

Cell Surface Glypicans Are Low-Affinity Endostatin Receptors

S. Ananth Karumanchi,^{1,7} Vivekanand Jha,^{1,7}
Ramani Ramchandran,^{1,7} Anil Karihaloo,^{1,9}
Leonidas Tsiokas,^{1,8} Barden Chan,¹
Mohanraj Dhanabal,¹ Jun-ichi Hanai,¹
Ganesh Venkataraman,³ Zachary Shriver,³
Nishla Keiser,³ Raghu Kalluri,¹ Huiyan Zeng,²
Debabrata Mukhopadhyay,² Robert L. Chen,⁵
Arthur D. Lander,⁵ Kazuki Hagihara,⁴ Yu Yamaguchi,⁴
Ram Sasisekharan,³ Lloyd Cantley,^{1,9}
and Vikas P. Sukhatme^{1,6}

¹Department of Medicine and the Cancer Center

²Department of Pathology

Beth Israel Deaconess Medical Center and
Harvard Medical School
Boston, Massachusetts 02215

³Division of Bioengineering and Environmental Health
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

⁴The Burnham Institute
La Jolla, California 92037

⁵Department of Developmental and Cell Biology
University of California
Irvine, California 92697

Summary

Endostatin, a collagen XVIII fragment, is a potent anti-angiogenic protein. We sought to identify its endothelial cell surface receptor(s). Alkaline phosphatase-tagged endostatin bound endothelial cells revealing two binding affinities. Expression cloning identified glypican, a cell surface proteoglycan as the lower-affinity receptor. Biochemical and genetic studies indicated that glypicans' heparan sulfate glycosaminoglycans were critical for endostatin binding. Furthermore, endostatin selected a specific octasulfated hexasaccharide from a sequence in heparin. We have also demonstrated a role for endostatin in renal tubular cell branching morphogenesis and show that glypicans serve as low-affinity receptors for endostatin in these cells, as in endothelial cells. Finally, antisense experiments suggest the critical importance of glypicans in mediating endostatin activities.

Introduction

Neovascularization is critical for tumor growth and metastasis. Angiogenesis, the formation of new capillaries through sprouting, is the primary process responsible for tumor neovascularization. This process depends upon the balance between pro- and anti-angiogenic molecules.

Vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) are two of the best-characterized proteins promoting angiogenesis. Inhibitors of this process include a wide array of compounds, with some of the most potent being proteolytic fragments of endogenous proteins. Notable among these is endostatin (ES), a 20 kDa C-terminal fragment of collagen XVIII, a relatively rare collagen present in vascular and renal tubular basement membranes (O'Reilly et al., 1997; Halfter et al., 1998). In vitro, ES blocks endothelial cell migration, promotes apoptosis, and induces cell cycle arrest with effects in the former assay at ng/ml doses (Dhanabal et al., 1999a; Dhanabal et al., 1999b; Sasaki et al., 1999; Yamaguchi et al., 1999). An in vivo role for ES has not been demonstrated, but exogenously administered recombinant ES is a potent angiogenesis inhibitor, causing tumor regression in several animal models (O'Reilly et al., 1997; Dhanabal et al., 1999a). In recent clinical trials, regression of bulky disease in a few patients has been reported with no toxicity.

Little is known about ES's mechanism of action, nor has there been a detailed structure-function analysis of the protein. Phosphorylation of intracellular adaptor protein Shb has been described (Dixelius et al., 2000), but no specific cell surface binding has been demonstrated. The ES crystal structure reveals an extensive basic patch of 11 arginines, which may serve as a binding site for heparin, and it was predicted that ES may inhibit angiogenesis by binding to heparan sulfate proteoglycans (HSPG) (Hohenester et al., 1998). ES mutants which lack heparin binding show decreased ability to inhibit FGF-2 and VEGF induced angiogenesis in chick chorioallantoic membrane angiogenesis assays (Sasaki et al., 1999), though other groups have not substantiated the importance of heparin binding in migration assays (Yamaguchi et al., 1999).

In this paper, we describe endothelial cell surface binding for ES with Scatchard analysis showing two binding affinities. Expression cloning identified glypican as the lower-affinity ES receptor. Glypicans are cell surface glycosyl-phosphatidylinositol (GPI) anchored HSPG, which bind various ligands and enhance formation of their receptor-signaling complexes (David and Bernfield, 1998; Bernfield et al., 1999). Furthermore, we demonstrate that the heparan sulfate-glycosaminoglycan (HSGAG) component of glypicans bind ES, and that glypicans are required for ES action in endothelial cells. Finally, we describe a role for endostatin in branching morphogenesis of renal epithelial cells. In addition to endothelial cells, ES binds renal tubular cells through glypicans with similar binding affinities, and glypicans are necessary for ES's role in inhibiting renal epithelial cell branching processes.

Results

ES Binds Endothelial Cells and Renal Tubular Cells and Is Functional in Both Cell Types

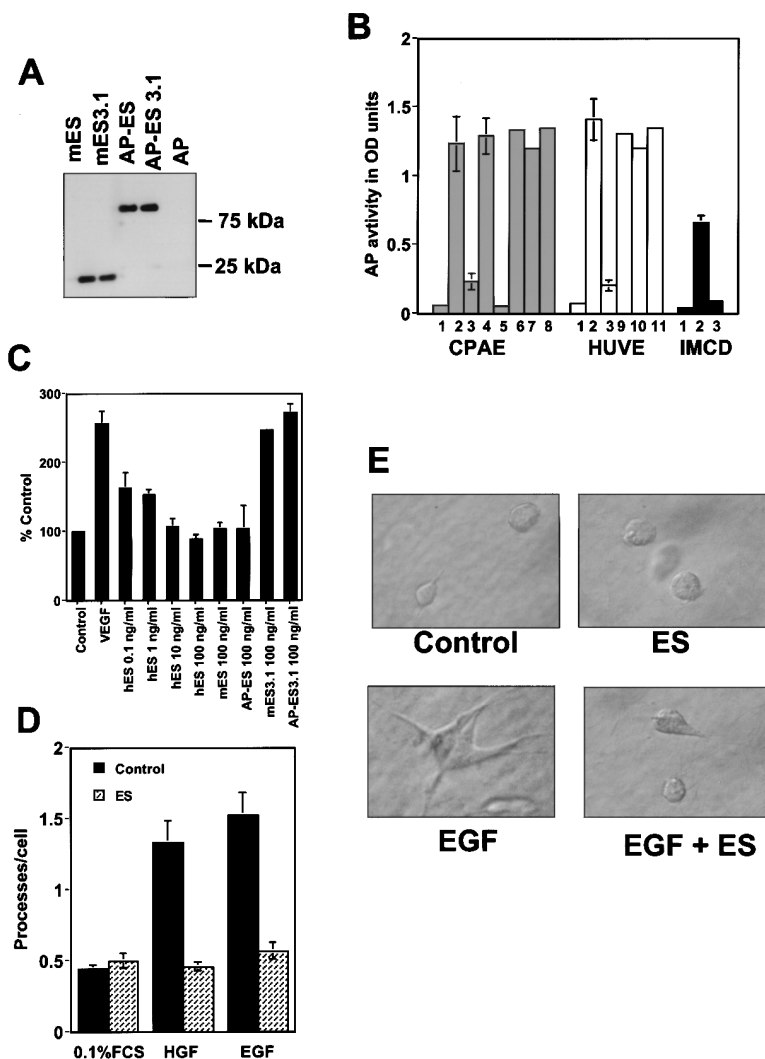
To identify cell surface binding molecules for ES, we generated alkaline phosphatase-tagged endostatin (AP-ES).

⁶ To whom correspondence should be addressed: (e-mail: vsukhatm@caregroup.harvard.edu).

⁷ These authors contributed equally to this study.

⁸ Present address: Department of Cell Biology and Warren Medical Research Institute, University of Oklahoma Health Science Center, 941 S. L. Young Blvd, Oklahoma City, Oklahoma 73104.

⁹ Present address: Department of Medicine, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06520.



The crystal structure of ES reveals a helix stabilized by two disulfide bonds which result in the hydrophobic side chains of phenylalanine residues 162 and 165 being presented to the outside of the molecule (Hohenester et al., 1998). We hypothesized that these side chains might form a high-affinity binding site in the hydrophobic environment of an ES receptor, and therefore changed these residues to alanines in an attempt to decrease the affinity for the receptor. The wild-type mouse endostatin (mES) and the mutant ES (mES3.1) were generated in soluble form in *Pichia* and as an AP-tagged form in mammalian cells (wild type referred to as AP-ES and mutant AP-ES3.1) (Figure 1A). We found that AP-ES, but not AP-ES3.1, bound endothelial cells specifically (Figure 1B). Also, mES competed for AP-ES binding, whereas mES3.1 failed to compete. Furthermore, several agents implicated in angiogenesis, like FGF-2, VEGF, heparin, antiangiogenic antithrombin III (ATIII), tumstatin, and arresten (the latter two noncollagenous fragments of type IV collagen), did not compete for AP-ES binding, suggesting that the binding was indeed specific (Figure 1B). Importantly, AP-ES was functional in endothelial migration assay in ng/ml doses similar to mES and hu-

man endostatin (hES), whereas the mutants mES3.1 and AP-ES3.1 did not inhibit migration of endothelial cells in response to VEGF₁₆₅, as predicted by the binding assay (Figure 1C).

During a survey of various cell lines that bound ES, we found that both inner medullary collecting duct (IMCD) (Figure 1B) and Madin-Darby canine kidney (MDCK) cells bound AP-ES. Since ES has recently been isolated in renal ureteric bud culture supernatants (J. Barasch, personal communications), we hypothesized that it may play a role in renal-epithelial cell branching morphogenesis. Figure 1D shows that mES indeed inhibited branching process formation of IMCD cells in response to both hepatocyte growth factor (HGF) and epidermal growth factor (EGF). The morphology of branching process formation is depicted in Figure 1E.

AP-ES Binds to Endothelial Cells and Renal Tubular Cells Revealing Two Binding Affinities

Scatchard plot analysis in cow pulmonary arterial endothelial (CPAE) cells showed two binding slopes with a high-affinity K_d of 18 pM (1.7×10^4 binding sites/cell) and a lower-affinity K_d of 200 pM (8.4×10^4 binding

Figure 1. Expression, Cell Surface Binding, and Activity of ES

(A) Western blot using polyclonal ES antibody shows expression of 85 kDa fusion protein in 20 μ l media supernatant from pAP-ES and pAP-ES3.1, but not pAP transfected 293T cells. 20 ng of *Pichia*-derived mES and mES3.1 are loaded in lanes 1 and 2.

(B) AP activity after binding to cow pulmonary arterial endothelial (CPAE), human umbilical vein endothelial (HUVE), and inner medullary collecting duct (IMCD) cells with AP (1), AP-ES (2), AP-ES+mES (3), AP-ES+mES3.1 (4), and AP-ES3.1 (5). 50 ng/ml of AP-ES or AP-ES3.1 was used in CPAE cells, and 200 ng/ml of AP-ES used in HUVE and IMCD cells. Five hundred-fold molar excess of mES or mES3.1 was used in the competition lanes (3 and 4). Vast excess (~200- to 300-fold molar excess) of FGF-2 (6), VEGF₁₆₅ (7), heparin (8), arresten (9), tumstatin (10), or antiangiogenic ATIII (11) did not compete with AP-ES binding to endothelial cells.

(C) mES, hES, and AP-ES, but not mES3.1 and AP-ES3.1, inhibit migration of HUVE cells in response to 10 ng/ml of VEGF₁₆₅. The anti-migratory effects of hES can be seen even at 0.1 ng/ml.

(D) IMCD cells develop branching processes in response to HGF (40 ng/ml) and EGF (20 ng/ml) but not to 0.1%FCS alone, and mES inhibits this effect at 1 μ g/ml.

(E) Branching process formation of IMCD cells in Type I collagen after 24 hr incubation with 0.1% serum (upper left), 0.1% serum + mES (upper right), 0.1% serum + EGF (lower left), or 0.1% serum + EGF + mES (lower right).

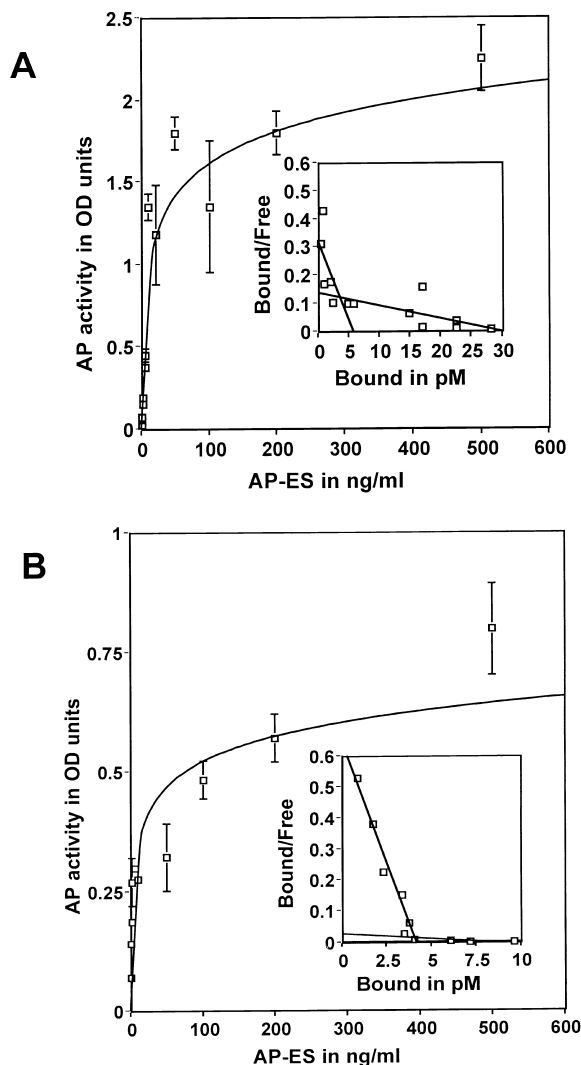


Figure 2. Binding Analysis of AP-ES Binding to CPAE and IMCD Cells. Binding assays were performed in CPAE (A) and IMCD (B) cells, with various concentrations of AP-ES protein ranging from 0.1–500 ng/ml. The inserts show Scatchard representation of specific binding.

sites/cell) (Figure 2A). Similar results were obtained in human umbilical vein endothelial (HUVE) cells with K_d s for the high- and low-affinity sites being 36 pM and 500 pM, respectively (data not shown). We next assessed binding to IMCD cells and found similar data: K_d s of 7 pM with 1.26×10^4 sites/cell and 333 pM with 2.80×10^4 sites/cell (Figure 2B).

Glypicans Are the Lower-Affinity Binding Partners for ES

To identify the ES receptor(s), we used an expression cloning strategy with AP-ES in situ binding as a screening assay. CPAE cells showed intense purple staining when bound with AP-ES (Figure 3A), whereas AP-ES3.1 did not bind (Figure 3B). Also, HUVE, human renal microvascular endothelial (HRME), human microvascular endothelial-lung (HMVE-L), MDCK and IMCD cells were

all strikingly positive for AP-ES binding. Lines negative for AP-ES binding included Chinese hamster ovary (CHO), NIH 3T3 (Figure 3C), and L (fibroblast) cells. Retroviral pools from a HUVE and HRME cDNA library were used to infect NIH 3T3 cells and expression cloning performed as described in Experimental Procedures. The clone that induced in situ binding of AP-ES was a 3.5 kb cDNA (likely of HRME origin) whose sequence matched identically with the region 205 to 3718 bases of the human glypican-4 sequence (Genbank accession number NM001448). To confirm that glypican-4 cDNA was indeed giving AP-ES binding, we infected NIH 3T3 cells with a retrovirus carrying the glypican-4 cDNA, and these cells (g4) showed purple staining (Figure 3D).

Glypican-4, or K-glypican as it was originally described, is highly expressed in developing brains and is thought to be the predominant glypican expressed in renal tubular cells (Watanabe et al., 1995). Glypican-1 is thought to be the major glypican in endothelial cells (Gengrinovitch et al., 1999). NIH 3T3 cells infected with a retrovirus carrying the glypican-1 gene (g1) showed purple staining after binding to AP-ES (Figure 3E), whereas AP-ES3.1 did not bind (Figure 3F) these cells.

Scatchard plot analysis of AP-ES binding to glypican-1 (g1) and glypican-4 (g4) infected NIH 3T3 cells appeared to show only one binding slope with a K_d of 200–300 pM (Figure 3G). These data suggested that glypicans were the lower-affinity binding partners for ES in endothelial and renal tubular cells. K_d s in this range have been described for glypican-VEGF₁₆₅ interaction (Gengrinovitch et al., 1999). Binding experiments done with NIH 3T3 cells overexpressing syndecan-4 revealed no specific binding (data not shown), suggesting that ES binds to glypicans with some specificity. In the presence of phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme that specifically cleaves the GPI anchor, AP-ES binding to endothelial cells was almost completely abrogated (Figure 3H).

ES Interacts with Glycosaminoglycan Chains of Glypican, an Interaction Necessary for ES Binding to Its High-Affinity Sites

Because of ES's affinity to heparin, we hypothesized that the interaction between ES and glypicans involved the Glycosaminoglycan (GAG) chains, which are predominantly composed of heparan sulfates. Chlorate treatment or heparinase I and III treatment of endothelial cells reduced AP-ES binding, suggesting that GAG chains are needed for ES to interact and that sulfation of GAG chains is critical for this interaction (Figure 4A). The decrease in AP-ES binding was more pronounced with heparinase I compared to heparinase III treatment (discussed later). To further define the type of sulfation necessary for binding and, in particular, the role of sulfation at the 2-O position, we resorted to the B16BL6 melanoma line (BL6), in which ES did not bind (Figure 4B). These cells were then stably transfected with a 2-O sulfotransferase (2-OST) antisense construct (BL6 OST-). Composition analysis of the GAGs between the two cell lines showed a greater than 90% reduction in 2-O sulfation in the antisense cells compared with wild type (Liu and Sasisekharan, our unpublished data). Importantly, BL6 cells infected with glypican-1 retrovirus

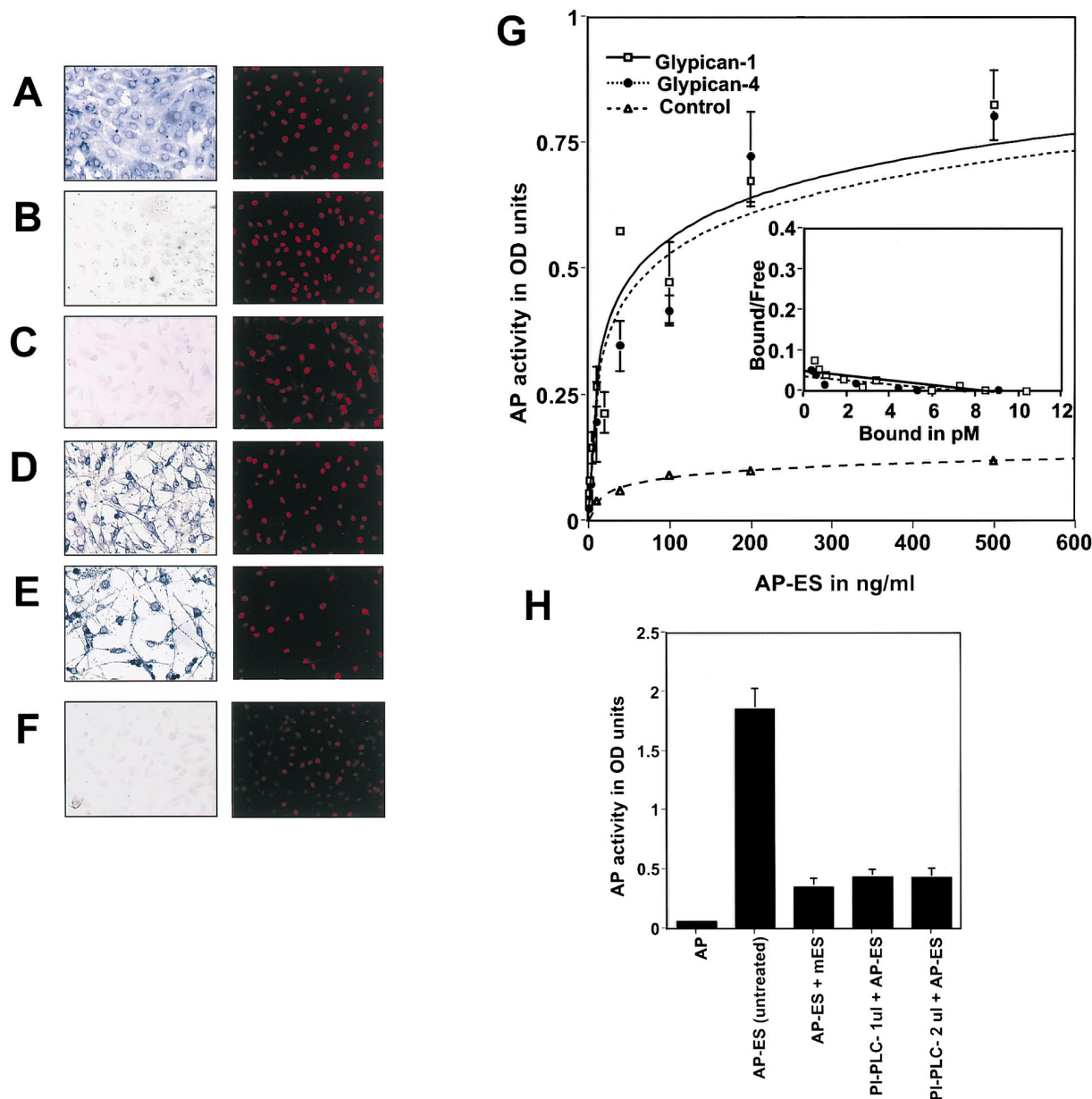


Figure 3. Identification of Glypicans as the Low-Affinity Receptor for ES

(A–F) CPAE cells show intense purple in situ alkaline phosphatase staining after binding with AP-ES (A) but not with AP-ES3.1 (B). Preincubation with excess mES abolished the staining, showing specific nature of the binding (data not shown). NIH 3T3 cells do not bind AP-ES (C), but NIH 3T3 overexpressing glypican-4 (g4) show positive staining (D). NIH 3T3 cells overexpressing glypican-1 (g1) bind AP-ES (E) but not AP-ES 3.1 (F). Shown on the right side are the same cells stained with propidium iodide.

(G) AP-ES binding curve in NIH 3T3 cells (control), NIH 3T3 cells overexpressing glypican-1 (g1) or glypican-4 (g4) cells. Scatchard transformation is shown as an insert.

(H) Treatment of CPAE cells with PI-PLC significantly reduces AP-ES binding, showing importance of the GPI-linkage. Five hundred-fold excess mES was used in the competition experiments (lane 3).

showed specific ES binding, but BL6OST- cells did not (Figure 4B). These experiments suggest that GAG residues with 2-O sulfation are necessary for binding. Though we believe that the major interaction between ES and glypican is through its GAG chains, we cannot exclude some interaction of ES with the protein core of glypican, as has been described between FGF-7 and perlecan (Mongiat et al., 2000).

To critically address the importance of the lower-affinity binding site for high-affinity binding, we performed detailed binding experiments in endothelial cells treated with heparinases or a 2 M salt wash to disrupt sugar-protein interactions. Scatchard plot analysis after heparinase or the 2 M salt wash revealed that AP-ES binding in endothelial cells was significantly reduced with loss of *both* high- and low-affinity binding indicating that

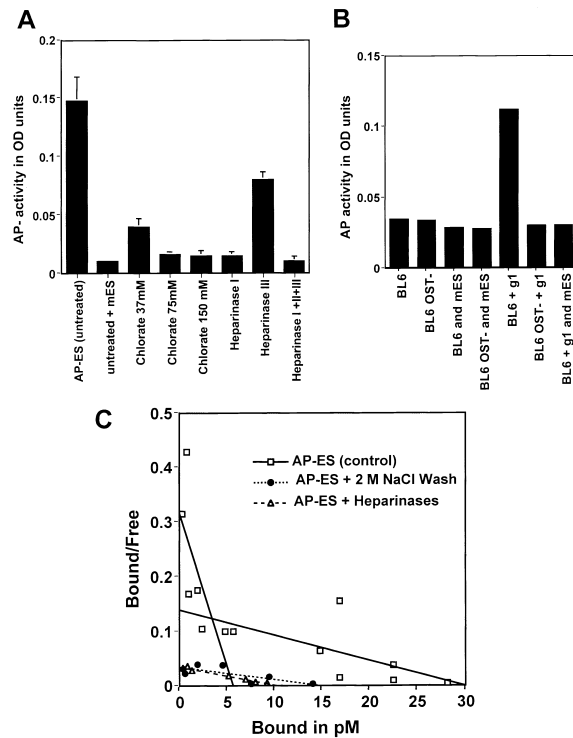


Figure 4. Glycosaminoglycan Components of Glypicans Mediate ES Binding to the Cell Surface

(A) Significant reduction of AP-ES binding to CPAE cells is seen after pre-treatment with sodium chlorate and 100 nM of Heparinase I, III, or I+II+III, as compared to untreated cells. Five hundred-fold excess mES was used in the competition experiments (lane 2).

(B) Comparison of AP-ES (10 ng/ml) Binding in B16BL6 Melanoma Cells (BL6), B16BL6 cells transfected with a 2-OST antisense construct (BL6 OST-), or with both these cell lines infected with glypican-1 retrovirus (BL6+g1 and BL6 OST- +g1). No significant baseline binding for AP-ES is seen in BL6 and BL6 OST- cells, whereas a significant increase of AP-ES specific binding is noted in BL6+g1 cells, but not in BL6 OST- +g1 cells.

(C) Scatchard transformation of AP-ES binding curves in CPAE cells pre-treated with Heparinase I+III or treated with 2 M NaCl wash show a marked reduction of binding with loss of both high- and low-affinity binding seen in untreated cells (control).

disrupting the sugar-protein interaction results in loss of high-affinity ES binding (Figure 4C).

Glypicans Bind Specifically to an Octasulfated Hexasaccharide Derived from Heparin

Next, we sought to examine the binding specificity of ES using mast cell heparin as a model heparin-like glycosaminoglycan. Binders to ES were selected using a surface noncovalent affinity mass spectrometry (SNA-MS) assay (Keiser et al., 2001). A hexasaccharide library derived from heparin was added to immobilized biotinylated mES on a hydrophobic surface (Figure 5A). Washing with salt removed the low-affinity binders leaving only a single high-affinity hexasaccharide moiety (Figures 5B and 5C). Importantly, this saccharide was not selected with heat-denatured mES or with mES3.1 (that does not bind glypican), indicating that the observed binder is highly specific for intact, native mES (data not shown). Sequence analysis of this saccharide using

previously published technology (Venkataraman et al., 1999) indicated that its sequence was $\Delta U_{2S}H_{NS,6S}I_{2S}H_{NS,6S}GH_{NS,6S}$ ($\pm DD-5$) (where I = α -L-iduronic acid, G = β -D-glucuronic acid, ΔU = 4,5 unsaturated uronic acid, H = glucosamine, 2S and 6S refers to 2-O sulfation and 6-O sulfation, respectively, and NS refers to N-sulfation). This sequence contains a 2-O sulfation site as predicted by our 2-OST antisense data. The presence of $GH_{NS,6S}$ in the hexasaccharide sequence suggests specific structural requirements in the heparin-ES interactions, as opposed to a fully sulfated oligosaccharide (present in the $I_{2S}H_{NS,6S}$ repeat) typically seen in heparin. Furthermore, the sequence of this hexasaccharide is entirely consistent with the efficacy and specificity of the heparinase cell culture experiments of Figure 4. Heparinase I, which cleaves glycosidic linkages containing a 2-O sulfate, is expected to readily cleave the hexasaccharide. Heparinase III, which cleaves glycosidic linkages without a 2-O sulfate, is expected to clip the reducing end disaccharide unit, albeit with less efficiency due to the high overall sulfate content of the hexasaccharide. Thus, these results demonstrate that mES binds with high-affinity to only certain saccharides, suggesting that in vivo ES binding to glypican at the cell surface is mediated primarily through the saccharide portion of glypican.

To confirm and extend these results, the hexasaccharide species that binds with high-affinity was purified and incubated with mES to determine the stoichiometry of the interaction (Figures 5D and 5E). The mass spectrometric results unambiguously point to the fact that mES forms a tight 1:1 complex with the hexasaccharide. As above, mES3.1 did not form a detectable 1:1 complex with the purified hexasaccharide, indicating that the 1:1 complex observed above is a specific structure (data not shown).

Glypicans Are Necessary for ES's Action on Endothelial Cells

Next, we sought functional evidence for a role for glypicans in mediating ES action. Toward this end, we constructed an antisense glypican-1 retrovirus, infected endothelial cells and tested their response to hES. Glypican-1 mRNA and protein were reduced by 70%–80% in the antisense glypican-1 cells compared to cells infected with vector alone; syndecan-4 levels were unchanged (Figures 6A and 6B), showing the specificity of our construct. Moreover, analysis of AP-ES binding to the antisense glypican-1 cells showed loss of both the low- and high-affinity binding sites (Figure 6C).

To understand glypican's role in ES action, we utilized two ligands (VEGF₁₂₁ and EGF) that could induce pro-angiogenic signals in the absence of glypicans. VEGF₁₂₁, in contrast to VEGF₁₆₅, lacks a heparin binding domain and does not require cell surface proteoglycan for its actions (Gengrinovitch et al., 1999). Migration experiments with HUVE cells showed that hES inhibited endothelial migration in response to VEGF₁₆₅ and VEGF₁₂₁ (Figure 6D). However, when these cells were infected with retroviruses carrying antisense glypican-1 cDNA, we found that VEGF₁₆₅ was unable to induce migration of endothelial cells, consistent with the fact that VEGF₁₆₅ requires glypicans to bind to its receptor and trigger

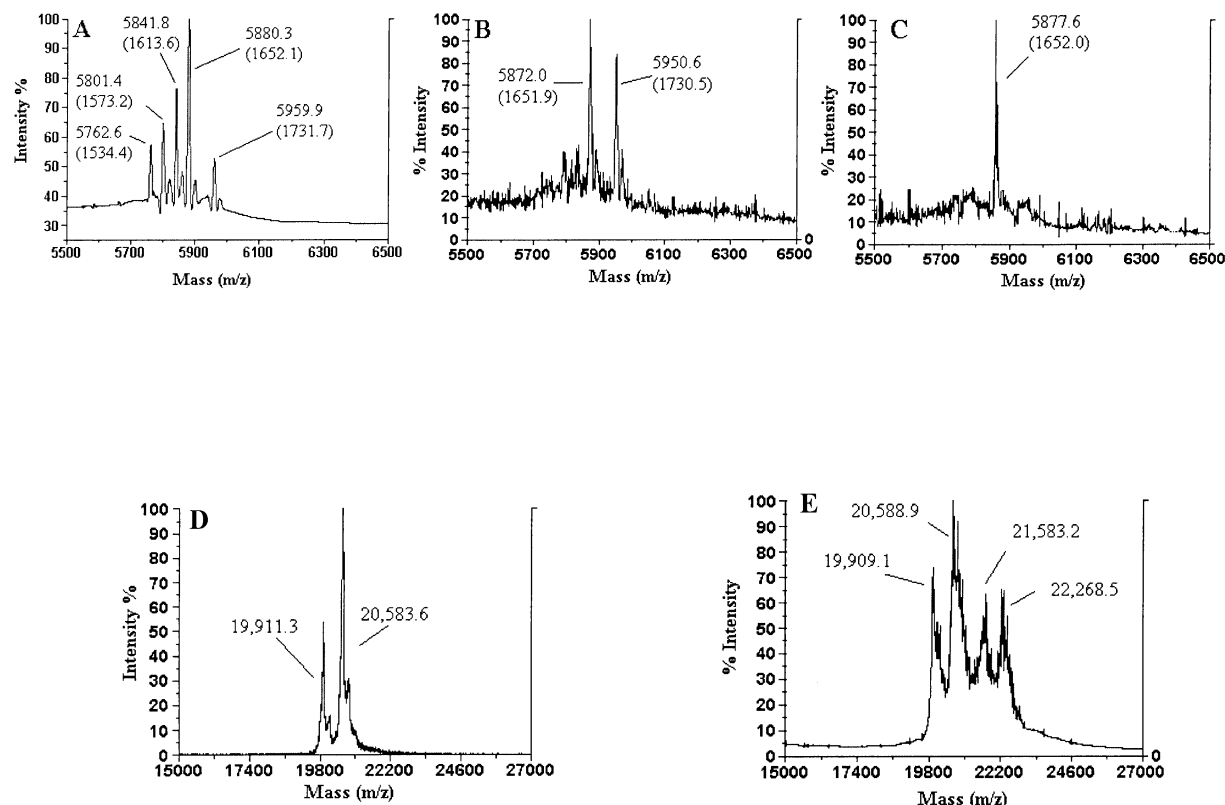


Figure 5. Selection of a Hexasaccharide from Porcine Heparin with High Affinity for ES and Detection of a Complex between the Octasulfated Hexasaccharide ($\Delta U_{25}H_{NS,6S}I_{2S}H_{NS,6S}GH_{NS,6S}$) and ES

All saccharides are observed as 1:1 noncovalent complexes with the basic peptide (RG)₁₉R. The indicated peaks show the m/z value of the (M+H)⁺ saccharide-peptide complex. The saccharide mass is shown in parentheses and is determined by subtracting the m/z value of the (M+H)⁺ uncomplexed peptide from this value.

(A) The hexasaccharide pool used in the selection procedure. Present in the mix are five major species corresponding to hexasaccharides with differential sulfation and/or acetylation.

(B) Binding of the hexasaccharide pool and washing with water indicates that only two species, an octasulfated (1651.9) and nonasulfated (1730.5) hexasaccharide, remain.

(C) Washing with 0.2 M NaCl to eliminate nonspecific binding resulted in the presence of only the octasulfated (1652.0) hexasaccharide. In separate solution experiments, we found this hexasaccharide to have the sequence $\Delta U_{25}H_{NS,6S}I_{2S}H_{NS,6S}GH_{NS,6S}$ (\pm DD-5).

(D) mES alone consists of two entities, one with a measured mass of 19,911 and another with a measured mass of 20,584.

(E) Introduction of an equimolar amount of hexasaccharide to mES in solution resulted in the formation of a detectable 1:1 complex between mES and the hexasaccharide. The peaks at 19,909.1 and 20,588.9 represent uncomplexed mES, and the peaks at 21,583.2 and 22,268.5 represent the 1:1 mES-hexasaccharide complex.

intracellular signaling (Gengrinovitch et al., 1999). As predicted, VEGF₁₂₁ was still able to cause migration of antisense glypican-1 infected endothelial cells, suggesting that glypicans are not necessary for VEGF₁₂₁ action.

Using this system, we next examined the importance of glypican expression for the effects of hES on VEGF₁₂₁ induced cell migration. In control cells, hES was able to inhibit VEGF₁₂₁ action. In conjunction with our earlier data that VEGF and ES do not cross compete for receptor binding, these results suggest that ES can transduce intracellular signals that interfere with signaling initiated by VEGF₁₂₁. Importantly, hES was unable to inhibit VEGF₁₂₁ induced migration in the antisense glypican-1 infected endothelial cells, demonstrating that glypicans are required for hES to block VEGF₁₂₁ signaling (Figure 6D). We obtained similar results in HMVE-L cells (data not shown).

To provide further evidence that glypican expression

is critical for ES action, and that ES can block intracellular pro-angiogenic signals, we created a retrovirus expressing cDNA for a fusion protein consisting of the extracellular domain of the EGF receptor in frame with the transmembrane and cytoplasmic domains of VEGF receptor-2 (kinase insert-domain containing receptor, KDR), referred to as EGDR and expressed in HUVE cells. Our choice of the EGF receptor was based on the fact that EGF does not interact with proteoglycans. KDR is known to mediate VEGF's pro-migratory action. Expression of EGDR in the HUVE cells was confirmed by FACS analysis using an N terminus EGF antibody (data not shown). Wild-type HUVE cells did not show an increase in cell migration in response to EGF, whereas HUVE cells expressing the chimeric EGDR molecule demonstrated a 3-fold increase (Figure 6E). This increase was abrogated by treatment with hES. These results indicate that hES inhibits VEGF's actions, not by competing for

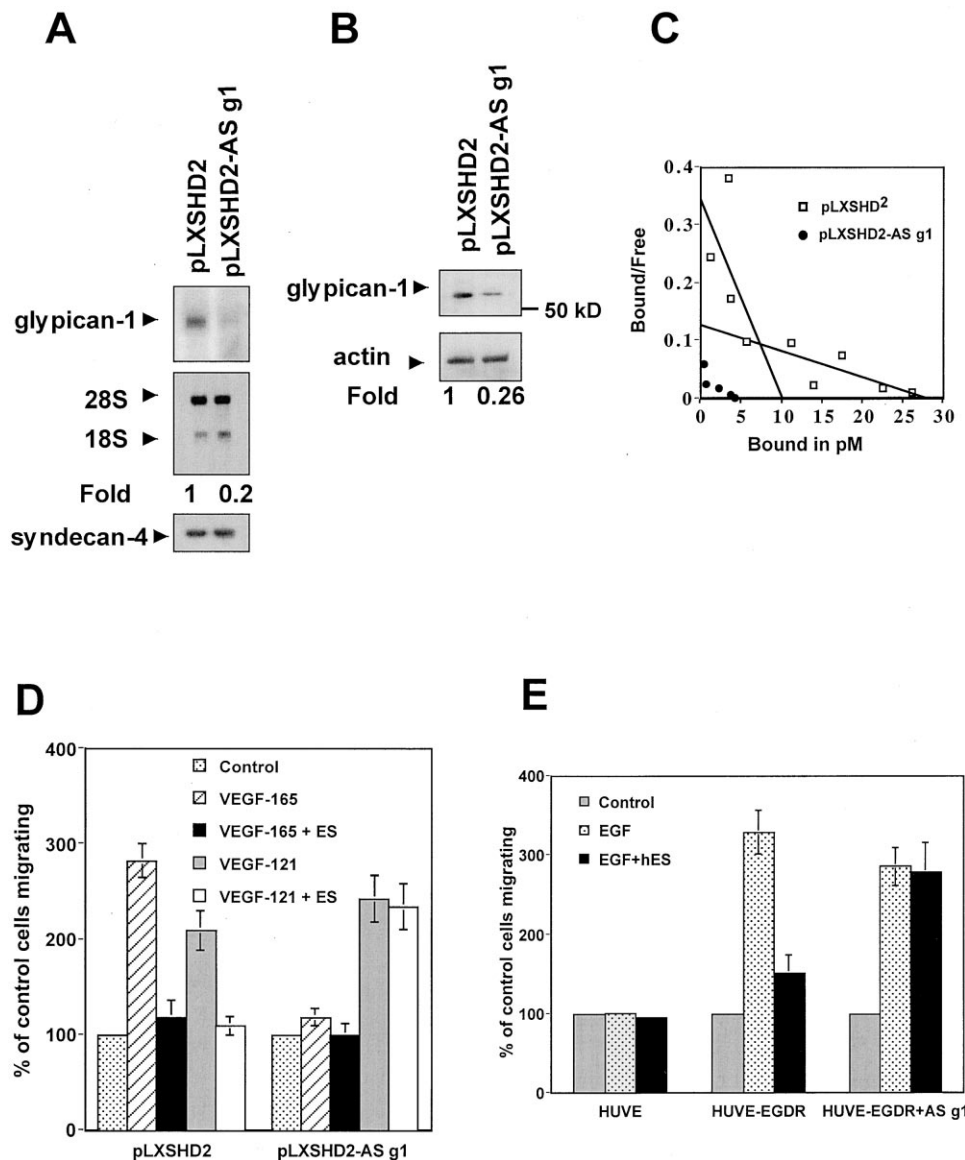


Figure 6. Effect of Antisense Glypican Expression on ES Binding and Action in Endothelial Cells

(A–B). HUVE cells infected with pLXSHD2 retrovirus carrying antisense glypican-1cDNA (pLXSHD2-AS g1) show an 80% reduction in glypican-1 RNA (A) and 75% reduction in glypican-1 protein as compared to empty virus infected HUVE cells (pLXSHD2³) (B).

(C) pLXSHD2-AS g1 cells show loss of both low- and high-affinity binding as compared to pLXSHD2 cells, when bound to AP-ES, as ascertained by Scatchard analysis.

(D) hES (100 ng/ml) inhibits VEGF₁₆₅ and VEGF₁₂₁ (10 ng/ml) induced migration of pLXSHD2 cells. pLXSHD2-AS g1 cells migrate in response to VEGF₁₂₁ but not VEGF₁₆₅, and hES is unable to inhibit VEGF₁₂₁ induced migration in these cells. Control wells did not contain growth factors or hES. The data are shown as % of control \pm SEM.

(E) EGF (20 ng/ml) can induce migration of HUVE cells infected with retrovirus carrying EGF-KDR chimera cDNA (HUVE-EGDR) and in HUVE cells infected with retroviruses carrying antisense glypican-1 and EGF-KDR chimera (HUVE-EGDR + AS g1), but not in wild-type HUVE cells. hES (100 ng/ml) inhibited this response in HUVE-EGDR cells but not in HUVE-EGDR + AS g1 cells. The control wells did not contain EGF or hES. The data are shown as % of control \pm SEM.

presentation to its KDR receptor, but by inhibiting intracellular VEGF signals mediated by KDR. We utilized this chimeric system to assess the importance of glypican-1 in mediating hES actions. With antisense glypican-1 infected cells, hES was unable to inhibit EGF induced migration (Figure 6E). These data, along with VEGF₁₂₁ results, indicate that glypicans are critical for ES action in endothelial cells.

Glypicans are Necessary for ES's Effect on Renal Tubular Cells

Next, we examined a nonendothelial system based on our observations of ES binding and function (inhibition of branching processes) in IMCD cells. Because glypican-4 is the predominant species in renal tubular cells, we infected IMCD cells with a retroviral vector carrying antisense glypican-4 cDNA and infected IMCD cells (AS g4).

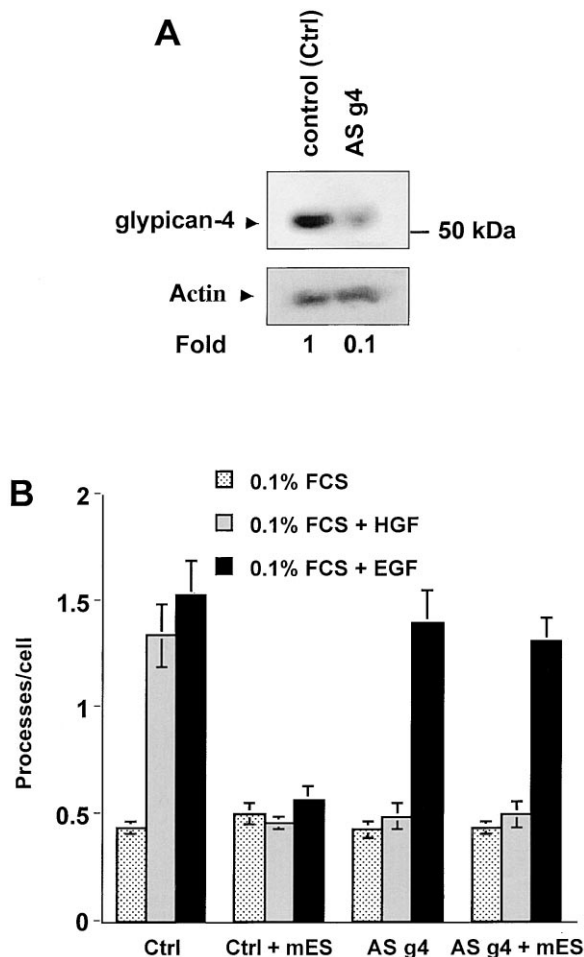


Figure 7. Effect of Loss of Glypicans on ES Action in IMCD Cells
(A) Western blot of heparinase-digested lysates from IMCD cells infected with pLXSHD² retrovirus vector carrying antisense glypican-4 cDNA (AS g4) shows 90% reduction in glypican-4 protein levels compared to IMCD cells infected with empty virus (control).
(B) mES (1 μ g/ml) inhibits HGF (40 ng/ml) and EGF (20 ng/ml) induced branching morphogenesis in control IMCD cells (Ctrl). AS g4 cells branch only in response to EGF, and mES is unable to inhibit this response. Data are shown as mean \pm S.E.

Expression of glypican-4 was confirmed by Western blot: control IMCD cells expressed abundant glypican-4, whereas AS g4 cells showed greater than 90% reduction (Figure 7A). mES inhibited HGF and EGF induced branching morphogenesis of IMCD cells (Figures 1D and 7B) and of ureteric bud cells at doses from 0.1 ng/ml to 1 μ g/ml (data not shown; Karihaloo and Cantley, personal communication). HGF, analogous to VEGF₁₆₅, requires GAGs for presentation to its receptor (Sakata et al., 1997). As expected, IMCD cells infected with antisense glypican-4 retroviruses (AS g4) did not branch in response to HGF; however, they did branch in response to EGF, which does not interact with HSGAGs. Analogous to the endothelial cell experiments, mES was unable to inhibit EGF induced branching morphogenesis in cells infected with antisense glypican-4 retrovirus, suggesting that glypican-4 is necessary for mES's action on IMCD cells (Figure 7B).

Discussion

This paper presents several novel observations. First, ES, but not a two amino acid mutant (ES3.1), binds endothelial cells with two affinities: a high-affinity site ($K_d \approx 15$ –35 pM) and a lower-affinity site ($K_d \approx 200$ –500 pM). Second, glypican family members serve as low-affinity cell surface binding partners for ES through their GAG chains, an interaction that shows fine specificity as demonstrated by biochemical and genetic experiments. Third, antisense glypican experiments demonstrate that glypicans are necessary for ES's anti-angiogenic actions. Fourth, ES antagonizes proangiogenic actions of VEGF not by cross-competition for receptor binding but rather via antagonizing intracellular signaling. Finally, ES regulates branching morphogenesis in response to HGF and EGF in renal tubular cell lines through binding to glypican-4, thus defining a role for ES in nonendothelial cells.

ES Cell Surface Binding

We utilized an AP-tag to label ES, after unsuccessful attempts at iodination. This molecule was functional in a migration assay at ng/ml levels, similar to our previously described Pichia-derived mES and hES (Figure 1C). We designed a two amino acid mutant (ES3.1) based on ES's crystal structure that might affect function. This mutant was produced in mammalian cells (AP-ES3.1) and in soluble form in Pichia (mES3.1). It bound heparin (eluting at 0.2–0.3 M NaCl for mES3.1 versus 0.3–0.6 M NaCl for mES), suggesting that it shared some conformational properties with wild-type ES. However, it was functionally inactive, did not show cell surface binding to endothelial cells or glypican expressing NIH 3T3 cells, and did not bind to the ES hexasaccharide binder (\pm DD-5). These data confirm the importance of the two phenylalanines for ES binding and function, illustrating that heparin binding does not equate with heparan sulfate binding. In this regard, it has been shown that while both HGF and chemokine stromal cell-derived factor (SDF)-1 α bind heparin with equal affinities, only HGF selectively binds to cell surface CD44-HS (van Der Voort et al., 2000). Importantly, the specificity of ES cell surface binding is also demonstrated by the fact that other anti-angiogenic factors, namely angiostatin (data not shown), arresten, tumstatin, and anti-angiogenic ATIII or proangiogenic agents such as FGF-2 and VEGF₁₆₅ do not compete for ES binding to endothelial cells.

Importance and Specificity of the Glypican HSGAG-ES Interaction

The binding of glypican, a cell surface HSPG, to ES is not surprising because ES was initially purified by heparin chromatography (O'Reilly et al., 1997; Sasaki et al., 1999). However, we have now shown a critical specificity to this binding. ES does not bind syndecans, nor does the functionally inactive ES3.1 mutant bind glypican, showing correlation of binding to function. Our biochemical (heparinase I versus III, chlorate and 2M salt wash), genetic (2-OST), and selection data (\pm DD-5) all point to a fine specificity of this interaction. It must be noted that our results contrast with previous findings that ES binding to blood vessels in situ is resistant to heparinase III treatment (Chang et al., 1999), and the finding in a

separate study of a lack of importance of heparin binding for ES function (Yamaguchi et al., 1999). In the former study, the assessment is not quantitative and cannot be compared to our quantitative analysis in cultured cells. Furthermore, only heparinase III, and not heparinase I, was used in this study. In the latter study, heparin column chromatography was used to assess binding when the interaction is predominantly ionic in nature, with only some element of specificity. As above, such a study is not quantitative and thus may not represent what is actually occurring at the cell surface.

Various ligands including FGF-2, HGF, platelet factor-4 (PF4), pigment epithelium-derived factor (PEDF), thrombospondin-1 (TSP-1), and ATIII bind selectively to specific GAG sequences (Alberdi et al., 1998; Feitsma et al., 2000; Tumova et al., 2000). Only recently, with the advent of mass spectrophotometric methods, has there been progress in refining these recognition sequences (Venkataraman et al., 1999; Keiser et al., 2001). We have used this methodology to select an ES binding hexasaccharide from a library of hexasaccharides derived from porcine heparin. Importantly, a single high-affinity moiety was found that was not selected by the mutant ES3.1, further pointing to the importance of this methodology, because both mutant and wild-type ES bound to heparin, but with differing affinity. Defining the fine specificity of sugar-protein interactions might lead to identification of small molecules to replace ES or the use of such GAGs as pro/anti-angiogenic agents with great specificity.

Our results indicate that ES binding to its high-affinity site critically depends on the presence of glypican and its GAGs. These data suggest cooperative binding by these two binding sites (possibly two receptors, see later) or between glypican and ES. For example, glypicans could effect conformational change in ES, enabling it to interact with its high-affinity site. Definitive confirmation of this model awaits identification of this high-affinity binder; work is now in progress.

Effects of ES on Renal Tubular Cells

We tested whether ES might affect branching morphogenesis of kidney IMCD cells, a model system for collecting duct development, for a number of reasons. First, collagen XVIII is present in the tubular kidney basement membrane (Halfter et al., 1998). Second, ES has been recently purified from the cultured supernatant of a ureteric bud cell line and can affect mesenchyme to epithelial differentiation (J. Barasch, personal communication). Third, glypicans (in particular K-glypican or glypican-4) are expressed in the developing kidney (Watanabe et al., 1995), and HSPGs play critical roles in kidney development. A 2-OST (-/-) mouse shows renal agenesis as a consequence of defects in mesenchymal condensation around the ureteric bud and lack of initiation of branching morphogenesis (Bullock et al., 1998). Interestingly, a human disease (Knobloch syndrome) which occasionally shows abnormalities in the collecting duct has recently been ascribed to a splice mutation in collagen XVIII, resulting in premature truncation of the short isoform (Sertie et al., 2000). The major phenotype in this disease is a neural one, as is also seen in *C. elegans* carrying mutant collagen XVIII (*cle-1* gene, Genbank ac-

cession number AF164959), suggesting possible roles for endostatin in axonal guidance and branching morphogenesis outside of the vascular system. Our data support a role for ES in control of ureteric bud branching, mediated through binding to glypicans.

Model for Glypican in Mediating ES Action

It is likely that specificity of ES resides in the restricted expression of a high-affinity receptor, because we were unable to obtain any gain of function in cells lacking high-affinity binding sites, e.g., in our NIH 3T3 cells expressing glypican-4 or 1 (data not shown). However, glypican core proteins do display tissue-specific expression patterns. Endothelial cells express glypican-1, primarily, whereas glypican-4 is highly expressed in kidneys and developing brains. Moreover, cell-specific isoforms of enzymes involved in HS biosynthesis can produce chains with various sequence specificities and overall organization. Though the overall affinity of fibroblast and endothelial glypicans for ES appears to be similar (200–500 pM), an analysis of the sugar sequences specifically binding to ES will ascertain whether cell type specific binding sequences exist.

We currently favor a model of two receptors binding to ES. In such a model, glypican acts as a “presenting” co-receptor by binding endostatin and presenting it to its high-affinity receptor, which then signals intracellularly. However, we cannot exclude a role for glypican in signaling as well, as reported with other GPI-anchored proteins (Solomon et al., 1996; Mouillet-Richard et al., 2000), nor can we exclude the possibility of a single receptor with multiple binding affinities (high and low) due to cell specific GAGs.

Because glypicans are known to interact with numerous ligands that control cell growth and differentiation, including VEGF₁₆₅, FGF-2, FGF-7, and HGF (Bernfield et al., 1999), ES could antagonize the proangiogenic actions of VEGF₁₆₅ or FGF-2 by simply competing with these ligands for binding to either glypican or their high-affinity receptor. Such a scenario is unlikely, because we have shown that endostatin can antagonize the actions of non-HSPG binding growth factors such as VEGF₁₂₁ and EGF (in our EGF-KDR chimera experiments) in endothelial cells and EGF in IMCD cells. This suggests that that ES can deliver intracellular signals to antagonize pro-angiogenic activity. Moreover, ES can function at low concentrations (<1 ng/ml), so that it would be impossible to saturate glypican binding sites. Furthermore, ES does not displace iodinated VEGF₁₆₅ cell surface binding (data not shown) nor do VEGF₁₆₅ and FGF-2 compete for ES cell surface binding. Finally, the sugar sequences bound by various proangiogenic factors, though poorly defined, do not overlap with the ES binding sequence. Collectively, our data suggest that ES initiates agonistic anti-angiogenic signals through a co-receptor complex, in which glypicans serve as the low-affinity receptor.

Experimental Procedures

Materials

Recombinant VEGF₁₆₅, VEGF₁₂₁, and EGF were obtained from R&D Systems (Minneapolis, MN), Cyquant reagent from Molecular Probes (Eugene, OR), alkaline phosphatase substrate PNP and

heparin from Sigma (St. Louis, MO). PI-PLC was obtained from ICN Biochemicals (Aurora, OH).

Cell Lines

CPAE, NIH 3T3, MDCK cells, 293T, and CHO cells were obtained from ATCC (Rockville, MD). HUVE and HMVE-L cells (Clonetics, Walkersville, MD) were maintained in EGM-2MV/5%FCS. Immortalized mouse IMCD cells were grown in DMEM-F12/10% FCS (Rauchman et al., 1993); B16BL6 melanoma cells (BL6) were grown in 10% FBS in MEM, and the same cells transfected with the 2-OST antisense construct had G418 in addition to the basal medium. HRME cells were obtained from T. Daniel and grown in DMEM/10%FBS and FGF. These cells display endothelial markers such as Factor VIII; however, unlike HUVE cells they do not express the angiogenic EGF ligand, LERK-1, but instead show LERK-2 expression characteristic of ureteric bud epithelia and mature collecting duct (Daniel et al., 1996). 293 GPG cells were obtained from R. Mulligan (Ory et al., 1996).

Plasmids and Constructs

Rat glypican-1 and rat syndecan-4 in the MSCV retroviral vector were obtained from M. Simons (Volk et al., 1999). The Aptag-1 expression vector was a gift from J. Flanagan (Flanagan and Leder, 1990). The K-cadherin leader sequence, followed by alkaline phosphatase, was cloned into pcDNA3, using PCR from the Aptag-1 vector to express a secreted version of alkaline phosphatase, and this plasmid was referred to as pAP3. The mouse ES sequence was excised from pPICZαA (Dhanabal et al., 1999a) and cloned in frame into the C terminus of AP in pAP-3, resulting in pAP-ES. mES cDNA was mutated at the two phenylalanines F162A and F165A (ES3.1) by PCR and cloned into pAP-ES by excising ES cDNA and was referred to as pAP-ES3.1. Full-length mouse glypican-4 cDNA with Not I sites was amplified from the pcDNA1 K-glypican plasmid (Watanabe et al., 1995) and was cloned into Not I digested retroviral vector pLXSHD² (gift from D. Miller and A. Kazlauskas). The antisense clone, referred to as pLXSHD²-AS g4, was confirmed by sequencing. Full-length human glypican-1 cDNA was amplified from placental library using primers with Eco RI sites and was cloned into Eco RI digested pLXSHD². The antisense clone (pLXSHD²-AS g1) was confirmed by sequencing. The pMMP-EGDR retroviral vector carrying the gene for the fusion protein—the extracellular domain of EGF receptor in frame with the transmembrane and cytoplasmic domains of KDR (VEGF receptor) is described elsewhere (Zeng and Mukhopadhyay, our unpublished data).

Expression and Purification of Recombinant Proteins

Recombinant mouse endostatin (mES) and human endostatin (hES) were expressed and purified from Pichia Pastoris as described previously (Dhanabal et al., 1999a; Dhanabal et al., 1999c). Mutant mES cDNA was excised from pAP-ES3.1 and cloned into pPICZαA and mutant endostatin (mES3.1) was also generated in Pichia. AP-ES and AP-ES3.1 were produced in mammalian cells by transient transfection of their cDNAs into 293T cells. The supernatant was collected after 4 days, concentrated and quantitated for ES amount by using a Western blot with known amounts of mES. AP activity was determined with known dilutions of AP-ES and a standard curve was plotted against AP activity and defined concentrations of AP-ES (data not shown). The curve was linear between AP activities of 0.05 and 1.5. Recombinant heparinase I, II, and III were produced as described previously (Natké et al., 2000). Anti-angiogenic ATIII was generated from recombinant ATIII as described by O'Reilly et al. (O'Reilly et al., 1999). Recombinant arresten and tumstatin was made as described elsewhere (Colorado et al., 2000; Maeshima et al., 2000).

AP Endostatin Binding Assays

Lysate Assays

Cells were grown to 80% confluency, trypsinized, and resuspended at 5×10^5 cells/ml DMEM/10%FBS containing varying amounts of AP-ES. For competition experiments, cells were preincubated with 500-fold excess of mES for 1 hr. After binding for 2 hr at 4°C, cells were pelleted and the supernatant removed to determine the unbound AP-ES. Cell pellets were then lysed in ice, using 150 μl AP

lysis buffer (1% Triton X-100, 10 mM Tris-Cl [pH 8], and 60 mM octyl glucoside) for 15 min and spun. 125 μl of supernatant was heated at 65°C for 10 min to inactivate endogenous alkaline phosphatase. Heat-inactivated lysates (100 μl) were incubated with equal volumes of 2× AP buffer (1 ml = 0.97 ml 2 M diethanolamine [pH 9.8], 1 μl 1 M MgCl₂, 20 μl 5% BSA and 10 μl 2.4 M PPNP) in a 96-well plate at room temperature for 1 hr, and AP activity was measured using a plate reader at 405 nm. AP activity was normalized to total protein present in the lysates. Samples that gave AP readings >1 were diluted and measured AP multiplied by the dilution factor. AP activity obtained after competition with excess mES was subtracted from the total AP activity to determine specific AP-ES binding.

In Situ Staining of Cells with AP-ES Fusion Protein

Cells were plated in 12-well plates at 70%–80% confluency, washed twice in Hank's balanced salt solution (HBSS), and incubated in DMEM/10%FBS containing AP-ES (200 ng/ml) ± 100 μg of mES (in wells with competition) at room temperature for 90 min. Cells were then washed six to seven times with HBSS, fixed in 60% acetone, 3% formaldehyde and 20 mM Hepes (pH 7.5), washed again in HBSS, and heated at 65°C for 10 min to inactivate endogenous phosphatases. Wells were rinsed twice in staining solution (100 mM Tris [pH 9.5], 100 mM NaCl, 5 mM MgCl₂) and incubated in BCIP/NTB substrate (Vector Lab, Burlingame, CA) until color developed. The cells were then washed twice with PBS, counterstained with propidium iodide (PI) by incubating in 0.5 mg/ml PI and 0.5 mg/ml RNase in PBS for 15 min, and viewed under fluorescent light.

Expression Cloning and Retrovirus Production

Retroviral production was done as described (Ory et al., 1996). Briefly, 20 μg of retroviral vector cDNA was transfected into 293 GPG packaging cells using the Calphos transfection kit. Forty-eight hours after transfection, the supernatant was collected and used to infect target cells in DMEM/10%FBS containing polybrene (8 μg/ml). Viral titer was calculated using the pLIB-GFP vector (Clontech, Palo Alto, CA), and we routinely obtained $1-2 \times 10^5$ cfu/ml. A unidirectional retroviral library was made from polyA RNA generated from HUVE and HRME cells in pLIB vector (Clontech, Palo Alto, CA). It contained 4.6×10^5 independent clones with cDNA sizes ranging from 0.5 to 5.0 kb (average 2.0 kb). For expression cloning, we subpooled the library into 40 pools of approximately 100,000 clones each. 60 μg of DNA from each pool was used to transfect three 10 cm 293-GPG dishes. About 18 ml of virus supernatant obtained from three such plates was concentrated to 1 ml using ultracentrifugation (Ory et al., 1996) and used to infect 2×10^5 NIH 3T3 cells. This procedure routinely gave us greater than 95% transduction efficiency. Seventy-two hours after retroviral transduction, we split the cells into 2 plates; one was used for in situ hybridization, whereas the cells from other plate were distributed into a 96-well plate. After achieving confluence, cells were spread into two duplicate 96-well plates. One plate was used for in situ hybridization. Cells from the wells corresponding to positive wells were pooled and distributed into a 48-well plate, and the process was repeated three times, at which time more than 90% of cells were positive for AP staining. The retroviral inserts were rescued by PCR (long-range PCR kit, Boehringer-Mannheim) from genomic DNA using primers flanking the cDNA inserts (5' primer 5'-AGCCCTCACTCCTCTCTAG3' and 3' primer 5'-ATGGCGTTACTTAAGCTAGCTTGCCAAACCTAC3') and then sequenced.

Migration Assay

This assay was performed using Transwells (Costar Corporation) with a polycarbonate membrane (8 μm pore size). HUVE cells were suspended at 500,000/ml in EBM-2 medium containing 0.5% BSA. The lower wells were filled with EBM-2 medium containing growth factors (VEGF₁₆₅, VEGF₁₂₁, or EGF at 10 ng/ml), whereas the upper chamber was seeded with 50,000 cells/well with different concentrations of recombinant ES or ES mutants. Each condition was done in triplicate. Cells were allowed to migrate at 37°C for 2.5 hr, and cells on the upper surface of the membrane were wiped off with a cotton swab. The membranes were cut, placed in a 96-well plate, and frozen overnight at −80°C. Next morning, plates were thawed to room temperature for 3 hr, 200 μl of CyQuant DNA stain solution was added to each well and read on a spectrofluorometer with

Deltasoft-3 software. The number of cells migrating were extrapolated from a standard curve generated by using a known amount of cells over a range of 1×10^3 cells– 1×10^5 cells per well.

Northern and Western Blots

These experiments were done as described previously (Knebelmann et al., 1998). The probes used for Northern blots were obtained by PCR and included a 1.6 kb fragment containing the coding region of glypican-1 cDNA, and 600 bp fragment in the coding region of syndecan-4 cDNA. For Western blots, cell lysates (20 μ g) were digested with heparinase I (100 nM) and heparinase III (100 nM) at 37°C for 2 hr and then resolved by SDS-PAGE (5%) gel. Rabbit polyclonal glypican-1 antibody (Litwack et al., 1998), affinity purified anti-glypican-4 antibody (R364) (Hagihara et al., 2000), and polyclonal ES antibody have been described elsewhere (Dhanabal et al., 1999a).

Surface Noncovalent Affinity Mass Spectrometry Assay

A Surface Noncovalent Affinity Mass Spectrometry (SNA-MS) assay was used as previously described (Keiser et al., 2001) to identify specific high-affinity saccharide binding to mES. Briefly, mES was biotinylated with EZ-link sulfo-NHS biotin (Pierce) in the presence of heparin. Excess biotin was removed by a spin column with a molecular weight cutoff of 10,000 (Millipore). mES was immobilized to the surface by first drying 4 μ g neutravidin (Pierce) on the film (Canon NP Type E transparency film), then adding biotinylated mES to the neutravidin spot after drying. To probe for oligosaccharide binding, 1 μ l of a 1.5 mg/ml hexasaccharide mixture generated by incomplete digestion of porcine intestinal mucosa heparin with heparinase I was added to immobilized mES and allowed to bind. Unbound saccharide was removed by washing with 0.2 M NaCl, followed by water washes. Then, a saturated caffeic acid matrix solution in 50% acetonitrile with 2 pmol/ μ l (RG)₃R was added to the protein/saccharide spot prior to MALDI analysis. All saccharides were detected as noncovalent complexes with (RG)₃R using MALDI parameters published previously (Venkataraman et al., 1999).

To confirm the results generated from the above series of experiments, analysis of heparin oligosaccharide binding to mES in solution was completed using a separate MALDI-MS methodology (Venkataraman et al., 1999).

Branching Morphogenesis Assay

IMCD cells were plated to 50% confluence and serum starved overnight. One group of cells was pretreated with mES for 2 hr at 37°C. Cells were then trypsinized, resuspended in rat type I collagen (Collaborative Biomedical), and cultured in the presence or absence of growth factor \pm mES. After a 24 hr period of incubation at 37°C, an average of 50 single cells were randomly selected and scored for the presence or absence of branching processes as previously described (Kjelsberg et al., 1997).

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