Structure of the LINGO-1-anti-LINGO-1 Li81 antibody complex provides insights into the biology of LINGO-1 and the mechanism of action of the antibody therapy

R. Blake Pepinsky*, Joseph W. Arndt, Chao Quan, Yan Gao, Omar Quintero-Monzon, Xinhua Lee, and Sha Mi

Departments of Drug and Molecular Discovery, Biogen Idec, Inc.,
14 Cambridge Center, Cambridge, MA 02142, USA

*Corresponding author
a) Running title: Structure and mechanism of action of anti-LINGO-1 antibody

b) R. Blake Pepinsky, Biogen Idec, Inc., 14 Cambridge Center, Cambridge, MA 02142
   Telephone: 617-679-3310, Fax: 617-679-3148, email: blake.pepinsky@biogenidec.com

c) text pages-36
   number of tables-1
   number of figures-9 (plus 7 supplementary figures)
   number of references-48
   number of words in Abstract-237
   number of words in Introduction-664
   number of words in Discussion-1776 (includes citations)

d) LINGO-1, leucine-rich repeat and Ig containing Nogo receptor interacting protein-1;
   CNS, central nervous system; MS, multiple sclerosis; mAb, monoclonal antibody; LRR,
   leucine-rich repeat; CHO, chinese hamster ovary; CV, column volumes; PAGE,
   polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; ELISA,
   enzyme-linked immunosorbent assay; DLS, dynamic light scattering; MR, molecular
   replacement; MBP, myelin basic protein; OPC, oligodendrocyte progenitor cell; HC,
   heavy chain; LC, light chain; CDR, complementarity determining regions; NgR1, Nogo
   receptor interacting protein-1; EM, Electron microscopy.

e) Neuropharmacology
ABSTRACT

Multiple sclerosis (MS) is an autoimmune-inflammatory induced disease of the central nervous system (CNS) with prominent demyelination and axonal injury. While most MS therapies target the immunological response, there is a large unmet need for treatments that can promote CNS repair. LINGO-1 (leucine-rich repeat and Ig containing Nogo receptor interacting protein-1) is a membrane protein selectively expressed in the CNS that suppresses myelination, preventing the repair of damaged axons. We are investigating LINGO-1 antagonist antibodies that lead to remyelination as a new paradigm for treatment of individuals with MS. The anti-LINGO-1 Li81 antibody (BIIB033) is currently in clinical trials and is the first MS treatment targeting CNS repair. Here, to elucidate the mechanism of action of the antibody, we solved the crystal structure of the LINGO-1/Li81 Fab complex and used biochemical and functional studies to investigate structure-function relationships. Li81 binds to the convex surface of the leucine-rich repeat domain of LINGO-1 within repeats 4-8. Fab binding blocks contact points used in the oligomerization of LINGO-1 and produces a stable complex containing 2 copies each of LINGO-1 and Fab that results from a rearrangement of contacts stabilizing the quaternary structure of LINGO-1. The formation of the LINGO-1/Li81 Fab complex masks functional epitopes within the Ig domain of LINGO-1 that are important for its biological activity in oligodendrocyte differentiation. These studies provide new insights into the structure and biology of LINGO-1 and how Li81 mAb can block its function.
Introduction

LINGO-1 (LERN1, LRRN6A) is a 581 amino acid membrane associated-glycoprotein selectively expressed on neurons and oligodendrocytes in the CNS (Mi et al. 2005). It contains a large extracellular domain with 12 leucine rich repeat (LRR) motifs flanked by N- and C-terminal capping modules, one immunoglobulin (Ig) domain of the I1 subtype, and a stalk region that is attached to a transmembrane region and a short cytoplasmic tail (Mi et al., 2004; Mosyak et al., 2006). LINGO-1 suppresses oligodendrocyte differentiation thereby preventing axonal myelination (Mi et al. 2007, 2009). Blocking its function leads to robust myelination in vitro and in animal models of demyelination (Mi et al. 2005, 2007, 2009). The biological effects of LINGO-1 antagonists have been validated using siRNA, a dominant negative construct lacking the cytoplasmic tail, a soluble LINGO-1-Fc fusion protein in which the LINGO-1 extracellular domain is genetically linked to the hinge region and Fc of human IgG1, and anti-LINGO-1 mAbs, Fab, Fab2s, and PEG-Fabs (Mi et al., 2005, 2007, 2009; Ji et al., 2006; Fu et al., 2008; Pepinsky et al., 2011; Cen et al., 2013). Of these, antibodies were preferred because of their potency, selectivity, and duration of treatment effects due to long serum half-life (Aires da Silva et al., 2008; Pepinsky et al., 2011). The anti-LINGO-1 antibody Li81 was isolated using Fab phage display technology and engineered into a human IgG1 aglycosyl framework (Pepinsky et al., 2011; Hoet et al., 2005). We are currently investigating Li81 mAb (BIIB033) in clinical trials as a potential treatment to repair neuronal damage that occurs in the CNS of individuals with MS (Mi et al., 2013). The clinical trial.gov number for the Phase 2 MS study is SYNERGY: NCT01864148.

The use of antibodies as drugs that target antigens expressed in the CNS is challenging because of poor exposure to the CNS following systemic administration due to the blood-brain barrier (Shen et al., 2004; Levites et al., 2006; Garg and Balthasar, 2009; Pepinsky et al., 2011). Current estimates are that only 0.1% of antibody drug levels in blood reach the CNS. Consistent with low levels reported by other labs, we determined that Li81 mAb levels in brain and spinal cord following systemic administration of the antibody in rats were 0.15% of blood levels, but despite the low
CNS exposure, Li81 treatment led to the repair of damaged CNS neurons with a direct dependence of dose on the extent of CNS repair (Pepinsky et al., 2011). These findings provided clear evidence that remyelination that results from blocking of LINGO-1 function is directly linked to the binding of Li81 to LINGO-1. Cellular responses that are mediated by LINGO-1 result from direct binding of LINGO-1 to growth factor receptors to block their function. To date four LINGO-1 signaling pathways have been elucidated (Mi et al., 2013). Structure activity relationship studies suggest that the Ig domain of LINGO-1 is sufficient for its activity (Mi et al. 2010, Bourikas et al., 2010, this study). The crystal structure of the LINGO-1 ectodomain revealed that the protein self-associates to form a ring-shaped tetramer (Mosyak et al., 2006). In the structure, potential function binding sites on the Ig domain are solvent exposed, providing a model for how LINGO-1 functions. How the Li81 antibody blocks LINGO-1 function is unknown.

Here, to investigate the mechanism of action of the Li81 antibody we solved the crystal structure of the LINGO-1 ectodomain/Li81 Fab complex and discovered that the binding of Li81 to LINGO-1 blocked contacts that allowed LINGO-1 to form the tetrameric state seen in the published LINGO-1 crystal structure. Li81 Fab treatment prevented the formation of LINGO-1 homo-oligomers in solution and on transfected cells expressing full length LINGO-1, and led to a rearrangement in the quaternary structure of LINGO-1, which provides a model for how the antibody inhibits LINGO-1 function in oligodendrocytes. These studies provide a detailed assessment of domains within the LINGO-1 structure that are important for function and expand our understanding of how they contribute to the complex biology of LINGO-1.
Materials and Methods

LINGO-1 production. LINGO-1-Fc produced by fusing the extracellular portion of human LINGO-1 (residues 1-488) to the hinge and Fc region of human IgGl was expressed in Chinese hamster ovary (CHO) cells and purified from clarified and filtered cell culture medium on recombinant Protein A Sepharose Fast Flow (GE Heathcare) at a loading density of 6 mg of fusion protein/ml of resin. The LINGO-1-Fc was eluted from the column with 25 mM NaH₂PO₄ pH 2.8, 100 mM NaCl and neutralized by adding 0.05 volume of 0.5 M NaH₂PO₄ pH 8.6. Peak protein-containing fractions were identified by absorbance at 280 nm, pooled, and dialyzed against PBS. The preparation was 0.2 µm filtered, aliquoted, frozen on dry ice, and stored at -70°C. The protein concentration was calculated using an extinction coefficient of \( \varepsilon_{280}^{0.1\%} = 1.04 \) using computer Beckman Coulter DU-800 Spectrophotometer NetA software that compensates for baseline shifting by subtracting absorbance due to turbidity from the value at 280 nm. LINGO-1 ectodomain was generated from LINGO-1-Fc by digestion with chymotrypsin or endoproteinase Lys-C as indicated. For limited proteolysis with chymotrypsin, LINGO-1-Fc (2.5 mg/ml) in PBS was diluted with 2 volumes of 100 mM Tris HCl pH 7.5, 10 mM CaCl₂. Chymotrypsin (Roche) was added to a 1:500 enzyme:protein ratio (w/w) and incubated for 3 h at room temperature. PMSF was added to a final concentration of 0.5 mM and an additional 100 mM NaCl was added. The sample was loaded onto a Protein A Sepharose column (8 mg protein/ml resin) equilibrated in PBS. The flow through fraction was pooled, concentrated using an Amicon ultra-15 centrifugal filter device (10 kDa membrane) and loaded onto a Superdex 200 size exclusion chromatography column. Peak fractions were pooled, aliquotted, and stored at -70°C. The protein concentration was calculated using an extinction coefficient of \( \varepsilon_{280}^{0.1\%} = 0.9 \). For limited proteolysis with endoproteinase Lys-C (Wako), LINGO-1-Fc at 7.5 mg/ml in PBS was incubated for 2 h at room temperature with a 1:1000 enzyme:protein ratio (w/w). Leupeptin was added to a final concentration of 0.2 mM and an additional 100 mM NaCl was added. The sample was loaded onto a Protein A Sepharose column (8 mg protein/ml resin) equilibrated in 20 mM Na₂HPO₄ pH 7.5, 250 mM NaCl. The flow through fraction was
pooled, and loaded onto a Ni-NTA Superflow column (Qiagen) at 3 mg protein/ml resin. The column was washed with 3 column volumes of 20 mM Na₂HPO₄ pH 7.5, 250 mM NaCl and the LINGO-1 was step eluted with the same buffer containing 20 mM imidazole. The elution pool was concentrated using Amicon ultra-15 centrifugal filter devices (10 kDa membrane) and loaded onto a Superdex 200 size exclusion chromatography column. Peak fractions were pooled, aliquoted, and stored at -70°C. The sequence of LINGO-1-Fc is shown below (ectodomain in black, Fc in green). Observed cleavage sites for chymotrypsin and endoproteinase Lys-C are shown with red and blue arrowheads, respectively. Predicted N-linked glycosylation sites are noted in red. N466 is not occupied with a glycan (experimentally determined).

For structural work, the LINGO-1-Fc producing CHO cells were cultured in the presence of kifunensine to reduce the complexity of the carbohydrates (Yu et al., 2011). The same methods were used for purification of LINGO-1-Fc and generation of the ectodomain fragments for LINGO-1-Fc produced in the presence and absence of kifunensine. Truncated versions of LINGO-1 ectodomain containing the LRR domain alone (residues 1-382), Ig domain alone (residues 383-460), and Ig domain (residues 383-460) with the RKH sequence (residues 423-425) mutated to EKV were fused to the hinge and Fc region.
of human IgGl, expressed in CHO cells, and purified from culture medium on Protein A Sepharose as described above.

**Li81 Fab production.** The Li81 mAb was purified from clarified and filtered Chinese hamster ovary (CHO) cell culture medium on recombinant Protein A Sepharose Fast Flow. The Li81 mAb was dialyzed into 10 mM sodium acetate pH 3.6. Li81 Fab2 fragment was generated from the mAb by digestion with pepsin at an enzyme:protein ratio of 1:500 for 4 h at 37°C. Tris-HCl was added to 300 mM, to quench the reaction and the sample dialyzed against 50 mM Tris-HCl pH 8.9, 50 mM NaCl. The sample was subjected to anion-exchange chromatography on a Fractogel EMD TMAE (M) (40-90 µm, Merck) column equilibrated in 50 mM Tris-HCl pH 8.9, 50 mM NaCl. The column was washed with 50 mM Tris-HCl pH 8.9, 50 mM NaCl and the Fab2 eluted with 20 mM Na2HPO4 pH 7.5, 150 mM NaCl (PBS). Li81 Fab fragment was generated from the Fab2 by digestion with papain at an enzyme:Fab2 ratio of 1:300. Fab2 in 10 mM Na2HPO4 pH 7.5, 5 mM EDTA pH 7.5, 20 mM cysteine-HCl, 20 mM NaOH was incubated with papain at 37°C for 2.5 h and quenched with the thiol protease inhibitor E64. The sample was dialyzed against PBS.

**SDS-Polyacrylamide Gel Electrophoresis.** Samples were subjected to SDS-PAGE on 4-20% Tris-glycine gradient gels (Invitrogen) and stained with Coomassie brilliant blue. Non-reduced samples were diluted with Laemmli non-reducing sample buffer, and heated at 75°C for 5 min prior to analysis. Reduced samples were treated with sample buffer containing 2% 2-mercaptoethanol and heated at 95°C for 2 min. Electronic images of stained gels were acquired with the true color setting on a HP Scanjet G4050 scanner (Hewlett Packard). For densitometry, the gel images were acquired and band intensities quantified using a GS-800 Calibrated Densitometer (Bio-Rad) and the Quantity One 1-D Analysis software (Bio-Rad). LINGO-1 and Li81 concentrations in the LINGO-1/Li81 Fab complex were calculated by interpolation of measured densities from standard curves of densities of LINGO-1 and Li81 Fab standards, which were run on the same gel.

**Analytical Size Exclusion Chromatography and Light Scattering.** Size Exclusion Chromatography (SEC) was carried out on a BioSep-SEC-S3000 column, 300 x 7.8 mm
(Phenomenex) in 20 mM sodium phosphate, 150 mM NaCl, pH 7.2, using a flow rate of 0.6 ml/min on a Waters Alliance instrument (Waters 2790, MA). The column effluent was monitored by UV detection at 280 nm. Static light scattering was synchronized with SEC and measured on-line using a Precision PD2100 Detector (Precision Detectors, MA). Molecular weights were calculated using Discovery 32 Light Scattering Analysis Software.

**Biacore measurements.** Solution-phase affinity measurements were performed on a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden). Human LINGO-1 ectodomain was immobilized on CM5 chips using amine-coupling chemistry in Biacore buffer (10 mM HEPES, pH 7.2, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20). Binding experiments were run in Biacore buffer containing 0.05% bovine serum albumin. For measuring LINGO-1-LINGO-1 interactions, serial 2-fold dilutions of the analyte human LINGO-1 ectodomain from 0.2 to 2 µM was used. For affinity of Li81 Fab, serial 2-fold dilutions of the Li81 Fab from 0.1 to 1 nM was tested. Data were analyzed with BIAevaluation 3.0 Software. Competition analysis of Li81 Fab with 1A7 were performed at a fixed concentration of 10 nM LINGO-1-ectodomain with 1 µM 1A7 Fab, or 25 nM and 1 µM 1A7 mAb on CM5 chips coated with Li81 and 1A7 mAbs using the amine-coupling method.

**Cross-linking studies.** LINGO-1 ectodomain (14 µM) alone in 20 mM sodium phosphate, 150 mM NaCl, 35 mM HEPES, pH 7.5, in the presence of 28 µM Li81 Fab, or within the SEC purified complex was incubated for 70 min at room temperature with 1 mM bissulfosuccinimidyl suberate (BS3) (Thermo Scientific). The reaction was stopped with 50 mM ethanolamine pH 8.0. LINGO-1 ectodomain was preincubated with Fab for 30 min at room temperature prior to treatment. For cross-linking on cells, stable CHO-cells expressing HA tag full length LINGO-1 (residues 1-581 with HA tag engineered at the N-terminus) were maintained at 37°C in Alpha plus Eagle Minimum Essential Medium containing 10% fetal bovine serum and 400 µg/ml Geneticin (Invitrogen). Cross-linking was performed on 6-well plates. Cells were seeded at 4 x 10^5 cells/well and incubated in medium without Geneticin for 2 days. Cells were washed twice with 2
mM Hepes, pH 7.5, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, and 25 µg/ml human serum albumin, then treated with 10 µg/ml Li81 Fab for 15 min at room temperature and cross-linked at 4°C for 1 h with 1 mM BS3. The reaction was stopped with 50 mM ethanolamine pH 8.0. Cells were lysed in 1 ml of RIPA lysis buffer (50 mM Tris HCl, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM MgCl2, 5% Glycerol) and samples were clarified by centrifugation in an eppendorf centrifuge. Cell supernatants were immunoprecipitated with 15 µl of anti-HA affinity Matrix (Roche, Cat. No. 1815016), washed 3 times with 0.3x lysis buffer, and analyzed by western blotting using horseradish peroxidase conjugated-rat-anti-HA antibody for detection. Similar results were generated when cells were treated with Li81 Fab for 3 h at 37°C prior to cross-linking (data not shown).

**Crystallization and Data Collection.** 5.6 mg of SEC-purified LINGO-1 ectodomain from LINGO-1-Fc cultured in the presence of kifunensine and digested with chymotrypsin was added to 4.2 mg of Li81 Fab. The sample incubated for 1 h at room temperature, then concentrated to 2 ml in an Amicon Ultra-15 centrifugal filter device and applied to a 1.6 cm x 60 cm Superdex 200 column. Fractions containing the LINGO-1/ Li81 Fab complex were pooled, dialyzed against 10 mM HEPES pH 7.5, 150 mM NaCl, and 0.2 µm filtered. The complex was concentrated to 4.5 mg/ml. The LINGO-1/Li81 Fab complex was crystallized by the nanodroplet vapor diffusion method at a temperature of 297 K by mixing 200 nl of 4.3 mg/ml LINGO-1/Li81 Fab solution (10 mM Tris pH 8.0, 150 mM NaCl) with 200 nl of the reservoir solution containing 36% pentaerythritol propoxylate (5/4 PO/OH), 0.2 M sodium thiocyanate, 0.1 M HEPES pH 7.0. LINGO-1/ Li81 Fab crystals were transferred to an oil-based cryoprotectant (66.5% paratone, 28.5 paraffin oil, 5% glycerol) prior to harvesting and flash freezing in liquid nitrogen.

Diffraction data were collected at a wavelength of 1.000 Å at the Advanced Photon Source (APS) on beam line LRL-CAT. The data set was collected at 100 K using a MARmosaic 225 detector. Data were integrated, reduced, and scaled using HKL2000.
The LINGO-1/Li81 Fab crystal was indexed in the hexagonal space group P6<sub>3</sub>22.

**Structure Determination and Refinement.** The LINGO-1/Li81 Fab structure was determined to 3.23 Å resolution by molecular replacement using the apo Li81 Fab structure and LINGO-1 structure (PDB# 2ID5) as the search models with the program Phaser (McCoy, 2007). The Li81/LINGO-1 model was manually built with Coot (Emsley and Cowtan, 2004). Structure refinement was performed using REFMAC (Vagin et al., 2004). The progress of the model refinement was monitored by cross-validation R<sub>free</sub>, which was computed from a randomly assigned test set comprising 5% of the data. The final stages of refinement employed TLS refinement (Winn et al., 2001) with anisotropic motion tensors refined for each of the LRR and Ig domains of LINGO-1 and each domain of Li81 Fab. The final model includes one Fab molecule (heavy chain residues 1-221, light chain residues 1-214) and LINGO-1 molecule (residues 3-477). No electron density was observed for residues 1-2 of LINGO-1. The final R factor is 19.6% with an R<sub>free</sub> factor of 25.9%. Refinement statistics are summarized in Table 1. Coordinates and structure factor data of the LINGO-1/Li81 Fab structure have been deposited with the RCSB protein data bank (PDB id code 4OQT).

**Structural analysis.** Hydrogen bonding, salt bridges and van der Waals contacts were identified with the program CONTACTS (CCP4 1994). Buried surface areas were calculated with Areaimol with a 1.4 Å probe radius and standard van der Waals radii. Analysis of the macromolecular assemblies of the LINGO-1<sub>2</sub> Li81 Fab<sub>2</sub> models was evaluated using the PISA algorithm (Krissinel and Henrick, 2007). Shape complementarity was calculated as described by Lawrence and Coleman (Lawrence and Colman, 1993). Figures were prepared with PyMOL (Schrodinger LLC).

**Dynamic Light Scattering.** The hydrodynamic radius of LINGO-1 in 50 mM Tris-HCl pH 7.5, 150 mM NaCl was measured at protein concentrations from 1.25-30 µM using a DynaPro DLS Plate Reader-II (Wyatt Technology). Samples were filtered through a 0.1 µm filter, transferred to a 384-well plate, analyzed using a 5 s acquisition time at 20°C, and 10-20 acquisitions were averaged per sample. Cross-linked LINGO-1 and LINGO-
1/Li81 Fab complex were analyzed using the same procedure. The Optimization Calculator tool in the DYNAMICS software package (Wyatt Technology) was used to determine the minimum protein concentration needed to obtain a good correlation function. The lowest recommended concentration for LINGO-1 was 1.18 µM.

**Oligodendrocyte Differentiation Assays.** Enriched populations of oligodendrocyte precursors (A2B5+) isolated from the forebrain of female Long Evans post-natal day 2 rats were grown in culture in high glucose Dulbecco’s modified Eagle medium containing FGF/PDGF (Peprotech) (10 ng/ml). To assess differentiation of the rat A2B5+ progenitor cells into mature myelin basic protein (MBP) positive oligodendrocytes, A2B5+ cells were plated into 24-well culture plates in FGF/PDGF free growth medium supplemented with N2, and treated with Li81 antibody or soluble LINGO-1 reagents for 72 h. The cells were lysed in RIPA buffer. The lysates were clarified by centrifugation, then boiled in Laemmli sample buffer and subjected to SDS-PAGE on a 4-20% gradient gel. Proteins were transferred to nitrocellulose membranes in a Trans-Blot Cell (Bio-Rad) at 30V for 2 h at 4°C in 50 mM Tris HCl, 400 mM glycine, 0.1% SDS, and 20% methanol. MBP expression was analyzed by Western blotting using a mixture of anti-MBP antibodies SMI-94 and SMI-99 (Calbiochem), and HRP conjugated-donkey anti-mouse antibody (Jackson ImmunoResearch) for detection. The same blot was probed with anti-actin antibody A5060 (Sigma Aldrich) as an internal control. For coculture studies, A2B5+ oligodendrocytes were added to dorsal root ganglion (DRG) neuron cultures as previously described (Mi et al., 2005). The cells were cultured for 14 days in the presence of the indicated treatments, then lysed with RIPA buffer and analyzed for MBP expression by Western blotting as described above.

**Negative stain EM.** A preparation of SEC purified endoproteinase Lys-C generated LINGO-1 ectodomain/Li81 Fab complex was cross-linked with 1 mM BS3 and SEC purified. The protein was adsorbed on ultrathin carbon, 400 mesh copper grids (Ted Pella, Inc.) and stained with 0.75% uranyl formate. Micrographs were recorded on a JEOL 1200EX electron microscope. To compare EM data to x-ray crystal structure models, a set of 2 dimension projections were generated from the 3 dimension x-ray
maps, on a coarse angular grid with ~15° spacing using the SPIDER software package (Shaikh et al., 2008). A 20 Å resolution cut off was applied to the high resolution models before projection to mimic the lower resolution EM images.
Results

Biochemical properties of LINGO-1 and LINGO-1/Li81 Fab complex. Anti-LINGO-1 Li81 (BIIB033) antibody is in clinical trials for treatment of MS. To understand the molecular basis of antigen recognition, we crystallized a Fab fragment of the Li81 monoclonal antibody (Li81 Fab) in complex with LINGO-1 ectodomain. LINGO-1-Fc was used as a source of LINGO-1 ectodomain. For these studies, LINGO-1-Fc was cultured in CHO cells in the presence of kifunensine to reduce glycan complexity and the purified protein was subjected to limited digestion with chymotrypsin to release the LINGO-1 ectodomain from the Fc. The chymotryptic fragment (amino acids 1-478) contained the entire LRR and Ig domains of LINGO-1, the same version that had been previously crystallized by Mosyak and coworkers in 2006. A schematic summarizing structural features of the chymotryptic fragment of the ectodomain is shown in Fig. 1A. LINGO-1-Fc was also produced from CHO cells grown in the absence of kifunensine in order to assess the biochemical characteristics of LINGO-1 containing complex, sialylated glycans routinely found on mammalian proteins. For these studies, LINGO-1 ectodomain was produced from LINGO-1-Fc following digestion with endoproteinase Lys-C. The endoproteinase Lys-C fragment contained the entire chymotryptic fragment of LINGO-1 plus 10 amino acids of the stalk region ending with amino acid residue 488. Figure 1B shows an analysis of key reagents by SDS-PAGE. LINGO-1-Fc migrated under reducing conditions as a single band (>95% pure) with an apparent mass of 90 kDa (lane 2 without, lane 3 with kifunensine). Under non-reducing conditions, LINGO-1-Fc migrated with an approximate mass of 180 kDa, consistent with the dimeric structure of the fusion protein that is stabilized by disulfide bonds in the IgG hinge (lane 4). The chymotryptic fragment of the LINGO-1 ectodomain fragment had an apparent mass of 70 kDa (lane 6 without, lane 7 with kifunensine). The sample produced from LINGO-1-Fc cultured in kifunensine (lane 7) showed a sharper gel band than the corresponding fragment from LINGO-1-Fc produced using standard culture conditions (lane 6), consistent with the reduced glycan heterogeneity. The endoproteinase Lys-C fragment of the LINGO-1 ectodomain also had an apparent mass of 70 kDa (lane 5). When the
endoproteinase Lys-C (lane 5) and chymotryptic (lane 7) fragments were mixed with Li81 Fab and the complexes were SEC purified, and analyzed by SDS-PAGE under non-reducing conditions we obtained the preparations shown in lanes 8 and 9, respectively. In the presence of SDS, the complexes completely dissociated into free LINGO-1 and Fab. Quantification of band intensities by densitometry revealed equimolar associations of LINGO-1 and Fab in the complex. Densitometry data for the sample shown in lane 8 is provided in Supplementary figure 1. The batches of the endoproteinase Lys-C LINGO-1 ectodomain fragment, Li81 Fab, and SEC purified LINGO-1/Li81 Fab complex shown in lanes 5, 8, and 10 were used in all the biochemical studies presented in the paper.

Biochemical studies shown in Fig. 2 were used to assess the oligomerization state of LINGO-1. SEC analysis of the LINGO-1 ectodomain showed concentration dependent change in molecular weight consistent with formation of reversible oligomers, which were stabilized into dimer and tetramer following cross-linking (Fig. 2A and 2B). Apparent molecular weights measured by light scattering ranged from 85-120 kDa in the absence of cross-linker to 160 and 230 kDa for cross-linked dimer and tetramer (Fig. 2A and 2B). The presence of cross-linked dimer and tetramer in the SEC elution fractions was confirmed by SDS-PAGE (Supplementary figure 2). The dependence of size on the LINGO-1 concentration in the absence of cross-linking is as was expected for a protein in a rapidly exchanging equilibrium and is indicative of low affinity interactions (Winzor and Scheraga, 1963; Zoltowski and Crane, 2008). Biacore studies established a more quantitative measure of the low affinity LINGO-1-LINGO-1 interactions observed by SEC (Fig. 2C) and revealed an apparent KD of 0.6 µM for binding of LINGO-1 to LINGO-1. Both association and disassociation of oligomers observed by Biacore occurred in seconds, which accounts for the rapid exchange characteristics seen by SEC. During the 15-20 min LINGO-1 migrates on the SEC column, interconversion between monomer and oligomer states can occur 1000 times. This leads to concentration dependent elution as single peaks intermediate between monomer and oligomer where the weight average migration is based on the equilibrium state. Concentration dependent oligomerization was also observed by DLS (Fig. 2D). With and without cross-linking the
hydrodynamic radius approached 6.5 nm, consistent with the formation of a tetramer. The oligomer state remained constant at concentrations >10 µM which is in close agreement with the DLS value of ≥ 16 µM reported by Mosayk et al (2006) in which a LINGO-1 ectodomain preparation lacking complex glycans had been characterized. DLS could not be used to investigate monomer-dimer conversion that occurs at low LINGO-1 concentrations because 1 µM LINGO-1 was the lower limit of detection of the method (see Materials and Methods). Cross-linking studies with CHO cells expressing full length human LINGO-1, as assessed by SDS-PAGE/western immunoblotting, were used to study LINGO-1-LINGO-1 interactions in its more relevant membrane bound form (Fig. 2E). Full length LINGO-1 migrated on SDS-PAGE gels with an apparent molecular weight of 80 kDa in the absence of cross-linker, consistent with the predicted size of the protein (Fig. 2E). After cross-linking, two prominent forms were detected with apparent masses of 200 and 400 kDa consistent with the formation of dimer and tetramer. Cross-linking was very efficient as evident by the near complete disappearance of the uncross-linked LINGO-1 80 kDa band. The large proportion of cross-linked 400 kDa product indicates that membrane associated LINGO-1 can assemble into a tetramer, which supports the model for oligomerization based on the published crystal structure of the LINGO-1 ectodomain (Mosayk et al, 2006).

Biochemical characteristics for binding of the Li81 antibody to LINGO-1 were also determined (Fig. 3). Biacore provided accurate measurements of binding affinities of the Li81 mAb and Fab for LINGO-1 (see Fig. 3A for Fab data). K_D values were ≤ 20 pM for binding of Li81 mAb and ≤50 pM for the binding of the Li81 Fab to the LINGO-1 ectodomain. Similar values were also measured for their binding to LINGO-1-Fc. When Li81 Fab was added to the LINGO-1 ectodomain and analyzed by SEC equipped with light scattering, the LINGO-1 ectodomain/Li81 Fab complex eluted with molecular weight of 250 kDa (Fig. 3B), twice the anticipated mass of 120 kDa assuming a 1:1 LINGO-1:Fab complex. Varying the ratio of LINGO-1 to Li81 Fab over a wide range of concentrations had no effect on the assembly of the 250 kDa complex (Fig. 3C). Furthermore, in sharp contrast to concentration dependent changes observed for LINGO-
1 alone in Fig 2A, there was no change in its association state when the purified 250 kDa complex was analyzed by SEC over a range of concentrations from 2 µM-3 nM (data not shown). 3 nM of the complex was lowest concentration that could be evaluated by SEC due to limits of detection of the method using absorbance at 280 nm for detection. By labeling the complex with Alexa-488 and using fluorescence detection we were able to extend the lower limit of quantification of the SEC method by 100-fold. Based on the stability data during SEC analysis, we can infer an EC50 value for dissociation of the complex of ~0.5 nM. Consistent with size measurements by SEC/light scattering, DLS analysis of the complex revealed a hydrodynamic radius of 6.95 nm, which is slightly larger than the 6.5 nm value observed for the LINGO-1 homotetramer.

Cross-linking studies were used to further assess the impact of Li81 Fab treatment on the assembly of LINGO-1 oligomers. Fig. 3D shows an analysis of cross-linked LINGO-1 ectodomain alone and cross-linked SEC purified LINGO-1 /Li81 Fab complex by SDS-PAGE. In the absence of Fab, cross-linking led to the formation of LINGO-1 dimer and tetramer in agreement with SEC results (Fig. 2B). The profile of cross-linked adducts closely resembled the data for cross-linked full length LINGO-1 on cells shown in Fig. 2E, indicating that protein-protein interactions of LINGO-1 in solution and on cells are similar. SDS-PAGE analysis of the cross-linked LINGO-1/Li81 Fab complex revealed a prominent diffuse band at an intermediate position between LINGO-1 dimer and tetramer and the disappearance of the tetramer band observed in the cross-linked LINGO-1 sample without Fab treatment (Fig. 3D). The formation of the cross-linked dimer was also impacted as evident by the decrease in the intensity of the cross-linked dimer band following Li81 Fab treatment. Although the cross-linking analysis was very sensitive to changes in the oligomerization state of LINGO-1, the analysis by SDS-PAGE did not allow us to determine the stoichiometry of LINGO-1 and Fab in the complex. Based on size by SEC/light scattering, hydrodynamic radius by DLS, equimolar stoichiometry determined by SDS-PAGE, and data from the crystal structure and EM analysis of the complex discussed below, we infer that the complex is a tetramer containing 2 copies each of the LINGO-1 ectodomain and of the Fab. The EM analysis
of the cross-linked SEC purified complex revealed a very homogeneous preparation in which ≥95% of the protein had assembled into a single LINGO-1<sub>2</sub>Li81<sub>2</sub>Fab<sub>2</sub> tetrameric state. Quantification of the cross-linked samples seen in Fig. 3D by densitometry revealed that in the SEC purified complex ≥90% of the LINGO-1 had been cross-linked as evident by reduction in free LINGO-1 whereas only 30% of the Li81 Fab was cross-linked evident by reduction in free Li81 Fab. The lower extent of reaction for the Li81 Fab can account for the complex banding pattern observed by SDS-PAGE as we would expect to see products that contain LINGO-1<sub>2</sub>Li81<sub>1</sub>Fab<sub>2</sub>, LINGO-1<sub>1</sub>Li81<sub>1</sub>Fab<sub>2</sub>, LINGO-1<sub>2</sub>Li81<sub>1</sub>Fab<sub>1</sub>, LINGO-1<sub>1</sub>Li81<sub>1</sub>Fab<sub>1</sub>, LINGO-1<sub>2</sub>, free LINGO-1, and free Li81 Fab that are released from the cross-linked complex following treatment with SDS.

When cells expressing full length LINGO-1 were treated with Li81 Fab, cross-linked and analyzed by SDS-PAGE with western detection (Fig. 2E), Li81 Fab treatment again prevented the formation of the tetramer observed in the absence of Fab. Instead bands migrating at the positions of LINGO-1 monomer, dimer, and intermediate between dimer and tetramer were detected. The absence of LINGO-1 tetramer in cross-linked LINGO-1 following Li81 Fab treatment indicates that Li81 interferes with the ability of the protein to form a homotetramer. Fab treatment of the cells over a range of concentrations from 1.2-40 µg/ml and with incubation times ranging from 0.25-48 h did not lead to a change in surface levels of LINGO-1 (Supplementary figure 3). The larger percentage of LINGO-1 monomer in the cross-linked LINGO-1/Li81 Fab sample on cells (Fig. 2E) versus in solution (Fig. 3D), may have been caused by a drop in the effective concentration of BS<sup>3</sup> due to reaction of the cross-linker with the large pool of amine containing lipids in the membrane. From titration studies of BS<sup>3</sup> with LINGO-1/Li81 Fab complex in solution, we found that a decrease in the BS<sup>3</sup> concentration from 1 to 0.6 mM could account for the change in the profile (data not shown).

**Structure of the LINGO-1/Li81Fab complex.** The structure of the LINGO-1/Li81 Fab ectodomain complex was solved to 3.23 Å resolution by molecular replacement (MR) using the coordinates of LINGO-1 as the search model (Mosyak et al., 2006). After placing the LINGO-1, it was possible to locate the Li81 Fab in a subsequent MR search.
There is a single copy of the LINGO-1/Li81 Fab complex in the asymmetric unit. Clear electron density was observed throughout the complex, with the exception of two N-terminal residues of LINGO-1 outside the binding interface. The structures of the LRR and Ig domains of LINGO-1 are essentially the same as in previous studies (Mosyak et al., 2006); however, the positioning of the Ig domain relative to the LRR is rotated by ~15°. The Li81 Fab binds to a discontinuous epitope located at the convex face of the LRR domain at LRRs 4-8 (Fig. 4A). A 2Fo-Fc electron density data map showing electron density for side chains at the LINGO-1/Li81 Fab interface is shown in Supplementary figure 4. There are 12 residues from LINGO-1 and 20 residues from the Li81 Fab that are within 4 Å of the binding partner. The epitope is formed by residues Y122, Q125, R146, G150, N152, H174, H176, K197, R198, L199, Y200, and R201 of LINGO-1 and contains a combination of hydrophobic, hydrophilic, and positively charged residues. The paratope of the Li81 Fab has 44% contributed by the heavy-chain variable domain and 56% by the light-chain variable domain. The key interactions made by the Fab are exclusively from the complementarity determining regions (CDRs), with the major specificity determining contacts coming from CDRs H2, H3, L1, and L3, though all of the CDRs contribute to binding. Cooperatively they form 18 direct antibody-antigen hydrogen bonds, 10 salt bridge interactions, and extensive hydrophobic contacts (Fig. 4B). Upon Li81 Fab binding about 796 Å² of solvent accessible surface is buried on each side of the LINGO-1/Li81 Fab interface with a shape complementarity statistic of Sc = 0.74. Collectively these metrics are all consistent with the high-affinity binding observed. The binding site of the Fab overlaps with contact regions in LINGO-1 seen in the tetramer structure (Fig. 4C, D), and therefore binding of the Fab would be expected to prevent the formation of the LINGO-1 tetramer.

**A model for the structure of the LINGO-12 Li81 Fab2 complex.** The crystal structure revealed two possible assemblies for the LINGO-1 Li81 Fab complex based on crystal symmetry (Fig. 5A). In Model 1, the N-cap of the LRR from one LINGO-1 molecule is bound to the C-cap of the LRR on a second molecule of LINGO-1, which leads to a buried surface area of 595 Å² at dimer interface whereas in the Ig to LRR version (Model
2), the buried surface area at dimer interface is nearly twice that at 1012 Å². Fig. 5B shows the LINGO-1-LINGO-1 contact sites from the two models and from the homotetramer structure overlaid onto the LINGO-1 protomer structure. Despite the large interfaces and their close proximity in the structure, none of the same contact sites are utilized for binding in the three structures. The most striking difference between the models is that the Ig domain is exposed in Model 1 and buried in Model 2. A series of biochemical and functional studies were designed to distinguish between these possibilities. First, we used limited proteolysis as a probe as proteolytic susceptibility can be a very sensitive method for detecting changes in structure (see for example Wen et al., 1996). Endoproteinase Lys-C selectively cleaves LINGO-1 within the stalk region at Lys488 at low enzyme concentrations (Fig. 1B, lane 5), but at higher concentrations and longer incubation additional cleavage occurs at two sites within the Ig domain, Lys392 and Lys424. The identity of these cleavage sites was first determined by mass spectrometry but can be readily monitored by SDS-PAGE. As shown in Fig. 6, when LINGO-1 ectodomain was treated with endoproteinase Lys-C approximately 80% of the protein was selectively cleaved at the sites within the Ig domain to generate the 55 kDa fragment. The 55 kDa fragment, which contains the entire LRR domain, was resistant to proteolysis by endoproteinase Lys-C under cleavage conditions used. In contrast, when the LINGO-1/Li81 Fab complex was treated with endoproteinase Lys-C, only 15% of the LINGO-1 was cleaved at these sites and there was no accumulation of the 55 kDa band at higher enzyme concentrations. This result supports the structure seen in Model 2 in which the Ig domain is buried and not accessible to the enzyme, as we would anticipate more extensive cleavage at the 55 kDa site if Model 1 were utilized.

We further investigated the structure of the LINGO-1/Li81 Fab complex using the non-competitive 1A7 antibody that binds within the Ig domain of LINGO-1 as a probe (Supplementary figure 5). The 1A7 binding epitope contains residue R386 noted in Fig. 4A as it is the single R386H amino acid change that leads to the 100-fold lower affinity of 1A7 for rat versus human LINGO-1 (data not shown). Residue R386 is in the solvent facing ABDE sheet of the Ig domain that is prominently exposed in the published
LINGO-1 crystal structure and far removed from the Li81 binding site (Fig. 7A). Biacore analysis confirmed that the Li81 and 1A7 mAbs were non-competitive as simultaneous binding of both antibodies to LINGO-1 ectodomain was observed (Fig. 7B). Consistent with the presentation of the Ig ABDE surface seen in the LINGO-1 crystal structure, 1A7 bound to the cross linked tetramer producing a shift in the SEC elution profile of cross-linked LINGO-1 from 270 kDa to 460 kDa (Fig. 7C). In contrast, when the LINGO-1/Li81 Fab complex was treated with 1A7, there was no shift in mobility indicating that 1A7 was unable to bind the complex (Fig. 7D). Thus 1A7 is capable of binding to the binary Li81 Fab-LINGO-1 1:1 complex used in the Biacore assessment, but not to the 2:2 complex generated by solution binding of Li81 to LINGO-1, further supporting the structure seen in Model 2. 1A7 was the first anti-LINGO-1 antagonist antibody we generated (Fu et al., 2008; Mi et al., 2009), but was later replaced with the higher affinity Li81 mAb when it became available. 1A7 Fab forms a 1:1 complex with the LINGO-1 ectodomain where it directly binds the Ig domain of LINGO-1 to block function, which is quite distinct from the mechanism of action of Li81 where the assembly of the 2:2 LINGO-1/Li81 Fab complex causes the ABDE surface of the Ig domain to be buried, thereby masking the 1A7 binding epitope (discussed below). In the same study, the binding of Li81 Fab to the cross-linked LINGO-1 homotetramer was also analyzed (Supplementary figure 6). Whereas the binding of 1A7 was very efficient and from the shift in mass we determined the LINGO-1 homotetramer bound 4 1A7 Fabs, the homotetramer only bound 1 Li81 Fab. The inefficient binding despite the higher affinity of Li81 for LINGO-1 than 1A7 is consistent with the overlap in contact sites for LINGO-1-Li81 Fab and LINGO-1-LINGO-1.

Third, we used oligodendrocyte progenitor cell (OPC) differentiation as a functional readout to probe the structure of the LINGO-1/Li81 Fab complex. The biology of LINGO-1 pathway modulators has been studied extensively using in vitro OPC differentiation and OPC/DRG coculture assays. LINGO-1-Fc treatment in both assays leads to dramatic morphological changes and increased expression of MBP that are characteristic of OPC maturation (Mi et al., 2005). Previously we showed that the
The RKH tripeptide sequence within the LINGO-1 Ig domain contributed to the binding of LINGO-1 to NgR1 (Mi et al., 2010). Here we expanded that analysis to investigate if the RKH sequence is also responsible for the activity of LINGO-1 in the OPC/DRG coculture assay. Figure 8A shows 3 new constructs that were generated containing the LINGO-1 LRR domain alone, Ig domain alone, and Ig domain in which the RKH sequence was replaced with EKV by mutagenesis. Figure 8B shows that the LINGO-1-Ig domain Fc fusion protein containing the RKH sequence promoted MBP expression whereas the same construct in which the RKH sequence was mutated was inactive. The same result was obtained using a cyclic peptide containing either RKH or the mutated sequence. The cyclic peptide containing RKH promoted MBP expression and the corresponding EKV containing peptide did not. The observations that the Ig domain-Fc construct was equipotent with full ectodomain version, whereas the LRR alone construct had minimal activity, indicates that the Ig domain is a key determinant for the OPC differentiation activity of LINGO-1 and that the RKH tripeptide is important for the response. The activity of the LINGO-1/Li81 Fab complex was evaluated in the 3 day OPC differentiation assay out of concern that the complex might dissociate or degrade during the longer 14 day incubations used in the coculture study. When the LINGO-1/Li81 Fab complex was assayed in the OPC differentiation assay, there was a >10 fold reduction in activity seen with LINGO-1 or Li81 Fab alone (Fig. 8C). The absence of a response at the 6 µg/ml dose of complex, which contains efficacious 3 µg/ml each amounts of the Fab and LINGO-1, indicates that the complex is very stable as dissociation to free Fab and LINGO-1 would lead to MBP expression. This finding also supports Model 2 where the complex would lead to the RKH motif being buried. Together these studies reveal that Li81 binding induces a rearrangement in the oligomeric state of LINGO-1 from the homotetramer seen in the crystal structure in the absence of the Fab, in which the Ig domain is exposed, to our heteromeric LINGO-1$_2$ Li81 Fab$_2$ complex where the Ig domain of LINGO-1 is buried, which was inactive when the complex was exogenously added to the OPC differentiation assay. The ability of the Li81 binding to inactivate LINGO-1 by assembling the LINGO-1$_2$/Fab$_2$ tetramer is a
novel mechanism of action that is distinct from typical blocking mechanisms used by most antagonist antibodies.

Transmission electron microscopy (EM) provided further evidence of the arrangement of the Fab and LINGO-1 in the LINGO-1/Li81 Fab complex. The EM image shown in Fig. 9A revealed a highly homogeneous preparation of the complex with an average particle size of ~15 nm, consistent with the predicted ~18 nm for either model based on the crystal structure. Different orientations of the complex were evident in the micrograph, making it amenable to more in depth analysis. Reference projections generated from 90°C transpositions of the two crystal structure models are shown in Fig. 9B and C. Manual inspection of the raw EM particles and the reference projections allowed us to correlate EM data with the crystallographic data. The three most represented particles in the EM image match projections a-c from Model 2. Conversely, the distinctive projections expected from Model 1 are absent in the micrograph. The high representation of structures a-c in the EM image support biochemical and functional analysis showing that LINGO-1 and Li81 Fab are organized in the complex shown in Model 2. Approximately 30% of the particles could not be assigned to any of the shown structures. When particle shapes were interpreted using the more extensive collection of 15° projections (24 per model), over 95% of the shapes were classified as Model 2 and none supported Model 1 (data not shown). The structure of the LINGO-12Li81 Fab2 complex was confirmed by elucidating its 3 dimensional volume by EM (Supplementary figure 7). The EM structure was produced by sub-tomogram averaging (single particle tomography) to a resolution of ~30Å and agrees with the Model 2 complex observed in the crystal structure (0.5 Fourier Shell Correlation criterion).
Discussion

Li81 mAb (BIIB033) is a fully human anti-LINGO-1 antibody in clinical trials for the treatment of MS. Here we used extensive biochemical, biophysical, and functional studies to investigate the mechanism of action of the antibody. The crystal structure of the LINGO-1/Li81 Fab complex revealed that the antibody bound to the convex surface of the LRR domain within LRRs 4-8. All of the LINGO-1-LINGO-1 contacts that contributed to the tetrameric structure in the absence of Fab were lost in the LINGO-1-Fab complex, thus revealing that Li81 binding interfered with the ability of LINGO-1 to oligomerize. Indeed when transfected cells expressing full-length LINGO-1 or soluble LINGO-1 were cross-linked alone or in the presence of the Fab, we found that Li81 Fab treatment prevented and/or disrupted the formation of LINGO-1-LINGO-1 oligomers. The high affinity of the Li81 for LINGO-1 and conversely the low affinity of LINGO-1 for itself would drive the disruption of LINGO-1 oligomers. Whereas the paper by Mosyak et al. (2006) demonstrated tetramer formation of LINGO-1 ectodomain in solution, our study is the first to show the presence of LINGO-1 tetramers on cells expressing full length LINGO-1. The ability of LINGO-1 to form tetramers on transfected cells in the absence of ligands or coreceptors indicates that oligomer formation is an intrinsic property of LINGO-1.

The oligomeric state of LINGO-1 in solution is dynamic. Monomeric, dimeric, and tetrameric states were readily detected using SEC, DLS, SDS-PAGE, and cross-linking analysis consistent with published results (Mosyak et al, 2006). A $K_D$ value of 0.6 µM for LINGO-1 dimerization was determined by Biacore and tetramer was observed by DLS at concentrations of >10 µM. In contrast, only a single species was observed in the presence of Li81 Fab. An unexpected feature of the LINGO-1/Li81 Fab complex was that it assembled into a tetramer containing 2 Fabs and 2 LINGO-1 subunits rather than the expected 1:1 association. Higher order binding was also apparent using the Li81 mAb, but since each Fab forms a 2:2 association with LINGO-1 and each mAb contributes 2 Fabs for binding, bivalent binding through the mAb produced a heterogeneous series of complexes with apparent masses by SEC/light scattering of 500,
1000, and 2000 kDa (data not shown). While the 500 kDa product is consistent with a 2 mAb to 4 LINGO-1 complex, we were unable to definitively identify the stoichiometry of this or of the higher mass forms.

The crystal structure revealed two possible arrangements of the LINGO-1/Li81 Fab subunits. Biochemical and functional studies support Model 2 in which the LRR-Ig domain contacts stabilize the structure and the LINGO-1 Ig domain is buried. In addition, we used the PISA algorithm (Krissinel and Henrick, 2007) as an interactive tool to explore the protein interfaces used in the two models. PISA analysis also predicted that the LINGO-1-LINGO-1 interface of Model 2 would be more stable in solution based on the more extensive buried surface area (595 and 1012 Å² for Models 1 and 2, respectively) and stabilization by 10 hydrogen bonds. The Model 2 interface extends beyond LINGO-1-LINGO-1 contacts with a significant contribution coming from a secondary interface between the LINGO-1 Ig domain and the constant region of the Fab (494 Å² buried surface area), which presumably participates in the formation and/or stabilization of the higher order complex. The 15° rotation in the LINGO-1 Ig domain observed in the LINGO-1/Li81 Fab complex is likely to be a structural prerequisite for the assembly observed in Model 2. Neither of the LINGO-1 dimeric assemblies observed in the LINGO-1/Li81 Fab structure is related to the ring-shaped LINGO-1 tetramer since the specific LRR-LRR and LRR-Ig contact points are different than those that stabilize the LINGO-1 tetramer (Fig. 4D and 5B), thus they are not expected to be intermediate conformations prior to activation. The LINGO-1-LINGO-1 contact points observed in the homotetramer and LINGO-1/Li81 Fab complex (Fig. 4D and 5B) may also contribute to interactions between LINGO-1 and its ligands and coreceptors. This type of situation where the same interface can be used for multiple interactions such as ligand binding and multimer formation has been reported for other LRR containing proteins (Nose et al., 1992; Karalanov et al., 2006; Seabold et al., 2008; Kajander et al., 2011).

A striking feature of LINGO-1 is the high sequence identity across species (human and rat LINGO-1 share 99.5% identity). In contrast, LINGOs 1-4 share only 44-61% identity. Despite low sequence identity, the predicted structures for LINGOs 2-4
are similar as all 5 major structural elements (LRR, Ig, stalk, transmembrane region, and cytoplasmic tail) are conserved. Many of the contact sites that contribute to LINGO-1-LINGO-1 binding are preserved in LINGOs 2-4 and one would thus infer that the dynamic oligomerization features we studied with LINGO-1 may apply to LINGOs 2-4. Similarly, the six N-linked glycosylation sites present within the LRR of LINGO-1, which restrict surfaces available for interactions between LINGO-1 with itself and its binding partners, are conserved. Whereas most LRR proteins utilize the concave surface for ligand binding and/or dimerization (Bella et al., 2008; Kajander et al., 2011), N-linked glycans on amino acid residues N254 and N302 in LINGO-1 block this surface and only the convex face is available for binding. We anticipate this will be true for LINGOs 2-4.

Structure-function studies using OPC differentiation to identify functional epitopes within the LINGO-1 ectodomain have confirmed and extended our understanding of how the LRR and Ig domains contribute to the biology of LINGO-1. Most significantly we discovered that the Ig domain is a potent pathway antagonist and that the Ig containing EKV mutant is inactive, revealing the importance of the RKH tripeptide to LINGO-1 dependent oligodendrocyte biology. One explanation for the RKH-mediated activity is that the soluble Ig domain competes for the binding of full length LINGO-1 for formation of its signaling complex. Consistent with this notion, Bourikas et al. (2010) showed that a construct containing the Ig domain, stalk region, transmembrane and cytoplasmic tail but lacking the LRR region retained the activity of full length LINGO-1 in inhibiting OPC differentiation. The same construct also supported homotypic LINGO-1-LINGO-1 binding (Stein and Walmsley, 2012). How the LRR domain contributes to the biology is less clear, but perhaps it contributes to oligomerization to increase avidity for signaling partners and/or directly binds and increases the affinity of the interactions. Mosyak et al. (2006) provided a detailed discussion of potential contributions of oligomer formation to LINGO-1 biology. The low level activity seen in the OPC/DRG coculture assay with the LRR alone construct (Fig. 8B) could reflect either of these scenarios. Recently Jepson et al. (2012) published that LINGO-1 forms intercellular contacts and can self-associate in trans, which is
consistent with OPC/DRG coculture data we previously published showing that loss of LINGO-1 on either cell type alone or both promoted myelination (Lee et al., 2007). How Li81 at a molecular level affects the ability of LINGO-1 to interact with itself in cis (intracellular) and trans (intercellular) remains to be determined.

Of known ligands and coreceptors of LINGO-1 (Mi et al., 2013), the binding to NgR1 and p75 to form the LINGO-1/NgR1/p75 signaling complex that regulates neuronal survival through RhoA activation has been most studied (Mi et al., 2004; Mosyak et al. 2006; Saha et al., 2011; McDonald et al., 2011). Epitope mapping data showed that the LINGO-1 LRR and Ig alone constructs bound NgR1 with low affinity (EC50 values of 120 and 60 nM respectively) versus 6 nM for the intact ectodomain, that the RKH to EKV mutation within the intact ectodomain led to a 20-fold reduction in EC50, and that an LRR only construct of NgR1 bound LINGO-1 weaker than longer versions of NgR1 containing the stalk region (Mi et al., 2010). Together these studies reveal that LINGO-1/NgR1 interactions utilize contacts over large surface areas of both proteins and that the RKH within the Ig domain contributes to the binding. Bourikas et al. (2010) also used a domain truncation analysis to map binding to p75 and showed that the LRR domain of LINGO-1 did not contribute but that the Ig domain and stalk region were sufficient. The apparent K_D of ~1 µM for monovalent binding of LINGO-1 ectodomain to soluble NgR1 deduced by Biacore (Mosyak et al., 2006) is of similar affinity to the binding of LINGO-1 to LINGO-1 we determined (Fig. 2). Higher affinities were reported using cell surface binding and ELISA methods where avidity can lead to higher apparent affinities (Mi et al., 2004, 2010; Shao et al., 2005). Other studies have shown that Troy can substitute with p75 to form a LINGO-1/NgR1/Troy signaling complex (Shao et al., 2005; Park et al., 2005). Recently, Amhed et al. (2013) showed that AMIGO3, a distantly related LRR-Ig protein involved in CNS axon growth inhibition, also forms NgR1/p75 and NgR1/Troy signaling complexes. While there is little sequence identity between LINGO-1 and AMIGO3, structural data for AMIGOs 1-3 suggest the importance of the Ig domain in ligand binding (Kajander et al., 2011).
Three notable differences were observed in the structure-activity relationship studies using NgR1 binding and OPC/DRG coculture readouts. First, the intact ectodomain had a 10-fold higher apparent affinity for NgR1 than the Ig domain only version, but was equipotent in the cell-based assay. Second, LINGO-1 Ig domain constructs containing the wildtype RKH sequence had a 2-fold higher apparent affinity for NgR1 than the EKV mutant, but produced a 100-fold increase in activity in the cell-based assay. Third, LINGO-1 full ectodomain constructs containing the wildtype RKH sequence had a 10-fold higher apparent affinity for NgR1 than the same reagent with the EKV mutation, but only a 2-fold higher activity in the cell-based assay. There is no simple explanation that can account for all these differences. Because the signaling partner that drives OPC differentiation is unknown, one possibility is that LINGO-1 binding epitopes for this receptor and NgR1 could be different, which can only be addressed when the relevant OPC receptor(s) are identified. While our studies provide structural evidence that Li81 binding disrupts contacts used in the assembly of LINGO-1 oligomers, further studies are needed to assess the effect of Li81 treatment on the heteromeric complexes that form between LINGO-1 and its other signaling components.

LRR and Ig domains are common structural motifs used in protein-protein binding (McEwan et al., 2006; Bella et al., 2008). An interesting feature of the LINGO-1 structure is the large number of LINGO-1-LINGO-1 contact sites in the tetramer structure and their ability to reassemble with alternative contacts in the LINGO-1/Li81 Fab complex. Studying low affinity LRR/Ig protein-protein interactions that utilize an avidity component for function is challenging. The data we have generated for LINGO-1 should provide a framework for further studies designed to understand the molecular interactions between LINGO-1 and its coreceptors.

Acknowledgements

We wish to thank Greg Thill, Joe Amatucci, and Shelly Martin for cell line selection and generating conditioned medium; Allan Capili, Lee Walus, David Mo, Sheng Gu, Dingyi Wen, and Craig Wildes, for biochemistry support and helpful discussion; Alexey
Lugovskoy for generating molecular models of the RKH epitope and LINGO-1/1A7 complex; Hernan Cuervo for preparation of the synthetic peptides; Dyax Corp. (Burlington, MA) for their antibody discovery efforts behind the identification of Li81; Kasim Sader, Lingbo Yu, and Erik Franken at FEI™ for generating the 3 dimensional EM structure of the LINGO-1\textsubscript{2} Li81 Fab\textsubscript{2} complex; and Ajay Verma and Diego Cadavid, Biogen Idec, for critical reading of the manuscript. Use of the Advanced Photon Source was supported by the U. S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. DE-AC02-06CH11357. Use of the LRL Collaborative Access Team (LRL-CAT) beam line facilities at Sector 31 of the Advanced Photon Source was provided by Eli Lilly & Company, which operates the facility.

**Authorship contributions**

*Participated in research design:* Pepinsky, Arndt, Quan, Gao, Quintero-Monzon, and Mi.

*Conducted experiments:* Pepinsky, Arndt, Quan, Gao, Lee, Quintero-Monzon, and Mi.

*Contributed new reagents or analytic tools:* Pepinsky, Arndt, Quan, and Gao.

*Perfomed data analysis:* Pepinsky, Arndt, Quan, Gao, Lee, Quintero-Monzon, and Mi.

*Wrote or contributed to the writing of the manuscript:* Pepinsky, Arndt, Gao, Quintero-Monzon, and Mi.
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Legends for Figures

Fig. 1. Characterization of LINGO-1 ectodomain by SDS-PAGE. A, schematic showing the LINGO-1 ectodomain construct that was crystallized. The twelve LRR repeats, N-terminal cap (NT), and C-terminal cap (CT) are in red, and the Ig domain in green. The position of the seven N-linked glycans in the protein are indicted with yellow ovals. B, samples (4 µg/lane) were subjected to SDS-PAGE and stained with Coomassie brilliant blue. Lanes 1-3 were analyzed under reducing and lanes 4-10 under non-reducing conditions. Lane 1, molecular weight markers and their apparent molecular masses; lane 2, LINGO-1-Fc; lane 3, kifunensine treated LINGO-1-Fc; lane 4, LINGO-1-Fc; lane 5, LINGO-1 ectodomain produced with endoproteinase Lys-C, 1; lane 6, LINGO-1 ectodomain produced with chymotrypsin; lane 7, kifunensine treated LINGO-1 ectodomain produced with chymotrypsin; lane 8, SEC purified LINGO-1 ectodomain (endoproteinase Lys-C treated) /Li81 Fab complex; lane 9, SEC purified LINGO-1 ectodomain (produced with kifunensine and chymotrypsin treatments) /Li81 Fab complex; lane 10, Li81 Fab.

Fig. 2. Concentration-dependent oligomerization of the LINGO-1 ectodomain. A and B, samples containing 0.15, 0.3, 0.6, 1.2, 2, 5, 10, 30 and 50 µM LINGO-1 ectodomain (A) or 14 µM LINGO-1 ectodomain alone or after cross-linking with BS3 (B) were subjected to analytical SEC on a BioSep-SEC-S3000 column using PBS as the mobile phase. The column effluent was monitored at 280 nm. Samples in A and B were loaded onto the column at a fixed volume of 80 µl. The y-axis values of samples in the overlaid SEC chromatograms in A and B were normalized to peak height of the largest peak. C, apparent KD of dimerization of LINGO-1 ectodomain measured by SPR. Dilutions containing 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, to 2 µM of the analyte LINGO-1 ectodomain were passed over a chip containing immobilized LINGO-1 ectodomain. Binding curves were expressed as resonance units (RU) as a function of time and fit to a
1 to 1 binding model using BIAevaluation. D, apparent hydrodynamic radius of LINGO-1 ectodomain samples at concentrations of 1.25, 2.5, 5, 10, 20, 25, and 30 µM (closed diamonds) and for SEC purified cross-linked LINGO-1 tetramer at 1.5 µM (open diamond) were determined by dynamic light scattering. Each data point is the average of 10-20 determinations ± standard deviation for non-cross-linked samples and 40 determinations for cross-linked samples. E, CHO-cells expressing HA tag full length LINGO-1 alone or cross-linked with 1mM BS3 in the absence or presence of Li81 Fab were subjected to immunoprecipitation with anti-HA affinity matrix and analyzed by western blotting. (L1), (L1), (L1)4 marked with arrows note the positions of the LINGO-1 monomer, dimer, and tetramer. Samples with and without BS3 treatment were run on the same gel. A, B, D, and E each show representative data from at least 3 separate experiments. Biacore samples for each experiment were analyzed in triplicate. For cross-linking, different preparations of cross-linked LINGO-1 and different cultures of cells expressing LINGO-1 were used for each experiment.

**Fig. 3.** Li81 Fab forms a LINGO-12Fab2 tetrameric complex. A, the affinity of Li81 Fab binding to LINGO-1 ectodomain was measured with a Biacore. Dilutions containing 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1 nM of the analyte Li81 Fab were passed over a chip containing immobilized LINGO-1 ectodomain. Binding curves were fit to a 1 to 1 binding model using BIAevaluation. B, SEC purified complex of LINGO-1 ectodomain and Li81 Fab (100 µg) was subjected to analytical SEC equipped with light scattering. C, 20 µg/ml LINGO-1 ectodomain was mixed with 0, 2, 6, 20, 60 and 200 µg/ml Li81 Fab, and subjected to analytical SEC. D, LINGO-1 ectodomain alone (50 µg in 50 µl), Li81Fab alone (50 µg in 50 µl) and a 1:1 mixture of LINGO-1 and Fab (100 µg in 100 µL) were cross-linked with 1 mM BS3. Aliquots of LINGO-1 and Fab (5 µg each) and 10 µg of the mixture with and without cross-linking were analyzed by SDS-PAGE under non-reducing conditions and stained with Coomassie brilliant blue. Samples with and
without BS\textsuperscript{3} treatment were run on the same gel. A-D each show representative data from at least 3 separate experiments. Biacore samples for each experiment were analyzed in triplicate. For SEC/light scattering studies, complex formation was assessed at the same, higher, and lower concentrations of LINGO-1 with Fab added at ratios from 10-fold lower to 10-fold over the concentration of LINGO-1 tested and there was no impact on the size of the complex as detected by SEC or light scattering. For cross-linking studies, different preparations of BS\textsuperscript{3} treated samples were used for each experiment.

**Fig. 4.** Li81 blocks LINGO-1 interface required for tetramerization. A, ribbon diagram showing the protomer structure of the LINGO-1/Li81 Fab complex. The two-module, kinked structure of LINGO-1 ectodomain is conserved in the complex. LINGO-1 structure with attached N-linked sugars is shown in yellow and the Fab structure in hot pink. Positions within the Ig domain of the RKH tripeptide and R386 (cyan) are denoted in the diagram. B, detailed view showing contacts between Fab CDR residues (black) and LINGO-1 (grey). LINGO-1 structure is in yellow and Fab structure in hot pink. H1, H2, and H3 and L1, L2, and L3 denote heavy (H) and light (L) chain residues and the specific CDRs. C, ribbon diagrams showing the tetramer structure of LINGO-1 (Mosyak et al., 2006) overlaid with the binding of Li81 Fab seen in the LINGO-1/LI81 Fab complex. LINGO-1 protomers are shown in yellow, green, cyan and grey, and Li81 Fab in hot pink. D, protomer structure highlighting contact sites within LINGO-1 used in LINGO-1 tetramer formation (blue), and Li81 binding (red). Overlapping epitopes are in purple.

**Fig. 5.** Possible assemblies of LINGO-1/Li81 Fab complex based on crystal symmetry. A, two models for the LINGO-1\textsubscript{2} Li81 Fab\textsubscript{2} based on contacts observed in the Li81 Fab LINGO-1 crystal structure. LINGO-1 is shown in yellow and the Fab in hot pink. The position of the RKH tripeptide (cyan) is denoted in the diagram. B, protomer structures
from two orientations highlighting LINGO-1-LINGO-1 contact sites utilized in the LINGO-1 tetramer (blue), the Model 1 interface (green), and the Model 2 interface (magenta).

**Fig. 6.** Li81 binding reduces the susceptibility of LINGO-1 Ig domain to proteolysis. LINGO-1/ Li81 Fab complex and LINGO-1 ectodomain with a corresponding concentration of control Fab were treated for 16 h at room temperature with 1:150, 1:300, and 1:600 enzyme:LINGO-1 ratios containing 4, 2, and 1 µg/ml endoproteinase Lys-C, respectively. Reactions were quenched with the addition of 0.2 mM leupeptin. Samples (4 µg/lane) were subjected to SDS-PAGE under reducing conditions and stained with Coomassie brilliant blue (left panel). Bands for the intact ectodomain (70 kDa) and 55 kDa fragments were quantified by densitometry (right panel). Studies used the endoproteinase Lys-C fragment of LINGO-1 and LINGO-1/ Li81 Fab complex shown in lanes 5 and 8 of Fig.1 respectively. Representative data from two separate experiments are shown using different preparations of protease treated samples.

**Fig. 7.** 1A7 binding as a probe of structural attributes of the LINGO-1/ Li81 Fab complex. A, ribbon diagram of LINGO-1 ectodomain showing contact sites for Li81 and 1A7 antibodies. B, Biacore competition analysis for the binding of Li81 Fab and 1A7 to LINGO-1 ectodomain. Li81 and 1A7 mAbs were immobilized on CM5 chips, and treated with 10 nM LINGO-1 ectodomain and then fixed concentrations of 1 µM 1A7 Fab, 25 nM and 1 µM 1A7 mAb. Binding curves were expressed as resonance units (RU) as a function of time. C, analytical SEC analysis of cross-linked tetramer of the LINGO-1 ectodomain alone and in the presence of 1A7 Fab. D, SEC/light scattering analysis of LINGO-1/ Li81 Fab complex alone and in the presence of 1A7 Fab. Studies in B, C, and D show representative data from at least 3 separate experiments. Biacore samples for
each experiment were analyzed in triplicate. Different preparations of cross-linked LINGO-1 with Li81 and 1A7 Fabs added were used for each experiment.

**Fig. 8.** Oligodendrocyte differentiation as a probe of functional activity of the LINGO-1/Li81 Fab complex. A, domain structure of full length LINGO-1 (a) and soluble LINGO-1-Fc containing the entire ectodomain (b), the LRR domain alone (c), the Ig domain alone (d), and the Ig domain alone in which the RKH tripeptide was mutated to EKV (e). B, the biological activity of soluble LINGO-1-Fc fusion constructs b-e from panel A and of cyclic peptides Acetylated-CLSPRKHC-NH₂ and Acetylated-CLSPEKVC-NH₂ in the OPC/DRG coculture assay. MBP levels were assessed by western blotting. Samples containing LINGO-1-Fc constructs b-e were from the same study and run on the same gel. The peptide samples were from a separate study. C, the biological activity of Li81 Fab, LINGO-1 ectodomain, and of the SEC purified LINGO-1/ Li81 Fab complex shown in Fig. 1, lanes 10, 5, and 8 in the OPC differentiation assay. Expression of MBP in OPC cultures was assessed by western blotting. Studies in B and C show representative data from at least 3 experiments using different preparations of cells for each study and in which each sample was analyzed with a dose-response.

**Fig. 9.** Analysis of the LINGO-1/Li81 Fab complex by EM. SEC purified LINGO-1/Li81 Fab complex that had been cross-linked with BS3 (3 µl at 5 µg/ml) was spotted on an EM grid and stained with uranyl formate. A, image of particles observed by EM and tabulated summary of the number of copies of each particle type detected in the image (left column) and in the entire micrograph (right column). The particles were classified into groups based on their shape. Shapes were interpreted from structural predictions shown in B and C that were generated by viewing the crystal structure at different orientations. B and C, 90° transpositions of Model 1 and 2 structures. For each structure
presented, a high resolution view (left) and its corresponding projection (right) are shown. Structures b and c, and e and f show 90° transpositions of the structures shown in a and d, respectively.
**TABLE 1**

Data collection and refinement statistics

**Data Collection**

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**Refinement**

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*a* Values in parenthesis are for highest resolution shell.  *b* TLS groups were used in the refinement.
Figure 1
Figure 2
Figure 3
Figure 5
Figure 6
Figure 8
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**Model 2**

![Image of model 2 structures]

**Model 1**

![Image of model 1 structures]

Figure 9