Antisense Inhibition of Syndecan-3 Expression during Skeletal Muscle Differentiation Accelerates Myogenesis through a Basic Fibroblast Growth Factor-dependent Mechanism*

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Syndecan-3 is a member of a family of transmembrane proteoglycans that possess highly homologous cytoplasmic and transmembrane domains and function as extracellular matrix receptors and low-affinity receptors for signaling molecules such as basic fibroblast growth factor (FGF-2). Syndecan-3 is transiently expressed in developing limb bud and absent in adult skeletal muscle. In this study we investigated the expression of syndecan-3 and its role on FGF-2-dependent inhibition of myogenesis. Syndecan-3 expression was down-regulated during skeletal muscle differentiation of C2C12 myoblasts, as determined by Northern blot analyses and immunoprecipitation. To probe the function of syndecan-3 during myogenesis, C2C12 myoblasts were stably transfected with a plasmid coding for antisense syndecan-3 mRNA. The resulting inhibition of syndecan-3 expression caused accelerated skeletal muscle differentiation, as determined by expression of creatine kinase and myosin and myoblast fusion. Expression of a master transcription factor for muscle differentiation, myogenin, was also accelerated in antisense syndecan-3-transfected myoblasts compared with control transfected and wild type cells. Reduced expression of syndecan-3 resulted in a 13-fold decrease in sensitivity to FGF-2-dependent inhibition of myogenin expression. Addition of heparin partially reversed this effect. These results demonstrate that syndecan-3 expression is down-regulated during differentiation and the level of expression of membrane-bound heparan sulfate on myoblast surface is critical for fine modulation of responsiveness to FGF-2. These findings strongly suggest a role for syndecan-3 in regulation of skeletal muscle terminal differentiation.

Skeletal muscle myoblasts are the precursors to skeletal muscle fibers. During development these cells are maintained in a proliferative, undifferentiated state until appropriate signals cause the cells to undergo conversion to multinucleated myotubes. The differentiation of myoblasts is also controlled in a negative manner by specific mitogens, such as basic fibroblast growth factor (FGF-2), hepatocyte growth factor/scatter factor, and transforming growth factor type β (TGF-β1–4). In the presence of these growth factors, myoblasts continue to proliferate and fail to fuse to myotubes or to express muscle-specific gene products. Although skeletal muscle cells are terminally differentiated, a small number of cells escape the differentiation process. These cells, termed satellite cells, persist in differentiated muscle tissue, where they lie between the muscle fiber and the basal lamina. Skeletal muscle cell differentiation is controlled at the transcriptional level by a network of muscle-specific regulatory factors (6–8). One of the factors responsible for the induction of terminal differentiation is myogenin, a member of the basic helix loop helix family, which activates transcription of skeletal muscle-specific genes, such as creatine kinase, myosin heavy chain, and acetylcholine receptor (9). Inhibitory growth factors exert their effects on muscle differentiation, at least in part, by inhibiting myogenin expression.

It has been demonstrated that the activities of FGF-2, hepatocyte growth factor/scatter factor, and TGF-β can be regulated by binding to proteoglycans (10–12). Cell surface heparan sulfate proteoglycans, in particular, have been suggested to play a role in modulating the activities of heparin-binding growth factors. They may do this by providing for storage of the growth factors in the extracellular compartment (13, 14) and by serving as obligatory receptors for growth factor receptor kinase activation. A model that has been proposed to explain the latter activity of heparan sulfate proteoglycans is that the growth factor, heparan sulfate proteoglycan, and receptor kinase interact to form an active ternary complex (15). An alternative proposal is that binding to heparan sulfate proteoglycans induces oligomerization of the growth factor, which promotes receptor dimerization and activation (16).

The syndecans are a gene family of transmembrane heparan sulfate proteoglycans that are thought to play a role in tissue morphogenesis and differentiation (13). Four syndecan core proteins that are the products of different genes are synthesized in cell type and development-specific patterns (14, 17). We have shown that expression of syndecan-1 is down-regulated during skeletal muscle differentiation and that its expression is regulated by FGF-2, TGF-β, and retinoic acid (18). Constitutive expression of syndecan-1 in myoblasts that are induced to differentiate results in a diminished capacity of the

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myoblasts to differentiate and a 7-fold increase in sensitivity to FGF-2-dependent inhibition of myogenesis (12).

Another potential modulator of growth factor activity is syndecan-3. In contrast to what has been reported for syndecan-1, syndecan-3 purified from newborn brain binds poorly to most extracellular matrix proteins, including fibronectin, laminin, and collagen I and III. However, the proteoglycan does bind with high affinity to FGF-2 (19). A potential role for syndecan-3 in myogenesis is suggested by the fact that it is expressed transiently during limb development, prior to myoblast differentiation to myotubes (20), whereas its expression is absent in adult skeletal muscle (21, 22).

In this study we investigated the expression of syndecan-3 during terminal differentiation of C2C12 cells, a skeletal muscle satellite cell line. The results obtained demonstrated that syndecan-3 expression is down-regulated during muscle differentiation. To investigate the function of syndecan-3 in muscle differentiation, C2C12 myoblasts were transfected with an expression plasmid containing antisense syndecan-3 cDNA. Transfection with antisense syndecan-3 accelerated terminal differentiation and produced a 13-fold decrease in sensitivity to FGF-2-dependent inhibition of myogenesis. These findings demonstrate that the responsiveness of myoblasts to FGF-2, an inhibitor of skeletal muscle differentiation, is directly modulated by syndecan-3 expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—The C2C12 cell line and the plasmid pSVlacZII phage-mid vector (β-galactosidase reporter) were purchased from ATCC. Trizol LS, LipofectAMINE, Dulbecco’s modified Eagle’s medium, chicken embryo extract, horse serum, fetal calf serum, Opti-MEM I, Hanks’ balanced salt solution, and G418 were obtained from Life Technologies, Inc. Wizard plus Maxypreps and Prime-a-Gene labeling system were from Promega, Madison, WI. F-12 medium, heparin, chondroitinase ABC, creatine kinase assay kit, monoclonal anti-myosin, TRITC-conjugated goat anti-rabbit IgG, human hepatocyte growth factor/scatter factor, and fluorescein isothiocyanate-conjugated TRITC-conjugated goat anti-rabbit IgG, human hepatocyte growth factor/scatter factor, and fluorescein isothiocyanate-conjugated anti-mouse IgG were from Sigma. Anti-mouse myogenin was from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Heparitinase and mAb 3G10 mouse IgG were from Sigma. Anti-mouse myogenin was from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Heparitinase and mAb 3G10 mouse IgG were from Sigma.

**Cell Culture**—The mouse skeletal cell line C2C12 was grown as described (18, 23).

**RNA Isolation and Northern Blot Analysis**—Total RNA was isolated from cell cultures using Trizol. RNA samples were electrophoresed through 1.2% agarose/formaldehyde gels, transferred to Nytran membranes, and exposed to Kodak x-ray film. For quantitative determination of the mRNA levels the intensity of the hybridization signals were measured by densitometric scanning (GS 300 Scanning Densitometer, Hoefer Scientific Instruments, San Francisco, CA).

**Proteoglycan Immunoprecipitation and GAG Lyases Assay**—Metabolic labeling of cell cultures with [35S]H2SO4 (24) or [35S]methionine (25) was done for 18 h. The cells were extracted with immunoprecipitation buffer (0.5% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.05 M Tris-HCl, pH 7.5) and aliquots of the extracts were incubated overnight at 4°C with affinity purified rabbit anti-syndecan-3 antibodies (18, 22). The immune complexes were harvested by the addition of Protein A–agarose beads followed by centrifugation. The amount of radiolabeled proteoglycan immunoprecipitated was determined by liquid scintillation counting. Some aliquots were analyzed by SDS-PAGE and Sepharose CL-4B. Treatment of the immunoprecipitates with chondroitinase ABC and heparitinase was carried out as described (24).

**Immunofluorescence Microscopy**—Cells to be immunostained were grown on coverslips. The medium was removed and the coverslips were rinsed with phosphate-buffered saline. For staining of myogenin and myosin the cells were rinsed with phosphate-buffered saline and fixed with 3% paraformaldehyde for 30 min at room temperature and permeabilized with 0.05% Triton X-100, and incubated with the primary antibodies. Then the cells were rinsed with Blotto and incubated for 1 h at room temperature with affinity purified TRITC- or fluorescein isothiocyanate-conjugated secondary antibodies diluted in Blotto. After rinsing, the coverslips were mounted and viewed with a Nikon Diaphot inverted microscope equipped for epifluorescence.

**Transfections and Analysis of Myogenin Reporter Activity**—The cells were plated in growth medium 1 day before transfection at a density of 6000 cells/cm2 in 60-mm plates. For transfection the cells were incubated for 6 h in Opti-MEM I containing 5 μg each of the myogenin reporter plasmid pMYOCAT (12, 18) and pSVlacZII phage-mid vector (β-galactosidase reporter) and 15 μg of LipofectAMINE. After transfection the cells were incubated for 14 h in Opti-MEM I containing 10% fetal calf serum and 0.5% chicken embryo extract. The cells were then rinsed twice with Hanks’ balanced salt solution and incubated for 2 days in growth medium. After 3 days the cells were switched to normal growth medium supplemented with G418 (400 μg/ml). After 2–3 weeks viable colonies were subcultured using cloning rings.

**Analysis of Creatine Kinase Activity**—Myoblasts and myoblasts induced to differentiate for the indicated days were washed twice with phosphate-buffered saline and lysed by incubation with phosphate-buffered saline containing 0.1% Triton X-100 for 10 min at 25°C and harvested by scraping. Creatine kinase activity was determined using the creatine phosphokinase assay kit. All data points represent the means of duplicate determinations from two independent experiments.

**RESULTS**

**Myoblasts Synthesize Syndecan-3**—To measure the expression of syndecan-3, total RNA was isolated from C2C12 myoblasts and from neonatal mouse brain, a tissue known to express syndecan-3 (22), and used for Northern blot analysis with a syndecan-3 cDNA probe. As shown in Fig. 1A, C2C12 myoblasts express a 5.6-kilobase syndecan-3 mRNA at a level comparable to that in neonatal brain. For comparison the levels of tubulin mRNA are also shown. Fig. 1B shows results of immunoprecipitation of detergent extracts obtained from [35S]H2SO4-labeled myoblasts using affinity-purified anti-syndecan-3 antibodies (22). The antibodies immunoprecipitated a radiolabeled product that migrated as a high molecular weight smear on SDS-PAGE. This smear was degraded by heparitinase treatment but was insensitive to chondroitinase ABC, demonstrating the presence of heparan sulfate. Fig. 1C shows the results of immunoprecipitation of detergent extracts obtained from myoblasts labeled with [35S]methionine. Incubation with heparitinase caused the disappearance of the high molecular mass smear with concomitant appearance of a band of 120 kDa, corresponding to the core protein of syndecan-3 (22). Fractionation of immunoprecipitated [35S]syndecan-3 on a Sepharose CL-4B column revealed a radiolabeled peak.
with a $K_{av} = 0.33$. Nitrous acid treatment of the immunoprecipitated material caused all the label to elute at the $V_e$ of the column (data not shown). To determine if syndecan-3 is shed from the surface of myoblasts, aliquots of cell extracts and incubation media obtained from $[^{35}S]$H$_2$SO$_4$-labeled myoblasts were immunoprecipitated with anti-syndecan-3 antibodies. As shown in Fig. 2, most of the radiolabeled syndecan-3 was present in the detergent extract. Less than 10% of total $[^{35}S]$syndecan-3 was released from the cell surface into the incubation medium. Together, these results demonstrate that myoblasts synthesize syndecan-3 that contains only heparan sulfate glycosaminoglycan chains and is localized mainly on the myoblast cell surface.

**Syndecan-3 Synthesis Decreases during Myogenes**—To measure the expression of syndecan-3 during myoblast differentiation total RNA was isolated from myoblasts and from myoblasts induced to differentiate for 1, 2, or 4 days and used for Northern blot analysis. As shown in Fig. 3A, there is a dramatic decrease in the steady state level of syndecan-3 mRNA during differentiation. This decrease was evident after 1 day of differentiation and essentially complete by day 4. Fig. 3A also shows the concomitant increase of myogenin mRNA during skeletal muscle differentiation. The synthesis of syndecan-3 during skeletal muscle differentiation was also determined by immunoprecipitation of detergent extracts of cells incubated with $[^{35}S]$H$_2$SO$_4$. As shown in Fig. 3B, the amount of syndecan-3 synthesized by cells that were allowed to differentiate for 4 days decreased approximately 65% compared with the amount synthesized by myoblasts. Together, these results demonstrate that the synthesis of syndecan-3 is significantly down-regulated during skeletal muscle differentiation.

**Stable Transfection of C$_2$C$_12$ Myoblasts with Antisense Syndecan-3 cDNA**—Because syndecan-3 binds FGF-2 we reasoned that down-regulation of syndecan-3 expression would affect FGF-2 activity and, therefore, promote muscle differentiation. In order to examine effects of syndecan-3 expression on muscle differentiation, C$_2$C$_12$ myoblasts were transfected with a selectable expression plasmid containing full-length syndecan-3 cDNA subcloned in the antisense orientation. Transfected clones were selected under growth conditions in the presence of G418. As a control, myoblasts were transfected with the same vector containing no cDNA insert. A total of 13 antisense-transfected G418-resistant clones were isolated. Several of these were chosen for more detailed studies. The clones were examined by Northern blot analysis to measure syndecan-3 mRNA levels. Fig. 4A shows the results of hybridization of a labeled syndecan-3 cDNA probe to total RNA isolated from wild type cells, control transfected cells, and three antisense-transfected clones (AN7, AN8, and AN10). Two of the antisense clones showed a significant decrease in the level of syndecan-3 mRNA compared with wild type cells and control transfected clones. The levels of tubulin mRNA are also shown for comparison of gel loading.

To analyze syndecan-3 synthesis in the stably transfected clones, myoblasts were labeled with $[^{35}S]$H$_2$SO$_4$ and detergent extracts were immunoprecipitated with anti-syndecan-3 antibodies. As shown in Fig. 4B, there was a substantial decrease in the amount of syndecan-3 synthesized by antisense clones 8 and 10 compared with wild type and control transfected cells. Northern blot analysis was carried out to determine the levels of syndecan-1 and glypican mRNAs. As shown in the Fig. 4C, the levels of transcripts coding for these two proteoglycans were not affected by transfection with antisense syndecan-3. The core protein of any proteoglycan that is substituted with heparan sulfate can be identified by staining the heparitinase-digested protein with the mAb 3G10 (28). Fig. 4D shows the presence in wild type cells of bands of $\sim 120, \sim 85$, and a doublet of $\sim 67–64$ kDa, corresponding to syndecan-3, syndecan-1, and glypican, respectively. In contrast, the antisense cells contain very low levels of syndecan-3, but levels of syndecan-1 and glypican similar to that in wild type cells. Therefore, the re-
duced expression of syndecan-3 in the antisense clones was specific.

**Skeletal Muscle Differentiation Is Accelerated in Antisense Syndecan-3-transfected Myoblasts**—The capacity of antisense-transfected clones to differentiate to myotubes was compared with that of wild type myoblasts. Both morphological and muscle-specific biochemical markers were evaluated. Fig. 5 shows the morphology of myoblasts induced to differentiate for 1, 2, and 3 days. Cells from antisense clones AN8 and AN10 produced visible myotubes much earlier that wild type myoblasts. Myotube formation was observed by day 2 of differentiation and was essentially complete by day 3. In contrast, in cultures of wild type cells (Fig. 5) or control transfected cells (data not shown) myotubes appeared only after 3 or 4 days of differentiation.

The expression of muscle-specific creatine kinase and myosin heavy chain was measured in antisense-transfected clones and compared with wild type cells. As shown in Fig. 6A, creatine kinase mRNA levels were much higher at 24 and 48 h of differentiation in both antisense clones than in wild type myoblasts. Creatine kinase activity was also detected earlier in the antisense-transfected cells than in wild type myoblasts (Fig. 6B). Myosin heavy chain expression was determined by indirect immunofluorescence. As shown in the Fig. 7, antisense-transfected cells expressed myosin in as early as 2 days of differentiation. In contrast, in wild type myotubes myosin expression was detected only after 4 days of differentiation. These results demonstrate that myotube formation and expression of skeletal muscle-specific markers are accelerated in antisense-transfected cells compared with wild type cells.

It is well established that induction of skeletal muscle differentiation is dependent on the expression of myogenin. To measure expression of myogenin total RNA was isolated from transfected clones and wild type myoblasts that were induced to differentiate for 24 or 48 h. As shown in Fig. 8A, myogenin mRNA expression was expressed earlier in antisense (AN8 and
AN10) cells that in wild type myoblasts. A similar result was obtained when the activity of a myogenin reporter (pMYOCAT) was determined (Fig. 8B). To unequivocally establish that expression of myogenin was indeed accelerated in antisense-transfected clones, indirect immunocytolocalization of myogenin was performed. As shown in Fig. 9, nuclear myogenin staining was detected in antisense-transfected cells 1 day after initiation of differentiation, whereas myogenin staining in nuclei of wild type myoblasts was detected only after 2 days of differentiation. Together, these results demonstrate that the expression of an early skeletal muscle marker like myogenin and late markers of differentiation such as fusion and myosin and creatine kinase are detected earlier in antisense syndecan-3 clones compared with wild type cells.

Inhibition of Syndecan-3 Expression Decreases the Sensitivity of Myoblasts to FGF-2-dependent Inhibition of Myogenin Expression—To determine whether the accelerated expression of myogenin and other skeletal muscle markers observed in the antisense-transfected cells resulted from a change in FGF-2 signaling activity, we measured the FGF-2-dependent inhibition of myogenin mRNA expression as a function of growth factor concentration. Wild type and antisense-transfected myoblasts (clone AN10) were triggered to differentiate for 30 h in the presence of different concentrations of FGF-2. As shown in the Fig. 10, exposure of the cells to FGF-2 resulted in a significant inhibition of myogenin mRNA expression. However, the antisense-transfected cells showed a marked shift in the dose-response curve, from an IC50 of 0.83 ng/ml for wild type cells to 11.01 ng/ml for AN10 cells (Table I). This shift in responsiveness to FGF-2 is specific, since no change in TGF-β activity was observed (IC50 = 0.04 ng/ml for myogenin expression in control and transfected cells). The levels of FGF-R1 and R-4, as determined by Northern and Western blot analyses, were the same in both antisense-transfected and wild type cells (data not shown). These results demonstrate, therefore, that the decrease in syndecan-3 expression caused by antisense transfection leads to lowered sensitivity to extracellular FGF-2.

Heparin Partially Restores FGF-2-dependent Inhibition of Myogenin Expression in Antisense Syndecan-3 Cells—We have shown previously in chlorate-treated cells that exogenous heparin can substitute for the absent heparan sulfate chains and increase FGF-2-dependent inhibition of myogenin expression (12). As shown in Fig. 11, addition of 1 μg/ml heparin increased FGF-2-dependent inhibition of myogenin expression in the an-

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**Fig. 5.** Myoblasts fusion is accelerated in antisense syndecan-3 clones. Wild type (WT) and antisense-transfected clones (AN8 and AN10) were grown on coverslips. The cells were photographed with phase-contrast optics after 1 (1d), 2 (2d), and 3 days (3d) in differentiation medium. The bar corresponds to 50 μm.

**Fig. 6.** Creatine kinase expression is accelerated in antisense syndecan-3-transfected clones. A, creatine kinase expression in wild type (WT) and antisense syndecan-3-transfected clones (AN8 and AN10) was evaluated by Northern blot analysis. 10 μg of total RNA isolated from myoblasts incubated in differentiation medium for 24 or 48 h was separated by electrophoresis, blotted onto nylon membranes, and hybridized with a 32P-labeled creatine kinase cDNA probe (CK). The transcript size is indicated. The ethidium bromide (EtBr) stained gel is shown in the lower half of the panel and the ribosomal RNAs are indicated. B, creatine kinase activity was quantitated as a function of time of differentiation as described under “Experimental Procedures.” ■, wild type; ▼, AN8; ●, AN10.

**Fig. 7.** Accelerated expression of myosin in antisense syndecan-3-transfected clones. Wild type (WT) and antisense syndecan-3 cells (AN10) were grown in differentiation medium for 2 (2d), 3 (3d), and 4 (4d) days. Cells were fixed and stained with anti-myosin and fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibodies to reveal intracellular myosin staining. The bar corresponds to 25 μm.
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The results presented in this paper demonstrate that the expression of syndecan-3 in wild type cells adds a shift to the right in the dose-response curve, increasing the IC50 almost 5-fold (Table I). These results demonstrate that the level of expression of heparan sulfate proteoglycans on the cell surface is a critical determinant of the biological activity of the growth factor.

DISCUSSION

The results presented in this paper demonstrate that the expression of syndecan-3, an integral membrane heparan sulfate proteoglycan (14, 17), decreases during differentiation of skeletal muscle cells. This conclusion is based on measurements of syndecan-3 mRNA levels and immunoprecipitation of the proteoglycan from cell extracts. The biochemical characteristics of syndecan-3 synthesized by skeletal muscle cells are in good agreement with the ones described for this heparan sulfate proteoglycan from other sources (20, 22); it contains mainly heparan sulfate glycosaminoglycans chains and a core protein of approximately 120 kDa. The down-regulation observed in cultured cells correlates well with the observation that syndecan-3 is present in limb buds prior to muscle differentiation (29) but absent from adult skeletal muscle tissue (22)

The function of syndecan-3 on the myoblast surface and the functional consequences of down-regulation during differentiation are not known. The findings presented here suggest several roles for syndecan-3 and its regulated expression. To investigate the function of syndecan-3 during skeletal muscle differentiation we inhibited the expression of proteoglycan by transfecting myoblasts with antisense cDNA. This produced an acceleration of skeletal muscle differentiation, as assessed by appearance of morphological and biochemical markers of muscle differentiation. It is well known that FGF-2 stimulates proliferation of myoblasts (30) and inhibits skeletal muscle terminal differentiation (1). Inhibition of muscle differentiation by FGF-2 is caused, at least in part, by inhibition of transcription of the gene coding for myogenin, a master regulator of expression of muscle-specific genes. Syndecan-3 binds FGF-2 with high affinity (19) and has been proposed to function as a co-receptor for FGF-2. Analysis of myogenin mRNA expression revealed that inhibition of syndecan-3 expression resulted in a 13-fold decrease in sensitivity to FGF-2-dependent inhibition of myogenin expression. These observations suggest that changes in the steady state level of syndecan-3 can modulate the action of this inhibitory growth factor on skeletal muscle cells.

We have proposed a similar mechanism for syndecan-1, which also appears to function as a co-receptor function for FGF-2 in myoblasts. Constitutive expression of syndecan-1 produces a strong inhibition of skeletal muscle differentiation (12). Maintenance of syndecan-1 expression on the myoblast plasma membrane produced a 7-fold decrease in the IC50 for FGF-2-dependent inhibition of myogenin expression (12). In this paper we show that inhibition of syndecan-3 expression produces a 13-fold increase in the IC50 for FGF-2 inhibitory activity. These results clearly demonstrate a significant modulating effect of syndecans on FGF-2 signaling activity. These results also suggest that more than one syndecan type can modulate FGF-2 activity on the myoblast surface. It is interesting that syndecan-1 and -3 are down-regulated during skeletal muscle differentiation. This phenomenon probably explains why syndecan-1 is not sufficient to overcome the absence of syndecan-3.

We have shown that decreasing the levels of heparan sulfate by chlorate treatment (12) abolishes FGF-2-dependent inhibition of myogenin expression, which is partially reversed by heparin. Here we shown that heparin reverses the effects of antisense syndecan-3 transfection on FGF-2 activity, as indicated by a shift to the left in the dose-response curve to values comparable to those observed for wild type cells. Heparin at the concentration used (1 μg/ml) presumably acts by presenting FGF-2 to the signaling receptors when endogenous syndecan-3 levels are low. In contrast, in wild type cells heparin produced a shift to the right in the dose-response curve, probably by sequestering the growth factor away from the receptor. These observations reflect the fine tuning of FGF-2 activity on the myoblast cell surface by syndecan-1 and -3, with the additional participation of glypican (see below).

The observed effects of antisense syndecan-3 transfection on myoblast differentiation are consistent with an effect of the proteoglycan on FGF-2 signaling. However, because of the inability of syndecans to interact with other polypeptides (14, 17), one cannot exclude additional functions for syndecan-3 in muscle differentiation. Antisense syndecan-3-transfected cells underwent fusion as early as 2 days after inducing differentiation. It has been shown that fusion is independent of myogenin expression (31). One of the molecules that participates in myoblast fusion is N-CAM, which colocalizes with N-cadherin (32, 33).
It has been shown that N-CAM binding activity is inhibited by heparan sulfate (34). Therefore, it is possible that the reduced levels of syndecan-3 might promote myoblast fusion by directly affecting cell-cell adhesion.

As our earlier work has shown, myoblast differentiation produces a concerted pattern of regulated expression of several heparan sulfate proteoglycans (35). Synthesis of soluble heparan sulfate proteoglycans that accumulate in conditioned medium decreases during terminal differentiation (36). In contrast, the synthesis of cell and substratum-associated proteoglycans increases when skeletal muscle cells are induced to differentiate (12). These results are in accordance with a previous report on primary cultures of embryonic chicken skeletal muscle cells (37). Specifically, the expression of syndecan-1 (18) and syndecan-3 decreases during differentiation. In contrast, the synthesis of glypican, a lipid anchored heparan sulfate proteoglycan (38), increase during skeletal muscle differentiation. This proteoglycan accounts for 20% of the total proteoglycans associated with myotube membranes and is the main heparan sulfate proteoglycan associated with the cell surface or the extracellular matrix (24).

### Table I

| Inhibitory activity | IC50 (ng/ml) |
|---------------------|--------------|
| IC50 FGF-2 WT       | 0.83 ± 0.18  |
| IC50 FGF-2 AN10     | 11.01 ± 3.04 |
| IC50 FGF-2 WT + heparin | 3.70 ± 1.09 |
| IC50 FGF-2 AN10 + heparin | 1.93 ± 0.76 |
| IC50 TGF-β WT       | 0.04         |
| IC50 TGF-β AN10     | 0.05         |

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FIG. 11. Dual effect of heparin on FGF-2-mediated inhibition of myogenin expression in control and antisense syndecan-3 cells. Antisense syndecan-3-transfected cells (AN10) and wild type (WT) were incubated for 30 h in differentiation medium containing the indicated concentrations of FGF-2 in the presence (circles) and absence (squares) of 1 μg/ml heparin. RNA was isolated from the cells and 10 μg of total RNA were analyzed by Northern blot with 32P-labeled myogenin and tubulin cDNA probes as described in the legend to Fig. 10. Graphical representations of FGF-2-dependent inhibition of myogenin expression in antisense syndecan-3 cells (A) and wild type cells (B) are shown. The values correspond to the mean and standard deviations of three independent experiments.

ulate that changes might contribute to regulation of myoblast differentiation.

These observations are also interesting in terms of the specificity of action of different heparan sulfate proteoglycans during differentiation. Both syndecans and glypican-1 have been shown to modulate FGF-2 activity (19) (12). Glypican, like syndecan-1 and syndecan-3, are synthesized as a plasma membrane-associated proteoglycans. These proteoglycans differ, however, in their modes of attachment to the plasma membrane. We have demonstrated an endogenous processing mechanism for glypican that occurs during skeletal muscle differentiation. This results in the release of most of this proteoglycan from the membrane, which becomes incorporated into the extracellular matrix (24). We have observed that removal of glypican from the myoblast surface by phosphoinositol-phospholipase C produces an increase in FGF-2-dependent inhibition of myogenin expression, suggesting the sequestration of FGF-2 in an inactive form on the myoblast membrane by glypican. It is also possible that the presence of glypican in the extracellular matrix sequesters growth factors away from the plasma membrane, rendering them unable to interact with transducing receptors. In contrast, as shown in this paper, syndecan-3 is retained on the myoblast surface.

Syndecan-3 is highly expressed in the developing nervous system, including the floor plate during neural tube development and the midbrain, spinal cord, and peripheral nerves during late embryonic and early postnatal development (20, 22, 39, 40). Syndecan-3 is also expressed during limb cartilage differentiation (29). During limb development, syndecan-3 is expressed in the distal mesenchymal cells of the limb bud which are undergoing outgrowth in response to the apical ectodermal ridge (41). Syndecan-3 is transiently expressed during the precartilage condensation of the skeletal elements of the limb and subsequently in differentiating osteoblasts of the periosteeum (42). It has been shown that anti-syndecan-3 antibodies inhibit limb cartilage differentiation in vitro (43). During limb formation, myoblasts migrate from the somites to the limb bud. In the bud the myoblasts proliferate and begin to express myogenin and differentiate (8). It is possible that migrating myoblasts express syndecan-3 in the proliferative stage but down-regulate their expression after arriving in the limb bud. This decrease in syndecan expression could trigger differentiation, as a consequence of a decrease in FGF-2 mediated signaling activity. The cells used in this study are derived from satellite cells isolated from adult mouse skeletal muscle (44). This cell type corresponds to committed myoblasts that proliferate in response to injury or external signals during regeneration (5). The present findings suggest that syndecan-3 functions as a co-receptor of FGF-2 on the satellite myoblast surface.

In summary our results support the idea that syndecan-3 plays an important role during skeletal muscle differentiation by modulating the ability of the differentiation-inhibitory growth factor FGF-2 to interact with its signaling receptor.

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