A Novel Modulatory Mechanism of Transforming Growth Factor-β Signaling through Decorin and LRP-1*

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Transforming growth factor-β (TGF-β) is a multifunctional cytokine that signals to the nucleus through cell surface transmembrane receptors with serine/threonine kinase activity and cytoplasmic effectors, including Smad proteins. Here we describe two novel modulators of this pathway, lipoprotein-receptor related protein (LRP-1) and decorin. Decorin null (Dcn null) myoblasts showed a diminished TGF-β response that is restored by decorin re-expression. Importantly, this reactivation occurs without changes in the binding to TGF-β receptors, Smad protein phosphorylation, or Smad-4 nuclear translocation. In wild type myoblasts, inhibition of decorin binding to LRP-1 and deletion of LRP-1 inhibited TGF-β response to levels similar to those observed in Dcn null myoblasts. Re-expression of decorin in Dcn null myoblasts cannot restore TGF-β response if the Smad pathway or phosphatidylinositol 3-kinase activity is inhibited, suggesting that this LRP-1-decorin modulatory pathway requires activation of the Smad pathway by TGF-β and involves phosphatidylinositol 3-kinase activity. This work unveils a new regulatory mechanism for TGF-β signaling by decorin and LRP-1.

Transforming growth factor-β (TGF-β) is an essential regulator during development, cell proliferation, and extracellular matrix (ECM) deposition (1). This growth factor, which belongs to a superfamily of structurally related proteins that comprises activins, inhibins, and bone morphogenetic proteins, presents three very similar mammalian isoforms, TGF-β1, TGF-β2, and TGF-β3 (2, 3). TGF-β regulates cellular processes by binding to high affinity cell surface receptors: TGF-β-R1, TGF-β-RII, and TGF-β-RIII (also known as betaglycan). A general mechanism for TGF-β signaling has been established in which TGF-β binds to type III receptors and is presented to type II receptors or binds to type II receptors directly (4). Once activated by TGF-β, type II receptors recruit, bind, and transphosphorylate type I receptors, thereby stimulating their protein kinase activity. Active type I receptor kinases then phosphorylate Smad-2 or -3, allowing their association with Smad-4. Smad complexes translocate to the cell nucleus and mediate the transcriptional regulation of TGF-β target genes. Smad-7, on the other hand, is a strong inhibitor of this pathway (5, 6). Besides Smad-mediated transcription, TGF-β activates other signaling cascades, including MAPK pathways. Some of these pathways regulate Smad activation, but others might induce non-transcriptional responses (7). TGF-β can activate the ERK (8), JNK (9), p38 MAPK kinase (10), and phosphatidyl-inositol-3-kinase (PI3K)-mediated pathways (9, 11).

Decorin is one of the most studied members of the family of small leucine-rich proteoglycans (12). Its core protein, which constitutes up to 80% of the protein moiety, is composed of 12 repeats of a 24-amino-acid-residue domain (leucine-rich repeats). In addition, decorin carries a single glycosaminoglycan chain at its NH₂ terminus. Decorin is able to interact with several growth factors and plasma membrane-located receptors. For instance, it is well known that decorin binds to the insulin-like growth factor-I (13) and interacts with tumor necrosis factor-α (14). Decorin is also known to cause rapid phosphorylation of the epidermal growth factor receptor and activation of the mitogen-activated protein kinase signaling pathway (15). Decorin also modulates TGF-β activity (16, 17). Several different functions, based in these interactions, have been established for decorin. Cells that do not express decorin show a decreased cell responsiveness to TGF-β, suggesting that decorin is required to activate the TGF-β signaling pathways (18). Interestingly, proteoglycans are modulators of the biological activity of several growth factors (19, 20), and its presence either in the ECM or in the plasma membrane seems to be critical for this modulation (19). Although decorin has been normally considered as an ECM proteoglycan, it has been shown to be able to associate to the plasma membrane through a specific decorin endocytic receptor in a variety of eukaryotic...
LRP-1-decorin Are Required for TGF-β Signaling

We have recently demonstrated that decorin binds and that it is endocytosed by the low density lipoprotein receptor-related protein (LRP-1) (23). LRP-1 is a giant receptor that binds, internalizes, and mediates the degradation of several ligands (24). The folding process of the receptor in the endoplasmic reticulum requires the participation of a 39-kDa receptor chaperone, the receptor-associated protein (RAP) (25). This receptor-related protein competes efficiently with the binding of decorin to the cell surface (23). To study the possible role of LRP-1-decorin in TGF-β signaling, we decided to study its role in myoblasts since it has been characterized with regard to TGF-β response (18, 26, 27).

Here we show that decorin and LRP-1 modulate TGF-β responses. This novel function for LRP-1 and decorin requires activation of the Smad pathway and is affected by PI3K inhibitors. This work unveils a new regulatory role for decorin and LRP-1 in TGF-β signaling.

EXPERIMENTAL PROCEDURES

Cell Culture—The mouse skeletal muscle cell line C2C12 (ATCC) (28) was grown and induced to differentiate as described (29). Decorin null (Dcn null) myoblasts have been previously described and characterized (18).

Adenoviral Infection—Myoblasts were plated at a density of 10,000 cells/cm² in 6-well plates. After 4 h, myoblasts were infected with 500 plaque-forming units/cell of an adenovirus containing the human sequence of decorin (Adv-Dcn) (18) in Dulbecco’s modified Eagle’s minimal essential medium containing 2% heat-inactivated fetal bovine serum (FBS). After 90 min of incubation, standard medium (Dulbecco’s modified Eagle’s minimal essential medium, 10% FBS) was added, and incubation was continued for an additional 24 h. Transfection assays were performed in fresh growth medium.

Transient Plasmid Transfection—The following TGF-β responsive constructs were used: p3TP-Lux (30), pCTGF-Luc (kindly donated by Dr. A. Leask, Royal Free and University College Medical School, London, UK), p4X-SBE-luc (31), and pMyo-Luc (18). pAct-EF Smad-7-Myc (kindly donated by Dr. K. Okazaki, Department of Molecular Biology, Biomolecular Engineering Research Institute, Furuuedai, Suita, Osaka, Japan) and pDN-TGF-βRII-HA (kindly donated by Dr. J. Massagué, Sloan Kettering, Howard Hughes Medical Institute, New York, NY) were used to overexpress Smad-7-Myc and a negative dominant mutant of TGF-β receptor II (DN-TGF-βRII-HA) respectively. Briefly, cells were plated in 24-well plates until they reached 60% confluence. Cells were then incubated in Opti-MEM containing 1 μg of each plasmid, 0.02 μg of pRL-SV40, 2 μl of PLUS reagent, and 1 μl of Lipofectamine. After 6 h, FBS was added to the medium, and the cells were cultured for a further 12 h. Medium was changed to fresh growth medium, and the following reagents were added: TGF-β1 dissolved in 0.5% FBS; GST or GST-RAP in phosphate-buffered saline (23), or inhibitors of p38MAPK activity (SB203580, from Pierce), TGF-β receptor I kinase activity

FIGURE 1. Dcn null myoblasts are less responsive to TGF-β, whereas re-expression of decorin recovers its sensitivity to TGF-β. Wild type (WT), Dcn null, and Dcn null myoblasts infected with Adv-Dcn were transiently transfected with plasmids containing sequence for p3TP-lux (A), pCTGF-luc (B), and p4X-SBE-luc (C), in all the cases together with a plasmid containing pRL-SV40 sequence. TGF-β1 (1.0 ng/ml for p3TP-lux and p4X-SBE-luc, or 5.0 ng/ml for pCTGF-luc) was added, and after 24 h, the cells were processed, and luciferase activities were determined. Wild type values correspond to 100%. The values for p3TP-lux and p4XSBE-luc, or 5.0 ng/ml for pCTGF-luc) was added, and after 24 h, the cells were

JUNE 29, 2007 • VOLUME 282 • NUMBER 26
LRP-1-decorin Are Required for TGF-β Signaling

A

| WT          | Dcn null |
|-------------|----------|
| TGF-β1 (ng/mL) |          |
| 0.1          | 0.048    |
| 0.3          | 0.052    |
| 1.0          | 0.060    |

Phospho-Smad-2

GAPDH

B

| WT       | Dcn null |
|----------|----------|
| TGF-β1 (ng/mL) | |
| 0        | 0.1      |
| 0.3      | 0.3      |
| 0.5      | 0.5      |
| 1.0      | 1.0      |

Phospho-Smad-2

GAPDH

C

| WT       | Dcn null |
|----------|----------|
| TGF-β1 (ng/mL) |     |
| -         | +        |
| -         | -        |
| -         | -        |
| -         | +        |
| -         | +        |

Phospho-Smad-2

GAPDH

FIGURE 2. Smad-2 phosphorylation and Smad-4 translocation to the nucleus is not affected in Dcn null myoblasts. A, Dcn null myoblasts were incubated with the indicated amounts of TGF-β1 for 30 min. Cells were lysed and immunoblotted for phospho-Smad-2 and GADPH. Standard molecular masses are indicated. WT, wild type. B, wild type and Dcn null myoblasts were incubated with the indicated amount of TGF-β1 for 60 min and processed for indirect immunofluorescence for Smad-4. The bar corresponds to 10 μm. C, wild type and Dcn null myoblasts were or were not infected with Adv-Dcn and treated or untreated with TGF-β1 (1.0 ng/ml) for 30 min. Cells were lysed and immunoblotted for phospho-Smad-2 or GADPH. Molecular mass standards are shown on the left.
described (23). Briefly, for transfection, myoblasts were seeded into six-well plates until they reached 70% confluence. Subsequently, cells were incubated during 6 h in Opti-MEM containing siRNA for LRP-1 or control siRNA plus Lipofectamine 2000 (Invitrogen). For experiments with p3TP-Lux or pMyo-Luc plasmid reporters, these were co-transfected with siRNA. Following transfection, FBS was added to the medium, and the cells were cultured for a further 24 h. Then, cells were analyzed through immunofluorescence assays of Smad-4 or LRP-1 or harvested for immunoblot analysis of LRP-1, myogenin, or integrin β1.

**Labeling of Cultures**—Cells were infected with Adv-Dcn and incubated for 18 h in sulfate- and serum-free Dulbecco’s modified Eagle’s minimal essential medium/F12 with 100 μCi/ml H$_{2}^{35}$SO$_{4}$ (25 mCi/ml, PerkinElmer Life Sciences) (18). These conditioned media were concentrated on a DEAE-Sephacel column pre-equilibrated in 10 mM Tris-HCl, pH 7.4, 0.2 M NaCl, and 0.1% Triton X-100, and samples were eluted with 1 M NaCl and analyzed by 4–12% SDS-PAGE. Gels were dried and exposed to phosphorimaging system.

**Protein Determination**—Proteins were determined in aliquots of cell extracts using the bicinchoninic acid protein assay kit (Pierce) using bovine serum albumin as standard.

**RESULTS**

**Decorin Is Required for TGF-β Response without Changes in the Smad-dependent Pathway**—We have previously shown that Dcn null myoblasts present a decreased response to TGF-β when compared with wild type myoblasts, (18). Now, we have investigated this phenomenon, evaluating three TGF-β-inducible reporters: p3TP-lux, which contains the promoter region of plasminogen activator inhibitor (34); pCTGF, a reporter containing the promoter region of CTGF (35); and p4X-SBE-luc, a reporter containing four specific binding sites for Smad proteins (31). The three reporter activities were reduced in response to TGF-β in Dcn null when compared with wild type myoblasts (Fig. 1, A–C). The same effect was observed for the amount of integrin β1, a TGF-β1-inducible protein (Fig. 1D). The decreased responses to TGF-β1 were specific to the absence of decorin

**FIGURE 3.** Decorin-dependent TGF-β signaling requires phosphorylation of Smad-2 or -3 through the TGF-β transducing receptors. Wild type (WT) and Dcn null myoblasts were transfected with plasmids containing sequence for p3TP-lux together with pRL-SV40. A, wild type and Dcn-null myoblasts were incubated with the specific inhibitor for TGF-β-dependent serine/threonine kinase activity of TGF-β-RI, SB431542 (5 μM), or vehicle alone Me$_{2}$SO (DMSO). In the right panel, the inhibitory effect on Smad-2 and -3 phosphorylation by SB431542 is shown by immunoblot for the corresponding Smad proteins and GAPDH in wild type and Dcn null myoblasts. B, the cells were transfected with increasing concentrations of a plasmid containing the sequence for Smad-7 containing a Myc epitope. In the right, the corresponding immunoblots for Myc epitope and tubulin are shown. C, myoblasts were transfected with increasing concentrations of a plasmid containing the sequence for a dominant negative form of TGF-β-RII containing a HA epitope (DN-TGF-β-RII-HA). Immunoblots for HA epitope and tubulin are shown (right). In A–C, the activities in response to TGF-β1 were determined after 24 h. Values correspond to mean ± standard deviation of three independent experiments. Non-transfected wild type and Dcn null myoblasts correspond to 100%. Right, top to bottom, the inhibitory effect on Smad-2 and -3 phosphorylation by SB431542 is shown by immunoblot for the corresponding Smad forms and GAPDH. The absence of recovery of luciferase activity by re-expression of decorin is shown in myoblasts overexpressing Smad-7 or DN-TGF-β-RII-HA. The corresponding immunoblots for Myc epitopes, HA, and tubulin are shown, and the migration positions of molecular mass standards are shown on the left.
LRP-1-decorin Are Required for TGF-β Signaling

since its re-expression, using an adenovirus containing Adv-Dcn, restored the TGF-β-dependent activity to wild type levels (Fig. 1, A–D). The diminished response was observed at different TGF-β1 concentrations (supplemental Fig. 1, A and B). Re-expression of decorin, after the infection with the Adv-Dcn in Dcn null myoblasts, shows this proteoglycan as the main radiolabeled biosynthetic product (supplemental Fig. 1C). These results suggest that decorin is required for part of the response to TGF-β in myoblasts. The proteoglycan biglycan, highly related to decorin, has been shown to be a target of TGF-β (36). Fig. 1E shows the amount of biglycan messenger transcripts in wild type and Dcn null myoblasts in response to TGF-β for 24 h. A diminished response to TGF-β, when compared with wild type myoblasts, was observed in Dcn myoblasts.

The levels of TGF-β-RI and TGF-β-RII, the binding of 125I-TGF-β1 to TGF-β-RI and TGF-β-RII, as well as the amount of Smad-2, -3, -4, and -7 were similar in the Dcn null when compared with wild type myoblasts (supplemental Fig. 2, A and B). The phosphorylation of Smad-2 induced by different amounts of TGF-β1, as well as the kinetic, are normal when compared with wild type cells (Fig. 2A, and see supplemental Fig. 2C). Indirect immunofluorescence analyses showed that the nuclear accumulation of Smad-4 in response to different TGF-β1 concentrations was also similar in both cell types (Fig. 2B). Importantly, the re-expression of decorin in Dcn null myoblasts by infection with Adv-Dcn did not affect the amount of phospho-Smad-2 (Fig. 2C), an experimental situation that totally recovers TGF-β responses in Dcn null myoblasts (Fig. 1, A–D). These results clearly indicate that although Dcn null myoblasts are less responsive to TGF-β, the Smad pathway is unaffected.

Decorin Requires an Active Smad Signaling Pathway to Allow Maximal TGF-β Response—To examine the contribution of the Smad pathway with regard to the role of decorin in the TGF-β response, we used a specific inhibitor of TGF-β-RI kinase activity (SB431542) (Fig. 3A), overexpression of Smad-7, an inhibitor of TGF-β phosphorylation of Smad-2 and -3 (Fig. 3B), or a dominant negative mutant of TGF-β receptor II (DN-TGF-β-RII) (Fig. 3C) in wild type and Dcn null myoblasts. These three approaches, independently, produced a strong decrease in the TGF-β1-dependent p3TP-Lux activity in wild type and Dcn null myoblasts, which was dependent on the expression levels of Smad-7 or DN-TGF-β-RII. Under these treatments, re-expression of decorin in Dcn-null myoblasts did not recover TGF-β-dependent activity (Fig. 3A, left panel, and B and C, right panels). The effect of the SB431542 inhibitor on Smad-2, -3 phosphorylation and the overexpression of Smad-7 and DN-TGF-β-RII are shown (Fig. 3A, right panel, and B and C, left panels). The above results suggest that decorin requires an active Smad pathway to allow maximal TGF-β response.

Decorin Requires LRP-1 to Exert Its Effect on TGF-β Signaling—Decorin is a soluble proteoglycan internalized by different cell types, including endothelial and myogenic cells. One possibility to explain how decorin positively regulates the TGF-β response in myoblasts is through its interaction with a specific cell surface receptor. A candidate for this function is LRP-1, the endocytic receptor for decorin, as we have recently shown (23). To evaluate whether LRP-1 participates in the regulation that decorin exerts in response to TGF-β, we determined TGF-β-dependent p3TP-Lux activity in the presence or absence of RAP, a protein that inhibits the binding and endocytosis of decorin by LRP-1 (23). RAP inhibited about 50% of TGF-β1-dependent p3TP-Lux activity in wild type myoblasts (Fig. 4A) similar to the decrease of TGF-β activity in Dcn null cells. Importantly, the inhibitory effect of RAP depends on decorin because in Dcn null myoblasts, RAP has no effect (Fig. 4A). The inhibitory effect by RAP was reversible (supplemental Fig. 3A). This effect was dependent on the presence of decorin because in Dcn null myoblasts that re-express this proteoglycan, RAP inhibited the induction of p3TP-Lux in response to TGF-β1 (Fig. 4A). RAP did not have any effect on Smad-2 phosphorylation in response to TGF-β1 in wild type and Dcn null myoblasts (Fig. 4B), supporting the idea that Smad-dependent TGF-β signaling is not affected in Dcn null myoblasts. Levels of LRP-1 in wild type and Dcn null myoblasts were equivalent (supplemental Fig. 3B).

To prove the participation of LRP-1 in the regulatory mechanism that decorin exerts in the TGF-β response in myoblasts, we used siRNA for LRP-1. A strong decrease of LRP-1 protein was observed when compared with siRNA control transfected cells (Fig. 5A). The strong decrease of LRP-1 was confirmed by indirect immunofluorescence (supplemental Fig. 3C). To determine whether the absence of LRP-1 has an effect in the response to TGF-β, we evaluated: p3TP-Lux activity; the amount of integrin β1; and the amount of CTGF messenger transcripts in wild type and Dcn null myoblasts transfected with
a siRNA for LRP-1 or siRNA control. The decrease of LRP-1 protein levels diminished these three parameters in wild type myoblasts (Fig. 5, B and C, and supplemental Fig. 4). Interestingly, in Dcn null myoblasts, the absence of LRP-1 did not affect any of the parameters evaluated (Fig. 5B and C, and supplemental Fig. 4). When Dcn null myoblasts re-express decorin, the absence of LRP-1 inhibited p3TP-Lux activity and the amounts of integrin β1 and CTGF messenger transcripts (Fig. 5, B and C, and supplemental Fig. 4). Therefore, decorin did not re-establish maximum levels of TGF-β response in the absence of LRP-1. Finally, the absence of LRP-1 did not have any effect on the nuclear accumulation of Smad-4 in response to TGF-β1 in wild type or Dcn null myoblasts (Fig. 5D). These results suggest that decorin could exert its effect on TGF-β signaling through LRP-1.

**Inhibitors of PI3K Pathway Affect the Decorin-LRP-1 Component of the TGF-β Response**—The above results indicate that part of the response to TGF-β is modulated by the presence of decorin and LRP-1. To study which pathway(s) might be implicated in this decorin-LRP-1 modulatory effect, we used specific inhibitors of PI3K, p38MAPK, MEK1/2, and IGFR-I kinase activities on the response to TGF-β of wild type and Dcn null myoblasts. Only the inhibitor of the PI3K pathway LY294002 affected the decorin-LRP-1 component of the response to TGF-β determined by the p3TP-lux reporter activity (Fig. 6A). Inhibitors of p38MAPK (Fig. 6B) did not affect the p3TP-lux activity, and inhibitors of MEK1/2 or IGFR-I kinase activity did affect the TGF-β-dependent reporter activity in both cell types independent of decorin expression (Fig. 6, C and D). Fig. 7A shows that re-expression of decorin using Adv-Dcn did not revert the effect of two PI3K inhibitors (wortmannin and LY294002) on the TGF-β response. Finally, the absence of LRP-1 did not revert the effect of LY294002 on the TGF-β1 response in wild type or Dcn null myoblasts (Fig. 7B). These results suggest that the PI3K pathway may be involved in the modulatory response to TGF-β mediated by the interaction between LRP-1 and decorin.

**DISCUSSION**

In this study, we have shown that TGF-β signaling depends on decorin and LRP-1. The proteoglycan decorin is a pleiotro-
LRP-1-decorin Are Required for TGF-β Signaling

Figure 6. PI3K inhibitors affect the decorin-LRP-1 component of TGF-β signaling. Wild type (WT) and Dcn null myoblasts were transiently transfected with plasmids containing p3TP-lux and pRL-SV40 reporters and co-incubated with TGF-β1 (1.0 ng/ml) for 24 h in the presence of LY294002 (10 μM) for PI3K activity (A), SB 203580 (20 μM) for p38MAPK activity (B), U0126 (10 μM) for MEK1/2 activity (C), and AG1024 (10 μM) for IGFRI activity (D). The values correspond to mean ± standard deviation of three independent experiments. DMSO, Me2SO.

Figure 7. Inhibition of PI3K pathway affect component of TGF-β when decorin and LRP-1 are present. A, wild type (WT) and Dcn null myoblasts were transiently transfected with plasmids containing p3TP-lux and pRL-SV40 reporters and co-incubated with TGF-β1 (1.0 ng/ml) for 24 h in the presence of wortmannin (1 μM) or LY294002 (10 μM). When corresponding myoblasts were infected with Adv-Dcn. Then all the cells were lysed, and luciferase activity was determined. The values correspond to mean ± standard deviation of three independent experiments. B, wild type myoblasts were transfected with siRNA control or LRP-1 and co-incubated with TGF-β1 (1.0 ng/ml) for 24 h in the presence of LY294002 (10 μM). After treatment, cells were lysed, and luciferase activity was determined. Values correspond to mean ± standard deviation of three independent experiments.

pic molecule that has the ability to interact with several ligands, among them TGF-β (12). It has been shown that as a result of this interaction, decorin can inhibit as well as activate TGF-β-dependent signaling depending on the cell type (12, 37). It inhibits TGF-β-dependent signaling in mesangial cells (38), glialoma cells (39), and differentiated myotubes (30) and stimulates this signaling in osteoblasts (16) and non-differentiated myoblasts (18). Several proteoglycans have a well established role as co-receptors (19, 40). Because decorin is required for TGF-β signaling activity (18) and LRP-1 has the ability to directly bind and subsequently internalize decorin (23), we hypothesized that to participate in TGF-β signaling, decorin interacts with LRP-1. In this study, we present experimental evidence that LRP-1, a receptor located on the myoblast surface, is required for this decorin-dependent TGF-β signaling. The role of decorin in this respect was evaluated in myoblasts that do not synthesize decorin, determining the transcriptional activity of four different promoters that respond to TGF-β; p3TP-Lux, pCTGF-luc, p4xSBE-luc, and pMyo-Luc; and a TGF-β target protein, integrin β1. TGF-β showed a decreased response in Dcn null myoblasts, but it returned to wild type levels when re-expressing decorin using the Adv-Dcn.

In Dcn null myoblasts TGF-β-R1 and TGF-β-RII, the amounts of Smad-2, -3, -4, and -7, the kinetics and titration curve of phosphorylation of Smad-2, together with the nuclear translocation of Smad-4, in response to TGF-β, were unaltered in Dcn null when compared with wild type myoblasts. Importantly, when Dcn null myoblasts were induced to re-express decorin, the amount of phosphorylated Smad-2 remained unaltered. All these results suggest that the Smad-dependent pathway was not affected in Dcn null myoblasts. These results suggest that another component might be regulating decorin-dependent TGF-β signaling. We evaluated the role of LRP-1 using RAP, an inhibitor of decorin binding to LRP-1 and siRNA for LRP-1 (23). RAP and inhibition of LRP-1 expression, using siRNA, inhibited TGF-β-dependent signaling in wild type myoblasts to the levels observed in Dcn null myoblasts, without effect in the remaining TGF-β response observed in this cell type. The inhibition of TGF-β-dependent transcription by RAP or siRNA for LRP-1 in Dcn null myoblasts that re-express decorin was attained at levels comparable with those observed in uninfected Dcn null myoblasts. In all these cases, no effect on the phosphorylation of Smad-2 or nuclear translocation of Smad-4 was detected, confirming that the Smad pathway was not directly involved in the decreased response to TGF-β observed in the absence of decorin. The dependence of TGF-β signaling on decorin and LRP-1 seems to be opposite to the described mechanism of decorin-mediated inhibition of TGF-β signaling with the subsequent phosphorylation of Smad-2 at a key regulatory site and the sequestration of Smad-4 in the nucleus (41).

Although TGF-β, a strong inhibitor of myogenesis (26, 27), is present during myogenesis in development or muscle regener-
ation, these processes occur successfully (42, 43), suggesting that mechanisms to attenuate TGF-β signaling might exist. One of these mechanisms could be LRP-1-dependent; we have determined that the amount of LRP-1 decreases substantially during skeletal muscle differentiation (23).

Decorin-LRP-1-dependent TGF-β signaling does not involve changes in the levels, phosphorylation, or nuclear translocation of several constituents of the Smad-dependent pathway, but our experiments overexpressing the inhibitory Smad-7, the use of a specific inhibitor of the kinase activity of TGF-βRI, or using a dominant negative form of the TGF-βRII strongly suggest that these components are required for this decorin-LRP-1 dependence. Thus, it is plausible to propose that the component of the response mediated by the TGF-β, which depends on decorin and LRP-1, requires the phosphorylation of Smad-2 and -3 and translocation to the nucleus associated with Smad-4.

There is growing evidence that indicates that TGF-β also signals in a Smad-independent fashion (7). Besides Smad-mediated transcription, TGF-β activates other signaling cascades, including MAPK pathways. Some of these pathways regulate Smad activation, but others might induce responses not related to transcription (7). TGF-β can activate the ERK (44), p38 MAPK (45), IGFR-I (13), and PI3K (46) kinase pathways. By the use of specific inhibitors of those kinases, we have found that only inhibitors of PI3K affected TGF-β-dependent signaling in wild type myoblasts without an effect in Dcn null myoblasts, suggesting that the PI3K pathway might be involved in this decorin-LRP-1-dependent TGF-β signaling. Furthermore, re-expression of decorin in Dcn null myoblasts, a situation that recovers TGF-β signaling, was affected by the use of PI3K inhibitors. There are few examples in which PI3K/Akt enhances expression of TGF-β target proteins in response to this growth factor (9, 47, 48). Further studies are required to clarify this point.

LRP-1, through its large ectodomain, which contains four ligand-binding domains, binds (among other proteins) multiple ECM molecules, metalloproteinases, and plasminogen activator (24). LRP-1 has generally been recognized as an endocytic receptor involved in clearance and cellular degradation of ligands. However, LRP-1 also regulates signaling cascades by binding growth factors such as platelet-derived growth factor (49), CTGF (50), and TGF-β (51, 52). Interestingly, several of these molecules also bind decorin (53–55). Type V TGF-β receptor was found to be identical to LRP-1 (56) and mediates its response upon stimulation of insulin-like growth factor-binding protein 3 (IGFBP-3) (56, 57). Many carcinoma cells produce little or no LRP-1 and do not respond to growth inhibition induced by IGFBP-3 or TGF-β. Besides TGF-β (17), decorin binds to the insulin-like growth factor-I (13) and interacts with tumor necrosis factor-α (14). Decorin is also known to cause rapid phosphorylation of the epidermal growth factor receptor and concurrent activation of the mitogen-activated protein kinase signaling pathway (15). Recent studies have shown that decorin binds to the IGFR-I, inducing its phosphorylation and activation, followed by receptor down-regulation (13). The use of a specific inhibitor to this kinase activity did affect TGF-β-induced activity in decorin-independent fashion in myoblasts.

The binding of growth factors to proteoglycans and the consequent modulation of growth factor activities represent an important conceptual advance in the field. In myoblasts, we have shown that the expression of decorin is up-regulated during skeletal muscle differentiation (58), and as shown in this study, it is a requirement for myoblasts to respond to TGF-β through the interaction with LRP-1. On the other hand, we have shown that decorin during skeletal muscle differentiation diminished TGF-β binding to its receptors and signaling (30). This apparent discrepancy can be explained as follows; in myoblasts, most of the decorin interacts with cell receptors, such LRP-1 (23). During skeletal muscle differentiation, the amount of LRP-1 on the cell surface diminished substantially (23), the ECM became more structured, and several proteoglycans (59, 60) including decorin are incorporated, sequestering TGF-β, and as a consequence, decreasing TGF-β binding to cell surface receptors signaling (30). Therefore, the binding of growth factors to proteoglycans and the interaction of these complexes with the plasma membrane or ECM would be critical for its regulatory action. It is provocative to suggest that the requirement of LRP-1 and decorin for TGF-β signaling, as shown in this study, is part of a situation, whereas these traditionally believed ECM molecules and endocytic receptors are involved in intricate signaling mechanisms that might be relevant to many cellular responses and be involved in many diseases.

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