Syndecan-1 is an integral membrane proteoglycan involved in the interaction of cells with extracellular matrix proteins and growth factors. It is transiently expressed in several condensing mesenchymal tissues after epithelial induction. In this study we evaluated the expression of syndecan-1 during skeletal muscle differentiation. The expression of syndecan-1 as determined by Northern blot analyses and immunofluorescence microscopy is down-regulated during differentiation. The transcriptional activity of a syndecan-1 promoter construct is also down-regulated in differentiating muscle cells. The decrease in syndecan-1 gene expression is not dependent on the presence of E-boxes, binding sites for the MyoD family of transcription factors in the promoter region, or myogenin expression. Deletion of the region containing the E-boxes or treatment of differentiating cells with sodium butyrate, an inhibitor of myogenin expression, had no effect on syndecan-1 expression. Basic fibroblast growth factor and transforming growth factor type β, which are inhibitors of myogenesis, had little effect on syndecan-1 expression. When added together, however, they induced syndecan-1 expression. Retinoic acid, an inducer of myogenensis, inhibited syndecan-1 expression and abolished the effect of the growth factors. These results indicate that syndecan-1 expression is down-regulated during myogenesis and that growth factors and retinoic acid modulate syndecan-1 expression by a mechanism that is independent of myogenin.
expression of gypcanic (26, 27) and perlecan (28) varies during skeletal muscle differentiation of the C_{12} myoblast cell line. Syndecan-1 is expressed transiently during limb development (13) but is absent in adult skeletal muscle (29), making this proteoglycan a good candidate for modulation of bFGF signaling during myoblast differentiation. The presence of E-boxes in the syndecan-1 promoter has led to the suggestion that MyoD or a related protein binds to these sites to cause down-regulation of syndecan-1 expression. The decrease in syndecan-1 expression would attenuate bFGF signaling and promote differentiation of myoblasts (30).

In this study we show that expression of syndecan-1 is down-regulated during skeletal muscle differentiation of C_{12} myoblasts but by a myogenin- and E-box-independent pathway. Syndecan-1 expression is controlled by a proximal region of the promoter which contains putative SP1, NF-

EXPERIMENTAL PROCEDURES

Cell Culture—The mouse skeletal muscle cell line C_{12} (31) was grown in Dulbecco and Braggan et al. (32) 3 days after confluence, the medium was changed to differentiation medium (Dulbecco's modified Eagle's medium supplemented with 5% horse serum). Two days later, 0.1 ml cytosine-

RNA Isolation and Northern Blot Analysis—Total RNA was isolated from cell cultures at the indicated times by guanidium thiocyanate/phenol/chloroform extraction and isopropyl alcohol precipitation using ISOLATE RNAzol B (Cinna/Biotecx Laboratories, Inc., Houston, TX) (34). RNA samples (15 μg) were electrophoresed through 1.2% agarose/formaldehyde gels, transferred to Nylon membranes (Sigma), and hybridized with probes for myogenin, syndecan-1, and creatine kinase. The probes were prepared as follows. For myogenin, a conserved 672-bp fragment of human myogenin cDNA was amplified by PCR (35). For syndecan-1 and creatine kinase a fragment of 529 bp (from 1505 to 2033 in the cDNA) and 486 bp from 389 to 874 in the cDNA), respectively, were amplified by reverse transcriptase PCR from total RNA obtained using the Sequenase kit. A 667-bp upstream fragment that begins 50 bp downstream from the TATA box and contains potential transcription binding sites for SP1 (GC and GT boxes), MyoD (E-boxes) and NF-

Immunofluorescence Microscopy—Cells to be analyzed by immunofluorescence microscopy were grown on glass coverslips, as described previously (27). For cell surface staining the live unfixed cells were incubated in the first antibody solution (1:20 of affinity-purified anti-syndecan-1 (36) in 5% non-fat milk, 0.1M sodium chloride, 0.02 M incubated in the first antibody solution (1:20 of affinity-purified anti-

Immunoblot Analysis—Conditioned medium from approximately 6 × 10^6 cells was concentrated in a Q-Sepharose column (Sigma) (2.5 × 15 cm) equilibrated with 50 mM Tris-HCl, pH 7.4. After application of the conditioned medium the column was washed with the equilibration buffer until the A_{280} of the effluent was 0. Bound proteins were eluted with the same buffer containing 1.0 M NaCl until phenol red was eluted. After dialysis against the equilibration buffer the sample was subjected to high-performance liquid chromatography on a 7.5 × 75-mm column of DEAE (Beckman Sphero gel TSK DEAE-5PW) equilibrated with the same buffer. The bound proteins were eluted with a linear gradient from 0 to 1 M NaCl in the equilibration buffer at a flow rate of 1 ml/min. For immunoblot analysis aliquots were subjected to sodium dodecyl sulfate-gel electrophoresis in 7.5% polyacrylamide gels, electrophoretically transferred to Immobilon membranes (Millipore, Bedford, MA), and stained with affinity-purified rabbit anti-syndecan-1 antibodies (36) and visualized by enhanced chemiluminescence (Pierce).

RESULTS

Myoblasts Synthesize Syndecan-1—To evaluate the expression of syndecan-1 in myoblasts, total RNA was isolated from C_{12} myoblasts and from 10T1/2 fibroblasts and NMuMG epithelial cells, two cell lines known to express syndecan-1. Fig. 1A shows that C_{12} myoblasts express both the major 2.5-kilobase and minor 3.1-kilobase forms of syndecan-1 mRNA at levels comparable to the mouse epithelial cell line 29. Fig. 1B shows Western blot analyses of conditioned medium from myoblasts. The proteoglycans were eluted from a DEAE column at different NaCl concentrations (lane 2, 0.85 and lane 3, 1.0 M NaCl) and stained with affinity-purified anti-syndecan-1 antibodies (38). The antibodies recognized a high molecular weight smear in the conditioned medium of myoblasts, which was eluted from the DEAE column at high salt concentration, consistent with staining of a proteoglycan. For comparison, lane 1 shows an immunoblot of syndecan-1 present in conditioned medium of Schwann cells transfected with syndecan-1 cDNA (36). Together, these results indicate that myoblasts synthesize and release syndecan-1.

Syndecan-1 Expression Decreases during Myogenesis—To evaluate the expression of syndecan-1 during myoblast differentiation total RNA was isolated from myoblasts and from myoblasts induced to differentiate for 2 and 5 days and evaluated by Northern blot analysis. Fig. 2 shows a significant
syndecan-1. Essentially no staining was observed after 5 days of differentiation (myotubes) (panel E). Together, these results clearly demonstrate that the synthesis of syndecan-1 during differentiation of C2C12 skeletal muscle cells is significantly diminished.

To characterize the mechanism underlying this down-regulation syndecan-1 transcriptional activity was measured in transient transfection experiments using a reporter vector consisting of 667 bp of the rat syndecan-1 promoter linked to a CAT reporter gene (p-667CAT). This promoter region contains a putative TATA box sequence and consensus binding sites for several transcription factors, including two E-boxes (414 and 289 bp upstream of TATA box). Fig. 4 shows the CAT activity in myoblasts transiently transfected with p-667CAT and induced to differentiate. The transcriptional activity decreases significantly in differentiating cells so that by 3 days the activity is essentially abolished. The inset in Fig. 4 shows transcriptional activity obtained with an expression construct that contains a myogenin promoter fused to a CAT reporter gene. As expected, and in contrast to syndecan-1 promoter activity, transcription driven by the myogenin promoter increases significantly after differentiation is triggered. These results demonstrate that the decrease in syndecan-1 expression which occurs during skeletal muscle differentiation results from a decreased rate of transcription.

The Decrease in Syndecan-1 Expression during Myogenesis Is Myogenin- and E-box-independent—The syndecan-1 promoter contains several E-boxes, which are DNA binding sites for MyoD and related proteins, including myogenin. Upon induc-
Syndecan-1 Expression during Skeletal Muscle Differentiation

The function of syndecan-1 in myoblasts and the consequences of syndecan-1 down-regulation during differentiation are not known. However, several roles for syndecan-1 and its downstream factors during muscle development are suggested. These roles include a mediator of muscle differentiation and a promoter of muscle differentiation. Together, these results suggest that RA inhibits syndecan-1 expression and abolishes the stimulatory effect of bFGF and TGF-β on the expression of syndecan-1.

RA Inhibits Syndecan-1 Expression—RA, a vitamin A metabolite, plays a major role in skeletal muscle development (41) and regulates the expression of syndecan-1 during differentiation. As shown in Fig. 7A, the presence of RA strongly inhibited the transcriptional activity of p-244CAT. Maximal inhibitory activity was observed at 10⁻⁶ M RA. Interestingly, as shown in Fig. 7B, the synergistic stimulatory effect observed for bFGF and TGF-β on syndecan-1 expression was totally abolished by RA. RA treatment also blocked the increase in syndecan-1 transcriptional activity caused by 10% FCS (Fig. 7B). These results indicate that RA, an inducer of skeletal muscle differentiation, inhibits the expression of syndecan-1 and abolishes the stimulatory effect of bFGF and TGF-β on the expression of syndecan-1.

DISCUSSION

The results presented in this paper demonstrate that the expression of syndecan-1, a transmembrane heparan sulfate proteoglycan (1), decreases during differentiation of skeletal muscle cells. This conclusion is based on analysis of syndecan-1 mRNA levels, immunofluorescent staining of cells, and the activity of a reporter construct containing a portion of the rat syndecan-1 promoter. These observations are potentially important in the context of the specificity of action of different heparan sulfate proteoglycans. It has been shown that addition of perlecan but not soluble syndecans or glypican restores bFGF signaling and biological activity to heparan sulfate-deficient fibroblasts (25). On the other hand, recombinant membrane-associated syndecans or glypican have been shown to promote bFGF signaling in a hematopoietic cell line that expresses low levels of heparan sulfate proteoglycans (42). It can be speculated that down-regulation of syndecan-1 expression, together with the bFGF receptor down-regulation (43), makes the cells refractory to the presence of bFGF and therefore allows differentiation of skeletal muscle (44).
Syndecan-1 expression and inhibition of endogenous syndecan-1 expression in myoblasts are in progress.

Another potential role for syndecan-1 on the surface of myoblasts is the binding of extracellular matrix adhesive proteins. Syndecan-1 has been shown to bind several extracellular matrix adhesive molecules, including fibronectin (4); thrombospondin (5); tenascin (6); and collagen types I, III, and V (7). Cell adhesion to these matrix proteins might not be required after differentiation when individual muscle fibers are in direct contact with basement membrane extracellular matrix. Furthermore, it has been shown that syndecan-1 can influence cell invasion (45). Myoblasts are able to migrate through basal lamina during early stages of differentiation (46). It is tempting to speculate that the presence of syndecan-1 on the surface of myoblasts may influence their migratory pathway to give rise to slow or fast primary myotubes (47).

Syndecan-1 expression in myoblasts appears to be regulated at the level of transcription. It has been suggested that down-regulation of syndecan-1 expression during muscle differentiation could result from the presence of E-boxes, target sites for the action of myogenic regulators such as MyoD and myogenin (39), in the syndecan-1 promoter (30). We tested this by transiently transfecting myoblasts with CAT reporter vectors containing the rat syndecan-1 promoter that contained or lacked E-boxes. Our results clearly demonstrate that the presence of the E-boxes is not required for the decrease in expression of syndecan-1 which is observed after differentiation is triggered. These results are supported by the finding that treatment of differentiating myoblasts with sodium butyrate, an agent known to inhibit myogenin expression (40, 48), had no effect on the pattern of syndecan-1 expression. Furthermore, treatment of the myoblasts with bFGF or TGF-β strongly inhibited myogenin expression (18, 19, and data not shown), without affecting syndecan-1 expression. Together, these results suggest that syndecan-1 expression in myoblasts is not directly regulated by myogenin.

In this study we also found that promoter activity was not significantly affected by bFGF or TGF-β. However, exposure to both growth factors resulted in a significant increase in syndecan-1 gene activity. These results are similar to the finding of Elenius et al. (33) on syndecan-1 expression in fibroblasts. On the other hand, treatment of the cells with RA, an inducer of skeletal muscle differentiation (21), inhibited the activity of the syndecan-1 reporter. Furthermore, RA was able to abolish the stimulatory effect of bFGF and TGF-β as well as FCS. This is particularly interesting because both growth factors and RA are known to be expressed in the vicinity of condensing mesenchymal cells (49, 50) and limb buds during early developmental stages (41).

These results indicate that the main regulatory elements responsible for the transcriptional regulation of syndecan-1 expression during myoblasts differentiation are contained in the proximal 277-bp segment of the syndecan-1 promoter. This region contains a putative TATA box sequence and consensus binding sites for several transcription factors such as SP1 and...
The inhibitory effect of RA is interesting not only as an explanation for the decrease of syndecan-1 expression during myoblasts differentiation, but also because suppression of syndecan-1 expression has been shown to be associated with malignant conversion (57). Tumor necrosis factor-α is the only other factor described which restricts the expression of syndecan-1 (58). The inhibitory effect of RA on syndecan-1 expression could be explained by the presence of putative RA response elements in the promoter region. This sequence is sufficient to cause the inhibitory effect of RA on the murine Oct4 promoter (59). The syndecan-1 promoter region contains sequences that are similar, but not identical to RA response elements. These are located 55, 80, and 100 bp upstream of the TATA box and are included in the p-244CAT reporter.

The expression of perlecan during myogenesis is also down-regulated (28). As indicated by Vihinen et al. (51), the promoter region of perlecan (60) resembles that of mouse syndecan-1. The upstream regions of these genes lack canonical TATA and CAAT boxes, but several SP1 transcription factor binding sites are present within the first 200 bp of the promoter. These observations suggest that similar regulatory mechanisms are involved in the regulation of expression of these cell surface macromolecules.

In summary, we have found that the expression of syndecan-1 is down-regulated during skeletal muscle differentiation. This phenomenon is modulated by growth factors and RA but is E-box- and myogenin-independent. The regulatory sequences responsible for this modulation are contained within a 277-bp segment of the gene which contains consensus binding sites for several transcription factors such as SP1, NF-κB, and RA-binding proteins. These results will help toward understanding the complex regulation during development and differentiation of this key integral membrane heparan sulfate proteoglycan.

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