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Nectin-like molecule 1 is a high abundance protein in cerebellar neurons

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Summary. Nectins and Nectin-like molecules belong to the Ca-independent immunoglobulin superfamily of cell adhesion molecules and are mandatory for various cellular functions such as morphogenesis, differentiation and proliferation. Among them, Nectin-like molecule 1 (Necl-1) is unique for its exclusive expression in the brain where it is localized at the contact sites among axon terminals and glia cell processes, cooperatively forming synapses.

We hereby aimed to unambiguously characterize Necl-1 at the protein level in rat brain. Rat cerebellar neurons were lysed, proteins extracted and run on two-dimensional gel electrophoresis with subsequent in-gel digestion and mass spectrometrical (MS/MS) analysis of protein spots. One spot at pI 5.96 with an observed molecular weight of 26 kDa was identified as Nectin-like molecule 1. MS/MS analyses of three matching peptides warranted unambiguous identification for the first time. Additionally, we verified the result by immunoblotting and detected two bands at about 48 kDa and 60 kDa.

The proposed roles of Necl-1 in cerebellar morphogenesis as well as plasticity of synapses challenge further research on its function in more detail and we hereby provide a fair analytical tool for the unequivocal determination of Necl-1, independent of antibody availability and specificity.

Keywords: Nectin-like molecule 1 – Cerebellum – Neuron – Mass spectrometry – Proteomics

Introduction

In multicellular organisms cell–cell adhesion plays important roles in various cellular processes including morphogenesis, differentiation, proliferation and migration. Especially in the brain, cell–cell junctions and contacts are essential for neuronal cell migration, axon bundle formation and plasticity of synapses (Redies and Takeichi, 1996; Kakunaga et al., 2005).

Adhesion sites in the central nervous system are mediated by homo- or hetero-trans-interactions of cell adhesion molecules (CAMs) comprising two families: the Ca-dependent cadherin-superfamily and the Ca-independent immunoglobulin (Ig) superfamily (Nakanishi and Takai, 2004).

Recently, nectins have emerged as a novel class of Ig-like CAMs, being involved in a variety of cell–cell junctions and contacts, particularly in forming puncta adherentia junctions at synapses of a certain set of neurons (Nakanishi and Takai, 2004). Nectins form a family of four members, nectin-1–4. They all consist of one extracellular region with three Ig-like loops, one transmembrane region and one cytoplasmic region linked to the actin cytoskeleton by binding afadin, a F-actin-binding protein. Nectin-1 and -3 are asymmetrically localized at the synapses of hippocampal neurons, forming hetero-trans-dimers at the presynaptic and postsynaptic side. Inhibition of these adhesion sites prevents formation of synapses in cultured neurons (Mizoguchi et al., 2002).

Five molecules possessing similar domain structures to those of nectins have recently emerged and are called nectin-like molecules 1–5. All of these molecules are composed of one extracellular region with three Ig-loops, one transmembrane region and one cytoplasmic tail, and appear to have roles different from those of nectins (Takai et al., 2003).

Among them, Nectin-like molecule 1 (synonyms: tumor suppressor of lung cancer like gene 1 (TSLL1); SynCAM3; Necl-1) is unique for its exclusive expression in adult and fetal brain and in neurogenic cells (Fukuhara et al., 2001; Takai et al., 2003; Kakunaga et al., 2005). Necl-1 is localized at the contact sites among axon terminals and glia cell processes that cooperatively form synapses, serving as a CAM at non-junctional cell–cell adhesion sites in the central nervous system (Kakunaga et al., 2005).

It shows Ca-independent homophilic cell–cell adhesion activity and Ca-independent heterophilic cell–cell adhesion

activity with Necl-2, nectin-1 and nectin-3, but not with Necl-5 or nectin-2 (Takai et al., 2003; Sakisaka and Takai, 2004).

The ability of Necl-1 to interact with nectin-1 and -3 suggests involvement in the process of remodeling synapses in a neural activity-dependent manner as well as in cerebellar morphogenesis (Takai et al., 2003; Sakisaka and Takai, 2004).

Since nectin-1 and -3 are involved in the formation of puncta adherentia junctions in the hippocampus, it is suggested that Necl-1 is recruited to the nectin-1 and -3-based cell–cell adhesion in the process of synapse formation. After Necl-1 is assembled to the primordial synapses, it may be translocated to the axonal shafts where Necl-1 may function to maintain axon bundle formation (Irie et al., 2004). Moreover, Necl-1 mediates segregation of synapses from neighboring axons, preventing neurotransmitters from diffusing and stimulating neighboring synapses (Irie et al., 2004).

Necl-1 does not bind afadin, but has the potential to bind MPP3, one of the human homologues of a drosophila tumor suppressor gene, Discs large (Dlg3), a novel membrane-associated guanylate kinase family member that is highly expressed in brain (Smith et al., 1996; Kakunaga et al., 2005). Recent work also suggests binding of Necl-1 to protein 4.1N. Since protein 4.1 is involved in the reorganization of actin cytoskeleton it is proposed that Necl-1 modulates the structure or function of cell–cell junctions through protein 4.1 (Zhou et al., 2005).

Furthermore, Necl-1 demonstrates structural homology to Tumor suppressor in lung cancer 1 (TSLC1) of 76% in the cytoplasmic domain as well as 37% in the overall amino acid sequence (Fukuhara et al., 2001) suggesting a possible role as a tumor suppressor gene.

The relevance of Necl-1 in basic neuroscience, development and tumorigenesis challenged the search for a reliable analytical tool (Oh et al., 2005). Necl-1 was described so far at the nucleic acid and immunochemical level. In this report we propose a proteomic method for the unambiguous determination of Necl-1, that was observed to be a high abundance protein in rat cerebellar neurons.

Materials and methods

Primary cultures of rat cerebellar neurons

Primary cultures were obtained from the Laboratory of Neurobiology, Fundación Valenciana de Investiganciones Biomédicas, Valencia, Spain. All animal procedures were approved by the Institute and met the guidelines of the European Union for care and management of experimental animals. Cultures enriched in granule neurons were obtained from cerebella of 7-day-old Wistar rats as previously described (Stein et al., 1987). Pups were killed by decapitation; cerebella were rapidly dissected in ice-cold phosphate-buffered saline (PBS) and placed in a Petri dish. After removal of PBS, tissue was cut into small fragments with a sterile surgical blade. To dissociate enzymatically, minced cerebella were incubated with 3 mg/ml Dispase, grade II (Boehringer Mannheim, Mannheim, Germany) for 30 min in a 5% CO_2 incubator at 37 °C. The supernatant was removed and the cerebellar tissue was rinsed with 1 mM EDTA in PBS. Once the fragments have settled, the supernatant was removed and basal Eagle's medium (BME) containing 40 µg/ml DNase I (Boehringer Mannheim) was added. The fragments were gently dissociated four times with a 10-ml plastic pipette and incubated for 20 min in DNase solution in a 5% CO_2 incubator at 37 °C. The tissue was then dissociated again three to four times with a 10-ml plastic pipette and the cellular suspension was filtered through a mesh with a pore size of 90 µm. The filtered cell suspension was centrifuged at $400 \times g$ for 5 min, and the cell bottom was rinsed twice with BME, by centrifuging at $400 \times g$ for 5 min each time. Finally the cells were resuspended in complete medium composed of BME, 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 µg/ml gentamycin, 5 µg/ml fungizone and 25 mM KCl. Cells were counted and cell viability was measured by using Trypan blue staining. Cells were seeded onto poly-L-lysine-coated plates (60-diameter) at 1.5×10^6 cells/ml of medium and, after 20 min at 37 °C the medium containing unattached cells was removed and fresh medium was added. Proliferation of non-neuronal cells was prevented by adding 10 µM cytosine arabinoside approximately 20 h after seeding. Glucose (5.6 mM final concentration) was added to the culture medium twice a week. Postnatal cerebellar neurons prepared in this manner adhere, extend processes soon after plating and survive for long periods of time with a high viability. Cultures were kept in humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The cultures were used after 10–13 days in vitro (DIV). All reagents for cell culture were from Gibco BRL (Life Technologies S.A., Barcelona, Spain).

Sample preparation

Harvested cells were washed three times with 10 mL PBS (phosphate buffered saline) (Gibco BRL, Gaithersburg, MD, USA) and centrifuged for 10 min at 800 g at room temperature. The supernatant was discarded and the pellet was suspended in 1.0 ml of sample buffer consisting of 7 M urea (Merck, Darmstadt, Germany), 2 M thiourea (Sigma, St. Louis, MO, USA), 4% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) (Sigma, St. Louis, MO, USA), 65 mM 1,4-dithioerythritol (Merck, Germany), 1 mM EDTA (ethylenediaminetraacetic acid) (Merck, Germany), protease inhibitors complete® (Roche, Basel, Switzerland) and 1 mM phenylmethylsulfonyl chloride. The suspension was sonicated for approximately 15 sec. After homogenisation samples were left at room temperature for 1h and centrifuged at 14,000 rpm for 1 h. The supernatant was transferred into Ultrafree-4 centrifugal filter unit (Millipore, Bedford, MA), for desalting and concentrating proteins. Protein content of the supernatant was quantified by Bradford protein assay system (Bradford, 1976). The standard curve was generated using bovine serum albumin and absorbance was measured at 595 nm.

Two-dimensional gel electrophoresis (2-DE)

Samples prepared from each cell line were subjected to 2-DE as described elsewhere (Oh et al., 2004; Shin et al., 2004, 2005; Weitzdoerfer et al., 2002).

1 mg protein was applied on immobilized pH 3–10 nonlinear gradient strips at their basic and acidic ends. Focusing was started at 200 V and voltage was gradually increased to 8,000 V over 31 h and then kept constant for a further 3 h (approximately 150,000 Vh totally). After the first dimension, strips (18 cm) were equilibrated for 15 min in the buffer containing

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  1 MGAPSALPLL LLLACSWAPG GANLSQDDSQ PWTSDETVVA GGTVVLKCQV
 51 KDHEDSSLQW SNPAQQTLYF GEKRALRDNR IQLVSSTPHE LSISISNVAL
101 ADEGEYTCSI FTMPVRTAKS LVTVLGIPQK PIITGYKSSL REKETATLNC
151 QSSGSKPAAQ LTWRKGDQEL HGDQTRIQED PNGKTFTVSS SVSFQVTRED
201 DGANIVCSVN HESLKGADRS TSQRIEVLYT PTAMIRPEPA HPREGQKLLL
251 HCEGRGNPVP QQYVWVKEGS EPPLKMTQES ALIFPFLNKS DSGTYGCTAT
301 SNMGSYTAYF TLNVNDPSPV PSSSSTYHAI IGGIVAFIVF LLLILLIFLG
351 HYLIRHKGTY LTHEAKGSDD APDADTAIIN AEGGQSGGDD KKEYFI
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Fig. 1. Amino acid sequence of rat Nectin-like molecule 1 (accession number Q99N28) with peptides matched (bold) demonstrating sequence coverage of 18%. Additionally, the trans-membrane domain is shown in italics (amino acids 327–349)

6 M urea, 20% glycerol, 2% SDS, 2% DTT and then for 15 min in the same buffer containing 2.5% iodo-acetamide instead of DDT. After equilibration, strips were loaded on 9–16% gradient sodium dodecyl-sulfate polyacrylamide gels for second-dimensional separation. Gels ($180 \times 200 \times 1.5$ mm) were run at 40 mA per gel. Immediately after the second dimension run gels were fixed for 18 h in 50% methanol, containing 10% acetic acid, the gels were then stained with Colloidal Coomassie Blue (Novex, San Diego, CA) for 12 h on a rocking shaker. Molecular masses were determined by running standard protein markers (Biorad Laboratories, Hercules, CA) covering the range 10–250 kDa. pI values 3–10 were used as given by the supplier of the immobilized pH gradient strips (Amersham Bioscience, Uppsala, Sweden). Excess of dye was washed out from the gels with distilled water and the gels were scanned with ImageScanner (Amersham Bioscience).

Electronic images of the gels were recorded using Adobe Photoshop and Microsoft Power Point Softwares.

MALDI-TOF and MALDI-TOF/TOF – mass spectrometry (MS)

Spots were excised with a spot picker (PROTEINEER sp™, Bruker Daltonics), placed into 96-well microtiter plates and in-gel digestion and sample preparation for MALDI analysis were performed by an automated procedure (PROTEINEER dp™, Bruker Daltonics). Briefly, spots were excised and washed with 10 mM ammonium bicarbonate and 50% acetonitrile in 10 mM ammonium bicarbonate. After washing, gel plugs were shrunk by addition of acetonitrile and dried by blowing out the liquid through the pierced well bottom. The dried gel pieces were reswollen with 40 ng/μl trypsin (Promega, Madison, WI, USA) in enzyme buffer (consisting of 5 mM octyl β-D-glucopyranoside (OGP) and 10 mM ammonium bicarbonate) and incubated for 4 hrs at 30 °C. Peptide extraction was performed with 10 μl of 1% TFA in 5 mM OGP. Extracted peptides were directly applied onto a target (AnchorChip™, Bruker Daltonics) that was loaded with α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics) matrix thinlayer. The mass spectrometer used in this work was an Ultraflex™ TOF/TOF (Bruker Daltonics) operated in the reflector for MALDI-TOF peptide mass fingerprint (PMF) or LIFT mode for MALDI-TOF/TOF with a fully automated mode using the FlexControl™ software. An accelerating voltage of 25 kV was used for PMF. Calibration of the instrument was performed externally with $[M+H]^+$ ions of angiotensin I, angiotensin II, substance P, bombesin, and adrenocorticotropic hormones (clip 1–17 and clip 18–39). Each spectrum was produced by accumulating data from 200 consecutive laser shots for PMF. Those samples which were analysed by PMF from MALDI-TOF were additionally analysed using LIFT-TOF/TOF MS/MS from the same target using two MS/MS modes: laser-induced dissociation (LID) and collision-induced dissociation (CID). In the LID-MS/MS mode using a long-lifetime N_2 laser, all ions were accelerated to 8 kV under conditions promoting metastable fragmentation in the TOF1 stage. After selection of jointly migrating parent and fragment ions in a timed ion gate, ions were lifted by 19 kV to high potential energy in the LIFT cell. After further acceleration of the fragment ions in the second ion source, their masses could be simultaneously analysed in the reflector with high sensitivity. In addition to the LID-MS/MS mode, a high-energy CID mode is adapted for distinguishing leucine and isoleucine by their different side-chain fragmentation. Argon gas as a collision gas was used and about 1500 shots were summed to achieve spectra. PMF and LIFT spectra were interpreted with the Mascot software (Matrix Science Ltd., London, UK). Database searches, through Mascot, using combined PMF and MS/MS datasets were performed via BioTools 2.2 software. A mass tolerance of 25 ppm and MS/MS tolerance of 0.5 Da and 0 missing cleavage site were allowed and oxidation of methionine residues was considered. The probability score calculated by the software was used as criterion for correct identification (http://www.matrixscience.com/help/scoring-help.html).

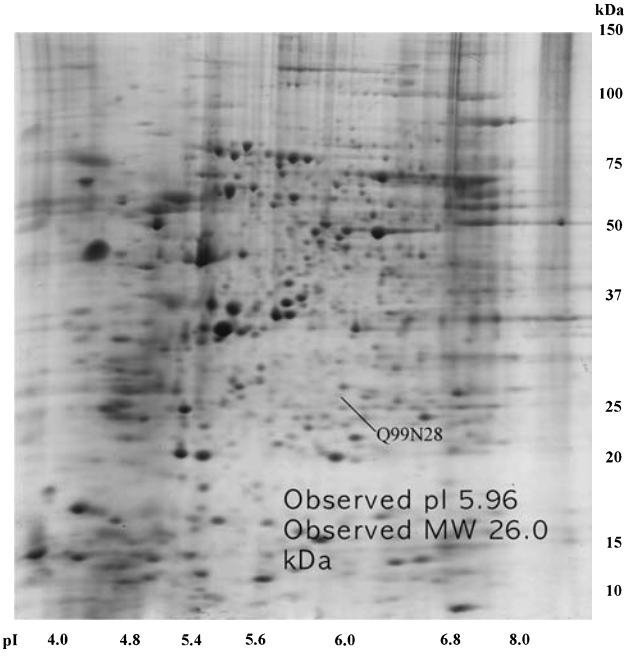

Fig. 2. 2-DE gel image of rat cerebellar neurons, showing rat Nectin-like molecule 1 (accession number: Q99N28). Proteins were extracted and separated on an immobilized pH 3–10 non-linear gradient strip followed by separation on a 9–16% gradient polyacrylamide gel. Gels were stained with Coomassie blue and spots were analysed by MALDI-MS and MS/MS

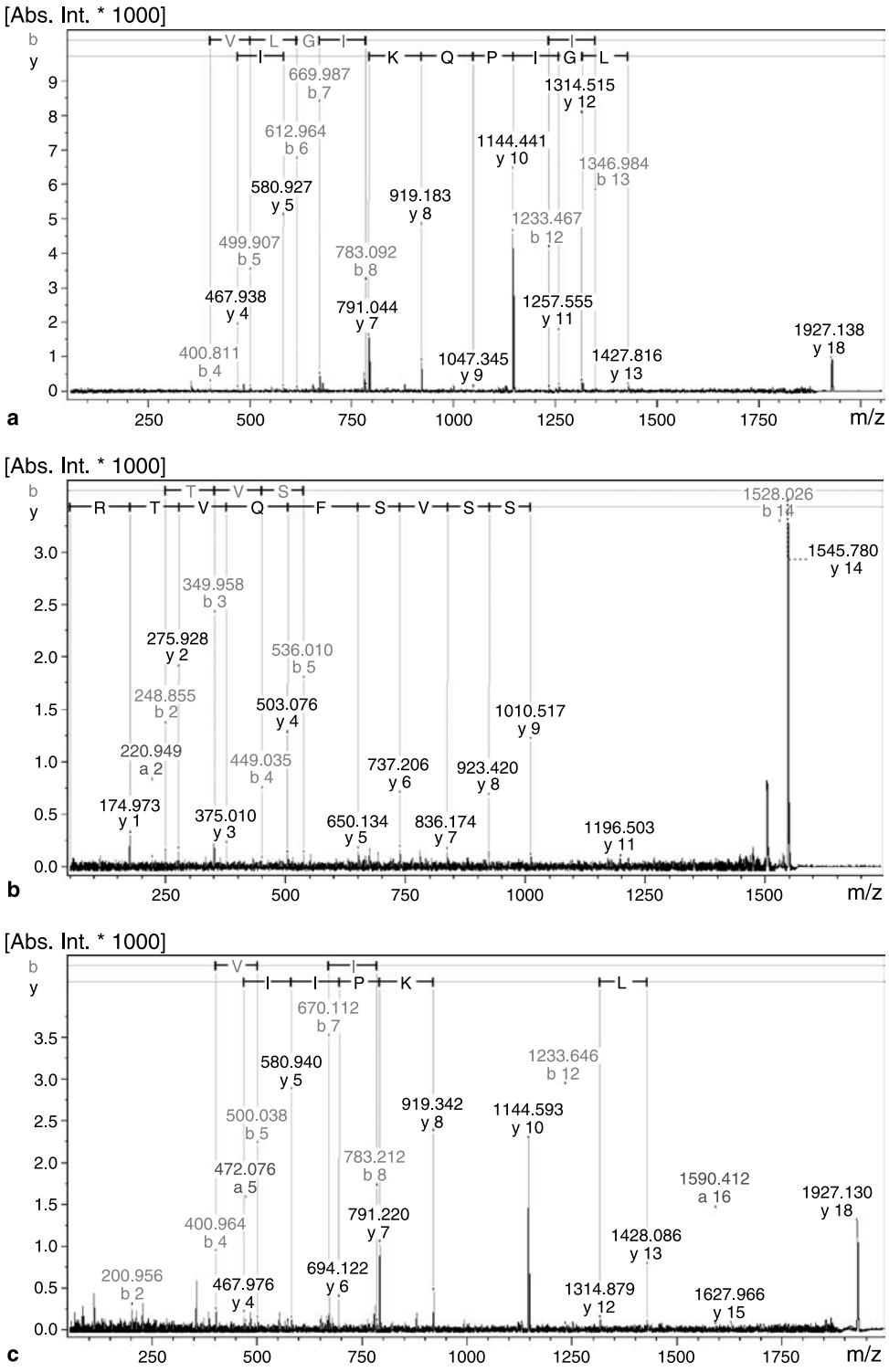

Fig. 3. MS/MS analysis of rat Nectin-like molecule 1: The parent ions (m/z: 1545.78, 1927.15, 1927.15) were selected for further analysis by MS/MS and the amino acid sequences TFTVSSSVSFQVTR (**a**), SLVTVLGIPQKPIITGYK (**b**), and SLVTVLGIPQKPIITGYK (**c**) were confirmed by analyzing the ions derived from the peptide ions. Ions a + b were analyzed by laser-induced dissociation (LID) and ion c by collision-induced dissociation (CID)

Western blot analysis

Different amounts of protein (10–50 µg/lane) were loaded onto SDS-polyacrylamide gels (10% acrylamide/bisacrylamide) separated at 80 V (constant) and electrophoretically transferred to 0.45 nitrocellulose membranes (Schleicher Schuell, Dassel, Germany). Filters were blocked with 5% skimmed milk, 0.5% Tween 20, followed by

incubation overnight at 4 °C with a polyclonal anti-Necl-1 antibody at a 1:1000 dilution (supplied by Yoshimi Takai; see Kakunaga et al., 2005). Secondary antibody reaction was carried out by incubating filters with horseradish peroxidase conjugated with donkey anti-rabbit antibody (Amersham International, UK) at a 1:5000 dilution for 1 h at room temperature. Signal was developed using the ECL kit according to the instructions of the manufacturer (Amersham International, UK).

Database searches

Bioinformatic characterization of Nectin-like molecule 1 (Necl-1) included following databases:

http://www.expasy.org/sprot
http://dip.doe-mbi.ucla.edu
http://string.embl.de
http://interweaver.i2r.a-star.edu.sg
http://www.bind.ca
http://www.ebi.ac.uk/interpro
http://elm.eu.org
http://www.cbs.dtu.dk/services/NetPhos
http://www.cbs.dtu.dk/services/NetOGlyc
http://www.cbs.dtu.dk/services/NetNGlyc
http://www.cbs.dtu.dk/services/TMHMM-2.0.

Results

In the protein extract from rat cerebellar neurons a single spot at an apparent molecular weight of 26 kDa and an observed pI of 5.96 was detected (Fig. 1). The theoretical molecular weight of a 43 337 Da Necl1 protein may not have been observed due to low abundance rather than fragmentation or posttranslational modifications and indeed one O-glycosylation site and two N-glycosylation sites were predicted. Sequence coverage was 18% but the five peptides matched did neither cover the cytoplasmic domain nor the transmembrane domain (Fig. 2).

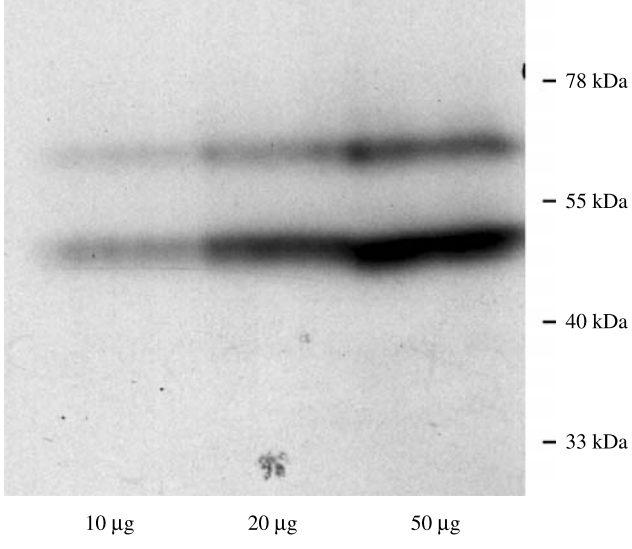

Fig. 4. Western blot images displaying Necl-1 protein expression in rat cerebellar neurons (10, 20, 50 µg protein)

Unambiguous identification was however, obtained by MS/MS analysis; three peptides were fragmented and PSD as well as CID characterisation was carried out (Figs. 3a–c).

In order to verify results from above immunoblotting was performed and two bands, a major band at about 48 kDa and a minor band at about 60 kDa were detected (Fig. 4).

Database searches for posttranslational modifications (ptms) predicted thirty-two phosphorylation sites (NetPhos 2.0 Server): 18 serine, 8 threonine and 6 tyrosine phosphorylation epitopes.

Furthermore, one possible O-glycosylation site (NetOGlyc 3-1 Server) and two N-glycosylation sites (NetNGlyc 1.0 Server) were predicted.

Domain searches in InterPro showed the presence of three functional domains: IPR003598 Immunoglobulin C2 type domain, IPR007110 Immunoglobulin-like domain and IPR003585 Neurexin/syndecan/glycophorin C-like domain.

Prediction of the transmembrane helix revealed involvement of amino acids 327–349 (TMHMM Server v. 2.0).

Motif searches revealed the presence of PDZ binding motifs LIG_PDZ_II,III recognizing short sequences at the carboxy terminus of target proteins and LIG_SH3_1 and 3, motifs recognized by the SH3 signaling structures. A tyrosine-based sorting signal, responsible for the interaction with mu subunit of an adaptor protein complex TRG ENDOCYTIC 2 was demonstrated in addition.

The search for protein-protein interactions using STRING failed to provide any information on interactions, DIP did not contain any experimental data about the protein and InterWeaver listed junctional adhesion molecule 1 (O88792) as a tentative interaction partner in the mouse as well as poliovirus receptor, protein-tyrosine kinase, cell recognition molecule, Caspr2 precursor, vascular endothelial growth factor receptor 2 precursor and Down syndrome cell adhesion molecule like 1 in humans.

The protein Q99N28 was identified as mouse Necl-1 and no entry for a corresponding rat protein was listed in databases. BLAST submission of Q99N28 on EXPASY/SIB revealed only 34% identity of the extracellular domains of a probable homolog Q6AYP5 in the rat, but 62% identities of the transmembrane and cytoplasmic domain (Fig. 5) (Altschul et al., 1997).

Homology between Q99N28 and its human counterpart TSLC1-like 1 (synonyms: Brain immunoglobulin receptor precursor, GAPA225, TSLL1) was 87%.

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Q6AYP5_RAT          Immunoglobulin superfamily, member 4A                476 AA
                    (Predicted)
                    [Igsf4a_predicted] [Rattus norvegicus (Rat)]

 Score = 173 bits (438), Expect = 7e-42
 Identities = 100/294 (34%), Positives = 157/294 (53%), Gaps = 8/294 (2%)

Query: 27  DDSQPWTSDETVVAGGTVVLKCQVKDHEDSSLQWSNPAQQTLYFGEKRALRDNRIQLVSS 86
           D    +T D TV+ G    + CQV   +DS +Q  NP +QT+YF + R L+D+R QL++
Sbjct: 46  DGQNLFTKDVTVIEGEVATISCQVNKSDDSVIQLLNPNRQTIYFRDFRPLKDSRFQLLNF 105

Query: 87  TPHELSISISNVALADEGEYTCSIFTMPVRTAKSLVTVLGIPQKPIITGYKSSLREKETA 146
           +  EL +S++NV+++DEG Y C ++T P + + + +TVL  P+  +I   K +  E E
Sbjct: 106 SSSELKVSLTNVSISDEGRYFCQLYTDPPQESYTTITVLVPPRNLMIDIQKDTAVEGEEI 165

Query: 147 TLNCQSSGSKPAAQLTWRKGDQELHGDQTRIQEDPNGKXXXXXXXXXXQVTREDDGANIV 206
            +NC +  SKPA  + W KG++EL G ++ ++E  +             +V +EDDG  ++
Sbjct: 166 EVNCTAMASKPATTIRWFKGNKELKG-KSEVEEWSD--MYTVTSQLMLKVHKEDDGVPVI 222

Query: 207 CSVNHESLKGADRSTSQRIEVLYTPTAMIR---PEPAHPREGQKLLLHCEGRGNPVPQQY 263
           C V H ++ G +  T + +EV Y P   I+   P     REG    L CE  G P P
Sbjct: 223 CQVEHPAVTG-NLQTQRYLEVQYKPQVQIQMTYPLQGLTREGDAFELTCEATGKPQPVMV 281

Query: 264 VWVKEGSEPPLKMTQESALIF-PFLNKSDSGTYGCTATSNMGSYTAYFTLNVND 316
            WV+   E P          +F   LNK+D+GTY C A++ +G   + + L V D
Sbjct: 282 TWVRVDDEMPQHAVLSGPNLFINNLNKTDNGTYRCEASNTVGKAHSDYMLYVYD 335


 Score = 92.4 bits (228), Expect = 2e-17
 Identities = 43/69 (62%), Positives = 49/69 (70%)

Query: 328 HAIIGGIVAXXXXXXXXXXXXXGHYLIRHKGTYLTHEAKGSDDAPDADTAIINAEGGQSG 387
           HA+IGG+VA             G Y  RHKGTY THEAKG+DDA DADTAIINAEGGQ+
Sbjct: 408 HAVIGGVVAVVVFAMLCLLIILGRYFARHKGTYFTHEAKGADDAADADTAIINAEGGQNN 467

Query: 388 GDDKKEYFI 396
            ++KKEYFI
Sbjct: 468 SEEKKEYFI 476
```

Fig. 5. Predicted rat protein immunoglobulin superfamily, member 4A (accession number Q6AYP5), revealing 34% identity of extracellular domains and 62% identity of transmembrane and cytoplasmic domain to Nectin-like molecule 1 when blasted at the SIB BLAST network service

Discussion

As shown in the results Necl-1 was unambiguously identified in the rat for the first time based upon the mouse amino acid sequence Q99N28 from the ExPASy Proteomics Database (www.expasy.org). Necl-1 was first described in humans as Necl-1 by direct submission to GenBank AF062733. This protein is 87% homologous to mouse Necl-1 which in turn shows homology to a rat nucleic acid sequence of Immunoglobulin superfamily member 4A.

The existence of Necl-1 in rat brain was reported by immunoreactivity using a polyclonal rabbit anti-mouse antibody against the cytoplasmic region of Necl-1 (Kakunaga et al., 2005).

MS and MS/MS showed peptides from extracellular regions and on immunoblotting immunoreactivity with the antibody given above was demonstrated. According to Kakunaga et al. (2005) two bands can be observed at 48 kDa and 60 kDa and this is in agreement with our data in rat cerebellar neurons. The 60 kDa band can be

observed during postnatal days 6–14 (Kakunaga et al., 2005) and corresponds to the neurons we used that were obtained from cerebella of 7-day-old Wistar rats.

Both bands may represent splicing variants that have not been described so far or reflect presence of ptm. No band at 26 kDa as revealed in the two-dimensional gel used for mass spectrometrical identification was observed on Western blots. The underlying cause for the apparent discrepancy may be that the cytoplasmic region was cleaved or modified and therefore the antibody directed against this domain did not recognise Necl-1. Second, migration in the 2D gel could have been changed by ptms as e.g. glycosylation. A fair explanation is that a truncated form of rat Necl-1 exists in analogy to human dendritic cell nectin-like protein 1 short isoform (Q8IZQ9). This isoform does not contain the cytoplasmic domain and therefore this structure cannot be immunoreactive with the afore-mentioned antibody on Western blotting.

We here provide the analytical basis for the first unambiguous determination of Necl-1 in rat brain. Its predominant expression in the CNS as well as high abundance expression in cerebellum and localization at cell–cell junctions in confluent cells strongly suggests a role for Necl-1 in neuronal membrane-cytoskeleton interaction (Zhou et al., 2005). This role may be critical for morphological development and dynamic plasticity of the central nervous system. Furthermore, the possible role of Necl-1 as a tumor suppressor in cancer pathogenesis remains to be elucidated. It will be of interest to study the proposed functional roles of Necl-1 in more detail and we here provide the analytical basis for its fair determination in future experimental designs.

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