Glypican-1 Is a VEGF\textsubscript{165} Binding Proteoglycan That Acts as an Extracellular Chaperone for VEGF\textsubscript{165}\* 

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Glypican-1 is a member of a family of glycosylphosphatidylinositol anchored cell surface heparan sulfate proteoglycans implicated in the control of cellular growth and differentiation. The 165-amino acid form of vascular endothelial growth factor (VEGF\textsubscript{165}) is a mitogen for endothelial cells and a potent angiogenic factor in vivo. Heparin binds to VEGF\textsubscript{165} and enhances its binding to VEGF receptors. However, native HSPGs that bind VEGF\textsubscript{165} and modulate its receptor binding have not been identified. Among the glypicans, glypican-1 is the only member that is expressed in the vascular system. We have therefore examined whether glypican-1 can interact with VEGF\textsubscript{165}. Glypican-1 from rat myoblasts binds specifically to VEGF\textsubscript{165} but not to VEGF\textsubscript{121}. The binding has an apparent dissociation constant of \(3 \times 10^{-10}\) M. The binding of glypican-1 to VEGF\textsubscript{165} is mediated by the heparan sulfate chains of glypican-1, because heparinase treatment abolishes this interaction. Only an excess of heparin or heparan sulfates but not other types of glycosaminoglycans inhibited this interaction. VEGF\textsubscript{165} interacts specifically not only with rat myoblast glypican-1 but also with human endothelial cell-derived glypican-1. The binding of \(^{125}\text{I}-\text{VEGF}_{165}\) to heparinase-treated human vascular endothelial cells is reduced following heparinase treatment, and addition of glypican-1 restores the binding. Glypican-1 also potentiates the binding of \(^{125}\text{I}-\text{VEGF}_{165}\) to a soluble extracellular domain of the VEGF receptor KDR/flt-1. Furthermore, we show that glypican-1 acts as an extracellular chaperone that can restore the receptor binding ability of VEGF\textsubscript{165} which has been damaged by oxidation. Taken together, these results suggest that glypican-1 may play an important role in the control of angiogenesis by regulating the activity of VEGF\textsubscript{165}, a regulation that may be critical under conditions such as wound repair, in which oxidizing agents that can impair the activity of VEGF are produced, and in situations where the concentrations of active VEGF are limiting.

Glypican-1 is the prototype member of the glycosylphosphatidylinositol anchored cell surface heparan sulfate proteoglycans (HSPGs)\(^1\) implicated in cell adhesion and migration, lipoprotein metabolism, anticoagulation, and modulation of growth factor activities (1, 2). The glypicans family includes the human and rat glypicans (3, 4), rat cerebroglycan (5), rat OCI-5 (6) and its human homologue glypican-3 (7), K-glypican (8), glypican-5 (9), and the Drosophila daily (10). Mutations in daily affect cell division in the Drosophila visual system and lead to morphological defects in the eyes, antenna, genitalia, and wings (10). In addition, mutations in glypican-3 are responsible for the Simpson-Golabi-Behmel syndrome, a disease that is associated with specific congenital malformations and a predisposition to tumors (2, 7). Recent studies in mammalian cells indicated that glypican-1 binds to members of the fibroblast growth factor (FGF) family. Thus, OCI-5 binds to FGF-2, and glypican-1 interacts with at least three FGFs: FGF-1, FGF-2, and FGF-7 (11–13). Furthermore, glypican-1 modulates the biological activities of these growth factors, and this modulation can be stimulatory or inhibitory depending on the FGF type (11, 12). Taken together, these findings indicate that the glypicans play an important regulatory role in the control of cellular growth, differentiation, and morphogenesis.

Vascular endothelial growth factors (VEGFs) are mitogens for endothelial cells and are potent angiogenic factors in vivo. Five VEGF isoforms, designated VEGF\textsubscript{121}, VEGF\textsubscript{145}, VEGF\textsubscript{165}, VEGF\textsubscript{189}, and VEGF\textsubscript{206}, are generated via an alternative splicing mechanism from a unique gene (14–16). The active form of the VEGFs is a homodimer, but active heterodimers have also been observed. Two VEGF tyrosine-kinase receptor types have been characterized. These tyrosine-kinase receptors do not differentiate between the various VEGF forms. The KDR/flt-1 receptor mediates the mitogenic activity of VEGF, whereas flt-1 stimulates VEGF-induced cell migration (17). In addition, endothelial cells express VEGF\textsubscript{165}-specific receptors of unknown function. These receptors do not bind VEGF\textsubscript{121} or VEGF\textsubscript{145} (16, 18).

The best characterized VEGF forms are VEGF\textsubscript{121} and VEGF\textsubscript{165} (165- and 121-amino acid-long polypeptide, respectively). VEGF\textsubscript{165} contains the peptide encoded by exon-7 of the VEGF gene, whereas VEGF\textsubscript{121} lacks this peptide. The presence of exon-7 confers on VEGF\textsubscript{165} the ability to bind heparin-like molecules. Removal of heparan sulfates (HS) from the surface of endothelial cells by heparinase digestion reduces the binding of \(^{125}\text{I}-\text{VEGF}_{165}\) to its receptors, and addition of heparin re-

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\[1\] The abbreviations used are: HSPG, heparan sulfate proteoglycan; BSA, bovine serum albumin; FGF, fibroblast growth factor; FGF-1, acidic fibroblast growth factor; FGF-2, basic fibroblast growth factor; FGF-7, keratinoctye growth factor; GAGs, glycosaminoglycans; HS, heparan sulfate; PBS, Dulbecco's phosphate-buffered saline; VEGF, vascular endothelial growth factor; ELISA, enzyme-linked immunosorbent assay; HUVEC, human umbilical vein endothelial cell; PF4, platelet factor 4.
stores $^{125}$I-VEGF$_{165}$ binding. We have recently shown that part of the effect of heparin involves restoration of $^{125}$I-VEGF$_{165}$ binding to KDR/flk-1 by a chaperone-like effect that recovers the biological activity of VEGF$_{165}$ that was damaged by oxidizing agents (18). Such a role of heparin-like molecules may be important under conditions like wound repair, hypoxia-induced angiogenesis, or inflammation, in which oxidants or free radicals are produced and may damage VEGFs (19–21). Unlike VEGF$_{165}$, VEGF$_{121}$ is irreversibly inactivated by oxidizing reagents, and heparin cannot restore its receptor binding ability (18).

Recent evidence strongly suggests that VEGFs play a critical role in the process of tumor angiogenesis. This process is essential for tumor progression and for the subsequent process of tumor metastasis. A number of molecules that display some structural similarity to heparin such as suramin and pentosan sulfates exert an anti-angiogenic effect. These observations, together with the findings that HS-degrading enzymes can inhibit tumor angiogenesis (22), suggest that HSGLPs play an important role in the angiogenic process. So far, HSPGs that can bind VEGFs and modulate their biological activities have not been identified. Among glypicans, glypican-1 is the only member expressed in the vascular system (23, 24). We have therefore examined whether glypican-1 can interact with VEGFs and modulate their interaction with VEGF receptors. We show here that purified glypican-1 binds VEGF$_{165}$, with high affinity and supports its binding to heparan sulfate-treated endothelial cells. Furthermore, glypican-1 can also restore the receptor binding ability of oxidized VEGF$_{165}$ and can therefore be viewed as a native, cell-surface-localized proteoglycan that displays a chaperone-like activity.

**Experimental Procedures**

**Materials**—Human recombinant VEGF$_{165}$ and VEGF$_{121}$ were produced and purified from SF-9 insect cells as described previously (25–27). Human recombinant FGF-2, FGF-1, PF4, and FGF-7 were produced and purified from Sf-9 insect cells as described previously (25–27). Human recombinant FGF-2, FGF-1, PF4, and FGF-7 were produced and purified from Sf-9 insect cells as described previously (25–27).

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Glypican-1 by testing its ability to enhance binding of FGF-1 and FGF-2 to FGF receptor 1 and to modulate the biological activities of FGF-7 and FGF-1 as described previously (11).

**Radiodination of Glypican-1 and VEGFs**—Radiodination was carried out utilizing the chloramine T method as described previously (11, 33, 34). $^{125}$I-VEGF$_{165}$ was separated from free iodine using heparan-Sepharose column, and $^{125}$I-VEGF$_{121}$ was separated from free iodine using size exclusion chromatography on Sephadex-G25 as described (34). The specific activities of the $^{125}$I-VEGF$_{165}$ and the $^{125}$I-VEGF$_{121}$ were about $10^5$ cpm/mg. Radiolabeled glypican-1 was separated from free iodine by chromatography on DEAE-Sephaloc (11). Specific activities of iodinated glypican-1 were in range of 6–12 $10^6$ cpm/mg.

**Binding of $^{125}$I-Glypican-1 to VEGF-coated Wells**—Binding of radioiodinated glypican-1 to wells coated with VEGF$_{165}$ and various other proteins was done essentially as described (35). Briefly, 0.2 $\mu$g of each protein in 100 $\mu$L of coating buffer were adsorbed to 96-well ELISA plates for 2 h at room temperature, and binding was performed with $^{125}$I-glypican-1 for 2 h at room temperature. Free $^{125}$I-glypican-1 was removed by three washes with wash buffer (35). Estimation of bound $^{125}$I-glypican-1 was done following solubilization with 0.2 M NaOH. All the experiments were done in triplicate and were repeated at least three times. Nonspecific binding was estimated as described under “Results,” and it was less than 10% of the total binding. Standard deviation between replicates in all the experiments was less than 10%.

**Purification of Glypican, Enzymatic Deglycosylation, and Isolation of Glypican-1-associated GAG Side Chains**—Glypican-1 was purified from salt extracts of subconfluent cultures of the rat myoblast cell line L6E9 by anion exchange chromatography with DEAE-Sephaloc followed by FGF affinity purification as described previously (11). Enzymatic deglycosylation was carried out in PBS containing $^{125}$I-glypican-1 and 0.5 units/ml of heparinase I and III. Incubation was for 2 h at 37°C. Degradation of the core protein of glypican-1 was carried out overnight with proteinase K ($0.5$ mg/ml). To ensure that digestion was complete, a parallel incubation was carried out in the presence of radioiodinated glypican-1, and digestion was monitored by SDS-polyacrylamide gel electrophoresis and autoradiography. The GAG side chains were then separated from the protease and degradation products by DEAE-Sepha-locel chromatography as described (11).

**Quantitation of GAG and HSPG**—The Safranin O dye that reacts with carboxyl and sulfate groups and can detect ng amounts of sulfated GAGs (32) was used to estimate GAG content.

The concentrations of glypican-1 are relative to its HS concentration, which accounts to about two-thirds of the total PG concentration (11). Prior to performing the study we examined the biological activity of glypican-1 by testing its ability to enhance binding of FGF-1 and FGF-2 to FGF receptor 1 and to modulate the biological activities of FGF-7 and FGF-1 as described previously (11).

**RESULTS**

**Glypican-1 Binds to VEGF$_{165}$**—A solid phase assay was utilized to investigate the ability of glypican-1 to bind VEGF$_{165}$ and VEGF$_{121}$. Increasing concentrations of radioiodinated glypican-1 were bound to microtiter plates precoated with 0.2 $\mu$g/well of VEGF$_{165}$ or VEGF$_{121}$, as well as FGF-7, which was previously reported to bind glypican-1 (11). As shown in Fig. 1, only VEGF$_{165}$ and FGF-7 bind similarly to $^{125}$I-glypican, whereas VEGF$_{121}$ or BSA did not exhibit any binding capacity. Binding was saturable and Scatchard analysis yielded an apparent dissociation constant of $1.2 \times 10^{-10}$ M for VEGF$_{165}$ and $3 \times 10^{-10}$ M for FGF-7 (Fig. 1B).

To test the specificity of this interaction we measured the ability of a panel of proteins to compete with $^{125}$I-glypican for binding to VEGF$_{165}$. As shown in Fig. 2, all three FGF mem-
BSA could not compete with 125I-glypican for binding to washed, and quantification of bound 125I-temperature, the wells were extensively described under “Experimental Procedures.” After 2 h of incubation at room temperature, the wells were extensively washed, and quantification of bound 125I-glypican-1 was done as described under “Experimental Procedures.”

To determine whether glypican-1-derived GAGs are involved in VEGF165 binding, we prepared heparinase-digested glypican. Heparinase treatment abolished maximal inhibition but about 10-fold lower than that of commercial HS. By contrast neither chondroitin sulfate nor hyaluronic acid were able to compete with glypican-1 for binding to VEGF165. These results establish that the HS side chains of glypican-1 mediate binding to VEGF165.

Glypican-1 Enhances the Binding of VEGF165 to VEGF Receptors—VEGF165 binds to three VEGF receptors on HUVEC-derived endothelial cells (28). The larger of these receptors is KDR/flk-1, whereas the identity of the other two is not yet known (18). Heparin enhances the binding of VEGF165 to all three receptors (28). Heparin is not a normal constituent of endothelial cells, whereas glypican-1 is known to be expressed in these cells (24). We have therefore examined whether glypican-1 can modulate the binding of VEGF165 to cell surface receptors in untreated and in heparinase-digested endothelial cells. Digestion of the endothelial cells by heparinase inhibited almost completely the binding of 125I-VEGF165 to the two smaller receptors, whereas the binding to KDR/flk-1 (18) was inhibited by about 80% (Fig. 4A, lane 3). Exogenously added glypican-1 restored the binding to all three receptors (Fig. 4A, lane 4). Glypican-1 also potentiated the binding of 125I-VEGF165 to the two smaller receptors in untreated cells (Fig. 4A, lane 2).

The ability of glypican-1 to potentiate the binding of VEGF165 to KDR/flk-1 was also confirmed utilizing a soluble extracellular domain of this receptor (flk-1/SEAP) in a cell-free assay (26). The potentiation of 125I-VEGF165 binding to the soluble receptor was concentration-dependent and was maximal at about 1 μg/ml of glypican-1 (Fig. 4B). These findings were also confirmed in cross-linking experiments (data not shown).

The ability of myoblast-derived glypican-1 to restore binding of VEGF165 to heparinase-treated HUVEC to a level that is higher than the binding observed in untreated cells strongly suggests that glypican-1 from endothelial cells also fulfills a similar function. We have therefore partially purified glypican-1 from HUVEC cells and have tested its ability to interact with VEGF165. VEGF165 was bound to nitrocellulose membranes and incubated with partially purified 35S-HSPGs. As shown in Fig. 5A, VEGF165 and FGF-2 bound 35S-HSPGs, whereas VEGF121, BSA, transferrin, and insulin did not exhibit any HSPG binding ability. Moreover, a monoclonal antibody directed against human glypican-1 interacted specifically with VEGF165 and FGF-2-bound HSPGs (Fig. 5B). These observations strongly suggest that HUVEC-associated glypican-1 is a candidate modulator of VEGF165 activity in these cells.

Glypican-1 Restores the Activity of Oxidized VEGF165—We have previously shown that oxidation of VEGF165 with agents such as H2O2 impairs its ability to bind to the KDR/flk-1

\footnote{D. Ron, unpublished results.}
receptor and that heparin restores the receptor binding capacity of VEGF165 (18). Because glypican-1 binds efficiently to VEGF165, we examined whether it displays a similar restorative capacity. As shown in Fig. 6, H2O2-treated VEGF165 lost the ability to efficiently compete with VEGF121 for binding to flk-1/SEAP, and addition of glypican-1 restored the receptor binding capacity of the oxidized VEGF165.

PF4 Inhibits the Binding of Glypican-1 to 125I-VEGF165 and Abrogates the Stimulatory Effect of Glypican-1 on 125I-VEGF165 Receptor Binding—PF4 is a heparin-binding protein that is synthesized by megakaryocytes, sequestered in platelets, and released from α-granules as a complex with chondroitin 4-sulfate proteoglycan (37, 38). PF4 displays an anti-angiogenic activity in vivo that is attributed in part to its heparin binding capacity. Thus, a peptide derived from the heparin-binding carboxyl-terminal domain of PF4 possesses anti-angiogenic properties (29). The receptor binding ability of VEGF165 and its mitogenic activity are inhibited by PF4 (35). Our results indicate that glypican may be one of the endothelial cell-associated heparan sulfate proteoglycans that bind VEGF165 and mediate its biological activity. We reasoned that PF4 may inhibit angiogenesis by preventing the interaction between VEGF and glypican. We have therefore examined whether PF4 can inhibit the binding of VEGF165 to glypican-1 and abrogate the stimulatory effect of glypican-1 on VEGF receptor binding. As shown in Fig. 7A, PF4 efficiently inhibited the binding of glypican-1 to VEGF165-coated wells. Half-maximal inhibition was obtained at a PF4 concentration of 100 ng/ml. Moreover, PF4 nullifies the stimulatory effect of glypican-1 on the binding of 125I-VEGF165 to flk-1/SEAP (Fig. 7B).

DISCUSSION

Heparin had been previously shown to bind to VEGF165 and to act as an accessory receptor that enhances the interaction of VEGF165 with its signaling receptors. Furthermore, heparin restores the bioactivity of damaged VEGF165 (18). However, because cells express several types of HSPGs on their surfaces but not heparin, it was important to identify native HSPGs that bind VEGF and modulate its activity. In the present study we have demonstrated for the first time an interaction of VEGF with the lipid anchored cell surface heparan sulfate proteoglycan glypican-1. Glypican-1 was found to interact with VEGF165.
and to modulate its receptor binding properties. We show that glypican-1 binds to VEGF165 via its heparan sulfate chains and that the binding is specific and saturable. The affinity of glypican-1 to VEGF165 is high and has an apparent dissociation constant of $1.2 \times 10^{-10}$ M. Glypican-1 not only binds to VEGF165 but is also capable of enhancing the interaction of VEGF165 with its signaling receptors both in cell-free binding assays and in heparinase-treated endothelial cells.

VEGF165 is a major angiogenic factor that is active in processes such as wound repair, hypoxia-induced angiogenesis, and inflammation (15), processes associated with the generation of oxidizing agents and free radicals. VEGF165 is inactivated by these agents (18), and therefore their presence may inhibit VEGF-induced angiogenesis. Therefore, repair mecha-

**Fig. 5.** Specific binding of HUVEC-derived glypican-1 to VEGF165, VEGF121 (V121), FGF-2, BSA, VEGF121 (V121), transferrin (TR), and insulin (INS) at concentrations of 2 µg/spot were dot-blotted on to a nitrocellulose filter. Binding of soluble 35S-HSPGs from HUVEC cells to the filters and immunodetection of bound glypican were carried out as described under “Experimental Procedures.” A, a specific binding of soluble 35S-HSPGs to nitrocellulose filters. B and C, immunodetection of bound glypican by monoclonal antibody S1 (B) or with a monoclonal anti-phosphotyrosine antibody (C). The filters were also autoradiographed at the end of the assay to confirm that bound 35S-HSPGs are detectable in both filters. D, a scheme indicating the positions of the various proteins on the filters.

**Fig. 6.** Glypican-1 restores the receptor binding ability of oxidized VEGF165. 125I-VEGF121 (20 ng/ml) was bound to ELISA dishes coated with flk-1/SEAP. The binding was performed in the absence or presence of 0.5 µg/ml of unlabeled VEGF121 (V121), VEGF165 (V165), H2O2-treated VEGF165 (V165-Gly), and H2O2-treated VEGF165 plus 3 µg/ml of glypican (V165-Gly).

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Fig. 7. PF4 inhibits binding of glypican-1 to VEGF165 and abrogates the stimulatory effect of glypican-1 on binding of VEGF to flk-1/SEAP. A, effect of PF4 on the binding of glypican-1 to VEGF165. Labeled glypican-1 (specific activity of 6 × 10⁶ cpn/μg) was bound to ELISA dishes coated with VEGF165 in the presence of increasing concentrations of PF4. Binding was quantified as described under "Experimental Procedures." B, PF4 abrogates the stimulatory effect of glypican-1 on VEGF165 binding to flk-1/SEAP receptor. Binding of 125I-VEGF165 (20 ng/ml) to flk-1/SEAP-coated wells was performed in the absence (open bars) or presence (shaded bars) of 2 μg/ml of PF4. When indicated, 1 μg/ml of glypican-1 (Gly) or heparin (Hep) were added.

Fig. 4A). These findings could also imply that endothelial cells express HSPG species that inhibit the binding of VEGF to its receptors.

Heparin-like molecules have long been implicated in the control of angiogenesis. The earliest indication that heparin may be involved in regulation of the angiogenic process was the finding that mast cells accumulate at the site of tumor angiogenesis before capillary ingrowth, that conditioned medium finding that mast cells accumulate at the site of tumor angiogenesis before capillary ingrowth may be involved in regulation of the angiogenic process was the control of angiogenesis. The earliest indication that heparin and HS play an important role in modulating the biological activity of the growth factors which bind to them. However, only recently were efforts taken to identify native HSPGs that bind and modulate the activity of these growth factors (11–13, 49). Our previous studies have shown that glypican-1 modulates the biological activity of FGF-2, suggesting a role for glypican-1 in the regulation of angiogenesis (11). The present study lends support to this hypothesis and further suggests that glypican-1 may play a more general modulatory role in angiogenesis by regulating the stability and activity of VEGFs.

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