Characterization of the Interaction of a Recombinant Soluble Neuroligin-1 with Neurexin-1β*

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Neuroligins, proteins of the α/β-hydrolase fold family, are found as postsynaptic transmembrane proteins whose extracellular domain associates with presynaptic partners, proteins of the neurexin family. To characterize the molecular basis of neuroligin interaction with neurexin-β, we expressed five soluble and exportable forms of neuroligin-1 from recombinant DNA sources, by truncating the protein before the transmembrane span near its carboxyl terminus. The extracellular domain of functional neuroligin-1 associates as a dimer when analyzed by sedimentation equilibrium. By surface plasmon resonance, we established that soluble neuroligins bind neurexin-1β, but the homologous α/β-hydrolase fold protein, acetylcholinesterase, failed to associate with the neurexins. Neuroligin-1 has a unique N-linked glycosylation pattern in the neuroligin family, and glycosylation and its processing modify neuroligin activity. Incomplete processing of the protein and enzymatic removal of the oligosaccharides chain or the terminal sialic acids from neuroligin-1 enhance its activity, whereas deglycosylation of neuroligin-1 did not alter its association capacity. In particular, the N-linked glycosylation at position 303 appears to be a major determinant in modifying the association with neurexin-1β. We show here that glycosylation processing of neuroligin, in addition to mRNA splicing and gene selection, contributes to the specificity of the neuroligin-1β/neuroligin-1 association.

Appropriate differentiation and maturation of neurons are essential requirements for synapse formation and achieving patterns of fidelity for cell signaling. Because of the multiplicity of potential targets for any given axon and because of the asymmetric nature of the synapse, partner recognition is critical for developing and maintaining functional synapses (1, 2). Neurexin and neuroligin appear to form heterologous cell contacts at synaptic connections and are suitable candidates for controlling synaptic recognition patterns. Neurexin-1β (NX1β) is a member of a large family of neuronal proteins, composed of at least three genes (neurexins 1 through 3) driven by two promoters (α and β), resulting in the expression of at least six neurexin forms. Alternative mRNA splicing confers additional complexity to the possible gene products (3). The neuroligins form a family of cell adhesion proteins encoded by at least four genes that are widely expressed in brain as well as in selected locations outside the central nervous system (4–7). Neuroligin-1 (NL1) has been localized at postsynaptic densities of glutamatergic synapses (8). Association between the neurexin-β and neuroligins appears to be Ca²⁺ dependent and selectively blocked by insertion of an alternatively spliced region in NX1β (4, 9, 10). Recently, mutations in neuroligin-3 and -4 were shown to be linked to autistic spectrum disorders in man (11). Consistent with the hypothesis that NX1β and NL1 can mediate the assembly of the pre- and postsynaptic terminals, a recent investigation delineates some of the cellular processes involved in neuroligin-mediated synapse formation (12).

Neuroligins have structural features typical of other type I cell surface receptors with an extended NH₂-terminal globular region, an O-linked carboxyhydrate-rich domain linked to the single transmembrane region, and COOH-terminal cytoplasmic domain containing a PDZ recognition sequence (13). The NH₂-terminal domain of NL1 consisting of 695 residues has an α/β-hydrolase fold that is characteristic of the cholinesterases and many other serine hydrolases. The NL1 extracellular domain contains two regions of alternative splicing: the first (residues 165–184) has a net charge of +8 and a potential disulfide formed between cysteine at positions 172 and 181; the second (residues 298–305) contains an N-glycosylation consensus sequence at Asn-303. NL1 contains a total of five potential N-glycosylation sites throughout the extracellular domain and, in the stalk region, a Ser-Thr-rich module that is likely to be highly O-glycosylated (10). The extracellular domain of NL1 between the leader peptide and residue 638 shares ~34% residue identity with acetylcholinesterase (AChE) and probably the same α/β-hydrolase fold (14).

To examine the molecular determinants of the neuroligin-neurexin interaction, we constructed and expressed several truncated forms of NL1 in HEK293 cells. The truncated proteins are secreted into the cell culture medium, and can be purified as soluble neuroligins in milligram quantities. This enabled us to analyze several molecular features of NL1 that govern selectivity in its interaction with neurexin. Our findings indicate that specific post-translational modifications may provide an additional level of control in the NX1β-NL1 interaction.

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§ The abbreviations used are: NX1β, neurexin-1β; NL1, neuroligin-1; AChE, acetylcholinesterase; PBS, phosphate-buffered saline; SPR, surface plasmon resonance.
**EXPERIMENTAL PROCEDURES**

**Plasmids, Mutagenesis**—The rat-NX1β-1-Hlg fusion protein construct (hereafter referred as NX1β) was described previously (9, 15). The coding region of the coding rat-NL1 was subcloned into a FLAG-tag vector (Sigma) for detection and purification, resulting in N-FLAG-NL1, encoding the FLAG octapeptide at the NH2 terminus, a linker peptide of 10 residues followed by the NL1 sequence beginning at Gln-46. Residue numbering begins at the methionine start site and includes the processed amino acid leader peptide (10). Five soluble mutants were also constructed by introducing stop codons: Tyr-692, Thr-695, Ile-743, His-634, and Leu-627 generating the proteins NL1–691, NL1–651, NL1–638, NL1–633, and NL1–626. To eliminate individual N-glycosylation consensus sequences Asn, Ser, or Thr were mutated to Ala generating the following proteins: T111A-NL1–638, N303A-NL1–638, N343A-NL1–638, and S549A-NL1–638. An N-FLAG-mouse-AChε expression plasmid, truncated at Pro-548 and lacking the most carboxy-terminal cysteine and thus incapable of intersubunit cross-linking (mA-Chε-548), was also constructed. Mutation sequences were introduced using the QuikChange mutagenesis kit (Stratagene, San Diego, CA). Mouse AChε truncated at Leu-539 was fused to the COOH-terminal part of NL1 starting at His-634, resulting in the AChε-NL1 chimera.

**Cell Culture and Transfections**—HEK293 cells were maintained at 37 °C and 10% CO2 in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and periodically tested to ensure the absence of mycoplasma. Cells were transfected by CaPO4 precipitation with 10 µg of plasmids and carboxymethylated dextran coated gene and were selected by growth in 800 µg/ml G418 (Geneticin, Sigma). After 2–3 weeks, surviving cells formed colonies suitable for clonal selection. The best producing colonies were further expanded for large scale production.

**NL1 and NX1β Expression and Purification**—To purify soluble neuroligins and NX1β, 2–4 liters of media were typically collected over a few days from triple layer flasks (120 ml/flask) maintained in Ultrace- lume serum-free medium (BioWhittaker, Walkersville, MD) at 37 °C and 5% CO2. The medium containing expressed NL1 was passed over a column containing 6 ml of M2 anti-FLAG affinity gel (Sigma) at ~1 ml/min. After washing, the protein was eluted in 2.5 bed volumes of Hepes buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM MgCl2, and 0.01% bacitracin, 0.04% benzamidine, 0.02% sodium azide, 1 mM CaCl2 and 0.005% (v/v) surfactant P20, on a Pharmacia FPLC system. Columns were run at room temperature with Hepes buffer, pH 7.4, at 0.5 ml/min. **Analytical Procedures**—Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry was performed on a PE Biosystems Voyager DE-STR instrument (Framingham, MA). Purified recombinant neuroligins at 1 mg/ml in 0.1% (v/v) trifluoroacetic acid were mixed 1:1 with a matrix of 10 mg/ml saturated sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) dissolved in 30% acetonitrile, 0.3% trifluoroacetic acid, pH 2.2. One-µl droplets of neuroligin/matrix mixture, containing ~10 pmol of protein, were spotted and dried by slow evaporation. Mass spectra were collected using the linear mode and external calibration was performed using Yeast Endolase protein (+dim, +1 and +2 monomer mass-to-charge species).

**Surface Plasmon Resonance Analysis**—Recombinant NL1 was expressed in 293 COS cells using the QuikChange mutagenesis kit (Stratagene, San Diego, CA) and 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 100 µg/ml G418 (Geneticin, Sigma). After 2–3 weeks, surviving cells formed colonies suitable for clonal selection. The best producing colonies were further expanded for large scale production.

**RESULTS**

**Expression and Characterization of Soluble NL1**—To express soluble recombinant forms of NL1, we introduced stop codons into the full-length NL1 cDNA at various positions NH2-terminal to the transmembrane spanning region (Fig. 1). The longest truncated neuroligin, NL1–691, contains a cleavable leader sequence that allows for export and processing of the proteins, an NH2-terminal FLAG epitope, the complete CH1-like do-
main, and the entire Ser-Thr-rich stalk region. All of the potential N- and O-linked glycosylation sites are retained. NL1–651 is devoid of 40 carboxyl-terminal residues that contain the Asn-662 glycosylation site and about half of the predicted O-glycosylation sites; NL1–638 lacks all the potential O-linked glycosylation sites. NL1–633 and NL1–626 further shorten the protein and terminate in the cholinesterase homologous domain. Residue homology comparisons indicate that NL1–638 is of comparable length to mouse AChE truncated at residue Pro-548 that expresses as a fully functional, soluble AChE. Recombinant NX1β, expressed as a soluble human-IgG fusion protein, appears in reducing SDS-PAGE gels as a broad band with an apparent Mr of 72,000 (data not shown).

In immunoblots after SDS-PAGE, the soluble forms show distinctive migrations depending on whether they were harvested from the cell extract or medium. FLAG full-length NL1, with a calculated peptide mass of 91,175 Da appeared in SDS-PAGE as a double band with molecular mass of 140,000 and 120,000 (Fig. 2, left, and Table 1). The relative density of the bands expressed in HEK293 cells, with the higher molecular mass band being more intense, resembles that seen in rat brain (4, 8, 10, 13) indicating that NL1 is constitutively expressed as two predominant, differentially glycosylated forms. NL1–631 appears in SDS-PAGE at a Mr of 98,000 when extracted from the cell lysate, whereas after being exported outside the cell, presumably after completion of post-translational processing, it appears as a broad band at 126,000 (Fig. 2). Similar differences in apparent Mr, depending on origin of harvest are visible in both NL1–651 and NL1–638.

Relative expression levels for each mutant were estimated from the amounts of purified protein recovered from serum-free media. The three longer mutants (NL1–691, NL1–651, and NL1–638) have comparable expression but appearance in the medium markedly decreased for NL1–633 and was not detectable for NL1–626 (Table 1). Because intracellular expression of the two shorter mutants revealed levels of antigenically reactive protein comparable with the intracellular expression of the exported mutants (data not shown), it is possible that folding of these mutants was compromised.

Hydrodynamic properties and the oligomeric state of the NL1s were initially monitored by size exclusion chromatography. The retention times of the NL1–691, NL1–651, and NL1–638 were compared with globular, non-glycosylated protein standards giving an apparent mass between 260 and 280 kDa, indicative of dimensionally asymmetric proteins or oligomers. Samples were analyzed by sedimentation equilibrium, where the counterbalancing influences of the diffusion and sedimentation should reflect the molecular masses. Using different concentrations of NL1–691 (30–100-300 µg/ml) and three different rotor speeds, molecular mass was determined to be 174,000 ± 5,000 Da (n = 9). Comparison with the determined mass spectrometry (MS) weight averaged mass (88,900 ± 200 for the monomer, 178,000 Da for the dimer) indicated that truncated NL1 was a dimer at these concentrations. NL1–638 had a mass of 160,000 ± 6,000 Da (n = 6) from sedimentation equilibrium data (MS data: 79,900 ± 200 Da of monomeric form, 160,000 Da for the dimer) (Fig. 3, A and B). The distribution of the residuals in the analysis appeared to be random, and the difference between the experimental and measured Mr is well within the 10% typically seen for ultracentrifugation sample columns of 2 mm in length.

Binding of Soluble Truncated NL1 Is Dependent on Ca2+ and the Amino-terminal NL1 Sequence Homologous to Acetylcholinesterase—To compare the relative NX1β binding of the different NL1 mutants, a range of NL1 concentrations extending between 62.5 and 2000 nM was injected and binding was monitored by SPR. NL1–651 and NL1–638 showed about 2.5-fold enhanced binding over NL1–691, indicating that the stalk region itself, when not associated to the cell membrane, reduces apparent binding of the soluble form (Fig. 4A). Rates of association and dissociation using recombinant, soluble entities provide an estimate of the kinetic parameters for the interaction. The calculated bimolecular association rate constant, kₐ = 5 × 10⁴ M⁻¹ s⁻¹; dissociation rate constant, kₐ = 0.015 s⁻¹, gave an equilibrium dissociation constant Kᵤ of ~300 nM. In SPR experiments we were unable to detect binding...
of the full-length NL1 to NX1β in either the absence or presence of detergent (0.5% Nonidet P-40). The detergent requirement for solubility of the full-length NL1 precludes our direct comparison with the soluble forms.

Mouse AChE, when tested for NX1β/H9252 binding over an extended concentration range (62.5 nM to 1000 nM) using NX1β/H9252, showed no binding for the soluble monomeric form of mAChE in the subtracted sensorgram. A similar result was found for NX1β-3 (4, 10) that represents the unspliced isoform at position 4 (cf. Fig. 4B). Similarly, no binding to the immobilized NX1β was observed for bovine serum albumin over the same concentration range.

To examine the Ca$^{2+}$ dependence of NX1β-NL1 binding reported previously (4, 9, 10), NL1–691 purified in Ca$^{2+}$-free Hepes buffer was diluted in either Hepes-Ca$^{2+}$ or Hepes-Mg$^{2+}$ buffer and injected over the NX1β surface. Specific binding was

![Equilibrium ultracentrifugation analysis of NL1](image1)

**Fig. 3.** Equilibrium ultracentrifugation analysis of NL1. The raw data are shown as circles; the solid line is the best fit. The distribution of the residuals of the best fit is shown above. Data were collected at 10,000 rpm, 20 °C, 100 μg/ml NL1 (10 mM Hepes, pH 7.4, 150 mM NaCl). A, NL1–691, mass determined by sedimentation equilibrium: $M = 179,000$. B, NL1–638, mass determined by sedimentation equilibrium: $M = 159,000$.

![Relative binding kinetics of three truncated NL1 mutants](image2)

**Fig. 4.** Relative binding kinetics of three truncated NL1 mutants. A, three neureligins-1, truncated in the extracellular domain (solid line, NL1–638; dashed line, NL1–651; dotted line, NL1–691), were injected at 125 nM over the surface of neurexin-1β attached to the BIAcore™ chip. Injections were in Hepes buffer at 20 μl/min. The injection interval was 150 s followed by a decay interval of 300 s in which running buffer alone runs through the surface. Table I gives maximum binding of the three neureligins. B, NX1β binding capacity of the four designated concentrations (nM) of NL1–638 (solid line) compared with identical concentrations of mAChE (dotted line).
detected only in the presence of Ca\(^{2+}\) (Fig. 5). Moreover, replacing the Ca\(^{2+}\) sample buffer with Mg\(^{2+}\) at the end of the injection resulted in a rapid diminution of the signal to baseline. Addition of a divalent cation chelator (EDTA, 5 mM) to the Hepes buffer showed a loss of specific binding that was more rapid than when Mg\(^{2+}\) simply replaced Ca\(^{2+}\), indicating that removal of Ca\(^{2+}\) does not require dissociation of the complex. Hence, the Ca\(^{2+}\) is not apparently sequestered at the NX1\(\beta\)-NL1 interface.

To determine whether the ionic strength of the buffer plays a role in the NX1\(\beta\)-NL1 association, NL1–691 was diluted in different buffers with NaCl concentrations ranging from 37.5 to 600 mM. As the sensorgram overlay in Fig. 6 shows, at NaCl concentrations higher than 75 mM the binding becomes markedly reduced and is virtually lost above 300 mM, indicating that a significant component of the interaction is electrostatic.

Removal of the NL1 N-Glycosyl Residues Leads to an Increased NX1\(\beta\) Binding—SPR and immunoblotting techniques were used to ascertain if the glycosylation processing that gives rise to apparent migration differences in SDS-PAGE (Fig. 2) influences NX1\(\beta\) binding. Unprocessed NL1–691 was purified from cell extract-bound NX1\(\beta\) with a specific activity 4–6-fold higher than NL1–691 purified from the cell culture medium (Fig. 7), indicating that the post-translational modifications dramatically decreased both binding affinity as well as mobility on SDS-PAGE. The slower apparent association and dissociation rates measurable for the NL1–691 from the cell extract, compared with the NL1–691 from the medium, are because of the influence of ligand depletion (mass transport) that becomes important at high surface density in the face of lower ligand concentrations. High density surfaces were necessary to detect the low signal generated by 16.25 nM NL1–691 purified from medium.

NL1–691 and NL1–638 were subjected overnight to enzymatic deglycosylation with PNGase F under non-denaturing conditions and analyzed by SPR (Fig. 8, A and B). Each mutant showed approximately a 3-fold increase in binding after PNGase F treatment that correlates with the increased mobility in SDS-PAGE. PNGase F alone did not bind NX1\(\beta\) and does not influence binding when mixed with NL1–638 immediately before injection over a NX1\(\beta\) surface (Fig. 8C). Proportional increases in binding following treatment of the two mutants indicated that the N-linked glycosylation site at position Asn-662, which is not present in NL1–638, is not involved in the decreased binding of NL1–691. No binding was detected for PNGase F-treated mAChE-548 (up to 10 \(\mu\)M). To determine whether sialic acid, which often terminally caps both N- and O-linked oligosaccharides, reduces binding affinity for NX1\(\beta\), NL1–691 and NL1–638 were treated under non-denaturing conditions with sialidase II either alone or in combination with PNGase F. For both truncations, sialidase II treatment alone increased binding about 2–2.5-fold. The combined treatment improved binding about 5-fold (Fig. 9). Because sialidase enhanced activity comparably in both truncations, we could rule out sialidation of the O-linked sugar set carried by the stalk region of the NL1–691 mutant as source of binding hindrance.

To ascertain whether the single N-linked glycosylation site in NX1\(\beta\) influences NX1\(\beta\)-NL1 interactions, NX1\(\beta\) treated with PNGase F was tested on NL1–691 and NL1–638. Enhanced migration in SDS-PAGE revealed that deglycosylation occurred; however, NL1 binding of deglycosylated NX1\(\beta\) was identical to untreated protein (data not shown).

N-Linked Glycosylation of Residue 303 Found in the Second Splice Site of NL1 Reduces NX1\(\beta\) Binding—To identify which of the N-linked glycosylation positions in NL1 was responsible for hindering the NX1\(\beta\) binding, we first demonstrated that all four potential N-linked glycosylation sites of NL1–638 are fully occupied by oligosaccharides.\(^2\) We then mutated the four N-glycosylation consensus sequences of NL1–638 separately. Each mutant at 500 nM concentration was divided in two identical aliquots, one of which was treated with PNGase F. NL1–638 wild type and three N-linked glycosylation mutants (T111A-NL1–638, N343A-NL1–638, and S549A-NL1–638) digested with PNGase F increased their SDS-PAGE migration rates and their NX1\(\beta\) binding activity by 229 ± 55%, whereas N303A-NL1–638, enclosed in the second region of alternative splicing, revealed SDS-PAGE migration consistent with deglycosylation but exhibited no significant increase its NX1\(\beta\) binding (17 ± 20%) (Fig. 10). The virtual lack of increase in activity after PNGase F treatment indicated that the mutation at position Asn-303 was sufficient to remove a large fraction of the inhibitory capacity of glycosylation of soluble NL1 on the association with NX1\(\beta\). This suggests that position 303 may play a primary steric role in inhibiting NX1\(\beta\)-NL1 interaction.

The rate constant for NL1 dissociation, being a unimolecular reaction, should be independent of the concentration of the reacting species. The crossover of dissociation traces (Fig. 10) shows that deglycosylation of all the mutant forms of NL1 except N303A results in an increase in dissociation rate. Because total binding in the mutants is enhanced, the association rate constants must also increase, but to a greater extent. We

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\(^2\) Hoffman, R. C., Jennings, L. L., Tsigelny, I., Comoletti, D., Flynn, R., Südhof, T. C., and Taylor, P. (2003) Biochemistry, in press.
note that the N303A mutant has greater intrinsic rates of both association and dissociation, and it is marginally affected by PNGase F treatment. The comparison of glycosylation mutations before and after PNGase F digestion using NL1–691 yielded a similar pattern of binding and enhancement with PNGase F (data not shown), confirming position 303 as the main region for steric hindrance.

Finally we investigated whether N303A-NL1 could stimulate presynaptic differentiation in hippocampal primary neurons co-cultured with NL1-transfected COS cells by determining co-localization of NL1 and synapsin expressed by the neurons at the contact points. Synapsin co-localized with cells expressing wild type NL1 as well as T111A-NL1 and N303A-NL1, whereas no co-localization was seen in co-cultures of an AChE-NL1 chimera, indicating that NL1 mutants were able to initiate presynaptic maturation in these cultures (Fig. 11).

DISCUSSION

By inserting a stop codon in the NL1 cDNA at various positions 5’ of the region encoding the transmembrane span, we have expressed and characterized soluble neuroligins with sequences replicating the extracellular domain of NL1. In stably transfected HEK293 cells, the protein is exported into the medium and can be purified in milligram quantities using the FLAG epitope attached at the amino terminus of the processed protein. The soluble protein is glycosylated at the predicted N-linked glycosylation signals and presumably at several of the candidate O-linked glycosylation sites. Hydrodynamic analyses indicate that the protein behaves as a dimer in solution.

The principal advantage of the soluble protein is that studies to measure NL1 binding with its cognate partner, NX1β, can be conducted in solution, in real time and using physiologic buffers. We have employed surface plasmon resonance to characterize the association of NL1 with NX1β and begun to rank order the affinities and binding capacities of the various members of the complex NL1 and NX1β gene families (4, 10). To this end, we have linked NX1β to the solid phase and injected NL1 in the flow cell. To confirm the findings for several complexes, we have reversed the order attaching NL1 to the solid phase, and observed virtually identical specificity patterns (data not shown). In all measurements the volume of the mobile phase is large to minimize rate limitations on binding through ligand depletion.

Our findings with surface plasmon resonance show the expected specificity, whereas binding of mouse AChE, a related α/β hydrolase fold protein, shows no binding to NX1β at concentrations up to 100-fold above where NL1 binding can be demonstrated. The absence of AChE binding does not appear to be a consequence of the different glycosylation pattern on AChE, because a glycosidase-treated AChE also shows no detectable NX1β binding.

The analysis shows the expected dependence on Ca²⁺ because when Mg²⁺ is substituted for Ca²⁺ no binding is evident (4, 9, 10). An analysis of the rates of NL1-NX1β dissociation by buffer perfusion (NL1 depletion) compared with Ca²⁺ depletion by EGTA shows the latter to be far more rapid. This would suggest that the Ca²⁺ site maintaining the conformation for
binding does not become inaccessible upon formation of the NL1-NX1β complex, rather when Ca^{2+} is removed the complex immediately dissociates (Fig. 5). However, the precise location of the Ca^{2+}-binding domain requires further definition, as does the exact role of Ca^{2+} in maintaining the NX1β-NL1 association (14, 19). Using the same Hepes buffer with different ionic strengths, we also noted that the binding of NL1 with NX1β is highly dependent on the NaCl concentration. Thus, high ionic strength wash procedures may yield different patterns of specificity than can be obtained through direct measures of binding in physiological buffers.

Sedimentation equilibrium analysis shows that soluble NL1 can associate as a homodimer with no indication that higher orders of associated oligomers are present. Dimer formation is
unaffected by the presence or absence of the stalk region at the COOH-terminal end of the extracellular domain, thus confirming recent findings on the involvement of a four-helix bundle region within the cholinesterase homology domain in maintaining adhesion activity (12). It is therefore likely that both NL1–633 and NL1–626, being unable to form homodimers, do not fold in or retain conformations appropriate for their expression.

Owing largely to their expression patterns, the cholinesterases have been proposed to have non-catalytic morphogenic activities during vertebrate central nervous system development, promoting both neuritogenesis and synaptogenesis (20–25). Because some cell adhesion molecules (neuroligins and tactins) have high sequence similarities with AChE, and over-expression of AChE in transgenic mice caused a drastic decrease in NX1β mRNA, it has been conjectured that there may be an interaction between these two proteins (21). Mouse-AChE-548 shares with the ChE-like domain of NL1 almost 34% amino acid identity, distributed over the extracellular region up to NL1 residue 638, however, no specific NX1β-AChE binding was evident using SPR, a technique that enables detection of complexes formed in the micromolar range (Fig. 4B). On the contrary, three NL1 truncation mutants bind NX1β, confirming that the binding surface in NL1 is located entirely in the ChE-like domain. This result is consistent with the domain swapping studies (26) that demonstrated that the AChE domain could not substitute for NL1 in triggering any pre-synaptic modification. Our findings, however, cannot exclude the possibility that AChE binds other NX1β forms.

NL1 purified from cell extracts, which is presumably incompletely processed, bound NX1β severalfold better than the secreted form. This led us to examine the role of the individual sugars of NL1 and their interaction with NX1β. The demonstration that the deglycosylated protein shows enhanced binding contrasts with many glycoproteins where removal of N-linked sugars produces loss of function (27–30). Possibly glycosylation of NL1 plays a regulatory role similar to that seen in the Notch receptor family, where addition of certain O-linked oligosaccharides causes the receptor to become more sensitive to Delta and less sensitive to Serrate/Jagged. Accordingly, Notch signaling is amplified in certain cells, but not in others (31). Additionally, in the neural cell adhesion molecule protein, polysialic acid oligosaccharides exhibit a highly regulated expression pattern and their presence in certain glycoproteins serves as a modulator of cell-cell interactions (32, 33).

Whether specific glycosylation processing is a physiologic mechanism controlling the NX1β-NL1 association in the central nervous system or whether the reduction in activity reflects unusual glycosylation patterns in our expression system is thus far unknown. However, recent studies (12, 26) reported that full-length NL1 expressed in HEK293 cells is

![Fig. 10. Influence of deglycosylation of wild type and mutant NL1 on NX1β binding.](image)

A, immunoblot of the wild type NL1–638 and the four N-linked glycosylation mutants shows the extent of PNGase F action. The respective samples were treated with PNGase F in non-denaturing buffer for 16 h and binding was measured by surface plasmon resonance. B, each graph shows the binding before and after deglycosylation of a single mutant over the same NX1β surface. The scale of each graph was chosen to optimally show the effect of the enzymatic treatment. Dotted line, native protein; solid line, PNGase F-treated protein.

![Fig. 11. Synapse formation experiments using NL1 glycosylation mutants and AChE-NL1 chimera.](image)

COS cells were transfected with NL1 wild type or with different mutants and co-cultured with primary hippocampal neurons as described. Immunostaining for synapsin is shown by the green fluorescence emission, for NL1 and mutants, in red emission, and co-localization of both shown by the composite yellow emission.
able to trigger morphological and functional presynaptic differentiation in contacting axons. Hence post-translational modifications of the recombinant protein expressed in the HEK293 cells yielded a NL1 with adhesive functions. We also show here activity with a mutant lacking a site for glycosylation at Asn-303, whereas AChE does not show adhesion activity.

Given that glycosylation processing inhibited the capacity of NL1 to bind to NX1β, sequential mutations eliminating single glycosylation signals may indicate the region of the NL1-NX1β association. That only the N303A-NL1 mutant is not or marginally influenced by PNGase treatment indicates that glycosylation processing at Asn-303 likely occurs in a region proximal to the NX1β binding site. The specific activity of the N303A mutant was not enhanced as might be anticipated, indicating that side chain substitution of the N303A mutant may alter folding of the protein domain, whereas PNGase F only removes exposed oligosaccharides without interfering with the basic peptide fold. Studies on conformational dynamics of oligopeptides indicate that glycosylation can allow a polypeptide to assume conformations originally not accessible to non-glycosylated peptides (34). Thus, a sequon may funnel a protein, or one of its domains, through a particular folding pathway producing a specific conformation not otherwise attainable. This role of structure is currently being explored with a series of mutations. Nevertheless, it is likely that the second alternative splicing region (insert B) and glycosylation processing of the product regulate association between NX1β and NL1.

In summary, we show here that truncations just beyond the cholinesterase homology domain yield a soluble NL1 whose binding to neurexin can be quantitated by surface plasmon resonance. This approach has allowed us to delineate the recognition domain for NL1 and establish a role for glycosylation processing in governing the interaction with NX1β.

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