

Identification of a Functional Epitope of the Nogo Receptor by a Combinatorial Approach Using Ribosome Display

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The Nogo receptor (NgR) plays a central role in mediating growth-inhibitory activities of myelin-derived proteins, thereby severely limiting axonal regeneration after injury of the adult mammalian central nervous system (CNS). The inhibitory proteins Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) all bind to the extracellular leucine-rich repeat (LRR) domain of NgR, which provides a large molecular surface for protein–protein interactions. However, epitopes within the LRR domain of NgR for binding Nogo, MAG and OMgp have not yet been revealed. Here, we report an evolutionary approach based on the ribosome display technology for detecting regions involved in ligand binding. By applying this method of “affinity fingerprinting” to the NgR ligand binding domain we were able to detect a distinct region important for binding to Nogo. Several residues defining the structural epitope of NgR involved in interaction with Nogo were subsequently confirmed by alanine scanning mutagenesis.

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Introduction

The inability of mammalian central nervous system (CNS) axons to regenerate can partly be attributed to growth-inhibitory proteins present in CNS myelin.¹ Three myelin proteins that are capable of inducing growth cone collapse and inhibiting neurite outgrowth *in vitro* have been identified as Nogo,^{2,3} myelin-associated glycoprotein (MAG)^{4,5} and oligodendrocyte-myelin glycoprotein (OMgp).^{6,7} Nogo occurs in three alternative splicing forms, termed Nogo-A, Nogo-B and Nogo-C,² each of which share a common C-terminal portion composed of two transmembrane domains and a short 66 amino acid residue extracellular domain (Nogo-66). Nogo-66 has been shown to be at least partly responsible for

the inhibitory activity of Nogo-A.^{8,9} MAG is a transmembrane protein whose extracellular region is composed of five immunoglobulin (Ig)-like domains. OMgp is a glycosylphosphatidylinositol (GPI)-anchored receptor with a large leucine-rich repeat (LRR) domain of eight repeats, followed by a serine/threonine-rich region. Even though these three inhibitory proteins do not share structural similarities based on primary sequence, each binds to the Nogo receptor (NgR).^{7,8,10,11} Hence, NgR has emerged as a focal point of convergence for mediating growth-inhibitory activities and axonal plasticity.¹² NgR is a GPI-linked surface receptor containing 473 amino acid residues, including an amino-terminal signal sequence. The largest portion of the protein is composed of a LRR domain (Figure 1), which contains a LRR core region of 8.5 repeat motifs as well as N and C-terminal “capping regions”.⁸ A unique C-terminal region is located between the LRR domain and the GPI anchorage site. While the LRR domain of NgR, including the capping regions, has been reported to be responsible for binding to all three inhibitory myelin proteins,^{7,8,11} the C-terminal region is believed to be involved in signal transduction upon binding to p75.^{13–15} An additional modulator of this receptor complex has been identified as Lingo-1, another

Abbreviations used: BSA, bovine serum albumin; CNS, central nervous system; GPI, glycosylphosphatidylinositol; HIC, hydrophobic interaction chromatography; Ig, immunoglobulin; LRR, leucine-rich repeat; MAG, myelin-associated glycoprotein; NgR, Nogo receptor; OMgp, oligodendrocyte myelin glycoprotein; Trx, thioredoxin.

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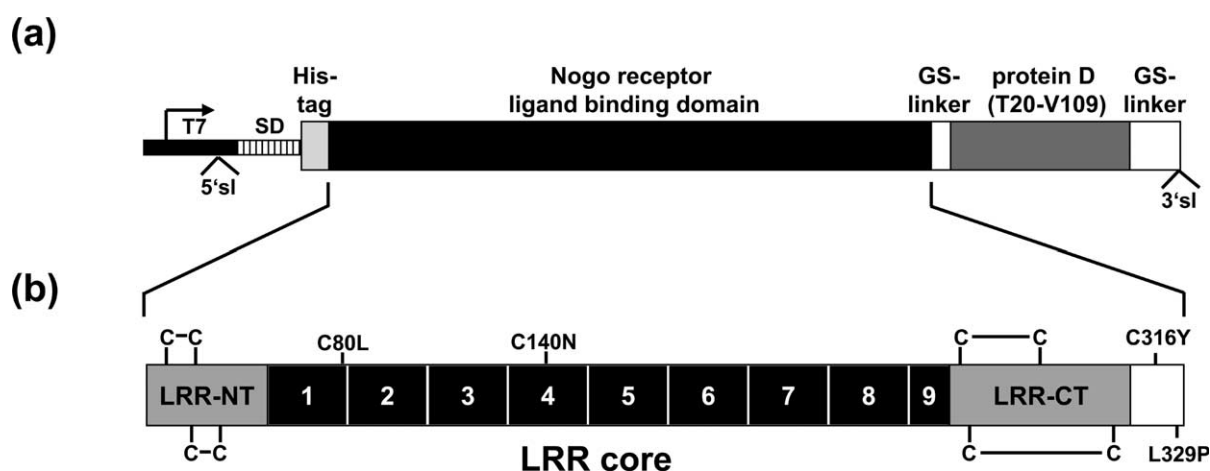


Figure 1. (a) Schematic representation of the ribosome display construct containing the Nogo receptor ligand binding domain (residues 24–331), including both the LRR core regions and the capping regions LRR-NT and LRR-CT. (b) Schematic and magnified view of the Nogo receptor ligand binding domain displayed on ribosomes. The conserved disulfide bridges in LRR-NT and LRR-CT as well as the individual repeats of the LRR core region and the positions of mutated residues are depicted. Mutation of the free cysteine residues in the LRR core region to the respective consensus residues has been shown to have no effect on binding activity.²⁷ The mutations in the far C-terminal overhang of 22 amino acid residues represent the changes of the macaque to the human receptor sequence.

neuronal LRR protein that interacts with NgR and p75.¹⁶ Upon binding to inhibitory myelin proteins, the resulting trimeric complex directs activation of the downstream RhoA signaling pathway.¹⁷

In spite of the rapid progress that has been made in identifying interaction partners of NgR, the molecular basis for the promiscuous interactions of NgR is still poorly understood. Furthermore, it is controversial whether the respective binding regions of Nogo-66, MAG and OMgp on NgR overlap.^{10,11} Therefore, a detailed understanding of the NgR epitopes responsible for ligand binding would provide highly valuable information for drug design in order to specifically target and block NgR interactions, thus offering a means for promoting axonal growth and functional recovery after CNS injury. The determination of the crystal structure of the ligand binding domain of NgR was an important step towards a future molecular understanding of the interaction.^{15,18} The typical elongated shape of the LRR domain provides a large molecular surface for interactions and a number of potential “binding hot spots”.

For the mapping of discontinuous functional epitopes, alanine scanning mutagenesis represents a very useful method.^{19,20} However, it is very laborious, even if accurate assumptions about the localization of interacting residues can be made, because each mutant has to be constructed, expressed and evaluated separately. More recently, combinatorial methods based on gene libraries and display systems, which establish a link between the displayed protein variants and their encoding DNA, have been successfully adapted to delineate residues involved in interactions,^{21–23} or even to fine-map epitopes.²⁴ Combinatorial methods present an important alternative to classical alanine scanning mutagenesis, because they allow for much

faster exploration of sequence space without the necessity of purifying and characterizing individual protein variants. However, many mammalian proteins are not amenable to bacterial expression and phage display.

Here, we report a novel combinatorial approach for the detection of discontinuous epitopes on large molecular surfaces, based on the ribosome display technology.^{25,26} We had previously found that NgR, even though not amenable to phage display due to its lack of soluble and functional expression in *Escherichia coli*, could be displayed in functional form on ribosomes and specifically bound to Nogo-66.²⁷ We used the combinatorial and evolutionary capabilities of ribosome display to obtain an “affinity fingerprint” of the molecular surface of NgR with respect to its interaction with Nogo-66. By subsequent alanine mutagenesis we were able to identify several residues of NgR that are directly involved in binding to Nogo-66 and define a functional epitope for Nogo-66 on the surface of NgR. We were also able to exclude other regions of NgR that have been proposed to serve as binding sites for myelin proteins. Our results suggest that ribosome display can be used as a general method for dissecting functionally important regions and residues on receptors, even for those that are extremely difficult to express in bacteria or to display on phage.

Results and Discussion

Ribosome display selections of Nogo receptor variants

The NgR is a member of the family of extracellular LRR domains,²⁸ the largest subfamily of LRR-type

proteins, which is defined by repeat lengths of 24–25 amino acid residues that are flanked by cysteine-rich capping regions. Many members of this class play important roles in a number of diseases, and they are involved in a large variety of different interactions. Nevertheless, structural and functional investigations of these domains have proven very difficult, as their heterologous expression in *E. coli* has often yielded only aggregated protein, and refolding attempts have had only very limited success.^{29,30} While attempts to produce soluble ligand binding domain of NgR by bacterial expression or by refolding from inclusion bodies failed, we surprisingly found that the ligand binding domain of the Nogo receptor is expressed in a soluble form in ternary ribosomal complexes, as formed in ribosome display.²⁷ Moreover, we were able to reconstitute the specific interaction between ribosomal complexes displaying NgR and Nogo-66 *in vitro*. In these complexes the receptor maintains a connection to the ribosome *via* a C-terminal tether and the peptidyl

tRNA. In addition, the mRNA also remains coupled to the ribosome, providing a link between genotype and phenotype of the displayed protein.

In addition to using the ribosome display technology (Figure 2) as a tool for *in vitro* protein evolution with the aim to evolve mutants with either favorable folding properties or increased affinity towards Nogo-66, we wished to gain insight into the molecular basis for the interaction between the two proteins. The initial gene library of randomized NgR genes was created by error-prone PCR of a gene fragment encoding the LRR portion of NgR and the capping regions LRR-NT and LRR-CT (residues 24–331). The two free cysteine residues of the LRR core region had previously been mutated to the respective consensus residues without affecting the binding to Nogo-66.²⁷ The theoretical diversity of the library was 2×10^{11} members with an experimentally determined average mutational load of nine base substitutions per gene (see Materials and Methods).

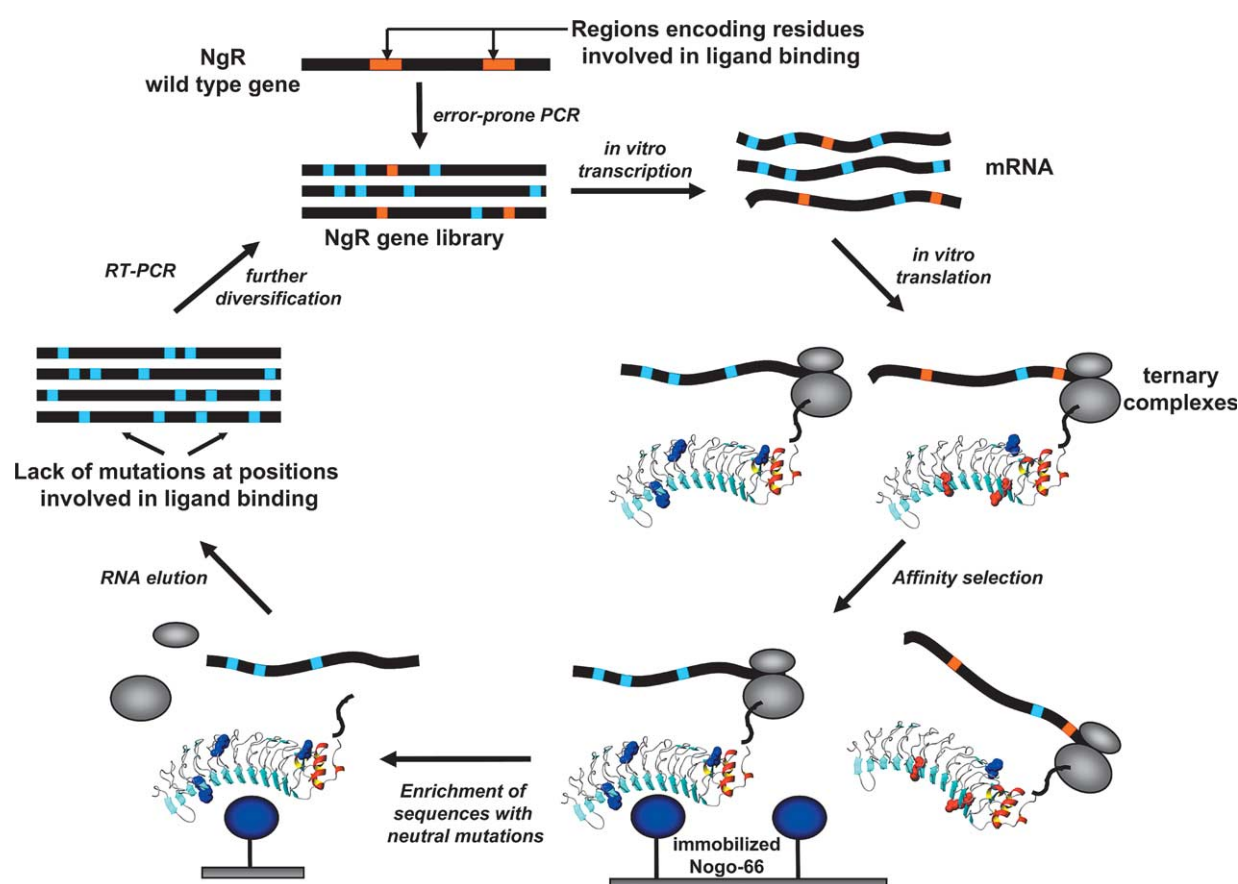


Figure 2. Principle of affinity fingerprinting of the Nogo receptor by ribosome display. A gene library of high diversity is generated by error-prone PCR, which contains mutations that are either “neutral” with respect to ligand binding (blue squares), or are directly involved in ligand recognition (red squares). Note that sequences with mutations severely interfering with folding would be lost. The ribosomal complexes stay intact as the mRNA has no stop codon. The LRR domain is followed by a C-terminal tether such that it can fold without interference with the ribosomal channel. In a standard ribosome display experiment, neutral (or very rare “improved”) mutations are enriched over deleterious ones by positive selection for binding. Importantly, the enriched pools are further diversified by errors occurring during PCR amplification or additional gene shuffling steps, leading to an overall high density of mutations accompanied by a continuous accumulation of neutral mutations at the expense of deleterious ones. The lower mutation frequencies in certain regions define the epitope.

Seven ribosome display selection cycles were carried out using Nogo-66 as an antigen, which was immobilized on paramagnetic beads as a C-terminal fusion protein to either lambda phage protein D or to *E. coli* thioredoxin (Figure 2). In order to prevent enrichment of mutants that attain binding affinity to either one of the fusion partners, the fusion proteins were alternated in each selection round. In addition, ribosomal complexes were pre-incubated with butyl-Sepharose beads prior to panning on Nogo-66. This hydrophobic interaction chromatography (HIC) step is expected to efficiently remove ribosomal complexes displaying misfolded proteins and potentially drive the selection process towards an enrichment of mutants with favorable folding properties.³¹

After three rounds of ribosome display selection, the resulting DNA pool was split into two parts, and DNA shuffling³² was performed on one part of the pool, which was again shuffled after the sixth selection round while the other part of the pool was carried on to the seventh round without performing DNA shuffling. The amount of PCR product obtained after RT-PCR in the last step of each selection cycle reflects approximately the amount of mRNA recovered from the panning step and thus allows monitoring of the enrichment process. The direct comparison of band intensities between the library pools and displayed wild-type NgR, used as a control in each selection cycle, showed that, even after seven rounds of selection, the amount of PCR product from the randomized library was still slightly less than for the wild-type protein (data not shown). This indicates that under the given low-stringency selection conditions no members with properties superior to wild-type NgR had been selected. Nevertheless, binding to Nogo-66 still seemed to be specific, as very little PCR product was detected upon panning on beads coated with either fusion protein alone or with protein G (Dyna-

Biotech). A more quantitative analysis by radioimmunoassay (RIA) showed that the binding properties of the selected pools remained essentially unchanged throughout the selection and affinity for Nogo-66 was retained (Figure 3). Thus, the majority of members in a selected pool apparently still contained the sequence information determining the ability to interact with Nogo-66.

Sequence analysis

Upon DNA sequence analysis of single members obtained after seven cycles of ribosome display, we observed that the average mutational load had significantly increased throughout the selection process from nine base mutations per gene in the initial library to 16 and 17 mutations per gene (1.5–2.0% of bp) in the non-shuffled and the shuffled pool, respectively. Thus, DNA shuffling neither led to a significant increase of the overall mutation rate nor to a change of the overall binding properties of the selected pool. The introduction of DNA shuffling steps was therefore not essential for the outcome of the performed selection, and the observed strong increase of the mutational load presumably resulted almost exclusively from the high number of PCR cycles performed during selection and the intrinsic error rate of the Taq polymerase.³³ Analyzed sequences showed a high degree of diversity, confirming that no preferential selection of single members had occurred. However, upon amino acid sequence alignment we observed that amino acid mutations were not evenly distributed over the NgR sequence. We therefore hypothesized that this uneven distribution of the mutation rate might reflect the functional importance of different regions with respect to the binding of Nogo-66 (Figure 2). Most of the occurring mutations might thus represent “neutral” mutations that accumulated during the

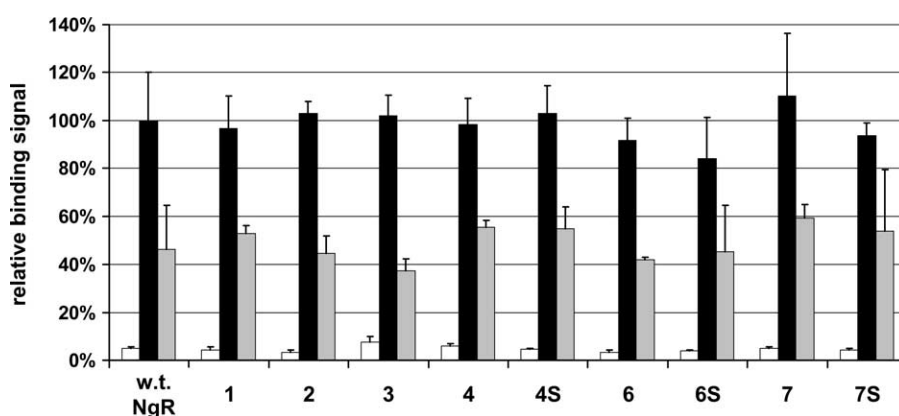


Figure 3. Radioimmunoassays of the library pools after different rounds of ribosome display selection in comparison with wild-type NgR. S refers to the pool shuffled after the third and the sixth selection round. Pooled RNAs were translated *in vitro* in the presence of [³⁵S]methionine. After purification of the ribosomal complexes by ultracentrifugation through a sucrose cushion, binding to the ligand Nogo-66 was examined. White bars represent background binding to paramagnetic beads coated with protein G. Black bars represent binding to pD-Nogo-66 and grey bars inhibition of binding by addition of 4 μ M pD-Nogo-66 as competitor. Each bar represents the average of two measurements.

randomization process and which do not have a negative effect on binding to Nogo. In contrast, replacements of amino acid side-chains that are involved in the interaction will reduce the free energy of binding and the respective protein variants will more likely be sorted out during the affinity selection step. The degree of conservation within certain regions of the protein and of specific amino acid residues is thus indicative for an involvement in binding.

In order to identify regions displaying higher degrees of conservation, we took advantage of the modular structure of the LRR domain, which is characterized by the two flanking regions (NT and CT), the regular arrangement of eight repeats of 24–25 amino acid residues length and an additional “half-repeat” only composed of the β -strand region on the concave side of the LRR domain (Figure 4(a)). By calculating the amino acid mutation frequency in each of the subdomains, regions of lower mutation frequencies can be assigned to the central LRR region and LRR-CT (Figure 4(b)–(d)). The statistics are based on 127 sequences derived from both sequence pools (one having undergone shuffling and the other not) after seven rounds of selection. The mutation rates, as well as the overall trends observed, were highly similar in both pools. The sequence data were therefore combined in order to achieve higher statistical significance.

LRR proteins are characterized by an extensive accessible surface area providing a high number of

potential interaction sites with different molecular partners.¹⁸ In most cases, protein–protein interactions are mediated by the concave surface of the LRR, while the convex side of LRR proteins is often defined by large surface areas containing many polar residues, but residues of functional importance could also be assigned to regions outside of the concave face.³⁴ In order to restrict the analysis to the surface residues of the NgR domain, we calculated the relative accessible surface area (RSA) of the side-chains for each residue. Surface residues were defined as residues with RSA > 5% and we distinguished between the concave and the convex side (Figure 4(c) and (d)). On the convex side, the mutation frequencies of repeat motifs are evenly distributed across the whole domain. In contrast, mutation frequencies on the concave side show a distinct pattern with all residues on the concave side of LRR5 being almost completely conserved. The neighboring repeats LRR3 and LRR4 exhibit comparably low mutation frequencies, whereas residues in LRR2 and LRR8 are highly variable. Intriguingly, residues of LRR4 and LRR5 have been proposed to be part of a potential binding “hot-spot”¹⁵ formed by two exposed tyrosine residues, which are flanked by a negatively charged patch of aspartate residues and two exposed histidine residues.

The statistical significance is determined by the mutational load and the total number of analyzed sequences. While the limited set of analyzed

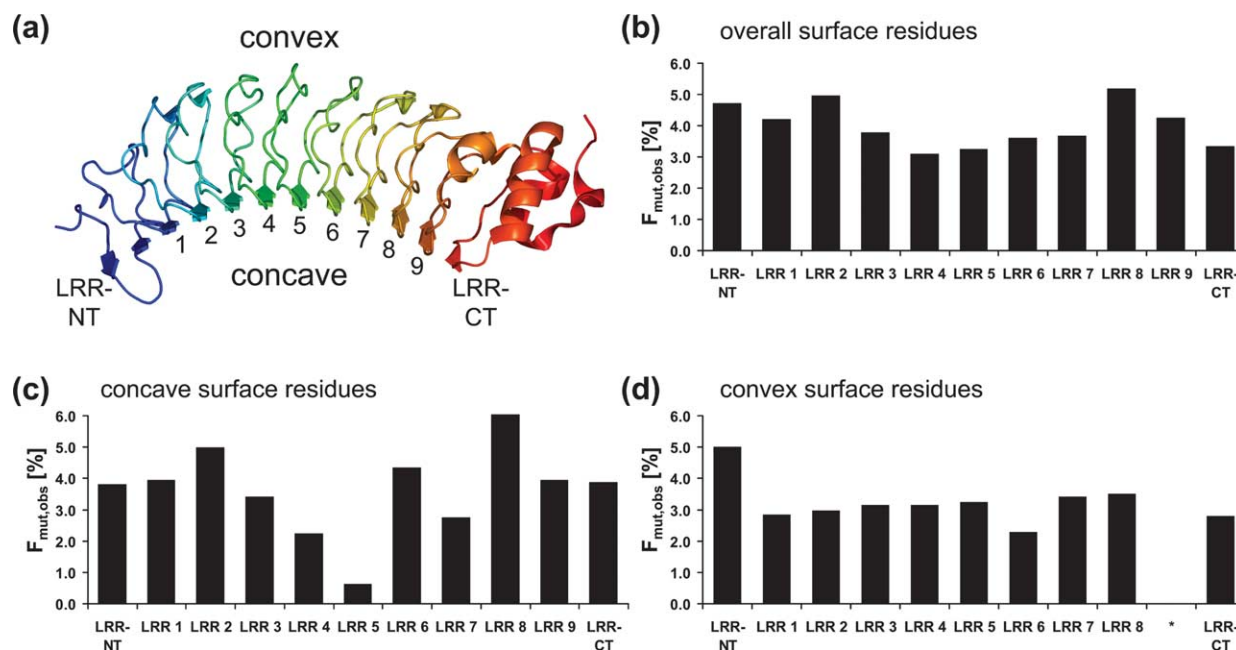


Figure 4. Distribution of mutation frequencies in the NgR subdomains after seven rounds of ribosome display selection on Nogo-66. (a) Ribbon structure of the NgR ligand binding domain.¹⁵ (b) Based on 127 sequences, mutation frequencies across the primary sequences were calculated after grouping them into the respective subdomains. (c) Mutation frequencies of surface residues on the concave face of NgR. (d) Mutation frequencies of surface residues on the convex face of NgR. Surface residues are defined as residues with relative side-chain accessibilities larger than 5%. Residues xLxxLxxLxLxN of the LRR motif²⁸ are defined as residues of the concave face and remaining residues of the LRR motif as convex face residues. LRR9 represents a “half-repeat” not contributing any residues to the convex face of the molecule (asterisk).

sequences does not necessarily provide a high enough statistical significance to make reliable statements on the single residue level, residues of functional significance would be expected to be visible as distinct clusters on the surface of NgR. Unfortunately, an even probability of mutating a given amino acid into other amino acids by "random" gene diversification is difficult to achieve for two reasons. Due to the degeneracy of the genetic code certain DNA mutations do not result in amino acid changes. In addition, errors resulting from gene diversification by error-prone PCR and the subsequent PCR amplifications are characterized by a strong bias for base transitions compared with base transversions,³⁵ thereby affecting different codons to a different extent. Therefore, in order to normalize the observed mutation frequencies, theoretical expectation values for mutating any amino acid residue at a given position were calculated for each codon of the genetic code as described in Supplementary protocols, using the overall transition and transversion rates observed across all sequences in this experiment.

The conservation score for each position is obtained by dividing the observed mutation frequency by the expectation value of the respective codon (Figure 5(a)). The resulting conservation scores were projected onto the surface structure of NgR (Figure 5(b)). The convex surface does not display extended clusters of conserved surface residues, except for a small region of charged residues adjacent to LRR-CT. Other conserved residues include mostly proline, forming small kinks in the center of the extended loop structure. In contrast, an extended region of high conservation can be identified in the region of LRR3-5 on the concave side, including all residues of LRR5 and the neighboring residues H133 and T134 in LRR4, as well as D163 and, with a somewhat lower degree of conservation, D111 and D114 in LRR3 and D138 in LRR4. These aspartate residues form an acidic cavity, which has already been proposed as a potential binding hot spot.¹⁵ Glutamine side-chains flanking this acidic patch (Q162 as well as Q211 in LRR7) also show a high degree of conservation. Residues of high variability also accumulate in certain regions, for example in the N-terminal area

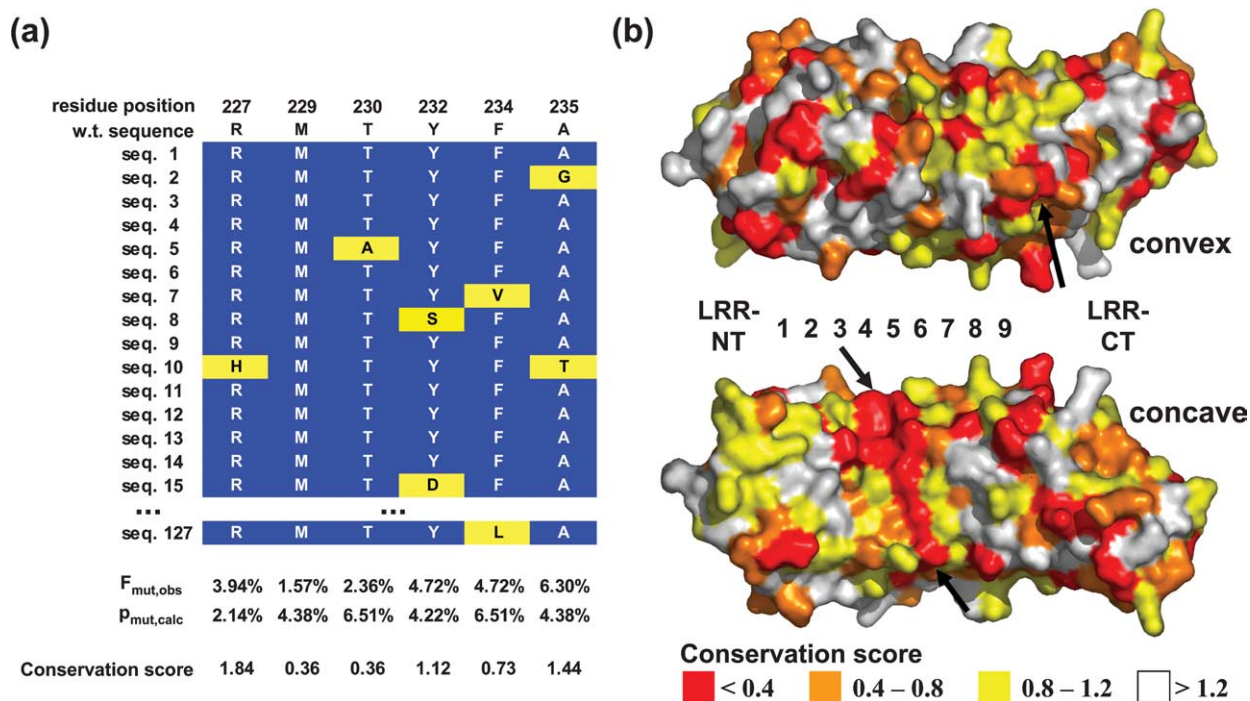


Figure 5. Distribution of conserved and variable amino acid residues after seven rounds of ribosome display. (a) The conservation score for each residue at a given position was calculated by dividing the observed mutation frequency at each position ($F_{mut,obs}$) by the expectation value for the respective codon ($p_{mut,calc}$) calculated from the sum of all mutations in the final sequences. A conservation score of 1.0 corresponds to a mutation frequency of statistical average, while a conservation score below 1.0 corresponds to a codon that is conserved more than average. As an example, 15 sequences of 127 are shown, in the region 227–235. Residues identical to the wild-type are shown in blue, mutated residues in yellow. For each residue, the expectation value $p_{mut,calc}$ and the observed frequency $F_{mut,obs}$ is shown, as is their ratio. (b) The conservation scores are projected onto the surface of NgR.¹⁵ Red and orange patches correspond to residues of high conservation, yellow and white patches represent residues of high variability. Coloring thresholds are indicated below. The positions of repeat subdomains are indicated in the middle panel. Extended clusters of conserved residues are indicated by arrows. Pictures were created using Pymol†.

† DeLano, W. L. (2002). The Pymol Molecular Graphics System. on World Wide Web <http://www.pymol.org>

defined by LRR-NT, LRR1 and LRR2 as well as in LRR7 and LRR8.

Residues involved in defining the binding site for Nogo

The identification of epitopes on NgR that are responsible for mediating the interaction with Nogo and other ligands is of high importance for the design and development of antagonists neutralizing the inhibitory effects of these myelin proteins. In order to test whether the observed statistical distributions indeed reflect a well-defined interaction site of NgR for Nogo-66, alanine scanning of several surface regions with a focus on the conserved residues in LRR3 to LRR5 was performed. The use of ribosome display offers the additional advantage of rapid production of many defined NgR mutants and a rapid evaluation of relative binding signals, which would be a far more laborious task if eukaryotic expression systems need to be employed. By using the ribosome display-based *in vitro* assay, we had previously detected weak binding activity for LRR-CT alone, but the major contribution to ligand interaction was provided by the LRR core region.

Interactions of other LRR proteins with their ligands have been shown to involve a rather large number of residues forming discontinuous epitopes on the LRR surface.^{36,37} We were therefore concerned that single amino acid replacements by Ala would only cause small differences in the binding free energy and the resulting changes of the absolute binding signal might be below the detection limit. Thus, a large set of single and multiple alanine mutants of NgR were generated by site-directed mutagenesis, *in vitro* transcribed and studied by RIA analysis (Figure 6(a)). The amounts of expressed NgR variants were investigated by SDS gel electrophoresis of the respective *in vitro* translations (Figure 6(b)) and by measuring the amount of radioactively labeled protein displayed on ribosomes (Figure 6(c)). All mutants, as well as the wild-type protein, were expressed and displayed to the same extent. The comparison of relative binding signals was therefore expected to serve as a proper measure of the relative binding activities towards Nogo-66.

Relative binding signals of an ensemble of mutants in comparison with wild-type NgR are shown in Figure 6(d). Several additional substitutions were made in order to verify the predictions resulting from the statistical analysis. The exposed side-chains of Y34, F63 and W87 form an extended hydrophobic surface area in the N-terminal region of the concave side, which forms a putative site for ligand binding.¹⁵ These three residues, however, displayed a high degree of variability after selection, indicating a lack of involvement in Nogo binding. Indeed, double alanine mutants as well as a triple mutant do not give rise to a decrease of the binding signal. Another extended region of highly randomized residues covers many positions in

LRR7 and LRR8. No signal decrease can be detected for any substitution in this region either, except for a very slight decrease for a quadruple mutant comprising four exposed residues of LRR8. Several multiple alanine substitutions within the only pronounced "conserved" surface region on the convex surface (see above) (defined by R196, R199, H202, H220, R223, R227 and R250) also did not result in changes of the detected signals.

The most extensive analysis was performed on the extended conserved region on the concave face defined by LRR3 to LRR6. Initially, we tested multiple alanine mutations of the most conserved residues in LRR4 and LRR5. Even though we did not detect loss of binding upon mutation of the exposed tyrosine residues Y157 and Y158, a combined mutation of H133 and T134 in LRR4 caused a moderate decrease of binding, which became more pronounced by additional substitutions of neighboring residues. The most obvious decrease of binding activity was, however, observed in a double mutant Q162A-D163A. The same is true, though to a lesser extent, for the respective single mutants. We therefore reasoned that the acidic cavity between LRR3 and LRR5 might indeed play a major role in defining the binding site for Nogo-66 and thereby subsequently replaced aspartate residues D111 and D114 as well as residues flanking this region. The reduction of binding indeed becomes more pronounced upon successive replacement of all aspartate residues. Additional mutations of the flanking glutamine residues Q162 and Q211 lead to a further decrease of signal in an additive manner to approximately 20% of the wild-type signal. Another residue adjacent to this region, which was found to be conserved in the statistical analysis, is residue D205, whose mutation to Ala also had an impact on binding. However, we have not yet produced double mutants including D205 to unambiguously support this finding.

Implications for the mode of interaction of NgR

In summary, we were able to identify several residues of the Nogo receptor directly involved in binding to Nogo-66. Several studies have investigated the influence of different regions of NgR on ligand binding by studying deletion mutants in cell-based assays, leading to controversial results.^{7,11,13} An epitope could not be identified in these studies. While weak binding of LRR-CT alone was reported in one study,⁷ no detection of binding was possible for any mutant with two adjacent repeat motifs deleted, including deletion of LRR-CT.¹³ However, deletion of whole subdomains in cell-based assays might have additional side-effects, such as inhibition of NgR multimerization on the cell-surface. In contrast, the *in vitro* assay in a ribosome display format most likely reflects interaction of 1:1 stoichiometry.

The major contribution to binding activity was found in the acidic cavity defined by a cluster of four aspartate residues. Of these four aspartate

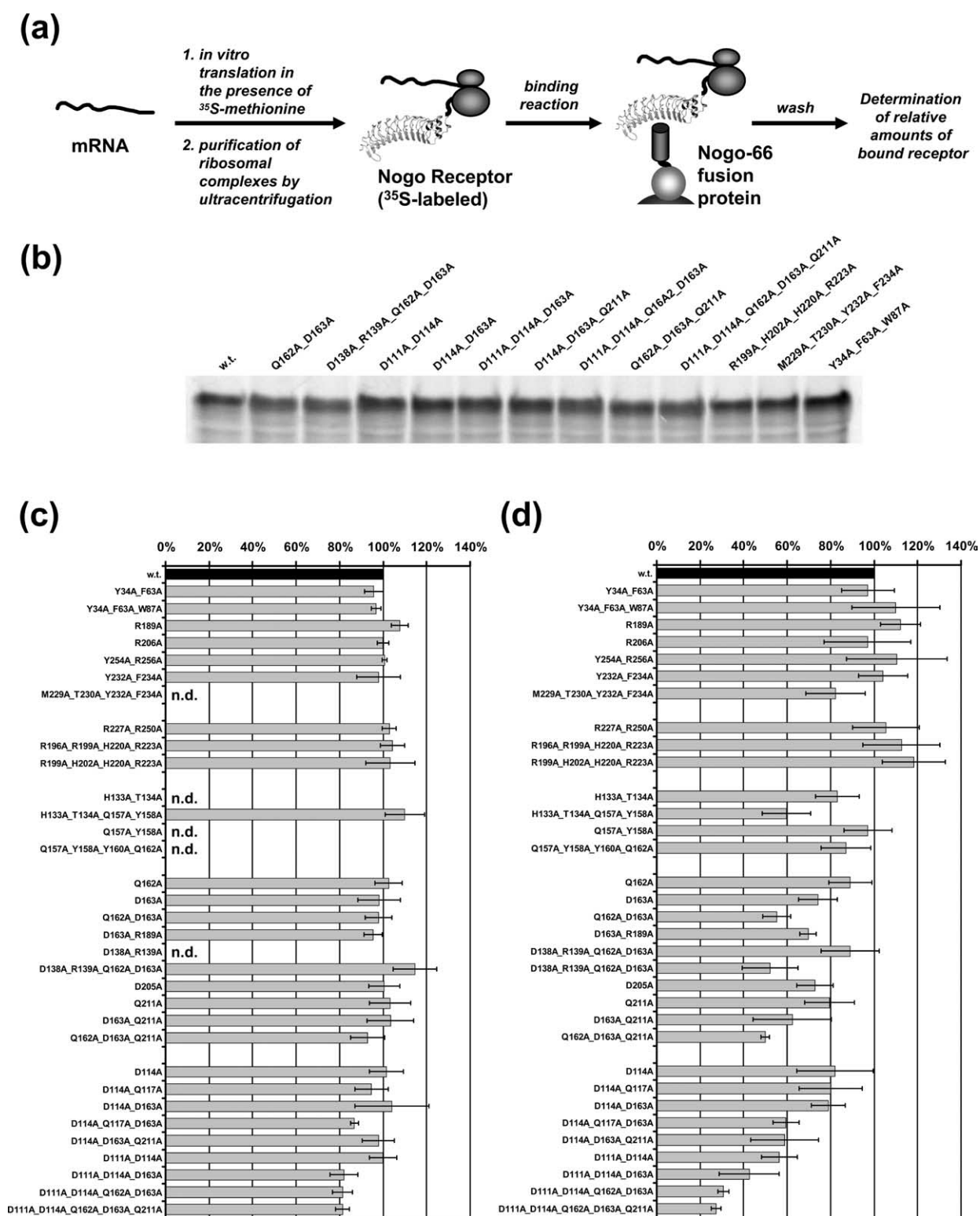


Figure 6. (a) Schematic representation of the radioimmunoassay used for investigating alanine mutants of NgR. (b) SDS gel electrophoresis of *in vitro* translations. Soluble fractions used for RIA were subjected to SDS gel electrophoresis and analyzed by autoradiography. Samples of mutants exhibiting different binding behavior (see Figure 5(d)) are shown. (c) In parallel to the binding reaction, relative amounts of translated protein variants were assayed by measurement of the relative amounts of [^{35}S] methionine incorporated in ternary complexes. After purification of ribosomal complexes by ultracentrifugation, radioactivity was measured by liquid scintillation counting. (d) Relative binding signals of Ala-mutants of NgR in comparison with the wild-type protein investigated by RIA. Bars represent average values from three independent experiments, each performed in triplicates. The upper group displays mutants whose substituted side-chains correspond to positions of low conservation after selection (except Y254). The second group corresponds to residues of a "conserved" cluster on the convex face of NgR, whereas the three lower groups show an ensemble of alanine substitutions in the extended conserved cluster of LRR3-LRR6.

residues, we detected decreased binding upon mutation of D111, D114 and D163, while point mutation of D138 showed no effect. Mutation of the flanking glutamine residues Q162 and Q211 lead to additional decreases of binding signals. Other residues adjacent to this region (H133, T134 and D205) were also found to affect the interaction (Figure 7). All observed effects on binding activity were found to be additive in double and multiple mutants. This is especially important because a quantitative determination of the binding constant is not possible in our assay for technical reasons.²⁷

Although the identified side-chains obviously constitute the major interaction site, minor

contributions also seem to reside in other regions of NgR, including the C-terminal capping domain.^{7,27} Very recently, Li *et al.*³⁸ reported the epitope of a monoclonal antibody 7E11, which efficiently inhibits the interaction of rat NgR with Nogo-66, thereby providing another experimental indication of which region of NgR the interaction site might be located. However, it remains unclear whether these residues are directly involved in the interaction with Nogo-66, MAG and OMgp or whether the observed effects are due to steric hindrance or induced conformational changes. In particular, the area affected by steric hindrance upon binding of an IgG antibody easily spans a region of two adjacent repeats. The contact residues on NgR that are responsible for the interaction with 7E11 were found to reside in the turn region of LRR3 (positions 114–119) including residue D114 which we identified. Our combinatorial approach and alanine scanning data are thus in agreement with these results and provide the first direct and detailed analysis of the Nogo-epitope on NgR.

Besides Nogo, NgR binds to other growth inhibitory proteins of the CNS myelin proteins MAG and OMgp. Epitopes for neither OMgp nor MAG on NgR have been defined and reports of cross-competition experiments of MAG with Nogo-66 on NgR have yielded conflicting results.^{10,11} Notably, 7E11 inhibits the interaction of all three myelin proteins with NgR,³⁸ suggesting that the binding sites might be either overlapping or in close proximity to each other. Our analysis has included residues in several distinct regions on the concave face of NgR. Structural analysis has revealed two additional extended hydrophobic patches on the concave face that constitute potential sites for ligand binding.¹⁵ Interestingly, we have neither detected an influence on binding to Nogo-66 of exposed aromatic residues located in the extended hydrophobic patch in the N-terminal region of NgR, nor of residues defining the hydrophobic region between LRR5 and LRR8. This leaves two distinct regions encompassing a high potential for ligand binding in close proximity to the Nogo-66 interaction site, which might thus be involved in binding to MAG and OMgp.

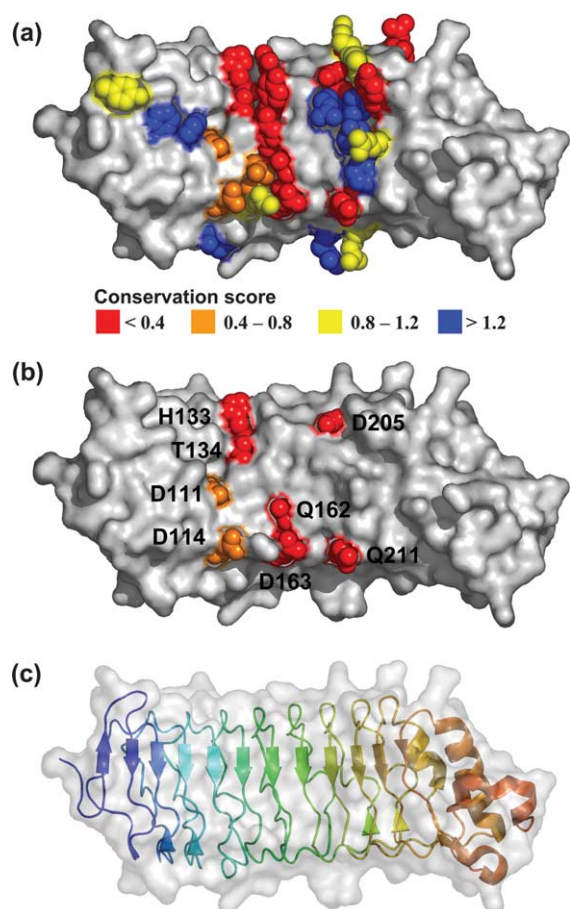


Figure 7. Residues contributing to the interaction of NgR with Nogo-66. (a) Positions of deliberately introduced alanine mutations investigated by RIA analysis. The coloring scheme corresponds to the respective conservation scores after ribosome display selection. (b) Binding to Nogo was affected upon replacement of the depicted side-chains. The most severe effects on binding signal are observed upon mutation of the three indicated aspartate side-chains that are part of the acidic cavity in the region of LRR3 to LRR5. None of the investigated residues that displayed a high degree of variability after selection showed any effect on binding, including residues of the hydrophobic surface patches in the N-terminal region and LRR8. (c) Ribbon representation of NgR in the same orientation.

“Affinity fingerprinting” as a tool for investigating protein–protein interactions

We present here a novel combinatorial method for the detection of discontinuous epitopes on proteins based on the ribosome display technology. Other combinatorial methods employing randomly mutagenized proteins for the detection of interacting residues and mapping of epitopes have been reported.^{21–23} These methods are based on phage display^{21,22} or yeast surface display²³ and either rely on several cycles of negative selection or high throughput screening in order to identify residues whose mutation leads to a loss of binding. In

contrast, our approach employs continuous rounds of positive selection in order to identify regions or even single residues of high conservation and functional importance. In this respect, our approach is similar to "alanine shotgun scanning", a highly versatile tool for the fine mapping of epitopes.²⁴ Instead of random mutagenesis, shotgun scanning uses oligonucleotides with degenerate codons to construct libraries of protein variants that carry preferentially alanine or the respective wild-type residue at defined positions. The protein variants are then displayed on phage and subjected to several rounds of positive selection for binding. At each varied position the ratio of alanine to wild-type residues is subsequently determined. The resulting statistical distribution at a given position has been shown to correlate with changes of the binding free energy upon mutating this position to alanine.

Affinity fingerprinting does not represent an alternative to shotgun scanning, but the two methods can complement one another. While shotgun scanning can reveal detailed maps of energetic contributions of single residues to the interaction, the investigator needs to have a rather precise model of the epitope's location, because the practically achievable library size limits the number of analyzable positions to 20–30 residues. Even though several libraries might be handled in parallel, the use of degenerate oligonucleotides makes library construction extremely laborious if non-contiguous stretches of sequence are to be investigated. In the case of repeat proteins, such as LRR-based receptors, library construction by using degenerate oligonucleotides is extremely difficult to implement.

By contrast, we scanned the entire sequence of a large protein domain for potential sites of interaction. The number of residues counted as "surface" was 196. However, this could only be achieved at the expense of a simultaneous loss of information. Even by analyzing very large sets of sequences, a direct correlation between the energetic contribution of a given residue to binding cannot be made, because not every residue is replaced by an equal set of different residues *via* single nucleotide substitutions (see Materials and Methods). Moreover, a crude simplification is made by neglecting the type of mutation for a given amino acid residue. Nevertheless, we were able to identify regions and even single residues contributing to the interaction within a highly discontinuous epitope with rather high accuracy.

The knowledge of the 3D structure of NgR¹⁵ was advantageous for correlating the statistical data with the spatial arrangement of surface residues. Even though structural knowledge is not a prerequisite, the interpretation of the statistical information might in some cases become ambiguous if no structural information is available. In the present case, however, the regular arrangement of protein motifs with a well-defined fold would have allowed a rather accurate prediction of adjacent surface residues even without prior structural information.

The advantage of using ribosome display in such a context is many-fold. Phage display is based on the expression of protein variants in bacterial host cells. However, many mammalian proteins are highly aggregation-prone when expressed in bacteria and a functional display on phages is often not possible. While NgR failed to be displayed on phages,²⁷ we were able to functionally display NgR using ribosome display. This observation has implications for another complication often occurring during statistical data evaluation of selected sequences. It is often very difficult to distinguish whether a given residue is conserved because of its structural or its functional relevance. This difficulty is likely to be more pronounced in phage-display or yeast-display-based methods, because of the more stringent requirements for proper folding and transport of the displayed protein.³⁹ In contrast, the requirements for protein folding and stability are lower in ribosome display for two reasons. First, the ribosome and the connected mRNA act as solubility-enhancing fusion partners and might even sterically block protein aggregation.^{27,31} Moreover, selection is carried out at low temperatures in order to stabilize the ternary ribosomal complexes. Thus, unless additional external selection pressures are introduced,⁴⁰ the selection process is likely to be more strongly directed towards functional activity of the displayed protein, rather than being biased by its expression levels and folding properties.

Another advantage of the ribosome display technology is its powerful built-in gene diversification process. A prerequisite for performing reliable statistical evaluations based on a limited set of sequences is a high density of mutations in a set of variants with retained function. This can be achieved by an iterative process of diversification and positive selection for binding. In ribosome display, this iterative process is automatically guaranteed, because mutations occur at low frequency, albeit continuously, during PCR amplification.

In summary, by employing the ribosome display technology to the ligand binding domain of NgR we were not only able to investigate the binding to Nogo-66 outside of its cellular context, but also to use the combinatorial and evolutionary aspects of this technique to identify the regions and key residues responsible for the binding activity. These findings might pave the way for specifically targeting this surface region to evolve antagonists that can neutralize the inhibitory activities of myelin proteins acting on NgR. Moreover, a soluble form of the NgR ligand binding domain itself has been shown to act as an efficient antagonist of these interactions and to promote axonal sprouting after spinal cord injury *in vivo*.⁴¹ Thus, detailed information about the key epitope also offers a framework for targeting this region by means of protein engineering in order to create soluble NgR variants with higher affinity for their natural substrates.

Materials and Methods

Library generation

Randomized NgR gene libraries were generated by error-prone PCR amplification³⁵ of the pRDV2-NRC[−] template²⁷ at a concentration of 1 fmol/μl. Error-prone PCR was performed under standard conditions using Taq polymerase, 200 μM of each dNTP and 20 cycles of amplification. In order to control the mutation rate dNTP analogs were added at different concentrations. 6-(2-deoxy-β-D-ribofuranosyl-3,4-dihydro-8H-pyrimidin-4,5-c[1,2]oxazin-7-one-triphosphate) (dPTP) and 8-oxo-2'-deoxyguanosine (8-oxo-dGTP) were added at 5 μM, 10 μM and 20 μM, resulting in three different libraries with average mutational loads of four, ten and 15 mutations per NgR gene, respectively. After PCR purification, aliquots of each library corresponding to a theoretical diversity of 2×10^{11} members were used for *in vitro* transcription and subsequent RNA purification as described.⁴² Aliquots of the purified RNA were mixed in equal amounts and used for the subsequent ribosome display selection.

In vitro selection by ribosome display

In vitro translations were carried out as described.⁴² After 12 minutes at 37 °C, the translation reaction was stopped by fivefold dilution with ice-cold buffer WBK₅₀₀T (50 mM Tris-acetate (pH 7.5), 150 mM NaCl, 50 mM magnesium acetate, 0.5 M KCl, 0.1% Tween-20) containing 2.5 mg/ml heparin. After centrifugation at 11,000g for five minutes, the ternary complexes of RNA, ribosomes and protein were further purified by ultracentrifugation through a sucrose cushion. For this purpose, 500 μl supernatant was applied to 2.5 ml 35% (w/v) sucrose in WBKT (50 mM Tris-acetate (pH 7.5), 150 mM NaCl, 50 mM magnesium acetate, 250 mM KCl, 0.1% Tween-20), and ultracentrifugation was carried out at 100,000g for 20 minutes. The pellet was resuspended in WBKT containing increasing concentrations of KCl (from 1.5 M in the first selection round to 3 M in the sixth selection round). The solution was incubated with 100 μl butyl-Sepharose for one hour at 4 °C and the supernatant containing the ternary complexes was then applied to a gel filtration column to exchange the buffer to WBKT.⁴³ Selection of binders to Nogo-66 was performed for one hour at 4 °C by adding half of the eluted ternary complexes to 10⁷ paramagnetic beads coated with either pD-Nogo-66 or Trx-Nogo-66 in the presence of BSA (final concentration 5 mg/ml) and *Saccharomyces cerevisiae* RNA (final concentration 250 μg/ml). After washing, RNA elution and purification, reverse transcription (RT) was performed with the ThermoScript-Kit (Invitrogen) and primer rdN2_L (5'-GGCGCGGTTGCGGTACCCATAG-3'), including a RNaseH digestion step after the RT reaction. PCR was carried out with oligonucleotides EP_f (5'-CACGGATCTGGTTCATGGG-3') and rdN2_L, which only amplify the NgR coding region. The pool of gene fragments was religated into pRDV2, and ligation products were PCR-amplified for the next round of selection.

After the third round, the selected pool was split, and DNA shuffling was carried out for one part of the pool. One μg of purified PCR-product obtained after reverse transcription and PCR of the selected pool was digested with 0.2 unit of RQ1-DNase (Promega) in 100 μl for ten minutes at room temperature (RT). DNA fragments of

100–200 bp length were extracted after separation on a 1.5% (w/v) agarose gel. Assembly PCR was performed as described.⁴² After purification, the full-length product was amplified by PCR with sequence-specific primers EP_f and EP_NR_b (5'-CCACCGGATCCCCCAGCGGC-3') and recloned into pRDV2-NRC[−]. The shuffled and the non-shuffled pool were selected in parallel to the seventh round. The pool shuffled after the third round of selection was again DNA shuffled after the sixth round while the non-shuffled pool was carried on to the seventh round without performing a DNA shuffling step.

RIA analysis

RIA analysis of selected pools and of single mutants was performed in the ribosome display format as described above, except that [³⁵S]methionine was added during *in vitro* translation, and pre-incubation on butyl-Sepharose beads was omitted. Binding reactions were performed for one hour at 4 °C. Before elution of labeled protein with 8 M urea, beads were washed four times with 600 μl WBKT over a period of 30 minutes. Eluted radioactivity was quantified by liquid scintillation counting, and the binding signals were normalized to signals obtained from parallel experiments with ternary complexes displaying NgR wild-type protein.

Statistical analysis

Based on 127 sequences derived from randomly picked clones of both pools after seven rounds of ribosome display selection, mutation frequencies were calculated as follows (for details, see Supplementary protocols): The probability for mutating an amino acid residue at a given position depends on the degeneracy of the genetic code and the bias introduced by the gene diversification process. For example, transitions are favored over transversions, and transitions of AT base-pairs are usually favored over those of GC base-pairs, thereby affecting different codons to a different extent.^{35,44}

The probabilities for all base mutations (two transitions and four transversions) were experimentally determined from the 127 sequences after seven selection rounds. Mutation expectation values could thus be assigned for each codon, calculated as described in Supplementary protocols. Observed mutational frequencies at a given position were then normalized by dividing them by the mutation expectation value of their respective codon to obtain a conservation score at each position in the sequence. Residues were defined as surface residues if the relative side-chain accessibility was larger than 5%. Accessibility calculations were performed using NACCESS[†] with a van der Waals radius of 1.4 Å and the coordinates of 1OZN.¹⁵

Gel electrophoresis of *in vitro* translation products

Aliquots from *in vitro* translations used for RIA analysis (35 μl *in vitro* translation mix) were centrifuged at 21,000g for five minutes at 4 °C to remove insoluble components and treated as described.²⁷ Samples were subjected to reducing SDS gel electrophoresis on a 12% (w/v)

[†] <http://wolf.bms.umist.ac.uk/naccess/>

polyacrylamide gel and checked by [³⁵S]methionine autoradiography on a Storm Phosphorimager (Molecular Dynamics).

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2005.06.073](https://doi.org/10.1016/j.jmb.2005.06.073)

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