^1H , ^{13}C , and ^{15}N resonance assignments of the second immunoglobulin domain of neurolin from *Carassius auratus*

Žarko Kulić · Günter Fritz · Heiko M. Möller

Abstract Neurolin is a member of the superfamily of immunoglobulin-like cell surface receptors. It is essential during neuronal development in the model organism *Carassius auratus* (goldfish) and involved in the guidance of the growing axon. Among the five extracellular immunoglobulin (Ig) domains, the second Ig domain is crucial for axon pathfinding. In the present study, we report the NMR assignment and secondary structure prediction of the second Ig domain of neurolin.

Keywords Neurolin · Goldfish · Neuronal receptor · Axonal pathfinding

Biological context

Neurolin is a cell surface receptor expressed in the growing axons of retinal ganglion cells of *Carassius auratus* (goldfish) and *Danio rerio* (zebrafish) and plays a key role in axonal pathfinding during embryonal development (Stuermer and Bastmeyer 2000). Moreover, in case of neuronal lesions, the expression of neurolin is upregulated contributing to the spontaneous neuronal regeneration capability in goldfish (Paschke et al. 1992). The mature receptor is composed of an extracellular moiety (23–498), a single transmembrane helix (499–522) and a short intracellular domain

(523–555). The extracellular region bears five immunoglobulin (Ig) domains: the two N-terminal domains belong to the V-type whereas the subsequent domains belong to the C-type Ig domains. Neurolin is glycosylated on domains 1, 2, 4 and 5 resulting in a total mass of approximately 90 kDa (Denzinger et al. 1999). The role of the individual domains in axonal pathfinding was examined using monoclonal antibodies directed against the different domains. Injection of a monoclonal antibody specific for the second domain of neurolin (NIg2) substantially disturbed the directed growth of the axons towards the optic disc in goldfish retinae. In contrast, antibodies blocking domains 1 and 3 perturbed the tight bundling of growing axons (Leppert et al. 1999). It was proposed that NIg2 recognizes an axon guiding component that occurs in a concentration gradient from the inner retinal area to the periphery. However, the detailed molecular mechanisms are still unclear. Noteworthy, the function of neurolin might be conserved in mammalia. It was shown recently that ALCAM (activated leukocyte cell adhesion molecule) a close homologue of neurolin is expressed on axons and potentiates nerve growth factor (NGF)-induced neurite outgrowth (Wade et al. 2012). In order to get insight into the structure and function of NIg2 isotope labelled protein was expressed heterologously in *E. coli*. Here we report the resonance assignment of NIg2 as a first step towards structural investigations of this protein by NMR.

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Methods and experiments

Expression and purification

A pET15b vector containing the cDNA coding for neurolin's Ig domain 2 (UniProtKB accession number Q90304, residues 130–240) was purchased from GENSCRIPT INC.

The construct comprised a cleavable N-terminal 12.2 kDa His₆-SUMO1-tag, serving as an expression enhancement (Malakhov et al. 2004) followed by the neurolin domain consisting of 111 residues. *E. coli* strain BL21 (DE3) Origami B (NOVAGEN) was transformed according to Inoue et al. (1990), and transformants were selected on DYT-Agar plates containing 100 µg/mL ampicillin. For expression 2 mL growth medium was inoculated with a single colony of the transformed *E. coli* strain and grown overnight at 37° C. This culture was used to inoculate 0.5 L medium. The growth medium contained M9 salts, supplemented with BME vitamin solution (SIGMA ALDRICH), 100 mg/L isotope labeled Celtone powder (CIL) and the selection markers for the plasmid and the strain, respectively (100 µg/mL ampicillin, 15 µg/mL kanamycin and 12 µg/mL tetracyclin). As carbon and nitrogen sources, 0.1 % (w/v) ¹⁵NH₄Cl and 0.4 % (w/v) ¹³C-glucose were added. The cells were grown at 37 °C to an OD₆₀₀ of 0.6. The temperature was decreased to 25 °C and gene expression was induced with 0.25 mM isopropyl-β-D-thiogalactoside. Cells were harvested by centrifugation after 16 h of expression.

The bacterial pellets were resuspended in purification buffer (50 mM Tris pH 8.0, 0.5 M NaCl and 5 % (v/v) glycerol) supplemented with a protease inhibitor cocktail tablet (ROCHE), 1 mM MgCl₂ and a spatula tip of DNaseI (ROCHE) and ruptured by three passages through a French pressure cell. Cell debris was removed by ultracentrifugation at 100,000g for 1 h. The supernatant was loaded on 5 mL Nickel-chelating-Sepharose matrix (GE). After extensive washing with purification buffer, the fusion protein was eluted with 0.5 M imidazole. To remove imidazole, the eluate was dialyzed against purification buffer in a 10 kDa cut-off membrane (SPECTRA/POR)

overnight. In order to cleave off the SUMO1 domain, 1 mg of His₆-tagged ULP1 protease was added per 10 mg of fusion protein to the dialysis tube. The relatively high amount of protease was used in order to take into account its decreased activity in 0.5 M NaCl buffer (Malakhov et al. 2004). The protease and the cleaved SUMO1 domain were separated from the Nlg2 domain by another nickel affinity chromatography step. Nlg2 was further purified by gel filtration using a HiLoad 16/60 Superdex75 prep grade column (GE). Except dialysis and cell rupture, all purification steps were performed at 4 °C. Fractions from each purification step were analyzed by SDS-PAGE for the content of Nlg2.

The ULP1 protease was prepared using a slightly modified protocol according to Malakhov et al. (2004). After purification of the protein at 4 °C in 40 mM HEPES pH 7.5, 150 mM KCl, 20 mM β-mercaptoethanol, glycerol was added to a final concentration of 8 % (v/v) and aliquots were frozen in liquid nitrogen and stored at -20 °C.

NMR data collection, processing and analysis

All NMR experiments were acquired at 302 K on a BRUKER AVANCE III 600 MHz spectrometer equipped with a TCI-H/C/N triple resonance cryoprobe with an actively shielded Z-gradient. NMR samples were prepared in H₂O containing 5 % D₂O for experiments detecting exchangeable protons, and in 100 % D₂O for experiments involving solely carbon bound protons. Sample solutions contained 50 mM NaPi pH 7.0, 100 mM NaCl, 0.1 mM PMSF, and 4 mM NaN₃. Chemical shifts were referenced with respect to internal TSP. ¹³C and ¹⁵N chemical shifts were referenced indirectly as described by Bax and Subramanian (1986) and Wishart et al. (1995), respectively.

Fig. 1 [¹H, ¹⁵N] HSQC spectrum of 0.9 mM ¹³C, ¹⁵N labelled Neurolin Ig2 in 50 mM NaPi pH 7.0, 100 mM NaCl, 0.1 mM PMSF, 4 mM NaN₃ in H₂O/D₂O 95/5 (v/v) acquired at 600 MHz and 302 K. Peak assignments are indicated with *one letter code* and residue number. Except for N⁹, N¹⁰, N³¹ and Q⁹⁸ all resonances of side chain carboxamides have been assigned and are connected by dotted horizontal lines

Fig. 2 Secondary structure prediction according to analysis of backbone chemical shifts by TALOS+ (Shen et al. 2009). β sheets and α helices are indicated by red arrows and blue cylinders, respectively. The chemical shift based prediction is consistent with $^3J_{\text{HNH}\alpha}$ coupling constants derived from an HNHA experiment

Backbone resonances were assigned by a combination of HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO and HNHA experiments. Side-chain chemical shifts were assigned by a combination of CC(CO)NH-TOCSY, H(CCCO)NH-TOCSY, ^1H , ^{15}N -TOCSY-HSQC, H(C)CH-TOCSY, (H)CCH-TOCSY, H(C)CH-COSY, ^1H , ^{15}N -NOESY-HSQC and ^1H , ^{13}C -NOESY-HSQC experiments. Resonances of the aromatic residues could be obtained by (HB)CB(CGCD)HD, (HB)CB(CGCDCE)HE and (HB)CB(CGCC-TOCSY) H_{ar} experiments.

Acquired data were processed and analyzed using Bruker Topspin (v3.0) and CARA (Keller 2004) software, respectively.

Assignments and data deposition

An almost complete assignment of Nlg2 could be achieved. Figure 1 shows the backbone assignment of Nlg2. Uniform peak width and large signal dispersion are suggestive of a globular, β -sheet-rich protein domain. This is supported by the distribution of secondary structure as determined from backbone chemical shifts (Fig. 2). In detail, we could assign 94.2 % of the backbone nuclei including H^{N} , N, C^{α} , H^{α} and C' . 92.9 % of the non-exchangeable ^1H resonances could be assigned, including 100 % of aromatic ^1H resonances and 92.6 % of the aliphatic ^1H resonances. 94.1 % of the ^{13}C resonances originating from protonated carbons could be assigned, including 100 % of aromatic ^{13}C resonances and 93.7 % of the aliphatic ^{13}C resonances. Deficiencies in the completeness of assignment arose from severe resonance overlap of the eight prolines, two of which are in a row, and five other amino acids (Lys¹, Asn⁹, Asn¹⁰, Asn³¹ and Lys⁹¹) that do not exhibit a ^1H , ^{15}N -HSQC peak presumably due to exchange broadening. A detailed list of chemical shifts of Nlg2 has been deposited to the BioMagResBank database with accession number 18155.

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