Decorin Interacts with Connective Tissue Growth Factor (CTGF)/CCN2 by LRR12 Inhibiting Its Biological Activity*

Cecilia Vial1, Jaime Gutiérrez2, Cristian Santander, Daniel Cabrera, and Enrique Brandon2
From the Centro de Regulación Celular y Patología, Centro de Regeneración y Envejecimiento, Departamento de Biología Celular y Molecular, MIFAB, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile

Fibrotic disorders are the end point of many chronic diseases in different tissues, where an accumulation of the extracellular matrix occurs, mainly because of the action of the connective tissue growth factor (CTGF/CCN2). Little is known about how this growth factor activity is regulated. We found that decorin null myoblasts are more sensitive to CTGF than wild type myoblasts, as evaluated by the accumulation of fibronectin or collagen III. Decorin added exogenously negatively regulated CTGF blasts, as evaluated by the accumulation of fibronectin or collagen III. Decorin added exogenously negatively regulated CTGF pro-fibrotic activity and the induction of actin stress fibers. Using co-immunoprecipitation and in vitro interaction assays, decorin and CTGF were shown to interact in a saturable manner with a $K_d$ of 4.4 nm. This interaction requires the core protein of decorin. Experiments using the deletion mutant decorin indicated that the leucine-rich repeats (LRR) 10–12 are important for the interaction with CTGF and the negative regulation of the cytokine activity, moreover, a peptide derived from the LRR12 was able to inhibit CTGF-decorin complex formation and CTGF activity. Finally, we showed that CTGF specifically induced the synthesis of decorin, suggesting a mechanism of autoregulation. These results suggest that decorin interacts with CTGF and regulates its biological activity.

Fibrotic disorders are the end point of many chronic diseases in different tissues such as kidney, skin, and skeletal muscle, among others (1–3). This complex biological process includes an inflammatory response and the activation of fibroblasts to myofibroblasts that have a highly contractile phenotype and are the principal source of connective tissue growth factor (21–24). In this way, fibrotic scarring ensues, and the regeneration of muscle is precluded by this fibrotic scar. Both TGF-β and CTGF are known to be overexpressed in this disease (23, 25, 26). Moreover, we have previously shown that myoblasts are able to participate in this fibrotic process because they are able not only to produce CTGF but also to respond to the growth factor by increasing ECM production and actin stress fiber formation (27).

* This work was supported by Research Grants FONDAP-Biomedicine 13980001, CARE PF12/2007, FONDECYT 103193 (to C. V.) and 21050032 (to J. G.), MDA 89419, and Fundación Chilena para Biología Celular Proyecto MF-100.
1 Both authors contributed equally to this work.
2 To whom correspondence should be addressed. Fax: 56-2-635-5395; E-mail: ebrandon@bio.puc.cl.
3 The abbreviations used are: ECM, extracellular matrix; CTGF, connective tissue growth factor; dcn, decorin; LRR, leucine-rich repeat; FN, fibronectin; GAG, glycosaminoglycan; CABC, chondroitinase ABC.

Because 45% of the deaths in developed countries are associated to a type of fibroproliferative illness, finding a way to diminish fibrosis has become an important issue (3). Because CTGF is the principal molecule responsible for fibrosis in many tissues, it would be of great interest to find an inhibitor for its action. A good candidate to regulate CTGF action is decorin, which is a proteoglycan formed by a core protein with 12 leucine-rich repeats (LRRs) and a chondroitin/dermatan sulfate glycosaminoglycan (GAG) chain (28). Decorin is synthesized and secreted to the ECM where it binds and organizes the collagen fibers (29, 30), and it interacts with other ECM proteins such as fibronectin (31). Decorin also interacts with some growth factors and growth factor-transducing receptors, regulating their actions (32, 33). The three isoforms of TGF-β also interact with decorin (34–36), and this interaction reduces fibrosis because of the relocalization of TGF-β to the ECM where it is sequestered by decorin (29, 37). Because CTGF is responsible for TGF-β fibrotic action, decorin could also be regulating this growth factor. In this study, we show the following: (i) decorin inhibits CTGF bio-

© 2011 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
logical activity in myoblasts and fibroblasts, in which a peptide derived from LRR12 is sufficient to regulate CTGF action; (ii) both molecules interact in vitro, and LRR12 is important for this interaction; and (iii) CTGF induces the synthesis of its negative regulator decorin.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The mouse skeletal cell line C2C12 (ATCC), the decorin null myoblasts (dcn null) (38), and the NIH-3T3 fibroblasts (ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with fetal bovine serum (Hyclone/Thermo Fisher Scientific) as described previously (38, 39). Cells were treated with recombinant purified CTGF containing a FLAG epitope (27) for 48 h prior to starvation in DMEM for 18 h. When CTGF was co-incubated with bovine cartilage decorin (Sigma), WT, and decorin deletion mutants or decorin-derived peptides (Peptide 2.0), these molecules were preincubated with CTGF for 30 min at room temperature.

**Western Blotting**—Cells were washed twice with PBS, lysed in lysis buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Triton X-100), and scraped. Cell extracts were electrophoresed through SDS-polyacrylamide gels and electrotransferred onto PVDF filters. Filters were blocked for 1 h at room temperature in Blotto (20 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 5% nonfat dry milk) and incubated with polyclonal anti-fibronectin (Sigma), anti-mouse decorin antibody LF-136 (kindly donated by Dr. L. Fischer, NIDR, National Institutes of Health, Bethesda) at room temperature for 1 h or at 4°C overnight (38, 40). The bound antibodies were visualized by enhanced chemiluminescence (Pierce).

**Adenoviral Infection**—Myoblasts were plated at a density of 20,000 cells/cm² in 55-cm² cell plates. After 6 h, cells were infected with an adenovirus that contained human decorin whole sequence (38) using 500 plaque-forming units/cell in 4 ml of DMEM supplemented with 2% of heat-inactivated fetal bovine serum (38). After 90 min of incubation, standard growing medium was added, and the incubation was continued for 24 h. Cells were incubated with growing factors or radiolabeled with H₂[³⁵S]SO₄ (23).

**Decorin and Deletion Mutants of Decorin**—The wild type decorin and the mutants that lack different LRRs were generated by overlapping PCR starting from human decorin (NM_133593) cloned in pcDNA 3.1 (kindly donated by Elke Schoenherr, Institute of Physiological Chemistry and Pathobiology, Muenster, Germany). All decorins generated had an HA epitope in their C terminus. The constructs generated were sequenced and then stably transfected into CHO-K1 cells using media supplemented with G418 (400 μg/ml). After 4 weeks, different clones were isolated. Decorin purification was performed from conditioned media of the CHO-K1 clones using a DEAE-Sephacel column (Bio-Rad) pre-equilibrated in 10 mM Tris-HCl, pH 7.4, 0.2 M NaCl, and 0.1% Triton X-100. Sequential washes with increasing NaCl concentrations were performed. All the decorins were eluted at 0.6 M NaCl. The purity was evaluated by 4–12% SDS-PAGE and Coomassie Blue staining of samples digested or not with chondroitinase ABC (CABC) or heparitinase (Seikagaku, Tokyo, Japan) (41). Decorin dimerization assay was performed as described previously (30). Briefly, decorin or its deletion mutants lacking LRR10–12 were maintained for 1 h at room temperature in PBS. Dithiobis(succinimidyl) propionate was added at a final concentration of 0.15 mM for an additional 30 min at room temperature. The reaction was stopped with a Tris-HCl, pH 7.4, buffer at a final concentration of 30 mM for 15 min. Loading buffer was added, and the decorin dimerization was evaluated by Western blot using anti-HA antibodies.

**Design and Synthesis of LRR Peptides**—Peptides were derived from the human decorin amino sequence (accession number P07565). LRR4 corresponds to the region between amino acids 142 and 162, LRR5 to the region between 163 and 186, LRR6 to the region between 187 and 212, LRR10 to the region between 282 and 304, LRR11 to the region between 305 and 334, and LRR12 to the region between 335 and 359. All peptides were obtained from Peptide 2.0.

**Iodination of Decorin and Fibronectin**—Carrier-free bovine decorin (R & D Systems, Minneapolis, MN) or fibronectin (Sigma) was radiolabeled using Na[¹²⁵I] and chloramine T as described previously (30, 39).

**Fibronectin Degradation Analysis**—The degradation of fibronectin was evaluated as described with minor modifications (39). Briefly, 20 ng of radioactive [¹²⁵I]-fibronectin (1 × 10⁶ cpm) were added to myoblast pretreated for 6 h with DMEM 1% FBS supplemented with CTGF, decorin, and TGF-β1 (R & D Systems) as indicated in the legend of the corresponding figure. After 42 h, the conditioned media were collected and fractionated by 7.5% SDS-PAGE. The radioactive bands were then visualized using a PerkinElmer Life Sciences phosphorimaging device (Cyclone model).

**CTGF-Decorin Binding Assay**—Recombinant CTGF (27) was immobilized in anti-FLAG M2 affinity gel (Sigma) for 3 h at 4°C; after five washes with PBS, the gel was blocked with 5% BSA in PBS for 1 h. After three washes with PBS, [¹²⁵I]-decorin and decorin deletion mutants with or without the indicated competitors described in the corresponding figures were incubated at room temperature for 3 h. After five washes with PBS, the bound material was eluted with protein loading buffer or was directly counted. Saturation isotherm and Scatchard analysis were performed with GraphPad Prism software, version 5.03, adjusting the curve with nonlinear regression assuming one binding site.

**Co-immunoprecipitation**—In vitro co-immunoprecipitation was performed as described previously (43). Briefly, purified recombinant CTGF was co-incubated with pure decorin or pure decorin core protein for 3 h at room temperature. Then the proteins were immunoprecipitated for 2 h at 4°C using an anti-mouse decorin antibody LF-136 that was previously attached to protein G beads (Pierce/Thermo Fisher Scientific). After washing, proteins were twice eluted in protein loading buffer, electrophoresed, and analyzed by Western blot.

**Immunofluorescence Microscopy**—The cells to be immunostained were grown on coverslips. The medium was removed,
and the coverslips were rinsed with PBS, fixed with 3% paraformaldehyde for 30 min at room temperature, then rinsed with Blotto, and further incubated for 1 h in Blotto. For actin filament staining, cells were incubated with 0.1 μm phalloidin conjugated with FITC (Sigma) for 40 min and rinsed with PBS. For nuclear staining, cells were incubated with 1 μg/ml Hoechst 33258 in PBS for 10 min. After rinsing, the coverslips were mounted and viewed under a Nikon Diaphot microscope, equipped for epifluorescence (27).

RNA Isolation and Reverse Transcription—Total RNA was isolated from cell cultures using TRIzol™ reagent according to the manufacturer’s instructions (Invitrogen).

Semi Quantitative RT-PCR—Reverse transcriptase reaction was performed using Moloney murine leukemia virus reverse transcriptase according to the manufacturer’s instructions (Invitrogen). The primers used in expression experiments for TGF-β1 and fibronectin and the PCR reactions were done as published (39, 44).

RESULTS

Decorin Null Myoblasts Are More Sensitive to CTGF than Wild Type—Many cell processes, including cell differentiation and fibrosis, are regulated by proteoglycans. To study if the proteoglycan decorin could be regulating CTGF, we incubated a C2C12 myoblast or C2C12 myoblast cell line that does not express decorin (38) with different concentrations of CTGF, and the amount of accumulated fibronectin was determined. Fig. 1A shows that dcn null myoblasts presented an increased basal level of fibronectin and an augmented sensitivity to CTGF compared with WT myoblasts. The incubation of dcn null myoblasts with low CTGF concentrations resulted in a strong increase in fibronectin accumulation, whereas at higher concentrations of CTGF, a reduction in fibronectin levels was observed. This reduction was also seen in wild type myoblasts incubated at even higher concentrations of CTGF (data not shown). To analyze if this CTGF effect is specific to decorin absence, we re-expressed decorin in dcn null myoblasts using an adeno virus with the complete human decorin sequence (38). Fig. 1B shows that wild type and dcn null myoblasts behaved as shown above, but when decorin is re-expressed in dcn null myoblasts, these cells behave more like wild type myoblasts, suggesting that dcn null sensitization to CTGF is specific to decorin absence. As a control, Fig. 1C shows decorin levels determined by the autoradiographic analysis of incubation media from wild type, dcn null, and dcn null infected with decorin adenovirus in the presence of H<sub>2</sub>[35S]SO<sub>4</sub>. Altogether, these results show that in the absence of decorin, myoblasts are more responsive to CTGF.

Decorin Inhibits CTGF-mediated Induction of Fibronectin, Collagen III, and Actin Stress Fibers—To analyze the effect of decorin on wild type myoblasts, decorin and CTGF were preincubated for 30 min at room temperature and then added to myoblasts for 48 h. Fig. 2A shows that fibronectin and collagen III levels induced by CTGF were inhibited when decorin is present. Decorin inhibited fibronectin accumulation at 60 and 80% at 1:1 and 1:4 CTGF/decorin molar ratios. Decorin inhibition of collagen III accumulation mediated by CTGF was even more pronounced. Another effect of CTGF on myoblasts is the induction of the formation of actin stress fibers (27). When myoblasts were incubated with CTGF, actin stress fiber formation was induced as reported previously (27), but when these myoblasts were co-incubated with CTGF and decorin, the induction was inhibited (Fig. 2B). To evaluate the mechanism of fibronectin accumulation induced by CTGF, we analyzed if it was due to an inhibition of its degradation and evaluated if the presence of CTGF and decorin affected the degradation of 125I-fibronectin. Fig. 2C shows that CTGF and decorin have no effect on fibronectin degradation. To further analyze the CTGF-mediated fibronectin accumulation mechanism, we analyzed if the presence of CTGF and decorin affected fibronectin expression. Fig. 2D shows that CTGF induces fibronectin mRNA and that this induction is inhibited in the presence of decorin. TGF-β1 was used as a positive control for the induction of fibronectin mRNA. So CTGF induction of fibronectin accumulation is due to an increase in fibronectin expression, because CTGF has no effect on fibronectin degradation. Next, we determined whether the inhibition of CTGF by decorin induced, in a compensatory fashion, the synthesis of another pro-fibrotic factor TGF-β1. Fig. 2D shows that neither CTGF nor decorin have an effect on mRNA levels of TGF-β1. The same figure shows that TGF-β1 slightly induced the expression of TGF-β1, as it has been shown in other systems (45). Because fibroblasts are the major source of the ECM in...
fibrosis and are one of the main cell targets of CTGF, we analyzed if this decorin inhibitory effect on CTGF activity was the property of myoblasts or was a general mechanism involving these other cell types. 3T3 fibroblasts were incubated with CTGF in the presence of increasing concentrations of decorin. Fig. 2E shows CTGF induced accumulation of fibronectin and decorin inhibition of 50 and 80% of fibronectin accumulation at a 1:1 and 1:4 CTGF/decorin molar ratio. Therefore, the decorin inhibition of CTGF could be a general mechanism of CTGF regulation. These results indicate that decorin is able to inhibit CTGF action inhibiting CTGF-mediated fibronectin accumulation and expression and stress fiber formation.

CTGF Activity Is Regulated by the Proteoglycan Decorin

CTGF and Decorin Interact in Vitro—To analyze the mechanism by which decorin is able to inhibit the action of CTGF, we studied if these two molecules were able to interact. We analyzed if CTGF interacted with decorin by binding its core protein or the GAG chains to the cytokine. CTGF was co-incubated with the whole molecule of decorin or with the decorin core and immunoprecipitated with decorin antibodies. Fig. 3A shows the absence of CTGF in both controls without antibody

FIGURE 2. Exogenously added decorin inhibits the fibrotic effect of CTGF on myoblasts and fibroblasts. A, C2C12 myoblasts were serum-starved for 18 h and then incubated with the indicated concentrations of CTGF and decorin (Dcn). B, C2C12 myoblasts were serum-starved and treated as indicated for 6 h, then 20 ng of 125I-FN (1 × 10^6 cpm) were added to each well. After 42 h, the conditioned media were collected, subjected to electrophoresis, and analyzed by phosphorimaging. Medium alone corresponds to a well with culture medium in the absence of cells. E, 3T3 fibroblasts were serum-starved for 18 h and then incubated with the indicated concentrations of CTGF and decorin. When both molecules were added, they were preincubated for 30 min. After 48 h, the FN levels were determined by Western blot. FN/tub corresponds to a quantification of FN levels normalized to tubulin levels of this representative experiment.
CTGF Activity Is Regulated by the Proteoglycan Decorin

or without decorin. It also shows that CTGF immunoprecipitated with either the decorin whole molecule or just the protein core. Therefore, it binds at least to the decorin core protein. To analyze CTGF and decorin interaction and to evaluate their affinity, we immobilized CTGF to beads and incubated it with different concentrations of the 125I-decorin core protein. The specific binding was derived from the subtraction of nonspecific binding to the total binding in each point. The dissociation constant \( K_d \) is 4.4 nM is indicated in the graph. The values correspond to one experiment performed in triplicate. Scatchard analysis of the presented data is shown.

**FIGURE 3. CTGF interacts with decorin core protein.** A, CTGF was incubated with complete decorin or with decorin core protein (Dcn core) and then immunoprecipitated with an anti-decorin (anti-Dcn) antibody. The presence of CTGF on the immunoprecipitate was determined by an anti-FLAG Western blot (WB). B, binding isotherm of increasing concentrations of 125I-decorin to immobilized CTGF. The specific binding was derived from the subtraction of nonspecific binding to the total binding in each point. The dissociation constant \( K_d \) is 4.4 nM is indicated in the graph. The values correspond to one experiment performed in triplicate. Scatchard analysis of the presented data is shown.

CTGF, and the different decorins determining the effects on the amount of fibronectin. Fig. 4C (top panel) shows that fibronectin accumulation by CTGF was inhibited by wild type and all decorin mutants except for the one lacking the LRR10–12. Fig. 4C (bottom panel) shows quantitative analysis of the Western blot indicating that only the decorin mutant lacking LRR10–12 was unable to inhibit the induction of fibronectin in response to CTGF. Because decorin lacking LRR10–12 was the only one that did not show inhibitory activity, we evaluated if this mutant decorin still has the capability to form dimers in solution as wild type decorin (49). Fig. 4D shows that wild type decorin and decorin lacking LRR10–12 were able to form a dimer in solution, indicating that the absence of LRR10–12 would not affect the capability to form dimers. These results suggest that LRR10–12 is important for decorin inhibition of CTGF.

**LRR10–12 of Decorin Is Essential for the Interaction with CTGF**—As shown above, different deletion mutants of decorin had different effects on CTGF action, so we wanted to determine the ability of these deletion mutants to interact with CTGF. We co-incubated the different decorin core proteins with CTGF and immunoprecipitated CTGF with the anti-FLAG antibody. Fig. 5 shows that wild type decorin as well as all the deletion mutants, except the one lacking LRR10–12, were able to interact with CTGF. The figure also shows that the same amount of CTGF was immunoprecipitated in each assay as control. Above the input of the different core proteins used in this assay are shown. To further evaluate if the LRR10–12 was critical to interact with CTGF, the latter was immobilized to beads containing anti-FLAG antibodies and was incubated with 125I-decorin and increasing concentrations of wild type decorin and decorin mutants lacking LRR4–6 and LRR10–12. Fig. 5B shows that decorin lacking LRR10–12 was unable to displace bound 125I-decorin from CTGF. In contrast, wild type and decorin lacking LRR4–6 substantially displaced bound decorin at a decorin/CTGF molar ratio of 2 and 4. These results suggest that LRR10–12 is critical for the interaction between CTGF and decorin.

**Peptide Derived from the Decorin LRR10–12 Region Is Able to Inhibit Decorin-CTGF Interaction and Repress Biological Effects Mediated by CTGF**—To further study the region of decorin responsible for the interaction with CTGF, we used different peptides derived from different regions of decorin, containing LRR4, -5, or -6 as controls and LRR12 that was shown to be important for the interaction and inhibition of CTGF biological activity. Fig. 6A shows the specific amino acid sequence of each peptide utilized. To analyze if any of these peptides affected the interaction of decorin and CTGF, the latter was immobilized to beads containing anti-FLAG antibodies and incubated with decorin and the different peptides, at a molar ratio of 2:1. Fig. 6B shows the eluate-containing decorin, determined by Western blot analysis of the precipitates using anti-HA antibodies. In the absence of peptides, decorin interacts with the immobilized CTGF. Of all the peptides used, only the one derived from the LRR10–12 region, peptide LRR12, was able to inhibit the interaction between decorin and CTGF in about 50%. As a control of the assay, Fig. 6B, bottom panel, shows that the same amount of CTGF was immobilized at the
different conditions and is observed in the eluate with anti-FLAG antibodies. Next, we titrated the amount of LRR12 required to inhibit the interaction between decorin and CTGF.

Fig. 6C (left upper panel) shows that LRR12 inhibits the interaction between CTGF and decorin in a dose-dependent manner. As a control, the effect of LRR4 is shown (left bottom panel). Fig. 6C (right panel) shows a quantitative analysis of this experiment. These results indicate that a molar ratio of LRR12/decorin of around 2 is required to inhibit in 50% the formation of the decorin-CTGF complex. In contrast, a small and rather erratic inhibition of the interaction between CTGF and decorin was observed when LRR4 was used. These results strongly suggest that the LRR12 region of the decorin core protein is involved in the in vitro interaction between decorin and CTGF.

To analyze if the interaction of LRR12 with CTGF had any effect on the biological activity of the growth factor, we incubated C2C12 myoblasts with CTGF and the different peptides. Fig. 7A shows fibronectin and collagen III accumulation by CTGF and how this is not affected when the control peptides were used. However, when the LRR12 was used along with CTGF, it inhibited CTGF action, as observed by the lack of fibronectin and collagen III accumulation in response to CTGF. The figure also shows the values of induction of fibronectin and collagen III over tubulin and the exclusive inhibition exerted by LRR12. To evaluate the actin stress fiber formation induced by CTGF in C2C12 myoblasts, we co-incubated them with CTGF and the different LRR peptides. As seen in Fig. 7B, when CTGF was co-incubated with the control peptides, CTGF induction of actin stress fibers was slightly increased when peptides 4 or 5 were used. However, when LRR12 was used, actin stress fiber formation was strongly inhibited. Finally, we also studied if the peptide could inhibit CTGF biological activity in fibroblasts. Fig. 7C shows the CTGF accumulation of fibronectin and collagen III in 3T3 fibroblasts as seen for myoblasts. Control peptides
CTGF Activity Is Regulated by the Proteoglycan Decorin

A, CTGF (0.4 nm) was preincubated with decorin (Dcn) or its deletion mutants Δ1–5, Δ4–6, Δ5–6, or Δ10–12. The proteins were immunoprecipitated with an anti-FLAG antibody and further analyzed by Western blot (WB) using an anti-HA antibody that recognizes the epitope present in all decorin constructions. Before loading the gel, the samples were treated with CABC to detect only the decorin core protein for a more accurate quantification. The bottom panel shows the levels of the different decorin constructions that were incubated with CTGF as an input control (each input was diluted 1:20 before being loaded onto the gel) and analyzed by Western blot with an anti-HA antibody. A quantification of the bound decorin or deletion mutant normalized to the corresponding input is presented as the percentage of the specific binding of decorin (% bound decorin).

B, 125I-decorin was incubated with immobilized CTGF in the absence or presence of increasing amounts of cold decorin, Δ4–6, or Δ10–12 as competitors at the indicated competitor/125I-decorin molar ratios. The bound 125I-decorin was determined in a gamma counter and presented as the percentage of the binding in the absence of competitors. Results are from one experiment done in triplicate. Values represent mean ± S.E.

had no effect on fibronectin accumulation, and the LRR12 peptide was able to inhibit CTGF action on fibroblasts. To further evaluate peptides present in LRR10–12, myoblasts were incubated with peptides LRR10–12, and the accumulation of fibronectin was determined. Fig. 7D shows that only LRR12 was able to inhibit the CTGF effect in the cells. These results suggest that LRR12 of decorin is necessary to inhibit CTGF action.

CTGF Increases the Amount of Decorin—Because decorin specifically interacts and inhibits CTGF-mediated activity, we evaluated the levels of decorin in myoblasts treated with CTGF. To analyze this, C2C12 myoblasts were incubated with increasing concentrations of CTGF in the presence of H2[35S]SO4. Fig. 8A shows that CTGF induces the synthesis of a proteoglycan that migrated as smear of 120 to 80 kDa. To determine the GAG nature of this proteoglycan, an aliquot of the radioactive medium from the myoblasts was incubated with two GAG lyases, CABC and heparitinase, that specifically degrade chondroitin/dermatan and heparan sulfate, respectively. Fig. 8B shows that most of the radioactive material induced by CTGF corresponds to proteoglycan containing the chondroitin/dermatan sulfate radioactive chain. The molecular weight as well as the GAG nature corresponds with the proteoglycan decorin previously described in the same types of cells (41). Interestingly, a proteoglycan of higher molecular weight also is induced by CTGF, and it contains chondroitin/dermatan GAG chain and likely corresponds with the proteoglycan biglycan (50). To confirm that CTGF specifically induces decorin, aliquots from myoblast incubation media were incubated with CABC followed by Western blot analyses with antibodies that specifically recognize the decorin core protein. Fig. 8C shows that CTGF induces the synthesis of decorin in a dose-dependent manner as determined by Western blot analyses. These results indicate that CTGF is able to induce the synthesis of decorin, which we have shown to be a specific inhibitor of CTGF-mediated biological activity.

DISCUSSION

Our results show that decorin can interact with CTGF and, moreover, can negatively regulate the action of CTGF. We also show that myoblasts that do not express decorin are more sensitive to the growth factor than are wild type ones. Finally, we show that CTGF is able to increase decorin accumulation in incubation media. This last result sheds light on how this could be a mechanism where CTGF autoregulates its own action by increasing the amount of its own inhibitor. In normal healing, CTGF is transiently increased, and once the scar is formed, it is down-regulated (51), but when this down-regulation does not occur, the tissue becomes fibrotic. One of the mechanisms that could be helping to regulate CTGF action so that the tissue does not become fibrotic could be decorin. In our experiments, when decorin null myoblasts were used, as CTGF induces decorin accumulation in wild type myoblasts and in the decorin null myoblasts this is not possible, CTGF cannot negatively autoregulate itself, and in this way these myoblasts were more sensitive to the growth factor. The finding that decorin interacts and inhibits CTGF action is critical because little is known about how CTGF is regulated. Decorin is located in the ECM of a cell (30, 52), so it could sequester the growth factor toward it as other growth factors such as TGF-β1 (29, 30). In this way, CTGF would not be available to interact with the transducing receptors. However, we have shown that decorin is able to bind to the endocytic LDL receptor-related protein receptor type-1 (LRP-1) and is endocytosed and degraded as a consequence of this binding (53). Because CTGF is also able to bind LRP-1 (54), decorin could be regulating this binding and increasing degradation of CTGF by LRP-1. In this way, it
would decrease the time that CTGF is available to exert its biological functions. We determined that the binding of decorin to CTGF was saturable, with a half-saturation occurring at 4.4 nM. As mentioned CTGF is a multiligand protein, and different values of interaction with receptors and growth factors have been described. Thus, it binds VEGF, BMP-7 and -4, and TGF-β with a $K_d$ of 26, 14, 15, and 30 nM, respectively (55–57). Regarding the binding of CTGF to putative cells, cell surface receptors, or molecules that mediate cell interaction, it has been described as interaction with osteoblasts, integrins $\alpha_{IIb}\beta_3$, and fibronectin with a $K_d$ of 17, 15, and 64 nM, respectively (58–60). As mentioned, particularly attractive is the binding of CTGF to LRP-1 with an observed $K_d$ of 0.5–1 nM (54). All these interaction values are generally in the same order of magnitude suggesting that competition between ligands and cell receptors for CTGF must result in variable biological effects, during physiological conditions and under pathogenic conditions. Whichever mechanism decorin is using to negatively regulate CTGF, it seems to be a general issue because the inhibition is seen in at least the two different cell types we used as follows: myoblasts, which have been shown to participate in muscle fibrosis by responding to CTGF (27), and fibroblasts, which are another important cell target for this growth factor and mainly responsible for ECM production in many fibrotic disorders (3, 61).

In this study, we also show that mutant decorins that lack different LRRs are all able to inhibit CTGF except for the one lacking LRR10–12. The models that predict decorin structure show that it has the shape of a horseshoe where the inner part would be formed by $\beta$-sheets composed of the different LRRs (49, 62, 63). The crystal structure of the dimeric protein core of decorin has been elucidated (49). It is suggested that decorin dimerizes through the concave surfaces of the LRR domains, which have been implicated previously in protein-ligand interactions (49). Through the cross-link assay, we demonstrated that decorin and the deletion mutant lacking LRR10–12 are able to dimerize in vitro, indicating that the ability of the deletion mutant lacking LRR10–12 to form a dimer is not lost by the absence of this LRR. If the dimeric or monomeric form of decorin is required to interact and inhibit the CTGF biological activities requires further investigation. It has been shown that the majority of the growth factors, collagen, and transducing receptors, which interact with the decorin core protein, do so through LRR4–8 (36, 64, 65). Therefore, the finding that the important region for inhibition of CTGF is not located within this region would help develop therapies that will not
interfere with the decorin regulation of other growth factors. In this study, we used a peptide derived from the LRR10–12 region that was able to inhibit CTGF by itself. Several investigators have used decorin as a therapeutic inhibitor of TGF-β (37, 66–70), a strong inductor of fibrosis (3, 71, 72). Because TGF-β has pleiotropic effects, this type of therapeutic approach is not very promising. Previously, we have shown that decorin interacts with TGF-β1 through a sequence present in LRR3–5 (36). Interestingly, some partial effects on myoblast fibronectin accumulation and actin stress fiber formation were observed when CTGF was co-incubated with LRR4 and -5. Previously, it has been suggested that CTGF could be an agonist of TGF-β (57). The role of these LRRs regions on TGF-β activity deserves further evaluation. The finding that LRR12 interacts and inhibits CTGF is attractive. These are potentially important findings because although fibrotic disorders are responsible for 45% of human deaths, an effective therapy for these fibrotic diseases does not exist (3). In Duchenne muscular dystrophy, all the therapies being developed try to either restore dystro-

FIGURE 7. Peptide derived from the LRR12 region of decorin inhibits CTGF action. A, C2C12 myoblasts were serum-starved for 18 h and then incubated with 0.8 nM CTGF and increasing concentrations of different LRR peptides for 48 h (1.6 and 3.2 nM), preincubated for 30 min at room temperature. FN and collagen III levels were determined by Western blot of the cell extracts, using tubulin (tub) as a loading control. The normalized relation between FN or collagen III levels to tubulin levels is shown below each blot. These experiments were done twice. B, C2C12 myoblasts were grown on coverslips, serum starved for 18 h and then incubated for 48 h with CTGF 0.8 nM and different LRR peptides at 3.2 nM, preincubated as in A. Actin stress fibers were observed by immunofluorescence using phalloidin coupled to FITC. Bar, 100 μm. C, 3T3 fibroblasts were treated as in A. FN and collagen III (Col-III) levels were determined by a Western blot of the cell extracts using tubulin as a loading control. D, C2C12 myoblasts were serum-starved for 18 h as in A and incubated with CTGF and the indicated LRR peptides (3.2 nM). FN was determined by a Western blot of the cell extracts using GADPH as a loading control.
phin expression or inject dystrophin-expressing cells that repair the injured muscle. However, these have not been effective, and one of the reasons is because the muscle tissue is already fibrotic (73, 74). In vivo experiments have shown that myostatin knock-out mice developed significantly less fibrosis and displayed better skeletal muscle regeneration compared with wild type mice at 2 and 4 weeks following gastrocnemius muscle laceration injury (42). The fibrotic scar in Duchenne muscular dystrophy muscles and other skeletal muscular dystrophies is large, and it would be of great help to decrease it before cell therapies are used to restore defective protein expression (74). In this way, muscle regeneration could be more effective. The LRR12 peptide could be of great help in this matter, and we are developing in vivo experiments to assess the effectiveness of this peptide in animal models of muscle fibrosis. Finally, we propose the model summarized in Fig. 9 in which, as shown previously, CTGF induces the accumulation of ECM proteins and actin stress fiber formation, but we now show that these are inhibited by decorin.

FIGURE 8. CTGF induces decorin in C2C12 myoblasts. A, C2C12 myoblasts were serum-starved for 18 h and then incubated with different concentrations of CTGF for 48 h. The last 18 h was in the presence of [35S]H2SO4. The cell media were concentrated by DEAE-Sephalac, electrophoresed, and the gel autoradiographed. Dcn, decorin. B, C2C12 myoblasts were treated as in A, and the conditioned media were incubated with the indicated GAG lyases, electrophoresed, and autoradiographed. The bracket in A indicates decorin migration and in B indicates decorin and biglycan (Bgn) migration. C, C2C12 myoblasts were serum-starved for 18 h and then incubated with different concentrations of CTGF for 48 h. Decorin levels from the media were determined after CABC digestion by Western blot using antibodies against mouse decorin that recognize the decorin core protein. Tubulin (tub) levels were used as a loading control. Heparitinase (Hase) and CABC enzyme hydrolyzes heparan sulfate and chondroitin/dermatan sulfate GAG chains, respectively.

FIGURE 9. Proposed model for CTGF regulation by decorin. CTGF is responsible for ECM accumulation and actin stress fiber formation. CTGF is also able to induce decorin (Dcn) accumulation, which would be a mechanism of autoregulation because decorin is able to inhibit CTGF action through its LRR12.

REFERENCES
1. Bolster, M. B., and Silver, R. M. (1993) Baillieres. Clin. Rheumatol. 7, 79–97.
2. Ziyadeh, F. N. (1993) Am. J. Kidney Dis. 22, 736–744.
3. Wynn, T. A. (2008) J. Pathol. 214, 199–210.
4. Hinz, B., Phan, S. H., Thannickal, V. J., Galli, A., Bochaton-Piallat, M. L., and Gabbiani, G. (2007) Am. J. Pathol. 170, 1807–1816.
5. Denton, C. P., and Abraham, D. J. (2001) Curr. Opin. Rheumatol. 13, 505–511.
6. Cicha, I., and Goppelt-Struebe, M. (2009) Biofactors 35, 200–208.
7. Holbourn, K. P., Acharya, K. R., and Perbal, B. (2008) Trends Biochem. Sci. 33, 461–473.
8. de Winter, P., Leoni, P., and Abraham, D. (2008) Growth Factors 26, 80–91.
9. Shi-Wen, X., Leask, A., and Abraham, D. (2008) Cytokine Growth Factor Rev. 19, 133–144.
10. Bork, P. (1993) FEBS Lett. 327, 125–130.
11. Abraham, D. (2008) Rheumatology 47, Suppl. 5, v8–9.
12. Leask, A. (2009) Front. Biosci. 1, 115–122.
13. Igarashi, A., Nashiro, K., Kikuchi, K., Sato, S., Ihn, H., Grotendorst, G. R., and Takehara, K. (1995) J. Invest. Dermatol. 105, 280–284.
14. Igarashi, A., Nashiro, K., Kikuchi, K., Sato, S., Ihn, H., Fujimoto, M., Grotendorst, G. R., and Takehara, K. (1996) J. Invest. Dermatol. 106, 729–733.
15. Ito, Y., Aten, J., Bende, R. J., Oemar, B. S., Rabelink, T. J., Weening, J. J., and Goldschmeding, R. (1998) Kidney Int. 53, 853–861.
16. Paradis, V., Dargere, D., Bonvoust, F., Vidaud, M., Segarini, P., and Bessada, P. (2002) Lab. Invest. 82, 767–774.
17. Lasky, J. A., Ortiz, L. A., Tongthab, H., Hoyle, G. W., Corti, M., Athas, G., Lungarella, G., Brody, A., and Friedman, M. (1998) Am. J. Physiol. 275,
