Available online at www.sciencedirect.com
ScienceDirect
Protein Expression and Purification 59 (2008) 47–54
Protein Expression & Purification
www.elsevier.com/locate/yprep

Expression and purification of neurolin immunoglobulin domain 2 from *Carrassius auratus* (goldfish) in *Escherichia coli*

Christina Drees [a], Claudia A.O. Stürmer [b], Heiko M. Möller [a,*], Günter Fritz [b,*]

[a] *Department of Chemistry, University of Konstanz, 78457 Konstanz, Germany*
[b] *Department of Biology, University of Konstanz, Universitaetsstrasse 10, Postfach M 665, 78457 Konstanz, Germany*

Received 26 September 2007, and in revised form 25 December 2007
Available online 26 January 2008

Abstract

The immunoglobulin superfamily protein neurolin plays a central role during differentiation and development of retina ganglion cells in goldfish. As shown in earlier work, blockage of the second immunoglobulin domain (Ig2) of neurolin with domain-specific antibodies causes severe pathfinding defects of growing axons in the retina. Thus Ig2 of neurolin was identified as the critical domain for axon guidance. In the present study we have developed a protocol for expression and purification of neurolin-Ig2 suitable for structure analysis, functional studies and ligand identification. Neurolin was expressed in Rosettagami and Origami strains of *Escherichia coli* which is deficient in glutathione- and thioredoxin reductase facilitating proper formation of the disulfide bond in the cytoplasm. The protein was purified via an N-terminal His_6-tag by Ni^{2+} affinity and size exclusion chromatography. After purification the His_6-tag was cut-off without loss of solubility. Analytical size exclusion chromatography revealed an apparent molecular mass for neurolin-Ig2 in agreement with a non-covalent homodimer. Analysis of CD and FTIR spectra gave a secondary structure content typical for Ig domains.

Keywords: Neurolin; Axonal growth; Axonal regeneration; Immunoglobulin; Neuronal development

Neurolin was identified as a growth-related neuronal cell surface protein in goldfish [1]. It is a 90 kDa glycosylated protein of the immunoglobulin (Ig) superfamily [2,3], consisting of five extracellular Ig domains, a single transmembrane helix, and a highly conserved cytoplasmic domain. Homologs, designated ALCAM, SC1/BEN, DM-GRASP, have been found in human, rodents, and chicken, respectively. Expression of neurolin is upregulated on axons of retinal ganglion cells during growth and axonal regeneration. In the retina neurolin is selectively expressed by the newly differentiated retinal ganglion cells and their growing axons [3]. In an earlier study, neurolin function was probed by *in vivo* studies with antibodies specifically directed against Ig domains 1, 2, and 3. Blockage of neurolin function led to severe pathfinding mistakes [4,5]. Instead of growing towards the optic disk, axons left their fascicle of origin and turned around or entirely lost orientation [4,5]. It was shown that the second Ig domain of neurolin is preferentially involved in this pathfinding function [4]. Neurolin function is not exclusive for goldfish. In zebrafish, neurolin has a similar function leading to massive defects of neuronal development upon application of neurolin-specific antibodies [6]. Recently, it was reported that a mouse lacking the neurolin-homolog BEN showed retinal pathfinding errors and dysplasia [7]. How neurolin exerts its function in these organisms is not well understood, and several alternative mechanisms are being discussed [4,5]. Like its closest homolog ALCAM in mammalia, neurolin might form homo- or heterophilic contacts by *cis* and/or *trans* interactions. However, this has not been shown for neurolin so far. The experimental data from *in vivo* experiments suggest that neurolin-Ig2, which is crucial for pathfinding function, interacts with a soluble or

* Corresponding authors. Fax: +49 7531 88 5174 (H.M. Möller), +49 7531 88 2966 (G. Fritz).
E-mail addresses: heiko.moeller@uni-konstanz.de (H.M. Möller), guenter.fritz@uni-konstanz.de (G. Fritz).

1046-5928/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2007.12.015

membrane-bound guiding component, and a gradient of the guiding molecule might direct axonal growth. Hence, neurolin plays a key role in the development of the visual system and is also involved in regeneration of the fish (goldfish, zebrafish) optic nerve representing a model system for neuronal development and regeneration. Characterizing structure and function of neurolin will improve our understanding of axon guidance and regeneration, particularly since the mammalian central nervous system has lost the capacity of regeneration, e.g. after spinal cord injury or stroke.

Here we report on an efficient protocol for the production of recombinant neurolin-Ig2 in *Escherichia coli*, which allows to express neurolin-Ig2 for structural characterization by X-ray crystallography and NMR spectroscopy. The expression of neurolin-Ig2 in *E. coli* has the advantage that the protein lacks glycosylation and is amenable for isotope-labeling with ^{15}N and ^{13}C for multidimensional NMR experiments or SeMet for phase determination in X-ray crystallography. Furthermore, the recombinant neurolin-Ig2 preparation is suitable for ligand capture experiments aimed at identifying a soluble guidance compound, and for extensive *in vivo* experiments which will lead to a better understanding of neurolins function.

Materials and methods

Bacterial strains

Escherichia coli JM109 was used for amplification of plasmids and *E. coli* BL21(DE3) Origami B (Novagen), *E. coli* Rosettagami BL21(DE3) B, *E. coli* BL21(DE3) B (Novagen), and *E. coli* BL21(DE3) Rosetta B (Novagen) were used for recombinant expression of neurolin-Ig2. Competent *E. coli* cells were prepared according to Inou et al. [8].

Cloning

Cloning of neurolin-Ig2 was performed by Trenzyme (Konstanz, Germany). The cDNA corresponding to the Ig2 domain (amino acid residues 132–228) of goldfish neurolin was amplified by PCR using a plasmid containing the whole cDNA [4]. The primers used for the cloning of neurolin-Ig2 were designed using the nucleotide sequence published in the GenBank database (Accession No. L25056) and contained the two restriction sites NdeI and XhoI. Furthermore, a stop codon was introduced before the XhoI site. The following forward (5′-AAA CAT ATG TCA GCC CCT GTA ATC AAA AAC AAC G-3′) and reverse primers (5′-ACT CGA GAT TAC TGG TCG GGG CCC ATC AC-3′) were used to amplify neurolin-Ig2 using the following PCR conditions: 2 min at 94 °C followed by 30 cycles of 15 s at 94 °C, 30 s at 60 °C, and 2 min at 72 °C; the final extension was for 7 min at 72 °C using Phusion polymerase (New England Biolabs). The PCR products were analyzed on 1.5% agarose gel and extracted from the agarose gel using the DNA extraction kit (Quiagen). The DNA was ligated blunt-end into vector pAlli10 (Trenzyme) and the sequence was checked by sequencing using standard M13 forward primer. The resulting pAlli10-neurolin-Ig2 was digested with NdeI and XhoI (New England Biolabs) separated on a 1% agarose gel and extracted from the agarose gel using QIAquick Gel Extraction Kit (Qiagen). The pET15b vector was digested with the same two enzymes and purified from 1% agarose gels. The digested insert from pAlli10-neurolin-Ig2 was ligated into the linearized vector pET15b at 25 °C for 5 min using the Rapid Ligation Kit (Roche Applied Science). Vector pET15b contains the sequence of an N-terminal hexa-histidine tag (His_6-tag) to facilitate purification of neurolin-Ig2 and a thrombin-cleavage site for post-translational removal of the His_6-tag. The resulting vector pET15b-neurolin-Ig2 was transformed into competent *E. coli* JM109 cells and amplified.

Expression and purification

Competent *E. coli* strains were transformed with 5 ng of plasmid DNA. Transformed cells were selected on DYT agar plates containing 100 μg ml^{-1} ampicillin (AppliChem), 15 μg ml^{-1} kanamycin (AppliChem), for *E. coli* BL21(DE3) Origami B or additionally 34 μg ml^{-1} chloramphenicol (Fluka) for selection of the *E. coli* Rosettagami BL21(DE3) B strain. A single colony was used to inoculate 25 ml of DYT medium containing 0.2% glucose and the same amount of antibiotics as listed above. The culture was incubated overnight at 37 °C and 220 rpm. Cells were pelleted by centrifugation at 5000*g* at 4 °C for 5 min and the supernatant culture medium was discarded in order to remove extracellular β-lactamases. The pellet was resuspended in 12 ml fresh DYT medium and 5 ml of this suspension were used to inoculate 500 ml DYT medium. Cells were grown at 37 °C to an OD_{600nm} of about 0.6. Then temperature was decreased to 25 °C and the culture was grown to an OD_{600nm} of about 1.2. Expression of neurolin-Ig2 was induced by addition of 0.5 mM IPTG[1] (AppliChem) and cells were allowed to grow at 25 °C for another 12–14 h. Cells were harvested by centrifugation at 8000*g* at 4 °C for 20 min. The cell pellets were used immediately for protein purification or frozen in liquid nitrogen and stored at −70 °C.

All purification steps were performed at 4 °C. Ten grams of wet weight cells were suspended in ice-cold 40 ml 50 mM KH_2PO_4, pH 7.4. Protease inhibitors (Complete, Roche Diagnostics) and 0.4 mM PMSF (AppliChem) were added. In order to remove large DNA fragments released upon cell rupture 5 mM $MgCl_2$ and a spatula tip of DNAse (Roche Diagnostics) were added. Cells were ruptured by three passages through a French pressure cell at

[1] *Abbreviations used:* IPTG, isopropyl-β-D-thiogalactopyranoside; FTIR, Fourier transformed infra red.