The Structure of the Lingo-1 Ectodomain, a Module Implicated in Central Nervous System Repair Inhibition

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Nogo receptor (NgR)-mediated control of axon growth relies on the central nervous system-specific type I transmembrane protein Lingo-1. Interactions between Lingo-1 and NgR, along with a complementary co-receptor, result in neurite and axonal collapse. In addition, the inhibitory role of Lingo-1 is particularly important in regulation of oligodendrocyte differentiation and myelination, suggesting that pharmacological modulation of Lingo-1 function could be a novel approach for nerve repair and remyelination therapies. Here we report on the crystal structure of the ligand-binding ectodomain of human Lingo-1 and show it has a bimodular, kinked structure composed of leucine-rich repeat (LRR) and immunoglobulin (Ig)-like modules. The structure, together with biophysical analysis of its solution properties, reveals that in the crystals and in solution Lingo-1 persistently associates with itself to form a stable tetramer and that it is its LRR-Ig-composite fold that drives such assembly. Specifically, in the crystal structure protomers of Lingo-1 associate in a ring-shaped tetramer, with each LRR domain filling an open cleft in an adjacent protomer. The tetramer buries a large surface area (9,200 Å2) and may serve as an efficient scaffold to simultaneously bind and assemble the NgR complex components during activation on a membrane. Potential functional binding sites that can be identified on the ectodomain surface, including the site of self-recognition, suggest a model for protein assembly on the membrane.

Injured neurons in mature organisms are unable to effectively regrow their axons after central nervous system damage. One of the many factors restricting axonal regeneration after injury is the growth-inhibiting components associated with damaged myelin. At least three of these components, Nogo-66, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein, either individually or collectively, have been shown to be potent inhibitors of neurite outgrowth (1, 2). All three signal inhibition through the Nogo receptor complex, composed of the ligand-binding Nogo-66 receptor (NgR) and two complementary co-receptors p75 and Lingo-1 that act as a signal-transducing pair on an axon’s cell membrane (3, 4). Although both NgR and the p75 nerve growth factor receptor have well documented roles in the context of myelin inhibition, reports exploring the role of Lingo-1 are more recent.

Human Lingo-1 is a central nervous system-specific transmembrane glycoprotein (Fig. 1) also known as LERN-1, which belongs to a larger family of LRR-Ig-containing proteins involved in central nervous system development and axonal growth (5). Its large extracellular or ectodomain is thought to be of functional importance in protein-protein recognition and is characterized by a tandem array of multiple LRRs and one Ig-like domain. The first studies examining the role of Lingo-1 demonstrated that in cultured neurons Lingo-1 directly associates with NgR and p75 and that whenever myelin-NgR/p75-mediated growth inhibition is observed, Lingo-1 is present, and is essential to this process (6). The functional capacity of the tripartite complex to launch the downstream RhoA-dependent signaling pathway that evokes the inhibition of neurite outgrowth has been reported. Of note is the finding that truncated Lingo-1 lacking the intracellular domain restores neurite outgrowth in vitro by interrupting the interaction of Lingo-1 with its binding partners. Direct physical interactions between the full-length Lingo-1 and either NgR or p75 have been reported, as have interactions of truncated soluble Lingo-1 with either NgR or p75 (6). More recently, our understanding of this inhibitory system has changed with the identification of yet another member of the Nogo receptor complex, designated TROY (7). The latter belongs to the same, tumor necrosis factor-receptor family as p75 but, unlike p75, is broadly expressed in adult neurons, where it can substitute for p75 in the signaling complex, allowing for RhoA activation and outgrowth inhibition in neurons lacking p75 (7, 8). The current model for myelin-mediated inhibition includes therefore one alternative signaling complex that involves NgR, Lingo-1, and TROY.

In addition to its expression on neurons, Lingo-1 is also receptor; LRR, leucine-rich repeat; CHO, Chinese hamster ovary; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; r.m.s., root mean square; NCAM, neural cell adhesion molecule; SIRAS, single isomorphous replacement with anomalous scattering.
Oligomeric Ectodomains of Lingo-1

Detecting and quantifying oligomeric domains in extracellular proteins using a novel approach

**Experimental Procedures**

**Protein Expression and Purification**—An extracellular portion of human Lingo-1 was expressed in lectin-resistant CHO Lec3.2.8.1 cells as a C-terminal 6-His-tagged protein (residues 1–549, signal sequence 1–33). The human Lingo-1-His was subcloned into pSMEG vector behind a murine cytomegalovirus promoter and verified by sequencing analysis. CHO cells were grown and maintained in a humidified incubator with 5% CO₂ at 37 °C. DNA transfection and large-scale production of conditioned cell culture media for Lec3.2.8.1 cells were performed as described previously (12).

The media expressing Lingo-1-His was exchanged into a buffer of 1 M Tris, 100 mM NaCl, pH 8.0, to which a mixture of protease inhibitors (complete inhibitors from Roche Applied Science) was added. The protein was captured by nickel-nitrilotriacetic acid resin and then purified by gel-filtration chromatography (Superdex-200). NgR 1D4 (residues 27–451) fused at the C terminus with the 10-amino acid 1D4 epitope tag was expressed in CHO-A2 cells using the honeybee melitin secretary leader, then purified from the media by anti-1D4 affinity chromatography followed by gel filtration.

**Neurite Outgrowth Assays**—96-well plates were coated with a thin layer of nitrocellulose (Bio-Rad) before incubating with Lingo-1-His or control IgG-Fc (R&D Systems) proteins in the presence of 2.5 µg/ml MAG-Fc (R&D Systems) at 4 °C overnight. 3-Tubulin antibodies were incubated overnight in Hanks’ balanced salt buffer with 1% fetal bovine serum, 20 mM HEPES for 2 h at 37 °C. Alkaline phosphatase-conjugated anti-β-tubulin antibody (TuJ1, Covance). The average of total neurite lengths from each neuron was quantitated by Cellomics’ Neurite Outgrowth Bioapplication from at least 400 neurons per well, in triplicate wells per experiment. Results have been repeated independently for more than three times.

**Cell-based Binding Assays**—25,000 CHO-DUKX cells stably expressing NgR and p75 were seeded overnight in 96-well plate and then incubated with various concentration of Lingo-1-His in Hanks’ balanced salt buffer with 1% fetal bovine serum, 20 mM HEPES for 2 h at 37 °C. Alkaline phosphatase-conjugated anti-His IgG was added, and the mixture was incubated for another hour. Bound Lingo-1-His was detected by incubation with AntiPhos substrate (Promega) at 0.6 mg/ml for half an hour and read on Flex Station (emission: 440 nm, excitation: 560 nm).

**Biacore Experiments**—Surface plasmon resonance with BIAcore was used to determine the equilibrium dissociation constant (**K_D**) between NgR 1D4 and Lingo-1-His. NgR 1D 4 was immobilized onto a CM5 chip using amine-coupling chemistry. A titration series using 2-fold serial dilutions was performed with the analyte Lingo-1-His ranging in concentration from 10 to 0.039 μM. A **K_D** of ~1 μM was determined for the NgR 1D4
and Lingo-1-His interaction by steady-state equilibrium analysis using BLAevaluation 3.0.

**Crystallization**—To obtain diffraction quality crystals, the 6-His tag and stalk region were removed by proteolytic treatment with chymotrypsin for 2 h at 18 °C. The cleaved Lingo-1 was further purified and analyzed by gel filtration, SDS-PAGE, and mass spectrometry (molecular mass ~ 66.4 kDa compared with a value of ~71.62 kDa obtained for Lingo-1-His). For crystallization, the protein was concentrated to 4–6 mg/ml in TBS (Tris-buffered saline, 50 mM Tris, pH 8.0, 150 mM NaCl). Crystals were obtained at 18 °C in hanging drops using 1.2–1.4 M (NH₄)₂SO₄, 0.1M sodium citrate, pH 5.0, as a precipitant. The protein crystallized in two forms, with both forms found in the same crystallization droplets: I222, with two molecules per asymmetric unit and 74% solvent content, and P2₁2₁2₁, with four molecules per asymmetric unit and 73% solvent content. For data collection, crystals were gradually transferred from the mother liquor to the stabilizing cryoprotecting solution containing 2.9 M sodium malonate, pH 5.2. This solution, in which crystals were found to be stable over the period of several days and over the pH range 5–7, was used for crystal derivatization. A single derivative that allowed structure determination by the SIRAS method was obtained from crystals were soaked in 50 mM K₂PtCl₆ and 2.9 M sodium malonate at pH 7.0 for 24 h. Prior to data collection, all crystals were flush cooled under a nitrogen stream at 100 K.

**Data Collection, Phasing, and Refinement**—Two data sets obtained from crystals of space group I22 were used for phase determination: the 3.5-Å native data set and the 3.6-Å data set for the platinum derivative, both measured in house with Saturn92 CCD mounted on an FR-E CuKα rotating anode source (Rigaku, Japan). The higher resolution native data set was collected to 2.7 Å at Advanced Photon Source beamline 22-ID of Southeast Regional Collaborative Access Team from a crystal that belongs to the P₂₁2₁2₁ space group. All data were integrated and scaled with HKL2000 (13).

The initial positions of platinum atoms in the derivative crystal were located with SHELDX (2001 Bruker-AXS, XM, version 6.12) using anomalous differences of platinum atoms at the CuKα edge. The input SAS coefficients were prepared with XPREP (2001 Bruker-AXS, version 6.12). Refinement of heavy-atom parameters, phase calculation, and density modification by SOLOMON, all were performed with SHARP (14) at 20- to 3.6-Å resolution, using both anomalous and isomorphous differences from the native and derivative data sets. The final 3.6-Å SIRAS maps produced with SHARP were of interpretable quality and revealed two Lingo-1 molecules in the asymmetric unit. SHARP phases were further improved by 2-fold NCS averaging and phase extension to 3.5 Å in DM (15). The resulting maps allowed us to build an initial, 90% complete (~855 residues) model with QUANTA. This model was then used for molecular replacement with the P₂₁2₁2₁ data set to utilize the higher 2.7-Å resolution data. A clear solution for four molecules in the asymmetric unit was identified with Phaser (16). The I222 and P₂₁2₁2₁ crystal forms share the same tetrameric packing, in which the tetramer can be built by replicating a dimer around the 2-fold axis. Subsequent rounds of rebuilding and refinement against the 2.7-Å data set were done with COOT (17) and REFMAC (18).

The final model contains 4 protein molecules (residues A1–477, B3–475, C2–477, and D3–476), 39 N-acetylgalactosamine and 12 mannose residues, and 310 water molecules. Residues 1–2 at the N termini of B, C, and D, 476–477 at the C termini of B and D, and residues D32–34 were not modeled into the structure due to the lack of adequate electron density, presumably because of disordering. Geometric analysis of the final refined structure performed with MolProbity (19) places 94% of all residues in favored regions and 0.16% as outliers. Statistics for data collection, phasing, and refinement are summarized in Table 1.

**Chemical Cross-linking**—The solution state of Lingo-1 (residues 1–478) was analyzed by chemical cross-linking and dynamic light scattering, and of Lingo1-His by analytical centrifugation. To achieve a substantial level of cross-linking, 50 μM Lingo-1 in 50 mM Tris, pH 7.5, 150 mM NaCl was buffer exchanged with 50 mM Tris, pH 8.7, 150 mM NaCl using a desalting column. The buffer exchanged Lingo-1 at 50 μM was incubated with 75 μM glutaraldehyde at 18 °C for 2 h. Cross-linked Lingo-1 was purified away from glutaraldehyde by washing the mixture several times with 50 mM Tris; pH 7.5, 150 mM NaCl, followed by concentration of the protein using a Microcon. As a negative control, Lingo-1 in 50 mM Tris, pH 8.7, 150 mM NaCl was incubated at 18 °C for 2 h. Both these samples were run on 4–12% Bis-Tris SDS-PAGE gel and protein bands were visualized by Coomassie staining.

**Dynamic Light Scattering**—The hydrodynamic radius of Lingo-1 was measured at various protein concentrations (ranged from 4 to 125 μM) using a DynaPro DLS instrument. Each protein sample was centrifuged at 13,000 rpm for 15 min to remove any particulates. The supernatant was transferred to a quartz cuvette, and 10–20 readings at 18 °C were averaged per sample.

**Analytical Ultracentrifugation**—Sedimentation equilibrium experiments were performed on a Beckman XLI/XLA analytical ultracentrifuge at 20 °C at three different rotor speeds (9,000, 12,000, and 18,000 r.p.m.) and four concentrations (1.0, 5.8, 16.6, and 49.3 μM). Samples were loaded into six-channel (1.2-cm path length) carbon-Epon centerpieces in an An-50 Ti titanium rotor. Scans were recorded at 230, 250, and 280 nm with a 0.001-cm spacing and ten replicates per point, and equilibrium was judged to be achieved when there was no deviation between successive scans taken 3 h apart. Data were analyzed by nonlinear regression with WinNONLIN (20). The solvent density and viscosity were calculated with the program Sednterp (21). The data were fit to different associating models (monomer-dimer-trimer, monomer-dimer-trimer-tetramer, and monomer-dimer-tetramer). The residual, variance of the fit, and R₂ were used to judge how well the data fit to the different models. Better fits were obtained for protein concentrations <=50 μM, and the best fit was for a monomer-dimer-tetramer equilibrium model. Association constants for monomer-dimer-tetramer equilibria obtained from WinNONLIN were converted from absorbance (Kₙₐ₅) to molar units (Kₙ₉₉₉) with the equation, Kₙ₉₉₉ = Kₙₐ₅(ε/l) / n, where l is the path length of the cell (1.2 cm), ε is the molar extinction at the wavelength.
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RESULTS AND DISCUSSION

Functional Characterization and Structure Determination of Recombinant Lingo-1—To obtain a homogeneous high mannose glycoform of the protein suitable for crystallography studies, the extracellular portion of glycosylated recombinant human Lingo-1 (amino acids 1–516, coding sequence 34–549, plus a C-terminal 6-histidine tag) was produced in lectin-resistant CHO Lec 3.2.8.1 cells and purified to homogeneity as the extracellular portion of glycosylated recombinant human Lingo-1 (amino acids 1–516, coding sequence 34–549, plus a C-terminal 6-histidine tag) was produced in lectin-resistant CHO Lec 3.2.8.1 cells and purified to homogeneity as human Lingo-1 (amino acids 1–516, coding sequence 34–549, plus a C-terminal 6-histidine tag) was produced in lectin-resistant CHO Lec 3.2.8.1 cells and purified to homogeneity as human Lingo-1 (amino acids 1–516, coding sequence 34–549, plus a C-terminal 6-histidine tag) was produced in lectin-resistant CHO Lec 3.2.8.1 cells and purified to homogeneity as human Lingo-1 (amino acids 1–516, coding sequence 34–549, plus a C-terminal 6-histidine tag) was produced in lectin-resistant CHO Lec 3.2.8.1 cells and purified to homogeneity as

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A key feature of the ectodomain conformation is a sharp, close to 90° angle relating the LRR and IgI1 modules and placing the latter at the back, convex side of the LRR coil. Such topological arrangement allows for the formation of a wide, 22-Å deep and 35-Å long, cleft that extends on a glycan-free surface as if poised for binding. The sides of the cleft are formed from the convex and recessed faces of the two domains: namely the convex curvature of repeats 10–12 plus helix α1 shapes one side and the concavity of the β-sheet A’CC’FG shapes the other. The opposed surfaces lie relatively far apart and have different chemical properties: the LRR face is polar and charged, whereas the CC’FG face is predominantly hydrophobic (Fig. 3B). Hence, the two modules appear not to interact with each other directly, except for few van der Waals contacts at the bottom cleft area. Of general note are the location of the Cys336–Cys382 disulfide bond right at the tip of the elbow and the absence per se of an interdomain linker region. Cys382 is the last residue in the C-cap, and Arg383, positioned immediately after, clearly belongs in the IgI1 domain (previously referred to as to the basic region (6)). These features may constrain the positioning of the two modules relative to each other and to the membrane.

Glycosylation—The glycoform of Lingo-1 in lectin-resistant CHO cells is expected to be predominantly high mannose type glycans such as Man5GlcNAc2, which is consistent with mass spectrometry data (not shown). The sequence of the crystallized protein contains eight potential N-glycosylation consensus sites. Six of them are located in the LRR domain (Asn105, Asn163, Asn225, Asn235, Asn254, and Asn302) and two in the IgI1 domain (Asn453 and Asn466). Of these eight predicted sites, all but one (Asn466) is found to be occupied. The putative site at Asn466-X-Ser468, located on the exterior A’CC’FG surface, is concluded unoccupied based on both the lack of electron density and

**FIGURE 2. Biological activity and functional characterization of recombinant Lingo-1.** A and C, block of neurite outgrowth inhibition induced by MAG (2.5 μg/ml) with Lingo-1 (40 μg/ml) but not with the control protein IgG-Fc of the same concentration. Neurite lengths were quantitated from >400 neurons/well in triplicate treatments. These results were repeated independently for more than three times. B, total neurite lengths from neurons cultured in the presence of MAG-Fc (2.5 μg/ml) were significantly increased by an application of Lingo-1 in a dose-dependent manner. D, Lingo-1 binding to CHO cells expressing either NgR or p75. The data show the difference between transfected and non-transfected control. The values for half-maximal binding of Lingo-1 to NgR and p75 were -3 and 1 μg/ml, respectively. E, surface plasmon resonance evaluation of the interaction between Lingo-1-His and NgR 1D4. Top, BIAcore sensograms of a titration series with analyte Lingo-1-His and the immobilized NgR 1D4 ligand. Binding curves are expressed as resonance units (RU) as a function of time. The experiments were performed in duplicate with one representative experiment shown. Lingo-1-His dissociation was allowed to proceed for 3 min returning to baseline between injections. The binding of Lingo-1-His to a reference surface was subtracted at each concentration of analyte examined. Bottom, a BIAevaluation 3.0 fit of equilibrium binding response (Req) versus the concentration of Lingo-1-His. The steady-state affinity model was used to determine the equilibrium dissociation constant (Kd).
on the observed interactions of Asn466 in the intermolecular interface that is described in more detail later. The glycan cores at the remaining seven sites, for which densities can be assigned unambiguously, are found throughout the structure as shown in Figs. 1 and 4. Remarkably, out of four LRR faces, only the convex surface is free of carbohydrate. By contrast, the concave

FIGURE 3. The protomer structure of Lingo-1. A, the $2F_o - F_c$ electron density map (cyan mesh) is contoured at 1.3 $\sigma$ in the region of the His$^{185}$–His$^{209}$–His$^{233}$ ladder on the concave face of LRR (yellow stick model). B, side view. Ribbon diagram showing the overall architecture of the Lingo-1 monomer, colored according to secondary structure: beige, coil; blue, $\beta$ strand; red, $\alpha$ helix. Disulfide bonds are shown in green, and the N-linked carbohydrates are yellow. LRRs are numbered. Selected loops, $\alpha$ helices, and $\beta$ strands of the IgI1 domain are labeled. To the right is a close-up view of the cleft surface, marked with a white line and colored by electrostatic potential (red for negative; blue for positive) to emphasize different chemical properties of the opposed surfaces. C, superposition of the LRR structures of Lingo-1 (red) and NgR (cyan). Structures were aligned to highlight the difference in the C-cap regions. The small circle marks the location of the $\beta$-bulge in the Lingo-1 structure; its comparison to the segment of glycoprotein GpIba (yellow), discussed in the text, is shown to the right. D, superposition of the IgI1 module of Lingo-1 (red) with the Ig3 module of NCAM (green). The view is from the face of the $\beta$ sheet ABDE.
and two major side surfaces are evenly glycosylated, each bearing two N-linked glycans. The last glycan maps to the A/H11032 CC/H11032 FG face of the IgI1 domain, but to the far membrane-proximal end of the domain, thereby rendering much of its surface still accessible.

In general, the presence of extensive glycosylation on glycoprotein surfaces limits their accessibility for interaction with ligands, and in most cases surfaces that are not covered with glycan chains are located where the ligands are predicted to bind. In most if not all LRR-related ligand complexes solved to date, the concave face of the LRR structure is essential for ligand-binding activities (29). In this regard, glycan decoration on the corresponding surface of Lingo-1 is quite unexpected though not unprecedented. The other LRR structure that revealed a high density glycosylation pattern on the concave face, which was otherwise thought to be a ligand-binding site, is that of human Toll-like receptor TLR3 (30).

To our knowledge, the role of glycosylation in Lingo-1 function has not been investigated. The importance and biochemical role of oligosaccharides in glycoproteins in general is well established, including protease protection, protein trafficking, folding, stability, and molecular geometry. In line with this, we found Lingo-1 to be consistently unstable upon deglycosylation in vitro, with a significant tendency to precipitate or aggregate over a wide pH range. Interestingly, of the seven occupied sites, two glycans that reside within the LRR concave sheet may have an effect on the structure. As shown in Fig. 4, the glycan chain attached at Asn302, just one strand away from the begin-

**TABLE 1**

Crystallographic statistics for data collection, phasing, and refinement

| Data Collection     | Native 1 | K2PtCl6 | Native 2 |
|---------------------|----------|---------|----------|
| Space Group         | I222     | I222    | P21212   |
| Unit Cell Dimensions|          |         |          |
| a (Å)               | 148.7    | 149.6   | 201.5    |
| b (Å)               | 158.6    | 157.3   | 149.7    |
| c (Å)               | 200.0    | 200.3   | 157.5    |
| Source              | FR-E CuKα| FR-E CuKα| APS ID-22|
| Max. resolution (Å) | 3.5 (3.63-3.5)| 3.6 (3.73-3.6)| 2.7 (2.8-2.7)|
| Reflections (total/unique) | 258,821/29,973 | 201,963/27,406 | 913,490/129,431 |
| Completeness (%)    | 98.2/95.3| 98.9 (99.9)| 98.9 (94.7) |
| Rsym* (%)           | 12.6 (62.2)| 11.9 (48.8)| 9.0 (61.9)  |
| lα(l)               | 18.2 (3.2)| 21.1 (4.7)| 21.5 (1.3)  |

**Phasing**

| Anom. Iα(l) (4.5Å /3.6Å) | 2.3 /1.2 |
| Riso (%)                  | 43.6     |
| Rano (%)                  | 8.6      |
| Number of Pt sites        | 15       |
| Phasing Power* (4.5Å /3.6Å) | anomalous /isomorphous |
| FOM d (4.5Å /3.6Å)        | 0.45 /0.25 |

**Model Refinement**

| Resolution (Å) | 50.0-2.7 |
| Number of Reflections | 122,982 |
| Completeness (%) | 98.7 (91.3) |
| Rfactor/Rfree* (%) | 21.5/25.5 |
| No. of protein atoms | 15,114 |
| No. of carbohydrate atoms | 700 |
| r.m.s. deviations | bonds (Å) angles (deg) |
|                      | 0.009    | 1.25     |

**a** \( R_{sym} = \frac{\sum|I(h)| - \langle I(h)\rangle}{\sum|I(h)|}\), where \( \langle I(h)\rangle \) is the mean intensity. Numbers in parentheses reflect statistics for the highest resolution shells.

**b** \( R_{iso} = \frac{\sum|F_{obs}(h)| - \langle F_{obs}(h)\rangle}{\sum|F_{obs}(h)|} \) and \( R_{ano} \) is calculated for the amplitudes of the positive and negative counterparts of the Bijvoet pairs.

**c** Phasing power is defined by \( (\langle FH\rangle)/\langle(lack-of-closure)\rangle \), where \( H \) represents heavy-atom.

**d** Mean figure of merit is the estimated mean cosine of the phase error.

* \( R_{free} \) is calculated with 5% of the data.

**Tetramerization**—The structural relatedness of the two individual modules reflects directly on their ability to assemble into a high order tetramer, which we observe both in the crystals and by studies in solution. In the reported structure (crystal form P2₁₂₁₂), the four crystallographically unique Lingo-1 molecules wind around each other in a circular ring-like fashion to form a closed tetramer (Fig. 5A). The resultant structure has an approximate 4-fold axis of symmetry (C₄) and approximate dimensions of 110 × 110 × 65 Å with a central hole of a diameter of ~45 Å. The four symmetrical partner molecules encircle the hole in a head-to-tail orientation, such that the N-terminal convex face of each LRR domain fills the opening of the ectodomain cleft on the next molecule. If we apply this geometry relative to a cell surface, then the protein rotation axis will lie approximately normal to the surface, the curved LRR domains will lie horizontally, back-to-back, whereas the IgI1 C-terminal ends will extend vertically as if to continue toward the membrane. To the best of our knowledge, such tetrameric assemblies have not been observed in LRR proteins before.
The observed mode of tetramerization yields four interfaces, all showing essentially the same geometry. The arrangement at each employs the bent cleft of one molecule as a binding site for another molecule (Fig. 5B), thus the packing of three surfaces: two from the cleft furnished as described above (C-terminal repeats 10–12, helix α1, and β sheet CC'FG) and one from the convex curvature of an adjacent monomer (N-terminal repeats 1–6). The convex-to-convex contacts provide half of the binding interface and primarily involve polar and charged residues. Sets of intricate hydrogen bonding (Fig. 5B, top) generate thirteen anchor points along the curve and contribute to the specificity of the interaction. Few van der Waals contacts (defined as <4 Å) were also observed; the most prominent include a stacking interaction between Tyr290 and Arg146 and close packing between Gly320 and Tyr128.

By contrast, the other half of the interface is largely through hydrophobic patches. The central elements of this interaction are four β strands C, C', F, and G and four consecutive backloop regions of repeats 3–6 that curve along the opposing strands roughly following their directions. There are in total fourteen direct carbon-to-carbon contacts (Fig. 5B, bottom) and only two potential hydrogen bond contacts (Lys424Nζ–Asn163' O; Asn466N82–Ile95'O). The potential glycosylation site Asn466-X-Ser468 is part of the interface, contributing a fairly large surface area of interaction (Asn466 has a total fractional accessibility index of 23%). Such important involvement is of apparent consequence: if it were utilized in the mature protein, glycosylation would cause a steric clash and prevent binding of a partner molecule. Our solution studies indicate that the lack of glycosylation at this position is not an artifact of the mutant CHO cell line, because we were able to detect similar size tetramers with the Lingo-1 HEK cell-derived material (data not shown), a result that would be unlikely if this very site were glycosylated.

Tight packing requirements favor the presence of several glycine residues on both sides of the interface (positions 320, 464, 465, 74', 98', and 102'). Other details of this interaction include a few buried water molecules, the role of which is presumably to optimize the electrostatic complementarity of binding regions. Small differences in the ectodomain tilt angle (∼3°) suggest that the bound geometry of this interaction utilizes surfaces charge and hydrophobic complementarity as an efficient means of achieving both specificity and plasticity in binding. In general, the plastic nature of protein-protein interfaces is often identified as an emerging hallmark of surfaces of signaling proteins and often cited as having a role in the mechanism of binding and release of signaling partners.

The many interactions between the two monomers bury a surface area of ∼2,300 Å², comparable to and above the average size interface observed for protein-protein complexes in general (∼2,000 Å² (31)). Accordingly, the total molecular surface area buried within the tetramer is ∼9,200 Å². Comparable values have been found from studies of large tetrameric protein complexes (∼10,000 Å² (32)). Such extensive contacts in the interface support the finding that Lingo-1 can exist as a tetramer. To explore the self-associating interactions of Lingo-1 in solution we employed chemical cross-linking, gel-filtration chromatography, dynamic light scattering, and analytical ultracentrifugation (Fig. 6). These experiments indicated that at concentrations >16 μM Lingo-1 is best modeled as a tetramer. Cross-linking of Lingo-1 with molar ratios of glutaraldehyde yielded a second band on a denaturing gel, with a molecular size expected for a tetramer ∼260 kDa (Fig. 6A). Upon gel filtration, Lingo-1 eluted as a single sharp peak with the retention time expected for a tetrameric species (200–300 kDa, Fig. 6B). Light scattering measurements showed that at concentrations of ∼16 μM the protein is predominantly in a single state (polydispersion of ∼10%), with a hydrodynamic radius of 6.2 nm and a corresponding molecular mass of ∼260 kDa (Fig. 6C). Sedimentation equilibrium analysis of Lingo-1-His, performed at four protein concentrations (1, 5.8, 16.6, and 49.3 μM), indicated that at 16.6 μM (Fig. 6D) Lingo-1 undergoes a monomer-dimer-tetramer self-association, with equilibrium association constants $K_{1,2}$...
2.65 \times 10^5 \text{ M}^{-1} \text{ and } K_{2,4} \sim 5.020 \times 10^3 \text{ M}^{-1} \text{ for the monomer-dimer and dimer-tetramer steps, respectively. The stability of tetrameric species was examined during gel filtration, analytical centrifugation, and by light scattering. The tetrameric protein remained stable over a wide pH range and at a very low ionic strength (data not shown). These studies, combined with the notion that similar tetrameric configurations are observed in two crystal forms (P2\_12\_12 and I222 symmetry), suggest that the formation of tetramers is not a consequence of crystal packing, but reflects tetramerization of Lingo-1 in solution.}

**FIGURE 5.** Structure of the Lingo-1 tetramer. A, view of the top and front surfaces of the Lingo-1 tetramer, rendered in red, green, magenta, and yellow. The two views are related by a 90° rotation about the horizontal axis. Carbohydrate are shown as yellow sticks. The LRR modules interlock the ring head-to-tail, back-to-back, with the IgI1s extend vertically. The bottom view illustrates the putative orientation of the tetramer relative to a cell surface. B, detailed view of molecular interfaces. The imprint of bound LRR (red ribbons) on the molecular surface of a neighboring monomer is colored blue. The top and bottom insets are close-up views of some of the interactions at the LRR-LRR' and IgI1-LRR' interfaces, respectively; the prime symbols denote the partner molecule. Molecular surfaces for the two interacting monomers are colored as in A, green and red. Side chains of interacting residues are shown as a ball-and-stick model, and hydrogen bonds are shown with dashed white lines. All interface residues are conserved apart from Ala461 (Ser in chicken, see also Fig. 7A).
Classification of Possible Ligand Binding Sites—The availability of the Lingo-1 structure may now facilitate a number of experiments, among which are site-directed mutagenesis and computational docking. To identify likely functional sites on Lingo-1, which could then help the strategies in future experiments, we analyzed its molecular structure in the context of both monomer and tetramer forms. This involved consideration of evolutionary conserved sequences, electrostatic surface potentials, carbohydrate exposure, and common characteristics that have emerged from the structures of other LRR- and Ig-related protein complexes.

Lingo-1 has a high degree of evolutionary conservation, with 92.7–99.8% extracellular sequence identity among human and homologous monkey, mouse, rat, and chicken (see “Experimental Procedures” for sequence accession information), which suggest a precise biologically relevant function. The seventeen non-conservative mutations are shown in Fig. 7A. The remained conserved patterns, with much of the concave face, the self-recognition motifs, and glycosylation motifs, identify surfaces that may be important for ligand binding, oligomerization, or the structural integrity of folding topology.

Various studies have documented the key role of both electrostatic attraction and hydrophobic interaction upon protein-protein complexation (32). When the calculated electrostatic potentials are mapped on the molecular surface of Lingo-1, it becomes clear that tetramerization of Lingo-1 creates a large change in surface electrostatic potential (Fig. 7B, see also Fig. 4). This indicates a higher surface charge density and less hydrophobic nature for the tetrameric Lingo-1 molecules as compared with the monomeric molecules. The exposure of large
hydropic patches on a protein surface is in general energetically unfavorable, and such is the case with intact Lingo-1 monomers. Upon tetramerization, ~4000 Å² of hydrophobic surface area become buried, which by itself can be a driving force for assembly. Although it is formally possible that the Lingo-1 self-recognition site could also correspond or overlap with its ligand binding sites, we feel that this is less likely.

Further, because the tetramer reveals relatively fewer solvent-exposed hydrophobic residues, the interaction between Lingo-1 and its non-self ligands would be expected to involve a significant electrostatic component. In this vein, we find that the charge distribution on the composite tetramer surface is more striking, leading to the concept that the specificity of Lingo-1 may be largely due to its oligomeric structure and not its individual binding sites. Specifically, a remarkably large area of continuous positive charge, which develops on the molecule front face, could constitute an essential binding site for acidic p75 or a still unidentified acidic protein. Fourteen basic residues combine here from two facing molecules (arginines and lysines; all conserved across the species analyzed) to give this surface a V-shaped positive potential (Fig. 7B). Another note of interest is a net positive potential of the interior of the ring, which is due to repetitive clusters of arginine and histidine residues.

Extrapolating from knowledge of structurally available LRR-ligand complexes (29), two other surfaces on Lingo-1, both unobscured in the tetramer, deserve special mention: the concave face of the LRR module and the ABDE face of the IgI domain. Examples of the LRR-ligand complexes suggest that, perhaps for Lingo-1, too, binding with ligands occurs at or near the concave LRR β sheet. The degree of the β-sheet binding differs for different ligand-receptor pairs. At one extreme is the LRR β-sheet of intercellular adhesion molecule 1 (ICAM-1), where it completely surrounds the ligand domain of human E-cadherin, hEC1 (33). Such intimate contacts cannot be made with the corresponding interior of Lingo-1, because it is filled with carbohydrate. At the other extreme is the LRR β-sheet of platelet receptor glycoprotein Ibα, GpIbα, that has minimal interaction with the ligand von Willebrand factor A1 domain; instead, it binds it with the two insertions, the β finger and the long β switch (34). The latter is also involved in the GpIbα-thrombin interaction (35). An insertion at this position, although only 6-residues long, is also present in Lingo-1 and corresponds to the aforementioned interhelical β-bulge (see Figs. 3C and 8A). Features that are centered around the β-bulge structure are a prominently exposed tryptophan (Trp³⁴⁰) and a forward protruding arginine (Arg⁻⁴³⁷). The latter two residues seem appropriate to consider for possible binding epitopes, especially in light of the fact that amino acids, particularly tryptophan and arginine (also tyrosine), are known to be more frequently encountered in protein-protein recognition sites or sites that are defined as hot spot residues (36). Interestingly, from the four LRR faces, the concave face shows the most contrasting set of differently polarized elements (see Figs. 8A). Among these, elements that may be of relevance to Lingo-1 binding activity are a conspicuous conserved histidine ladder and a continuous area of exceptionally negative charge (Fig. 8A), which comes mainly from glutamic, but also aspartic acids (nine in total, all strictly conserved), extending diagonally across the β sheet to the N-terminal β-hairpin (“β-finger” in GpIbα).

Finally, the structure of the Ig-like module of Lingo-1 evokes comparison with that of the Ig3 module of NCAM. Because both Lingo-1 and NCAM are part of a larger integrated family of cell adhesion-modulating molecules, the topological similarity of the referred domains can be interpreted in terms of evo-
Oligomeric Ectodomains of Lingo-1

The Lingo-1 structural data described here revealed the unusual concept of an LRR protein tetramerization, which we further examined and confirmed by studies of Lingo-1 in solution. This unexpected architecture of Lingo-1 gives a framework for the design of experiments to address the biological relevance of oligomerization, and in particular, the still unresolved questions about the stoichiometry of the Lingo-1 receptor complex. Many studies on signaling-receptor proteins have shown evidence that oligomerization has a role in regulating receptor functions by influencing ligand binding and signaling properties (23). In its association with itself, Lingo-1 may provide a mechanism for assembly of the receptor complex components, localizing their signaling functions to the sites of neuronal pathways that terminate axon growth. Accordingly, Lingo-1 may need to form tetramers at the cell surface to have distinct binding sites, relative to the monomer. For example, the signaling complex may consist of four of each binding receptor components (4:4:4) rather than one (1:1:1). Given, however, that Lingo-1 molecules may not necessarily be present as oligomers in the basal state, prior to receptor binding, the mode of Lingo-1 self-interaction observed in the crystal may occur during activation. Lingo-1 appears to be integrated in several central nervous system processes, with the ability to bind multiple signaling molecules, and so its functional roles may vary, and different oligomerization mechanisms may be used in different contexts.

Although the precise arrangement of the inhibitory complex awaits the solution of a crystal structure, the molecular structure of Lingo-1 obtained by the current work can provide useful information about potentially important functional binding sites that can already be integrated into therapeutic discovery programs. Functional validation of these sites should help the design of therapeutic strategies to block signals of axon growth inhibitors and to encourage remyelination.

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