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Syndecan 3: A member of the syndecan family of membrane-intercalated proteoglycans that is expressed in high amounts at the onset of chicken limb cartilage differentiation

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ABSTRACT **A partial cDNA that encodes a newly discovered member of the syndecan family of integral membrane proteoglycans, which we have termed syndecan 3, has been isolated from an embryonic chicken limb bud cDNA library. Syndecan 3 is distinct from but structurally related to syndecan and fibroglycan, two previously characterized members of this family of membrane-intercalated proteoglycans. Syndecan 3 contains a cytoplasmic domain potentially associated with the cytoskeleton that is 85% identical in amino acid sequence to the cytoplasmic domain of syndecan. Syndecan 3 also possesses a hydrophobic transmembrane domain and an extracellular domain containing several clustered potential glycosaminoglycan attachment sites. Like syndecan, the ectodomain of syndecan 3 has a single dibasic protease-susceptible site adjacent to the transmembrane domain, which might be involved in shedding the ectodomain from the cell surface. A striking feature of syndecan 3 is an extensive (182 amino acid) threonine, serine, and proline (T+S+P)-rich domain that closely resembles T+S+P-rich regions in several mucin-like proteins in which O-linked oligosaccharides are bound to the threonine and serine residues. Syndecan 3 is expressed in high amounts during a critical phase of chicken limb chondrogenesis in which limb mesenchymal cells condense, round up, and interact with one another before depositing a cartilage matrix. The multiple functional domains of syndecan 3 provide potential sites for mediating the adhesive cell–matrix interactions and cytoskeletal reorganization involved in this critical condensation process.**

The onset of cartilage differentiation in the developing vertebrate limb is characterized by a transient cellular condensation process in which prechondrogenic mesenchymal cells become closely juxtaposed before initiating cartilage matrix deposition. During this condensation process, critical cell–cell and cell–matrix interactions occur that are necessary to trigger chondrogenic differentiation (1). Regulatory events occurring during condensation result in initiation of the expression of cartilage-specific genes, including the genes for type IX collagen (2) and cartilage proteoglycan core protein (3), and an increase in the expression of the gene for cartilage-characteristic type II collagen (4). Several regulatory factors have been implicated in controlling cartilage-specific gene expression during condensation, including cAMP (5, 6), peptide growth factors (7, 8), gap junctional communication (9), and a cytoskeletal-mediated change in the shape of the cells from a flattened morphology to a rounded configuration (10).

Precartilage condensation is thought to be mediated by adhesive interactions between several cell-surface and extracellular matrix molecules that are transiently expressed at high amounts during the process, including fibronectin (11, 12), heparan sulfate proteoglycans (HSPGs) (13), tenascin (14), type I collagen (15), cell-surface galactosyltransferases (16), and the chondroitin sulfate-rich proteoglycan PG-M (versican) (17). In the present study, an embryonic chicken limb bud cDNA library has been screened with a cDNA to murine syndecan (18), an integral membrane proteoglycan that links the cytoskeleton to extracellular matrix molecules in several cell types and that is present at sites of mesenchymal cell condensation in the developing tooth and kidney (19). A partial cDNA has been characterized that encodes a newly discovered member of the syndecan family of membrane-intercalated proteoglycans, which we have termed syndecan 3 to distinguish it from syndecan (18, 20, 21) and fibroglycan (22), distinct members of this family of integral membrane proteoglycans.‡ Syndecan 3 contains a cytoplasmic domain potentially associated with the cytoskeleton that is 85% identical in amino acid sequence to the cytoplasmic domain of syndecan. Syndecan 3 also possesses a hydrophobic transmembrane domain and an extracellular domain containing several clustered putative glycosaminoglycan (GAG) attachment sites, as well as an extensive mucin-like domain potentially containing numerous O-linked oligosaccharide chains that is not present in other members of the syndecan family. Syndecan 3 is expressed in high amounts during the critical condensation phase of chicken limb chondrogenesis. The multiple functional domains of syndecan 3 provide potential sites for mediating the adhesive cell–matrix interactions and cytoskeletal reorganization involved in this critical condensation process.

MATERIALS AND METHODS

Isolation of Syndecan 3 cDNA and Genomic Clones. An oligo(dT)-primed cDNA library prepared from poly(A)$^+$ RNA isolated from 4- to 6-day embryonic chicken limb buds (23) was screened with a cDNA for murine syndecan (clone 4-19b) (18) at reduced stringency as described (24). Ten positive clones were obtained, nine with inserts of equal electrophoretic mobility and one containing a larger insert. One of the smaller clones and the larger clone were sequenced and were found to be overlapping cDNAs with identical 3′ ends that contain sequences highly similar to the cytoplasmic and transmembrane regions of syndecan (clones pCon-1 and pCon-2 in Fig. 1). Since the 136-base-pair (bp) 3′ nontranslated regions of both clones lack a consensus poly(A) attachment signal (see Fig. 2), the clones were likely primed on short poly(A) sequences upstream of the true poly(A) tail. To obtain clones extending further 5′, an aliquot of a cDNA library prepared from 10-day chicken embryos

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Abbreviations: GAG, glycosaminoglycan; HSPG, heparan sulfate proteoglycan.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M84910).

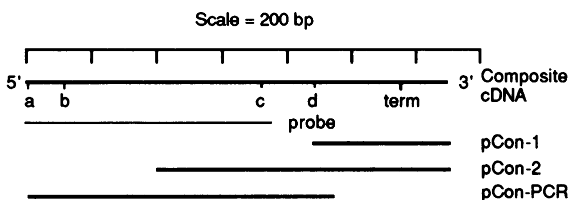

FIG. 1. Map of the partial syndecan 3 cDNA derived from cDNA clones and relative locations of the cDNA clones in the map. The position of the termination codon (term) is indicated. a, b, c, and d indicate the sites at which introns are located in the syndecan 3 genomic clone. The probe used for genomic screening and in Northern and dot blot hybridization analyses is indicated.

(Clontech) was screened by the polymerase chain reaction (PCR) using as a 5′ primer a vector-specific oligonucleotide, 5′-TGGTGGCGACGACTCCTGG-3′, and as a 3′ primer an oligonucleotide, 5′-GCTGCCCGACTCTATTGTGT-TGTCG-3′, complementary to a portion of the putative transmembrane domain encoded by pCon-1 and pCon-2 (nucleotides 910–934 in Fig. 2). PCR was performed as described (23) except that primer annealing was carried out at 50°C. A major 989-bp product was isolated from a 1% agarose gel and reamplified by PCR as described above, except that another vector-specific oligonucleotide, 5′-TGGAGCCCGTCAGTATCG-3′, was used. The 974-bp product was ligated into Bluescript SK− (Stratagene) as described (25). This clone (pCon-PCR; Fig. 1) was sequenced and found to overlap with pCon-1 and pCon-2. The 5′ sequence unique to the PCR-generated clone was confirmed by sequencing a syndecan 3 genomic clone (see below). DNA sequencing of both strands was done on double-stranded plasmid DNA by the dideoxynucleotide chain-termination method using Sequenase (United States Biochemical).

A genomic clone corresponding to the syndecan 3 gene was isolated by screening a chicken genomic library in EMBL3 (Clontech) (24) with a fragment (probe in Fig. 1) of plasmid pCon-PCR. A 6-kilobase (kb) *Bam*HI fragment of this clone was subcloned into Bluescript SK− and partially sequenced to define intron/exon boundaries.

Preparation of Cultures. High-density micromass cultures were prepared from the distal subridge mesenchymal cells of the wing buds of stage 25 (26) chicken embryos as described (27).

Determination of Syndecan 3 RNA Levels and *in Situ* Hybridization. For Northern blot analysis, total RNA was isolated by a modification (28) of the guanidine isothiocyanate/cesium chloride centrifugation procedure of Chirgwin *et al.* (29). Northern blots were prepared as described by Maniatis *et al.* (25) and were prehybridized, hybridized, and washed as described (4), except that hybridization and washing were performed at 50°C. Steady-state cytoplasmic levels of syndecan 3 mRNA were determined at various times during the progression of micromass culture by a modification of the cytoplasmic dot hybridization procedure (4). Levels of syndecan 3 RNA sequences were quantified as described (23), and syndecan 3 mRNA levels were normalized to the total poly(A)$^+$ mRNA content of samples by hybridizing portions of the same RNA samples used for determination of syndecan 3 mRNA with ^{32}P-labeled oligo(dT)$_{20}$ as described by Harley (30). *In situ* hybridization was performed as described by Mallein-Gerin *et al.* (31).

RESULTS

Characterization of Syndecan 3 cDNA. Fig. 2 shows the composite nucleotide and deduced amino acid sequence derived by sequencing the three overlapping clones shown in Fig. 1. The portion of the protein encoded by this 1273-bp

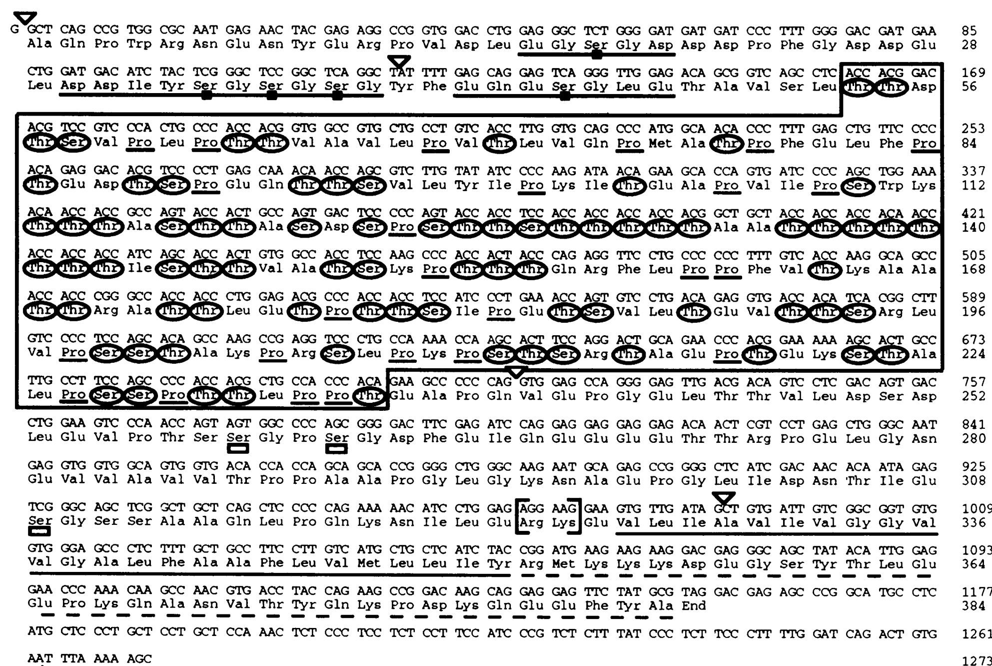

FIG. 2. Nucleotide and deduced amino acid sequence of the partial syndecan 3 cDNA. The putative cytoplasmic domain is indicated by dashed underlining, and the hydrophobic transmembrane domain is indicated by solid underlining. The dibasic protease-susceptible site adjacent to the transmembrane domain is indicated by brackets. The five potential GAG attachment sites toward the N-terminal end (amino acids 18, 34, 36, 38, and 45) are indicated by solid squares, and adjacent sequences are underlined. The serine residues of the three SG dipeptides located toward the transmembrane domain are indicated by open rectangles. The T+S+P-rich domain is enclosed by a box; the threonine and serine residues are circled and proline residues are underlined. This region closely resembles highly glycosylated T+S+P-rich regions of several mucin-like proteins in which O-linked oligosaccharides are bound to the threonine and serine residues. Open triangles indicate the sites at which introns are present in the syndecan 3 genomic clone.

partial cDNA, which we have termed syndecan 3, possesses a 33-amino acid cytoplasmic domain, a 25-amino acid hydrophobic transmembrane domain, and a 326-amino acid extracellular domain (Fig. 2).

The cytoplasmic domain of syndecan 3 is highly similar in amino acid sequence (85% identical) to the cytoplasmic domain of mammalian syndecans (18, 20, 21), and it is also quite similar in sequence (55% identical) to the cytoplasmic domain of human lung fibroblast HSPG (fibroglycan) (22), another member of the syndecan gene family of integral membrane proteoglycans (19) (Fig. 3). The hydrophobic transmembrane domain of syndecan 3 is also quite similar in sequence to the transmembrane domains of syndecan and fibroglycan (52% and 68% identical, respectively). The cytoplasmic domain of syndecan 3 possesses a sequence, KKKDEGSY, that corresponds to the tyrosine kinase phosphorylation consensus sequences KXXEXXY or KXXDXXXY as revealed by searching the Prosite data base (32) using the MOTIFS program of the Genetics Computer Group software package (33) (Fig. 3). Also contained in the cytoplasmic domain of syndecan 3 is a sequence, TLEE, that corresponds to a casein kinase II phosphorylation consensus sequence TXXE, in which the threonine residue is the potential site of phosphorylation (32). Interestingly, the potentially phosphorylated threonine and tyrosine residues are immediately adjacent to one another.

Like syndecan (18), the ectodomain of syndecan 3 has a single dibasic (RK) protease-susceptible site located immediately adjacent to the transmembrane domain (Fig. 2) that might be involved in cleavage of the ectodomain from the cell surface. However, the remainder of the ectodomain encoded by our partial syndecan 3 cDNA is very different from the ectodomains of syndecan and fibroglycan. The portion of the syndecan 3 ectodomain encoded by our cDNA has 5 SG dipeptides clustered toward the 5′ end of the cDNA (positions 18, 34, 36, 38, and 45 in Fig. 2), which represent potential sites of attachment of GAG side chains. The 3 serine-glycine repeats at positions 34, 36, and 38 are components of a sequence (DDIYSGSGSG) that closely resembles putative GAG attachment sequences (DDXXSASGSG and DDXSGSGSG) in fibroglycan (22) and phosphatidylinositol-anchored fibroblast cell-surface HSPG (34), which are proteoglycans that contain only heparan sulfate GAG chains. In contrast, the potential GAG attachment site at position 18 (EGSGD) corresponds to five known chondroitin sulfate attachment sites in aggrecan (35) and the site at position 45 (EQESGLE) of syndecan 3 corresponds to potential GAG attachment sequences in both aggrecan and the chondroitin sulfate-rich proteoglycan versican (36). These observations suggest the possibility that, like syndecan, syndecan 3 may be a hybrid proteoglycan containing both heparan sulfate and chondroitin sulfate GAG chains. The ectodomain of syndecan 3 also has three SG dipeptides (positions 259, 262, and 309; Fig. 2) located toward the transmembrane domain. However, these SG dipeptides are not surrounded by sequences typically associated with GAG

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syndecan 3   RMKKKDEGSYTLEEPKQANVT-YQKPDKQEEFYA*

syndecan     ..........S........GGA.....T.......
fibroglycan  ..R.......D.G.R.PSSAA-...APTK-.....
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FIG. 3. Comparison of the cytoplasmic domains of syndecan 3, mammalian syndecans (18, 20, 21), and fibroglycan (22). Only those amino acids that differ from syndecan 3 are shown. Gaps in the sequence are shown by hyphens, and conserved tyrosine (Y) residues are indicated by overlying dashes. The tyrosine kinase and casein kinase II phosphorylation consensus sequences of syndecan 3 are indicated by single and double underlining, respectively. The threonine residue potentially phosphorylated by casein kinase II is indicated by an overlying circle.

attachment sites and are therefore less likely to represent such sites.

It is of particular interest that the ectodomain encoded by our syndecan 3 cDNA possesses an extensive (182 amino acid) threonine, serine, proline (T+S+P)-rich domain located between the putative GAG attachment sites and the transmembrane domain of the core protein (Fig. 2). This region contains 32% threonine, 12% serine, and 14% proline residues including a 44-amino acid sequence that consists of 75% threonine and serine. This T+S+P-rich domain of syndecan 3 closely resembles highly glycosylated T+S+P-rich regions of several mucin-like proteins in which O-linked oligosaccharides are bound to the threonine and serine residues (37–41). This T+S+P-rich domain of syndecan 3 is not present in other members of the syndecan family.

Intron/Exon Organization. Using exon-specific primers, we sequenced regions of a syndecan 3 genomic clone to define intron/exon boundaries, which were confirmed by the presence of consensus splice donor and acceptor sequences. The portion of the deduced syndecan 3 core protein we have characterized is encoded by four exons (Fig. 2). Four of the five potential GAG attachment sites are encoded in one exon (Fig. 2). The adjacent 3′ exon encodes the other potential GAG attachment site and the entire T+S+P-rich domain of the core protein; the next 3′ exon includes the dibasic protease-susceptible site; and the most 3′ exon encodes most of the transmembrane domain and the entire cytoplasmic domain (Fig. 2). Thus, distinct potential functional domains of the portion of the deduced syndecan 3 core protein we have characterized are encoded by individual exons.

Expression of Syndecan 3 mRNA During Limb Cartilage Differentiation. A cDNA probe corresponding to the nonconserved 5′ portion of syndecan 3 (probe in Fig. 1) hybridizes to a major mRNA species of ≈7 kb on Northern blots of RNA isolated from 5-day (stage 25) chicken wing buds, a stage of development in which chondrogenic differentiation is being initiated in the proximal central core of the limb bud (Fig. 4). An ≈7-kb mRNA is also detectable in 5-day heart, although the amount of syndecan 3 mRNA is considerably less in heart than in limb buds. A syndecan 3 mRNA of the same size is also detectable in 12-day brain, but no syndecan 3 mRNA is detectable in 12-day liver.

To evaluate the expression of syndecan 3 during the progression of chicken limb cartilage differentiation, we examined changes in the steady-state cytoplasmic levels of syndecan 3 mRNA during the course of chondrogenic differentiation of the distal subridge mesenchymal cells of stage 25

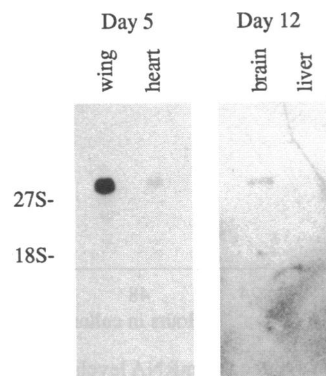

FIG. 4. Northern blot analysis of syndecan 3 mRNA expression in 5-day (stage 25) wing buds and 5-day heart, and, in a separate blot, in 12-day brain and 12-day liver. The size of the hybridizable mRNA in limb buds, heart, and brain is ≈7 kb. The significance of the very weakly hybridizing smaller band in the Northern blot of wing bud RNA is not clear. Positions of the 18S and 27S ribosomal RNAs are shown.

wing buds in high-density micromass culture. Stage 25 subridge mesenchymal cells comprise a homogeneous population of undifferentiated chondrogenic precursor cells, which uniformly progress through the phases of chondrogenesis in micromass culture and form a virtually uniform sheet of cartilage with little or no nonchondrogenic tissue detectable (27). In these cultures, widespread prechondrogenic condensations of cells are formed during the first day of culture, after which there is a uniform and progressive accumulation of cartilage matrix (27).

At 3 hr after the initiation of culture, which is before overt morphological indications of differentiation and the onset of condensation, relatively low levels of syndecan 3 mRNA are detectable in the cytoplasm of the cells (Fig. 5). Between 3 and 24 hr, during which time the cells are undergoing the formation of precartilage condensations, there is a progressive and striking (>5-fold) increase in the cytoplasmic levels of syndecan 3 mRNA. During the next 3 days of culture, as cells are initiating the synthesis of cartilage matrix, syndecan 3 mRNA declines to levels that are ≈2-fold lower than during the condensation phase of chondrogenesis. During this same period of culture, steady-state levels of mRNA for cartilage-characteristic type II collagen progressively increase (data not shown).

Syndecan 3 transcripts detectable by *in situ* hybridization are present in high amounts in the proximal chondrogenic central core of embryonic limb buds where cartilage differentiation is being initiated (Fig. 6). Thus, syndecan 3 is expressed at the onset of chondrogenesis *in vivo* as well as *in vitro*. In contrast, little or no syndecan 3 mRNA is detectable in the nonchondrogenic periphery of limb bud or in the overlying ectoderm (Fig. 6).

DISCUSSION

Characterization of Syndecan 3. In the present study, we have isolated a partial chicken cDNA that encodes an integral membrane proteoglycan possessing multiple potential functional domains that is a newly discovered member of the syndecan family of membrane-intercalated proteoglycans. The molecule encoded by this cDNA has been termed syndecan 3 to distinguish it from syndecan (18, 20, 21) and fibroglycan (22), previously characterized members of this gene family. Syndecan 3 shares several of the structural

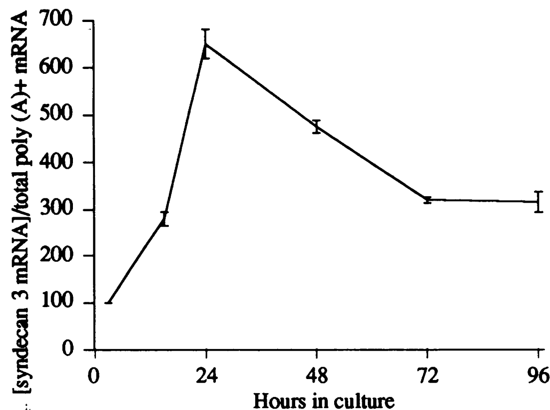

FIG. 5. Relative syndecan 3 mRNA levels at various times during the course of chondrogenic differentiation of distal subridge mesenchymal cells of stage 25 wing buds in high density micromass culture. The levels of syndecan 3 mRNA and total poly(A)$^+$ RNA at each time point were determined as described (23). The amount of cytoplasmic syndecan 3 mRNA/total poly(A)$^+$ RNA at each time point is presented as an amount relative to that at 3 hr of culture, which was arbitrarily set to 100. Values are the means and range of two determinations.

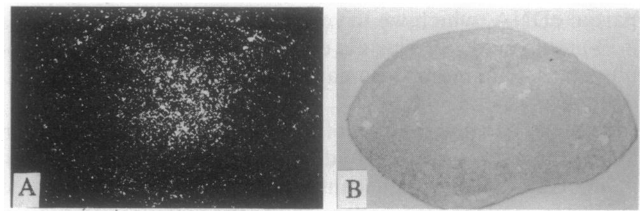

FIG. 6. Dark-field (*A*) and corresponding bright-light (*B*) autoradiograph of a cross-section through the proximal portion of a stage 28 wing bud hybridized with a specific ^{32}P-labeled syndecan 3 cDNA probe. High amounts of syndecan 3 transcripts are present in the chondrogenic central core, whereas little or no hybridization is detectable in the nonchondrogenic periphery or in the overlying ectoderm. The rim of silver grains along the edge of the section is not over tissue and presumably results from the accumulation of emulsion between the edge of the section and the slide. (×48.)

characteristics of syndecan (18, 20, 21) and fibroglycan (22). The amino acid sequence of the cytoplasmic domain of syndecan 3 is 85% and 55% identical, respectively, to the cytoplasmic domains of syndecan and fibroglycan. The transmembrane domains of syndecan 3, syndecan, and fibroglycan are also quite similar in sequence, and, like syndecan, the ectodomain of syndecan 3 has a single dibasic putative protease-cleavage site immediately adjacent to its transmembrane domain. However, the extracellular domain of syndecan 3 differs considerably from the ectodomains of these other molecules in amino acid sequence, as well as in the location of potential GAG attachment sites. A distinctive characteristic of syndecan 3 is its extensive (182 amino acid) T+S+P-rich domain that closely resembles similar regions of several mucin-like proteins that are highly substituted with O-linked oligosaccharides (37–41). This potentially highly O-glycosylated region of syndecan 3 represents a domain that could interact with lectin domains on other molecules (see below). Thus, syndecan 3, syndecan (18, 20, 21), and fibroglycan (22) appear to be distinct members of a family of membrane-intercalated proteoglycans possessing similar cytoplasmic domains.

Expression and Potential Functions of Syndecan 3 During Chicken Limb Cartilage Differentiation. The onset of cartilage differentiation in the developing vertebrate limb is characterized by a transient cellular condensation process in which prechondrogenic mesenchymal cells become closely juxtaposed and round up before initiating cartilage matrix deposition. During this condensation process, critical cell–cell and cell–matrix interactions occur that are necessary to trigger chondrogenic differentiation (1). Precartilage condensation formation is thought to be mediated by adhesive interactions between several cell-surface and extracellular matrix molecules that are transiently expressed at high amounts during the process (11–17). It is therefore of considerable interest that a striking progressive increase in syndecan 3 gene expression occurs during the critical condensation phase of limb chondrogenesis. The multiple functional domains of syndecan 3 provide potential sites for mediating the adhesive cell–matrix interactions and cytoskeletal reorganization involved in the critical condensation process.

Heparan sulfate and/or chondroitin sulfate chains bound to the GAG attachment sites in syndecan 3 would provide potential sites of interaction with the fibronectin, type I collagen, and/or tenascin expressed during precartilage condensation. Indeed, the heparan sulfate GAG chains of murine syndecan have been shown to interact with fibronectin (42), type I collagen (43), and tenascin (44). The putative highly O-glycosylated T+S+P-rich mucin-like region of syndecan 3 represents a domain that might bind to the 126-amino acid lectin domain in PG-M (versican) (17, 45), a chondroitin sulfate-rich proteoglycan transiently expressed during con-

densation (17). Terminal sugars in the mucin-like domain of syndecan 3 also provide potential sites for adhesive interactions with cell-surface galactosyltransferases that are expressed during condensation and that have been suggested to promote adhesive cell–cell interactions by interacting with cell-surface glycoconjugates containing terminal GlcNAc residues (16).

The cytoplasmic domain of syndecan 3 is highly similar to the cytoplasmic domain of murine syndecan and thus, like murine syndecan, may associate with the cytoskeleton (19). Thus, interaction of syndecan 3 with other matrix ligands might be involved in the cell shape changes that occur during condensation that facilitate chondrogenesis. Phosphorylation of the tyrosine kinase and/or casein kinase II phosphorylation sequences of the cytoplasmic domain of syndecan 3 in response to such interactions could provide a mechanism for modulating association of the cytoplasmic domain with the cytoskeleton, thus leading to cell shape changes. In addition, the regulated proteolytic shedding of the ectodomain of syndecan 3 at its dibasic protease-cleavage site and the resultant dissociation of its ectodomain from the cytoplasmic domain might also aid in maintaining the rounded cell configuration that is conducive to chondrogenesis. Indeed, the rounding of mammary epithelial cells in response to suspension culture is accompanied by shedding of the ectodomain of membrane-intercalated syndecan (46). Furthermore, the regulated shedding of the ectodomain of syndecan 3 might break the adhesive interactions that promoted condensation, thus facilitating the deposition of cartilage matrix molecules.

Interestingly, murine syndecan protein exhibits essentially a reciprocal pattern of expression during mouse limb cartilage differentiation to that exhibited by chicken syndecan 3 transcripts during chicken limb chondrogenesis. Murine syndecan protein expression ceases in areas of cartilage differentiation (47), whereas syndecan 3 mRNA is expressed in high amounts at the onset of chondrogenesis. This suggests that these two distinct members of the same family of integral membrane proteoglycans may be involved in different phases of limb chondrogenesis.

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