

# Molecular characterization of chicken syndecan-2 proteoglycan

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A partial syndecan-2 sequence (147 bp) was obtained from chicken embryonic fibroblast poly(A)<sup>+</sup> RNA by reverse transcription-PCR. This partial sequence was used to produce a 5'-end-labelled probe. A chicken liver cDNA library was screened with this probe, and overlapping clones were obtained encompassing the entire cDNA of 3 kb. The open reading frame encodes a protein of 201 amino acids. The cytoplasmic domain is identical with that of mammalian syndecan-2, and highly similar to those of *Xenopus laevis* and zebrafish syndecan-2. The transmembrane domain is identical with that of mammalian and zebrafish syndecan-2, and highly similar to that of *Xenopus laevis* syndecan-2. The ectodomain is 45–62% identical with that of zebrafish, *Xenopus laevis* and mammalian syndecan-2. Two coding single nucleotide polymorphisms were observed. *In vitro* transcription and translation yielded a product of 30 kDa. Western blotting of chicken embryonic fibroblast cell lysates with species-specific

monoclonal antibody mAb 8.1 showed that chicken syndecan-2 is substituted with heparan sulphate, and that the major form of chicken syndecan-2 isolated from chicken fibroblasts is consistent with the formation of SDS-resistant dimers, which is common for syndecans. A 5'-end-labelled probe hybridized to two mRNA species in chicken embryonic fibroblasts, while Northern analysis with poly(A)<sup>+</sup> RNAs from different tissues of chicken embryos showed wide and distinct distributions of chicken syndecan-2 during embryonic development. This pattern was different from that reported for syndecan-4, but consistent with the roles of syndecan-2 in neural maturation and in mesenchymal-matrix interactions.

**Key words:** embryonic development, glycosaminoglycan, heparan sulphate.

## INTRODUCTION

Syndecans are transmembrane heparan sulphate proteoglycans. There are four genes in the vertebrate syndecan family, which encode syndecan-1, -2, -3 and -4. The amino acid sequences of some syndecan genes have been deduced from their cDNAs [1,2], including those of syndecan-1 from mouse, human, golden hamster, Chinese hamster and rat; syndecan-2 from human, rat, mouse, *Xenopus laevis* and zebrafish; syndecan-3 from human, rat, mouse, chicken and *Xenopus laevis*; and syndecan-4 from rat, mouse, human and chicken. The ectodomain of all syndecan core proteins has three or four putative glycosaminoglycan (GAG) attachment sites near the N-terminus that are usually heparan sulphate substituted. Additionally, syndecan-1 and syndecan-3 proteins have two or three putative membrane-proximal GAG attachment sites. The transmembrane domains show a high degree of conservation across all four syndecans, and exact or almost exact conservation within an individual syndecan across species. Within the small cytoplasmic domains, there are two regions of high identity (conserved regions; C1 and C2) flanking a variable (V) region [3]. The V region is quite different across the syndecans, but usually identical across species for any single member. In addition to vertebrate syndecans, those from *Ciona savignyi*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Anthocidaris crassispina* have been identified [1,2,4].

The expression of the four syndecan genes depends on cell type, tissue type and developmental stage [5]. The proteins have multiple biological functions. Their heparan sulphate GAG chains can be involved in growth factor binding, cell-matrix

adhesion and cell-cell interactions [1,2]. The heparan sulphate chains of both syndecan-2 and its closest homologue syndecan-4 interact with laminin [6] and the HepII domain of fibronectin [7,8]. Although syndecan-2 and -4 may bind similar extracellular ligands, they have distinct functions. For example, syndecan-2 controls fibronectin matrix assembly [9], while binding of fibronectin by syndecan-4 drives focal adhesion formation [8]. Possible functions of syndecan-2 ectodomain protein have not been elucidated, but the region between the GAG attachment sites and the transmembrane domain of syndecan-4 can mediate cell adhesion [10]. All syndecans can oligomerize [1,2], but it is not known whether this is needed for the extracellular interactions of syndecan-2. However, multiple heparan sulphate chains of syndecan-1 can synergize to inhibit cell invasion into collagen gels [11].

Binding partners for the cytoplasmic domain of syndecans are beginning to be determined. The C-terminal sequence Glu-Phe-Tyr-Ala, which is identical in the C2 region of all syndecans, binds PDZ-domain-containing proteins, which form membrane-associated networks. Syntenin [12], CASK/LIN-2 [13] and synbindin [14] bind this sequence in syndecan-2. Binding of synbindin may be regulated by the oligomerization of syndecan-2. Activated EphB2 receptors phosphorylate Tyr<sup>189</sup> in the C1 region and Tyr<sup>201</sup> in the V region of the cytoplasmic domain of rat syndecan-2. This induces syndecan-2 clustering and association with EphB2. The clustered syndecan-2 then appears to recruit cytoplasmic ligands such as CASK and synbindin to synaptic locations [15]. The recruitment of these ligands is needed for the maturation of dendritic spines [14,16]. Oligomerization of syndecan-2 can also

Abbreviations used: C1 and C2 regions, conserved regions 1 and 2; cSNP, coding single nucleotide polymorphism; GAG, glycosaminoglycan; GST, glutathione S-transferase; RT-PCR, reverse transcription-PCR; V region, variable region.

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The nucleotide sequence data reported in this paper have been submitted to the GenBank™/EMBL/DDBJ/GSDB databases under accession numbers AF508228 and AF508229.

determine the phosphorylation status of syndecan-2 cytoplasmic serine residues [17], although the biological consequences are not known. The activation of protein kinase C $\alpha$  by the V region of syndecan-4 is also dependent on oligomerization [18,19], which is promoted by binding to phosphatidylinositol 4,5-bisphosphate [20,21] and prevented by serine phosphorylation in the C1 region [18]. The C1 region of syndecan-2 can bind ezrin, an actin binding protein [22], and syndecan-2 can mediate stress fibre [7] and filopodia [23] formation.

Since the oligomerization of syndecan-2 may regulate its extracellular and intracellular interactions, it is important to determine self-association sites and their regulation. Attempts to 'tag' syndecans have been unsuccessful, in that tagged constructs have altered biological activity. In addition, sequence alterations may affect oligomerization *per se*. An alternative is to use species-specific antibodies to study hetero-oligomerization of transfected constructs with endogenous syndecan-2. We have cloned the entire cDNA of chicken syndecan-2, compared this sequence with that of syndecan-2 from other vertebrates and with that of syndecan-4, characterized this gene with respect to its polymorphism, analysed the tissue distribution and glycanation of chicken syndecan-2, and made a species-specific antibody, mAb 8.1, a powerful tool for studies of oligomerization status and other biological functions of syndecan-2.

## EXPERIMENTAL

### Materials

General chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Tween was from Bio-Rad (Richmond, CA, U.S.A.). Coverslips and cultureware were from Fisher (Atlanta, GA, U.S.A.). RNazol™ B was from Leedo Medical Laboratories (Houston, TX, U.S.A.). *EcoRI*, Turbo *NarI*, PolyTract mRNA Isolation System, Access reverse transcription-PCR (RT-PCR) System, 5'-End Labeling System and TNT-Coupled Reticulocyte Lysate System were all from Promega (Madison, WI, U.S.A.). ECL® Western blotting reagents, mRNA Purification Kit, Sequenase Version 2.0 DNA Sequencing Kit, pGEX-5X-2 vector, Factor Xa and Hybond-N nylon membranes were all from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). TOPO TA Cloning Kit and pcDNA3 vector were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Adult chicken (Leghorn rooster) liver poly(A)<sup>+</sup> RNA was purchased from Clontech (Palo Alto, CA, U.S.A.). A chicken (broiler breeder, male, 7 weeks old) liver  $\lambda$  ZAP cDNA library was purchased from Stratagene (La Jolla, CA, U.S.A.). *Bsu36I* was purchased from New England Biolabs (Beverly, MA, U.S.A.). [<sup>35</sup>S]Methionine (1175 Ci/mmol) and [ $\gamma$ -<sup>32</sup>P]ATP (6000 Ci/mmol) were purchased from NEN Life Science (Boston, MA, U.S.A.). Heparitinase (EC 4.2.2.8) and chondroitinase ABC (EC 4.2.2.4) were purchased from Seikagaku America (Falmouth, MA, U.S.A.). Rabbit polyclonal antibodies against glutathione S-transferase (GST) were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Fertilized White Leghorn eggs were purchased from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.). FITC-conjugated F(ab')<sub>2</sub> fragments of goat anti-rabbit or anti-mouse were purchased from Cappel (Durham, NC, U.S.A.).

### Purification of poly(A)<sup>+</sup> RNA

Total RNA was isolated from chicken embryonic fibroblasts using RNazol™ B. Poly(A)<sup>+</sup> RNA was prepared from total RNA with a PolyTract mRNA Isolation System according to the manufacturer's protocol (Promega). Poly(A)<sup>+</sup> RNA from chicken embryonic tissues was prepared with an mRNA Purification Kit.

### RT-PCR

Using various combinations of primers encompassing part of the transmembrane domain and the total cytoplasmic domain of syndecan-2, RT-PCR with poly(A)<sup>+</sup> RNA from chicken embryonic fibroblasts was performed. The primers that gave rise to a product containing part of the transmembrane domain and the total cytoplasmic domain sequences of syndecan-2 were the forward primer 5'-GTG ATT GGC TTT CTC TTT GCA ATT-3' in combination with the reverse primer 5'-TTA TGC ATA AAA CTC CTT AGT AGG-3'. RT-PCR reactions were carried out with the Access RT-PCR System following the manufacturer's protocol (Promega). Each RT-PCR product of approx. 147 bp was extracted from the agarose gel, ligated and transformed with a TOPO TA Cloning Kit. Sequencing was performed with Sequenase Version 2.0 DNA Sequencing Kit. A 0.1  $\mu$ g portion of adult chicken (Leghorn rooster) liver poly(A)<sup>+</sup> RNA was also used for RT-PCR, and the product was extracted, ligated, transformed and sequenced by the same procedures. The resulting product containing part of the transmembrane domain and the total cytoplasmic domain of syndecan-2 was used in cDNA library screening.

### Screening of a chicken liver $\lambda$ ZAP cDNA library

A chicken liver  $\lambda$  ZAP cDNA library was screened with 8 pM of the 147 bp RT-PCR product, labelled with the 5'-End Labeling System. Hybridization was performed using the same procedure as that for Northern analysis. After the first round of screening, 32 positive plaques were randomly selected for two further cycles of selection and screening until 100% of plaques on each plate were positive. After isolation of the phage, the cDNAs were recovered in Bluescript using R408 helper phage. Plasmids were prepared from the miniculture of positive clones. Restriction reactions were performed with *EcoRI*. Seven plasmids were selected and sequenced.

### Polymorphism in the chicken syndecan-2 gene

Fertilized eggs of the White Leghorn strain were incubated for 15 days at 37 °C, and then livers were dissected from the embryos. Genomic DNAs were isolated from the tissues as described previously [24], or were purchased (New Hampshire Brown strain) from Clontech. Genomic DNAs were used in PCR reactions, with primers encompassing the GAG attachment region (which corresponds to exon 2 of human syndecan-2), or part of the ectodomain plus part of the transmembrane domain (which corresponds to exon 4 of human syndecan-2). Purified PCR products were subjected to restriction digestion overnight at 37 °C with *Bsu36I* or Turbo *NarI*, which cleave specifically at the polymorphism sites. The digested products were run on a 3% (w/v) wide-range agarose gel (Sigma) and photographed.

### Chromosomal localization of chicken syndecan-2 by fluorescence *in situ* hybridization

Chicken metaphase chromosome spreads were prepared from chicken embryo fibroblasts after treatment with colcemid. Cells were swollen in hypotonic solution, fixed in methanol/acetic acid (3:1, v/v), dropped on to methanol-cleaned slides and allowed to air dry. The cDNA probe was labelled with digoxigenin-11-dUTP by nick translation. A 100 ng aliquot of labelled probe precipitated with 6  $\mu$ g of salmon sperm DNA was hybridized to chromosomes overnight at 37 °C in a moist chamber. Hybridization was detected by incubation with anti-digoxigenin-FITC. Chromosomes were stained with DAPI (4,6-diamidino-2-phenylindole) and images were captured using a Nikon Microphot-SA

microscope fitted with a cooled CCD camera (Digital Pixels, Brighton, U.K.) and analysed with IPLab software (Scanalytics Inc., Fairfax, VA, U.S.A.).

### Sequence analysis and construction of a phylogenetic tree

Sequence analysis and construction of a phylogenetic tree were performed using programs from SeqWeb of the Genetics Computer Group (GCG). Similarity and identity analyses were performed using the PILEUP and GAP programs. Distances were corrected using the Kimura distance method. The phylogenetic tree was constructed using UPGMA. Database searches were performed using the BLAST network service at the National Center for Biotechnological Information (NCBI).

### In vitro transcription and translation of chicken syndecan-2

Full-length chicken syndecan-2 cDNA was ligated between the *Hind*III and *Bam*HI sites of the pcDNA3 vector. Then 1 µg of this construct in a 50 µl reaction mixture was subjected to *in vitro* transcription and translation with the TNT Coupled Reticulocyte Lysate System following the manufacturer's protocol. A 2 µl aliquot of [<sup>35</sup>S]methionine was added to each 50 µl reaction, and 1 µg of luciferase DNA in a 50 µl reaction was used as a positive control. Portions of 1 µl of the transcription and translation reaction mixtures or the positive control were loaded on to a 10% (w/v) polyacrylamide SDS/PAGE gel. The gel was run, dried and exposed to the Kodak BioMax film for 24 h at -70 °C.

### Expression and purification of recombinant proteins, and monoclonal antibody generation

cDNAs encoding the extracellular domain of the core protein lacking the signal peptide or the core protein lacking the signal peptide were subcloned into the GST expression vector pGEX-5X-2. Fusion proteins were expressed in BL21 using 0.1 mM isopropyl β-D-thiogalactoside. After purification, some fusion protein preparations were digested with factor Xa to release the syndecan-2 core protein: 50 units of factor Xa and 950 µl of 1 × PBS were added per ml of glutathione-Sepharose bed volume. A fusion protein containing the extracellular domain of the core protein was used for monoclonal antibody production [25]. One antibody (mAb 8.1) was used in further experiments.

### Characterization of chicken syndecan-2

For Western blotting of recombinant syndecan-2, 0.5 µg of recombinant protein (GST fused to the extracellular domain of chicken syndecan-2 core protein, the extracellular domain of chicken syndecan-2 core protein, GST fused to the rat syndecan-2 core protein or GST alone) was resolved by SDS/PAGE [3–15% (w/v) gradient gel]. Gels were transferred on to 0.45 µm nitrocellulose membranes, which were immunoblotted with a 1:2500 dilution of mAb 8.1 or 1 µg/ml rabbit polyclonal anti-GST in PBS containing 1% non-fat milk and 0.1% Tween, followed by goat anti-mouse or goat anti-rabbit antibodies conjugated to horseradish peroxidase. Detection was carried out with ECL<sup>®</sup> Western blotting detection reagents. For Western blotting of native syndecan-2, chicken or rat fibroblasts were grown in 10 cm dishes and scraped into 1 ml of PBS<sup>+</sup> containing 0.4 mM PMSF, 1 mM benzamide/HCl, 10 µg/ml leupeptin and 10 mM *N*-ethylmaleimide. Cells were sedimented for 5 min at 4 °C and 400 g, resuspended in 30 µl of heparitinase buffer (0.1 M sodium acetate/0.1 mM calcium acetate, adjusted to pH 7.0 with acetic acid) with 2.5 m-units of heparitinase and/or 25 m-units of chondroitinase ABC, and incubated for 2 h at 37 °C. Further

aliquots of heparitinase and/or chondroitinase ABC were added to the cell suspension and incubation was extended for another 2 h at 37 °C. After incubation, 20 µl of 5 × SDS sample buffer with reducing agent was added to each cell suspension, followed by heating for 5 min at 100 °C. Samples were passed through a 23 G syringe, centrifuged at 10000 g for 1 min at room temperature, and the supernatants were resolved by SDS/PAGE [3–15% (w/v) gradient gel]. The gels were transferred on to nitrocellulose membranes, and syndecan-2 was detected by mAb 8.1 as for recombinant syndecan-2.

### Northern analysis of syndecan mRNA

Portions of 30 µg of total RNA from chicken embryonic fibroblasts, or of 5 µg of poly(A)<sup>+</sup> RNA from liver, gizzard, kidney, eye, brain, heart, sternum, intestine and skin at embryonic days 9, 12 and 15, were isolated. RNA was loaded on a 1% denaturing agarose gel and the gel was run for 4 h at 55 V. Following transfer to Hybond-N nylon membranes, the membranes were prehybridized for 1 h at 37 °C with 5 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.002% BSA), 0.2% SDS and 50% (v/v) formamide solution containing 100 µg/ml denatured salmon sperm DNA and hybridized for 24 h at 37 °C in 5 × SSC, 10% (w/v) dextran sulphate and 50% (v/v) formamide with the 5'-end-labelled probe. The probe and the 5'-end-labelling procedure were the same as for library screening. Membranes were washed for 2 × 5 min at room temperature in pre-warmed (37 °C) 2 × SSC/0.1% SDS, then for 2 × 5 min in pre-warmed (37 °C) 0.1 × SSC/0.1% SDS, and exposed to Kodak BioMax film for 48 h at -70 °C.

### Immunohistochemical analysis

To monitor the cell surface distribution of syndecan-2 and syndecan-4, chicken embryonic fibroblasts were grown in αMEM containing 5% (v/v) fetal calf serum on uncoated 10 mm diameter glass coverslips for 2 days. Cells were washed with warm PBS<sup>-</sup>. Cells for syndecan-4 detection were fixed with cold methanol at -20 °C for 20 min. Cells for syndecan-2 detection were fixed with fresh 3.5% paraformaldehyde in PBS<sup>-</sup> at 37 °C for 20 min. After being washed with PBS<sup>-</sup>, incubated with 0.1 M NH<sub>4</sub>Cl for 15 min and re-washed, cells were stained with rabbit polyclonal antibody R1891 (1:50 dilution) against the syndecan-2 ectodomain or mouse monoclonal antibody 150.9 (1:50 dilution) against the syndecan-4 ectodomain N-terminus. Primary antibodies were detected with FITC-conjugated F(ab')<sub>2</sub> fragments of goat anti-rabbit or anti-mouse at 1:50 dilution. Cells were monitored with a Nikon Optiphot microscope and photographed with Ilford HP5 film.

## RESULTS

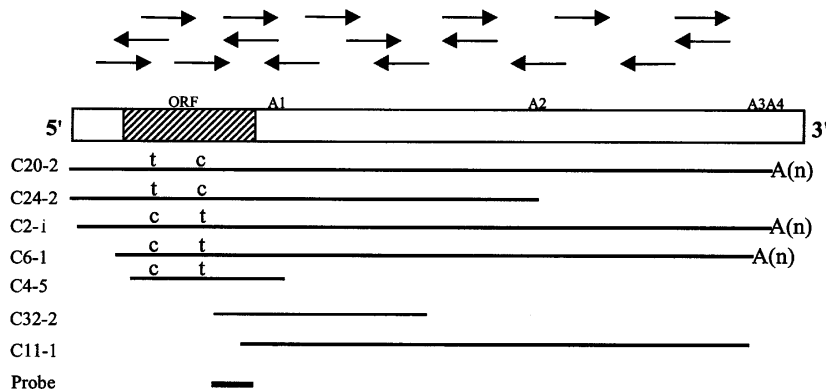
### Isolation of chicken syndecan-2 cDNA

Primers predicted to encompass part of the transmembrane domain and the total cytoplasmic domain of syndecan-2 were used in RT-PCR of poly(A)<sup>+</sup> RNA from chicken embryonic fibroblasts. Upon sequencing, a product was found that corresponded to part of the transmembrane domain plus the total cytoplasmic domain of syndecan-2. Using this RT-PCR product as a probe, 211 positive plaques from 1.25 × 10<sup>6</sup> plaque-forming units were obtained on the first round of library screening. After a further two cycles of dilution and re-screening of positive

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1   gcggggggaaggaaggagagaagcgcgagcgagggggggcgaggaggaggaggaggcgcagcagcagcagccg 79
80   ggagcccccagagccgagcgcagcgttgtgcccgccgagcgcggagcggagcggcagcagcagcggagcggagcc 158
159  ggagggcccccggccgctgcccgcgccccggggcgctgcccagcgcggcggccccgcttctcctttgtgcccggcc 237
238  acagggcggccgcccgcggctcccggggaagaatgcggtgctgtggctcgcgctcaccctcagcttcggtggcctgtg 316
1   M R C V W L A L T L S F V A C 15
317  cctccgggagccgagagcagatgttgacctctgataaagatttacctggacaacagcctctgttgaagagccatcg 395
16   A S G Q P R A D L T S D K D L Y L D N S S V E E A S G 42
396  cgc<t>ctatcccacgatgatgatgatttcttctgggtcgggttcaggctgctgaggaagatgagatagcacagtg 471
43   A<V> Y P I D D D D Y S S G S G S G A E E D E D S T V 67
472  acaacaacgtccagaatagttcccaagttaccaacaacgagtgatgctgtagagctgagaccaccacagtgaaaaatgc 550
68   T T T S R I V P K L P T T S D A S R A E T T T V K M 93
551  agaccaagatgctgcacaaacaaagtcacctgaagaatcgataaagaggaaagacct<c>gaggtggattccaagaa 626
94   Q T K M P A Q T K S P E E I D K E E R P E V D S K K 119
627  aaagagcaatgagccaggggatgacactgatgtgttcaactgaaaagcattcagaaaaacctctccagaggacagaagt 705
120  K S N E P G D D T D V F T E K H S E N L F Q R T E Y 145
676  ctggcagctgttattgctggcggagttatgttttctgttcaatctcctcatcctgctgctgttatatcgcatga 784
146  L A A V I A G G V I G F L F A I F L I L L L V Y R M 171
785  gaagaagatgaaggcagttacgccttggtgaacgtaaacctgtatgtgctgcctatcagaaggcacctactaagga 863
172  R K K D E G S Y D L Y S S R K P S S A K A Y Q K A P T G K A 198
864  gttttatgcataaaatcctatttagtctctatttagatgactgaaactttttatataaagcttttgcatagaa 942
199  F Y A 201
943  taatgaagatcttttttttttttttattcattaaagacatcacggcacctttatgataaaatccattgt attttaaact 1021
1022  ttacagtatcctttaaataatgataaaacttaacatttgcagtgcttctgtgaataacagtggaagcatttatt 1100
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1565  cctaagcaaacccaagactctaacagtatgttgagaagtgtcgcagatttctatggccactgtatttgttaattat 1643
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1881  cactactgataaaalcatgtgacctaatcactgaaaatccattgtgtagactttgtcagtgctcgttttggctgt 1959
1960  cgggtgtaagtgttttaagtccaccgttcgctgagtaggtcttcaaaagccaccgcaaacaaaacaaaaggctgt 2038
2039  gataaggctgacctcagaccagatttactgctgaagcggcagagaagcctggaatgagaagcgttacatctggcgc 2117
2118  acgagttatgagtcatttggcctttctgttatataataatggcagfattgacacagacctgttctcttgcgaatggt 2196
2197  tttcagtagctgtaaaatccaaggagtagccgggttctatttattgttatatcggttttggattgtgttcttttt 2275
2276  ttccctggccttttggagaaattgttttattctgctcggactccggcagaagattgagacgagtggaatatcttcg 2354
2355  taagagaagaagaaatattccaagtagaagataagaggctctgttcttagattgcagataggtgttggagagagga 2433
2434  caagtgagagaagcagtagtctgcctcctatattacgcttctgcacagcaaaagggcttctgctctctgtctgtaac 2512
2513  ctccgctcttcgattttgaaattccagagttagatgttcaggatgtcagagctgaacctctgcttccagaaatggcaa 2591
2592  cgtgttgaataaagagctgttagctctcagataaagattgctcactgtatttgcgctgcccaggttcagtggt 2670
2671  catttagaaaacaaacgacgctatcctgtgctaaaat atttaatatattgcttttaaataactataactactcggcag 2749
2750  gttttcaagccatacaagttcaacaagtaaaacagggtaaga atttaacaggtatatttattgtgcaat atttag 2828
2829  tgaattcaatttagtaaaataactgcttgaagtaactgtgccaagaaaagaaatctcggcaaatgctgtgccactgc 2917
2918  tgaataaataagcatttggtaagtgccaa (aaaaaaaaaaaaaaaa) laataaagctccatgctcctaaactaaaaaaaa 2978
2979  aaaaaaaaaaaaaaaaaaaaaaaaaa 3002

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**Figure 1** Nucleotide and deduced amino acid sequences of chicken syndecan-2

Boxed nucleotides are polyadenylation signal sequences. Underlined nucleotides are mRNA destabilization sequences. Bracketed single nucleotides (e.g. <c>) are alternatives to the preceding nucleotides in some cDNA clones. The bracketed poly(A) sequence is the poly(A) tail of cDNA clone C6-1. The bold italic amino acid sequence is the putative signal peptide. The underlined amino acid sequence is the transmembrane domain. The bold italic **N** is a putative N-glycosylation site, and bold italic **S** denotes putative GAG attachment sites. Bracketed amino acids (e.g. <V>) are alternative residues in some cDNA clones to the preceding amino acid. ORF, open reading frame; A1–A4, polyadenylation signal sequences. The seven clones sequenced are indicated below the representation of the full-length gene. A(n), poly(A) sequences; t and c, nucleotides that differ among the seven cDNA clones. Arrows represent primers used for sequencing. The GenBank™ accession numbers for the two cDNA sequences are AF508228 and AF508229.

plaques, 32 high-purity cDNA clones were recovered, seven of which were selected and sequenced (Figure 1). The full-length cDNA was 3 kb long, containing a predicted open reading frame that encodes a protein of 201 amino acids with the characteristics of syndecan-2 (Figure 1). The predicted protein sequence contains an 18-amino-acid signal peptide, a 126-amino-acid

ectodomain, a 25-amino-acid transmembrane domain and a 32-amino-acid cytoplasmic domain. In the ectodomain there are four putative GAG attachment sites. The calculated molecular mass of the predicted mature core protein excluding the signal peptide is 22.1 kDa. Compared with syndecan-2 from other species, the 5' non-coding region of chicken syndecan-2 cDNA is much more

GC-rich, suggesting that its translation is strictly controlled [26]. In contrast, the 3'-end contains 10 mRNA destabilization sequences (ATTTA) [27], suggesting that the mRNA is not stable.

### Coding single nucleotide polymorphisms (cSNPs) of chicken syndecan-2

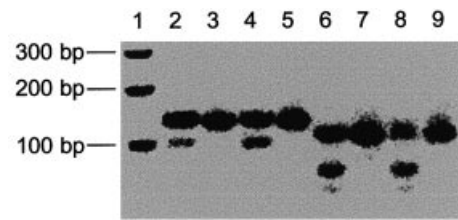
In the predicted open reading frame of chicken syndecan-2, there are two positions that have nucleotide variations in the cDNA clones (Figure 1). One position (CCC/T) is membrane-proximal in the protein, and does not change the residue Pro<sup>114</sup>. The other position (GT/CC) is immediately C-terminal of the first putative GAG attachment site, and results in a switch between Ala<sup>43</sup> and Val<sup>43</sup>. Human syndecan-2 and mouse syndecan-2 have valine at this position. GCC for Val<sup>43</sup> always accompanies CCT for Pro<sup>114</sup>, while GTC for Ala<sup>43</sup> accompanies CCC for Pro<sup>114</sup>. Of the five sequenced cDNAs, three had GCC and CCT at these positions, while two had GTC and CCC. To confirm the presence of cSNPs at these positions in the chicken syndecan-2 gene, we performed restriction digestions of two PCR products of chicken genomic DNA. One PCR product is equivalent to exon 2 of human syndecan-2, and the other is equivalent to exon 4 of human syndecan-2. One restriction enzyme used was *Bsu361*, which can cut the sequence CCTNAGG but not CCCNAGG (here both CCT and CCC encode Pro<sup>114</sup>). The other restriction enzyme used was Turbo *NarI*, which can cut the sequence GGCGCC but not GGCGTC (here GCC encodes Ala<sup>43</sup>, while GTC encodes Val<sup>43</sup>). Both restriction enzymes partially, but not totally, digested their respective sites, confirming the presence of cSNPs at these positions of the chicken syndecan-2 gene (Figure 2).

### Chromosomal localization of chicken syndecan-2

Using fluorescence *in situ* hybridization, chicken syndecan-2 was mapped to chicken chromosome 2q31 (results not shown), which is syntenic with human chromosome 8 and mouse chromosome 15. Human syndecan-2 was mapped to 8q23, and mouse syndecan-2 to chromosome 15 [2].

### Sequence analysis of syndecan-2 and construction of a phylogenetic tree

Sequence alignment (Figure 3) and identity analysis (Figure 4) were used to compare the (sub)domains of syndecan-2 from different species, as well as syndecan-2 and -4 (sub)domains. The cytoplasmic domain of chicken syndecan-2 is identical with that of human, mouse and rat syndecan-2, and highly similar to that of *Xenopus laevis* and zebrafish syndecan-2, except for one residue difference. The transmembrane domain of chicken syndecan-2 is identical with that of human, mouse, rat and zebrafish syndecan-2, and highly similar to that of *Xenopus laevis* syndecan-2, except for differences at two positions. The percentage identity of the ectodomain is 62% with human and mouse, 58% with rat, 47% with *Xenopus laevis* and 45% with zebrafish syndecan-2. The ectodomain can be divided into four subdomains. The N-glycosylation and GAG attachment subdomain of syndecan-2 is highly conserved among all vertebrate species, with identity from 60% to 92%, while the charged subdomain is highly variable, with identity from 11% to 84%. Syndecan-2 from both *Xenopus laevis* and zebrafish has no N-glycosylation site, while syndecan-2 from the higher vertebrates, including chicken, has one potential N-glycosylation site. The sequences of GAG attachment sites in chicken syndecan-2 are SG and SG-SGSG, which are the same as those in *Xenopus laevis* and zebrafish syndecan-2, while human, mouse and rat syndecan-2



**Figure 2** cSNPs occur in chicken syndecan-2

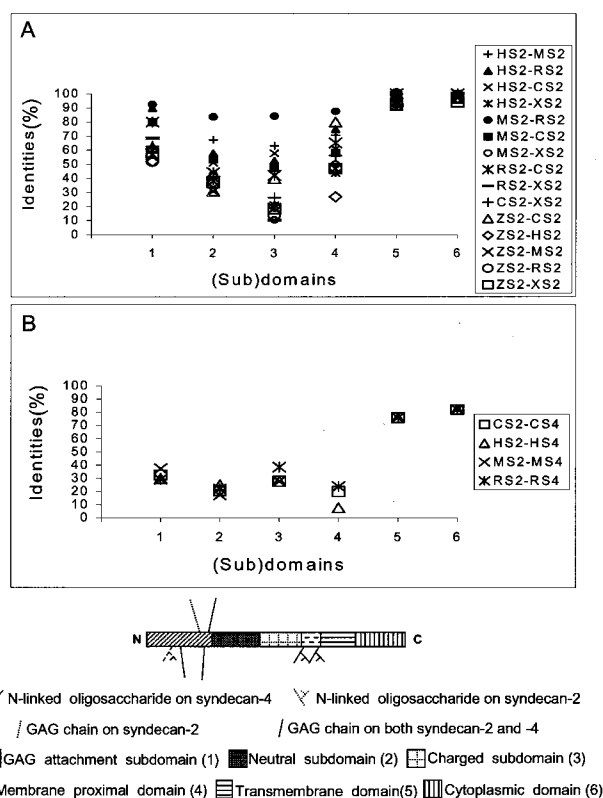
PCR reactions were performed with DNAs from the New Hampshire Brown strain (lanes 2, 3, 6 and 7) and the White Leghorn strain (lanes 4, 5, 8 and 9). PCR products corresponding to exon 4 (lanes 2–5) or exon 2 (lanes 6–9) of human syndecan-2 were undigested (lanes 3, 5, 7 and 9), or were digested with *Bsu361* (lanes 2 and 4) or Turbo *NarI* (lanes 6 and 8), and then were run on a 3% wide-range agarose gel. Lane 1 is a 100 bp DNA ladder.

	Signal Peptide	N-glycosylation and GAG-		
MS2	MQRAWILLTL GLMACVSAE	TRTELTSDDK	.MYLDN <del>SS</del> IE	EA. <del>SG</del> VYPID
RS2	MQRAWILLTL GLMACVSAE	TRAE <del>L</del> TSDDK	.MYLDSS <del>SS</del> IE	EA. <del>SG</del> LYPID
HS2	MRRAWILLTL GLVACVSAE	SRAELTSDDK	.MYLDN <del>SS</del> IE	EA. <del>SG</del> VYPID
CS2	MRCVWLALTL SFVACASGQ	PRADLTSDDK	.LYLDN <del>SS</del> VE	EA. <del>SG</del> AYPID
XS2	MRNVWLIVPF ALLAAFSGE	TWAQ. <del>ADR</del> D	.LYIDST...	ES. <del>SG</del> NYPVD
ZS2	MRNLWMLTL GLTAF <del>L</del> SGER	ERISVSAAKS	PST <del>TD</del> LLYLE	EAG <del>SG</del> GYPED
	-Attachment Subdomain	Neutral-		
MS2	DDDYS <del>S</del> ASGS GA...DED..	IESHVLITTSQ	LIPRIPLTSA	ASPKVETMTL
RS2	DDDY <del>S</del> SASGS GA...YED..	KGS <del>H</del> DLTTSQ	LIPRISL TSA	A.PEVETMTL
HS2	DDDY <del>S</del> ASGS GA...DED..	VESPELITTSR	PLPKILLTSA	A.PKVETMTL
CS2	DDDY <del>S</del> SGSGS GA...EED..	EDSTVITTSR	IVPKLPTSD	AS.RAETTTV
XS2	DDDY <del>S</del> SGSGS GIPAHDD..	EDNVVLTTSQ	TL...ISSPSS	EMPVYDTTTL
ZS2	DDDF <del>S</del> SGSGS GAGEVIEDPV	TVNLTFFVPK	AEPTQDSTKD	FTPKVETVTS
	-Subdomain	Charged Subdomain	Membrane-	
MS2	...KTQSIT PAQTESPEET	DKEVDISEA	EKGLGPAIKS	TDVYTEKHS
RS2	...KTQSIT PTQTESPEET	DKKEFEISEA	EKQDPVAVKS	TDVYTEKHS
HS2	...NIQNKI PAQTKSPEET	DKEKVLHSDS	ERKMDPAEED	TNVYTEKHS
CS2	...KMQTKM PAQTKSPEET	DKEERPEVDS	KKKSNPEGDD	TDVYTEKHS
XS2	...KTQTKM APE <del>T</del> KPEGEV	ESTNTVLVHE	NKNI.....	..IQTATHT
ZS2	QDAPKDSKRR RIEVAVPVTE	DSRRNPVTS	TSIPRPMPD	QDV...QSE
	-Proximal Subdomain	Transmembrane Domain	Cytoplasmic-	
MS2	NLF <del>K</del> RTEVLA AVIAGGVIGF	LFAIFLILLL	VYRMRKKDEG	SYDLGERKPS
RS2	NLF <del>K</del> RTEVLA AVIAGGVIGF	LFAIFLILLL	VYRMRKKDEG	SYDLGERKPS
HS2	SLF <del>K</del> RTEVLA AVIAGGVIGF	LFAIFLILLL	VYRMRKKDEG	SYDLGERKPS
CS2	NLF <del>F</del> RTEVLA AVIAGGVIGF	LFAIFLILLL	VYRMRKKDEG	SYDLGERKPS
XS2	NLF <del>F</del> RTEVLA AVIAGGVIGF	LFAIFLILLL	VYRMRKKDEG	SYDLGERKPS
ZS2	NLF <del>F</del> RTEVLA AVIAGGVIGF	LFAIFLILLL	VYRMRKKDEG	SYDLGERKPS
	-Domain	C1 Region V-		
MS2	SAAYQKAPTK EFYA			
RS2	SAAYQKAPTK EFYA			
HS2	SAAYQKAPTK EFYA			
CS2	SAAYQKAPTK EFYA			
XS2	SAVYQKAPTK EFYA			
ZS2	GAAYQKAPTK EFYA			
	-Region C2 Region			

**Figure 3** Sequence alignment shows that chicken syndecan-2 is highly similar to that from other vertebrates

MS2, mouse syndecan-2; RS2, rat syndecan-2; HS2, human syndecan-2; CS2, chicken syndecan-2; XS2, *Xenopus laevis* syndecan-2; ZS2, zebrafish syndecan-2. The bold italic **N** is the putative N-glycosylation site. Bold italic **S** are putative GAG attachment sites. The putative shedding sites are boxed. The signal peptide, subdomains and domains are indicated above their respective sequences. The C1, V and C2 regions are indicated below their respective sequences.

have SG and SASGSG sequences at the corresponding regions. A mono- or di-basic amino acid sequence in the membrane-proximal region of the ectodomain has been suggested to be a shedding site, through protease-mediated cleavage [1,2]. Chicken syndecan-2 has a monobasic amino acid at this site, as has syndecan-2 from *Xenopus laevis* and zebrafish, while mammalian syndecan-2 has a dibasic sequence.

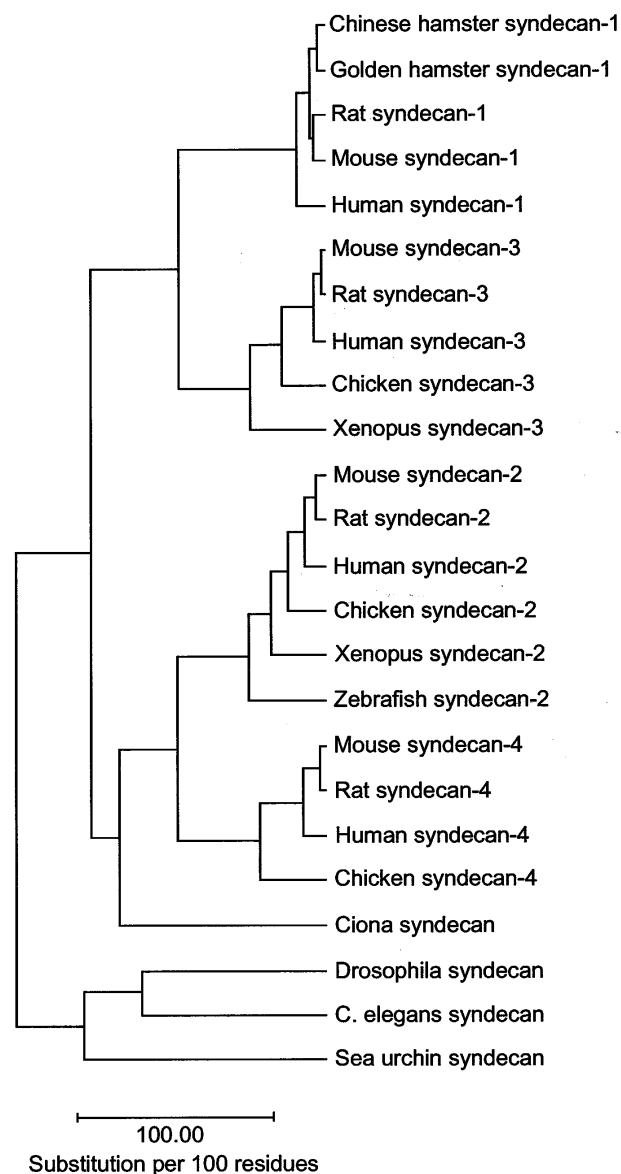


**Figure 4** Identities of syndecan-2 (sub)domains between different species (A), and identities between syndecan-2 and -4 (sub)domains within species (B)

Identities were calculated with Gap program (SeqWeb). HS2 and HS4, human syndecan-2 and -4 respectively; MS2 and MS4, mouse syndecan-2 and -4; RS2 and RS4, rat syndecan-2 and -4; CS2 and CS4, chicken syndecan-2 and -4; XS2, *Xenopus laevis* syndecan-2; ZS2, zebrafish syndecan-2. The bracketed numbers are (sub)domain numbers.

The overall sequence of chicken syndecan-2 is 44% identical with that of chicken syndecan-4. The transmembrane and cytoplasmic domains of chicken syndecan-2 and -4 are quite similar, with identities of 76% and 82% respectively. For the ectodomain, the GAG attachment subdomains of the two proteins show 33% identity, while the remaining regions have lower identities. Comparisons between human syndecan-2 and -4, mouse syndecan-2 and -4, and rat syndecan-2 and -4 give rise to similar results. Similarity analysis (which also includes conservative substitutions) between syndecan-2 (sub)domains from different species and syndecan-2 and -4 (sub)domains (results not shown) showed trends very similar to those with the identity results.

A phylogenetic tree was generated using all known syndecan sequences (Figure 5), which are from protostomia such as the arthropod *Drosophila* and the nematode *C. elegans*, and from deuterostomia such as the echinoderm sea urchin, the invertebrate chordate *Ciona savignyi* and the vertebrates zebrafish, *Xenopus laevis*, chicken, hamster, mouse, rat and human. Interestingly, the *Drosophila*, *C. elegans* and sea urchin syndecans are each similar to all four vertebrate syndecans, suggesting that the latter originated from a single ancestral gene. Since the reported *Ciona* syndecan is homologous to both syndecan-2 and -4, it is possible that there is another *Ciona* syndecan gene that is homologous to both syndecan-1 and -3.

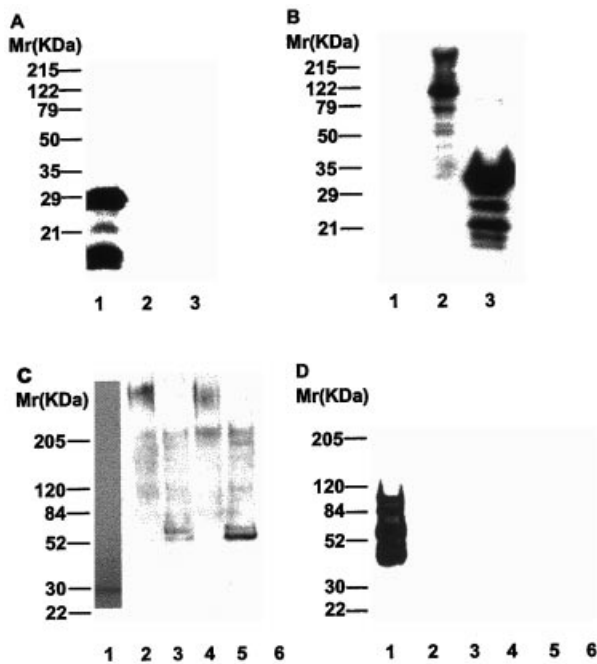


**Figure 5** Phylogenetic tree of the syndecan family

The phylogenetic tree was derived from the GrowTree program of SeqWeb. GCG. Kimura distance was used as the distance correction method. UPGMA was used as the tree construction method.

#### Immunochemical analysis of chicken syndecan-2

Monoclonal antibody mAb 8.1 was raised against a recombinant GST fusion protein containing the chicken syndecan-2 ectodomain. It reacts with the recombinant chicken syndecan-2 ectodomain, but not with recombinant rat syndecan-2. It also reacts with syndecan-2 from chicken embryonic fibroblasts, but not with that from rat embryonic fibroblasts (Figure 6). With chondroitinase ABC treatment, or without any enzyme treatment, a very-high-molecular-mass heterogeneous product of > 200 kDa was seen in Western blots. Treatment with heparitinase or heparitinase plus chondroitinase ABC eliminated this smearing and gave rise to two polypeptides of 53 kDa and 58 kDa. This suggests that chicken syndecan-2 is substituted with heparan sulphate, but not with chondroitin sulphate. The



**Figure 6** Chicken syndecan-2 is synthesized as a heparan sulphate proteoglycan

In (A) and (B), recombinant proteins were blotted with mAb 8.1 and anti-GST polyclonal antibody respectively. Recombinant proteins are the extracellular domain of chicken syndecan-2 after cleavage with factor Xa and separation (lane 1), GST–(rat syndecan-2) fusion protein (lane 2) and GST protein only (lane 3). In (C), chicken syndecan-2 was *in vitro* transcribed and translated (lane 1), and chicken embryonic fibroblasts were lysed and blotted with mAb 8.1, without digestion (lane 2), or digested with heparitinase (lane 3), chondroitinase ABC (lane 4) or heparitinase plus chondroitinase ABC (lane 5); lane 6, enzyme only without lysate. In (D), rat embryonic fibroblasts were lysed and blotted by mAb 8.1. Lane 1, GST fusion with the extracellular domain of chicken syndecan-2; lanes 2–6 correspond to those in (C).

larger 58 kDa polypeptide may be an N-glycosylated core protein. *In vitro* transcription and translation of chicken syndecan-2 (Figure 6C) gave rise to an apparent 30 kDa polypeptide by SDS/PAGE, larger than the predicted size of 22.1 kDa. This apparent discrepancy may result from the proline-rich ectodomain. Proline is bulky and inflexible, and can cause retardation on SDS/PAGE. The 53 kDa and 58 kDa species from cells could be dimers. All syndecans can form SDS-resistant dimers [1,2]. Moreover, dimer formation is independent of cysteine, since the chicken syndecan-2 mature core protein, like many other syndecans, contains no cysteine residues.

#### Spatial and temporal distribution of chicken syndecan-2 mRNAs during embryonic development

In tissues from chicks at embryonic day 9, Northern analysis showed a wide distribution of chicken syndecan-2 mRNAs, which are approx. 3 kb and 2 kb in size. Most tissues examined, including those originating from the ectoderm (eye and skin), the mesoderm (sternum, kidney and heart) and the endoderm (intestine, liver and gizzard), contained syndecan-2 mRNAs. However, the mRNAs were not detectable in brain. The intestine, skin and sternum had the highest syndecan-2 mRNA levels. The eye and gizzard had intermediate levels, while the liver, kidney and heart had low levels. On embryonic day 12, almost all the tissues had similar syndecan-2 mRNA levels, except for liver, where the mRNAs were not detectable. On day 15, however, the

mRNAs were not detectable in liver or kidney, and were at low levels in heart and intestine. Expression in the sternum also appeared low, although, in general, less mRNA was extractable from this tissue. The skin and gizzard contained high levels, while the highest syndecan-2 mRNA levels were in the brain and eye (Figure 7).

#### Chicken syndecan-2 and syndecan-4 are differentially localized on the cell surface

Staining of syndecan-2 and -4 showed that they have distinct localizations on the surface of chicken embryonic fibroblasts (Figure 8). Syndecan-2 was punctate on the cell surface, while syndecan-4 was localized at focal adhesion sites and in membranes overlying stress fibres. This is consistent with their localization on the surfaces of other cells [9,28,29], and suggests that they have distinct functions in chicken embryonic fibroblasts.

## DISCUSSION

### cDNA cloning and evolutionary analysis

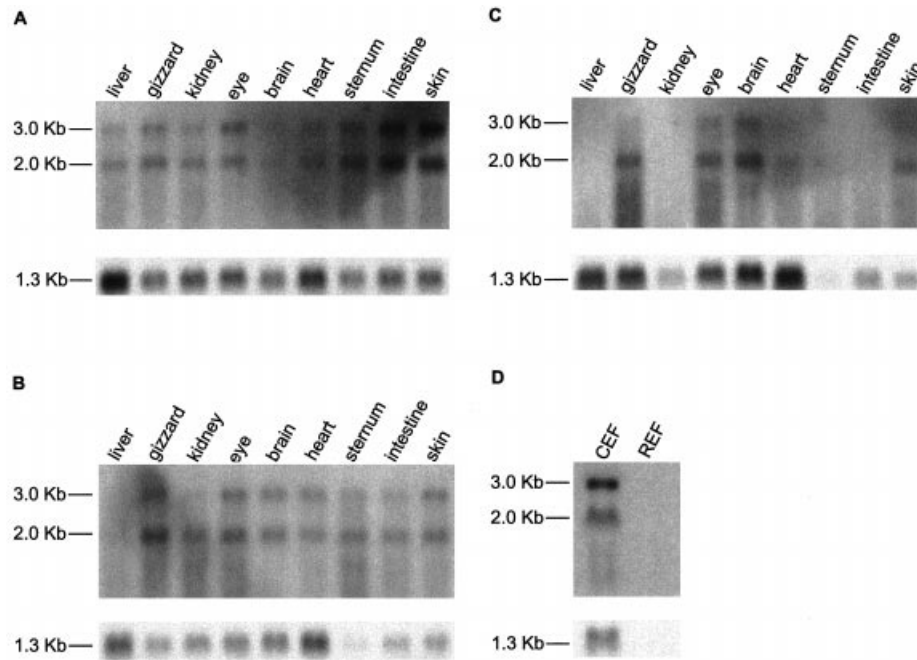
Here we report the cDNA cloning of an avian syndecan with clear homology with mammalian, amphibian and fish syndecan-2 proteins. The cytoplasmic domain of chicken syndecan-2 is identical with or highly similar to that of syndecan-2 from other vertebrates, suggesting highly conserved biological functions. In contrast, the ectodomains are less conserved, except for the GAG attachment sites. There may be structural conservation, even though primary sequences are divergent. Syndecans from different species are interchangeable for some functions. For example, microinjection of GST fusion proteins of either the cytoplasmic domain of *Drosophila* syndecan or the cytoplasmic domain of murine syndecan-1 prevents *Drosophila* embryo gastrulation, while GST alone had no effect [30]. This and other evidence, including phylogenetic analysis, suggest that the four syndecan genes were derived from a single ancestral gene, which underwent two sets of duplications.

### Polymorphisms of chicken syndecan-2

So far, alternative splicing has only been found for mouse syndecan-1 [31]. Two cSNPs in syndecan-2 and three cSNPs each in both syndecan-3 and -4 have been found (<http://www.ncbi.nlm.nih.gov/SNP/>). SNPs are very common in genomic DNA. In humans, approx. 90% of sequence variation results from single-base differences. The coding regions, however, being only about 5% of the human genome, have a low probability of SNPs. The number of cSNPs in the human genome is around  $5.0 \times 10^5$ , i.e. approx. six per gene [32]. cSNPs may or may not affect gene functions. It is well known that cSNPs can be associated with some human diseases [33]. The cSNPs of chicken syndecan-2 are common in both strains tested. Whether these cSNPs are functionally significant is not known. It will be interesting to study whether the alanine<sup>43</sup> amino acid switch affects GAG attachment at serine<sup>41</sup> and, therefore, has physiological significance.

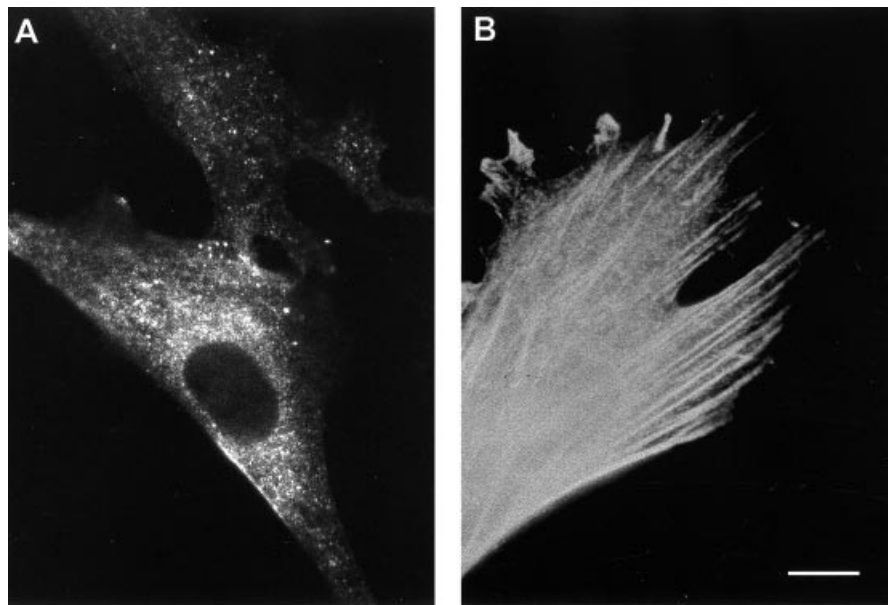
### Syndecan-2 and development

The tissue distribution of chicken syndecan-2 mRNAs during embryonic development is quite distinct from that of its closest homologue, chicken syndecan-4 [34]. In intestine or heart, syndecan-4 mRNAs increase from embryonic day 8.5 or 10 to 15, while syndecan-2 mRNAs decrease from day 9 or 12 to 15. Chicken syndecan-4 mRNAs are undetectable in brain and liver after embryonic day 8.5, but syndecan-2 mRNAs are increased in



**Figure 7** Regulated chicken syndecan-2 mRNA expression during chick embryonic development

mRNAs were isolated from chicken embryonic tissues on embryonic days 9 (**A**), 12 (**B**) and 15 (**C**). In (**D**), total RNAs were isolated from chicken embryonic fibroblasts (CEF) and rat embryonic fibroblasts (REF). The 1.3 kb panels are glyceraldehyde-3-phosphate dehydrogenase controls.



**Figure 8** Distributions of syndecan-2 and syndecan-4 on the surfaces of chick embryonic fibroblasts

Cells were stained with R1891 polyclonal antiserum against rat syndecan-2 (**A**) or monoclonal anti-syndecan-4 antibody (**B**). Bar = 10  $\mu$ m.

brain after day 12, and are detectable in liver on day 9. Syndecan-3 is expressed mainly in the developing avian brain, neural tube and limb bud, with more limited expression in the developing lens, optic vesicle, genital ridge, sclerotome and feather buds [35]. Although avian syndecan-1 has not yet been cloned, chicken

syndecan-2, -3 and -4 appear to play distinct roles in embryonic development.

The developmental roles of cell surface proteoglycans, including glypicans and syndecans, are being increasingly studied. Studies suggest that glypican(s) may be involved in morphogenesis,



growth regulation and tumour suppression [36]. Syndecan-2 acts as a Vg1 cofactor to mediate early left–right axis formation via a mechanism distinct from its regulation of matrix assembly [37]. Mice deficient in syndecan-1, -3 or -4 show no obvious phenotype. However, syndecan-1-deficient mice indicate a role for syndecan-1 in the regulation of Wnt-1 signalling [38]. Syndecan-3 expression in the hypothalamus normally controls feeding behaviour, and deficient mice lack the usual increase in food consumption following food deprivation [39]. In syndecan-4-deficient mice, the fetal vessels in the placental labyrinth [40], wound repair and angiogenesis [41] are all impaired, and the kidneys of deficient mice are more easily damaged by  $\kappa$ -carrageenan treatment [42]. Syndecan-4-deficient mice are also more susceptible to endotoxin shock after lipopolysaccharide injection [43].

During murine embryonic development, syndecan-2 mRNA and protein levels are high in mesenchymal cells, especially in areas where morphogenesis occurs [44]. We now report that chicken syndecan-2 mRNA decreases throughout embryonic development in the liver and kidney. Rat syndecan-2 mRNA is highest in adult liver and kidney in comparison with other tissues [45]. Chicken syndecan-2 was cloned from an adult chicken liver cDNA library with a very high hybridization rate (211 positive plaques out of  $1.25 \times 10^6$  plaque-forming units), so adult chicken liver may re-express syndecan-2. Syndecan-2 in the brain gradually increases during chicken embryonic development, and increases postnatally in the rat [46], suggesting that it may be involved in both embryonic and postnatal brain development.

### Roles of syndecan-2 and syndecan-4 in cells

Syndecan-4, which localizes at focal adhesion sites, can coordinate with phosphatidylinositol 4,5-bisphosphate to super-activate the serine/threonine kinase protein kinase C $\alpha$  [19,20], which is needed for focal adhesion formation [47]. Also, syndecan-4 binding to fibronectin contributes to focal adhesion formation [8]. In contrast, syndecan-2, which has a punctate distribution on the cell surface, has multiple roles in extracellular matrix assembly [9], cell adhesion [7] and dendritic spine morphogenesis [15]. The common denominator may be the regulation of the microfilament cytoskeleton [7,22]. However, our current understanding of syndecan-2 function is rudimentary, but will be enhanced with the aid of avian-specific reagents characterized here. This will allow transfection and analysis of oligomerization, phosphorylation and downstream signalling in mammalian cells.

The generation of mAb 8.1 was performed by the Hybridoma Core Facility of the Multipurpose Arthritis Center, University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A., with the generous help of Dr Mary Ann Accavitti. We thank Dr Anna Erickson and Dr Ssang-Taek Lim in our laboratory for very helpful discussions during the whole study. This work was supported by NIH grant DK54605 to A.W.

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Received 6 May 2002/29 May 2002; accepted 31 May 2002

Published as BJ Immediate Publication 31 May 2002, DOI 10.1042/BJ20020711