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# Tetraspanins expressed in the embryonic chick nervous system

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**Abstract Proteins of the tetraspanin superfamily participate in the formation of plasma membrane signaling complexes; recent evidence implicates neuronal tetraspanins in axon growth and target recognition. We used a degenerate PCR screen to identify cDNAs encoding tetraspanins expressed in the embryonic spinal cord. Two cDNAs identified apparently represent chick homologues of NAG-2 (cnag) and CD9 (chCD9). A third clone encodes a novel tetraspanin (neurospanin). All three mRNAs are widely expressed but exhibit developmentally distinct patterns of expression in the nervous system. Both neurospanin and cnag exhibit high relative expression in nervous tissue, including brain, spinal cord and dorsal root ganglia (DRG).**



*Key words:* Tetraspanin; Development; Spinal cord; Brain; Avian; Sensory neuron

## 1. Introduction

The tetraspanin superfamily, also known as the tetraspans or the transmembrane 4 superfamily, is a newly described family of integral membrane proteins comprising more than 20 members [1]. The tetraspanins are small, 220–280 amino acid glycoproteins, which share 20–45% sequence identity. These proteins have been conserved during evolution and are expressed in a wide variety of eukaryotic species including human, mouse and *Drosophila* [2–5].

Although initially identified as leukocyte surface antigens, many tetraspanin family members are expressed in a wide variety of cell types [6,7]. In the adult nervous system, both central and peripheral glia strongly express the tetraspanin CD9 [8]. Early in development, CD9 is expressed in restricted neuronal populations [4,8,9]. Another tetraspanin, the *Drosophila* protein *late bloomer* (*lbl*), exhibits an expression pattern nearly exclusive to a specific subset of motorneurons, with weak transient expression in some peripheral sensory neurons [5].

Tetraspanin family members may have roles in the regulation of axon growth. First, CD9 has been localized to neurons early in development, coincident with a period of active axon growth for many neuronal populations [4,8]. Second, $\beta_1$ integrins are commonly found in complexes with tetraspanins [10]; these integrins are known to mediate axon growth induced by numerous extracellular matrix proteins [11,12]. Enhanced integrin-mediated neurite outgrowth in response to CD9 activation suggests a functional relationship between integrins and tetraspanins [4,13]. Recent evidence suggests that tetraspanins may serve as a link between integrin subunits and various intracellular signaling molecules, such as phosphatidylinositol 4-kinase and PKC [14–16]. Third, tetraspanin activation can result in increases in intracellular calcium and enhanced tyrosine kinase and phosphatase activity [4,17,18], each of which has been implicated in the regulation of neurite outgrowth e.g. [19–21].

Tetraspanins may be important in the regulation of motor axon growth and target recognition [5,8]. To date, only a few tetraspanins have been shown to be expressed in the developing vertebrate nervous system, with only CD9 known to be expressed by developing neurons [13,34,35]. We hypothesized that specific populations of developing neurons express specific complements of tetraspanins and that these are involved in the regulation of axon growth and synapse formation. To begin to examine this issue for the developing spinal cord, we screened chick spinal cord mRNA for expression of tetraspanins at embryonic day 6 (E6), a time when motor axons are actively growing towards and synapsing with their targets [22,23]. Through this screen, we have identified and characterized a novel tetraspanin as well as two chick homologues of previously described mammalian tetraspanins.

## 2. Materials and methods

### 2.1. Materials

Fertilized eggs from SPAFAS (Preston, CT, USA) were incubated at 38°C until use. Various chick tissues were dissected from the embryos, immediately frozen in liquid nitrogen, and stored at −80°C until use. Restriction enzymes used for subcloning were purchased from New England Biolabs, (Beverly, MA, USA). All other reagents were from Sigma (St. Louis, MO, USA) unless otherwise noted.

### 2.2. Reverse transcription-polymerase chain reaction (RT-PCR) assays

Complementary DNA (cDNA) was reverse transcribed from total RNA isolated from various chick tissues and developmental ages using the Ultraspec RNA isolation system (Biotecx Laboratories, Houston, TX, USA). Equivalent aliquots of the cDNA synthesis reactions were used for each PCR assay. Superscript II reverse transcriptase, *Taq* DNA polymerase, and all PCR primers were obtained from Gibco-BRL (Grand Island, NY, USA). Random hexamers were purchased from Promega (Madison, WI, USA) and dNTPs were purchased from Stratagene (La Jolla, CA, USA).

### 2.3. Degenerate PCR screen of E6 spinal cord

RT-PCR assays were carried out on embryonic day 6 (E6) spinal cord mRNA using degenerate primers designed based on conserved regions of transmembrane domains 1 and 2 in the following tetraspanins: CD9, CD81, CD82, CD63 and PETA-3. Because of the limited sequence identity among tetraspanins even in this region, our primers contained both degeneracy and inosine residues ('I' in primer sequences). The 5′ primer corresponded to the sequence at the beginning of transmembrane domain 1 (5′-AAR TGY III AAR TWY YTI YTI TW-3′) and the 3′ primer corresponded to the sequence at the end of

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transmembrane domain 2 (5′-GC NCC III RCA NCC IAR RAA ICC-3′).

*2.4. Isolation of tetraspanin clones*

An E10 chick retinal library [24] and an E13 chick brain library [25] were screened with tetraspanin DNA fragments, obtained from the degenerate PCR assay, essentially as described [24]. Probes were 3′-end labeled as described [26].

Rapid amplification of cDNA ends (RACE) was performed using the Marathon cDNA Amplification kit (Clontech, Palo Alto, CA, USA). E8 brain mRNA was used to generate an adapter-ligated cDNA library for the RACE protocol. A 5′ cnag RACE product and a 3′ chCD9 RACE product were obtained using standard protocols. Alternative strategies were necessary to obtain the remaining sequences for cnag and chCD9. The 3′ end of cnag was amplified from E8 brain cDNA using a primer based on the amino acid sequence (AMTMYC) at the 3′ end of the fourth transmembrane domain of human and mouse NAG-2. Similarly, the 5′ end of chCD9 was amplified from E8 brain cDNA using a primer based on the completely conserved N-terminal amino acid sequence (MPVKGG) of several CD9 species homologues.

Dideoxy sequencing of clones was performed as described [24]. Sequences were also obtained from the University of Miami DNA Core Lab Sequencing Service. Sequence analysis was performed using the Genetics Computer Group (GCG) sequence analysis software (Madison, WI, USA).

*2.5. Northern blotting*

Total RNA or poly $A^+$ mRNA, isolated from total RNA using the Poly A-Tract mRNA isolation system (Promega, Madison, WI, USA), was separated on formaldehyde gels, transferred to nitrocellulose membranes and hybridized with full-length tetraspanin cDNA clones as described [24]. After exposure to film, membranes were stripped and re-probed with a random primer-labeled, 1.8 kb fragment of chick β-actin [25]. Intensity measurements of hybridization signals were obtained using SigmaScan Pro3 software from Jandel Scientific (San Rafael, CA, USA). Relative tetraspanin expression levels were obtained by normalization to β-actin levels.

*2.6. In situ hybridization*

Lumbar spinal cords from E8 chick embryos were dissected and treated exactly as described [27]. Sense and antisense probes were generated, using SP6 polymerase, from the full length neurospanin cDNA cloned into pSP73 and pSP72, respectively (Promega, Madison, WI, USA). Slides were exposed for 4–6 weeks at 4°C in the dark with desiccator. Following development of the autoradiographic emulsion, sections were counterstained with 0.02% toluidine blue and coverslipped for analysis.

## 3. Results

*3.1. Cloning of three tetraspanins expressed in the embryonic spinal cord*

The expression pattern of CD9 in the mammalian nervous system, together with the discovery of *lbl* in the fly [5,8,28], led us to hypothesize that a number of tetraspanins would be expressed in cell populations of the embryonic brain and spinal cord. To examine the tetraspanins expressed in the developing spinal cord, we performed a degenerate PCR screen of E6 chick spinal cord mRNA, using primers complementary to conserved regions in tetraspanin transmembrane domains 1 and 2. Sequence analysis of 42 clones revealed 5 putative

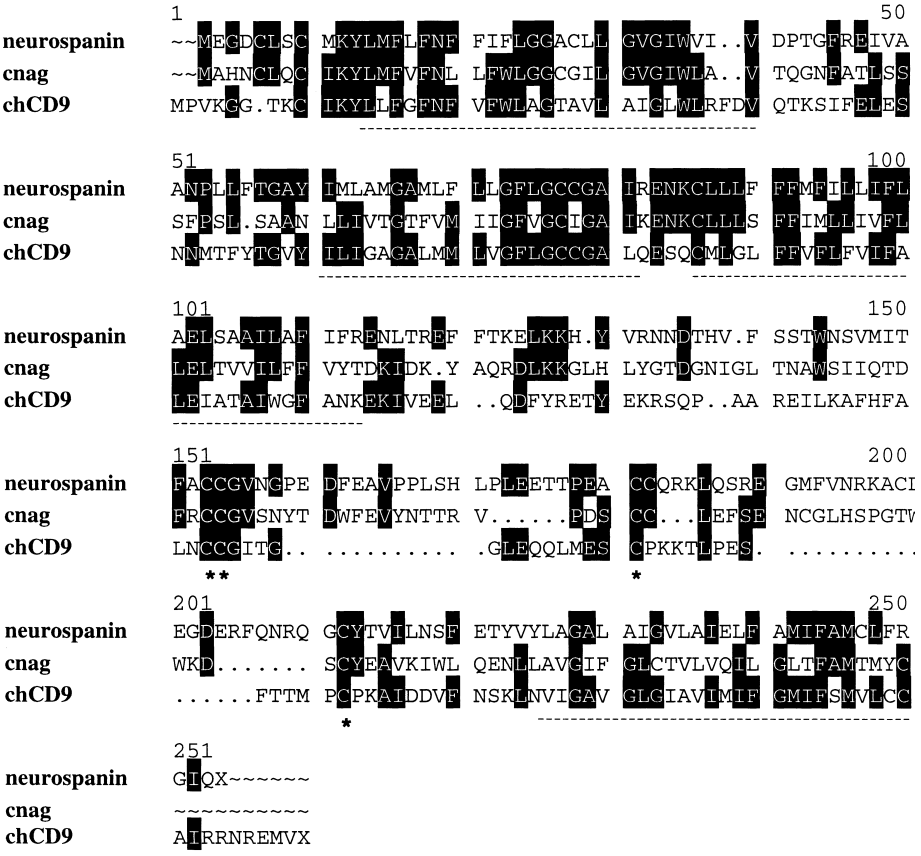


Fig. 1. Amino acid sequences of neurospanin, cnag, and chCD9. Black boxes in the figure indicate identical residues. Dashed lines indicate the putative transmembrane domains. Asterisks denote the four conserved cysteines found in the large extracellular domains of tetraspanin family members. The novel tetraspanin, neurospanin, shares up to 32% identity with other tetraspanin superfamily members. The cnag sequence shares 86% identity with human and mouse NAG-2 and chCD9 shares 70% to 74% amino acid identity with known mammalian CD9 isoforms. The DNA sequences for neurospanin, cnag and chCD9 have the GenBank accession numbers XX, YY and ZZ, respectively.

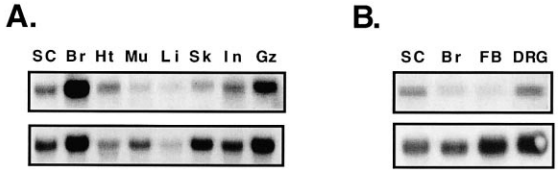


Fig. 2. A: Northern blot analysis of neurospanin expression in various embryonic chick tissues including E8 chick spinal cord (SC), brain (Br), heart (Ht), skeletal muscle (Mu), liver (Li), skin (Sk), intestine (In) and gizzard (Gz). B: Northern blot analysis of cnag expression in various embryonic neuronal populations including E8 spinal cord (SC), E8 brain (Br), E7 forebrain (Fb) and E9 dorsal root ganglia (DRG). 25 μg of total RNA, isolated from the indicated tissues, was probed with a full length neurospanin (A) or cnag (B) cDNA (upper panels), and re-probed with a random primer-labeled 1.8 kb cDNA fragment of chick β-actin as a loading control (lower panels).

tetraspanin clones, identified on the basis of significant sequence identity to known tetraspanins. Two clones were identified twice and were tentatively identified as the chick orthologues of previously identified tetraspanins, NAG-2 and CD9. Sequence obtained for a fifth clone apparently represented a novel tetraspanin. Because it was cloned from nervous tissue, we have named this novel clone neurospanin. Full-length sequences for neurospanin, cnag and chCD9 were obtained by a combination of RT-PCR, screening of cDNA libraries [24,25], and RACE protocols (Fig. 1).

### 3.2. *Embryonic tissue distribution of neurospanin and cnag mRNA expression*

We examined the expression of neurospani, cnag, and chCD9 in various E8 chick tissues (spinal cord, brain, heart, skeletal muscle, liver, skin, intestine and gizzard) by RT-PCR; mRNAs encoding all three tetraspanins are widely expressed in the chick embryo (data not shown). Relative levels of neurospanin mRNA in various E8 tissues were examined by Northern blot analysis (Fig. 2A). The highest level of neurospanin mRNA in the E8 embryo was detected in the brain, with high levels also in the heart. Moderate levels of neurospanin mRNA were found in the spinal cord, intestine, and gizzard, with low levels in skeletal muscle, liver, and skin.

Relative expression levels of cnag mRNA in various regions of the embryonic nervous system were also examined (Fig. 2B). Expression of cnag mRNA in embryonic brain was low relative to the spinal cord, and levels in forebrain were lower than in total brain. These data suggest a rostro-caudal gradient of expression for cnag, with lowest levels in the rostral brain. Relatively high levels of cnag mRNA were also detected in the peripheral dorsal root ganglia (DRG) at E9.

### 3.3. *Developmental expression of neurospanin, cnag and chCD9 in chick brain*

We also examined the developmental pattern of expression for neurospanin cnag, and chCD9 mRNAs in chick brain. Neurospanin mRNA was expressed in E5 brain and expression levels increased 7-fold between E5 and E10 (Fig. 3A). Neurospanin mRNA expression remained roughly constant from E10 until about E17, then increased about two-fold toward the end of embryogenesis. Levels of cnag mRNA in brain increased gradually throughout embryonic development and were highest at the time of hatching (Fig. 3B). The expression of chCD9 mRNA is evidently very low in whole brain and was not detected using total RNA. Northern blots using poly $A^+$-selected mRNA revealed that brain levels of chCD9 mRNA increased dramatically ($>300$-fold) between E14 and hatching (Fig. 3C). Previous studies have shown that mammalian CD9 is expressed in restricted neuronal populations at early developmental ages [4,8]. Similar restricted expression of chCD9 in specific populations of neurons in the chick brain could explain the low levels of expression we detected at early developmental stages.

### 3.4. *In situ hybridization analysis of neurospanin expression in the embryonic spinal cord*

We used in situ hybridization to examine the distribution of neurospanin mRNA expression in the E8 spinal cord. Expression was observed in portions of the dorsal horn throughout the lumbosacral enlargement (Fig. 4BB). In the dorsal horn, strongest expression was observed at more rostral and caudal aspects of the enlargement (Fig. 4B and F(high)), with weaker expression in intervening segments (data not shown and Fig. 4F(avg)). Expression appeared confined to laminae 2 and 3 of the dorsal horn [29]; in central segments of the enlargement expression was mainly in lamina 2 (data not shown). Hybridization signals could be seen on large cells (presumptive neurons); we could not determine whether glia also expressed neurospanin. No expression was detected in the ventral portion of the spinal cord, including the motor columns. Neurospanin mRNA expression was also found in the dorsal root ganglia of the lumbosacral region (Fig. 4E), but no differential pattern of rostral-caudal expression was detected, as evidenced by a single population of hybridization grain density (Fig. 4G).

## 4. Discussion

Several lines of evidence suggest that tetraspanins play roles in axonal growth and guidance processes. First, two tetraspanin family members, CD9 and *lbl*, have been localized to the nervous system, exhibiting restricted expression in subpopula-

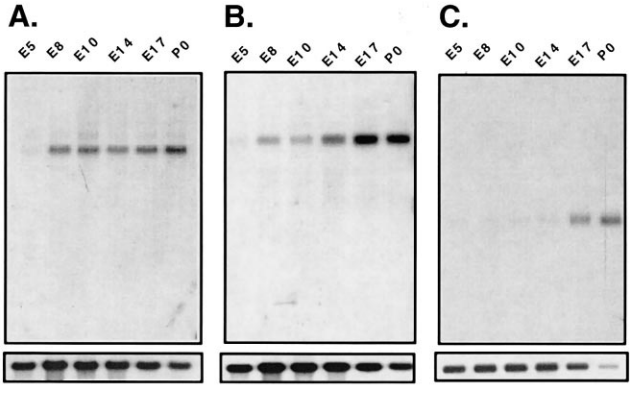


Fig. 3. Northern blot analysis of neurospanin, cnag, and chCD9 expression in the developing chick brain. (A,B) 40 μg of total RNA or (C) 5 μg of poly $A^+$ mRNA was isolated from brain at the indicated embryonic ages and probed with a full length neurospanin (A), cnag (B), or chCD9 (C) cDNA (upper panels). The blots were re-probed with a β-actin cDNA probe as a loading control (lower panels). The neurospanin, cnag and chCD9 transcripts are expressed as 3.4 kb, 5.3 kb, and 1.1 kb mRNAs, respectively. A shorter transcript was not detected in the cnag blot at 1.5 kb, as has been reported for NAG-2 in various adult human tissues [7].

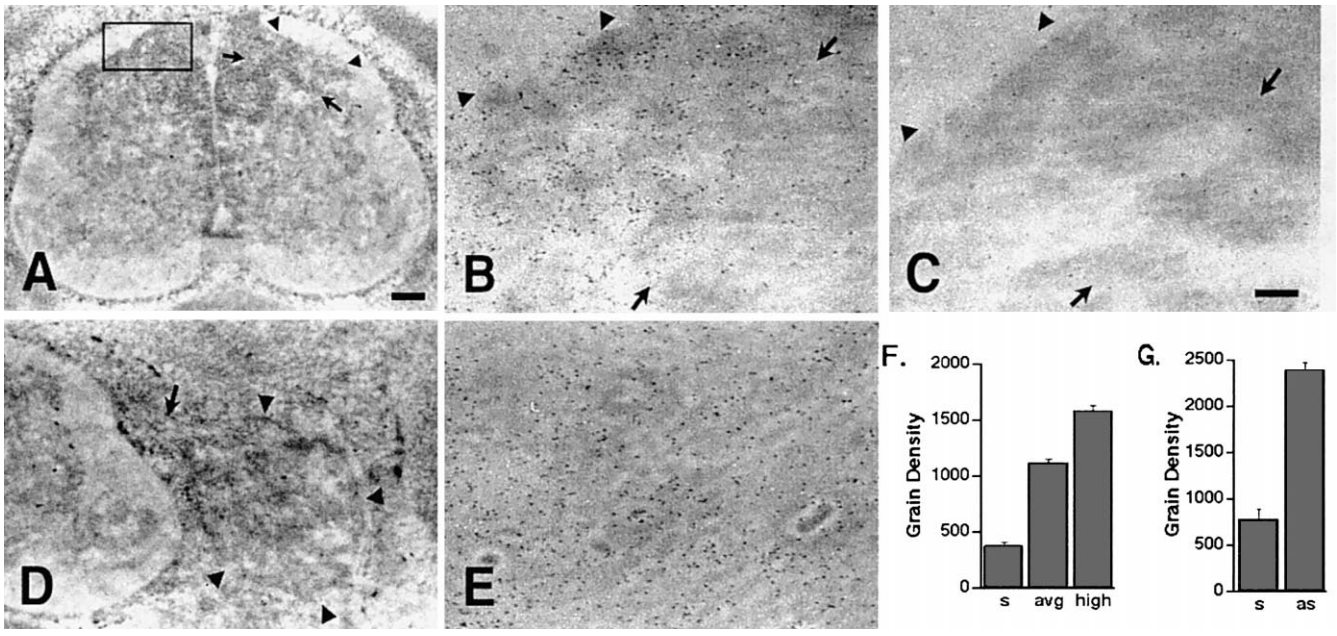


Fig. 4. In situ hybridization analysis of neurospanin mRNA in the embryonic chick spinal cord and DRG. A: Transverse section of E8 lumbar spinal cord. The black box represents area enlarged in panels B and C. B,C: Phase-contrast images of E8 dorsal horn hybridized with an antisense (B) or sense (C) neurospanin cRNA probe. Panel B represents those sections that exhibited stronger hybridization signals commonly found at more rostral and caudal aspects of the lumbar spinal cord. In panels A–C, arrowheads delineate the border between white and gray matter and arrows indicate the end of the specific hybridization band, approximately laminae 2 and 3 of the dorsal horn [29]. D: Transverse section of E8 lumbar DRG. The nerve root is indicated by the arrow and the ganglion is outlined by arrowheads. E,F: Phase-contrast images of E8 DRG hybridized with an antisense (E) neurospanin cRNA probe. F,G: Quantitative data for hybridization grain densities in the dorsal horn (F) and DRGs (G) of the lumbosacral spinal cord. Bar in A, 100 μm for A and D. Bar in C, 10 μm for B, C and E.

tions of motorneurons early in development [4,5,8]. Second, tetraspanins are known to form complexes with axonal growth-promoting proteins, such as integrins and cell adhesion molecules [4,6,10,14,15]. Third, antibody stimulation of tetraspanins results in calcium mobilization and tyrosine phosphorylation events [1], which are important features of signal transduction mechanisms regulating neurite outgrowth [4,20,21]. Finally, functional studies have implicated CD9 and *lbl* in axon growth and target recognition [4,5,13]. Taken together, the data suggest that neuronally expressed tetraspanins could function as facilitators of neurite outgrowth-dependent signal transduction through their association with growth promoting molecules.

In attempts to identify neurally-expressed tetraspanins, we cloned a cDNA encoding a novel tetraspanin, neurospanin. Although widely expressed in the embryo, neurospanin mRNA was found at highest levels in the developing brain. Further, neurospanin mRNA is upregulated in brain during the peak time of axon growth [30–33]. Expression of neurospanin in both the DRG and the dorsal horn was observed at embryonic day 8, a time when lumbosacral sensory afferents are forming synapses with their central targets [32,33]. Our data suggest that neurospanin may play an important role in early processes relevant to the development of the nervous system, perhaps in neuron-neuron interactions in the developing spinal cord.

We have identified a neuronally expressed chick tetraspanin that is highly similar to mammalian CD9. As mentioned, the sequence identity shared between the putative chick CD9 homologue and mammalian CD9 isoforms is relatively low. Mammalian CD9 isoforms share 84% to 99% sequence identity across species, whereas the putative chick CD9 homologue shares only 70% to 74% identity with mammalian CD9 isoforms. In contrast, the chick NAG-2 homologue shares 86% identity with the human and mouse NAG-2 isoforms. Our chick CD9 clone is sufficiently different from the known CD9 isoforms, especially in the large extracellular loop, to suggest that chCD9 may be a CD9 splice variant or a related gene product. Alternative splicing of the CD9 gene has been proposed [9].

The demonstration of cnag expression in chick brain appears to contradict the report on mammalian NAG-2 [7]. One obvious difference between the latter study and our own is the age of the tissue samples (adult vs. embryonic). However, the levels of cnag mRNA increased during embryonic development, with the highest levels associated with the day of hatching. This finding suggests continued expression in the adult animal. A more likely explanation is suggested by the low levels of cnag mRNA in brain compared to spinal cord and peripheral ganglia; expression in brain could simply be below the level of detection in the earlier study [7]. It is also conceivable that cnag is not the ortholog of mammalian NAG-2, despite the close sequence similarity.

DRGs express at least three different tetraspanin family members: neurospanin, cnag, and CD9 (present study; [8,28]). Indeed, it is possible that a number of tetraspanins are expressed in DRGs, as well as other populations of neurons throughout the developing nervous system. Thus expression of particular complements of tetraspanins in neurons could support the assembly of multimolecular signaling complexes with proteins that influence the growth and guidance of axons, such as β1 integrins and cell adhesion molecules, regulating the formation of specific sensory connections in the spinal cord.

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