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A Novel Mechanism of Sequestering Fibroblast Growth Factor 2 by Glypican in Lipid Rafts, Allowing Skeletal Muscle Differentiation▽

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Heparan sulfate proteoglycans (HSPGs) are critical modulators of growth factor activities. Skeletal muscle differentiation is strongly inhibited by fibroblast growth factor 2 (FGF-2). We have shown that HSPGs present at the plasma membrane are expressed in myoblasts and are downregulated during muscle differentiation. An exception is glypican-1, which is present throughout the myogenic process. Myoblasts that do not express glypican-1 exhibit defective differentiation, with an increase in the receptor binding of FGF-2, concomitant with increased signaling. Glypican-1-deficient myoblasts show decreased expression of myogenin, the master gene that controls myogenesis, myosin, and the myoblast fusion index. Reversion of these defects was induced by expression of rat glypican-1. Glypican-1 is the only HSPG localized in lipid raft domains in myoblasts, resulting in the sequestration of FGF-2 away from FGF-2 receptors (FGFRs) located in nonraft domains. A chimeric glypican-1, containing syndecan-1 transmembrane and cytoplasmic domains, is located in nonraft domains interacting with FGFR-IV- and enhanced FGF-2-dependent signaling. Thus, glypican-1 acts as a positive regulator of muscle differentiation by sequestering FGF-2 in lipid rafts and preventing its binding and dependent signaling.

Heparan sulfate proteoglycans (HSPGs), key components of cell surfaces and extracellular matrices (ECM), can influence cell growth and differentiation processes by interacting with a large number of macromolecules. One of the most recognized functions of HSPGs is the ability to modulate different growth factor activities. In this context, cell-surface HSPGs bind soluble ligands, increasing their local concentration and modulating ligand-receptor encounters (5). Levels of fibroblast growth factor 2 (FGF-2) and hepatocyte growth factor (HGF) signaling are markedly enhanced by HSPGs. In particular, FGF-2 completely depends on heparan sulfate to transduce an intracellular signal through its receptors (FGFRs) (48, 65, 86), through the formation of the ternary complex HSPG–FGF-2–FGFR (61). However, when HSPGs are localized at the ECM they can decrease FGF-2 signaling by sequestering it away from the transducing receptors (9).

Skeletal muscle formation and regeneration is a complex and regulated process that involves activation, proliferation, and differentiation of a muscle precursor, involving the participation of heparan binding growth factors, such as FGF-2 (13), HGF (2), and transforming growth factor type β (TGF-β) (49). Skeletal muscle differentiation is regulated by the expression of specific combinations of muscle regulatory transcription factors. Among them, a family of basic helix-loop-helix transcription factors, called muscle regulatory factors (MRFs), is critical for muscle differentiation (22, 72). The activity of MRFs, particularly myogenin, the master gene involved in skeletal muscle differentiation, is highly depressed in the presence of FGF-2, HGF, or TGF-ß (2, 13, 49).

Understanding of the role of HSPGs in skeletal muscle physiology, as well as in the skeletal muscle differentiation process, has been previously revised (40). In mature skeletal muscle tissue, HSPGs also act as coreceptors for the asymmetric form of acetyl-cholinesterase, increasing its concentration at the neuromuscular junction (10, 62). Inhibition of proteoglycan sulfation in cultures of C2C12 (51, 57), a satellite cell line derived from regenerating adult mouse skeletal muscle undergoing *in vitro* terminal myogenic differentiation or from intact myofibers (21), affects the proper progression of the *in vitro* myogenic program. Syndecans and glypicans are the two families of HSPGs that localize to the plasma membrane. Syndecans are bound to the plasma membrane through a highly conserved transmembrane domain and are composed of four separate genes in mammals (5, 25, 64), whereas glypicans are bound to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) linkage corresponding to six separate genes (30, 31).

We have shown that during C2C12 myogenesis, the expression levels of all syndecan forms are downregulated (32, 36, 43, 54), whereas the expression of glypican-1, which is the only glypican expressed in myoblasts, remains constant throughout the process (8, 36). This differential expression may reflect different functions or macromolecular specificity during myogenesis. Syndecans have been reported to modulate FGF-2 activity during *in vitro* myogenesis (32, 42, 65) and to participate in cell-cell and cell-matrix adhesion in development and adult wound repair (64). It has been reported that syndecan-3 and syndecan-4 are expressed during embryonic limb skeletal muscle formation by developing myocytes (21, 54) and that expression continues in adult muscle tissue restricted to satellite cells (21). Knockout mice used for syndecan-3 studies exhibit a novel form of muscular dystrophy, while syndecan-4 satellite cells fail to reconstitute damaged muscle, which re-

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veals the importance of these macromolecules in the skeletal muscle differentiation process (24).

Little is known about the localization of HSPGs to specific plasma membrane subdomains. In this context, it can be speculated that glypicans might be associated with "lipid raft" domains, corresponding to sphingolipid and cholesterol-rich domains forming phase-separated "lipid rafts" in the membrane (7, 12). Lipid raft domains might be involved in signal transduction processes through specific receptors and proteins anchored by GPI (11, 44). Since all the syndecans are downregulated during myogenesis (32, 36, 43, 54), it is believed that their absence facilitates the silencing of FGF-2-mediated signaling, helping the process of skeletal muscle differentiation and allowing the expression of myogenin. The presence of glypican-1 on myoblast surfaces during the entire process of skeletal muscle differentiation is puzzling, since glypican-1 is also able to form the ternary complex HSPG–FGF-2–FGFR when glypican-1 and FGFR-I are overexpressed in the same cell (78). This paradoxical situation led us to study the role of glypican-1 in the modulation of FGF-2 signaling during the process of skeletal muscle differentiation. We hypothesized that glypican-1, located in lipid raft domains, sequesters FGF-2, avoiding the interaction with its transducing receptors. This, in turn, allows the expression of myogenin, subsequent myoblast fusion, and expression of late muscle differentiation markers. In this study, we evaluated glypican-1 localization at the plasma membrane related to FGFRs and its direct participation in the modulation of FGF-2 activity. Our results strongly suggest that glypican-1 localizes to lipid raft domains, where it interacts with FGF-2, sequestering it away from the FGFRs, which prevents signaling and results in a strong positive effect on skeletal muscle differentiation.

MATERIALS AND METHODS

Cell culture. The mouse skeletal muscle cell line C2C12 (ATCC) (84) was grown, and differentiation was induced (42). Myoblasts were treated with FGF-2, HGF, TGF-β1, or platelet-derived growth factor (PDGF) (R&D, Minneapolis, MN), heparin and methyl-beta-cyclodextrin (MβCD) (Sigma Chemical, St. Louis, MO), and phosphatidylinositol-specific phospholipase C (PI-PLC) (Invitrogen, Carlsbad, CA) (14), as indicated in the corresponding figures. MβCD (1 mM) or PI-PLC (0.5 U/ml) treatments were performed using phosphate-buffered saline (PBS) containing 0.1 mM $CaCl_2$ and 1 mM MgCl (PBS Ca^{+2}/Mg^{+2}) for 1 h at 37°C in a humidifying chamber. The heparin wash involved the incubation of cells in PBS Ca^{+2}/Mg^{+2} containing 10 μg/ml of heparin and gentle agitation for 10 min at 4°C. This procedure was repeated twice. Heparitinase (Hase) myoblast treatment (Seikagaku, Tokyo, Japan) was undertaken as previously described (36). For the phosphorylation experiments, the cells were serum starved for 6 h and then treated for the indicated times.

For the inactivation of FGF-2, 1 or 10 μg/ml of the soluble form of FGFR-I [rhFGF R1a(IIIc)/Fc chimera; R&D, Minneapolis, MN] or 1, 5, or 10 μg/ml of a blocking antibody for FGF-2 (anti-FGF-2-neutralizing antibody [catalog no. 05-117; Millipore, Bedford, MA]) was exogenously added, and the inhibitory effects were analyzed by determining the FGF-2-dependent activation of extracellular signal-regulated kinase 1 (ERK1) and ERK2. In both cases, the indicated concentrations of FGF-2 were preincubated for 30 min at 37°C. In the case of the soluble receptor, 0.1 μg/ml of heparin was added. The differentiation medium (2% of horse serum) was preincubated with 10 μg/ml soluble FGFR-I or 10 μg/ml of the neutralizing antibody for 30 min at 37°C prior to being added to the cells. The FGF-2-neutralizing antibody was replaced daily.

Transient transfection and generation of stable clones. The myogenin reporter plasmid pMyo-Luc (67), short hairpin RNA (shRNA) for glypican-1 and its corresponding control (scrambled shRNA [shCtrl]), pEGFP-N1, pRL-SV40, and pcDNA3.0 empty vector (all from Invitrogen, Carlsbad, CA), and pcDNA3.0–rat-glypican-1 (courtesy of Ralph D. Sanderson, Department of Pathology, University of Alabama at Birmingham, Birmingham, AL) were transfected using Lipofectamine Plus transfection reagent (Invitrogen, Carlsbad, CA) according to the supplier's protocol. For the reporter experiments, the cells were plated in 24-well plates. At 48 h after transfection, the cells were induced to differentiate for 30 h in the presence of FGF-2. The samples were subsequently assayed for dual luciferase activity (Promega, Madison, WI).

shRNA expression vectors were constructed and packaged into recombinant lentiviruses by the use of a BLOCK-iT lentiviral RNA interference expression system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The target sequence for the shRNA against mouse glypican-1 (shGly) (GenScript ORF NM_016696) was as follows: 5′-GACCATCCGCCAGCAGA TTATGC-3′. pU6-shGly was used in transient transfection experiments. From this plasmid, we generated the pLenti6-U6-shGly vector, which was cotransfected with the packaging plasmid mixture into 293FT cells to produce recombinant lentiviruses. The conditioned medium of the lentivirus-producing cells was harvested and titrated using C2C12 myoblast cells. Transductions were performed at a multiplicity of infection of 50. The cells were maintained in normal growth medium for 48 h after transduction and supplemented with 30 μg/ml blasticidin (selection antibiotic). The target gene knockdown in transient transfections and in selected clones was confirmed by Western blot analysis. A stable cell line expressing a scrambled sequence was used as the control.

A FLAG epitope in the amino terminal of extracellular domain was incorporated into a chimeric HSPG containing the extracellular domain of rat glypican-1 and the cytoplasmatic domain of mouse syndecan-1 (GlySyn) (45, 85) (donated by Ralph D. Sanderson, Department of Pathology, University of Alabama at Birmingham, Birmingham, AL).

Isolation of lipid rafts. Lipid rafts were prepared as described previously (90), with some modifications. All of the buffers and instruments used in the procedure described below were maintained at 4°C. Briefly, C2C12 myoblasts from a 150-mm-diameter dish, either control or treated, were collected in cold PBS and resuspended in 400 μl of lysis buffer containing 25 mM MES [2-(*N*-morpholino)ethanesulfonic acid] (pH 6.5) and 150 mM NaCl with a mixture of protease inhibitors and 1 mM phenylmethylsulfonyl fluoride (PMSF) supplemented with 1% Triton X-100, 1% Lubrol, or 1% Brij 35, as indicated in each case. Cells were homogenized by passing them through a 21-gauge needle three times, incubated for 20 min on ice, and then homogenized with 10 strokes of a loose-fitting Dounce homogenizer. Homogenates were mixed with 400 μl of 90% sucrose (final concentration, 45%), loaded at the bottom of a Sorvall 4-ml centrifuge tube, and overlaid with 1.6 ml of 35% sucrose and 1.6 ml of 5% sucrose, both in the lysis buffer (79). The samples were centrifuged at 45,000 rpm for 18 h at 4°C in an AH-650 rotor. Twelve fractions (330 μl each) were collected from top to bottom and designated fractions 1 to 12.

SDS-PAGE, Western blotting, slot blotting, and coimmunoprecipitation. Aliquots from each gradient fraction were separated using 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Mini Protean II; Bio-Rad, Richmond, CA) and electrotransferred onto Immobilon membranes (Millipore, Bedford, MA). Western blots were probed using various primary antibodies: rabbit anti-mouse FGFR-I (Cell Signaling, Danvers, MA) (1:500); biotinylated anti-mouse FGFR-IV (R&D, Minneapolis, MN) (1:500); rabbit anti-caveolin-1 (anti-Cav-1) (Santa Cruz Biotechnology, Santa Cruz, CA) (1:500); anti-glypican-1 M95 (Santa Cruz Biotechnology, Santa Cruz, CA) (1:500); and mouse anti-sodium potassium ATPase (anti-Na^+/K^+ATPase) (Upstate Biotechnology, Lake Placid, NY) (1:1,000). To reveal the distribution of the ganglioside GM1 in the gradients, 30 μl of each fraction was spotted onto nitrocellulose membranes by the use of a Bio-Rad Slot Blot apparatus and probed with horseradish peroxidase-coupled CTX (Sigma-Aldrich, St. Louis, MO) (38) (1:10,000).

To identify HSPG core proteins, samples containing equivalent amounts of protein were incubated with Hase and chondroitinase ABC (Seikagaku, Tokyo, Japan) (78) and were analyzed by Western blotting using anti-Δ-heparan sulfate monoclonal antibody (anti-stub) (Seikagaku, Tokyo, Japan), as described previously (17, 36, 54), and visualized by enhanced chemiluminescence (ECL).

For immunoblot analysis, myoblasts were lysed in 50 mM Tris-HCl (pH 7.4)–0.1 M NaCl–0.5% Triton X-100 with a mixture of protease inhibitors and 1 mM PMSF. For analysis of phosphorylated proteins, cell extracts were prepared using radioimmunoprecipitation assay (RIPA) buffer (57). Aliquots with equivalent amounts of proteins were subjected to SDS-PAGE using 8% polyacrylamide gels, electrophoretically transferred onto Immobilon membranes (Millipore, Bedford, MA), and probed with rabbit anti-phosphorylated ERK1/2 (anti-phospho-ERK1/2) (1:1,000); mouse anti-FLAG (Stratagene, La Jolla, CA) (1:5,000); rabbit anti-ERK1/2 (1:1,000); rabbit anti-phospho-AKT (Calbiochem, La Jolla, CA) (1:1,000); mouse anti-α-tubulin (1:5,000); mouse antimyosin (Sigma-Aldrich, St. Louis, MO) (1:5,000); mouse anti-glyceraldehyde-3-phosphate-dehydrogenase (anti-GAPDH) (Chemicon, Temecula, CA) (1:2,000); rabbit antimyo-

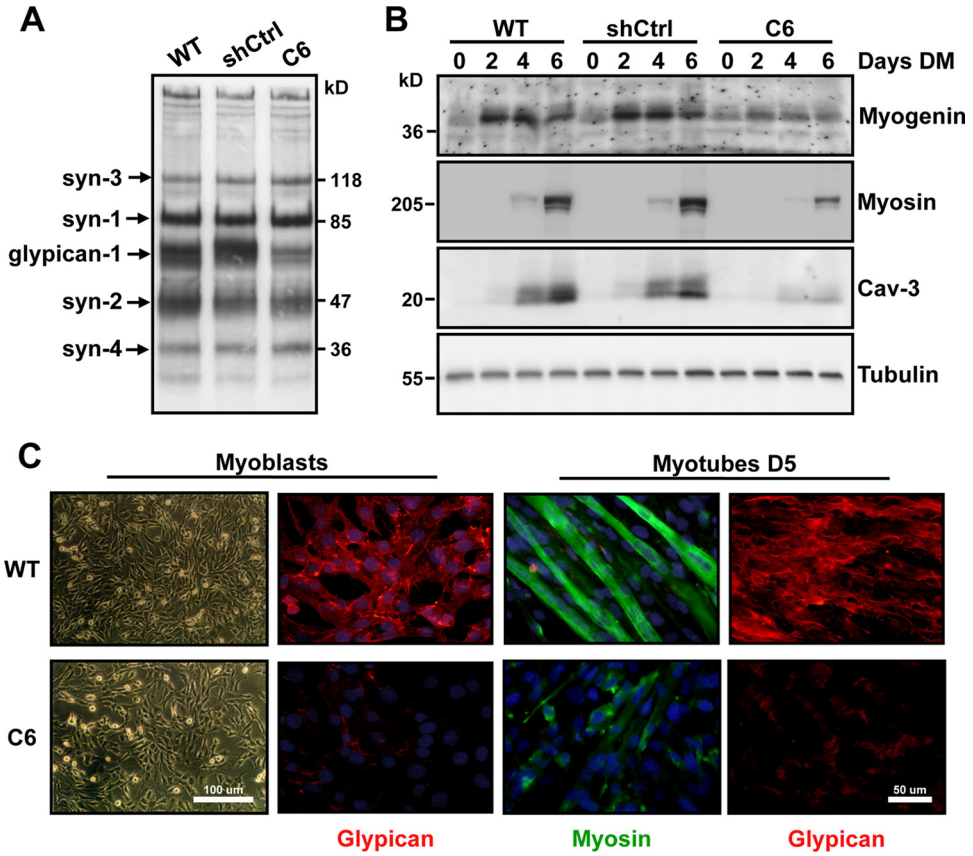

FIG. 1. Glypican-1 is required for a successful muscular differentiation process. (A) C2C12 myoblasts (WT) were infected with a lentiviral vector to generate a stable clone that expresses an shRNA control (shCtrl) or an shRNA specific for mouse glypican-1 (C6). Glypican-1 levels were determined by Western blot analysis using anti-stub antibodies that recognize a neoepitope generated in the heparan sulfate chains after digestion with Hase, enabling the core proteins of any HSPG to be visualized. syn-3, syn-1, syn-2, and syn-4 represent syndecan-3, -1, -2, and -4, respectively. (B) WT, shCtrl, and C6 myoblasts were induced to differentiate for 0, 2, 4, and 6 days in the differentiation medium (Days DM). Cell extracts were analyzed by Western blotting for myogenin, myosin. and caveolin-3. Tubulin levels are indicated as a loading control. In A and B, the molecular weights are indicated in thousands. (C) In a parallel experiment, WT and C6 myoblasts were fixed and analyzed by phase contrast microscopy or indirect immunofluorescence for glypican-1 (red) and myosin (green) after 5 days of differentiation (Myotubes D5). Nuclei were subjected to Hoechst staining (blue).

genin (1:500); and mouse anti-Cav-3 (Santa Cruz Biotechnology, Santa Cruz, CA) (1:1,000).

All immunoreactions were visualized by ECL (Pierce, IL) using a ChemiDoc-It HR 410 imaging system (Upland, CA).

For the coimmunoprecipitation experiment, wild-type (WT) and glypican-1-deficient (C6) myoblasts were transiently transfected as indicated in the corresponding figure. At 48 h after transfection, the cells were incubated with Dulbecco's minimal essential medium (DMEM)–0.1% bovine serum albumin (BSA) supplemented with FGF-2 (20 ng/ml) for 3 h on ice with gentle agitation. The cells were lysed in TS buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$)–0.1% Triton X-100 and precleared by centrifugation. The extracts were immunoprecipitated for 3 h at 4°C using 10 μg of a mouse anti-FLAG antibody or 10 μg of rabbit anti-mouse syndecan-4 (nS4ED; kindly donated by Alan C. Rapraeger, Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison), electrophoresed, and analyzed by Western blotting with rabbit anti-FGFR-IV or rabbit anti-glypican-1 as described above or with rabbit anti-mouse syndecan-4 (1:1,000 from a 1 mg/ml stock solution).

Immunofluorescence microscopy. Cells to be immunostained were grown on coverslips. The medium was removed, and the coverslips were rinsed with PBS. Cells were fixed with 3% paraformaldehyde for 15 min at 4°C, incubated for 30 min in PBS containing 3% BSA, and incubated with the primary antibody for 1 h in the same buffer. Rabbit anti-glypican-1 (1:300) (courtesy of David Carey, Sigfried and Janet Weis Center for Research, Danville, PA), mouse anti-FLAG (1:1,000), mouse antilaminin (Telios Pharmaceuticals, San Diego, CA) (1:50), and mouse antifibronectin (1:300) were used, as indicated in the corresponding figures.

For the detection of myosin, the cells were permeabilized with PBS containing 0.05% Triton X-100 (2 min at 4°C) and incubated for 30 min in PBS–3% BSA and subsequently with the primary mouse antimyosin antibody (Sigma-Aldrich, St. Louis, MO) (1:300). Cells were rinsed with PBS–3% BSA and further incubated for 1 h with Alexa 488-conjugated antibodies (1/1,000) or Alexa 568-conjugated antibodies (1/1,000) (Invitrogen, Carlsbad, CA).

Nuclear staining was done using 0.1 μg/ml of Hoechst 33258–PBS for 10 min (57). After rinsing, the coverslips were mounted and viewed under a Nikon Diaphot inverted microscope equipped for epifluorescence.

FGF-2 affinity labeling and cross-linking assay. Carrier-free FGF-2 was radiolabeled using Na[^{125}I] and chloramine-T. Binding and cross-linking of ^{125}I–FGF-2 to cell surfaces were assayed (26). In some experiments, the cells were pretreated with Hase (36) or subjected to competition with a 200 M excess of cold FGF-2.

Protein analysis. Protein was analyzed with the bicinchoninic acid protein assay kit (Pierce) with BSA as the standard.

RESULTS

Glypican-1 is required for successful skeletal muscle differentiation. To evaluate the role of glypican-1 during myogenesis, we studied the effect of its absence in this process. By lentiviral infection of an shRNA specific for mouse glypi-

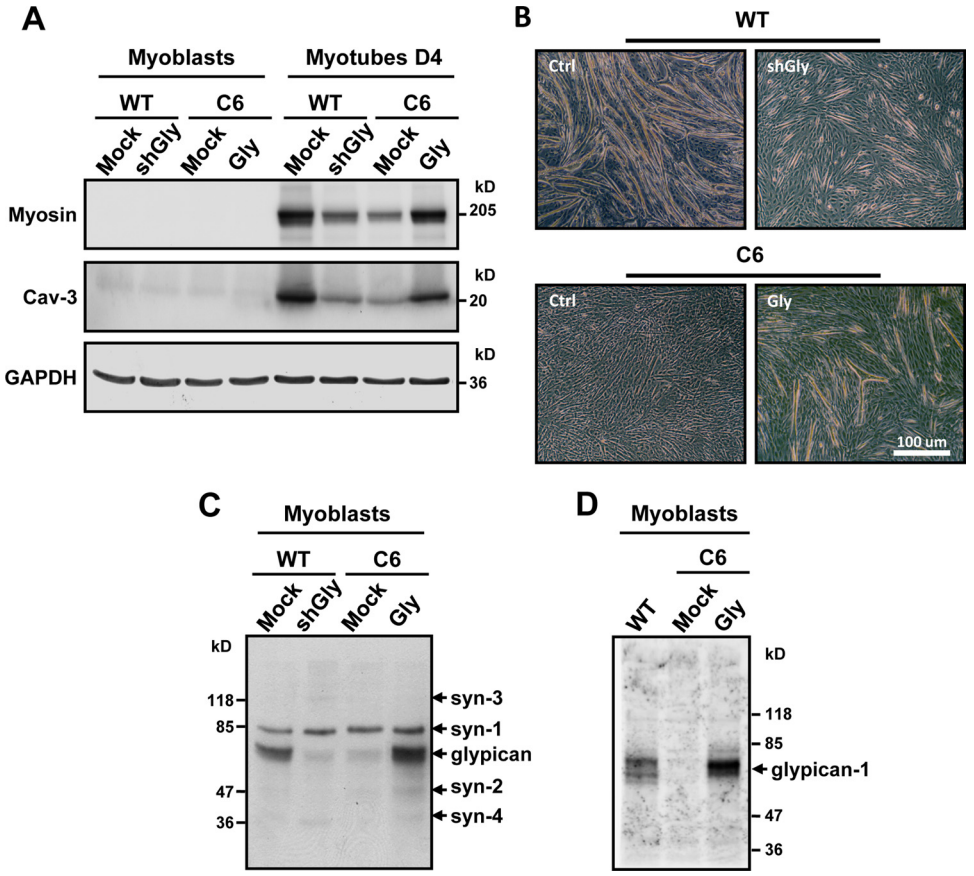

FIG. 2. Reexpression of glypican-1 restores the impaired muscular differentiation observed in glypican-1-deficient myoblasts. (A) WT and C6 myoblasts were transiently transfected with shGly and with rat glypican-1 (Gly), respectively. At 48 h after transfection, the myoblasts were induced to differentiate into myotubes for 4 days (Myotubes D4). The extracts were analyzed by immunoblotting for the late muscle differentiation markers, myosin, and Cav-3. GAPDH levels were used as a loading control. (B) Phase contrast images of each experimental condition of A, at day 4 of differentiation. (C) The glypican-1 protein levels of the myoblast transfected as described in the Fig. 1A legend were determined after 48 h by immunoblot analysis using the anti-stub, as described in the same legend. (D) The glypican-1 protein levels of the myoblast transfected as described for panel A were determined after 48 h by immunoblot analysis with a glypican-1-specific antibody.

can-1 in C2C12 myoblasts, we produced a stable clone (C6) that expressed low levels of glypican-1, as determined by Western blot analysis of the corresponding HSPG core protein after Hase digestion (Fig. 1A). The core proteins of any proteoglycan that is replaced with heparan sulfate can be traced using the monoclonal antibody 3G10 (anti-stub) (78), which recognizes a neoepitope generated after Hase treatment. shRNA-mediated knockdown of glypican-1 has no effect on the protein levels of other HSPGs, since infection with a lentiviral vector to express a scrambled shRNA (shCtrl) in a stable manner results in HSPG core protein levels that are equivalent to those present in WT extracts (Fig. 1A). Figure 1B shows the kinetics expression of myogenin, the master gene that controls skeletal muscle differentiation, and the myosin and Cav-3 late skeletal muscle differentiation markers (33) in WT, shCtrl, and C6 myoblasts under differentiation conditions. The latter showed diminished expression of myogenin concomitant with a significant reduction in levels of myosin and Cav-3 by day 5 of differentiation compared to the results seen with the WT and shCtrl. Immunofluorescence analysis (Fig. 1C) confirmed that myoblasts (left panels) not expressing glypican-1 were unable to form myosin-expressing myotubes after 5 days of differentiation (Myotubes D5; right panels).

Figure 2A shows that muscle differentiation is modulated specifically by glypican-1, since the deleterious effect on the expression of muscle-specific proteins in the C6 myoblasts was restored after reexpression of this HSPG by transient transfection with rat glypican-1, which is not recognized by the shGly, with expression of myosin and Cav-3 similar to the WT levels. This figure also shows that the transiently transfected WT myoblasts with shGly express very low levels of myosin and Cav-3, as seen with the C6 clone. In this sense, the lack of myotube formation observed in the C6 clone (as shown in Fig. 1C) is glypican-1 specific too, since it can be partially restored by reexpression of rat glypican-1, as indicated in Fig. 2B. In concordance, myoblasts transiently transfected with the shGly exhibited a considerably diminished amount of myotube formation (Fig. 2B). Figure 2C shows the level of glypican-1 core protein in myoblasts under each set of experimental conditions, as determined with the anti-stub antibody after Hase treatment. Figure 2D shows the glypican-1 levels of WT, C6, and C6 reexpressing glypican-1 myoblasts, as determined with an antibody specific against glypican-1.

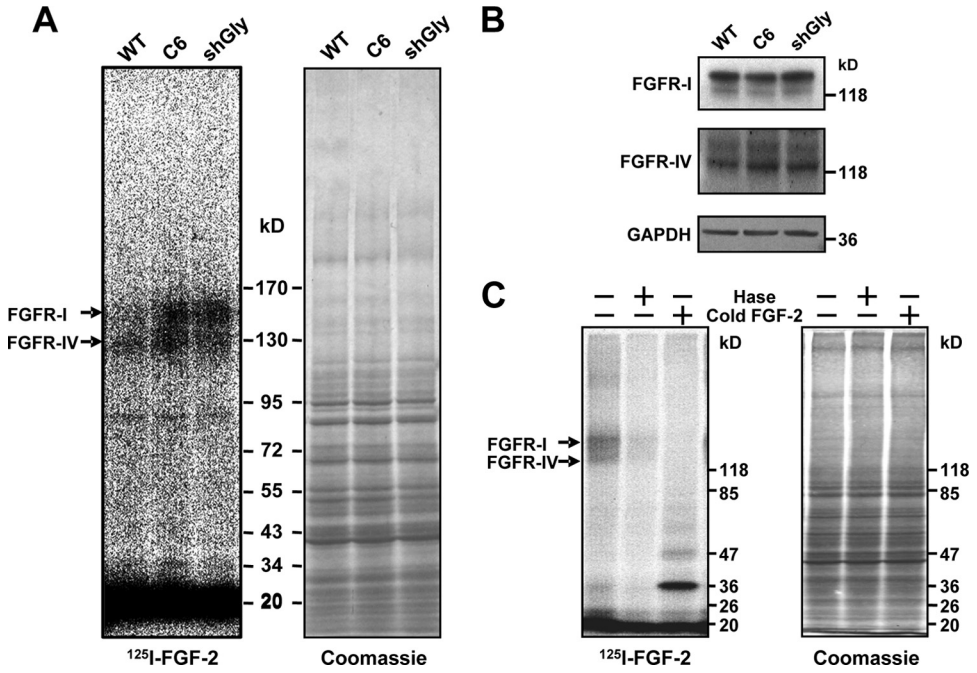

FIG. 3. The binding of FGF-2 to its receptors is augmented in glypican-1-deficient myoblasts. (A) FGF-2 cell surface receptors of WT myoblasts transiently transfected with or without shCtrl and shGly, and of C6 myoblasts transiently transfected with or without rat glypican-1 (C6-Gly), were affinity cross-linked to ^{125}I–FGF-2 at 4°C. Cell extracts were separated on SDS-PAGE and then exposed to a phosphorimager (left). As shown on the right, the gel was stained with Coomassie blue as a loading control. (B) The same extracts described for panel A (left) were analyzed by Western blotting to determine the total protein levels of FGFR-I and FGFR-IV. GAPDH levels were used as a loading control. (C) Myoblasts were treated with or without Hase, and then FGFRs were affinity cross-linked to ^{125}I–FGF-2 at 4°C in the presence or absence of an excess of cold FGF-2. As shown on the right, the gel was stained with Coomassie blue as a loading control.

The sum of these results indicates that glypican-1 is critical for skeletal muscle differentiation, as evaluated by the induction of myogenin, myosin, Cav-3, and myotube formation.

The binding of FGF-2 to its receptors and its consequent signaling are augmented in glypican-1-deficient myoblasts. HSPGs are essential for FGFR activation by FGF-2, acting as coreceptors of this muscle differentiation inhibitory growth factor. It is possible that glypican-1, in contrast to syndecans, might be sequestering FGF-2 away from its transducing receptors. To determine this, we evaluated the binding of radiolabeled ^{125}I–FGF-2 to FGFRs, in the presence or absence of glypican-1, through affinity labeling experiments. Figure 3A shows that the cross-linking of ^{125}I–FGF-2 to FGFR-I and -IV increased in C6 myoblasts, as well as in WT myoblasts transiently transfected with shGly. Importantly, the levels of FGFRs were unaffected by glypican-1 silencing (Fig. 3B). The observed binding of FGF-2 to its receptors was specific, since it was totally eliminated by an excess of cold FGF-2, and dependent on HSPGs, since it was abolished when the cells were pretreated with Hase (86) (Fig. 3C).

Subsequently, we evaluated whether such increased binding of FGF-2 would result in augmented FGF-2-dependent signaling. The extent of phosphorylation of phospho-ERK1/2 in response to this growth factor was determined (82). The left panel of Fig. 4A shows that C6 myoblasts required lower FGF-2 concentrations to induce phospho-ERK1/2 compared to WT myoblasts. The right panel of Figure 4A shows quantification of two independent experiments. The increased response to FGF-2 in the absence of glypican-1 was specific, since reexpression of rat glypican-1 in C6 myoblasts resulted in reversion of such heightened sensitivity to FGF-2, as shown in the left panel of Fig. 4B. A quantification of this experiment is shown in Fig. 4B (right panel). Similar results were obtained after inducing the phosphorylation of AKT by FGF-2 (20) (data not shown). Then we asked whether the absence of glypican-1 could alter the cellular response to other heparin-binding growth factors such as TGF-β1 (47, 66, 75), PDGF (34, 58, 68), and HGF (3, 18, 46). Figure 4C shows that the extent of phosphorylation of Smad-2 in response to TGF-β1, or the phosphorylation of ERK1/2 in response to PDGF, was unaltered in the C6 glypican-1-deficient myoblast compared to the WT myoblast. Interestingly, the induction of phospho-ERK1/2 in response to HGF was diminished in the glypican-1-deficient myoblast. These results suggest that glypican-1 is not involved in the regulation of TGF-β and PDGF signaling, but they do not exclude the possibility that other signaling pathways, such as HGF, could be regulated directly or indirectly by glypican-1.

Since the FGF-2-dependent inhibition of myogenin expression depends on the activation of the mitogen-activated protein kinase (MAPK) pathway (82), we determined the inhibitory effect of FGF-2 on the expression of myogenin. Figure 5A (upper panel) shows the inhibitory effect of FGF-2 on the activity of pMyo-Luc, which is a reporter plasmid containing the promoter region of myogenin coupled to the luciferase gene (67). Exposure of the cells to FGF-2 resulted in significant inhibition of pMyo-Luc activity. However, C6 cells showed a marked shift in the dose-response curve from a 50% inhibitory concentration (IC_{50}) of 2.0 ng/ml for wild-type myoblasts

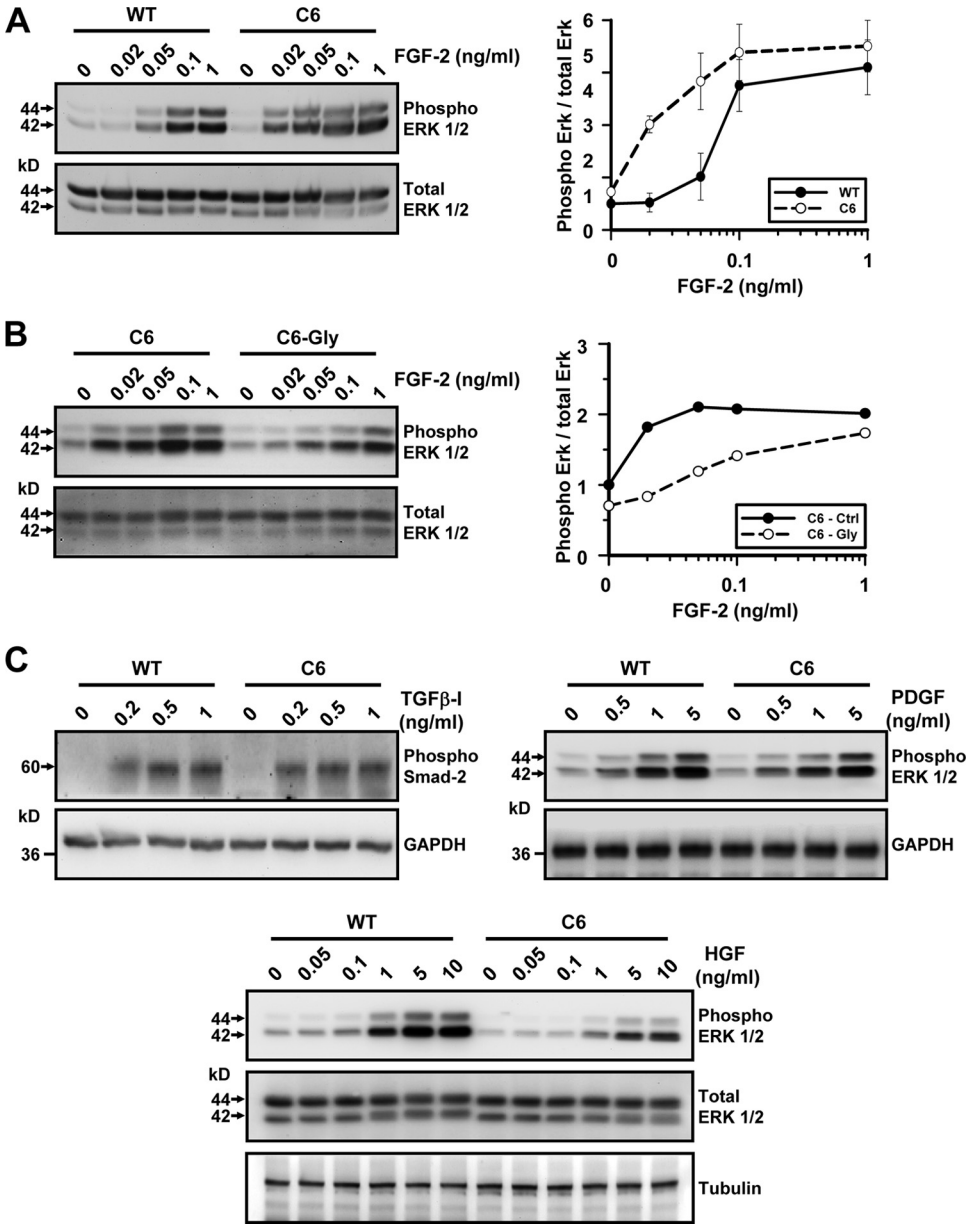

FIG. 4. Myoblasts deficient in glypican-1 are more sensitive to FGF-2 but not to other heparin binding growth factors. (A) WT and C6 myoblasts were treated with the indicated concentrations of FGF-2 for 5 min. Cell extracts were analyzed for phospho-ERK1/2 by immunoblotting. The levels of total ERK1/2 were used as a loading control. On the right, a quantification of two independent experiments is shown. (B) C6 myoblasts were transiently transfected with or without rat glypican-1 (C6-Gly), and at 48 h after transfection, the cells were treated as described for panel A. A quantification of this experiment is shown on the right. (C) WT and C6 myoblasts were treated with the indicated concentrations of TGF-β-1 and PDGF for 15 min or HGF for 5 min. The levels of phospho-Smad 2 or phospho-ERK1/2 were determined by immunoblot analysis. GAPDH or total ERK1/2 levels were used as a loading control.

to 0.5 ng/ml for nonexpressing glypican-1 myoblasts. Reexpression of rat glypican-1 in C6 myoblasts shifted the FGF-2 sensitivity to values closer to WT (~1.3 ng/ml). Similar results were obtained when the expression of glypican-1 in wild-type myoblasts was diminished by transient transfection of shGly (Fig. 5B). A shift in the dose-response curve from an IC_{50} of 2.0 ng/ml for WT cells to 0.6 ng/ml for myoblasts transfected with shGly was observed. When the WT myoblasts were transfected with the scrambled shRNA, no effect on the IC_{50} was observed (Fig. 5B).

The results described above suggest that the altered muscle differentiation process observed with the glypican-1-deficient myoblasts could be explained by an augmented sensitivity to the inhibitory signaling of FGF-2. To probe this, we decided to block the FGF-2 present in the differentiation medium that is produced by the myoblast itself (data not shown) (42), through its inactivation with a soluble form of FGFR-I or by the use of a neutralizing antibody against FGF-2. Figures 5C and E show that the phospho-ERK1/2 levels induced by exogenously added FGF-2 in WT and C6

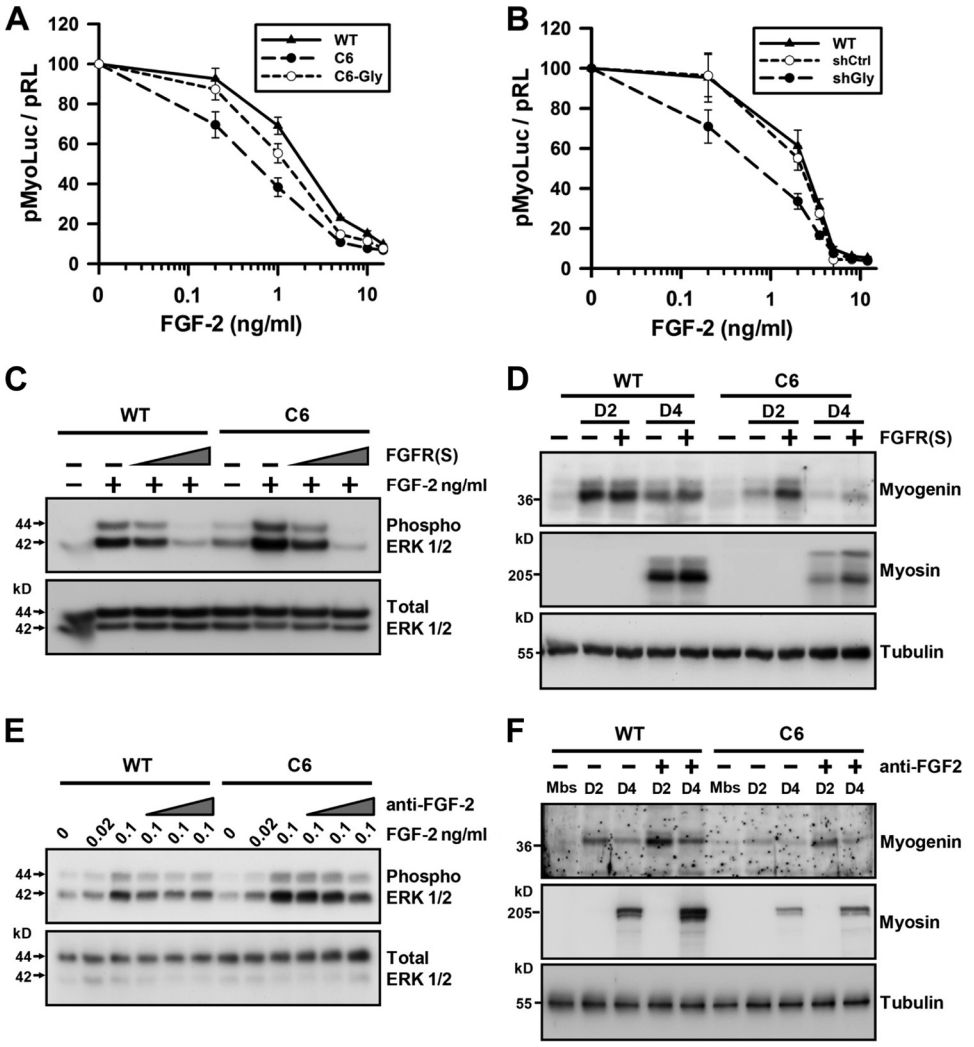

FIG. 5. The absence of glypican-1 induces an increase in the FGF-2-dependent inhibition of the muscular differentiation process. (A) WT and C6 myoblasts were transiently cotransfected with pMyo-luc, the transfection control plasmid (pRL-SV40), an empty pcDNA3.1 plasmid as a control, or rat glypican-1 to reexpress glypican-1 in the C6 myoblasts (C6-Gly). At 48 h after transfection, the cells were induced to differentiate for 30 h in the presence of FGF-2 at the indicated concentrations. The values (pMyo-Luc/pRL-SV40 activity) are expressed as percentages of reporter activity in the absence of FGF-2 for each set of experimental conditions. (B) WT myoblasts were cotransfected with the reporter system as described for panel A and with shCtrl or shGly. After 30 h in the differentiation medium, pMyo-Luc and pRL-SV40 activities were determined and are expressed as described for panel A. The values shown in panels A and B are the results obtained from three independent experiments performed in triplicate and correspond to the mean and standard deviations. (C and E) WT or C6 myoblasts were treated with FGF-2 preincubated with or without increasing concentrations of a soluble form of the FGFR-I [FGFR(S)] or a neutralizing antibody against FGF-2 (anti-FGF-2), respectively. The phospho- and total ERK1/2 levels were determined by immunoblot analysis. (D and F) WT and C6 myoblasts were induced to differentiate in the presence or absence of the FGFR(S) and the anti-FGF-2, respectively. The anti-FGF-2 was replaced daily. Myosin and myogenin levels were analyzed by immunoblotting after 2 or 4 days. Tubulin levels were used as a loading control in both cases.

myoblasts were diminished in the presence of the soluble receptor or the neutralizing antibody in a dose-dependent manner. Figure 5D shows that the myogenin and myosin levels of WT myoblasts after 2 or 4 days in differentiation media were slightly augmented in the presence of the soluble FGFR-I. In the C6 myoblasts, however, the presence of the soluble receptor significantly restored the diminished levels. Similar results were obtained with the neutralizing antibody against FGF-2. Under these conditions, myogenin and myosin levels were augmented in the WT and C6 myoblasts, respectively, when the corresponding cells were treated with the neutralizing antibody, as indicated in Fig. 5F.

Altogether, these results clearly indicate that glypican-1 inhibits the binding of FGF-2 to its transducing receptors, thus diminishing the FGF-2-dependent signaling, and that the blockage of endogenous FGF-2 increased the expression of myogenic markers in glypican-1-deficient myoblasts. These results indicate that glypican-1 inhibits FGF-2-dependent signaling in myoblasts, modulating the muscle differentiation process.

Glypican-1 is the only HSPG localized in myoblast lipid raft domains, binding FGF-2 and not colocalizing with FGFRs. The previous results demonstrate that glypican-1, in contrast to the syndecans that act like FGF-2 coreceptors, negatively regu-

lates FGF-2-dependent signaling. To act as a coreceptor, HSPGs require a set of spatial and structural conditions to allow the formation of the signaling ternary complex HSPG–FGF-2–FGFR (53, 63, 86). In this sense, it is strictly necessary that the HSPGs physically interact with the FGFRs on the plasma membrane. Hence, we decided to evaluate the distribution of HSPGs in myoblast plasma membrane domains and to compare their distribution with that of the FGFRs. For this, myoblasts were solubilized in Triton X-100 and fractionated in sucrose density gradients. Figure 6A shows that only glypican-1 was enriched in low-density fractions together with classical markers of raft domains, such as GM-1 and Cav-1, suggesting that glypican-1 localizes in lipid raft domains. On the other hand, all the members of the syndecan family, and some glypican-1, comigrated at high-density fractions together with Na^+/K^+ATPase, which is a nonraft domain marker. The upper panel of Figure 6B shows, by indirect immunocytolocalization analysis, that glypican-1 presents a punctuated pattern on the cell surface, suggesting its association with membrane microdomains (arrows), as well as glypican-1 localized at the ECM with a fibrillar pattern, which is a typical feature of ECM proteins (arrowheads) (8, 57). When lipid rafts were disrupted by MβCD treatment (59), glypican-1 and Cav-1 were mostly displaced from lipid raft to nonraft domains (Fig. 6C). Under these conditions, the punctuated staining of glypican-1 changed to more even staining on myoblast cell surfaces (Fig. 6B, middle panel). When myoblasts were incubated with PI-PLC to remove the plasma membrane-associated glypican-1 (8), only a single remaining stain associated with the ECM was observed (Fig. 6B, lower panel). Then, we asked whether the transiently transfected rat glypican-1 in the C6 clone was associated with lipid raft domains. For this, C6 myoblasts transfected with rat glypican-1 containing a FLAG epitope, or the empty vector as a control, were fractionated as shown in Fig. 6A. The fractions were harvested in three groups: group I (fractions 1 to 4), group II (fractions 5 to 8), and group III (fractions 9 to 12). Then, the distribution of rat glypican-1 was determined with an anti-FLAG antibody. Figure 6D (left panel) reveals that rat glypican-1 was mainly associated with the lipid raft domains (group II), whereas some fractionated in nonraft domains (group III) as well. Figure 6D (right panel) shows that rat glypican-1 expressed in C6 myoblasts exhibited a punctuated distribution pattern on the cell surface, which was disrupted after MβCD treatment. All these results strongly suggest that glypican-1 associates with membrane microdomains.

If glypican-1 present in lipid rafts regulates the binding and signaling of FGF-2, it must interact with the ligand. WT and C6 myoblasts were incubated with ^{125}I–FGF-2 and then solubilized with Triton X-100 and fractionated in sucrose density gradients. Figure 7A (left panel) shows that ^{125}I–FGF-2 cofractionated in raft and nonraft domains, which is probably evidence of binding to heparan sulfate chains present in glypican-1 and syndecan members, respectively. To determine that the binding of ^{125}I–FGF-2 to raft domains was indeed to glypican-1, C6 myoblasts were incubated with the radioactive ligand and fractionated as described for the WT cells. As expected, no ^{125}I–FGF-2 migrated in raft domains, whereas all detectable ^{125}I–FGF-2 migrated together with the nonraft domain markers (Fig. 7A, right panel). These results clearly indicate that glypican-1 is the only HSPG associated with lipid raft domains in myoblast cell surfaces, where it binds and concentrates FGF-2.

Next, we determined the plasma membrane localization of the transducing FGFRs. Western blot analysis of fractionated WT myoblasts showed that FGFR-I and FGFR-IV, the main FGF-2 receptors expressed in skeletal muscles (41), were found only in nonraft domains, cofractionating with syndecans (Fig. 7B). To determine whether the plasma membrane FGFRs were located in nonraft membrane microdomains specifically, we analyzed the distribution of the affinity-cross-linked FGFRs to ^{125}I–FGF-2 followed by Triton X-100 solubilization and sucrose density fractionation. Figure 7C shows that both receptors bound to FGF-2 fractionated only at the higher-density fractions, together with nonraft markers.

These data indicate that the plasma membrane FGFRs, as the FGF-2 coreceptor syndecans, are present only in nonraft domains, where they can interact and facilitate FGF-2 signaling.

Since glypican-1 localized in raft domains would be responsible for the sequestering of FGF-2, we expressed a chimeric form of a HSPG containing the extracellular domain of rat glypican-1 and the transmembrane and cytoplasmic domains of mouse syndecan-1 containing a FLAG epitope (F-GlySyn). C6 myoblasts were transfected with F-GlySyn, lysed, and subjected to sucrose density fractionation. Figure 8A shows that the chimeric HSPG revealed by an anti-FLAG immunoblot migrated only at high-density fractions. The signaling mediated by FGF-2 in C6 myoblasts expressing F-GlySyn form was evaluated. Figure 8B shows that in F-GlySyn-transfected myoblasts, FGF-2 induces phospho-ERK1/2 to levels even higher than observed with the mock-transfected or glypican-1-rescued C6 myoblasts. Consistently, the induction of myogenin and myosin diminished when the chimeric HSPG form was expressed compared to that seen with control transfected or glypican-1-rescued C6 myoblasts, as shown in Fig. 8C. These results suggest that the F-GlySyn form, present in nonraft domains, acts as a presenter of FGF-2 to its transducing receptors. If so, F-GlySyn should interact with the FGFRs. Figure 8D shows that in coimmunoprecipitation experiments with anti-FLAG antibodies, FGFR-IV was coimmunoprecipitated with F-GlySyn. As expected, rat glypican-1 containing a FLAG epitope as well as mock myoblasts did not coimmunoprecipitate any FGFR-IV. As a positive control, syndecan-4 was coimmunoprecipitated with FGFR-IV (Fig. 8D). The same figure shows expression of rat glypican-1 and the chimeric F-GlySyn, as determined by immunoreactivity with the anti glypican-1 antibody. Finally, the presence of syndecan-4 coimmunoprecipitated from control C6 myoblasts is shown. The results described above clearly indicate that glypican-1 modulates muscle differentiation processes, most likely by sequestering FGF-2 in lipid raft domains, avoiding interaction of the ligand with its receptors.

Glypican-1, present on the plasma membrane, is required for successful skeletal muscle differentiation independent from the ECM. Previously it has been shown that glypican-1 is present on the plasma membrane and on the ECM (8). Glypican-1 that is present in nonraft domains (Fig. 6A), as evidenced by its core protein after Hase treatment, likely corresponds to ECM-associated glypican-1, since most of it disappears after a cell surface heparin wash. This competes for the heparan sulfate chain binding sites without affecting the levels of glypican-1 in the lighter fractions associated with raft domains (Fig. 9A). The heparin wash effectively removes most

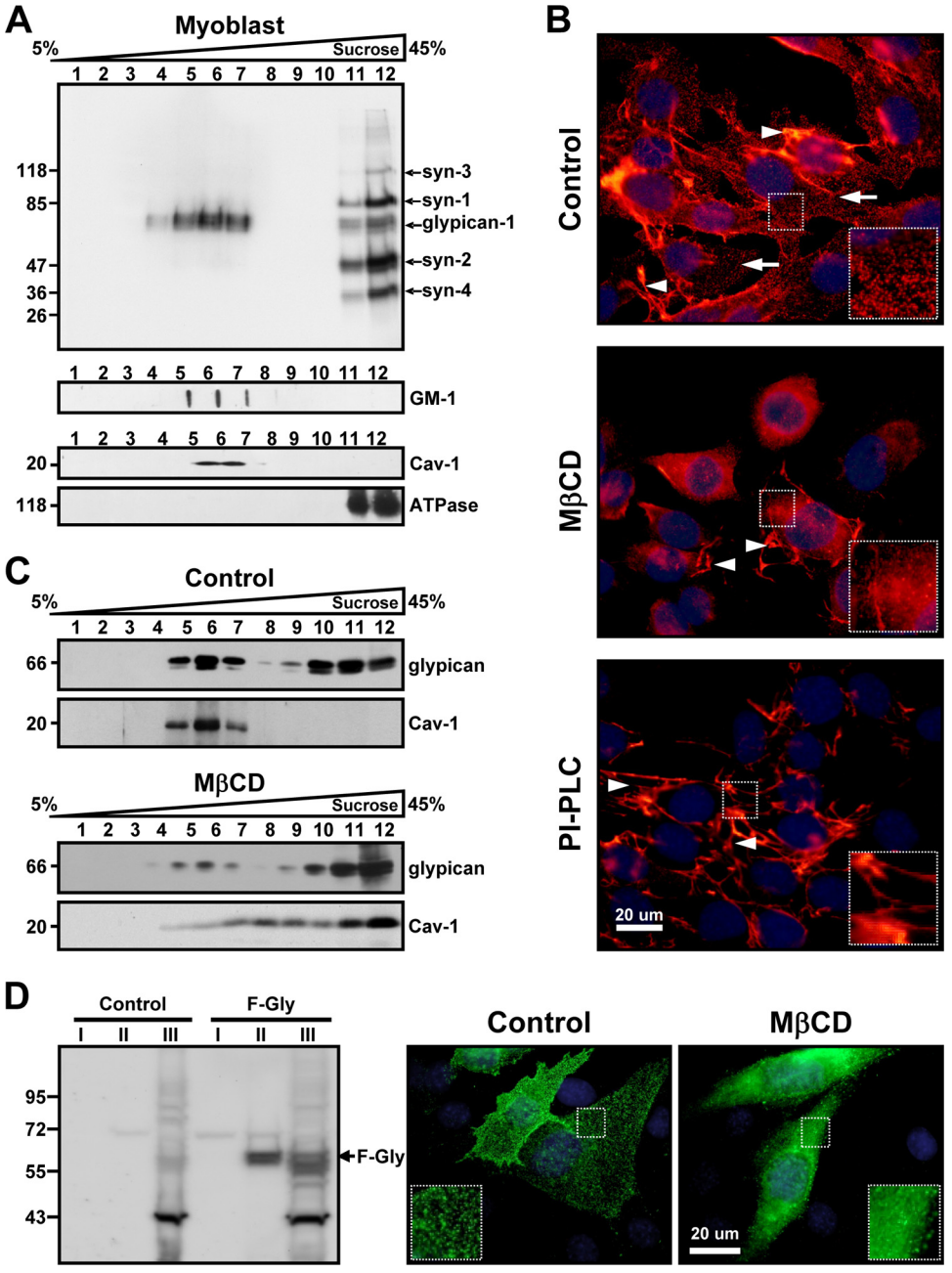

FIG. 6. Glypican-1 is the only HSPG associated with lipid rafts. (A) C2C12 myoblasts were lysed and then fractionated in sucrose density gradients (5 to 45%). The 12 fractions collected were analyzed by immunoblotting for HSPGs, as explained in the legend of Fig. 1A, as well as the lipid raft membrane protein markers, GM-1 (ganglioside GM-1),. and Cav-1. Na^+/K^+ATPase (ATPase), were used as a nonlipid raft domain marker. On the left, the molecular weight standards are indicated in thousands. (B) Indirect immunocytolocalization analysis for glypican-1 (red) in C2C12 myoblasts treated with or without MβCD or PI-PLC. The nuclei were subjected to Hoechst staining (blue). The arrows indicate the punctuated pattern of glypican-1 on the cell surface, and the arrowheads point at the ECM-associated glypican-1. (C) C2C12 myoblasts were treated with MβCD or left untreated and then fractionated as described for panel A. The fractions were analyzed for glypican-1 and Cav-1 distribution by Western blotting. (D) As shown on the left, C6 clone myoblasts were transiently transfected with rat glypican-1 containing a FLAG epitope in its amino terminal (F-Gly) or the empty vector. After 48 h, the cells were fractionated as described for panel A. The 12 fractions were pooled into three groups: group I (fractions 1 to 4), group II (fractions 5 to 8), and group III (fractions 9 to 12). The fractions in each group were analyzed for the distribution of rat glypican-1 by using an anti-FLAG antibody. As shown on the right, in a parallel experiment, C6 myoblasts transfected with F-Gly were treated with MβCD or left untreated and then fixed and analyzed by immunofluorescence for the presence of the FLAG epitope. syn-3, syn-1, syn-2, and syn-4 represent syndecan-3, -1, -2, and -4, respectively.

of the ECM-associated glypican-1, since its fibrillar pattern over the cell disappears after the wash, without affecting the cell-associated glypican-1, as shown by the immunofluorescence analysis presented in Fig. 9B. To confirm that part of glypican-1 was associated with the ECM, we determined the colocalization of glypican-1 and the ECM protein laminin. Figure 9C shows that a fraction of glypican-1 colocalized with laminin. All these results suggest that the glypican-1 that frac-

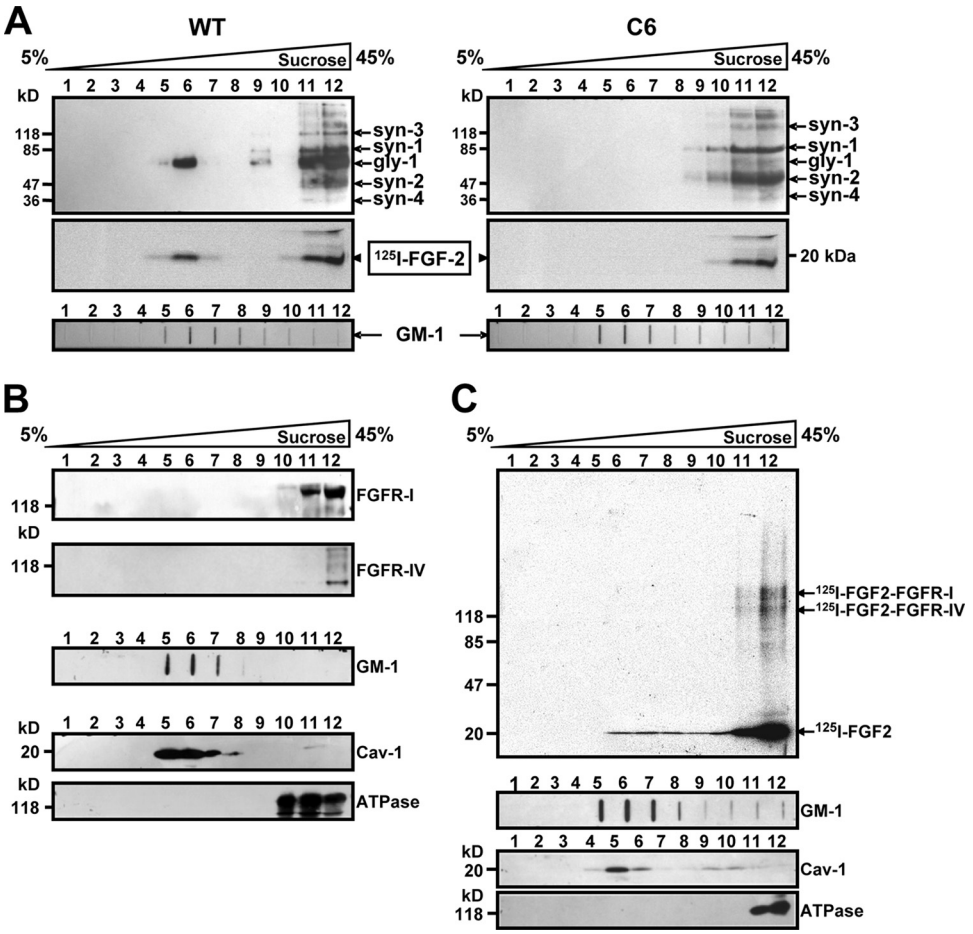

FIG. 7. Glypican-1 concentrates FGF-2 in lipid raft microdomains that exclude FGF-2 signaling receptors. (A) WT and C6 myoblasts were incubated with [^{125}I]–FGF-2 for 3 h at 4°C and then fractionated as described for Fig. 1. The fractions were analyzed for HSPGs by using anti-stub, as described for Fig. 1A, or exposed to a phosphorimager to detect the distribution of ^{125}I–FGF-2. (B) C2C12 myoblasts were fractionated as explained for Fig. 7A. The fractions were analyzed for the distribution of FGFR-I and FGFR-IV as well as membrane distribution markers Cav-1, GM-1, and Na^+/K^+ATPase (ATPase). (C) FGF-2 cell surface receptors in C2C12 myoblasts were affinity cross-linked to ^{125}I–FGF-2 at 4°C. The cells were lysed and fractionated as explained for Fig. 7A. Aliquots of each fraction were separated on SDS-PAGE (4 to 10%). The gel was dried and exposed to a phosphorimager (upper panel) or analyzed by Western immunoblotting for Cav-1, GM-1, and ATPase. ^{125}I–FGF-2–FGFR-I and ^{125}I–FGF-2–FGFR-IV correspond to ^{125}I–FGF-2 cross-linked to FGFR-I and FGFR-IV, respectively.

tionates in nonraft domains corresponds to an ECM-associated HSPG.

Since glypican-1 was found to be associated with lipid raft domains and was also found to be present at the ECM, we investigated the issue of whether myoblast glypican-1 associated with the plasma membrane, or with the ECM, is required for its negative role with respect to FGF-2-dependent signaling and skeletal muscle differentiation. Figure 10 shows that in myoblasts transiently transfected with an shCtrl and an expression vector for enhanced green fluorescent protein (E-GFP), myogenin expression was detected in the nuclei (Fig. 10A, D, and G). Consequently, these cells were also able to fuse with forming myotubes (Fig. 10I). In contrast, WT myoblasts transiently cotransfected with shGly and an expression vector for E-GFP were unable to express nucleus-associated myogenin after 2 days under differentiation conditions (arrows in Fig. 10B, E, and H), confirming that in the absence of glypican-1 this early step in the muscle differentiation process is abolished. These glypican-1-deficient cells, after 4 days under differentiation conditions, were unable to fuse with forming myotubes that expressed myosin (Fig. 10C and F). Interestingly, these results occurred despite the fact that the glypican-1-negative E-GFP-expressing myoblasts (green) were surrounded by glypican-1 present at the ECM network, as evidenced by specific glypican-1 immunostaining (arrowheads). Figure 10J shows quantification of this experiment. The left panel indicates that almost 40% of the nuclei of control transfected E-GFP-expressing myoblasts were positive for myogenin, whereas in the shGly-transfected myoblasts, this value was less than 5%. The right panel shows that almost 35% of the control transfected myoblasts were able to fuse with myosin-expressing myotubes, as revealed by the coexpression of myosin and E-GFP. In contrast, less than 10% of the shGly-transfected myoblasts were able to fuse with myosin-expressing myotubes. These results strongly suggest that glypican-1 present on the plasma membrane in lipid raft domains is required for successful skeletal muscle differentiation independent from the ECM-associated glypican-1.

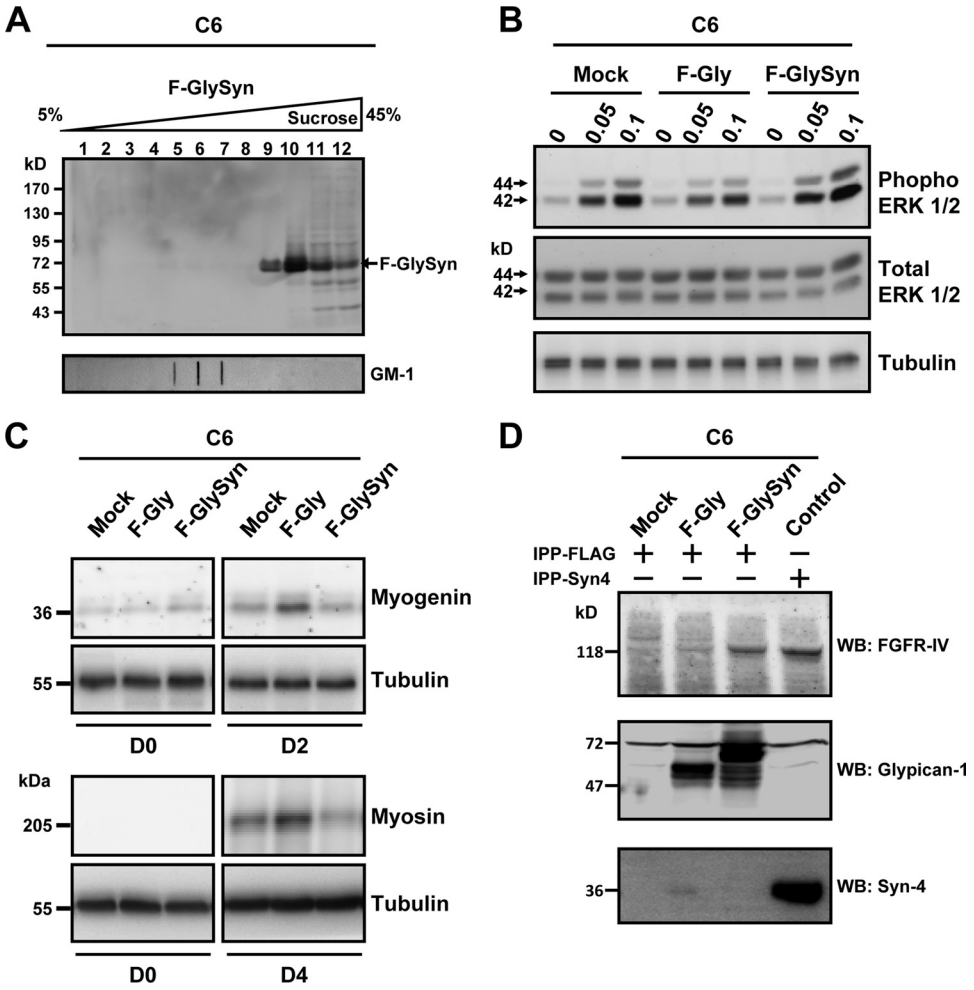

FIG. 8. Expression of glypican-1 outside the lipid raft acts like that of a FGF-2 coreceptor. (A) C6 myoblasts were transiently transfected with a chimeric HSPG (F-GlySyn) composed of the extracellular domain of rat glypican-1 and the transmembrane and cytosolic domain of mouse syndecan-1, containing a FLAG epitope. After 48 h, the cells were lysed and fractionated as described for Fig. 6A. The distribution of F-GlySyn was evaluated by anti-FLAG analysis. (B) C6 myoblasts were transfected with or without rat glypican (F-Gly) or F-GlySyn and treated with increasing concentrations of FGF-2. The phospho-ERK1/2, total ERK1/2, and tubulin levels were determined by immunoblot analysis. (C) C6 myoblasts were transfected with or without rat glypican (F-Gly) or F-GlySyn and induced to differentiate for 2 (D2) or 4 (D4) days to determine the myogenin, myosin, and tubulin levels by immunoblot analysis. (D) WT or C6 myoblasts were transiently transfected as described for panel C. After 48 h, the cells were lysed and the extracts immunoprecipitated with an anti-FLAG antibody or anti-mouse syndecan-4. The immunoprecipitates were analyzed by Western blotting for the presence of FGFR-IV, F-Gly, and F-GlySyn with an anti-anti-glypican-1 or syndecan-4.

DISCUSSION

In this paper we have shown that glypican-1 is required for a proper skeletal muscle differentiation process. Myoblasts with low levels of glypican-1, produced by transient transfection with either shGly or a stable clone that constitutively expresses this shRNA (C6 myoblast clone), showed low levels of myogenin and myosin with a diminished fusion index compared to WT myoblasts after having been induced to differentiate. Another marker of skeletal muscle differentiation, namely, Cav-3 (33), presents the same behavior. These defective consequences are glypican-1 dependent, since reexpression of glypican-1 in the C6 myoblasts with rat glypican-1 restores myosin and Cav-3 expression as well as myotube formation. The muscle differentiation process depends on the expression levels of glypican-1, since other clones, which expressed intermediate levels of glypican-1 compared to WT and C6 myoblasts, expressed medium levels of myosin when induced to differentiate (data not shown).

It has been well established that FGF-2, a strong myogenesis inhibitor (13, 42, 57, 77), diminishes the expression of the master gene myogenin (69, 70). Thus, its signaling must be finely controlled. Skeletal muscles mainly express FGFR-I and FGFR-IV (41), which both have a high affinity for FGF-2 (56). FGFs are normally present in muscle tissue and appear to be released upon injury and are expressed at higher levels during regeneration (22, 23, 74). The two receptors have different expression patterns during the muscular differentiation process. Expression of FGFR-I is temporally unchanged during the initial days and diminishes later, but it is still present during muscle differentiation (39, 41, 60). In contrast, expression of FGFR-IV is upregulated during this process (41, 87) and has

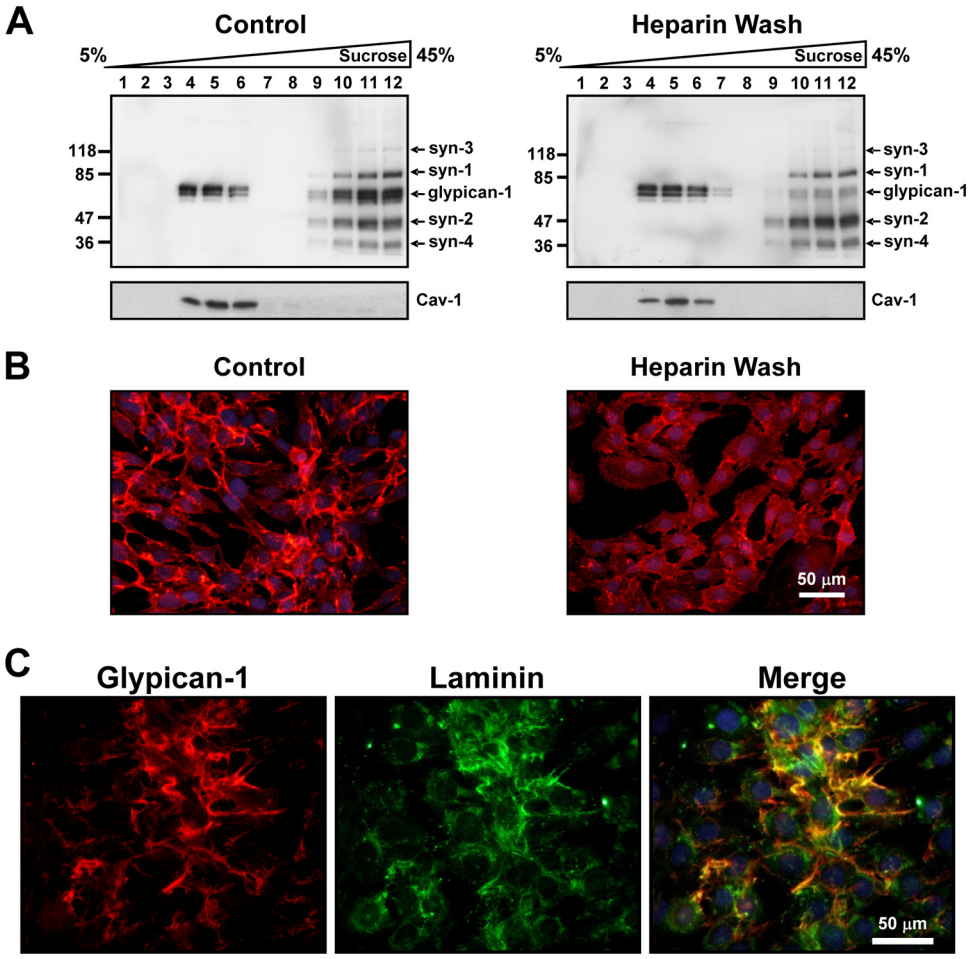

FIG. 9. Glypican-1 is present in the plasma membrane lipid rafts and in the extracellular matrix. (A) WT myoblasts were fractionated as shown in Fig. 7 after being washed with heparin in PBS Ca^{2+}/Mg^{2+} (Heparin Wash) or PBS Ca^{2+}/Mg^{2+} alone (control). The fractions were analyzed for HSPG core proteins and Cav-1 distribution. (B) Indirect immunofluorescence for glypican-1 (red) of cells treated as described for panel A. (C) Indirect immunofluorescence for glypican-1 (red) and laminin (green) in WT myoblasts.

been proposed to be essential for muscle regeneration (89). Since the signaling of FGF-2 through its receptor depends on the presence of HSPGs (65, 86), regulation by these FGF-2 coreceptors seems to attenuate FGF-2-dependent signaling, thus allowing myogenesis. In different systems, it has been shown that syndecans (6, 19, 28, 32, 42, 83, 88) and glypicans (52, 76, 80) have the ability to bind FGF-2, modulating its binding and signaling.

We, among others, have previously shown that HSPGs are essential for FGF-2-mediated signaling in skeletal muscle cells (17, 21, 32, 42, 55, 65). It has been demonstrated that syndecan-1 and -3 are directly involved in this phenomenon, acting as coreceptors of FGF-2 in myoblasts (32, 42). This is crucial, since myoblasts that do not express HSPGs, or are deficient in some of its forms, represent an affected process of skeletal muscle formation.

The expression of all syndecans is downregulated during the skeletal muscle differentiation process (32, 36, 43), suggesting that this might be associated with diminished sensitivity to the inhibitory effect of FGF-2. In contrast, the expression level of glypican-1 is constant through this process; hence, it represents the main HSPG present during myogenesis (8, 36).

Our results unequivocally demonstrate that glypican-1 is required for terminal myogenesis, which raises the issue of how glypican-1 regulates FGF-2-dependent signaling during the muscle differentiation process. Our experimental evidence indicates that in the absence of glypican-1, the binding of FGF-2 to its receptors augments, increasing the activation of the MAPK ERK1/2 and PI3K/AKT pathways and the FGF-2-dependent inhibition of myogenin expression. These effects were directly associated with the absence of glypican-1, since rat glypican-1 reexpression restored FGF-2-dependent signaling to near-WT levels. The blockage of FGF-2 activity present in the differentiation medium resulting from the use of a soluble form of FGFR-I or a neutralizing antibody against FGF-2 partly restored the altered muscle differentiation process in the glypican-1-deficient myoblast, suggesting that the deleterious effect of the absence of glypican-1 during myogenesis is a consequence of an increased sensitivity to FGF-2.

The formation of the ternary signaling complex involving

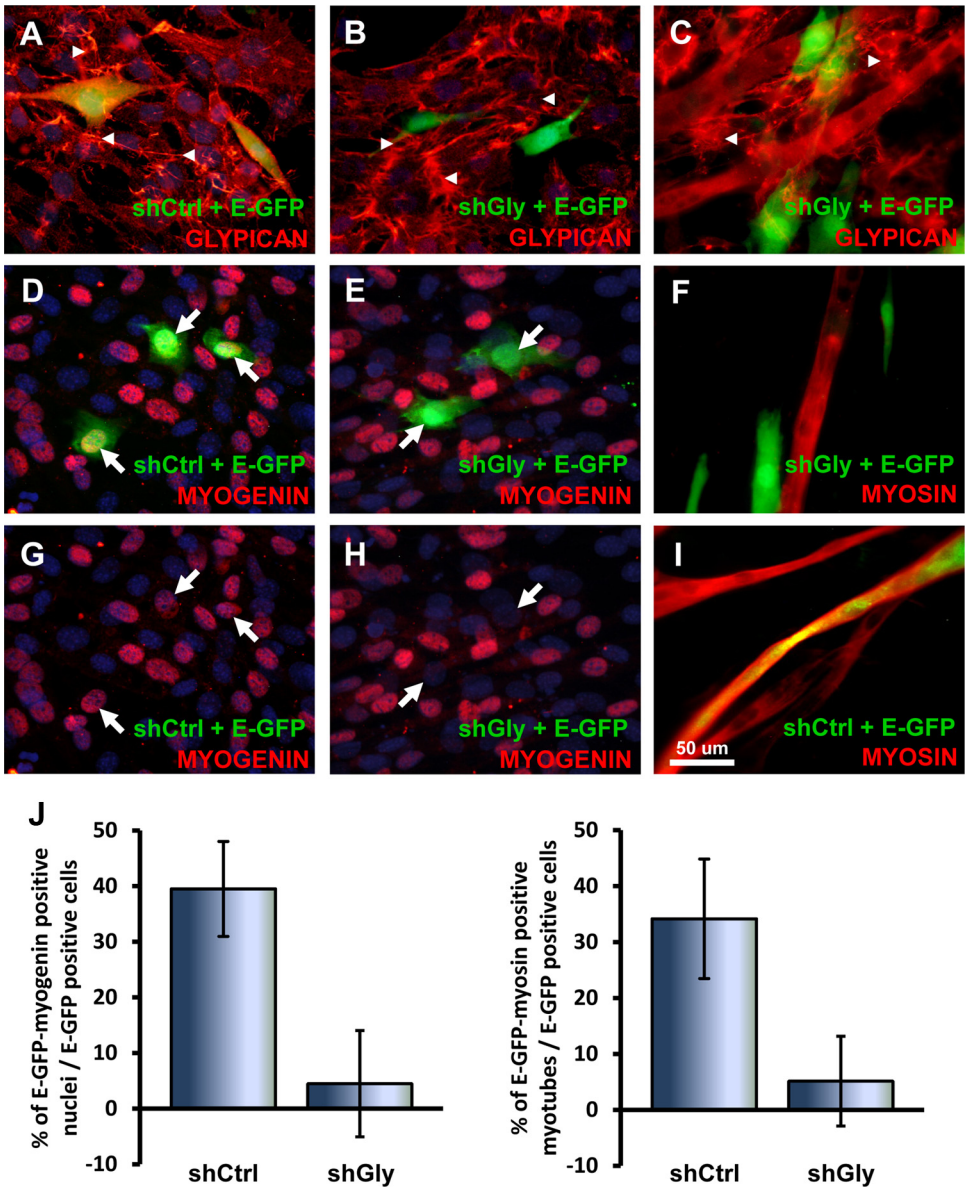

FIG. 10. Glypican-1 is required on the plasma membrane for a proper muscular differentiation process independent from extracellular matrix-associated glypican-1. C2C12 myoblasts were transiently cotransfected with scrambled shRNA (shCtrl) and a plasmid containing the sequence for E-GFP (A, D, and I) or shGly (B, E, C, and F). At 48 h after transfection, the myoblasts were induced to differentiate for 2 days (A, B, D, and E) or 4 days (C, F, and I). The cells were fixed and analyzed by immunofluorescence for glypican-1 (red) (A, B, and C), myogenin (red) (D and E), or myosin (red) (F and I). Panels G and H present the same images as panels D and E, respectively, but without the E-GFP signal, in order to better visualize myogenin nuclear staining. The arrowheads indicate the ECM-associated glypican-1. The arrows indicate the nuclei of transfected cells. (J) Shown on the left is a quantification of the cotransfected myoblasts (shCtrl/E-GFP or shGly/E-GFP) containing myogenin-positive nuclei compared to the total cotransfected cells (E-GFP positive) after 2 days of differentiation of 10 random fields. Shown on the right is a quantification of the E-GFP-myosin-positive myotubes compared to the total number of E-GFP-expressing cells after 4 days of differentiation. The data correspond to the means ± standard errors of the results obtained with 10 random fields.

HSPG, FGF-2, and FGFR (71) requires that these three components physically interact on the plasma membrane. We show that in myoblasts, glypican-1 is the only HSPG found associated with lipid raft membrane domains, away from all the syndecans and FGFRs. The localization in raft domains is sustained by low-density fractionation in the sucrose gradients and cofractionation with specific lipid raft domain markers. Glypican-1 shows a punctuated appearance, which typically characterizes lipid raft domain localization, and disappears after MβCD treatment (4). This pattern corresponds to plasma membrane glypican-1, since it totally disappears after treatment with PI-PLC. Glypican-1 localization in raft membrane domains is reinforced by the total abolishment of staining in glypican-1-deficient cells and the reappearance of the punctuated staining after reexpression of rat glypican-1. In contrast, all the syndecan forms and FGFRs cofractionated in nonlipid

raft domains, as determined by cofractionation of specific markers at high-density sucrose fractions. Other authors have suggested that clustering of syndecan-1 and -4 with antibodies, or after treatment with FGF-2, induces a relocalization of part of this HSPG from nonraft to raft microdomains in lymphoid and epithelial cells, respectively (50, 81). We did not observe any change in the distribution of HSPGs when myoblasts were treated with FGF-2 (data not shown). This indicates that glypican-1 remained the only HSPG associated with lipid rafts under our experimental conditions.

These results suggest that glypican-1 could be sequestering FGF-2 in lipid rafts, away from its transducing receptors. Our experiments indicate that FGF-2 binds and comigrates with glypican-1 in myoblast isolated membrane raft domains, since no FGF-2 was found in lipid rafts of myoblasts deficient for glypican-1. FGF-2 bound to FGFRs located at the cell surface fractionated in nonraft membrane domain fractions, as determined by cross-link assays. Furthermore, glypican-1 does not interact with FGFR-IV, as determined by coimmunoprecipitation experiments. This suggests that the FGF-2–FGFR complex is formed and maintained in a different spatial localization than glypican-1. The notion that colocalization of HSPG with FGFRs is critical for FGF-2 signaling is reinforced by the experiments involving expression of the F-GlySyn chimeric form. Despite containing a glypican-1 ectodomain, this HSPG was expressed in nonraft domains, most likely as a consequence of the presence of syndecan cytoplasmic and transmembrane domains on its structure. This chimeric form increased FGF-2-dependent signaling, interacting with FGFR-IV at least.

Since glypican-1 is endogenously processed to a soluble form that is incorporated in the ECM (8) and colocalizes with laminin, the possibility that glypican-1 present in the ECM is sequestering FGF-2 cannot be excluded. Glypican-1-deficient myoblasts did not express myogenin or fuse with control myoblasts, which expressed myogenin and later formed elongated myotubes. These processes occurred in an ECM enriched with glypican-1 and synthesized and processed by the control myoblast. This suggests that glypican-1 present in raft membrane domains is the required form for proper muscle differentiation processes and is probably responsible for the inhibitory effect on FGF-2 bioavailability.

Other functions for glypican-1, besides the inhibitory effect on FGF-2 availability, cannot be excluded. HSPGs interact with several ECM constituents (5), and glypican-1-deficient myoblasts present a diminished capability for fusing and forming elongated myotubes. This might reflect the possibility of other functions of glypican-1. Since HSPG can bind several ligands (27), such as Wnt (15), bone morphogenic protein (BMP), FGF (52, 76, 80), sonic hedgehog (16, 29), distinct members of TGF-β (47, 66), PDGF (34, 35, 58, 68), and HGF (3, 46), it is highly possible that glypican-1 might have other functions in the raft membrane domain. We tested the signaling response to TGF-β-1, PDGF, and HGF in the presence or absence of glypican-1. We did not detect any differences between glypican-1-deficient and WT myoblasts in the experiments performed with TGF-β and PDGF, suggesting that the response to these growth factors does not depend on glypican-1. However, we detected a decrease in the signaling response to HGF in glypican-1-deficient compared to WT myoblasts. This might indicate that glypican-1 is directly or indirectly involved in the signaling response to HGF. Nevertheless, evidence concerning localization of c-Met, the receptor for HGF, in membrane microdomains is controversial (73). It is worth mentioning that it has been shown that in myoblasts, BMP receptor type II (BMP-RII) is located in lipid raft membrane domains (37) and that BMP-RII and BMP-RIA are upregulated during myogenesis (1), although there is no functional evidence for this colocalization.

In summary, we have shown that glypican-1 located in membrane raft domains diminishes the bioavailability of FGF-2, sequestering this growth factor away from its transducing receptors. As a consequence, a decrease in FGF-2-dependent signaling occurs, allowing skeletal muscle differentiation to succeed. This novel mechanism of sequestering FGF-2 in lipid rafts, together with the downregulation of the syndecans which coreside with FGFRs, might be essential to ensure successful skeletal muscle differentiation during development and muscle regeneration.

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