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cDNA Cloning and Expression of the *Xenopus* Homologue of the Neural Adhesion Molecule, Contactin (F3/F11)

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ABSTRACT—Contactin (F3/F11) is an immunoglobulin superfamily cell surface glycoprotein predominantly expressed in the nervous system. To examine the structure and tissue distribution of *Xenopus* contactin, a cDNA clone was isolated based on the amino acid sequences conserved among chicken and mammalian contactin proteins. The conceptual translate of the cDNA consists of 1005 amino acid residues that have 70% identity to those of chicken and mammalian contactin. Northern blot hybridization using a labeled cDNA fragment revealed specific expression of 6.5 kb mRNA in the brain. Monoclonal and polyclonal antibodies prepared to the recombinant *Xenopus* contactin peptides detected a single 135 kD band on Western blots of the brain and spinal cord extracts. Differential extraction and phosphatidylinositol-specific phospholipase C (PI-PLC) digestion experiments showed that the immunoreactive 135 kD proteins bind, at least in part, to the membrane by GPI anchor. On brain tissue sections, strong contactin immunoreactivities were detected on nerve fibers of a subset of cerebral and cerebellar neurons. These results suggest that the basic structure and tissue distribution of *Xenopus* contactin are similar to those in other vertebrates.

INTRODUCTION

Morphogenesis of the nervous system involves sequential cellular interaction that regulates cell differentiation, tissue organization, and formation of the highly stereotyped neuronal network. Recent extensive studies on molecular mechanisms of such cellular interaction have pointed out critical roles of several groups of membrane glycoproteins. The immunoglobulin superfamily is one of the major groups of such membrane glycoproteins. A growing number of immunoglobulin superfamily proteins have been identified in the nervous system of diverse vertebrate and invertebrate species, suggesting conserved structural and functional properties of this superfamily (see reviews, Hynes and Lander, 1992; Sonderegger and Rathjen, 1992; Vaughan and Bjorkman, 1996).

Contactin (also called F3 in mammals and F11 in chickens) is a member of the immunoglobulin superfamily predominantly expressed in the nervous system of chickens (Ranscht, 1988; Brümmendorf *et al.*, 1989) and mammals (Gennarini *et al.*, 1989; Reid *et al.*, 1994; Hosoya *et al.*, 1995; Watanabe *et al.*, 1995). The molecules are 135 kD glycoproteins that belong to the immunoglobulin/fibronectin type III subfamily, having 6 immunoglobulin C2-like domains and 4 fibronectin type III-like domains (Rathjen and Jessel, 1991; Brümmendorf and Rathjen, 1993; Vaughn and Bjorkman, 1996). Although chicken contactin was first reported to be a transmembrane protein with a cytoplasmic domain (Ranscht, 1988), the later studies have shown that mammalian and chicken contactin bind to the membrane by a C-terminal glycosylphosphatidylinositol (GPI) anchor. A soluble form of contactin was also reported to occur in the brain and cerebrospinal fluid (Gennarini *et al.*, 1989; Durbec *et al.*, 1992; Moss and White, 1992).

Contactin mediates cell-to-cell adhesion and neurite extension in cultured neural cells by homophilic interaction (Gennarini *et al.*, 1991). In addition, it heterophilically interacts with various membrane glycoproteins such as Ng-CAM (Brümmendorf *et al.*, 1993), Nr-CAM/Bravo (Morales *et al.*, 1993) and receptor tyrosine phosphatase β (Peles *et al.*, 1995), which are expressed on neurons or neuroglial cells. These heterophilic interactions also cause neural cell adhesion and neurite outgrowth in vitro. In addition, contactin mediates molecular recognition of extracellular matrix glycoproteins such as restrictin/janusin and tenascin, which leads to adhesion of neural cells or repulsion of growing neurites (Rathjen *et al.*, 1991; Norenberg *et al.*, 1992; Pesheva *et al.*, 1993; Zisch *et al.*, 1992; Vaughan *et al.*, 1994). These homophilic and

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heterophilic interactions with various molecules are likely to represent functional diversity of the domains of this molecule. In mouse and rat brains, contactin proteins distribute preferentially on the axonal membrane and the amounts of its expression markedly increase after birth (Gennarini *et al.*, 1990; Faivre-Sarrailh *et al.*, 1992; Yoshihara *et al.*, 1995; Watanabe *et al.*, 1995). All these findings seem to imply that contactin may have major roles in cell-to-cell and cell-to-extracellular matrix interactions of post-mitotic neurons which regulate neurite outgrowth, axonal path finding, fasciculation, myelination and synaptogenesis. The detailed molecular mechanisms and the physiological roles of such contactin's actions are, however, remained to be determined in the developing nervous system.

We have introduced the South African clawed frog *Xenopus laevis* as a model to study roles of contactin in the developing nervous system, since this animal provides advantageous experimental approaches to investigate gene expression and cellular interactions in the vertebrate embryonic development. Thus, the pattern and the inductive tissue interactions in early neurogenesis were described in detail, and many genes, some of which are vertebrate homologue of the *Drosophila* neurogenic genes, are defined to regulate these processes (Lee *et al.*, 1995). We report here cloning of the cDNA encoding the *Xenopus* homologue of contactin and expression of its mRNA and proteins in brains of young adult frogs. Besides a well-known immunoglobulin superfamily member, NCAM (Kintner, 1988; Kreig *et al.*, 1989; Tonissen and Kreig, 1993), this is, to our knowledge, the first study to report the structure and tissue distribution of the neural adhesion molecule of this superfamily in amphibia.

MATERIALS AND METHODS

Animals

Six to 10 month-old J strain *Xenopus laevis* (Tochinai and Katagiri, 1975) were used for cDNA library construction and Southern blot analysis. In the other experiments outbred or J strain frogs at ages of 6-12 months were used.

Isolation and characterization of the contactin cDNA

To amplify cDNA fragments encoding the *Xenopus* homologue of contactin by PCR, a set of degenerate oligonucleotide primers was designed from the amino acid sequences conserved among chicken (Brümmendorf *et al.*, 1989), mouse (Gennarini *et al.*, 1989), rat (Hosoya *et al.*, 1995), bovine (Watanabe *et al.*, 1995) and human (Reid *et al.*, 1994) contactin proteins: (5')-TGGGTNGARCAYAT-HAATGA-(3') for amino acid residues 323-329 of chicken contactin, WVEHIND and (5')-CKRAAYTCRTAYTCCATCCA-(3') for amino acid residues 664-670, WMEYEFR. The total RNA was isolated from frog brains as described (Chomczynski and Sacchi, 1987), and the template cDNA was synthesized using AMV reverse transcriptase according to the manufacturer's instruction (Takara shuzo Co., Ohtu, Japan). Using these template and primers, a DNA fragment of about 1 kb, which was the size expected from the contactin cDNA sequences, was amplified and cloned in pBluescript. The partial sequence of this fragment showed about 70% nucleotide identity to the chicken sequence. The cloned fragment was labeled with digoxygenin through PCR amplification (Boehringer Mannheim, Mannheim, Germany) and used as the screening probe. Poly(A)$^+$ RNA was isolated from frog brains using the FastTrack mRNA isolation kit (Invitrogen, San Diego, CA), and an oligo (dT)-primed cDNA library in the λZapII vector (Stratagene, La Jolla, CA) was constructed. After screening approximately 2×10^6 recombinant plaques, 21 hybridizing clones were isolated and converted from recombinant phages to plasmids. One of the clones containing an entire long open reading frame was used for further characterization. Nucleotide sequences were determined by the dideoxy chain termination method using fluorescent primers and an automated DNA sequencer (Pharmacia Biotech, Uppsala, Sweden).

Southern and Northern blotting

Southern blot analysis was performed according to the previously described method (Kreig *et al.*, 1989). The genomic DNA was isolated from peripheral blood cells, digested with restriction endonucleases, fractionated by electrophoresis on a 0.8% agarose gel and transferred to the nitrocellulose membrane. The probes used were ^{32}P-labeled cDNA fragment a and b shown in Fig. 1A. For Northern blot analysis, total RNA preparations from various frog tissues were fractionated on a 1.2% agarose gel containing formaldehyde. The blots on nitrocellulose membranes were hybridized with the labeled cDNA fragment. The hybridized Southern and Northern blots were autoradiographed using X-ray film for several days at -70°C.

Antibodies and Western blotting

The cDNA fragments c and d were amplified by PCR and cloned into the bacterial expression vectors, pET-3b (Novagen Inc., Madison, WI) and pGEX-3X (Pharmacia Biotech), respectively. The transformed bacteria were induced to produce the recombinant peptides according to the instruction of the vector providers. On SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the bacterial lysate, the recombinant peptide of amino acid residues 18-292 (peptide I) of the *Xenopus* contactin homologue (cDNA fragment c in Fig. 1A) was identified as a 33 kD band and the GST-fusion peptide containing amino acid residues 319-665 (peptide II) as a 66 kD band. To purify recombinant peptides, the bacterial inclusion bodies were isolated (Sambrook *et al.*, 1989), fractionated by SDS-PAGE and the peptides were eluted from the gel by using the Electroeluter (BioRad Laboratories, Herculus, CA).

New Zealand white rabbits were injected subcutaneously with 500 μg of purified GST-peptide II in the complete Freund adjuvant, followed by 2 monthly injections in the incomplete adjuvant. Antibody production was monitored by Western blotting and the high titer antisera were harvested. The rabbit antiserum to the recombinant rat contactin (F3) peptide was produced similarly (Takeuchi *et al.*, unpublished). The sera were affinity-purified using an antigen-bound nitrocellulose membrane as an adsorbent according to the described method (Harlow and Lane, 1988). Hybridoma cell lines secreting monoclonal antibodies to the recombinant peptide I (1H1; subclass IgG1(κ)) and peptide II (5C9; IgG1(κ)) were produced according to the established method (Harlow and Lane, 1988). Hybridomas were injected into the Balb/c mice and the tumor ascites containing monoclonal antibodies were harvested.

Western blotting was performed as described previously (Watanabe *et al.*, 1995) and the blots were developed using ECL Western Blotting Detection Reagent (Amersham Life Science, Inc., Little Chalfont, England).

Brain membrane fractions and PI-PLC digestion

Preparation and PI-PLC digestion of the brain membrane fraction were performed essentially as described previously (Gennarini *et al.*, 1989). In brief, the membrane fraction was resuspended at a concentration of 5 mg protein/ml in 50 mM Tris-HCl containing protease inhibitors. One hundred μl of the membrane fraction were incubated with 0.5 U of PI-PLC from *B. cereus* (Boehringer Mannheim) for 40 min at 37°C, freeze-and-thawed once, and then reincubated with the fresh enzyme. The controls were incubated in the buffer with or without

0.5% NP-40. After incubation, the samples were centrifuged at 140 k ×g for 90 min to separate the pelleted membrane and the supernatant, and the membrane was washed twice with the buffer. Contactin in the membrane and the supernatant was detected by Western blotting.

Immunocytochemistry

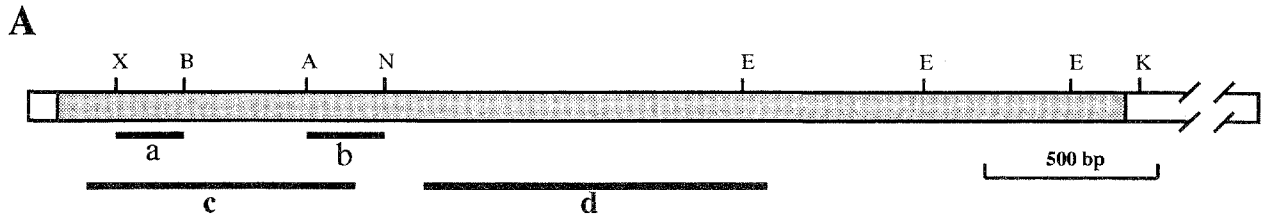
Frogs were anesthetized in 0.2% MS-222 (ethyl 3-aminobenzoate methane sulfonic acid; Aldrich Chem. Co., Milwaukee, WI), and perfused with PBS and then with 4% paraformaldehyde in PBS. After perfusion, the brains were dissected and immersed in the fixative overnight. They were washed with PBS, immersed in 30% sucrose, and embedded in the OCT compound (Miles Inc., Elkhart, IL). Frozen sections of about 10 μm thick were cut using a cryostat. The sections on glass slides were successively incubated with the affinity-purified rabbit antiserum (dilution 1:2000) and Vectastain ABC reagents (Vector Lab., Burlingame, CA). Control sections were incubated with a normal rabbit serum or the antiserum preabsorbed with either the recombinant GST-contactin peptide II or GST.

RESULTS

Structure of Xenopus *contactin*

The isolated cDNA clone is about 6.5 kb in size and contains an open reading frame of 3015 nucleotides. The conceptual translate consists of 1005 amino acids (Fig. 1B). Examination of the translate demonstrates that the N- and C-terminal regions contain stretches of hydrophobic amino acid sequences (amino acid residues 1-17 and 972-1005) which are a typical signal peptide and a GPI-anchorage motif, respectively. The mature peptide therefore consists of 988 amino acid residues, having a molecular weight of 109517 D.

Alignment of the translate with the chicken and mammalian proteins reveals great similarities between them, with identical amino acids at about 70% of the residues (Fig. 1B). However, amino acid residues in the N-terminal signal sequence and the C-terminal GPI anchor motif are not conserved well among the species. The signal sequence in the *Xenopus* peptides is 17 amino acid residues in contrast to



B

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X   1: MWPLVTQLV-IYFFFC------WAEEGKR-YGPVFEEQPIDTIFPEESTEGKVSVNCRARGNPPPIYKWRKNNLEIDLLNEHYSLVGGNLVINNPDKYKDSGRYVCIVSNIYGKVWSREA
C   1: .RFFISH..TLC.I..VADSTHFS...NKG.............Y....SD.Q..M.....AV.F.T...KL..WD...TKDR..M...R...S..E.SR.A.K...V....F.T.R.S..

X 113: TLNFGYLDPFPTEERYEVKVKEGTGAVLICDPPPHVPDDLNYRWLLNEFPVFLSPDNRRFVTQTNGNLYIAKVEHLDRGNYSCFVSSPSVTKSVFSKFISLLPQTDTSEKPKRYTPVINV
C 121: ..S.........P..H.....R..V....I.E..Y.Y....S...........IAL.R....S.........N..AS.K.....I.........P.I..A.---RA.V.PAD.K.

X 233: KFKDTRALKDQNVTLECFALGNPVPVITWRKINEAMPASADISMSGAVLKIFNVQPEDEGTYECEAKNIKGLDNHRAYVYVEGVPEWVEHINDTERDIGSDLHWACVARGKPQPSIRWLK
C 238: .....Y..LG.....ELR.S.YL.P...T.E.............I.Y....L...E.Y..K.K.Q.R...QAS.....K......Y.P...T...I.T.....

X 353: DGAPYGKGDIIKKGLTLEDAGMYGCIAENIHGIIYANAELKILALAPTFEFTPMRKKVLAAKGGRVIIECKPKAAPKAKFSWSKGTELLINNSRVSIWDDGSLEITNITKFDEGSYTCFA
C 358: N.VSFR..ELRIQ...F.....A.............V.SP....LN..K..I.....P.....V.S..IH.....I.V..L...R.....

X 473: ENDRGKANSTGIVSVTAATKITLAPSNADVTVGENATMQCHASHDPTLELTFIWALNGFPIEPDKEDGHYERAIRNVVGSELVIKNAQLIHAGRYTCTAQTIVDNSSASADLVVRGPPGP
C 478: ..N.......VLEM.E..R.....L.V.....I.....D.....S....V.D.E..HE....NVMIKSNG..L...V..R.....

X 593: PGGVRVEEIRDTSVKLTWSSGTDNHQPISKYNIQAQNILSEDWKDVKTENPNIEGNMEMARVIDLIPWMDYEFRVIATNTLGVGEPSLPSPKIRTEGAAPIVAPAEVGGGGGSNRELTIT
C 598: ...I.I......A.A....R.....S.....T..SKTF...E...A...PSD......S..........E....I.......T....M..QR......P.N...SD.............

X 713: WQPLSREYHYGDGFGYGVAFKPFNGREWRKVIVTNPESGHYVHKDDTITPATQFQVKVKAFNKIGEGPYSSTVVVYSAEDVPTEAPTAVVYNILSSSEVSVAWHPVYEKSIEGYQVRYWR
C 718: .M.........NN...I......GEK...R.T.....I.R.....ESMP.S..Y.........SK.D..F.L.A.I...Q.A...V..D.SVKV.....I..S..H.T...V....I...A

X 833: IQDKEAAAHRVQVKASDITTRLEGLMPNTHYNIEVRAFNSAGDGPPSKTLTFLTKKAPPSQIPQITSAVRYGSQYIITWEHVVPLSNESAVNGYKVLYRQDGQRDGKLYSTRKHSVELPV
C 838: AH......Q....SNQEYS.K..N.K...R.H.D.S......Y....R.IDII.R......R.R.I.S..S..R.....D..KAM.....E.......P...HE...F..G..TI.V..

X 953: PEEGVYVVEVRAHSEGGDGAVGFITISGVATGILPSVLGVLLPILSLLMYLEF
C 958: .SD.E.............E.AQ.K...ATA.VPTLL..LV..A.GV.A.SG.
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Fig. 1. (A) Restriction map of the cDNA encoding *Xenopus* contactin. The coding and non-coding sequences are represented by shaded and open boxes, respectively. The sites are: A, *Apa* I; B, *Bam* HI; E, *Eco* RI; K, *Kpn* I; N, *Nde* I; X, *Xho* I. Lines a and b represent fragments used as probes for Northern and Southern hybridization, and lines c and d for construction of the expression vectors (see text). (B) Amino acid sequence comparison of *Xenopus* contactin (X) with the chicken homologue (C). Dots and dashed lines in the chicken sequence indicate identical amino acid residues and gaps, respectively. Cysteine residues marked with closed circles are conserved to form immunoglobulin-like intrapeptide disulfide bonds, and asparagine residues marked with open squares potential N-linked glycosylation sites. The asparagine residue 616 (closed triangle) in the *Xenopus* sequence is not a glycosylation site because of the substitution of S to Q at the residue 618 (asterisk). Boxed sequences at N- and C-terminal regions indicate putative signals for secretion and GPI anchorage, respectively. The cDNA sequence data reported here will appear in the DDBJ, EMBLE and GenBank Nucleotide Sequence Databases with the accession No. D86505.

24-26 residues in chicken and mammals. The 12 cysteine residues, which can form intrapeptide disulfide bonds of 6 immunoglobulin type C2-like domains, are conserved in all the species. In addition, 4 fibronectin type III-like repeats contain conserved characteristic tryptophan residues (residues 600, 713, 815, and 911) and tyrosine/phenylalanine residues (residues 613, 663, 728, 766, 826, 863, 926 and 958). Chicken and mammalian contactin peptides have 9 possible N-glycosylation sites (NXT/S). The *Xenopus* peptide conserves 8 of them. However, one of the sites (N at amino acid residue 616) does not have the consensus sequence by substitution of S to Q (618) . There is an insertion of 3 amino acid residues in the second immunoglobulin C2 domain as compared with the chicken translate (or 2 residue insertion over the mammalian translates).

Contactin sequences in the Xenopus *genome*

Xenopus laevis is known to be pseudotetraploid (Kobel and Du Pasquier, 1986) and so has two functional non-allelic loci for many genes. To estimate the copy number of the contactin genes in the *Xenopus* genome, Southern blot analyses were performed using two separate cDNA fragments as probes. Both of the probe hybridized to two bands or a single broad band in each digest of 5 restriction enzymes (Fig. 2), suggesting that *Xenopus* has two non-allelic loci for the contactin gene as for the NCAM genes (Tonissen and Kreig, 1993).

Expression of the contactin mRNA and proteins

Northern blot hybridization using the radio-labeled cDNA fragment as a probe detected in the brain a single 6.5 kb band, which is approximately the same in size to the isolated cDNA (Fig. 3). No band was found in the other tissues examined even after an extended exposure period. The previous papers describe expression of a major 6.5 kb and minor 3.2-4.4 kb contactin mRNAs in chicken and mammalian brains (Ranscht, 1988; Gennarini *et al.*, 1989; Reid *et al.*, 1994), suggesting alternative polyadenylation of the transcript. Our effort was unsuccessful to detect such a shorter mRNA in *Xenopus* brains at various developmental stages (data not shown).

All the monoclonal and polyclonal antibodies detected a single 135 kD band on the Western blot of brain extracts (Fig. 4A-C). A similar 135 kD band was also detected in the spinal chord extract. However, as in above-described Northern blot analysis, no band was found on the blots of other tissues examined (data not shown). The rabbit antiserum prepared against rat contactin also detected a similar 135 kD band in the brain (Fig. 4D). From the difference between the molecular weight estimated by Western blotting and that deduced from the cDNA sequence, *Xenopus* contactin is, similar to mammalian and chicken contactin proteins, probably highly glycosylated.

The differential extraction and PI-PLC digestion experiments were performed to assess subcellular localization of contactin proteins in *Xenopus* brains (Fig. 5). A majority of immunoreactive contactin proteins was recovered in the membrane fraction and a part of them was solubilized from the membrane in the buffer containing Nonidet P-40. A smaller amount of contactin proteins was also detectable in Nonidet P-40-insoluble fraction, which may represent a "membrane skeleton"-associated form (Moss and White, 1992). The PI-PLC treatment clearly increased the release of contactin proteins from the membrane fraction as compared with the control incubated without PI-PLC. This implies that in *Xenopus* brains contactin molecules bind, at least in part, to the membrane by GPI anchor.

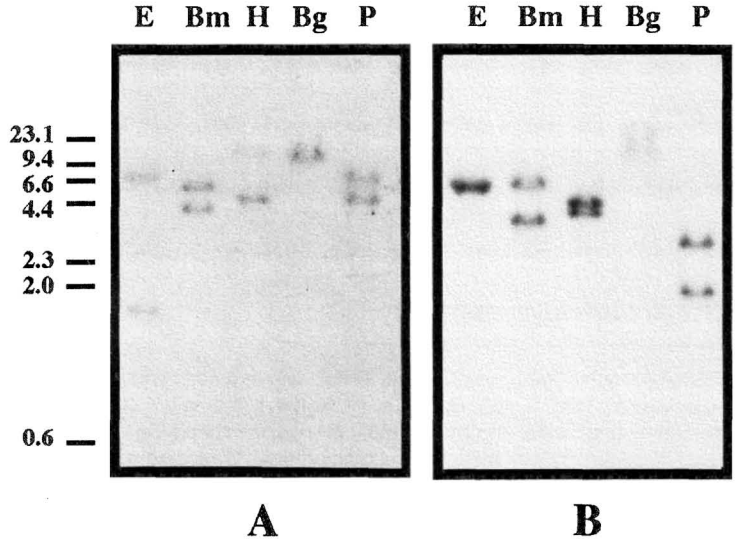

Fig. 2. Southern blot analysis of contactin sequences in *Xenopus* genomic DNA. Genomic DNA isolated from blood cells were digested with *Bam* HI (Bm) , *Bgl* II (Bg), *Eco* RI (E), *Hind* III (H) and *Pst* I (P), separated by agarose gel electrophoresis and transferred to the nitrocellulose membrane. (A) and (B) were probed with labeled cDNA fragment a and b in Fig. 1A, respectively.

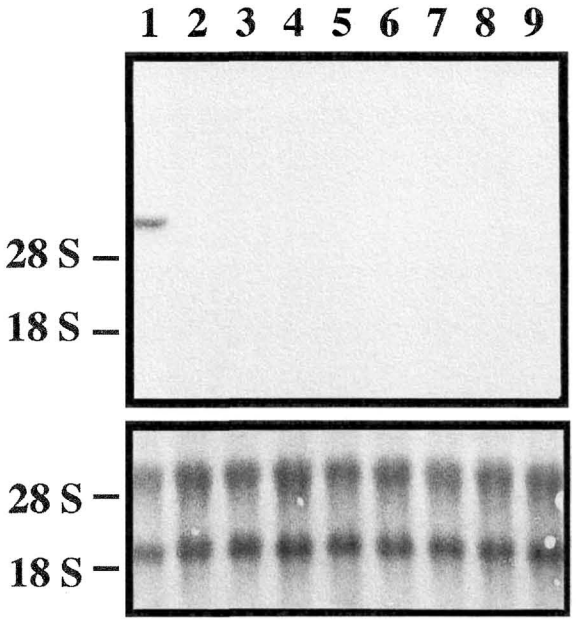

Fig. 3. Northern blot analysis of contactin expression in various *Xenopus* tissues. Each lane was loaded with 5 μg of total RNA and the blot was hybridized with the radiolabeled cDNA probe (upper panel). Expression of the 6.5 kb contactin transcript was detected in the brain (lane 1), but not in the other tissues: lanes 2-9, heart; kidney; liver; lung; skeletal muscle; spleen; testis; thymus. The same membrane was stained with methylene blue as a control for RNA loading (lower panel).

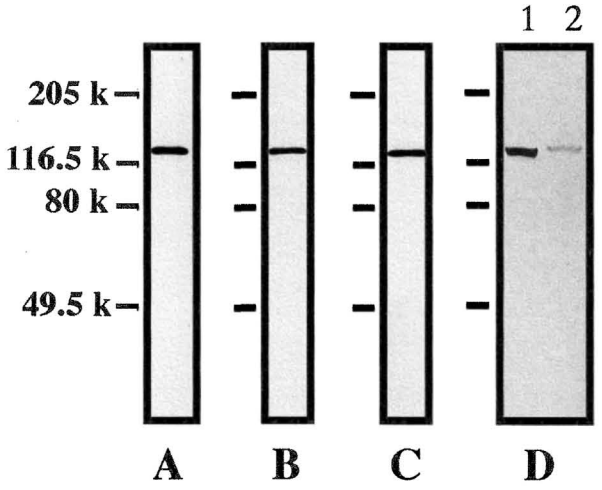

Fig. 4. (A-C) Western blot analysis of contactin proteins in *Xenopus* brains. The *Xenopus* brain extracts were fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with an affinity-purified rabbit antiserum (A), monoclonal antibodies 5C9 (B) and 1H1 (C) prepared against recombinant *Xenopus* contactin peptides. (D) Western blot analysis of contactin proteins in rat (lane 1) and *Xenopus* (lane 2) brain extracts using the rabbit anti-rat contactin antiserum.

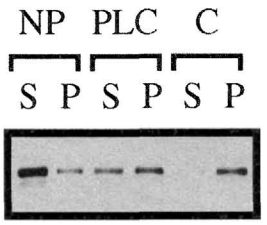

Fig. 5. PI-PLC digestion of the membrane fraction of *Xenopus* brains. The membrane fraction was incubated with NP-40 (NP), PI-PLC (PLC) or the buffer alone (C), and the supernatants (S) and the precipitates (P) were separated by centrifugation. Contactin in each fraction was detected by Western blotting using monoclonal antibody 5C9.

Tissue distribution of contactin in the brain

To examine tissue distribution of contactin proteins in the brain, frozen sections were immunocytochemically stained using the affinity-purified rabbit antiserum to recombinant GST-contactin peptides. Figure 6 shows a part of typical transverse sections through the cerebellum and the brain stem which are not clearly demarcated. Similar to other frog species (Kemali and Braitenberg, 1969; Sotelo, 1976), the layered arrangement of *Xenopus* cerebellum differs from that of the mammalian cerebellum, consisting of the dorsal molecular layer, rather diffuse Purkinje cell layer and granular layer intermingled with white matter. No prominent contactin immunoreactivity was detected in the Purkinje cell layer and the most part of the molecular layer (Fig. 6A, C). Sparsely-distributed immunoreactive fibers were found in the dorsal midline area of the molecular layer. Accumulation of the immunoreactive fibers was found in the granular layer and brain stem. In the brain stem, the strongly immunoreactive fibers are accumulated in the medulla.

Weak staining was occasionally found on the neuronal cell bodies. The immunoreactivities on the nerve fibers were eliminated by preabsorption of the antiserum with GST-contactin peptides (Fig. 6B), while the weak staining on neuronal cell bodies remained after the preabsorption. Similar staining of the neuronal cell bodies was obtained using a normal rabbit serum (data not shown), indicating the staining to be nonspecific. The preabsorption of the antiserum with GST did not affect the staining.

The contactin immunoreactivity was also found on the nerve fibers in the optic tectum (Fig. 6D), white matter of the medulla oblongata and cerebral cortex and medulla (data not shown).

DISCUSSION

The results described above show that *Xenopus* contactin is a highly glycosylated 135 kD protein that has 6 immunoglobulin C2-like domains and 4 fibronectin type III-like repeats. It is also shown from the cDNA sequence and the PI-PLC digestion experiment that the proteins are, at least in part, associated with the membrane by GPI anchor. The

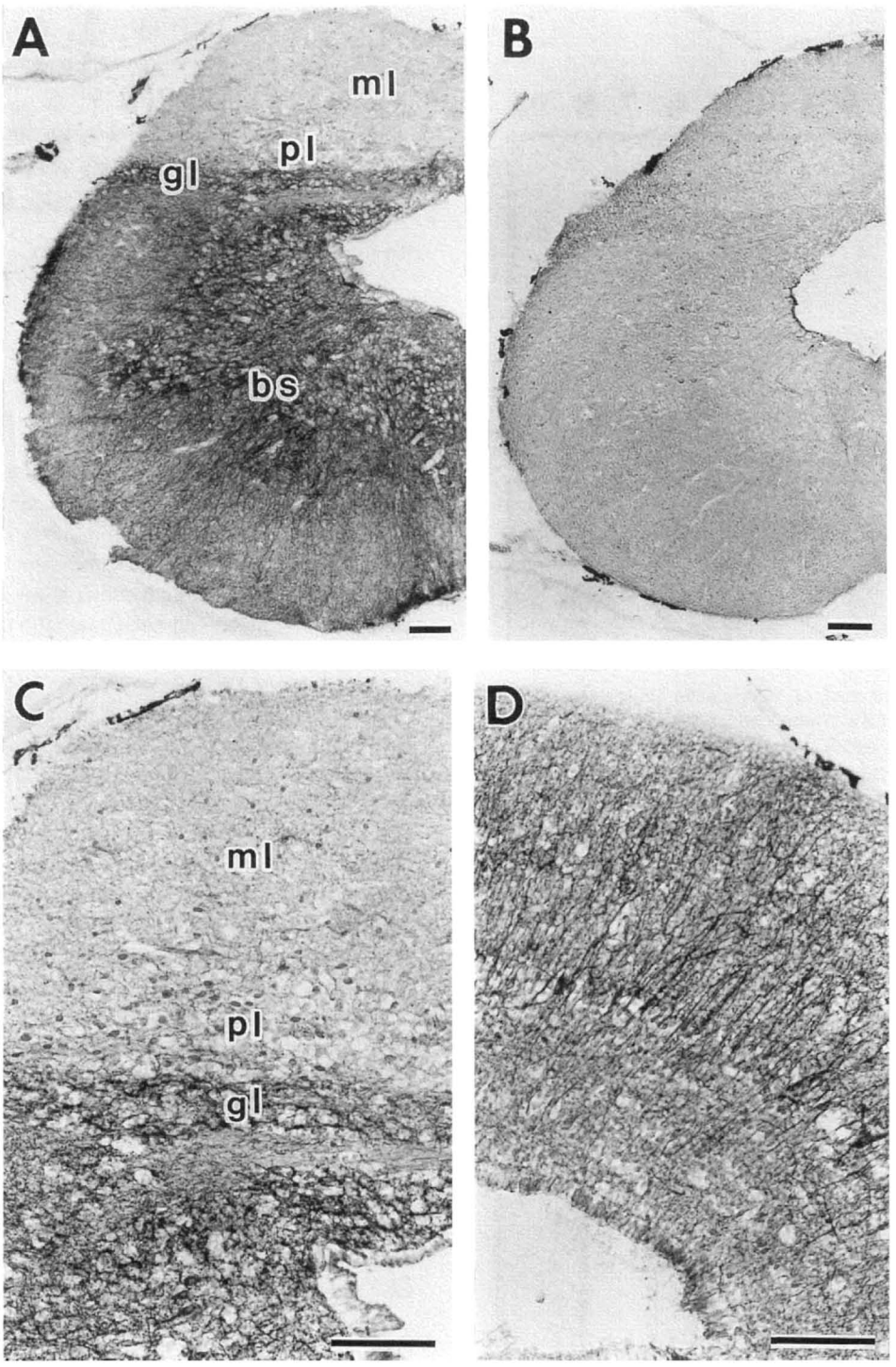

Fig. 6. Distribution of contactin immunoreactivities in *Xenopus* brain sections. (A) The frontal section through the cerebellum which is stained with an affinity-purified rabbit antiserum to *Xenopus* contactin. (B) The control section at the similar portion to A incubated with the antiserum preabsorbed with the GST-contactin peptide. (C) An enlargement of a parts of the figure A. (D) Distribution of immunoreactive fibers in the dorso-lateral portion of the optic tectum. bs, brain stem; gl, granular layer; ml, molecular layer; pl, Purkinje cell layer. Bars represent 100 μm.

mRNA and proteins are preferentially expressed in the brain. The immunocytochemical analysis using an affinity purified antiserum localizes contactin proteins to the fibers of a subset of cerebral and cerebellar neurons. In cerebellum, the contactin-positive fibers are densely accumulated in the granular layer, whereas they are sparsely distributed in the molecular layer and virtually absent in the Purkinje cell layer. Although the present study does not define the types of neurons that extend contactin-positive fibers, these observations are consistent with the report that contactin expression

in the mouse cerebellum is restricted to mossy fibers, granule cells and Golgi neurons, while Purkinje, stellate and basket cells and non-neuronal cells are contactin negative (Faivre-Sarrailh *et al.*, 1992). Thus, the structural characteristics and tissue distribution of *Xenopus* contactin are basically similar to those of previously reported chicken and mammalian contactin.

Contactin is thought to be involved in cell-to-cell and cell-to-extracellular matrix interactions that regulate neurite outgrowth, axonal path finding, fasciculation and synaptogenesis. In chicken and mammalian contactins, these interactions are shown to be based on homophilic and heterophilic recognition of several ligands by specific domains of the contactin molecule (Gennarini *et al.*, 1991; Brümmendorf *et al.*, 1993; Morales *et al.*, 1993; Peles *et al.*, 1995). From the identical domain structures and the highly conserved amino acid sequences in each domain, similar molecules may serve as ligands for *Xenopus* contactin.

Contactin binds to the membrane phospholipid by GPI anchor and has neither membrane spanning nor cytoplasmic domains. It is unknown how the ligand-binding signal can be transduced to the interior of the cell. The recent papers suggest direct or indirect non-covalent association of contactin proteins with the intracellular Fyn tyrosine kinase (Olive *et al.*, 1995; Cervello *et al.*, 1996). This may pose an important possibility that this tyrosine kinase is an intracellular component of contactin signaling. More detailed studies are obviously required to characterize molecular components and their interaction which transduce contactin signaling. A soluble form of contactin is reported to occur in the brain and cerebro-spinal fluid (Gennarini *et al.*, 1991; Durbec *et al.*, 1992). These proteins have been shown to induce neurite outgrowth in cultured neurons, suggesting their roles as growth or trophic factors in the brain. However, it is unknown how the GPI-anchored contactin proteins are released from the membrane (Moss and White, 1992). An alternative possibility that the secreted form of contactin is produced from the alternatively-spliced transcript has not been substantiated by cDNA cloning studies including the present one.

As assumed from the functional activities, contactin is thought to play a major role in postmitotic neurons after their migration and settlement in the specific sites of the nervous system. In fact, in mouse and rat brains contactin expression markedly increases after birth (Gennarini *et al.*, 1990; Faivre-Sarrailh *et al.*, 1992; Watanabe *et al.*, 1995). This makes sharp contrast with other immunoglobulin superfamily cell surface molecules such as NCAM. In *Xenopus* and chicken embryos, NCAM proteins are expressed in the cells of the neural plate and play an important role in early neural morphogenesis (Kintner, 1988). However, little is known about mechanisms regulating cell type- and developmental stage-specific expression of the contactin gene. The structure of the contactin gene in the mouse genome was reported recently (Buttiglione *et al.*, 1995). This study pointed out possible binding sites for homeodomain- and bHLH-containing transcription factors in the 5' regulatory region, postulating that contactin gene expression is regulated under a global developmental program.

Our preliminary study shows that contactin gene transcripts become detectable in *Xenopus* embryos before the closure of the neural tube (Nagata *et al.*, unpublished data), which is much earlier than the stages previously reported in other systems (Faivre-Sarrailh *et al.*, 1992; Vaughan *et al.*, 1994; Hosoya *et al.*, 1995; Watanabe *et al.*, 1995). The *in situ* hybridization experiment to locate the transcripts in embryos is under way. Furthermore, the contactin protein is immunocytochemically detectable in the restricted areas of embryonic brain and spinal cord (Nagata *et al.*, unpublished data). Thus, *Xenopus* may provide experimental approaches to examine the mechanisms of developmentally regulated contactin gene expression and its roles in the development of the vertebrate nervous system.

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