 was further studied. If L1 and CD24 were associated by trans-interaction, it should be possible to disrupt this association either with EDTA or sodium bicarbonate at alkaline pH. For this reason, membranes were subjected to further sucrose gradient centrifugation after those treatments. Western blot analysis of residual membranes showed that the three glycoforms were present in the mock-treated membranes. The EDTA-treated membranes also showed all glycoforms, indicating that the membrane association of CD24 does not depend on divalent cations. In contrast, in the membranes treated with alkaline bicarbonate, only the 27-kDa glycoform was detectable, whereas the 30- and 33-kDa glycoforms were absent (Fig. 5B). The amount of L1 did not change significantly in the differently treated membranes (Fig. 5B). From these results, we infer that the 30- and 33-kDa glycoforms of CD24 and L1 are located in the trans-position, while the 27-kDa glycoform and L1 are located in the cis-position on the same membrane.

In parallel experiments, the membrane fraction containing L1 and CD24 was treated with 1% Triton X-100 at 4 °C, and detergent-insoluble material was separated from detergent-soluble proteins. Part of the detergent-insoluble material was applied to a sucrose floatation gradient to isolate lipid raft microdomains (for a review, see Ref. 15). Western blot analysis of the different membrane subfractions showed that the 27-kDa glycoform is predominantly detergent-insoluble and thus operationally present in rafts (Fig. 5C), while the 30- and 33-kDa glycoforms of CD24 are completely detergent-soluble.
Fig. 5. Analysis of membrane subfractions from adult mouse brain. A, membranes from the 1.2/1.0 M sucrose interface (1/1.2M) and a crude myelin preparation (my) were subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis using antibodies against CD24, L1, gial fibrillary acidic protein, and MAG. B, membranes from the 1.2/1.0 M sucrose interface were incubated in the absence (1/1.2M) or the presence of either 10 mM EDTA (+EDTA) or 150 mM NaHCO3, at pH 10 (+NaHCO3/pH 10), separated on sucrose gradients, and subjected to Western blot analysis using antibodies against CD24 and L1. C, after incubation of membranes from the 1/1.2 M sucrose interface (1/1.2M) in the presence of 1% Triton X-100, detergent-insoluble material (Tx-insoluble) was separated from detergent-soluble proteins (Tx-soluble), which were subsequently subjected to methanol precipitation. Part of the Triton X-100-insoluble fraction was used for sucrose floatation gradient centrifugation, and the material floating to low density was collected (raft). The different subfractions were run on SDS-polyacrylamide gels and analyzed with antibodies against CD24 and L1. D, membranes from the 1/1.2 M sucrose interface (1/1.2M) were treated with 1% Triton X-100, and detergent-soluble material (Tx-soluble) was isolated. For immunoprecipitation, antibodies against L1 and protein A/G beads were added to part of this fraction. The different fractions and the immunoprecipitates (aL1-IP) were subjected to Western blot analysis (WB) using an antibody against CD24. The immunoprecipitate was also analyzed for binding of the lectin M. amurensis (MAA), which reacts specifically with α2,3-linked sialic acid residues.

DISCUSSION

In this study, we present evidence that the interaction between L1 and CD24- and CD24-induced neurite outgrowth depends on the presence of α2,3-linked sialic acid residues on CD24. Lectin blot analysis of CD24 affinity-purified from mouse brain indicates that the three major CD24 glycoforms differ in their glycans; the 30- and 33-kDa glycoforms, but not the 27-kDa glycoform, carry glycans substituted with terminal α2,3-linked sialic acid, recognized by these glycoforms of CD24 in the L1 immunoprecipitate (Fig. 5D), indicating that these glycoforms carry α2,3-linked sialic acid residues (see also Fig. 1, B and C).

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sequences contain a crucial arginine residue in the F-strand of the immunoglobulin fold that is flanked by hydrophobic amino acids (see Ref. 17 and references therein). Sialoadhesin, CD33, and MAG, which prefer NeuAc(2,3)-Gal contain the characteristic YXFR motif, while CD22, which only binds to NeuAc(2,6)-Gal, contains LXFR. The importance of a conserved arginine in the binding of sialic acid has been reported for several lectins, such as galectin-3 (18) and the siglecs (17), since the guanidinium group of the arginine forms a salt bridge with the carboxylate group of sialic acid. The sequence found in NeuAc(2,3)-Gal preferring siglecs is present in the first fibronectin type III (FNIII) domain of L1. This short sequence motif (YXFR) is present in all members of the L1 superfamily, including F3/T11/contactin, TAG-1/TAX-1/axonin, BIG-1/PANG, NB-2/NB-3, the close homologue of L1 (CHL1), NgCAM, Nr-CAM, neurofascin, and neuroglian (see Ref. 19 and references therein). The L1 family appears to be distinct from the original siglec family of adhesion molecules by the feature that the FNIII but not the immunoglobulin-like domains contain the consensus sequences for sialic acid binding. Three-dimensional structure analysis of a neuroglian fragment comprising the first and second FNIII domain indicates that the YXFR sequence is part of a β-sheet (Fig. 6A) (20), which, together with the flanking sequences, is highly conserved within the L1 family of several animal species and comprises the consensus sequence (LV/MMXF/W/Y/F)/V/A/MXYXFR (V/)/XAXNXXG (Fig. 6B). Mutations of the conserved alanine to glutamic acid or threonine were found in L1 from patients with MASA syndrome (mental retardation, aphasia, shuffling gait, and adducted thumbs) (21, 22). However, no gross morphological abnormalities were detectable in L1- or CD24-deficient mice in the cerebellum (23) or dorsal root ganglia (for abnormalities in the sensory nerves of L1 deficient mice, see Ref. 16, and for a new nomenclature, see Ref. 9). These consensus...
and identification of a consensus sequence. A surprising amino acids 610–814 of Drosophila neuroglian (1CFB) is shown. (The proteins depicted in this region are highlighted.) Arginine (box) amino acids and the highly conserved (gray) and similar (light gray) amino acids are shown. The position number within the sequence and the SwissProt accession numbers are given. The alanine mutated in human L1 and the asparagine mutated in human anosmin are marked by a circle.

The binding of α2,3-linked sialic acid residues on CD24 to L1 is likely to play a functional role in neurite outgrowth. CD24 but not asialo-CD24 inhibits neurite outgrowth of dorsal root ganglion neurons and promotes neurite outgrowth of cerebellar neurons. Sialic acid residues on L1 are not required for binding to CD24 and promotion of neurite outgrowth, since desialylation of L1 did not interfere with neurite outgrowth and since proaryctically expressed L1 fragments also promote neurite outgrowth, although less efficiently on a molar basis than L1 isolated from eucaryotic systems (see Ref. 32 and references therein). It is interesting that the mixture of L1 and CD24 substrate-coated proteins together reduces the levels of neurite outgrowth from an L1-mediated strong enhancement of neurite outgrowth of both dorsal root ganglion and cerebellar neurons to the level of substrate-coated CD24 alone. It is thus possible that CD24 competes with L1 in trans-interaction with L1 on neuronal membranes. It is also conceivable that binding of CD24 to L1 leads to a conformational change or to a steric hindrance of homophilic binding of L1 to the six immunoglobulin-like domains of L1 that comprise the decisive domains for cell binding and neurite outgrowth (32). That carbohydrates may be important for protein-protein interaction has been proposed for P0 (33) or L1-NCAM interactions (11, 34). A striking dependence of glycans in trans-interactions has recently been reported for notch, which interacts with its ligands delta and jagged/serrate. These interactions are modulated through the glycosyltransferase fringe. It has been proposed that a cell type-specific modification of glycosylation may provide a general mechanism to regulate ligand-receptor interactions in the nervous system (for discussion, see Ref. 35).

The effects on neurite outgrowth of dorsal root ganglion or cerebellar neurons by substrate-coated CD24 are neutralized by antibodies to L1 and are not observed when neurons are taken from L1 knockout mice, whereas neurons from CD24 knockout mice are not different in neurite outgrowth from wild type mice. These complementary findings show that substrate-coated CD24 generates its neurite outgrowth-inhibitory and -promoting effects via a trans-interaction with L1 at the neuronal cell surface and exclude the possibility that a homophilic interaction of CD24 mediates the effects on neurite outgrowth. Our biochemical analysis indicates that the α2,3-sialic acid-containing 30- and 33-kDa glycoforms are involved in this trans-interaction with L1, which was not only seen in the developing, 7-day-old brain but also in adult animals. These observations indicate the importance of this putative sialic acid binding motif for nervous system development.

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glycoforms can be removed from L1-containing membranes by alkaline treatment and co-distribute with L1 in a detergent-soluble membrane subfraction, and they are co-immunoprecipitated with L1 antibodies. Interestingly, the α2,3-sialic acid-depleted 27-kDa glycoform cannot be removed from membranes by treatment with alkaline buffer, and since it does not co-distribute with L1 in lipid raft microdomains, it is unlikely to interact in cis- or trans-positions with L1 (Fig. 7).

The observation that CD24 is highly expressed in the spinal cord where corticospinal axons decussate (36) suggests that repellent interactions between L1 and CD24 may play a role in axon guidance as has recently been shown for the functional connection between the repellent extracellular matrix glycoprotein semaphorin 3A and L1, which associates with the semaphorin 3A receptor neuropilin-1 (36). A striking reversion protein semaphorin 3A and L1, which associates with the connection between the repellent extracellular matrix glycoforms of CD24 and L1 posed for L1 and CD24 interactions.ing partners could be cytokine or neurotrophin receptors (e.g., fibroblast growth factor receptor (37) or neurotrophin tyrosine kinase activity levels of cis-interacting partner molecules, such as L1, in conjunction with trans-interacting partner molecules, such as CD24. The dissection of the network of glcan-dependent cis- and trans-interacting partners in terms of their signal transduction machineries remains an exciting topic for future investigations.

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