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Biochimica et Biophysica Acta 1669 (2005) 142–154

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Nectin-like molecule 1 is a protein 4.1N associated protein and recruits protein 4.1N from cytoplasm to the plasma membrane

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Received 21 September 2004; received in revised form 24 January 2005; accepted 28 January 2005
Available online 25 February 2005

Abstract

Nectins are immunoglobulin superfamily adhesion molecules that participate in the organization of epithelial and endothelial junctions. Sharing high homology with the poliovirus receptor (PVR/CD155), nectins were also named poliovirus receptor-related proteins (PRRs). Four nectins and five nectin-like molecules have been identified. Here we describe the cloning and characterization of human and mouse nectin-like molecular 1 (NECL1). Human and mouse NECL1 share 87.3% identity at the amino acid level. NECL1 contains an ectodomain made of three immunoglobulin-like domains, and a cytoplasmic region homologous to those of glycophorin C and contactin-associated protein. RNA blot and in situ hybridization analysis showed that *NECL1* predominantly expressed in the central nervous system, mainly in neuronal cell bodies in a variety of brain regions including the cerebellum, cerebral cortex and hippocampus. In vitro binding assay proved the association of NECL1 with protein 4.1N. NECL1 localizes to the cell–cell junctions and recruits protein 4.1N to the plasma membranes through its C-terminus, thus may regulate the function of the cell–cell junction. We propose that the NECL1 and protein 4.1N complex is involved in the morphological development, stability, and dynamic plasticity of the nervous system.

Keywords: Nectin; NECL1; Protein 4.1N

1. Introduction

Cell–cell adhesion mediated by cell adhesion molecules allows cells to adhere to one another, interconnect the cytoskeletons of adjacent cells, and give tissues strength and resistance to shear forces. Cell adhesion molecules generally fall into five classes: the cadherins, the selectins, the integrins, the mucins and the immunoglobulin (Ig) superfamily. Among them, the Ig superfamily cell adhesion molecules (IgCAMs) are the largest, with over 100 members in vertebrates. These well-characterized molecules include NCAMs, L1 family CAMs, and nectins. Nectins were originally described as molecules homologous to the poliovirus receptor (PVR/CD155) and called poliovirus related proteins (PRRs). Four members have been described: nectin-1/PRR1/CD111, nectin-2/PRR2/CD112, nectin-3/PRR3 and nectin-4 [1–7]. Their ectodomains are composed of three immunoglobulin (Ig)-like domains of V, C, C types and share between 30 and 55% amino acid identity. Nectin-1/PRR1 or the herpesvirus entry mediator A (HVEM/HveA)

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doi:10.1016/j.bbamem.2005.01.013

serve as the herpes simplex virus (HSV) entry receptor [1], whereas Nectin-2/PRR2 serve as the receptors for a limited range of alpha-herpes virus strains [2,7]. Nectin-1 and nectin-2 are also involved in the cell-to-cell spreading of the virus [8,9].

Nectins are a family of Ca^{2+}-independent immunoglobulin-like cell–cell adhesion molecules which homophilically and heterophilically trans-interact and cause cell–cell adhesion. Each member of the nectin family forms a homo-cis-dimer, in which the monomers are aligned in a parallel orientation, and then followed by the formation of a homo-trans-dimer, in which cis-dimers from opposing cell surfaces interact in a anti-parallel orientation [3,4]. Nectin-3 furthermore forms a hetero-trans-dimer with either nectin-1 or -2, and the formation of each hetero-trans-dimer is stronger than that of each homo-trans-dimer [3]. Nectin-4 also forms a hetero-trans-dimer with nectin-1, and this formation is also stronger than that of the homo-trans-dimer [4]. Each member of the nectin family except nectin-4 has two or three splicing variants. Most members of nectins have a carboxyl-terminal conserved motif of four amino acid residues (E/A-X-Y-V) that interacts with the PDZ domain of afadin, an F-actin binding protein. This binding of afadin to nectins links nectins to the actin cytoskeleton [10]. Nectin-based cell–cell adhesion is involved in the formation of cadherin-based adherens junctions in epithelial cells and fibroblasts [11]. Nectins also play roles in the formation of a variety of cell–cell junctions in cooperation with, or independently of, cadherins, such as synapses in neurons and Sertoli cell–spermatid junctions in the testis [12].

Five nectin-like molecules have been identified recently. They also contain one extracelluar domain containing three Ig-like loops, one transmembrane domain and one short cytoplasmic domain. These include NECL1/TSLL1/SynCAM3 (hereinafter refer as NECL1) [13,14], NECL2/TSLC1/SynCAM1/IGSF4/RA175/SgIGSF (hereinafter refer as NECL2) [15–18], NECL3/SynCAM2, NECL4/TSLL2/SynCAM4 and NECL5/Tage4 [19]. The existence of many nomenclatures for the same nectin-like molecule is confusing due to their diverse roles reported by independent groups.

The roles of NECL2 have been partly elucidated, but those of other NECLs are still elusive, except that the *NECL1* mRNA is exclusively expressed in the human and mouse nervous system [13,14]. NECL1 can also form Ca^{2+}-independent heterophilic dimer with NECL2 [20], but little is known about NECL1's cellular distribution and its functional capability.

We isolated human and mouse *NECL1* cDNA and have shown that (i) NECL1 is structurally related to the nectin family members; (ii) *NECL1* is expressed mainly in brain in human and mouse fetal and adult tissues, and is also expressed in E14.5 mouse embryo; (iii) *Necl1* mRNA is primarily expressed in neuronal cell bodies in a variety of brain regions, and its protein is located in neurites; (iv) similar to glycophorin C and Caspr, NECL1 associates with the FERM domain of protein 4.1N in vitro; (vi) NECL1 mediates the translocation of protein 4.1N to the sites of cell–cell junctions through its C-terminus.

2. Materials and methods

2.1. Isolation of human and mouse NECL1 cDNA

Unigene Hs.6164 (National Center for Biotechnology Information) represents a human neuronal-predominant gene according to the tissue origins of its ESTs. By searching GenBank, we found that the coding region of EST R88252 and H14720 in Hs.6164 share homology to the second and the third Ig-like domains of poliovirus receptor. IMAGE clone 192716, which was also in the list of Unigene Hs.6164, was sequenced and analyzed. To obtain the full-length cDNA, the 5′ end of 192716 cDNA was used as probe to screen human adult brain cDNA library. To obtain the mouse *Necl1* cDNA, human cDNA fragments (86–836) was used to screen a mouse brain Lamda ZAP Express™ cDNA library constructed by using the Lamda ZAP Express™ cDNA library construction kit (Stratagene).

2.2. Northern blot and dot blot analysis

NECL1 mRNA size and its tissue distribution were determined by hybridization to human and mouse Multiple Tissue Northern blot and Human RNA Master Blot (BD Clontech) according to the manufacturer's manual. The 787 bp cDNA probe (933–1719) for human Northern blot and RNA dot blot was amplified by PCR. The 773 bp cDNA probe (98–870) for mouse Northern blot was obtained through restriction digestion of mouse *Necl1* cDNA by *Bgl*II and *Xho*I. Control blots were performed using the *beta-actin* or the *ubiquitin* probe supplied by the manufacturer.

2.3. In situ hybridization

Experiments were carried out using digoxigenin-labeled cRNA probes corresponding to part of the 3′-untranslated region of mouse Necl1 cDNA. Control sections were hybridized with identical quantities of sense cRNA, and no signal was observed. The whole-mount in situ hybridization in mouse embryos was performed as described [21].

2.4. Antibody production

The human *NECL1* cDNA fragment corresponding to the cytoplasmic 47 amino acids was cloned into a modified pET-30a-DHFR prokaryotic expression vector with a 26-kDa dihydrogen folic reductase (DHFR) at the N-terminal of the fusion proteins (Novagen). The fusion protein was expressed in *Escherichia coli* BL21 (DE3) bacteria and then purified over nickel columns (Novagen) according to the

manufacturer's protocol. New Zealand white rabbits were immunized with the fusion protein according to established protocols.

2.5. Western blot analysis of mouse tissues and immunohistocemistry

Mice were perfused free of blood via left ventricular/ascending aortic perfusion with PBS. Protein extracts were prepared as described [22]. 30 μg of protein was loaded per lane in a 10% polyacrylamide gel. Proteins were wet transferred to an ECL nylon membrane (Amersham Bioscience) using an electroblotter (Bio-Rad). Nonspecific binding sites on the membranes were blocked by incubation for 1 h at room temperature in 10% skimmed dry milk in TBST (10 mmol/L Tris–HCl, pH 7.4; 150 mmol/L NaCl; and 0.1% Tween 20). Blots were washed three times with TBST and then incubated overnight with 1:5000 NECL1 antibodies at 4 °C in blocking buffer (TBST with 2% BSA and 2% normal goat serum). Immunoreactive bands were visualized using ECL Western blotting detection reagents (Amersham Bioscience). Immunohistocheimstry staining was performed as described [23].

2.6. In vitro binding assay

The cytoplasmic domain of glycophorin C contains 47 amino acid residues and that of NECL1 contains 46 amino acid residues. Peptide GPC-23 (Biotin-RYMYRHKGTYHT-NEAKGTEFAES-OH), NECL1-23 (Biotin-HYLIRHKG-TYLTHEAK GSDDAPD-OH), and NECL1-23mt (Biotin-HYLIAAAGTYLTHEAKGSDDAPD-OH) were synthesized at Research Genetics and coupled to biotin at their amino termini. Competitive peptide NECL1-14 (H-HYLIRHKGTYLTHE-OH) was also synthesized but not coupled to biotin. The synthetic peptides were purified by analytical reverse phase high-pressure liquid chromatography. The cDNA encoding the 30-kDa membrane binding domains (FERM domain) of protein 4.1N was amplified using PCR and cloned into pGEX-4T-1 prokaryotic expression vector (Amersham Bioscience). The glutathione *S*-transferase fusion proteins were expressed in *E. coli* strain BL21 (DE3) and purified as described in the manual.

Two methods were used to carry out the in vitro binding assay, ELISA and GST pull-down assay. ELISA binding assay was performed as described [24]. Briefly, the purified 30-kDa FERM domain of protein 4.1N was absorbed onto the Immuno 2 plate (Dynatech Labs. Inc) for 2 h. The plate was blocked with blocking buffer (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20, 3% bovine serum albumin and 0.02% sodium azide) for 3 h at room temperature. The biotinylated peptides (20 μg in each well) with/without 10- or 30- fold of competitive peptide were dissolved in the blocking buffer and incubated overnight in the ELISA plate at 4 °C. The plate was washed extensively with the blocking buffer, and the amount of bound peptide was measured at 450 nm using streptavidin-conjugated Horseradish peroxidase and *o*-phenylenediamine as substrate. In the GST pull-down assay, purified GST and fusion proteins of GST-4.1N FERM domains were coupled to glutathione-Sepharose beads and incubated with NECL1 in a binding buffer (5.0 mM sodium phosphate, pH 7.6, 1.0 mM 2-mercaptoethanol, 0.5 mM EDTA, 120 mM KCl, 0.02% sodium azide and 1.0 mg/ml bovine serum albumin) at 4 °C overnight; after extensive washing, the beads were re-suspended in 30 μl of sample buffer separated by SDS-PAGE followed by immunoblotting using the NECL1 antibody.

2.7. Cell culture and confocal microscopy

Expression vectors were constructed in pEGFP-N1 and pHA-CMV (BD Clontech). NECL1 with C-terminal green fluorescent protein (GFP) tag were constructed by cloning the full-length and cytoplasmic domain deleted mouse Necl1 cDNAs into the pEGFP-N1 vector as pEGFP-N1-NECL1 and pEGFP-N1-NECL1ΔC respectively. The cDNA encoding the FERM domain of protein 4.1N was cloned into pHA-CMV as pHA-CMV-4.1N, which is used for the in vivo expression of N-terminal hemagglutin (HA) tagged FERM domain of protein 4.1N. COS-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. For transfection, the cells were grown in 6-well plates (3–4×10^5 cells/dish) and then were switched into Opti-MEM I media before transfection. The cells were transfected with 1 μg of DNA/dish using Lipofectamine Plus (Invitrogen). Four hours post-transfection, the medium was replaced with complete DMEM. 20–24 h after transfection, the cells were fixed with 2% paraformaldehyde for 10 min and stained with antibodies. Briefly, the cells were permeabilized with 0.1% Triton-100 for 10 min and blocked with 5% BSA and normal goat serum. The cells were immunostained using rat monoclonal anti-hemagglutin tag antibody (3F10, Roche Molecular Biochemicals) to visualize the 4.1N protein, and then followed by a Cy3-conjugated goat anti-rat secondary antibody (Jackson), and/or Rhodamine-phalloidin or Alexa 488-phalloidin (Molecular Probes). After overlaying the coverslips, the slides were imaged using a Nikon PCM 2000 confocal microscope with a PA 60× oil/1.4 immersion objective. All experiments were performed three times with the same results.

3. Results

3.1. Cloning of human and mouse NECL1 cDNA

A novel human cDNA was identified and isolated by a combination of computer analysis of genetic databases and traditional cDNA library screening. This new cDNA of 2540 bp was designated NECL1 (GenBank Accession No.

AF062733). The gene was mapped to human chromosome 1 and spans about 30 kb with 10 exons. The genomic sequence and exon–intron boundary of human NECL1 were determined by the alignment of NECL1 cDNA sequence to the known genomic sequence of a BAC clone 134p22 (GenBank Accession No. AL035403). Mouse Necl1 cDNA was isolated by screening a mouse brain cDNA library using a human 5′ cDNA probe (GenBank Accession No. AF195662). The predicted protein product of human NECL1 is 47.1 kDa, while that of mouse Necl1 is 42.9 kDa. The identity of their amino acid sequence is 87.3%. Mouse Necl1 protein lacks the region encoded by the exon 2 of human NECL1, which lies between the signal peptide and the first Ig-like domain (Fig. 1A). The amino acid sequences of the cytoplasmic region of human and mouse NECL1 are identical. The overall molecular module of NECL1 displays typical immunoglobulin superfamily features, an NH_2-signal peptide, and an extracellular domain containing three immunoglobin-like loops with several potential *N*-glycosylation sites, one transmembrane region and a short cytoplasmic tail. They are also the structural modules of nectin and nectin-like family (Fig. 1C). NECL1

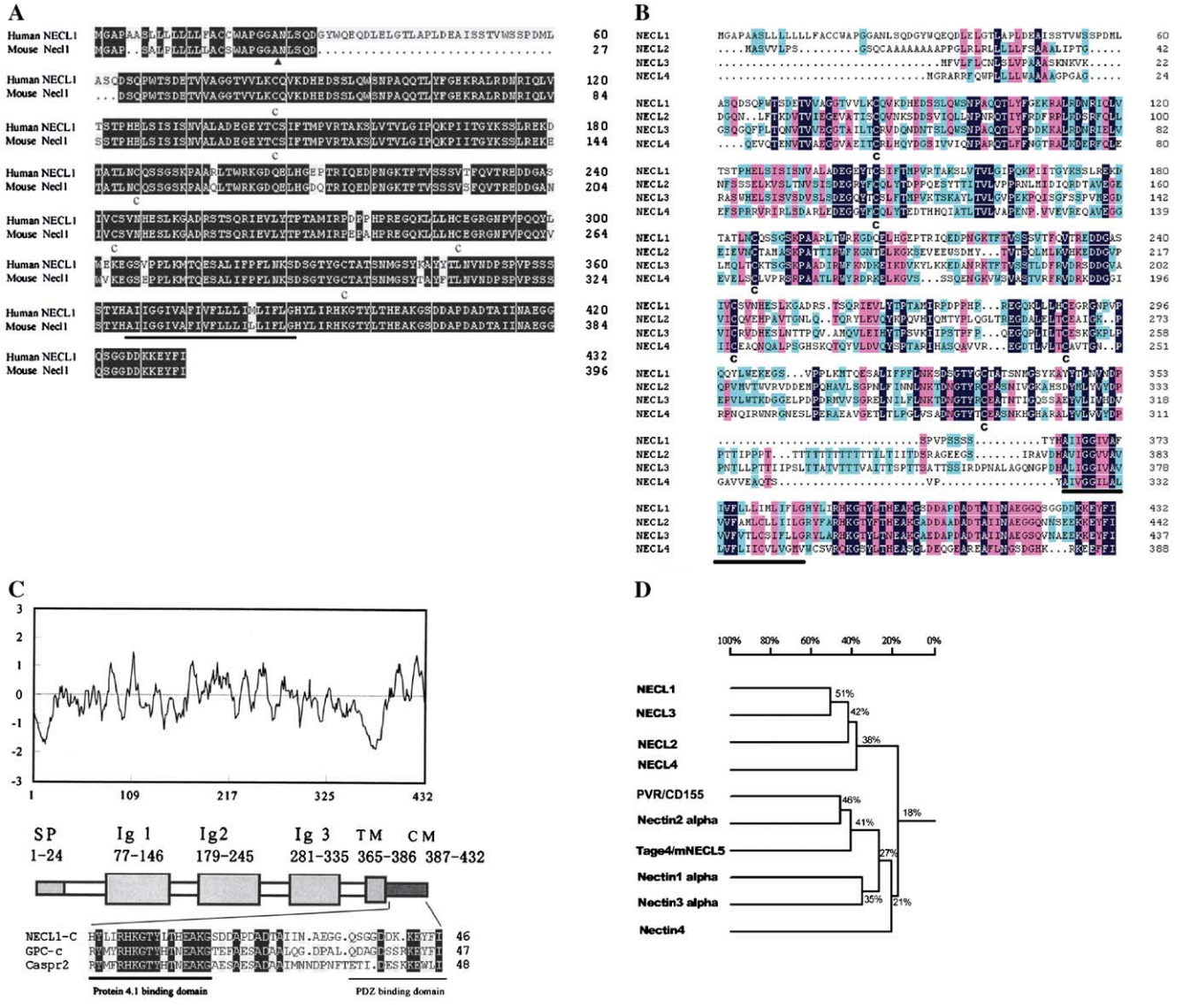


Fig. 1. Sequence analysis of NECL1. (A) Amino acid sequence alignment between human and mouse NECL1. Sequences show 87.3% identity. Mouse NECL1 protein lacks the region encoded by the exon 2 of human *NECL1*, which lies between the signal peptide and the first Ig-like domain. Arrowhead indicates the signal peptide cleavage sites. The transmembrane region is underlined. Consensus cysteines for the disulfide bonds formation are indicated. Shaded are identical amino acids. (B) Multiple alignment of human NECL1, NECL2, NECL3 and NECL4 amino acid sequences. The transmembrane region is underlined. Consensus cysteines for the disulfide bonds formation of Ig-like domains are indicated. Shaded are identical amino acids. (C) The hydrophilic analysis and domain organizations of NECL1. The cytoplasmic region of nectin-like molecule 1 (NECL1) is highly homologous to that of glycophorin C (GPC) and contactin-associated protein (Caspr). The Protein 4.1 binding domains and PDZ binding domains are marked. SP, signal peptides; Ig, immunoglobulin like domain; TM, transmembrane domain; CM, cytoplasmic domain. (D) Phylogenic tree of nectins and nectin-like molecules. The amino acid sequences of nectins and nectin-like molecules were analyzed through DNAMAN 4.0 (Lynnon BioSoft.) The percentages of the identity of amino acid sequences were marked.

shares significant homology to other members of nectin-like molecules (Fig. 1B). The predicted amino acid sequence of NECL1 cDNA displayed 39% identity and 56% similarity with that of NECL2, while the cytoplasmic similarity is 85%. Human NECL2, identified from the LOH region of chromosome 11q23.2, is transcribed into a 1.6- or a 4.4-kb mRNA encoding a 442-amino-acid protein and is widely expressed. We've also cloned human and mouse NECL2 cDNA (GenBank Accession No. AF138903 and AF061260, unpublished). NECL3 and NECL4 (GenBank Accession AF538973 and AF363368) also have high sequence and module similarity to NECL1 and NECL2 both in their extracellular, transmembrane and intracellular regions (Fig. 1B, C).

The cytoplasmic region of NECL1 protein exhibits 52% identity and 65% similarity to that of red blood cell membrane protein glycophorin C. The most conserved sequence motif spans residues 387–400 and 421–432 residues representing the functional domains of glycophorin C (GPC). They are protein 4.1 binding domain and p55-PDZ binding domain respectively, which suggests a similar function involving interaction with one or more scaffold proteins in the brain. The three consensus amino acids (Y/F-F-I) at the COOH end for PDZ domain binding are all conserved in NECL1, NECL2 and NECL3. The cytoplasmic region of NECL1 also displayed homology with neuronal glycoprotein contactin-associated protein (Caspr) in its protein 4.1 binding domain, while no apparent homology was found among the extracellular regions of NECL1, GPC and Caspr (Fig. 1C).

Phylogenic analysis showed that NECL1 is more closely related to NECL2, 3 and 4 than Nectin-1, -2, -3 and -4, while human Poliovirus receptor (PVR/CD155) and its

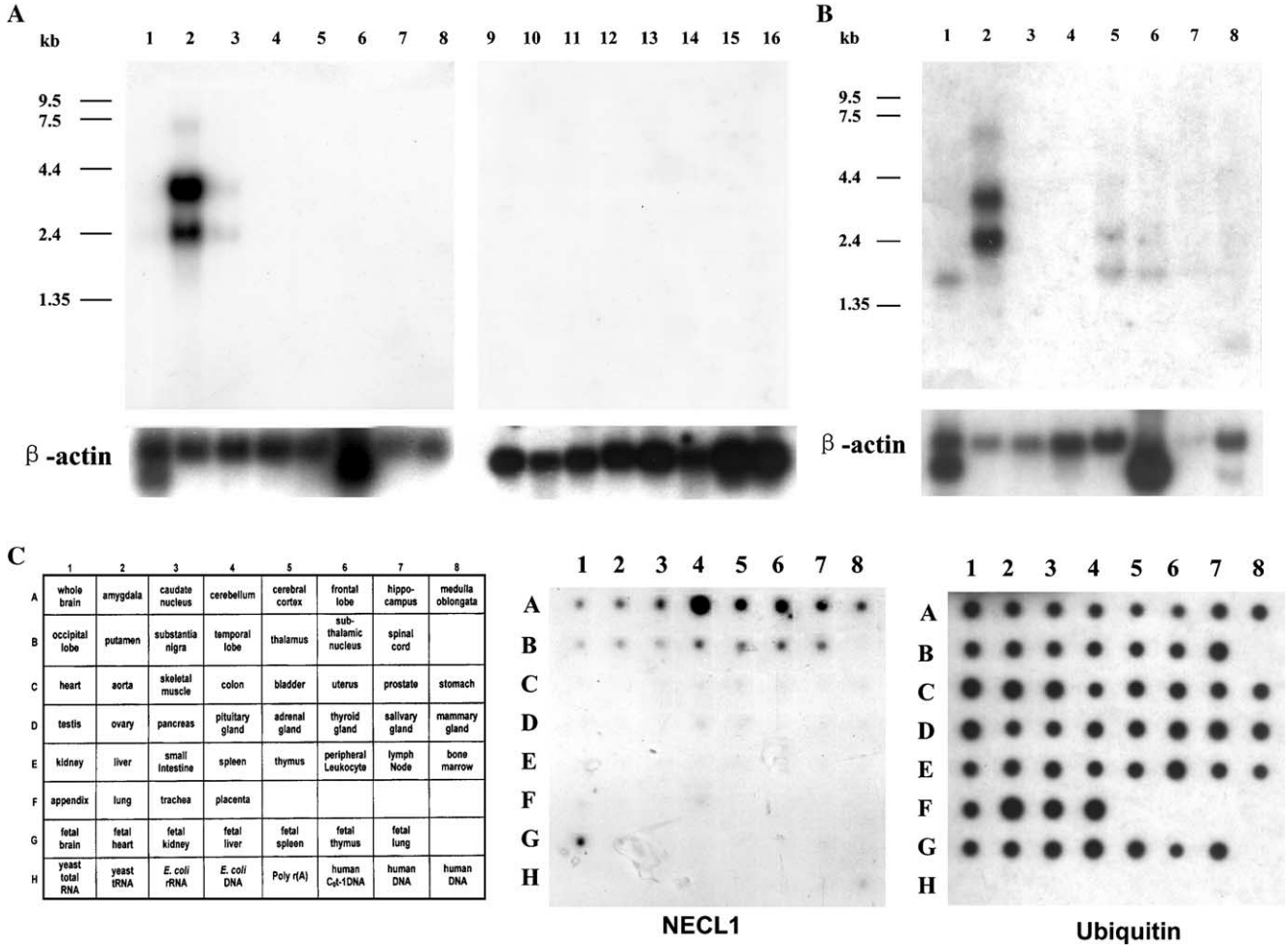


Fig. 2. NECL1 mRNA expressed predominantly in the central nervous system. (A) Human *NECL1* is highly expressed in adult brain with three transcripts of 2.5 kb, 4.0 kb and 7.5 kb. Human placenta also exhibited lower levels of 2.5 kb and 4.0 kb *NECL* transcripts. The result of beta-actin is included in the lower panel. Sources of RNAs: lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas; lane 9, spleen; lane 10, thymus; lane 11, prostate; lane 12, testis; lane 13, ovary; lane 14, small intestine; lane 15, colon; lane 16, peripheral blood leukocytes. (B) Mouse brain also exhibits three transcripts of 2.5 kb, 4.0 kb and 7.5 kb, with a lower levels of 1.8 kb transcript at the heart, liver and skeletal muscle. Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, testis. (C) RNA dot blot analysis indicates that the predominant expression of *NECL1* in the central nervous system and that the expression mount in the cerebellum (A4) is relatively higher than in other parts of the brain. *Ubiqutin* cDNA probe was used as a positive control. Grid shows the identification of RNA sources on the RNA master blot (BD Clontech).

mouse ortholog Tage4/mNecl5 are more closer to the nectin family but not to the nectin-like family (Fig. 1D).

3.2. Central nervous system-specific expression of NECL1 mRNA and protein

A Northern blot analysis of various human and mouse tissues was performed with *NECL1* cDNA. As shown in Fig. 2A, human brain exhibited high-level expression of a 2.6-kb and a 4.0-kb *NECL1* mRNA and somewhat lower levels of a 7.5-kb transcript. Human placenta also exhibited lower level of 2.6 kb and 4.0 kb *NECL* transcripts. Mouse brain also exhibited three transcripts of 2.6 kb, 4.0 kb and 7.5 kb, with a lower levels of a 1.8-kb transcript at the heart, liver and skeletal muscle (Fig. 2B). The 2.6-kb mRNA is consistent with the length of human and mouse *NECL1* cDNAs isolated from our lab. A more extensive survey of *NECL1* mRNA expression was performed by two additional approaches. First, a human *NECL1* probe was hybridized to a dot blot containing RNA from many human adult and fetal tissues (Fig. 2C). High expression was observed in different regions of adult brain (row A and B) and fetal brain (G1), and very high expression was observed in the cerebellum (A4). Very low expression was also detected in pituitary and placenta (D4 and F4). To obtain a higher resolution of *NECL1* expression patterns, we performed in situ hybridization. Whole-mount in situ hybridization in E14.5 mouse embryo indicated that *Necl1* was predominantly expressed in the nervous system (Fig. 3A). High-power views of *Necl1* in situ hybridization in adult mouse brain confirmed the association of *Necl1* mRNA with neuronal cell bodies in a variety of brain regions including the cerebellum, cortex, hippocampus, hypothalamus and spinal cord. Robust staining was observed in the pyramidal cells of the hippocampus, granule cells of the cerebellum and motor neurons of the spinal cord (Fig. 3B–J). Although in most brain regions *NECL1* mRNA occurred in all neurons, notable exceptions were the thalamus and Purkinje cells of the cerebellum. In the spinal cord, *Necl1* mRNA was predominantly localized to the motor neurons. In situ hybridization in adult rat brain and spinal cord showed similar staining (data not shown).

A fusion protein containing the C-terminus of NECL1 was used to generate a NECL1-specific polyclonal antibody. Western blot analysis of different mouse tissues revealed multiple bands only in mouse brain, with a 47-kDa main band. The other bands may be due to *N*-glycosylation. No apparent band was found at other mouse tissues (Fig. 4A).

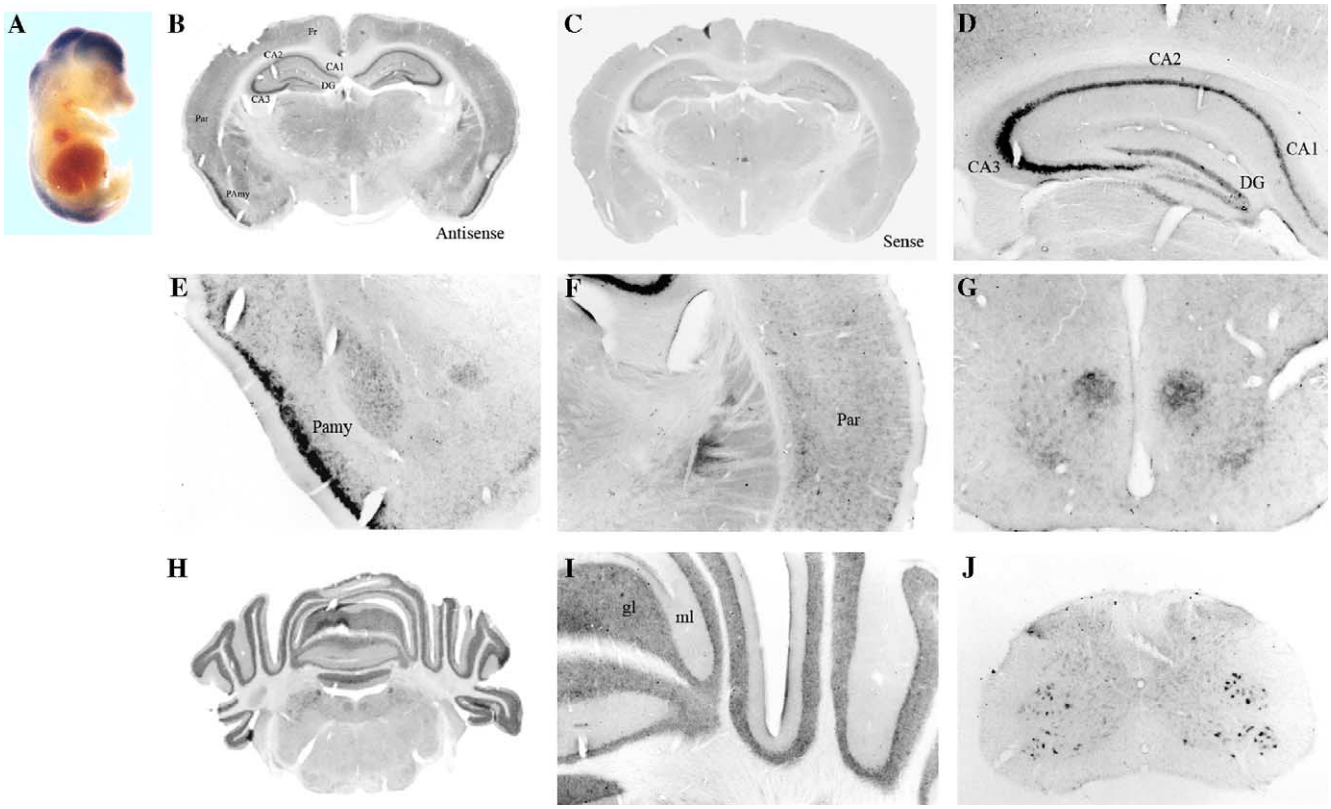


Fig. 3. In situ hybridization of NECL1. (A) Whole mount in situ hybridization in E14.5 mouse embryo indicated the neuronal predominant expression of *Necl1*. (B–I) High-power views of in situ hybridization in adult mouse brain confirmed the association of *Necl1* mRNA with neuronal cell bodies in a variety of brain regions including the cortex, hippocampus, hypothalamus (B), cerebellum (H) and spinal cord (J). Robust staining was observed at the pyramidal cells at the hippocampus (D) and periamygdaliod cortex (E), granule cells at the cerebellum (I) and motor neurons at the spinal cord (J). Positive signals are also found in the parietal cortex (F) and hypothalamus (G). No positive signals were observed when sections were hybridized with identical quantities of sense cRNA (C). Abbreviations: DG, dentate gyrus; Par, parietal cortex; Pamy, periamygdaliod cortex; ml, molecular cell layer; gl, granule cell layer.

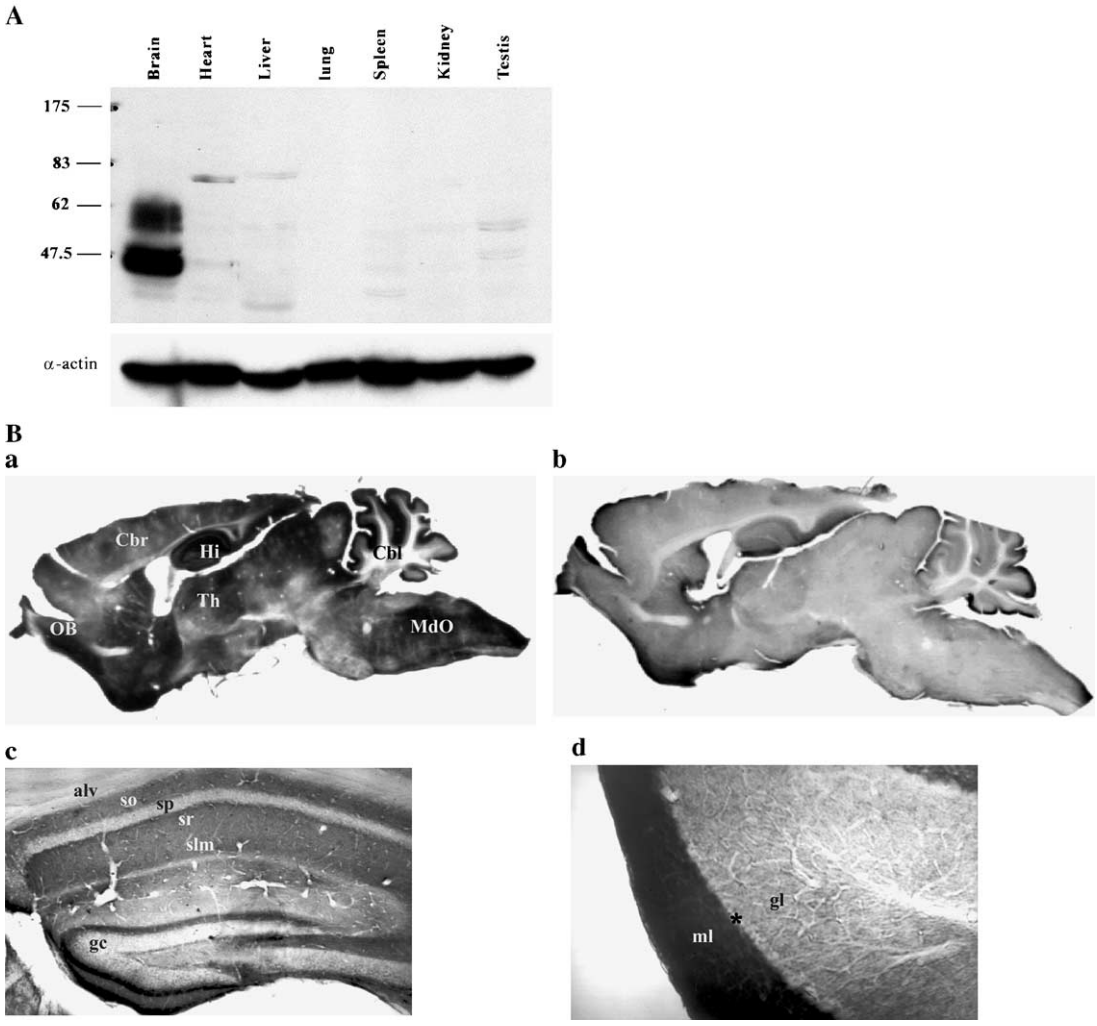


Fig. 4. Protein distribution of NECL1. (A) In Western blot analysis, a major 47-kDa band and several larger bands were detected only in adult mouse brain but not other tissues. The result of alpha-actin is included in the lower panel. (B) In immunohistochemistry staining, the NECL1 protein is widely distributed in mouse brain and highly expressed in the hippocampus, cerebellum, cerebral cortex and medulla oblongata (B-a). No staining was found by using pre-absorbed NECL1 antibody (B-b). Strong signals were found in fibrous structures but not cell bodies in hippocampus and dentate gyrus (B-c). In the cerebellum, robust staining was found in molecular layer, and punctate staining in granule layer, but no staining in the Purkinje cell layer (B-d). Abbreviations: OB, olfactory bulb; Cbr, cerebral cortex; Hi, hippocampus; Th, thalmus; Cbl, cerebellum; MdO, Medulla oblongata; alv, alveus hippocampi; so; stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; slm, substantia laculosum moleculare; gc, granule cells; ml, molecular cell layer; gl, granule cell layer.

Immunohistochemistry using the NECL1 antibody revealed strong immunoreactivity in the cerebral cortex, cerebellum, hippocampus and medulla oblongta, which coincided with mRNA distribution (Fig. 4B-a). In a control experiment, pre-absorption of the antibody with the NECL1 fusion protein completely abolished NECL1 immunoreactivity (Fig. 4B-b). High-power views revealed the fibrillous staining of NECL1 protein. In the dentate gyrus and all regions of the hippocampus, strong staining was observed on both sides of the granule cells and the pyramidal cells in the area of the stratum oriens and molecular layer. But cell somas were not labeled (Fig. 4B-c). In mouse cerebellar sections, the antibody exhibited very strong staining throughout the cerebellar molecular layer (Fig. 4B-d). The granule cell layer was more weakly stained and exhibited a punctate pattern of immunostaining. Because granule cells were the only cerebellar cells containing *Necl1* mRNA, the robust labeling of the cerebellar molecular layer must derive from NECL1 in granule cell axons. No obvious staining of white matter or Purkinje cells was observed.

3.3. NECL1 localizes to the cell–cell junction

The homology between NECL1 and some other adhesion molecules suggests that it might regulate the structure of cell–cell junctions. NECL1 with C-terminal GFP tag were constructed by cloning the full-length and cytoplasmic domain deleted mouse Necl1 cDNAs into pEGFP-N1 vector. The C-terminal GFP tag does not affect the localization of NECL1 as compared to the untagged NECL1 (unpublished data) and reported for the same family members [25]. In isolated single cells, GFP-NECL1 mainly

localized to the perinuclear vesicles (Fig. 5A-a). However, significant amounts of GFP-NECL1 moved to the cell–cell junctions when the cells were grown at a higher density and had contacted with each others (Fig. 5A-b). The deletion of the majority of cytoplasmic domain (GFP-NECL1ΔC) didn't change the intracellular localization pattern of NECL1 in isolated single cells. Similar to full-length protein, the GFP-NECL1ΔC still moved to cell–cell junctions in confluent cells, although GFP-labeled vesicles were also seen on the perinuclear regions (Fig. 5A), suggesting that the C-terminus of NECL1 does not determine its plasma membrane targeting.

3.4. NECL1 targets proteins 4.1 to the cell–cell junctions through its C-terminus

The homology among the cytoplasmic tails of NECL1, GPC and Caspr suggested putative binding of NECL1 to

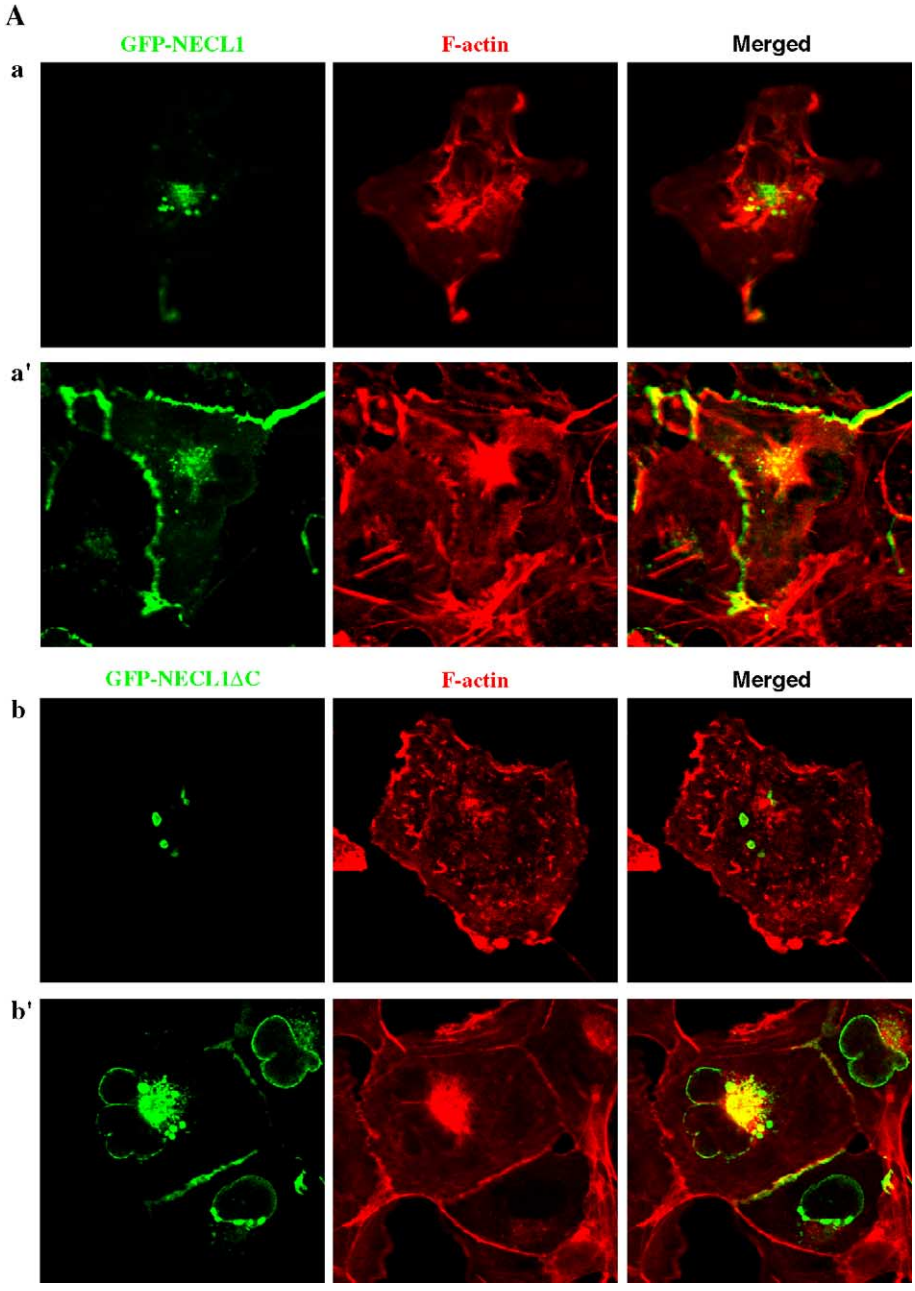


Fig. 5. Subcellular localization of NECL1 and its co-localization with protein 4.1N. (A) The full-length GFP-NECL1 localized to the cell–cell junctions in confluent cells (A-a′) but to the perinuclear vesicles in isolated cells (A-a). The deletion of cytoplasmic domain did not change its localization pattern (A-b, A-b′). (B) Protein 4.1N is cytosolic in isolated single cell. NECL1 and 4.1N were translocated to, and co-localized at the sites of cell–cell contact when cells expressing full-length NECL1. Upper row: an isolated cell; lower row: confluent cells. (C) Protein 4.1N is cytosolic in either isolated single cell or confluent cells. The deletion of C-terminus abolished the translocation of protein 4.1N to the sites of cell–cell junctions. Upper row: an isolated cell; lower row: confluent cells.

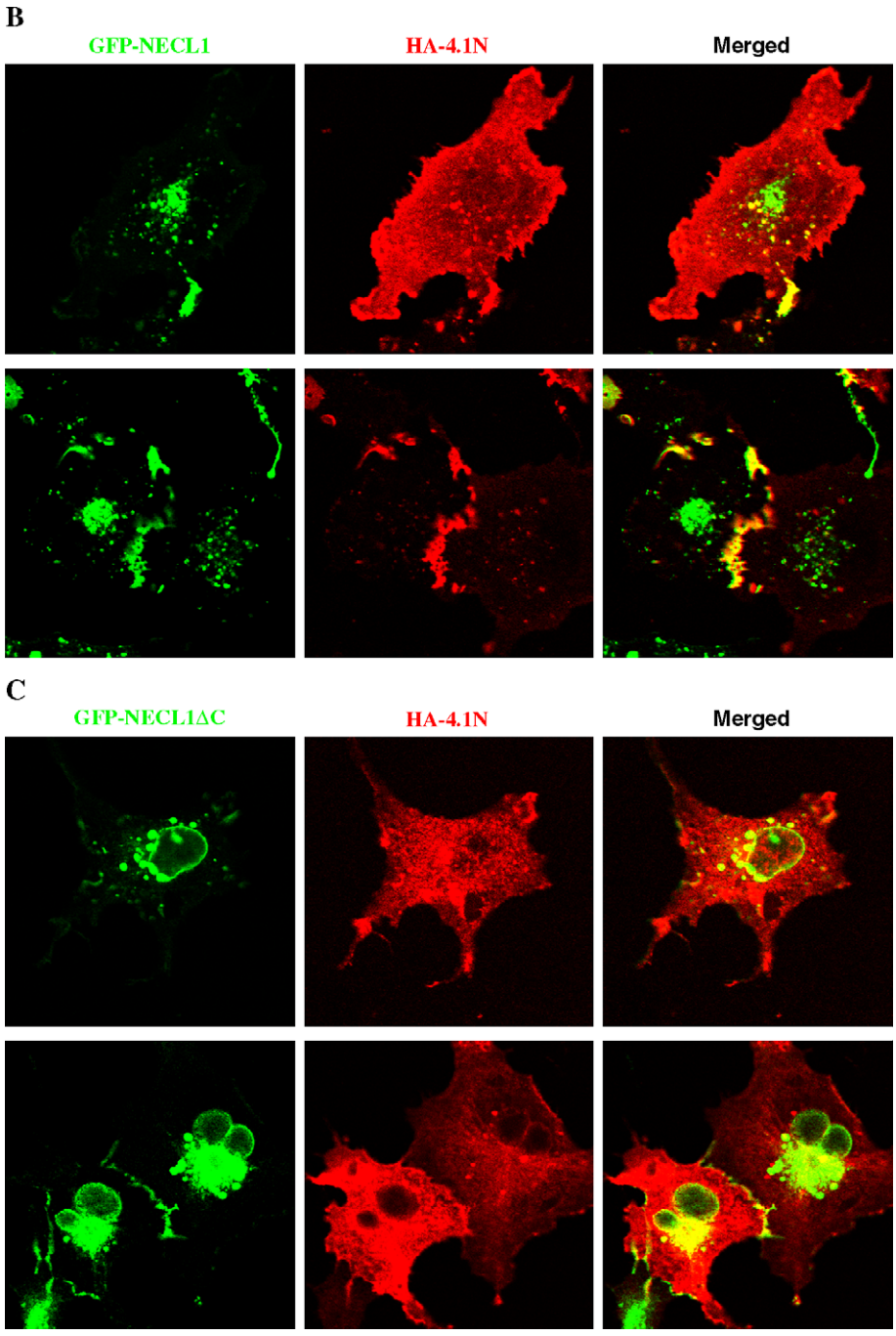


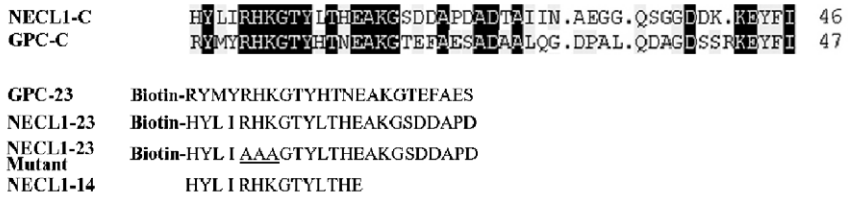
Fig. 5 (*continued*).

protein 4.1. Among the five members of protein 4.1 family, protein 4.1B/DAL-1 is known to be the binding protein of NECL2 and the two proteins shared very similar expression pattern. The expression pattern of NECL1 is very close to protein 4.1N and partially overlaps protein 4.1R. Thereby the binding assay and co-localization assay were focused on protein 4.1N. In vitro binding assay by ELISA showed that the biotinylated peptides GPC-23 and NECL1-23 corresponding to the cytoplasmic regions of glycophorin C and NECL1 can bind to the 30-kDa membrane binding domain (FERM domain) of 4.1N. 10-fold unlabeled competitive NECL1-14 peptide can partially inhibit this binding, and 30-fold competitive peptide totally abolished this binding. The mutant peptide (the core RHK were replaced with AAA) can't bind to protein 4.1N (Fig. 6B).

GST pull-down analysis indicated that the fusion protein of NECL1 cytoplasmic domain can be co-purified with the GST fusion protein of the 30-kDa membrane binding domain of protein 4.1N, but not the GST protein alone (Fig. 6C).

Since protein 4.1 is involved in the reorganization of actin cytoskeleton, we propose that NECL1 modulates the

A



B

Biotinylated peptides	Competitor	Binding(Protein 4.1N)	
	NECL1-14	A_{450nm}	%
A. GPC-23	None	0.4±0.04	100
	10 fold	0.213±0.02	53
	30 fold	0	0
B. NECL1-23	None	0.382±0.04	100
	10 fold	0.208±0.02	54
	30 fold	0.02±0.01	5
C. NECL1-23 Mutant	None	0	0

C

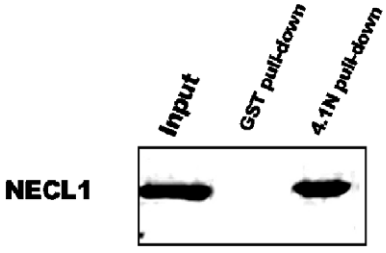


Fig. 6. Association of NECL1 with protein 4.1N in vitro. (A) The locations and amino acid sequences of synthesized NECL1 and glycophorin C C-terminal peptides. (B) Purified 30-kDa FERM membrane-binding domain of protein 4.1N were absorbed to Immulon 2 ELISA plates. The binding of biotinylated glycophorin C and NECL1 wild type or mutant peptides to protein 4.1N was determined by measuring OD at 450 nm with or without competitive peptide. The data are representative of four independent experiments carried out in quadruplicate. (C) In the GST pull-down assay, the fusion protein of NECL1 cytoplasmic domain can be co-purified by the GST fusion protein of the 30-kDa FERM domain of protein 4.1N, while the control GST alone can't pull down NECL1.

structure or function of cell–cell junctions through protein 4.1. COS-7 cell were co-transfected with pHA-CMV-4.1N and pEGFP-N1-NECL1, or pEGFP-N1-NECL1ΔC. In the cells transfected with only 4.1N (not shown), or the isolated cells transfected with both NECL1-GFP and 4.1N, protein 4.1N diffused in the cytoplasm. However, NECL1 at the cell–cell junction recruited the majority of 4.1N from the cytoplasm to the plasma membrane when cells were confluent (Fig. 5B). The interaction of 4.1N FERM domain and NECL1 C-terminus is required for the recruitment of 4.1N to the cell–cell junctions by NECL1 since the deletion of cytoplasmic domain abolished the NECL1's ability to relocate protein 4.1N (Fig. 5C).

4. Discussion

In this study, we characterized mouse and human forms of nectin-like molecule 1 (NECL1). NECL1 shares homology with a number of proteins in its Ig-like domain especially other nectin-like molecules and nectins, which suggests that NECL1 is a member of the immunoglobulin superfamily and may play a role as a cell adhesion molecule. The Ig superfamily encompasses diverse molecules that share a common structural homology. This superfamily includes cell-adhesion molecules (CAMs) and receptors for cytokines and growth factors. Members of the superfamily are involved in a variety of functions including cell–cell recognition, cell–cell signaling, muscle structures and the immune system.

Phylogenic analysis showed that NECL1 is more closely related to NECL2, 3 and 4 than to Nectin1, 2, 3 and 4, while Poliovirus receptor (PVR/CD155) and its mouse ortholog Tage4/Necl5 are closer to the nectin family but not to the nectin-like family [19,26]. But in the review of Takai et al., PVR/CD155/Tage4/Necl5 was categorized in the nectin-like family [12]. NECL1, 2, 3 and 4 share high homology among their cytoplasmic tails and the putative binding domains for protein 4.1 and PDZ domain are highly conserved. The cytoplasmic tails of the members of the nectin family all bind to afadin, a PDZ containing cytoskeleton protein, while NECL2, PVR/CD155/Tage4/Necl5 do not [4,27]. So it may

be more rational to re-categorize the nectin-like molecule family with only NECL1, 2, 3 and 4; nectin family with nectin1, 2, 3 and 4; while PVR/CD155/Tage4/Necl5 into a new sub-family, which we tentatively designated nectin-related molecules, due to their homology to nectin molecules but inability of binding to afadin.

Among members of the nectin-like family, NECL2/TSCL1/SynCAM1 is the best characterized one. NECL2 is a tumor related suppressor gene in human non-small cell lung cancer and encodes a cell adhesion molecule [28]. NECL2 can mediate cell adhesion through Ca^{2+}-independent homophilic binding and/or heterophilic interaction with NECL1 and nectin3 [20]. The majority of NECL1 protein is localized on the membrane of contacted cells, which suggests that NECL1 is a cell adhesion molecule and it may form trans-homo-dimer. NECL2 was also identified to be a synaptic adhesion molecule that can induce functional synapse formation in non-neuronal cells. *NECL1* mRNA is specifically expressed in a certain group of neurons and NECL1 protein is localized in the nerve terminals such as the axons of cerebellar granule cell. This indicated that, like NECL2, NECL1 is a synaptic protein and may participate in synaptogenesis and synaptic structural/functional maintenance.

The reports for the protein distribution of NECL2 are not consistent. Biederer et al. showed that NECL2/SynCAM protein is specifically expressed in the brain [18], while Shingai et al. indicated that NECL2 protein is more generally distributed [20]. The inconsistency may be due to the peptide for NECL2 antibody generation in the former study being from the C-terminal 11 amino acids, which are highly conserved between NECL1 and NECL2. Our Western blot showed that NECL1 protein is dominantly expressed in brain, which is consistent with RNA analysis. *N*-glycosylation may be responsible for the higher molecule weight bands, which was also shown in NECL2.

The conserved protein 4.1 binding domain and PDZ binding domain of NECL1 and NECL2 were shown by biochemical and cell biology means. NECL2 can directly associate with another lung tumor suppressor—DAL-1/Protein 4.1B, which belongs to the protein 4.1 family [15]. The protein 4.1 family comprises a group of skeletal proteins structurally related to the erythroid membrane skeletal protein, protein 4.1R. These proteins are characterized by the presence of three main conserved structural/functional domains. A 30-kDa N-terminal membrane binding domain (MBD; also called the FERM domain) possesses binding sites for the cytoplasmic tails of integral membrane proteins such as band 3, glycophorin C, CD44, and contactin-associated protein (Caspr) [29]. An internal 8–10 kDa domain contains the critical spectrin–actin binding activity required for membrane stability, and the C-terminal 22–24 kDa domain been reported to bind the immunophilin FKBP13 and nuclear mitotic apparatus protein (NuMA). The prototypical protein 4.1R has been characterized most extensively in the erythrocyte, where it plays a critical role in maintaining the erythrocyte's morphology and mechanical integrity via interactions with the cytoskeletal proteins spectrin and F-actin and with the band 3 and glycophorin C membrane proteins [24,30,31]. Among the five members of the protein 4.1 family, protein 4.1N is expressed in cerebellar granule cell, neurons of the hippocampus and cerebral cortex but not cerebellar Purkinje cells [32]. This expression pattern is very similar to that of NECL1. Protein 4.1N is enriched at regions of synaptic contact between neurons, where it could potentially play an important role in synaptic architecture and function [33]. In PC12 cells, treatment with nerve growth factor (NGF) elicits the translocation of 4.1N to the nucleus and promotes its association with NuMA. Nuclear 4.1N can then mediate the anti-proliferative actions of NGF by antagonizing NuMA in mitosis [34]. Protein 4.1N can also associate with glutamate alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors subunit GluR1 in vivo and colocalizes with AMPA receptors at excitatory synapses. The disruption of the interaction of GluR1 with 4.1N or the disruption of actin filaments decreased the surface expression of GluR1 in heterologous cells. Moreover, the disruption of actin filaments in cultured cortical neurons dramatically reduced the level of surface AMPA receptors [35]. These results suggest that protein 4.1N may link AMPA receptors to the actin cytoskeleton. The in vitro and in vivo interaction of NECL1 and 4.1N indicate that NECL1 may also play important roles in synaptic architecture and function by associating with F-actin cytoskeleton though protein 4.1N.

The neuronal cell-selective expression of this immunoglobulin-like molecule, coupled with its in vitro and in vivo functional profile, strongly suggests a role of NECL1 in membrane-cytoskeleton interaction and this role may be critical for the plasticity and function of the central nervous system. NECL1 and protein 4.1N may form complex with F-actin cytoskeleton and are involved in the morphological development, stability, and dynamic plasticity of the nervous system. The roles of NECL1 in cell–cell adhesion require further investigation. The *NECL1*-null mouse may help to elucidate the in vivo roles of NECL1 played in the development and functional maintenance of nervous system.

Acknowledgements

We thank Mr. Petur Petersen and Mr. Joshua Atkins (Yale University, New Haven, USA) for reading this manuscript. These investigations were supported by grants from The National Program for the Key Basic Research Project ("973" No. G19980510022 and 001cb510206); the National High Technology Research and Development Program ("863" No. 2001AA22 1041, 2002BA711A01-03); and the National Sciences Foundation of China (No.39830070 and 30170518).

References

[1] R.J. Geraghty, C. Krummenacher, G.H. Cohen, R.J. Eisenberg, P.G. Spear, Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor, Science 280 (1998) 1618–1620.

[2] M. Lopez, F. Cocchi, L. Menotti, E. Avitabile, P. Dubreuil, G. Campadelli-Fiume, Nectin2alpha (PRR2alpha or HveB) and nectin2-delta are low-efficiency mediators for entry of herpes simplex virus mutants carrying the Leu25Pro substitution in glycoprotein D, J. Virol. 74 (2000) 1267–1274.

[3] K. Satoh-Horikawa, H. Nakanishi, K. Takahashi, M. Miyahara, M. Nishimura, K. Tachibana, A. Mizoguchi, Y. Takai, Nectin-3, a new member of immunoglobulin-like cell adhesion molecules that shows homophilic and heterophilic cell–cell adhesion activities, J. Biol. Chem. 275 (2000) 10291–10299.

[4] N. Reymond, S. Fabre, E. Lecocq, J. Adelaide, P. Dubreuil, M. Lopez, Nectin4/PRR4, a new afadin-associated member of the nectin family that trans-interacts with nectin1/PRR1 through V domain interaction, J. Biol. Chem. 276 (2001) 43205–43215.

[5] M. Lopez, F. Eberle, M.G. Mattei, J. Gabert, F. Birg, F. Bardin, C. Maroc, P. Dubreuil, Complementary DNA characterization and chromosomal localization of a human gene related to the poliovirus receptor-encoding gene, Gene 155 (1995) 261–265.

[6] F. Eberle, P. Dubreuil, M.G. Mattei, E. Devilard, M. Lopez, The human PRR2 gene, related to the human poliovirus receptor gene (PVR), is the true homolog of the murine MPH gene, Gene 159 (1995) 267–272.

[7] M.S. Warner, R.J. Geraghty, W.M. Martinez, R.I. Montgomery, J.C. Whitbeck, R. Xu, R.J. Eisenberg, G.H. Cohen, P.G. Spear, A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus, Virology 246 (1998) 179–189.

[8] F. Cocchi, L. Menotti, P. Dubreuil, M. Lopez, G. Campadelli-Fiume, Cell-to-cell spread of wild-type herpes simplex virus type 1, but not of syncytial strains, is mediated by the immunoglobulin-like receptors that mediate virion entry, nectin1 (PRR1/HveC/HIgR) and nectin2 (PRR2/HveB), J. Virol. 74 (2000) 3909–3917.

[9] T. Sakisaka, T. Taniguchi, H. Nakanishi, K. Takahashi, M. Miyahara, W. Ikeda, S. Yokoyama, Y.F. Peng, K. Yamanishi, Y. Takai, Requirement of interaction of nectin-1alpha/HveC with afadin for efficient cell–cell spread of herpes simplex virus type 1, J. Virol. 75 (2001) 4734–4743.

[10] K. Takahashi, H. Nakanishi, M. Miyahara, K. Mandai, K. Satoh, A. Satoh, H. Nishioka, J. Aoki, A. Nomoto, A. Mizoguchi, Y. Takai, Nectin/PRR: an immunoglobulin-like cell adhesion molecule recruited to cadherin-based adherens junctions through interaction with Afadin, a PDZ domain-containing protein, J. Cell Biol. 145 (1999) 539–549.

[11] M. Miyahara, H. Nakanishi, K. Takahashi, K. Satoh-Horikawa, K. Tachibana, Y. Takai, Interaction of nectin with afadin is necessary for its clustering at cell–cell contact sites but not for its cis dimerization or trans interaction, J. Biol. Chem. 275 (2000) 613–618.

[12] Y. Takai, K. Irie, K. Shimizu, T. Sakisaka, W. Ikeda, Nectins and nectin-like molecules: roles in cell adhesion, migration, and polarization, Cancer Sci. 94 (2003) 655–667.

[13] H. Fukuhara, M. Kuramochi, T. Nobukuni, T. Fukami, M. Saino, T. Maruyama, S. Nomura, T. Sekiya, Y. Murakami, Isolation of the TSLL1 and TSLL2 genes, members of the tumor suppressor TSLC1 gene family encoding transmembrane proteins, Oncogene 20 (2001) 5401–5407.

[14] T. Fukami, H. Satoh, Y.N. Williams, M. Masuda, H. Fukuhara, T. Maruyama, M. Yageta, M. Kuramochi, S. Takamoto, Y. Murakami, Isolation of the mouse Tsll1 and Tsll2 genes, orthologues of the human TSLC1-like genes 1 and 2 (TSLL1 and TSLL2), Gene 323 (2003) 11–18.

[15] M. Yageta, M. Kuramochi, M. Masuda, T. Fukami, H. Fukuhara, T. Maruyama, M. Shibuya, Y. Murakami, Direct association of TSLC1 and DAL-1, two distinct tumor suppressor proteins in lung cancer, Cancer Res. 62 (2002) 5129–5133.

[16] T. Wakayama, K. Ohashi, K. Mizuno, S. Iseki, Cloning and characterization of a novel mouse immunoglobulin superfamily gene expressed in early spermatogenic cells, Mol. Reprod. Dev. 60 (2001) 158–164.

[17] K. Urase, A. Soyama, E. Fujita, T. Momoi, Expression of RA175 mRNA, a new member of the immunoglobulin superfamily, in developing mouse brain, NeuroReport 12 (2001) 3217–3221.

[18] T. Biederer, Y. Sara, M. Mozhayeva, D. Atasoy, X. Liu, E.T. Kavalali, T.C. Sudhof, SynCAM, a synaptic adhesion molecule that drives synapse assembly, Science 297 (2002) 1525–1531.

[19] W. Ikeda, S. Kakunaga, S. Itoh, T. Shingai, K. Takekuni, K. Satoh, Y. Inoue, A. Hamaguchi, K. Morimoto, M. Takeuchi, T. Imai, Y. Takai, Tage4/Nectin-like molecule-5 heterophilically trans-interacts with cell adhesion molecule Nectin-3 and enhances cell migration, J. Biol. Chem. 278 (2003) 28167–28172.

[20] T. Shingai, W. Ikeda, S. Kakunaga, K. Morimoto, K. Takekuni, S. Itoh, K. Satoh, M. Takeuchi, T. Imai, M. Monden, Y. Takai, Implications of nectin-like molecule-2/IGSF4/RA175/SgIGSF/TSLC1/SynCAM1 in cell–cell adhesion and transmembrane protein localization in epithelial cells, J. Biol. Chem. 278 (2003) 35421–35427.

[21] D.S. Rice, G.M. Northcutt, C. Kurschner, The Lnx family proteins function as molecular scaffolds for Numb family proteins, Mol. Cell. Neurosci. 18 (2001) 525–540.

[22] J. Sambrook, E. Fritsh, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2 ed., Cold Spring Harbor, NY, 1989.

[23] T. Kaneko, M.A. Caria, H. Asanuma, Information processing within the motor cortex: II. Intracortical connections between neurons receiving somatosensory cortical input and motor output neurons of the cortex, J. Comp. Neurol. 345 (1994) 172–184.

[24] S.M. Marfatia, R.A. Lue, D. Branton, A.H. Chishti, In vitro binding studies suggest a membrane-associated complex between erythroid p55, protein 4.1, and glycophorin C, J. Biol. Chem. 269 (1994) 8631–8634.

[25] C. Krummenacher, I. Baribaud, R.J. Eisenberg, G.H. Cohen, Cellular localization of nectin-1 and glycoprotein D during herpes simplex virus infection, J. Virol. 77 (2003) 8985–8999.

[26] I. Ravens, S. Seth, R. Forster, G. Bernhardt, Characterization and identification of Tage4 as the murine orthologue of human poliovirus receptor/CD155, Biochem. Biophys. Res. Commun. 312 (2003) 1364–1371.

[27] Y. Takai, H. Nakanishi, Nectin and afadin: novel organizers of intercellular junctions, J. Cell. Sci. 116 (2003) 17–27.

[28] H. Gomyo, Y. Arai, A. Tanigami, Y. Murakami, M. Hattori, F. Hosoda, K. Arai, Y. Aikawa, H. Tsuda, S. Hirohashi, S. Asakawa, N. Shimizu, E. Soeda, Y. Sakaki, M. Ohki, A 2-Mb sequence-ready contig map and a novel immunoglobulin superfamily gene IGSF4 in the LOH region of chromosome 11q23.2, Genomics 62 (1999) 139–146.

[29] K.B. Hoover, P.J. Bryant, The genetics of the protein 4.1 family: organizers of the membrane and cytoskeleton, Curr. Opin. Cell Biol. 12 (2000) 229–234.

[30] N. Alloisio, E. Dorleac, R. Girot, J. Delaunay, Analysis of the red cell membrane in a family with hereditary elliptocytosis—total or partial of protein 4.1, Hum. Genet. 59 (1981) 68–71.

[31] S.M. Marfatia, J.H. Morais-Cabral, A.C. Kim, O. Byron, A.H. Chishti, The PDZ domain of human erythrocyte p55 mediates its binding to the cytoplasmic carboxyl terminus of glycophorin C. Analysis of the binding interface by in vitro mutagenesis, J. Biol. Chem. 272 (1997) 24191–24197.

[32] L.D. Walensky, Z.T. Shi, S. Blackshaw, A.C. DeVries, G.E. Demas, P. Gascard, R.J. Nelson, J.G. Conboy, E.M. Rubin, S.H. Snyder, N. Mohandas, Neurobehavioral deficits in mice lacking the

erythrocyte membrane cytoskeletal protein 4.1, Curr. Biol. 8 (1998) 1269–1272.
[33] L.D. Walensky, S. Blackshaw, D. Liao, C.C. Watkins, H.U. Weier, M. Parra, R.L. Huganir, J.G. Conboy, N. Mohandas, S.H. Snyder, A novel neuron-enriched homolog of the erythrocyte membrane cytoskeletal protein 4.1, J. Neurosci. 19 (1999) 6457–6467.
[34] K. Ye, D.A. Compton, M.M. Lai, L.D. Walensky, S.H. Snyder, Protein 4.1N binding to nuclear mitotic apparatus protein in PC12 cells mediates the antiproliferative actions of nerve growth factor, J. Neurosci. 19 (1999) 10747–10756.
[35] L. Shen, F. Liang, L.D. Walensky, R.L. Huganir, Regulation of AMPA receptor GluR1 subunit surface expression by a 4. 1N-linked actin cytoskeletal association, J. Neurosci. 20 (2000) 7932–7940.