Syndecan-3 Is a Selective Regulator of Chondrocyte Proliferation*

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Chondrocyte proliferation is important for skeletal development and growth, but the mechanisms regulating it are not completely clear. Previously, we showed that syndecan-3, a cell surface heparan sulfate proteoglycan, is expressed by proliferating chondrocytes in vivo and that proliferation of cultured chondrocytes in vitro is sensitive to heparitinase treatment. To further establish the link between syndecan-3 and chondrocyte proliferation, additional studies were carried out in vivo and in vitro. We found that the topographical location of proliferating chondrocytes in developing chick long bones changes with increasing embryonic age and that syndecan-3 gene expression changes in a comparable manner. For in vitro analysis, mitotically quiescent chondrocytes were exposed to increasing amounts of fibroblast growth factor-2 (FGF-2). Proliferation was stimulated as much as 8–10-fold within 24 h; strikingly, this stimulation was significantly prevented when the cells were treated with both fibroblast growth factor-2 (FGF-2) and antibodies against syndecan-3. This neutralizing effect was dose-dependent and elicited a maximum of 50–60% inhibition. To establish specificity of neutralizing effect, cultured chondrocytes were exposed to FGF-2, insulin-like growth factor-1, or parathyroid hormone, all known mitogens for chondrocytes. The syndecan-3 antibodies interfered only with FGF-2 mitogenic action, but not that of insulin-like growth factor-1 or parathyroid hormone. Protein cross-linking experiments indicated that syndecan-3 is present in monomeric, dimeric, and oligomeric forms on the chondrocyte surface. In addition, molecular modeling indicated that contiguous syndecan-3 molecules might form stable complexes by parallel pairing of β-sheet segments within the ectodomain of the core protein. In conclusion, the results suggest that syndecan-3 is a direct and selective regulator of the mitotic behavior of chondrocytes and its role may involve formation of dimeric/oligomeric structures on their cell surface.

Chondrocytes constitute the embryonic cartilaginous skeleton, the growth plates of fetal and postnatal skeletal elements, and permanent cartilages such as articular cartilage, nasal septum, and tracheal rings. In these various structures and locations, chondrocytes exhibit characteristic and strictly controlled mitotic activity, and this activity has diverse roles and implications. For example, in the growth plate chondrocyte proliferation is topographically limited to a narrow zone flanked by a resting zone of quiescent cells and an underlying zone of postmitotic prehypertrophic cells (1). A main role for chondrocyte proliferation in the growth plate is to counterbalance the loss of hypertrophic chondrocytes occurring at the chondro-osseous border and their replacement by endochondral bone cells. A second important role is to determine the overall rates of skeletal growth, as illustrated by the different rates of proliferation displayed by chondrocytes in long bones elongating at different rates within the same organism (2). In other cartilaginous structures such as adult articular cartilage, chondrocytes are mitotically quiescent. However, proliferation can be reactivated in a vigorous manner when the tissue is mechanically injured or is targeted by pathological events such as osteoarthritis (3, 4). In these instances, chondrocyte proliferation is thought to represent a repair process and an attempt by the tissue to compensate for structural and functional losses. Indeed, chondrocyte proliferation in affected articular cartilage is accompanied by increased production of extracellular matrix components (3, 4). Thus, it is clear that chondrocyte proliferation is an event with multiple roles and important implications. It is a process tightly controlled both spatially and temporally and is modulated according to specific physiological or pathological conditions and requirements. Given its importance, it would be imperative to know precisely how it is regulated, but information in this area, although extensive, is far from complete.

Studies have established that chondrocyte proliferation is responsive to and regulated by a number of factors and agents; these include members of the fibroblast growth factor (FGF) family, insulin-like growth factors (IGFs), parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP), hepatocyte growth factor/scatter factor, Indian hedgehog, interleukin-1, and transforming growth factor-β (5–10). Some of these factors are potent stimulators of chondrocyte proliferation in culture; PTHrP and FGF-2 at nanomolar concentrations increase chondrocyte proliferation severalfold (7, 9). Other agents, such as interleukin-1, are inhibitors (11). There is evidence also that several of these factors and their respective cell surface receptors regulate chondrocyte proliferation and behavior in vivo as well. Receptors for PTHrP and IGFs are found to influence proliferation in a positive manner (12–14), whereas receptors for FGFs are found to be negative regulators (15). A category of potentially important cell surface receptors/cotransducers, which has received far less attention in this area,
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is the heparan sulfate proteoglycans (HSPGs). Yet, it is quite well established that HSPGs have important roles in mediating the responses of several cell types to growth stimuli, particularly the FGFs and IGFs, and are also involved in developmental events associated with or related to proliferation, including cell differentiation, maturation, and morphogenesis (16–20).

HSPGs consist of a large number of different proteins. Two families of HSPGs, which are associated with the plasma membrane, are the syndecans and the glypicans. Four isoforms have been cloned in the syndecan family and six in the glypican family. The syndecans are type I cell membrane components in which the core protein contains a single pass transmembrane domain, a long and extended extracellular domain bearing heparan sulfate chains, and a relatively short intracellular domain with roles in signal transduction (16, 17). Glypicans instead are associated to the cell surface via a glycosylphosphatidylinositol anchor and their core proteins have a more globular configuration with heparan sulfate chains close to the cell surface (21). In previous studies from our laboratories, we showed that a member of the syndecan family, syndecan-3, is expressed in the proliferative zone of the growth plate in chick embryo tibia, but is undetectable in other zones (22–24). We observed similar expression in the mitotic areas of other developing skeletal structures, such the sternum, in which proliferating chondrocytes occupy specific regions rather than a narrow and well defined zone as seen in long bones. In addition, we found that proliferation in cultured chondrocytes was enhanced by treatment with FGF-2 and that this response was counteracted by co-treatment with heparinase or heparitinase. These results were the first to link syndecan gene expression to chondrocyte proliferation, and indicated that the mitotic response of chondrocytes to FGF-2 is mediated by a HSPG(s), in line with our previous studies, however, had left several questions unanswered, the main one being the nature of the syndecan-3 role in chondrocyte proliferation. The present study was carried out to address this important issue. We provide evidence that syndecan gene expression. Data from previous related studies had suggested that in developing long bones, the location of proliferating chondrocytes might change with increasing embryonic age (15, 24). These cells appear to occupy initially the most epiphyseal extremities adjacent to the incipient joints at early embryonic stages; the cells then appear to shift to a more metaphyseal location once articular cartilage forms and the growth plate acquires a more definitive organization. Because this shift in the location of proliferating chondrocytes has not been studied in a systematic manner, we carried out a more

EXPERIMENTAL PROCEDURES

In Situ Hybridization and 5-Bromodeoxyuridine (BrdUrd) Labeling—Hindlimbs were isolated from chick embryos ranging in age from 5 to 18 days. Half of the specimens were directly processed for immunohistochemical detection of incorporated BrdUrd using a commercial kit (Vector Laboratories, Burlingame, CA).

Chondrocyte Cultures—Immature chondrocytes were isolated from the proximal part of day 14 chick embryo sternum as described (24). Cells were plated at a density of 1 × 10^6 cells/12-mm well plates and cultured in Dulbecco’s modified high glucose Eagle’s medium (Invitrogen, Gaithersburg, MD) containing 10% fetal bovine serum (HyClone, Logan, UT), 2 mM l-glutamine, and 50 units/ml penicillin and streptomycin. After cells reached confluence at days 3 or 4, they were switched to Dulbecco’s modified Eagle’s medium containing 0.3% fetal bovine serum for 24 h to induce mitotic quiescent. Cultures were treated over the following 24 h with FGF-2 at final concentrations ranging between 0.5 and 10 ng/ml, IGF-1 at final concentrations of 100 and 10 ng/ml, or PTH at final concentrations ranging between 1 and 500 ng/ml. FGF-2 was a generous gift from Dr. Denis Gospodarowicz (Chiron Co.), whereas human IGF-1 and PTH were obtained from R&D (Minneapolis, MN). When indicated, cultures were co-treated with one of the above growth factors and syndecan-3 antibodies (total IgG; see below) or preimmune rabbit IgG at final concentrations ranging between 0.5 and 20 μg/ml.

For the proliferation assays, cells were labeled with 10 μCi/ml [3H]thymidine during the last 4 h of incubation. After labeling, cultures were washed three times with phosphate-buffered saline (pH 7.4), and unincorporated [3H]thymidine was removed by extraction with 10% trichloroacetic acid followed by ether and ethanol. This extraction step was repeated twice. The resulting extracted cell layers were then solubilized in 500 μl/well of 4 μm guanidinium solution, and 10-μl aliquots were used to determine incorporated radioactivity by scintillation counting.

For immunoblot analysis chondrocyte cultures were extracted with lysis buffer containing 0.01 M sodium phosphate buffer (pH 7.4), 0.1 M NaCl, 1% Triton X-100, 0.1% SDS, 0.2% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride for 24 h at 4 °C. Extracts were centrifuged to remove particulate matter. Proteins in the supernatants were precipitated with methanol, re-dissolved in SDS sample buffer, separated by SDS-PAGE, and processed for Western blot as described (26).

Chemical Cross-linking Studies—For the cross-linking experiments, cell cultures were first incubated with 1 unit/ml of heparinase I, followed by incubation with 200 μg of the reducible cross-linker dimethyl suberimidate for 3 h at 37 °C in Hank’s saline. After incubation, cells were rinsed three times with phosphate-buffered saline and extracted as described above. Samples were then processed for SDS-PAGE and immunoblots before and after reduction (see below).

Proteins and Antibodies—Recombinant extracellular region of syndecan-3 (amino acids 215–315) was prepared using the pGEX expression vector (Amersham Biosciences) as we described previously (27). This region was chosen because it shows no homology to other syndecans and because it has been suggested to have important roles in function (16). Recombinant syndecan-3-glutathione S-transferase fusion protein was expressed in Escherichia coli DH5αF’ and purified. Rabbits were injected with 200 μg of purified syndecan-3-glutathione S-transferase fusion protein four times. Total IgGs from the antisera were obtained by affinity chromatography using a Protein A-Sepharose column (Amersham Biosciences). Preimmune IgGs isolated from the same rabbits prior to immunization with syndecan-3 fusion protein were used as controls. Specificity of the immune IgGs for syndecan-3 was determined by enzyme-linked immunosorbent assays (not shown) and immunoblottting.

SDS-PAGE and Immunoblotting—Samples were dissolved in 3% SDS sample buffer with or without 5-mercaptoethanol, denatured at 100 °C for 3 min, and separated by SDS-PAGE using 8% (w/v) gels (26). Samples were electroblotted onto nitrocellulose filters after electrophoresis. After blocking with a solution of low-fat milk protein, blotted proteins were immunostained with primary syndecan-3 antibodies followed by peroxidase-conjugated secondary antibodies and color development (26).

Molecular Modeling—A molecular model of chick syndecan-3 was constructed according to the following protocol. A prediction to the secondary structure of syndecan-3 core protein was prepared by the method of Ross and Golub (28). The calculated secondary structure map identified 10 N-terminal (1–16), 9 C-terminal (186–248), transmembrane (197–324), and periplasmic (350–383). Based on this analysis, three-dimensional models of each domain were constructed using the Homology module of the Insight II Program (Accelrys, Inc., San Diego, CA). The sequence of each domain was imported as a linear molecule, and the predicted secondary structure was then imposed on the chain. The structure was finally subjected to numerous rounds of energy minimization using the CHARMM force field and algorithm (29). The complete structure model was created by joining the domains, and minimizing the entire structure.

RESULTS

Syndecan-3 and Chondrocyte Proliferation in Vivo—In a first set of experiments, we sought to extend our previous in vivo observations correlating chondrocyte proliferation to syndecan-3 gene expression. Data from previous related studies had suggested that in developing long bones, the location of proliferating chondrocytes might change with increasing embryonic age (15, 24). These cells appear to occupy initially the most epiphyseal extremities adjacent to the incipient joints at early embryonic stages; the cells then appear to shift to a more metaphyseal location once articular cartilage forms and the growth plate acquires a more definitive organization. Because this shift in the location of proliferating chondrocytes has not been studied in a systematic manner, we carried out a more
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Fig. 1. Co-localization of proliferating chondrocytes and syndecan-3 gene expression. Tibial rudiments from days 6, 7, and 17 chick embryos were pulse-labeled with BrdUrd for 4 h and longitudinal sections were processed for histochemical detection of BrdUrd-labeled proliferating cells (B, E, and H). Similar sections from companion specimens were processed for in situ hybridization detection for syndecan-3 transcripts (C, F, and I). A–C, day 6; D–F, day 7; G–I, day 17. Brackets point to co-location of proliferating chondrocytes and syndecan-3 expression; arrows in E and F point to epiphyseal end areas containing few proliferating chondrocytes; arrowheads in B and E point to lack of proliferating chondrocytes in diaphysis. ac, articular cap; phz, prehypertrophic zone. Bar, 100 μm.

A thorough analysis of this phenomenon using the developing chick embryo tibia as a model. We then asked whether the predicted shift in distribution of proliferating chondrocytes is associated with a parallel shift in syndecan-3 gene expression. To this end, hindlimbs isolated from chick embryos ranging in age from 5 to 18 days were placed in organ culture and were immediately pulse-labeled with BrdUrd for 2 h to label proliferating cells. Serial longitudinal sections were processed for immunohistochemical detection of BrdUrd-labeled cells and for in situ hybridization detection of syndecan-3 transcripts. Systematic analysis of embryonic tibiae differing in age by 1 day revealed that BrdUrd-labeled proliferating chondrocytes were located at the epiphyseal extremity in day 6 chick embryos (Fig. 1, A and B, brackets); no incorporation was seen in the incipient diaphyseal region containing post-proliferative prehypertrophic chondrocytes (Fig. 1B, arrowhead). By day 7, BrdUrd-labeled cells were already less numerous at the epiphyseal extremity near the forming joint (Fig. 1E, arrow), but were prominent in a slightly more metaphyseal region immediately below (Fig. 1, D and E, brackets). This trend continued and became obvious at later time points such as day 17, when the entire population of proliferating chondrocytes was now restricted to a well-defined and narrow zone of the growth plate (Fig. 1, G and H, brackets); these cells were flanked by quiescent chondrocytes in articular cap (ac) above and in the prehypertrophic zone (phz) below (Fig. 1H).

In situ hybridization clearly revealed that the patterns of syndecan-3 gene expression changed in a comparable manner and syndecan-3 expression was a constant feature of proliferating chondrocytes at each stage examined (Fig. 1, C, F, and I). Thus, syndecan-3 transcripts were present throughout the proliferating chondrocyte-rich epiphysis in day 6 specimens (Fig. 1, B and C, brackets), whereas in day 7 samples the transcripts were reduced at the epiphyseal end (Fig. 1, E and F, arrows) and prominent in metaphyseal proliferating chondrocytes (Fig. 1, E and F, brackets). Day 17 embryos displayed a clear, narrow and overlapping zone of syndecan-3 expression and chondrocyte proliferation (Fig. 1, H and I, brackets). Note that at early stages syndecan-3 was also strongly expressed by perichondrium, which is rich in proliferating cells (Fig. 1, C and F) (22).

Antibody Interference Assays in Cultured Chondrocytes—In previous studies by others, antibodies raised against syndecan-3 were used to interfere with its function and determine its role in developmental processes (30). Thus, we used a similar approach to test the role of syndecan-3 in chondrocyte proliferation. Antibodies were raised in rabbits against a 98-amino acid long recombinant protein corresponding to amino acids 215–313 of the extracellular domain of chick syndecan-3 core protein. This portion of the core protein lies immediately after the membrane-spanning segment and was selected because it has no homology to other syndecans and may have important roles in syndecan-3 function (16). Chondrocytes isolated from the caudal portion of day 14 chick embryo sternae were reared in monolayer culture for 3 to 4 days; we showed previously that the cells are highly homogenous and express syndecan-3 strongly (24). Detergent extracts of day 4 semiconfluent chondrocyte cultures were processed for immunoblot before and after treatment with heparinase I. Two major immunoreactive bands were detected in samples not treated with heparinase, one broad band above the 200-kDa molecular mass marker corresponding to intact syndecan-3 molecules and one below the 66-kDa marker corresponding to the predicted size of its core protein (Fig. 2, lane 1) (31). After heparinase treatment, the high molecular weight band was no longer appreciable, whereas the lower band was still strong (Fig. 2, lane 2).

To analyze proliferation, similar semiconfluent day 4 chondrocyte cultures were switched to medium containing 0.3% fetal calf serum for 24 h to induce mitotic quiescence; cultures were then treated with increasing amounts of FGF-2 over the following 24 h and were labeled with [3H]thymidine during the last 4 h of culture (Fig. 3). FGF treatment resulted in a dose-dependent increase in proliferation, which was maximal at 5 ng/ml and represented 8–10-fold stimulation over control (Fig. 3, open circles). Strikingly, when companion cultures were simultaneously treated with FGF-2 and syndecan-3 antibodies (total IgG fraction, 10 μg/ml), the increase in proliferation was significantly dampened, amounting to about 50% of that seen in cultures treated with FGF-2 only (Fig. 3, filled triangles). When cultures were treated with FGF-2 and preimmune IgGs (from the same rabbit used for immunization), no neutralizing effect and no interference of proliferation were seen (Fig. 3, filled squares). In a parallel experiment, companion cultures were treated with a fixed dose of FGF-2 (10 ng/ml) and increasing amounts of syndecan-3 antibodies for 24 h and were labeled with [3H]thymidine during the last 4 h of culture as above (Fig. 4). Clearly, there was a dose-dependent inhibition of proliferation, which was maximal at about 10 μg/ml of antibodies.
and represented a decrease of about 60% over control values (Fig. 4).

To determine whether syndecan-3 action is growth factor-specific, semiconfluent chondrocyte cultures were treated for 24 h with FGF-2, IGF-1, or PTH in the absence or presence of 10 μg/ml syndecan-3 antibodies and were then pulse labeled with [3H]thymidine during the last 4 h. IGF-1 and PTH were selected for this comparative analysis because they have been proposed that syndecan-3 may form dimeric/oligomeric structures on the cell surface, aided by specific juxtamembrane amino acid residues or other mechanisms; these structures were suggested to be important for function including growth factor responsiveness (16, 32). To determine whether such structures are present in chondrocytes, semiconfluent cultures similar to those used above were treated with heparinase I and then incubated with the reducible cross-linker dimethyl suberimidate. Detergent extracts were separated by SDS-PAGE under reducing or nonreducing conditions and processed for immunoblotting with syndecan-3 antibodies. Interestingly, unreduced samples produced three strong immunoreactive bands (Fig. 6, lane 1). These bands displayed apparent sizes of over 220, 110, and 55–60 kDa (Fig. 6, lane 1) and may thus correspond to monomeric, dimeric, and oligomeric forms of syndecan-3. When samples were reduced prior to electrophoresis, only the 55–60-kDa band was prominent (Fig. 6, lane 2).

**Molecular Modeling**—To gain further insights into how syndecan-3 molecules may associate into dimeric/oligomeric structures, we used molecular modeling to analyze the configuration of syndecan-3 core protein and identify possible domains involved in intermolecular associations. We first obtained a predicted secondary structure map of syndecan-3 core protein using methods we described previously (28). This analysis revealed a core protein monomer with five clearly distinct domains (Fig. 7). The N-terminal domain (amino acids 1–123), the perimembrane domain (197–324), and the cytoplasmic domain (350–383) displayed globular configurations and were thus termed G1, G2, and G3, respectively (Fig. 7). However, domain 124–196 was predicted to have an extended stalk-like configuration consisting of several β-strand segments (Fig. 7) and reflecting its high threonine-serine-proline content (31). As those segments cannot exist in isolation, the protein may tend to exist as a dimer with corresponding secondary structure map of syndecan-3 core protein using methods we described previously (28). This analysis revealed a core protein monomer with five clearly distinct domains (Fig. 7). The N-terminal domain (amino acids 1–123), the perimembrane domain (197–324), and the cytoplasmic domain (350–383) displayed globular configurations and were thus termed G1, G2, and G3, respectively (Fig. 7). However, domain 124–196 was predicted to have an extended stalk-like configuration consisting of several β-strand segments (Fig. 7) and reflecting its high threonine-serine-proline content (31). As those segments cannot exist in isolation, the protein may tend to exist as a dimer with corresponding β-strands forming a parallel β-sheet. This possibility was modeled by juxtaposing two copies of the monomers and energy minimizing the resulting dimeric structure (Fig. 8). Interestingly, alignment of the β-strands of the stalk brought the globular N-terminal, per-
imembrane, and cytoplasmic domains of the two monomers into close proximity, forming cohesive structural domains and stabilizing the dimeric molecule as a whole. The exact orientation of the transmembrane domain is less exact, as no attempt was made to impose a membrane environment on this structure during the energy minimization calculations. Furthermore, as the actual sites of GAG chain attachment are currently not known, no attempt was made to include GAG chains in the structure.

DISCUSSION

The results of this study indicate that syndecan-3 is intimately associated with chondrocyte proliferation and plays a role in regulating this important process. Our immunohistochemistry and in situ hybridization data show that syndecan-3 gene expression is continuously associated with proliferating chondrocytes and changes according to the topographical redistribution of these cells with increasing embryonic age of developing skeletal elements. This association may be causal in nature, as clearly suggested by our data with chondrocyte cultures. The strong mitotic response of the cells to FGF-2 is significantly reduced by syndecan-3 antibodies, but not preimmune IgGs. This is very much in line with our previous report that the response to FGF-2 is antagonized by simultaneous

FIG. 5. Comparison of growth factor effects on chondrocyte proliferation. Serum-starved chondrocyte cultures were treated for 24 h with the indicated concentrations of FGF-2, IGF-1, or PTH in the absence or presence of 10 μg/ml syndecan-3 antibodies. Cultures were pulse-labeled with [3H]thymidine during the last 4 h of treatment and radioactivity was determined by scintillation counting. Data are from three separate cultures and are expressed as mean disintegrations/min per sample aliquot ± S.D.

FIG. 6. Protein cross-linking analysis of syndecan-3. Chondrocyte cultures were treated with heparinase I and were then incubated with the reversible cross-linker dimethyl suberimidate for 3 h. Cultures were extracted with nonionic detergents and samples were processed for immunoblot with syndecan-3 antibodies before (−) and after (+) reduction. Lane 1, note the presence of three strong and distinct immunostained bands, probably corresponding to monomeric, dimeric, and oligomeric forms of syndecan-3. Lane 2, note that a single band above the 55-kDa marker is detectable after reduction.

FIG. 7. Secondary structure prediction of syndecan-3 core protein. N-terminal, perimembrane, and cytoplasmic globular domains are termed G1, G2, and G3, respectively. Stalk and transmembrane (TM) domains are indicated. Hydrophilic residues are in brown-red and hydrophobic residues are in green.
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**Fig. 8. Syndecan-3 dimer model.** One monomer is in red and one in blue. Note that the juxtaposition of the stalk domains brings the G1, G2, and G3 globular domains in close proximity. The transmembrane (TM) domains appear far apart from each other as a possible consequence of lack of inclusion of a lipid environment in calculations. There are five potential chain attachment sites in the N-terminal domain and three in the perimembrane domain. However, as the exact location and number of heparan sulfate chains are not clear at present, the chains were not included in the model. See text for further details.

Treatment with heparinase I (24). We find also that the response to other mitogens, PTH and IGF-1, is not interfered by the syndecan-3 antibodies. This is in agreement with the fact that PTH does not require the intervention of HSPGs for receptor binding, and that interaction of IGFs with their receptors may be favored by glypicans rather than syndecans (18). Together, the data suggest that syndecan-3 may be one of the cell surface receptors regulating and modulating the mitotic activity of chondrocytes in response to specific local growth factors and cues in developing skeletal structures.

The neutralizing effects of the syndecan-3 antibodies seen in our study are not unprecedented, as pointed out above. Kosher and co-workers (30) used a similar strategy to determine the role of syndecan-3 in elongation and growth of the early chick embryo limb bud. Syndecan-3 is expressed in the progress zone of the limb buds, which contains undifferentiated mesenchymal cells actively proliferating in response to mitogenic stimuli, namely FGF-2, FGF-4, and FGF-8, from the adjacent apical ectodermal ridge (33). Syndecan-3 antibodies were injected into the progress zone and caused a marked decrease in mesenchymal cell proliferation and limb elongation. The data in those studies and those in our study do not suggest a definitive mechanism by which the antibodies exert their interfering activity and by which syndecan-3 actually operates. However, they do point to several plausible and interesting possibilities. An obvious possibility is that the antibodies may have interfered with the syndecan-3 co-receptor role and did so by either restricting the mobility of syndecan-3 molecules on the plane of the membrane or preventing syndecan-3 molecules from approaching signaling receptors (such as FGF-Rs). A second related possibility is suggested by our cross-linking data, which point to the occurrence of dimeric and oligomeric syndecan forms on the chondrocytes membrane. Occurrence of similar forms of syndecan-3 or other syndecans has been seen in other systems and cell types previously (32, 34). Such dimeric/oligomeric structures are thought to enhance the ability of syndecans to exert their co-receptor function, for example, by increasing the probability of interactions with other membrane proteins (35) or synergism among denser HS chains (36). They are also needed by syndecans, such as syndecan-4, for transmission of intracellular signals (34). Thus, if the syndecan-3 antibodies interfered with oligomerization, they could have led to a decrease of syndecan-3 co-receptor function. A third possibility is that the antibodies may have lowered FGF-2 binding to syndecan-3. This possibility seems to be unlikely, because FGF-2 binds to the HS chains and not the core protein of syndecan-3 (37). It is worth mentioning, however, that FGF-2 does bind with high affinity to the core protein of the transmembrane chondroitin sulfate proteoglycan NG2 (38) and another family member, FGF-7, binds to the core protein of perlecan (39).

Previous immunohistochemical studies have shown that FGF-2 is present in the proliferative zone of growth plate where it displays a nuclear, cytoplasmic, and extracellular distribution, and in the hypertrophic zone where it is present extracellularly (40). It is thus possible that FGF-2 may serve as a ligand for syndecan-3 in the proliferative zone and influence proliferation in a positive manner. This possibility would correlate well with the finding that overexpression of FGF-2 in transgenic mice causes a marked expansion of the proliferative zone as well as the reserve zone (40); this expansion is accompanied by inhibition of chondrocyte hypertrophy, a long known consequence of excessive FGF-2 signaling (9, 41). On the other hand, several studies have indicated that FGF-2 treatment inhibits chondrocyte proliferation in cartilaginous long bone rudiments in organ culture (42) and expression of a constitutively active form of FGF-R3 inhibits chondrocyte proliferation in transgenic mice (15). How can these apparently contrasting results be reconciled? Admittedly, this is not easy at this time. It is difficult to say which of the experimental approaches used in the different studies including ours, be it chondrocyte cultures, organ cultures, or in vivo models, reflects the in vivo condition most faithfully and most physiologically. Second, the nature of endogenous growth factors serving as ligands for FGF-Rs and syndecans in the growth plate in vivo are not known as is the status of activation of the receptors. FGF-18 expressed in perichondrium has very recently been suggested to serve as an endogenous ligand for FGF-Rs expressed by growth plate chondrocytes, but no data on FGF-18 distribution were provided (43, 44). Third, chondrocyte proliferation is controlled not only by syndecans and FGF-Rs, but also a multiplicity of pathways as indicated in the Introduction. As an example of the complexity of these issues and current uncertainties, it is worth mentioning that in the transgenic mice expressing the constitutively active FGF-R3 cited above, chondrocyte proliferation was found to be inhibited only postnatally but not in the embryo (15). In addition, a different constitutively active mutant of FGF-R3 used in another study was found to have no obvious consequence on chondrocyte proliferation (45), and syndecan-3 null mice were described as largely normal (46). Thus, more work will be needed to define precisely how chondrocyte proliferation in the growth plate is regulated by the concerted action of several receptors, co-receptors, and ligands.

In their biochemical and molecular studies on syndecan-3 core protein-core protein interactions, Carey and coworkers (17, 32) suggested that glycine residues within the transmembrane domain and 4 charged residues (ERKE) in the juxtamembrane segment together mediate the dimerization process. The glycine residues would favor an extended conformation and formation of a double-helical structure between two contiguous transmembrane domains. This interaction would be stabilized by electrostatic interactions between juxtamembrane-charged residues on one polypeptide with those in the contiguous molecule. Although quite attractive, this model may...
not account entirely for the dimerization/oligomerization process. For example, syndecan ectodomains shed from cultured cells are free of transmembrane domain, but are still able to dimerize as indicated by electrophoretic migration (16). In addition, *Drosophila* syndecan lacks basic juxtamembrane residues but still forms multimeric structures (47). Our molecular modeling data, then, offer an additional mechanism by which syndecan-3 could self-associate. The stalk domain of the core protein exhibits several β-strand segments, which would require interactions for stabilization; thus, this domain is likely to promote intermolecular interactions among β-strands and subsequent interactions among globular domains. The calculated thermodynamic stability of the dimer is high, indicating a low free energy. In constructing the dimer model, monomer models were first energy minimized, and these monomers were juxtaposed to align the β sheet stalks and the transmembrane helices, and the dimer model was further minimized. The dimer model rapidly snapped into a stable conformation, with a decrease in overall free energy of 100-fold in the first 300 iterations. To ascertain whether the dimer model had enhanced stability over the separated monomers, we continued energy minimization for a total of 1600 iterations, and extrapolated the asymptotic curves of free energy *versus* iteration to their plateau values. This analysis showed a small (~1000 cal/dimer), but persistent decrease in free energy for the dimer as compared with that seen for the monomers alone. Taken together, the large and rapid decrease in free energy observed as the monomers reached a stable conformation, and the increased stability of the dimer as compared with the monomer suggest that the dimer model is a reasonable one for this molecule. Our cross-linking data indicate the presence of oligomeric syndecan-3 structures in addition to dimers. We have made no attempt to envision oligomeric structures because our dimer model awaits experimental validation. In addition, we have not taken into consideration the possible roles of heparan sulfate chains, which are able of self-association (48) and are thus likely to participate in initiation, promotion, and/or stabilization of syndecan dimers/oligomers. Despite these limitations, our modeling, cross-linking, and antibody interference data all point to the possibility that syndecan-3 molecules form supramolecular structures on the surface of the chondrocyte. These structures would be part of mechanisms whereby syndecan-3 exerts its co-receptor function and regulates the selective responses of chondrocytes to growth factors.

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