OCI-5/Rat Glypican-3 Binds to Fibroblast Growth Factor-2 but Not to Insulin-like Growth Factor-2*

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OCI-5 encodes the rat homologue of glypican-3, a membrane-bound heparan sulfate proteoglycan that is mutated in the Simpson-Golabi-Behmel overgrowth syndrome. OCI-5 and glypican-3 are 95% identical. It has been recently suggested that glypican-3 interacts with insulin-like growth factor-2 (IGF-2) and that this interaction regulates IGF-2 activity. We report here that we have transfected OCI-5 into two different cell lines, and we have not been able to detect an interaction between the OCI-5 proteoglycan produced by the transfected cells and IGF-2. On the other hand, we have found that OCI-5 interacts with FGF-2, as has already been shown for glypican-1. This interaction is mediated by the heparan sulfate chains of OCI-5 because it can be inhibited by heparin or by heparitinase.

Glypicans are a family of heparan sulfate proteoglycans that are bound to the cell membrane through a glycosylphosphatidylinositol anchor (1). In mammalian cells four members of this family have been characterized (2–5). Although the function of glypicans is still poorly understood, David and collaborators have recently provided convincing evidence showing that glypicans can interact with fibroblast growth factor-2 (FGF-2) and stimulate occupancy and signaling of the FGF receptor-1 (6). This is not surprising because it is well established that many heparan sulfate chains can interact with heparin-binding growth factors such as FGF-2 (7–9). Recently, it was reported that glypican-3 can interact with IGF-2, suggesting that the SGBS phenotype is the result of altered regulation of IGF-2 activity. In this study we have transfected OCI-5, the rat homologue of glypican-3 (2, 13) into WI-L2, a human lymphoblastic cell line, and into HT-29, a human colo-

rectal carcinoma cell line. We report that OCI-5 produced by these cells binds to FGF-2. We have not been able, on the other hand, to show any interaction with IGF-2.

MATERIALS AND METHODS

OCI-5 Expression Vectors and Transfections—The introduction of the hemagglutinin A (HA) epitope into the OCI-5 cDNA has been previously described (2). The HA-tagged OCI-5 cDNA was introduced into the pEFP-BOS vector (14). WI-L2 cells were transfected by electroporation and selected in the presence of 800 μg/ml of geneticin. Cells expressing OCI-5 were selected by fluorescence-activated cell sorter after immunostaining with an anti-HA antibody. HT-29 cells were transfected with Lipofectin (Life Technologies, Inc.) and selected in the presence of 800 μg/ml of geneticin. Individual clones were isolated with cloning cylinders.

Cell Culture—WI-L2 cells were cultured in RPMI 1640, 10% fetal calf serum, and HT-29 cells in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum. Both cell lines were obtained from ATCC.

Characterization of OCI-5 in Transfected Cells—Confluent OCI-5-transfected media of WI-L2 and HT-29 cells were collected as described below and immunoprecipitated using the anti-HA 12CA5-I monoclonal antibody (20 μg/ml) and protein-Sepharose beads. The immunoprecipitates were then rinsed twice with wash solution (0.1% Nonidet P-40, 5 mM EDTA, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 mM EDTA). Once resuspended in 100 μl of enzyme buffer, the immunoprecipitates were incubated in the presence or the absence of 20 milliunits chondroitinase ABC (Sigma) or 2.5 milliunits of heparitinase (Seikagaku) for 3 h at 37 °C. The digested immunoprecipitates were then transferred to reducing sample buffer, boiled for 5 min, and run on a 4–9% polyacrylamide gradient gel. The proteins were transferred to a PVDF membrane (DuPont NEN) and subjected to Western blotting. Briefly, the membranes were blocked in blocking buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20, 5% nonfat dry milk) for 2 h at room temperature (or overnight at 4 °C) and then incubated for 2 h with 1 μg/ml 12CA5-I antibody at room temperature. After incubation with the anti-mouse secondary antibody for 1 h, bands were detected using ECL reagents (Amersham Corp.).

Cellular Adhesion Assay—96-microwell plates were coated a day in advance. Briefly, 20 μg/ml stock solutions of BSA, IGF-2 (Vector Bio-systems), and basic FGF (ID Labs) were serially diluted 1:2 in PBS. Wells were left overnight at 4 °C, and then the unbound proteins were removed by aspiration. Each well was then washed twice with PBS and blocked with 100 μl of blocking solution (2.5 mg/ml BSA in serum-free RPMI 1640) for at least 2 h at 37 °C. In the meantime, cells were harvested, washed three times in the blocking solution, and then diluted in blocking solution at 8 × 10⁴ cells/ml. 100 μl of the cell suspension was added to each well, and cells were allowed to attach to the coated wells for 1 h at 37 °C. For glycosaminoglycan inhibition assays, blocking was allowed to progress for another hour in the presence of 100 μg/ml heparin, dermatan sulfate, or chondroitin sulfate (all from Sigma) prior to cell attachment. Unattached cells were removed by gently aspirating the blocking solution with a micropipettor, and wells were washed twice with 100 μl of PBS. Attached cells were fixed with 100 μl of 3% paraformaldehyde in PBS for 20 min at 37 °C and then stained with 100 μl of 3% toluidine blue overnight at room temperature. The following day the dye was dumped out, and the wells were washed with water. The plates were dried and then read spectrophotometrically at 570 nm.

Collection of Conditioned Media—Conditioned media were collected from cells that were grown at high density overnight under serum-free conditions and concentrated to desired volumes using Centricon-10 filters (Amicon) at 4 °C. Proteinase inhibitors and Nonidet P-40 (final concentration of 0.1%) were added to reconstitute prior to immunoprecipitation. For ligand blots, nonreducing sample buffer was added, and the samples were stored at −20 °C.

Ligand Blotting—Concentrated conditioned media were run on a 4–9% polyacrylamide gel gradient, and proteins were then transferred to a PVDF membrane. The transferred membrane was immediately stained with 0.1% amido black in 10% acetic acid. The blots were washed three times with 100% methanol and dried overnight. The membranes were stained with 100 μl of PBS. Attached cells were fixed with 100 μl of 3% paraformaldehyde in PBS for 20 min at 37 °C and then stained with 100 μl of 3% toluidine blue overnight at room temperature. The following day the dye was dumped out, and the wells were washed with water. The plates were dried and then read spectrophotometrically at 570 nm.

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§ The abbreviations used are: FGF, fibroblast growth factor; SGBS, Simpson-Golabi-Behmel overgrowth syndrome; IGF, insulin-like growth factor; HA, hemagglutinin A; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PVDF, polyvinylidene difluoride.

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immersed in TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) with 3% Nonidet P-40 for 30 min at 4 °C. The membrane was then blocked for 3 h with 1% BSA in TBS. After a brief rinse with TBS-T (TBS with 0.1% Tween 20), the membrane was incubated with 1 μCi/ml of \(^{125}\text{I}\)IGF-II (ICN, 214 μCi/ug) in TBS-T overnight at 4 °C. The membrane was then washed twice with TBS-T for 20 min each and then three times with TBS for 30 min each. After drying the membrane was exposed to a PhosphorImager screen.

**Binding Assay**—Conditioned media, collected as described above, were immunoprecipitated with a 1:10 v/v ratio of a 12CA5-I/protein A-Sepharose slurry (12CA5-I antibody prebound to protein A beads) overnight at 4 °C. When indicated, immunoprecipitates were then subjected to heparitinase digestion as described above. Digested beads were washed three times with binding buffer (0.5% BSA in PBS) and resuspended in a final volume of 500 μl. 50 ng/ml of FGF-2 or 1 μg/ml of IGF-2 were then added, and binding was permitted for an additional 2 h at 4 °C on an orbital shaker. The immunoprecipitates were then washed three times in PBS, reducing sample buffer was added, the samples were boiled for 5 min, and electrophoresis was performed with 15% polyacrylamide gel. Proteins were transferred to a PVDF membrane and Western blotted as described above with either a primary mouse anti-FGF-2 or anti-IGF-2 antibody (both from Upstate Biotechnology Inc.).

**RESULTS**

WI-L2 and HT-29, two cell lines that do not express OCI-5/ glypican-3, were transfected with an expression vector containing an HA-tagged OCI-5 cDNA. WI-L2 is a human lymphoblastic cell line that does not bind significantly to FGF-2-coated cultured dishes (15). Based on this property this cell line was previously used to clone syndecan-1 with a ligand affinity cloning procedure designed to identify molecules that are able to interact with FGF-2. We have previously reported that OCI-5-transfected COS-1 cells secrete detectable amounts of this proteoglycan into the medium (2). Fig. 1 shows that this is also the case with OCI-5-transfected WI-L2 and HT-29 cells. It can also be seen in this figure that heparitinase, but not chondroitinase, is able to reduce the molecular weight of the secreted OCI-5, indicating that, like in COS-1 cells (2), OCI-5 expressed by WI-L2 and HT-29 cells display only heparan sulfate chains. It is important to note that because reducing conditions were used for the electrophoresis, Fig. 1 shows that the conditioned media from WI-L2 and HT-29 cells contain significant amounts of a 41-kDa band recognized specifically by the anti-HA antibody. Under nonreducing conditions, however, the amount of the 41-kDa band is significantly reduced, and a parallel increase in the glycanated OCI-5 is observed (compare Fig. 1 with

**Fig. 1. Characterization of OCI-5 secreted by WI-L2 and HT-29 cells.** OCI-5 from conditioned media were immunoprecipitated with 12CA5 antibody, incubated with buffer alone (lanes 1, 4, 7, and 10), heparitinase (lanes 2, 5, 8, and 11), or chondroitinase (lanes 3, 6, 9, and 12) and analyzed by Western blot with the 12CA5 antibody. WI-L2 wild type (lanes 1, 2, and 3), WI-L2 + OCI-5 (lanes 4, 5, and 6), HT-29 wild type (lanes 7, 8, and 9), and HT-29 + OCI-5 (lanes 10, 11, and 12) are shown. The 70-kDa OCI-5 core protein (filled arrowhead) and 41-kDa degradation product (open arrowhead) are indicated. The numbers on the right represent molecular mass markers.

**Fig. 2. Binding assays.** Conditioned media from WI-L2 + OCI-5 (lanes 2–5) and WI-L2 wild type (lanes 6–9) were immunoprecipitated with the anti-HA antibody, incubated at 37 °C for 3 h in the presence (lanes 2, 4, 6, and 8) or the absence (lanes 3, 5, 7, and 9) of heparitinase, and then 1 μg/ml of IGF-2 (lanes 2, 3, 6, and 7) or 50 ng/ml of FGF-2 (lanes 2, 3, 6, and 7) were added at 4 °C for 2 h. Immunoprecipitates were analyzed by Western blot with anti-FGF-2 and anti-IGF-2 antibodies. Lane 1 contains recombinant FGF-2 (5 ng), lane 10 contains recombinant IGF-2 (25 ng). The 70-kDa OCI-5 core protein (filled arrowhead) and 41-kDa cleavage product (open arrowhead) are indicated. The numbers on the right represent molecular mass markers.

Fig. 4A). Similar findings have been reported for K-glypican (5). These results suggest that there is a proteolytic cleavage site within the OCI-5 core protein and that at least a pair of cysteine residues form a disulfide linkage between the NH₂- and COOH-terminal sides of the cleavage site. Because the HA epitope is located at the NH₂ terminus, only this part of OCI-5 can be detected under reducing conditions with the anti-HA antibody. This 41-kDa cleavage product, however, does not contain the potential glycosaminoglycan attachment sites.

To investigate the interaction of OCI-5 with FGF-2 and IGF-2, conditioned media from OCI-5-transfected WI-L2 cells were collected, and OCI-5 was immunoprecipitated by using the anti-HA antibody bound to protein A beads. FGF-2 or IGF-2 were then added, and binding of these growth factors to the immobilized OCI-5 was determined by Western blot using antibodies against FGF-2 and IGF-2. Fig. 2 shows that although significant amounts of FGF-2 bound to the immunoprecipitated OCI-5, no detectable IGF-2 is found in the precipitates. If OCI-5 is previously incubated with heparitinase, the amount of bound FGF-2 is significantly reduced, indicating that the interaction of FGF-2 with OCI-5 is mediated by the heparan sulfate chains (Fig. 2).

We and others have shown that glypicans can be found both in the conditioned medium and on the cell surface (2–5). It was decided, therefore, to investigate whether cell-bound OCI-5 is able to interact with FGF-2 and IGF-2. A procedure previously used by others to demonstrate that syndecans interact with FGF-2 was used (15). WI-L2 cells transfected with OCI-5 were tested for attachment on microwells covered with FGF-2 or IGF-2. Although a significant dose-dependent attachment of WI-L2 cells to FGF-2 was observed (Fig. 3B), concentrations of IGF-2 ranging between 2.5 and 20 μg/ml failed to produce cell attachment above the nonspecific levels seen with BSA. The attachment to FGF-2 was completely inhibited if wells were precoated with heparin but not with chondroitin sulfate or dermatan sulfate (Fig. 3A). These results indicate that, like in the binding assay, the interaction between OCI-5 and FGF-2 is mediated by the heparan sulfate chains.

Because we were unable to detect any interaction between IGF-2 and OCI-5 in our cell-attachment and binding assays, we decided to determine if this interaction could be observed by ligand blot analysis. This is the experimental approach that
was used by Pilia et al. to show an interaction between glypican-3 and IGF-2 (10). For this assay we collected conditioned media from WI-L2 and HT-29 cells transfected with OCI-5. Fig. 4A shows a Western blot analysis under nonreducing conditions of OCI-5 found in the conditioned media of transfected WI-L2 and HT-29 cells. An IGF-2 ligand blot performed in parallel using the same conditioned media is shown in Fig. 4B. It can be seen that despite the presence of large amounts of glycanated and nonglycanated OCI-5 bands in the Western blot, the only bands detected in the ligand blot displayed molecular masses similar to isoforms of IGFBP4 (33–29 kDa), an IGF binding protein known to be produced by HT-29 cells (16) (Fig. 4B).

**DISCUSSION**

Here we show that OCI-5, the highly conserved rat homologue of glypican-3, can interact with FGF-2 and that this interaction is mediated by the heparan sulfate chains of OCI-5. In this respect, therefore, OCI-5/glypican-3 behaves like glypican-1 (6). We have been unable, on the other hand, to show an interaction between OCI-5 and IGF-2. This result differs from a previous report that showed by ligand blot analysis that glypican-3 can interact with IGF-2 (10). Besides failing to detect OCI-5/IGF-2 interaction by ligand blot analysis, we have also shown here that immobilized OCI-5 does not bind to IGF-2 and that it does not promote cell attachment to IGF-2-covered microwells. Currently we do not have an explanation for the different results obtained by us and Pilia et al. (10). These investigators have shown an interaction between glypican-3 and IGF-2 using conditioned medium from CaCo-2, a human colorectal carcinoma cell line. Because the type of GAG chains attached to proteoglycans can be cell type-specific (17), it could be argued that the difference in the results is due to the fact that we have not used the same cell line. However, the ligand blot shown by Pilia et al. (10) seems to indicate that IGF-2 interacts with the protein core of glypican-3, because the binding increases after digestion with chondroitinase AC. This would exclude cell specificity of GAG chains as an explanation for the different results. We have also performed ligand blot analysis with IEC-18, a cell line that expresses OCI-5 endogenously, but, like with WI-L2 and HT-29 cells, we were unable to detect any interaction between IGF-2 and OCI-5 (data not shown). It is also important to note that whereas we have been able to detect IGFBP-4 in our ligand blot from HT-29 cells, Pilia et al. (10) did not detect any of the IGFBPs that are reported to be produced by CaCo-2 cells (18).

The reason that prompted the investigation on the potential interaction of glypican-3 with IGF-2 is that there is considerable clinical overlap between SGBS patients and patients with the Beckwith-Wiedemann syndrome, an overgrowth syndrome in which the overexpression of IGF-2 is thought to play an important role (19). However, it also evident that there are numerous distinguishing features between both syndromes (11, 20).

Certainly the fact that OCI-5 can interact with FGF-2 does not rule out the possibility of OCI-5 interacting with other proteins, including molecules that regulate IGF-2 activity. In this respect, it is also important to note that the core proteins of glypicans are highly conserved during evolution (5, 10) and that the position of 14 cysteine residues is conserved in all glypicans. This raises the possibility that the core proteins of glypicans have functions other than just acting as anchors for GAG chains.

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