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Isolation, Primary Structure Characterization and Identification of the Glycosylation Pattern of Recombinant Goldfish Neurolin, a Neuronal Cell Adhesion Protein†

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Neurolin is a growth-associated cell surface glycoprotein from goldfish and zebra fish which has been shown to be involved in axonal path-finding in the goldfish retina and suggested to function as a receptor for axon guidance molecules. Being a member of the immunoglobulin superfamily of cell adhesion proteins, neurolin consists of five *N*-terminal extracellular immunoglobulin (Ig)-like domains, a transmembrane and a short cytoplasmatic domain. Repeated injections of polyclonal Fab fragments against neurolin and of monoclonal antibodies against either Ig domains cause path-finding errors and disturbance of axonal fasciculation. In order to obtain a complete structural characterization and a molecular basis for structure–function determination, recombinant neurolin with the complete extracellular part but lacking the transmembrane and cytoplasmatic domain was expressed in Chinese hamster ovary (CHO) cells (CHO-neurolin). The isolation of CHO-neurolin was carried out by Ni-affinity chromatography and subsequent high-performance liquid chromatography (HPLC). An exact molecular mass determination was obtained by matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS) and revealed 60.9 kDa, which suggested that ~10 kDa are due to glycosylation. The predicted molecular mass is 51.5 kDa, whereas sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) yielded an apparent molecular mass of 72 kDa. Gel shift assays using SDS-PAGE and Western blot analysis with anti-neurolin antibodies provided consistent molecular mass data. The complete primary structure and *N*-glycosylation patterns were identified using specific lectin assays, MALDI/MS peptide mapping analysis by proteolytic and in-gel digestion, electrospray ionization MS and MALDI/MS in combination with specific glycosidase degradation. HPLC isolation of glycosylated peptide fragments and MS after selective deglycosylation revealed heterogeneous glycosylations at all five *N*-glycosylation consensus sites. All attached *N*-glycans are of the complex type and show a mainly biantennary structure; they are fucosylated with α(2,3)-terminal neuraminic acid. These data serve as a first detailed model to characterize the molecular recognition structures exhibited by the extracellular domains.

KEYWORDS: neurolin; neuronal cell adhesion protein; glycosylation; structure

INTRODUCTION

During nervous system development, axons are guided to their targets by cues in their environment and express on their surface recognition/receptor components.[1] Several proteins involved in axon growth and guidance have been identified in the goldfish and zebra fish central nervous system (CNS), where they are re-expressed during axon regeneration in the adult CNS.[2,3] Cell adhesion proteins (CAMs) of the immunoglobulin (Ig) superfamily play a prominent role in the axon path-finding[2,4–7] and can function as axonal surface receptors for guidance cues.[2]

The neuronal cell adhesion protein neurolin has been identified as an integral membrane protein and a member of the Ig superfamily[4,6,8] with the characteristic Ig extracellular domain structure and a single transmembrane and short *C*-terminal cytoplasmatic sequence (Fig. 1).[9,10] Neurolin is the homologue of mammalian and avian DM-GRASP/SC-1/BEN, expressed in growing retinal ganglion cell axons of the goldfish, and has been shown to be involved in axonal

† In memoriam, Professor Wilhelm Richter.

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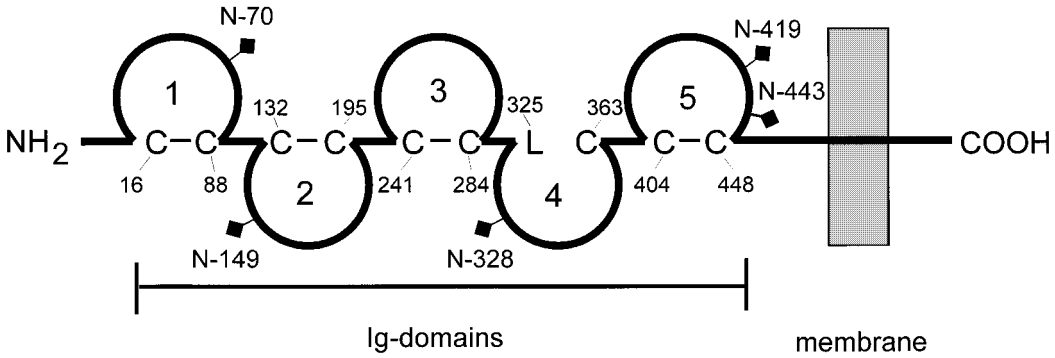


Figure 1. Model of the Ig domain structure of native neurolin. The location of cysteinyl residues and *N*-glycosylation sites is marked. The extracellular part of neurolin is depicted with five Ig domains, stabilized by their characteristic pair-bonded cysteinyl residues, except the fourth Ig domain.

path-finding.[5,10] The *N*-terminal, extracellular part consists of five Ig-loop domains[4,6,8] in which all five potential *N*-glycosylation sites are located (Fig. 1). Native goldfish neurolin has been shown to be a glycoprotein.[10] Its predicted molecular mass from the amino acid sequence is 57 832 Da. The apparent molecular mass determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is ~86 kDa, suggesting that the protein is extensively glycosylated.[10] However, despite the biochemical significance of glycosylation,[11] and other post-transitional modifications for cellular recognition and interaction, only a few structural details for cell adhesion proteins have so far been determined.[8,12–14]

The primary structure of native neurolin immunopurified from goldfish brain has been characterized in a recent study,[9] chiefly by mass spectrometric (MS) peptide mapping of tryptic peptides using matrix-assisted laser desorption/ionization (MALDI). For this native protein, however, no glycosylated peptides were detected by MS peptide mapping, although the complete sequence could be identified and indirect evidence was obtained for a heterogeneous and/or partially truncated *N*-glycosylation.[10] The usefulness of both MALDI/MS[15–18] and electrospray ionization (ESI) MS[19,20] for the characterization of glycosylated peptides and proteins has recently been demonstrated and several MS approaches successfully employed for the identification of post-translational modifications and even higher order structures in proteins.

In the present study, recombinant neurolin, encompassing the complete extracellular domain (including potential modifications, see Fig. 1), was expressed in Chinese hamster ovary cells (CHO; CHO-neurolin) and purified by Ni-affinity chromatography utilising a *C*-terminal oligo-His-tag sequence (see Fig. 4). The extracellular disulphide-loop pattern of CHO-neurolin was ascertained by MS disulphide mapping.[12] Disulphide-linked peptides were deglycosylated with PNGase F and analysed by MALDI/MS before and after selective reduction with dithiothreitol (DTT). MALDI/MS peptide mapping, in combination with high-performance liquid chromatography (HPLC), and mass spectrometry (MALDI/MS and ESI-MS) of isolated glycopeptides were performed before and after selective glycosidase degradation using neuramidase and PNGase F. The results revealed heterogeneous glycosylation at all five potential glycosylation sites. The complete pattern of *N*-glycans was identified to be of complex type with predominantly biantennary structures. In addition, a direct MALDI/MS analysis of the intact protein was obtained and revealed a molecular mass determination in good agreement with the sum of amino acid sequence and structural modifications.

EXPERIMENTAL

Recombinant expression of neurolin in CHO cells

The cDNA coding for the five extracellular Ig-like domains of neurolin, including the signal and the Kozak sequences, was amplified in a standard PCR with the full-length neurolin cDNA clone P19 as template and the primers 5′-ATTAGAGCTCACCATGCAATCGGTTGTCTGCCTTATC and 5′-CTAGTGATGGTGATGGTGATGAAACACGCTGATCTCCTTCGTGTC. The antisense primer was extended at the 5′-end by a stop codon (TAG) and six triplets coding for the amino acid histidine (CAG and CAC, respectively) to facilitate purification of the protein. The PCR product (1470 bp) was directly ligated into the eukaryotic expression vector pCR3 (Invitrogen, Leek, The Netherlands) and the integrity of the construct was confirmed by double stranded sequencing with a T7 Sequencing Kit (Amersham Pharmacia Biotech, Freiburg, Germany).

The neurolin expression clone and the pCR3 vector without an insert (mock control) were transfected into CHO cells using calcium phosphate precipitation. Stable transfectants were selected by their resistance to geneticin (500 μg ml^{-1}) (Gibco BRL, Eggenstein, Germany). Expression of soluble neurolin by the transfected CHO cells was confirmed by immuno-staining and immuno-blot experiments using polyclonal and monoclonal anti-neurolin antibodies.

Purification of CHO-neurolin

CHO cells were originally cultivated at 37 °C and 5% CO_2 in Ham's F12 medium (Gibco BRL) supplemented with 10% foetal calf serum (FCS) (Gibco BRL) and 100 U ml^{-1} penicillin–streptomycin (Gibco BRL). For the

purification of the recombinant neurolin protein, transfected CHO cells were converted to serum free growth using UltraCHO medium (BioWhittaker/Serva, Heidelberg, Germany). Conditioned cell culture medium was adjusted to 0.5 M NaCl–20 mM imidazole, pH 7.3, filter sterilized and loaded on to an Ni-NTA Superflow column (Qiagen, Hilden, Germany). The resin was washed with 10 column volumes of 0.5 M NaCl with imidazole concentrations increasing up to 50 mM. The recombinant protein was eluted with 0.5 M NaCl–200 mM imidazole and collected in 1 ml fractions. The appropriate fractions containing neurolin were pooled and concentrated to approximately 10-fold with 10 000 molecular mass cut-off membranes (Amicon, Beverly, MA, USA). For mass spectrometric analysis, neurolin was further purified by HPLC.

Gel electrophoresis

Standard SDS-PAGE was carried out in Laemmli buffer using 0.5 μg aliquots of HPLC-purified neurolin dissolved in sample denaturation buffer. Gels were evaluated after 1.5 h by staining (Coomassie Brilliant Blue, silver) and destaining as described previously[12] using Sigma-7B mass markers from 36 to 205 kDa. For in-gel digestion/peptide mapping, gel plugs were excised and stored at −20 °C for further analysis.

Lectin assays

Lectin assays of the glycosylated neurolin were carried out using a Glyco differentiation kit (Boehringer Mannheim, Penzberg, Germany). The kit uses glycan-specific lectins conjugated to the steroid hapten digoxigenin, detected by an anti-digoxigenin antibody. Purified neurolin was blotted directly on nitrocellulose membranes and incubated with the lectins *Gelanthus nivalis* agglutinin (GNA) *Sambucus nigra* agglutinin (SNA), peanut agglutinin (pna), *Maackia amurensis* agglutinin (MAA) and *Datura stramonium* agglutinin (DSA). The specificity of detection was ascertained by using the following glycoproteins as controls: transferrin with *N*-glycans of complex type (positive for SNA), fetuin (complex *N*-glycans) and *O*-glycans (positive for SNA, MAA and DSA), asialotransferrin with *O*-glycans (positive for PNA) and carboxypeptidase Y with high-mannose-type *N*-glycans (positive for GNA).

In-gel digestion and peptide mapping

Proteolytic digestion in the gel matrix after silver training was essentially performed according to a previously published procedure[21] with slight modifications. The excised gel plug was dehydrated in acetonitrile, the solvent was removed and the plug was rehydrated in 50 mM ammonium hydrogencarbonate solution. This procedure was repeated twice in order to remove staining agents and buffer components. The plug was then dried under vacuum for 15 min and 0.01 μg of protease (TPCK-trypsin or Asp-N protease) in 20 μl of digestion buffer was added. Excess protease buffer was removed after 10 min and the plug was overlaid with the digestion buffer, 100 μl of 50 mM NH_4HCO_3. All enzymatic digests were carried out for a total of 12 h at 37 °C. The digestion buffer was then removed and the mixture of proteolytic fragments extracted twice with 100 μl of aqueous acetonitrile (1:1, v/v, containing 0.1% trifluoroacetic acid (TFA) for 1 h: the solution was combined with the digestion buffer and lyophilized for MALDI/MS.

Proteolytic degradation in solution

Proteolytic digestion in solution was carried out using the endoproteinases Asp-N and Lys-C of sequencing grade (Boehringer Mannheim). TPCK-treated trypsin was obtained from Sigma (St Louis, MO, USA). All other reagents and chemicals were of analytical grade or the highest available purity. Approximately 15 μg of lyophilized neurolin was solubilized in 100 μl of 50 mM NH_4HCO_3–10% acetonitrile (pH 8.0) under nitrogen. An enzyme-to-substrate ratio of ~1:50 was used for Asp-N digestion. The samples were incubated for 5 h at 37 °C. The unreduced peptide fragment mixture was directly separated by HPLC or subsequently reduced with dithiothreitol (DTT) (1 h at 37 °C, 50-fold excess over disulphde bond) and alkylated with iodacetamide (1 h at 37 °C, 2.2-fold excess over DTT). A sample aliquot of 5 μl was lyophilized and redissolved for MALDI/MS analysis; the remaining sample was subjected to HPLC separation.

HPLC separation of proteolytic peptides

All HPLC separations were performed with a Waters-Millipore M590/510 solvent delivery instrument equipped with an M-490 UV detector system. The peptide mixture was separated on a 25 × 0.4 cm i.d. Vydac-C_8 Nucleosil column (Macherey-Nagel, Duisburg, Germany) using a linear binary gradient of 0.1% aqueous TFA (A) and 0.1% TFA in acetonitrile (B), from 5 to 60% B in 60 min. The HPLC fractions were lyophilized for analysis by MALDI/MS and ESI-MS. A 25 × 0.4 cm i.d. Vydac RP-C_4 column was employed for the purification of intact neurolin (see Fig. 3).

Enzymatic deglycosylation

Degradations by neuraminidase and other glycosidases (Boehringer Mannheim) were performed with aliquots of the isolated peptide fraction lyophilized and redissolved in 10 mM CH_3COONH_4–10% acetonitrile–1 mM EDTA (pH 5.0). Reactions were carried put by incubation for 48 h at 25% with an enzyme-to-substrate ratio of 1:50. The samples were then lyophilized and analysed by MALDI/MS. For PNgase F cleavage, an aliquot of the isolated peptide fraction was lyophilized and redissolved in 50 mM NH_4HCO_3–10% acetonitrile–1 mM EDTA (pH 8.0) and incubated for 4 h at 37 °C

with an enzyme-to-substrate ratio of 1:50. The samples were then lyophilized and analysed by MALDI/MS.

Mass spectrometry

MALDI/MS analysis was carried out with a Bruker-Biflex linear time-of-flight mass spectrometer equipped with a Scout source and video system, a nitrogen UV laser and a dual-channel plate detector (Bruker-Franzen, Bremen, Germany). Sample preparation was generally performed by crystallization of a saturated solution using the matrix 4-hydroxy-α-cyanocinnamic acid (HCCA). Lyophilized samples were dissolved in 20 µl of acetonitrile–water (40:60); a 0.5 µl aliquot was mixed on the stainless-steel target with 0.5 µl of the matrix solution and the droplet was then dried at 20 °C. Spectra were acquired at an acceleration voltage of 20 kV, using an initial 2 kV deflection pulse of 1–4 µs perpendicular to the ion path for removal of matrix ions; 60–300 single laser shots were accumulated to give the spectrum. Calibration was performed with singly and doubly protonated ions of insulin as internal standard.

ESI-MS was performed with a Vestec-A201 quadrupole mass spectrometer equipped with a Vestec-ESI source and a laboratory built nano-ESI-MS source; instrumental conditions, nano-ESI capillary and sample preparations were as described previously.[13,22]

RESULTS

Expression and purification of neurolin from CHO cells

The over-expressed neurolin was pre-purified from the CHO cell culture medium by Ni-NTA affinity chromatography utilising an oligo-His-tag sequence attached to the *C*-terminus of the protein. With a single concentration step by ultrafiltration, the protein could be isolated from the Ni-NTA eluate by RP-C_4 HPLC as shown in Fig. 2(a). Neurolin eluted from the HPLC column as a major fraction after 40 min, as indicated by the bracket in Fig. 2(a). The purified protein was then analysed by SDS-PAGE and identified by Western blot analysis using a monoclonal anti-neurolin antibody [Fig. 2(b)]. SDS-PAGE revealed an apparent molecular mass of 72 kDa, suggesting that substantial glycosylation had occurred, compared with the molecular mass of 51.5 Da calculated from the amino acid sequence (see Table 1). The presence of protein isoforms due to heterogeneity in the glycan structure leads to an observed extended band pattern, as indicated by the small arrows in the SDS-PAGE and Western blot analysis [Fig. 2(b)]. The MALDI mass spectrum of the purified CHO-neurolin is shown in Fig. 2(c), revealing a molecular mass of 60.9 kDa. Compared with the calculated sequence molecular mass of 51.5 kDa (see Table 1), this indicated a mass proportion of ~10 kDa due to glycosylation (including contributions from other post-translational

Table 1. Molecular masses of CHO-neurolin by SDS-PAGE and MALDI/MS

Method	Molecular mass (kDa)
Amino acid sequence	51.535
SDS-PAGE	72
MALDI/MS	60.920

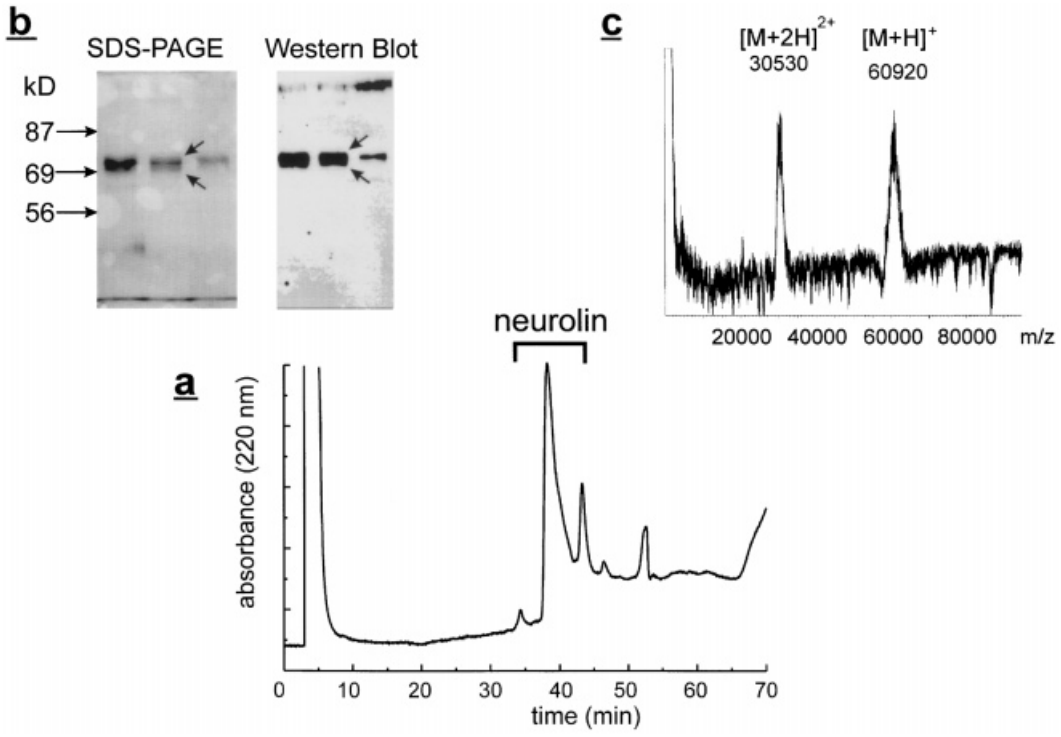


Figure 2. (a) HPLC isolation of CHO neurolin. The protein was concentrated from the cell culture medium by Ni-NTA affinity chromatography. (b) SDS-PAGE analysis and Western blot analysis of the HPLC-purified protein. (c) MALDI/MS analysis of CHO-neurolin. The comparison with the calculated molecular mass by sequence from the amino acid sequence suggests glycosylation of ~10 kDa.

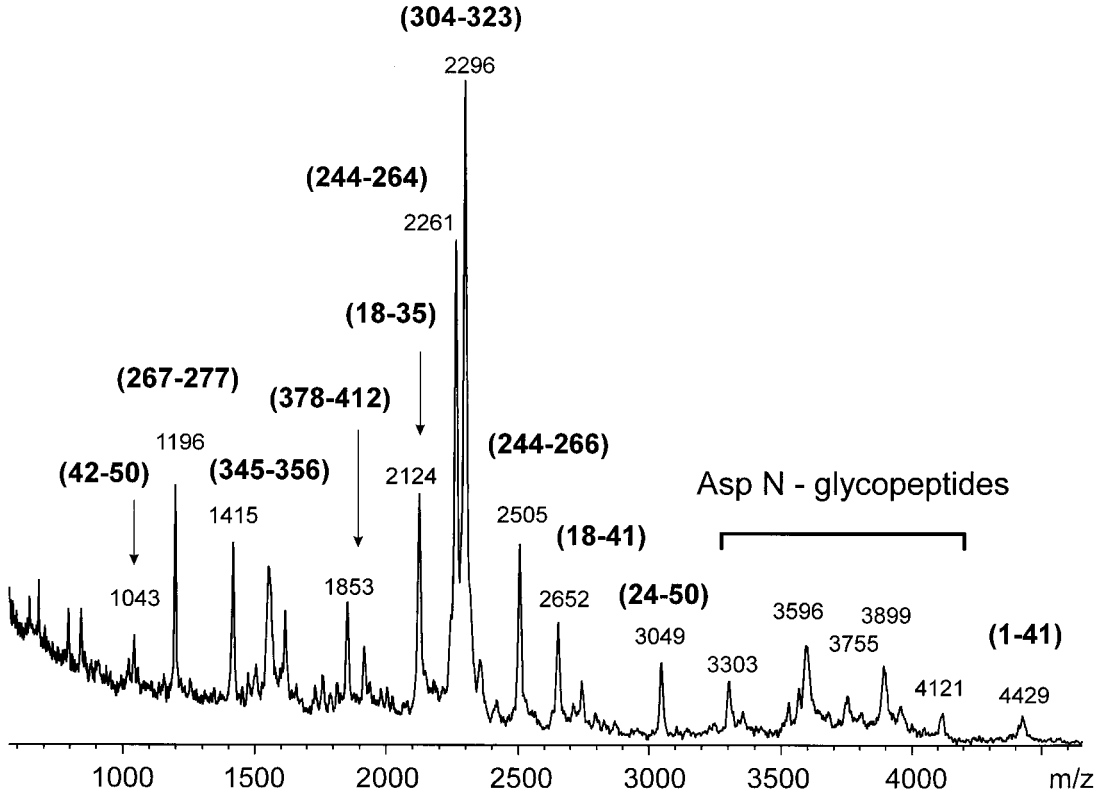


Figure 3. MALDI/MS peptide mapping analysis of a reduced and alkylated Asp-N peptide mixture. The molecular ions are assigned to proteolytic peptides and listed in Tables 2 and 5.

Table 2. Asp-N peptide fragments identified after HPLC isolation and MALDI/MS peptide mapping

Peptide fragment	Partial sequence	$[M+H]^+_{exp}$	$[M+H]^+_{calc}$
A_{1-2}	(1–23)	2419	2418.0
A_{1-3} [a]	(1–35)	3898	3897.6
A_{1-5} [a]	(1–41)	4429	4427.0
A_{2-3} [a]	(18–35)	2124	2124.5
A_{2-5}	(18–41)	2652	2653.0
A_{3-4}	(24–36)	1613	1612.9
A_{3-5}	(24–41)	2027	2026.3
A_{3-6} [a]	(24–50)	3049	3050.5
A_6	(42–50)	1043	1042.3
A_{6-7}	(42–58)	1818	1817.1
A_7	(51–58)	793	792.8
A_{11-12}	(154–167)	1505	1505.7
A_{13}	(168–187)	2242	2241.5
A_{17} [a]	(244–264)	2261	2261.6
A_{17-18} [a]	(244–266)	2505	2504.8
A_{17-19} [a]	(244–286)	3682	3682.2
A_{17-22}	(244–303)	6633	6633.5
A_{19}	(267–277)	1196	1195.4
A_{20}	(278–287)	1155	1155.3
A_{22}	(290–303)	1617	1618.8
A_{23} [a]	(304–325)	2296	2296.7
A_{29} [a]	(364–395)	3529	3529.1
A_{29-30}	(364–395)	5364	5364.1
A_{30}	(396–412)	1853	1853.1
A_{33} [a]	(457–470)	1762	1760.9

[a] Fragments identified by MALDI/MS peptide mapping after in-gel digestion.

modifications). No single protein isoform could be resolved in the molecular ion signal. The sequence of neurolin contains five potential *N*-glycosylation sites (see Fig. 1), suggesting an average modification by ~2000 Da if glycosylation is assumed to be present at all consensus sites. Mass spectrometric peptide mapping was used as the initial method for the detailed analysis of the distribution and *N*-glycan pattern of neurolin.

Primary structure characterization of CHO-neurolin

For the initial structural analysis of neurolin, enzymatic degradation in solution and 'in-gel' digestion were performed using the proteolytic enzymes Asp-N-protease and trypsin. MALDI/MS peptide mapping analyses of the reduced and alkylated Asp-N peptide fragments after digestion in solution are shown in Fig. 3. The molecular ions that could be directly assigned to unmodified Asp-N proteolytic peptides are indicated in the spectrum. The molecular ions at *m/z* 3303, 3596, 3755, 3899 and 4121 (marked by the bracket in Fig. 3) were identified as glycosylated Asp-N peptides which were analysed in detail by selective deglycosylation, and by ESI-MS after isolation by HPLC (see below and Table 5). The unmodified proteolytic peptides identified by Asp-N digestion experiments in solution and in the gel are summarized in Table 2, and are underlined in the neurolin sequence in Fig. 4. No unmodified peptides could be identified in the sequence areas encompassing

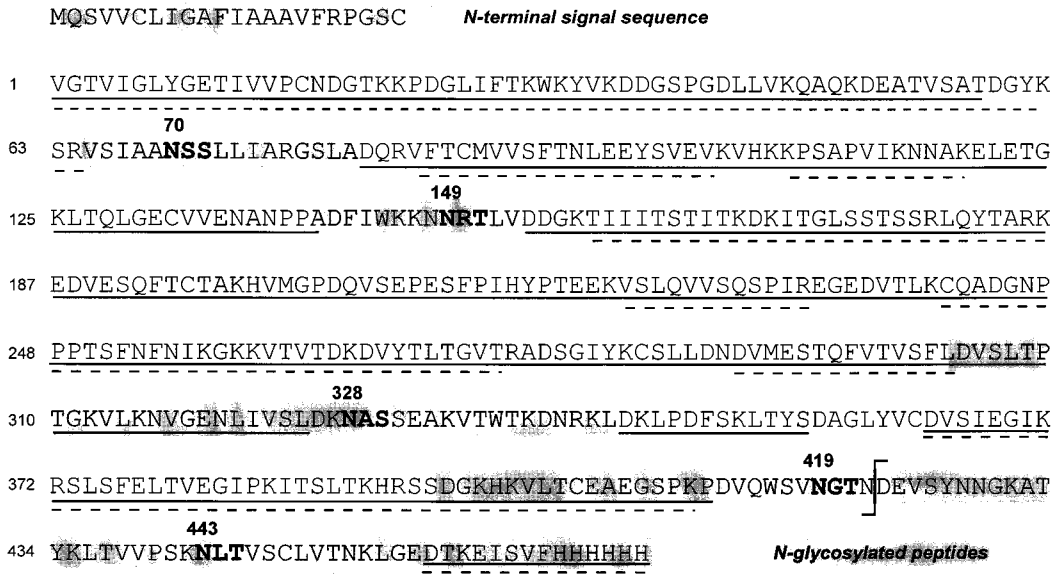


Figure 4. Primary structure characterization and *N*-glycosylation pattern of CHO-neurolin by MS peptide mapping. Unmodified proteolytic peptides from digestion in solution (Asp-N) and in-gel (Asp-N, trypsin) are underlined (dotted lines for in-gel digestion) as listed in Tables 2 and 3. The glycopeptides containing the five *N*-glycosylation sites (marked in bold letters with their sequence numbers) are coloured. The bracket at N-422 separates the glycopeptides comprising N-419 from N-443. The found glycopeptides with their asparagine-attached oligosaccharide structures are assigned in Table 5.

the *N*-glycosylation consensus sites, which was in agreement with the molecular mass data and strongly corroborated the assumption that all five possible sites of glycosylation in neurolin are indeed modified. When compared with the Asp-N digestion in solution, the in-gel digestion with Asp-N yielded a lower sequence coverage. The tryptic peptides identified by in-gel digestion are listed in Table 3 and underlined in Fig. 4.

Table 3. Tryptic peptide fragments identified by MALDI/MS peptide mapping after in-gel digestion

Peptide fragment	Partial sequence	$[M+H]^+_{exp}$	$[M+H]^+_{calc}$
T_{1-2}	(1–30)	3196	3193.7
T_{4-8}	(33–64)	3445	3443.8
T_{11}	(85–104)	2383	2382.7
T_{13-14}	(108–119)	1265	1266.3
T_{19-22}	(158–185)	3058	3057.5
T_{26}	(221–232)	1313	1312.5
T_{28}	(241–257)	1908	1908.0
T_{30-32}	(260–276)	1893	1894.0
T_{35-36}	(313–325)	1640	1641.0
T_{45}	(373–385)	1419	1419.6

In summary, all digestion proteolytic procedures identified the amino acid sequence, the expected *N*-terminus after complete processing of the signal peptide and the *C*-terminal oligo-His tag sequence. For the analysis of the detailed *N*-glycan structure, the proteolytic peptide mixtures were separated by HPLC, with the main focus on the isolation and elucidation of glycopeptides. The identified Asp-N glycopeptides are indicated in Fig. 4 by colours and summarized later in Table 5.

Determination of glycosylation pattern

In order to characterize the *N*-glycosylation structure of neurolin in detail, gel shift assays were initially per-

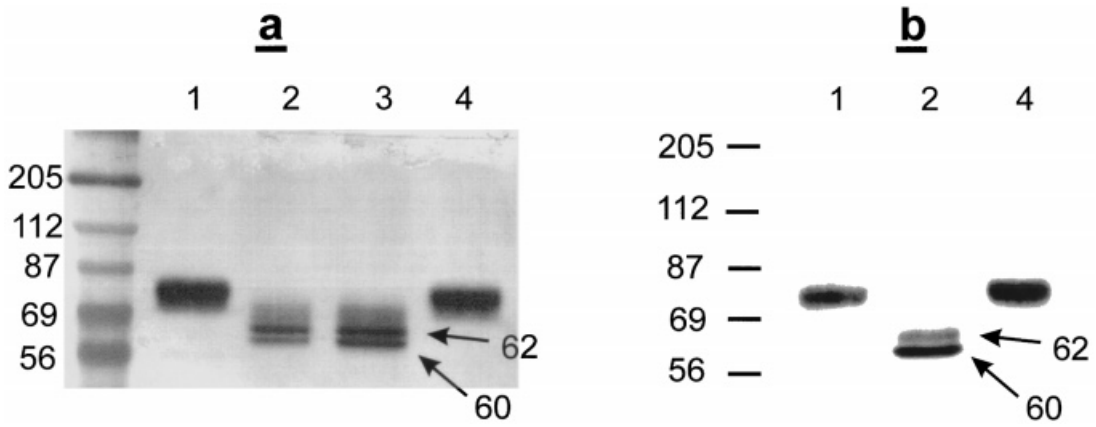


Figure 5. (a) SDS-PAGE and (b) Western blot analysis of CHO-neurolin upon deglycosylation with PNGase-F. Lane 1, purified and glycosylated neurolin; lanes 2 and 3, deglycosylation with PNGase F (24 and 48 h at 37 °C); lane 4, control, glycosylated neurolin kept for 24 h at 37 °C.

Table 4. Oligosaccharide structures in CHO-neurolin detected by specific lectins

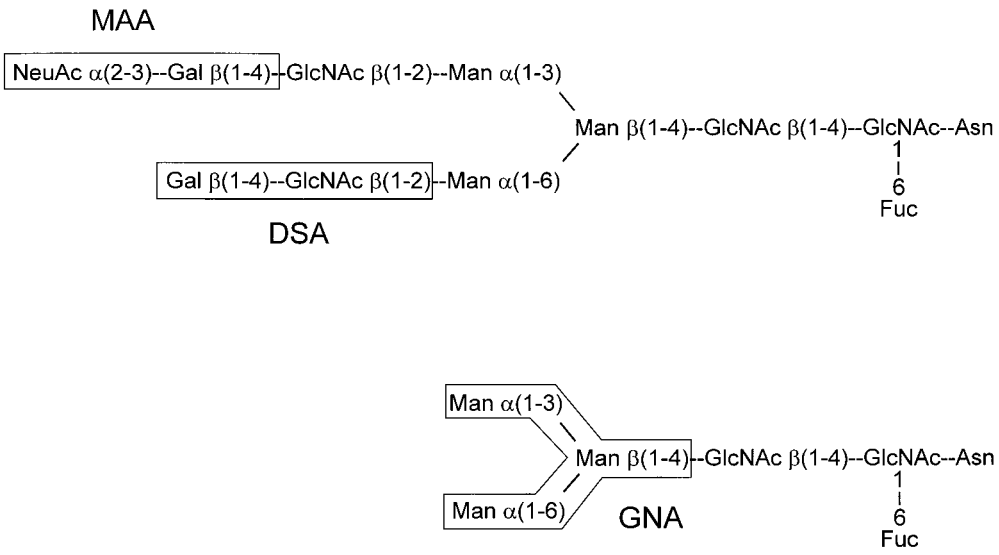
Lectin	Detection for CHO-neurolin[a]	Lectin-specific glycan structure	Glycan type
		Man-α(1,2)-Man	High-mannose *N*-glycan
GNA	+	Man-α(1,3)-Man	Complex *N*-glycan
		Man-α(1,6)-Man	Hybrid *N*-glycan
SNA	−	NeuAc-α(2,6)-Gal	Complex *N*-glycan
PNA	−	Gal-β(1,3)-GalNAc	*O*-Glycan
MAA	+ +	NeuAc-α(2,3)-Gal	Complex *N*-glycan
			O-Glycan
DSA	+ +	Gal-β(1,4)-GlcNAc	Complex *N*-glycan

[a] + +, Strong signal; +, weak signal; −, no signal.

formed (Fig. 5) after deglycosylation with PNGase F, thus specifically cleaving asparagine-linked glycans of all possible structural types. The deglycosylation products obtained after reaction times of 24 and 48 h were analysed by SDS-PAGE [Fig. 5(a)] and Western blot analyses [Fig. 5(b)], revealing an apparent mass shift of ~12 kDa of the molecular mass from 72 kDa (lane 1) to a band pattern of around 60 and 62 kDa (lanes 2 and 3 in Fig. 5). Deglycosylation was not quantitative as judged from the 62 kDa band in both SDS-PAGE and Western blot analysis. Complete deglycosylation of neurolin should lead to a molecular mass of 51.5 kDa as calculated from the amino acid sequence (Table 1). Since only apparent molecular masses are observed by SDS-PAGE, this mass difference may be related to the particular modified structure of neurolin. As a control, CHO-neurolin heated for 24 h at 37 °C did not reveal any unspecific proteolytic degradation under the conditions employed (Fig. 5, lane 4).

Preliminary dot-blot lectin assays of CHO-neurolin were carried out to reveal the pattern and type of the attached glycans. The specificity of the lectins used and the oligosaccharide structures/types identified for CHO-neurolin are summarized in Table 4. The results revealed that terminal neuraminic acid α(2,3)-linked to galactose and galactose β(1,4)-linked to *N*-acetylglucosamine were recognized by the lectins MAA and DAS, which is consistent with glycan structures of complex type as shown in Scheme 1. Furthermore, these data are strongly corroborated by the very weak or lack of response to GNA, which detects any terminal mannose, as part of the structural core of complex *N*-glycans (see Scheme 1). Complex glycoforms presenting a monosubstituted core structure are hence present in only small amounts, probably due to the glycan heterogeneities and/or partial truncation. In contrast, any significant *N*-glycans of high-mannose or hybrid type should provide a similarly strong response for GNA as for MAA and DAS. No terminal α(2,6)-linked neuraminic acid was detected by the SNA assay. Furthermore, the negative response to PNA was confirmed throughout by the MS peptide mapping data, indicating the absence or presence of traces only of *O*-glycosylation in CHO-neurolin.

For the detailed analysis of structural type and distribution of *N*-glycans in CHO-neurolin, proteolytic



Scheme 1. Terminal oligosaccharide structures in CHO-neurolin recognized by specific lectins. Glycan structures detected by the lectins MAA, DSA and GNA are marked by boxes (see Table 4). GNA detects a terminal mannose residue; the other mannose residues may be substituted according to the glycan structure type.

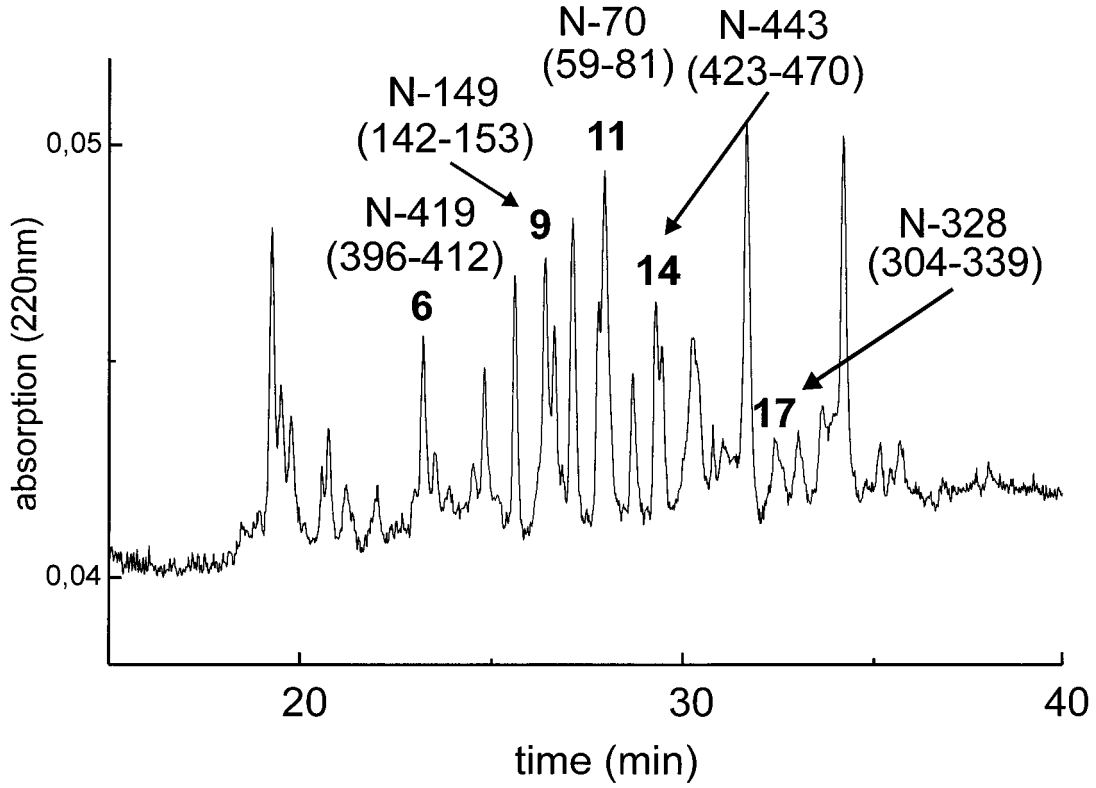
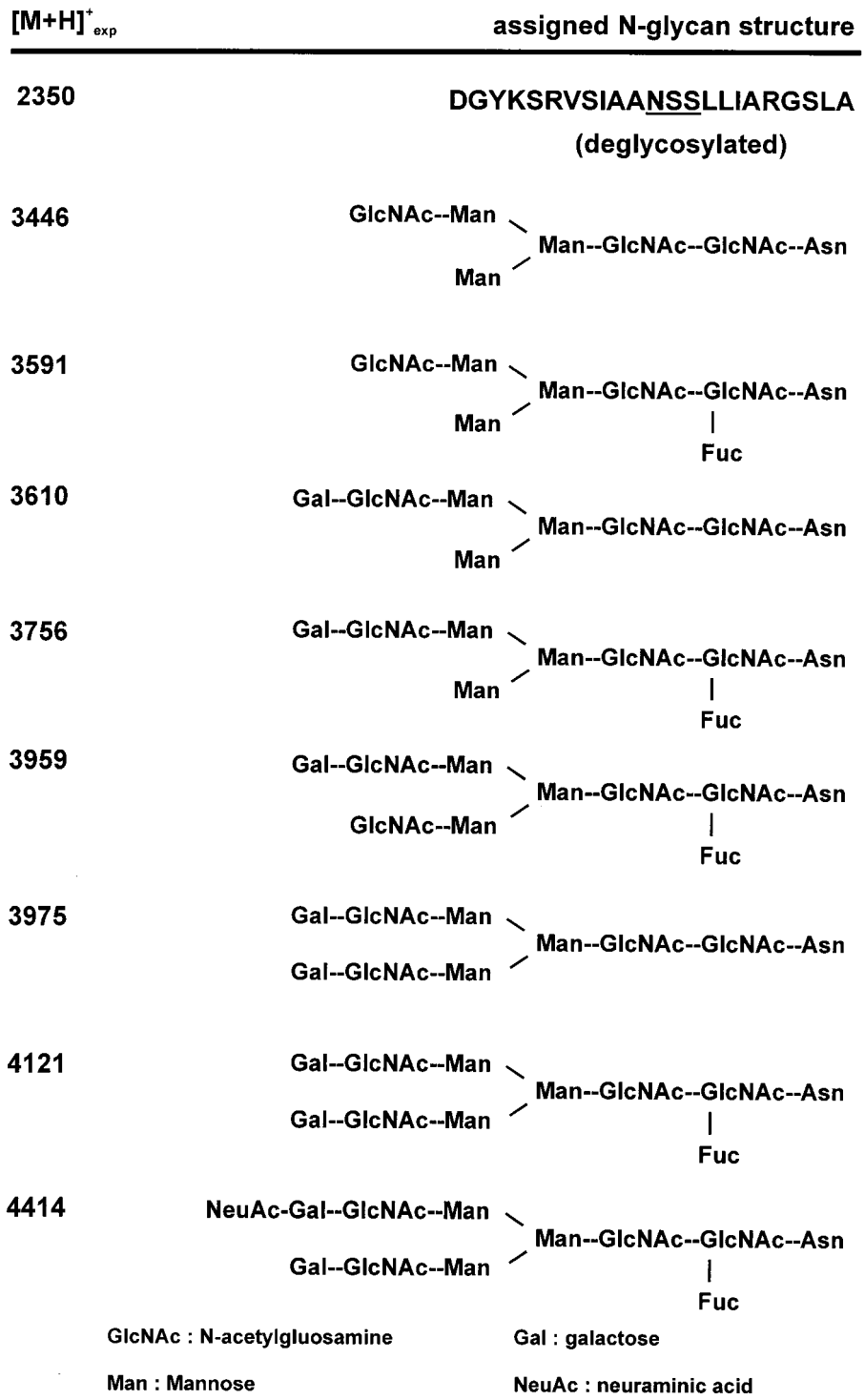


Figure 6. HPLC separation of peptide fragment mixture from Asp-N protease digestion. The fractions were analysed by MALDI/MS before and after PNGase F. The eluting Asp-N glycopeptides corresponding to all five *N*-glycosylation sites are assigned in the chromatogram.

peptide mixtures were separated by HPLC and the glycopeptides were isolated. Figure 6 shows the HPLC separation of the reduced and alkylated peptide mixture after Asp-N digestion, as characterized by MALDI/MS peptide mapping analysis (Fig. 3). The fractions, containing the glycopeptides linked at all five corresponding Asn residues (Asn-70, -149, -328, -419 and -443) are assigned in the HPLC trace (Fig. 6). All fractions were subjected to MS analysis, then treated with neuramidase and PNGase F and re-analysed by MALDI/MS. Glycopeptide-containing fractions and the glycosylation pattern could be identified by the series of molecular ions due to oligosaccharide heterogeneities revealed by mass differences of 162 Da (corresponding to mannose/galactose, 146 Da (fucose), 203 Da (*N*-acetylglucosamine) and 292 Da (neuraminic acid). The presence of terminal neuraminic acid was in all cases established by specific neuramidase digestion yielding the desialyated glycan forms. These results and additional ESI-MS analyses (data not shown) confirmed the correct mass assignments due to the neuraminic acid glycan structures as shown in Scheme 1, thus excluding a possible presence of two fucose units. Treatment of the glycopeptide fractions with PNGase F resulted in the complete cleavage of the *N*-linked glycan structures to yield the deglycosylated peptides which were identified as newly formed product ions in the spectra. The MALDI/MS analyses of the heterogeneous glycan structure types at Asn-70 (HPLC fraction 11 in Fig. 6) and assignments to the complex type glycan structures provided by glycosidase degradation (see Scheme 2) are illustrated in Fig. 7. The molecular ions of the sialyated form *m/z* 4416) were generally found with lower abundances than the desialyated forms (*m/z* 4121, 3959, 3756 and 3610). This may be explained by a negative charge of the corresponding glycopeptides causing a lower ionization efficiency in MALDI/MS analysis.[15] The terminal neuraminic acid residues (*m/z* 4416) were specifically identified by neuramidase cleavage [Fig. 7(b)]. Upon treatment with PNGase F, all *N*-linked glycans were cleaved off to yield the peptide ion at *m/z* 2350 [Fig. 7(c)], identified as the Asp-N peptide (59–81). Figure 8(a) shows the corresponding MALDI/MS analysis of the heterogeneous glycan structure at Asn-149 (HPLC fraction 9 in Fig. 6). The molecular ions could be assigned to the complex type glycans and confirmed by ESI-MS and glycosidase data, whereas small amounts of co-eluting unmodified Asp-N peptides (244–264, 244–266 and 24–50) were also found to be present in this fraction. Terminal neuraminic acid residues present (forms *m/z* 3899 and 3596) were specifically cleaved off by neuramidase digestion [Fig. 8(b)]. Complete deglycosylation by PNGase F provided the new ion at *m/z* 1535 which is assigned to the Asp-N peptide (142–153), hence confirming N-149 as the *N*-glycosylation site. This glycosylation pattern was fully consistent by ESI-MS [Fig. 8(d)] and ESI fragment ions (not shown). ESI-MS confirms the mass of the triply and quadruply charged molecular ions [Fig. 8(d)] which are assigned to the *N*-glycan forms, abbreviated as in Fig. 8(a).

In summary, the lectin assays and residue masses of the monosaccharide cleavage products provided the assignment of composition and glycosylation patterns



Scheme 2. *N*-Glycosylation pattern at Asn-70 and biantennary *N*-glycans of complex type identified for peptide (59–81) of CHO-neurolin (see also Table 5).

for all five glycosylation sites as summarized in Table 5. All the data suggest that biantennary complex structure types predominate, and that all *N*-glycans are fucosylated with terminal galactose and neuraminic acid residues.

DISCUSSION

The primary structure and complete glycosylation pattern of recombinant neurolin expressed in CHO cells and purified to molecular homogeneity by HPLC were characterized by MS molecular mass determination, MALDI/MS peptide mapping, MS analysis of HPLC-isolate proteolytic peptides, enzymatic deglycosylation and detection by specific lectins. Taken together, these data consistently reveal heterogeneous *N*-glycosylations throughout the extracellular protein domain at all five consensus glycosylation sites (see Fig. 1). All glycosylations found are of complex type with biantennaric structures predominating as shown in Schemes 1 and 2, whereas other glycan forms should be absent or present

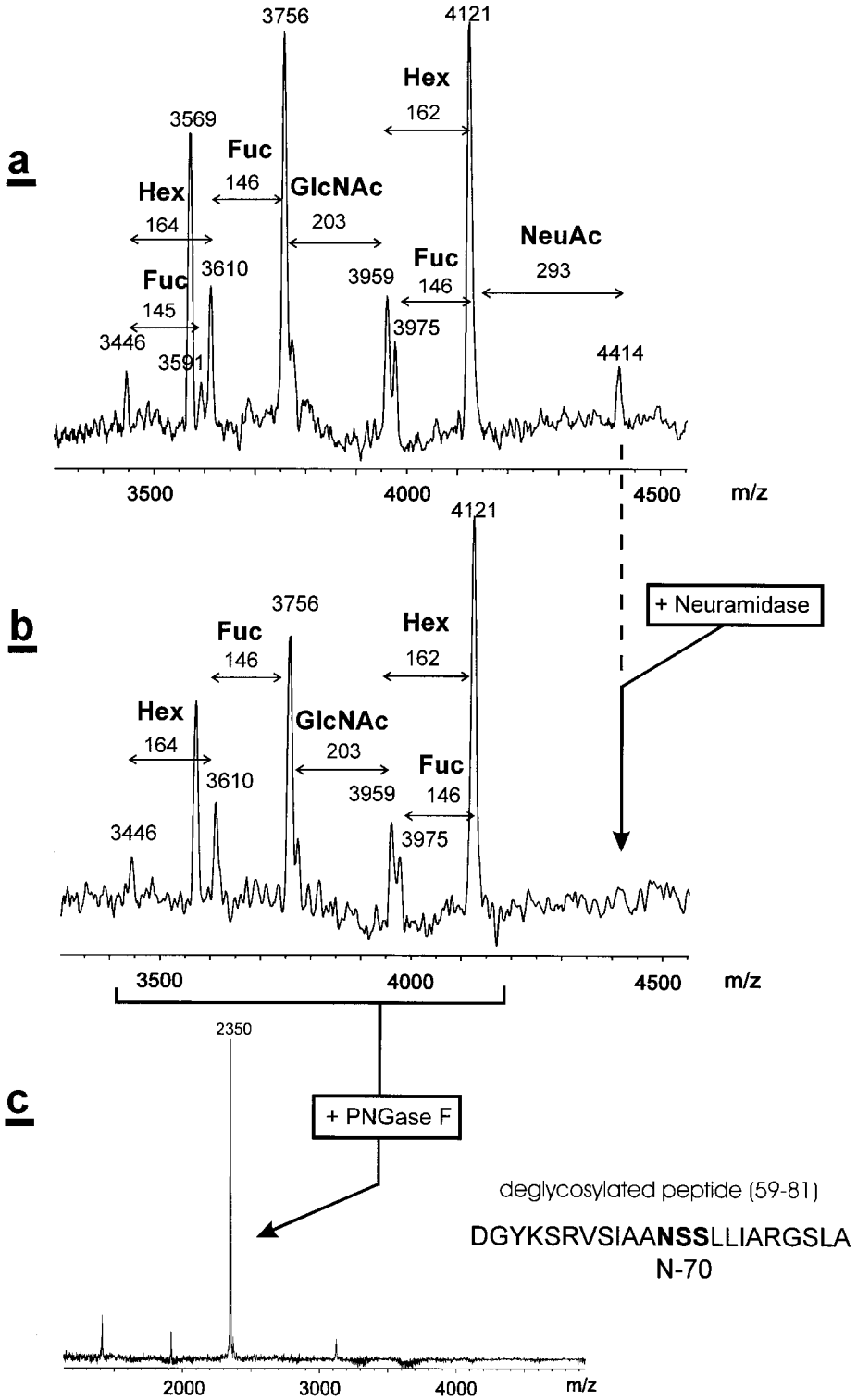


Figure 7. Characterization of *N*-glycosylated Asp-N peptide (59–81) by MALDI/MS and specific glycosidase cleavage (a) MALDI/MS of intact glycosylated peptides. Mass differences between adjacent molecular ions due to the glycan heterogeneity are indicated: 162 (mannose/galactose), 146 (fucose); 203 (*N*-acetylglucosamine) and 292 (neuraminic acid). (b) MALDI/MS after treatment with neuramidase. (c) MALDI/MS upon treatment with PNGase F, showing molecular ion of the deglycosylated peptide (59–81).

in only small amounts. These results are in agreement with the previously suggested extracellular domain structures of cell adhesion proteins,[16] i.e. the alignment of glycosylated Ig domains as illustrated in Fig. 1. They will allow the refinement of a detailed structure–function evaluation of the different Ig domains, such as their role in specific recognition processes.

The results also demonstrate the high efficiency of MS peptide mapping in combination with specific recognition assays and enzymatic digestion for the rapid

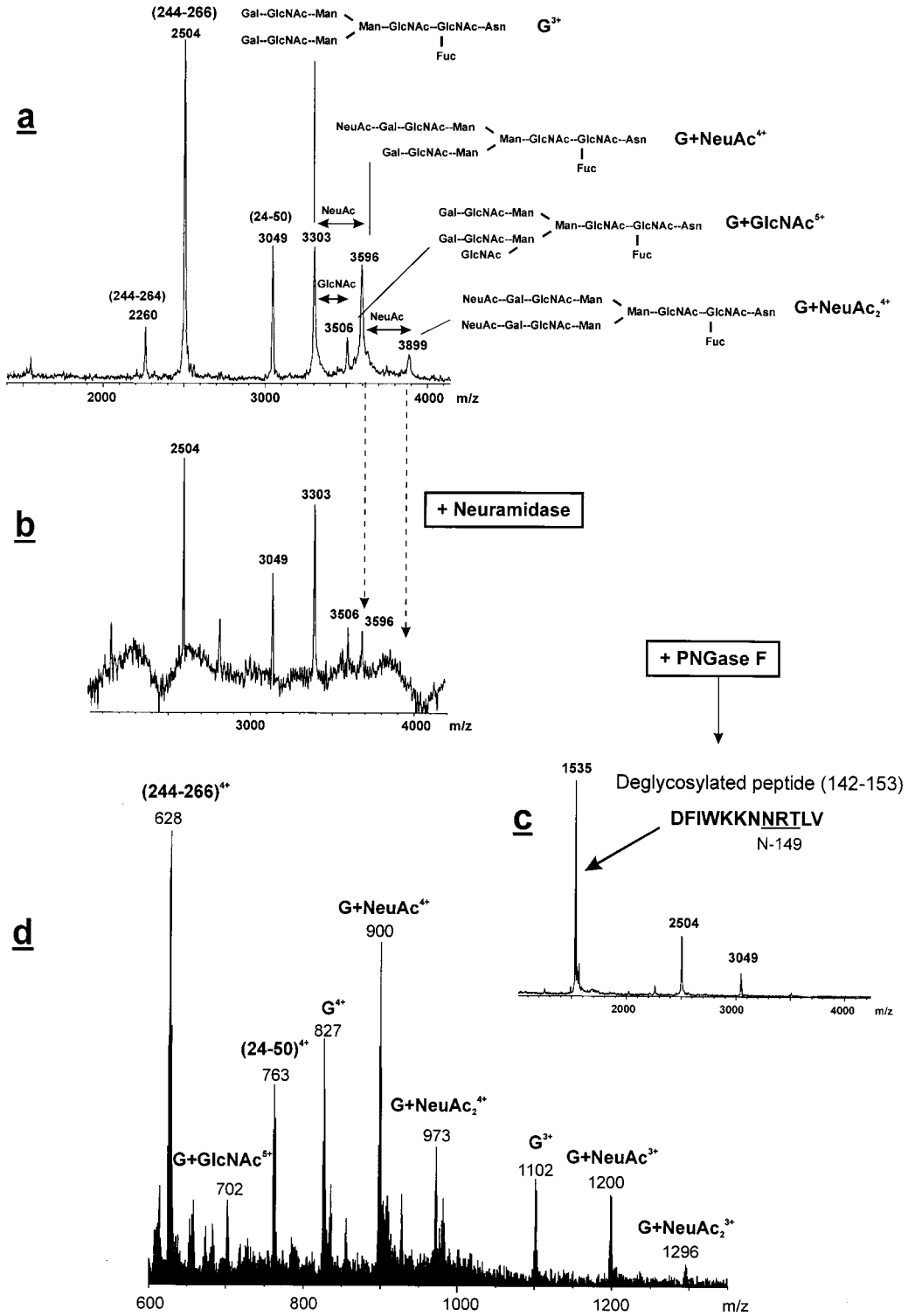


Figure 8. Structural characterization of *N*-glycosylated Asp-N peptide (142–153). (a) MALDI/MS of intact *N*-glycosylated peptide. Oligosaccharide heterogeneities are indicated by the glycan mass differences between adjacent signals: 203 (*N*-acetylglucosamine) and 292 (neuraminic acid). The glycan structures are assigned with G for the biantennary glycan core and the terminal sugar residues, respectively. Three unmodified Asp-N peptides (244–264, 244–266, 24–50) co-eluting in this HPLC fraction are also assigned. (b) MALDI/MS upon treatment with neuramidase. (c) MALDI/MS upon treatment with PNGase F. (d) ESI-MS analysis of *N*-glycosylated Asp-N peptide (142–153). The multiply charged signals are labelled as described above for the heterogeneous glycan structures.

and specific characterization of multiple and heterogeneous glycosylation structures. Furthermore, these methods provide a basis for a molecular comparison of the glycosylation pattern for proteins from different expression systems. Corresponding studies of different cell adhesion proteins (axonin, neurolin) and specific recombinant domains are currently being performed in our laboratory.

Table 5. Glycosylation pattern for the *N*-glycosylation sites of CHO-neurolin

N-Glycosylated peptide fragment[a]	Asn residue	$[M+H]^+$ after PNGase F cleavage	$[M+H]^+$ ions of assigned *N*-glycan peptide	Assigned glycan composition[b]
A_8 (59–81)	N-70	2350	—	—
			3446	$(GlcNAc)_3(Man)_3$
			3591	$(Fuc)_1(GlcNAc)_3(Man)_3$
			3610	$(Gal)_1(GlcNAc)_3(Man)_3$
			3756	$(Fuc)_1(Gal)_1(GlcNAc)_3(Man)_3$
			3959	$(Fuc)_1(Gal)_1(GlcNAc)_4(Man)_3$
			3975	$(Gal)_2(GlcNAc)_4(Man)_3$
			4121	$(Fuc)_1(Gal)_2(GlcNAc)_4(Man)_3$
			4414	$(Fuc)_1(Gal)_2(GlcNAc)_4(Man)_3(NeuAc)_1$
A_{10} (142–151)	N-149	1535	—	—
			3303	$(Fuc)_1(Gal)_2(GlcNAc)_4(Man)_3$
			3506	$(Fuc)_1(Gal)_2(GlcNAc)_5(Man)_3$
			3596	$(Fuc)_1(Gal)_2(GlcNAc)_4(Man)_3(NeuAc)_1$
			3899	$(Fuc)_1(Gal)_2(GlcNAc)_4(Man)_3(NeuAc)_2$
A_{23-24} (304–339)	N-328	3844	—	—
			5450	$(Fuc)_1(Gal)_1(GlcNAc)_4(Man)_3$
			5615	$(Fuc)_1(Gal)_2(GlcNAc)_4(Man)_3$
			5910	$(Fuc)_1(Gal)_2(GlcNAc)_4(Man)_3(NeuAc)_1$
			6201	$(Fuc)_1(Gal)_2(GlcNAc)_4(Man)_3(NeuAc)_2$
A_{30-31} (396–422)	N-419	2957	—	—
			4557	$(Fuc)_1(Gal)_1(GlcNAc)_4(Man)_3$
			4719	$(Fuc)_1(Gal)_2(GlcNAc)_4(Man)_3$
			5011	$(Fuc)_1(Gal)_2(GlcNAc)_4(Man)_3(NeuAc)_1$
			5303	$(Fuc)_1(Gal)_2(GlcNAc)_4(Man)_3(NeuAc)_2$
A_{32-33} (423–470)	N-443	5488	—	—
			7840	$(Fuc)_1(Gal)_2(GlcNAc)_4(Man)_3(NeuAc)_2$

[a] Asp-*N* proteolytic peptide.
[b] GlcNAc, *N*-acetylglucosamine; Man, mannose; Gal, galactose; NeuAc, neuraminic acid.

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