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Determination of Functionally Relevant Cell-Type Specific Differences in Heparan Sulfate Glycanation

Key Terms:

- Heparan Sulfate Proteoglycans (HSPG)
- Glypican
- Glycanation
- Alkaline Phosphatase Tagging (AP)
- Affinity Co-Electrophoresis (ACE)

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The purpose of this study is to examine the functional significance of cell-type specific glycanation patterns of cell-surface heparan sulfate proteoglycans (HSPG). The proteoglycan (PG) of interest, glypican, was fused with alkaline phosphatase (AP) and transfected into various cell lines. Subsequent binding assays of the fusion protein were carried out with affinity co-electrophoresis (ACE), a method that allows rapid determination of a PG's binding affinity by taking advantage of its high electrical mobility. This study demonstrates that combining AP-tagging and ACE offers a convenient, systematic method of measuring the binding affinity of a specific PG, and that HS glycanation patterns influence the binding affinities of PGs against their ligands. We find that even though glypican-AP fusion proteins from Chinese hamster ovary (CHO) and 293 cells bind basic fibroblast growth factor (bFGF) and laminin-1 with similar affinities, they bind Type I collagen with significantly different affinities.

NEXT

Abstract

Heparan sulfate is an important cell-surface carbohydrate that interacts with and regulates the activities of many molecules that influence cell growth and behavior. It is known that subtle variations in the structure of heparan sulfate control which molecules it can associate with, but little is known about how cells decide what heparan sulfate structures to make. Huy found a new way to approach this question, by using the tools of molecular biology to streamline the generation and analysis of heparan sulfate-bearing molecules (called proteoglycans) in multiple cell types.

BACK NEXT

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Arthur Lander

Heparan sulfate is an important cell-surface carbohydrate that interacts with and regulates the activities of many molecules that influence cell growth and behavior. It is known that subtle variations in the structure of heparan sulfate control which molecules it can associate with, but little is known about how cells decide what heparan sulfate structures to make. Huy found a new way to approach this question, by using the tools of molecular biology to streamline the generation and analysis of heparan sulfate-bearing molecules (called proteoglycans) in multiple cell types.

BACK NEXT

School of Biological Sciences

Huy Pham - Determination of Functionally Relevant Cell-Type... [1] [2] [3] [4] [5] [6] [7]

Back to Journal 1998 Index

Introduction

Proteoglycans (PGs), proteins bearing glycosaminoglycan (GAG) side chains and present in the extracellular matrix and plasma membranes of almost all cell types, are thought to play important roles in cell growth, morphogenesis, and cancer (Bonneh-Barkay et al. 1997). The biological activities of PGs are mediated in part by their associated GAG chains, polysaccharides having varying patterns of sulfation and consisting of two types: chondroitin sulfate and heparan sulfate (HS). The most abundant cell-surface PGs are those that bear HS side chains which interact with a variety of heparin-binding proteins such as extracellular matrix components and growth factors (Bonneh-Barkay et al. 1997). The biological importance of heparan sulfate proteoglycans (HSPGs) and the potential contribution of their GAG chains has prompted many attempts to correlate HS glycanation patterns to PG functions, in particular their binding against potential ligands. However, these attempts have often been frustrated by difficulties in isolating or labeling any specific PG; chemical analyses have often been done on relatively heterogeneous PG populations or mixtures of GAG chains obtained from such populations. The binding affinity of a heterogeneous PG population does not accurately reflect the contribution of the GAG chains due to potential variability in the core proteins. Measuring the binding of GAG mixtures, on the other hand, fails to consider the native spatial configuration of the GAG chains, and ignores the core protein altogether. To accurately assess the effect of glycanation patterns on the binding activity of a PG, one needs to keep the core protein constant and vary only its glycanation pattern.

One way of achieving this is to express the same PG in different cell types that glycanate it differently. In this study, the gene coding sequence for the PG of interest is first fused with the gene sequence for heat-stable alkaline phosphate (AP); (Flanagan and Leder 1990) the resultant fusion plasmid is transfected into various cell lines. AP-tagging allows convenient tracking of the fusion protein in subsequent biochemical assays. The binding affinity of the fusion protein is determined with affinity co-electrophoresis, or ACE (Herndon and Lander 1997), a method that allows rapid determination of the binding affinity of moderately to highly charged proteins such as PGs. The combination of AP-tagging and ACE allows one to conveniently determine the binding affinity of a specific PG.

Glypican is a cell-surface HSPG that is highly expressed in brain and skeletal muscle (Litwack et al. 1994; Karthikeyan et al. 1994), and that has been implicated in regulating cellular responses to fibroblast growth factors (Bonneh-Barkay et al. 1997) and the transforming growth factor decapentaplegic (Jackson et al. 1997). We find that the glypican-AP fusion proteins expressed in two mammalian cell lines, 293 and Chinese hamster ovary (CHO) cells, exhibit similar binding affinities against bFGF and laminin-1; however, they have significantly different affinities against Type I collagen.

Materials and Methods

Production of glypican-AP fusion plasmid:
The glypican-AP fusion plasmid was constructed as described by Flanagan and Leder (1990). The extracellular domain of glypican was inserted into the expression vector APtag-2, immediately upstream of the AP coding sequence (Figure 1). APtag-2 confers ampicillin and tetracycline resistance to transformed bacterial cells and contains a SV40 origin of replication that selectively enhances the plasmid's production in certain cell lines. *E. coli* transformed with the fusion construct were used to inoculate 500 ml of Luria Broth medium containing 40 μg/ml ampicillin and 10 μg/ml tetracycline, and were grown for 12 to 24 h at 37°C. The expressed plasmid was collected according to the Quiagen Maxi Protocol (Quiagen, Santa Clarita, CA) and its concentration determined with ultraviolet (UV) spectrophotometry.

Expression of the glypican-AP fusion plasmid in cell lines:
Cos-7 and 293 cells were grown in an environment of 100% humidity, 5 to 8% CO_2, at 37°C, and to 50 to 60% confluence in Dubelco's Modified Eagle Medium containing 8 to 10% fetal bovine serum (Hyclone Inc., Logan, UT), penicillin, and streptomycin. Five μg of DNA in 250 μl optimem (Life Technologies Inc., Gaithersburg, MD) were mixed with 30 μl lipofectamine (Life Technologies Inc., Gaithersburg, MD) in 250 μl optimem and incubated for 20 to 40 min. The mixture was then added to each 100 mm plate of cells along with 10 ml of optimem. The next day, the cell media were replaced with normal growth medium; the media harvested 3 to 4 d after the start of transfection. The media were sterile-filtered and stored at 4°C. Transfection

We look at the effects of cell-type dependent glycanation on the binding affinities of the PG glypican against three common extracellular molecules: Type I collagen, basic fibroblast growth factor (bFGF), and laminin-1.

of CHO cells, using protocols obtained from Life Technologies (http://www.lifetech.com), was similar except that 1) the medium used was Ham's F-12 (Life Technologies) and 2) only 5 ml of optimem were added at the time of transfection, followed by an additional 5 ml, 5 h later.

BACK NEXT

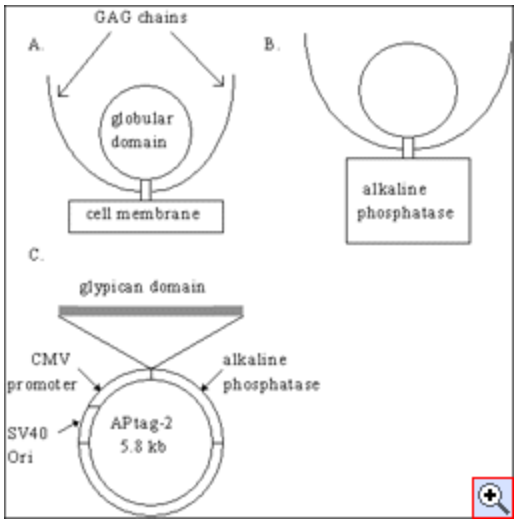

Figure 1
Overview of the construction of a glypican-AP fusion plasmid. In its native state (A), glypican consists of an extracellular domain attached to the cell-surface through a glycosylphosphotidyl (GPI) anchor (not shown). From its DNA sequence data (notably the presence of 14 cysteine residues) and anomalously fast migration on non-reducing gels, glypican's extracellular domain is thought to consist of a compact globular core and a short linear segment that contains varying number of glycosaminoglycan (GAG) attachment sites near the C-terminus. In the fusion protein (B), the GPI anchor is removed and replaced with heat-stable alkaline phosphatase. To generate the fusion plasmid (C), the appropriate glypican DNA sequence (nucleotides 1 to 1641) is inserted into APtag-2 immediately upstream of the AP-coding sequence. APtag-2 contains an SV40 origin of replication that selectively enhances the production of the plasmid in certain mammalian cell lines as well as a CMV promoter that promotes the expression of the plasmid. The expressed fusion protein, lacking a GPI anchor, is secreted into the extracellular medium and can be conveniently harvested.

Assaying for the presence of glycanated glypican-AP fusion protein in the cell media:
AP activity in the media was assayed by adding an equal volume of the phosphatase substrate p-nitrophenyl phosphate (PNP) from Sigma Inc. (St. Louis, MO; made in 2M diethanolamine and 1 mM $MgCl_2$) to 50 µl of cell medium and then, incubated at 37°C for 5 min. AP activity is signaled by a change in the color of the solution. The type of GAG chains present—HS or chondroitin sulfate—was determined by treating the cell media with heparitinase, chondroitinase ABC, or both, and separating the digested products

Purifying the fusion proteins with diethylaminoethyl (DEAE) chromatography:
A binding column, with a volume equal to 30% of that of the protein sample to be purified, was made using DEAE Sephacel beads (Pharmacia Biotech Inc., Piscataway, NJ) and equilibrated with 10 column volumes of 50 mM Tris-Cl buffer (pH 8) containing 150 mM NaCl. After 30 to 40 ml of cell medium passed through, the column was washed with two column volumes of 50 mM Tris-Cl (pH 8) containing 150 mM NaCl, followed with the same buffer containing 250 mM NaCl. The column was eluted with two column volumes of 750 mM NaCl made in the same buffer, and collected in 1.5 to 1.75 ml fractions. The fractions with the highest amount of AP activity, as determined by a visual inspection of their color (using AP substrate PNP) after 5 to 10 min of incubation at 37°C, were pooled. Protease inhibitors (250 µg/ml NEM, 1 µg/ml Pepstatin A, and 0.5 mM PMSF) were added to the combined fractions, which were then dialyzed against ACE buffer (see below) and stored at -80°C.

Measuring binding by affinity co-electrophoresis:
The binding affinities of the glypican fusion protein were determined by ACE (Herndon and Lander 1997; Figure 2). ACE gels were developed by incubation with the AP substrate NBT/BCIP at 37°C for several hours or overnight, analyzed using the software ImageQuant (Molecular Dynamics, Inc. Sunnyvale, CA) and quantified essentially as described (San Antonio et al. 1993), except that a colorimetric rather than radioactivity-based method was used to determine mobility. For each lane, relative AP-staining intensity as a function of the distance from the top of the lane was measured

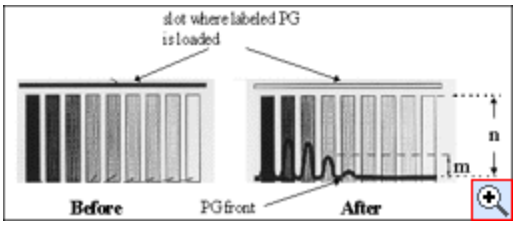

Figure 2
Schematic diagram of ACE. The illustrations represent an ACE gel before and after electrophoresis. A labeled PG, loaded into the top horizontal slot, migrates through lanes containing the test ligand at various concentrations, embedded in 1% low melting point agarose. The extent to which the PG migration is retarded, as a function of ligand concentration, can be

on a 1% low melting point agarose gel made in 50 mM Tris-Cl (pH 8) and 1 mM $MgCl_2$. The AP-containing proteins on the gel were detected with the addition of an AP substrate (NBT/BCIP), made from Sigma Fast tablets (Sigma). The solution turns blue in the presence of AP. The cos-7 cell medium was also analyzed by Western blotting, where the protein was probed with a mouse anti-AP antibody according to the ECL Western blotting protocol (Life Science).

used to calculate the dissociation constant K_d for the interaction. If the concentration of labeled PG is much less than K_d, the value of K_d may be calculated from the equation $R = R_{\infty}/(1+ K_d/[P])$ where R is the retardation coefficient, calculated as m, the amount of retardation, divided by n, the distance that unimpeded PG travels, R_{∞} is the value of R approached at saturating ligand concentrations. [P] is the concentration of the ligand embedded in each lane. Reprinted with the permission of author (Herndon and Lander 1997).

BACK NEXT

along a 2 mm wide vertical band in the middle of the lane. The mobility was taken to be the point that divides the intensity curve into halves of equal areas. In cases where non-binding AP-positive degraded products were significant, the intensity curve for each lane was first corrected by subtracting from it the intensity curve for the degraded product alone, obtained from the intervening lanes. The intensity curve for the degraded product was assumed to follow a Gaussian distribution and determined using the software CurveExpert (Daniel Hyams, Starkville, MS). Non-linear, least-squares curve fitting was used to fit ACE data to the equilibrium equation (Figure 2) using GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Results

Expression and Purification of the Glypican-AP Fusion Protein in Cell Lines:

The glypican-AP fusion plasmid obtained from transformed *E. coli* cells (at concentrations of 0.5 to 2 g/ml, as determined with UV spectrophotometry) was used to transfect three different cell lines: cos-7, 293, and CHO cells. The media of transfected cells, before and after heat treatment, all showed significant levels of AP activity, compared to unobservable AP activity in untransfected cell media. Separating the transfectant media on agarose gels resulted in highly extended bands characteristic of glycanated proteins, indicating the presence of the glypican-AP fusion proteins. This was also confirmed by a Western blot showing a band corresponding to the molecular weight of the fusion protein (done for cos-7 cells only; Figure 3B). The pooled DEAE fractions with high AP activity yielded an extended band (Figure 3A, arrow) that corresponds only to the lower portion of the band from the original medium, indicating that the pooled fractions contain a more highly charged subpopulation of the original glypican-AP sample. Heparitinase, but not chondroitinase ABC, treatment replaced the extended band with a compact band. This latter band corresponds to a de-glycanated protein (Figure 3C); this confirms that the glypican-AP fusion protein contains mostly, if not all, HS chains rather than chondroitin sulfate chains.

Affinity Co-electrophoresis Binding Assays of Glypican-AP Fusion Proteins:

The ACE electrophoretograms for the binding of glypican-AP fusion proteins obtained from 293 and CHO cells against three common HS-binding extracellular binding, AP-positive degraded products that probably consist of AP fragments resulting from proteolytic degradation of the fusion protein. To account for the degraded products, the intensity curve for each lane was corrected as described in Materials and Methods (Figure 5). After the necessary corrections, the mobility for each lane was determined and used to calculate R-values, which were plotted against ligand concentrations for each binding assay (Figure 6). A best-fit curve based on the equilibrium equation (Figure 2) was determined, and a K value was obtained for each binding assay (Figure 7).

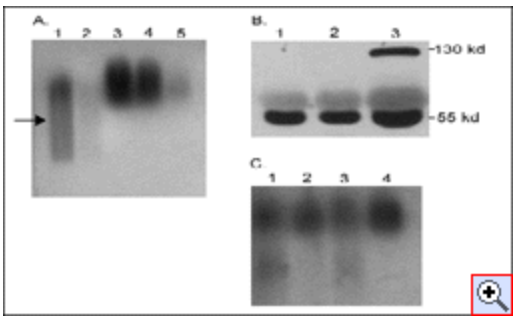

Figure 3
DEAE purification and identification of glypican-AP fusion proteins. (A) Wash and eluted DEAE fractions of transfectant cell medium were separated on a 1% agarose gel and the protein bands were developed by incubation in an AP substrate overnight. Lanes 1 and 2 show 750 mM NaCl eluted fractions with high AP activity, diluted 1:2 and 1:10, respectively; lane 3 shows sample flow-through; lane 4 is a 150 mM NaCl wash; lane 5 is a 250 mM NaCl wash. (B) Cell media from cos-7 cell cultures, transfected with APtag-2 containing only coding sequence for AP (lane 1), coding sequence for neither AP nor glypican (lane 2), or coding sequences for both AP and glypican (lane 3), were heparitinase-treated, separated on a 12% SDS-PAGE gel, and probed with an anti-AP antibody. The top band in lane 3 corresponds to 130 kd, the MW of the glypican-AP fusion protein. The bottom bands correspond to 55 kd, the MW of AP. The AP in sample 2 is believed to be endogenously produced. (C) DEAE-purified fusion proteins were treated with heparitinase (lane 2), chondroitinase ABC (lane 3), or both (lane 4), separated on a 1% low-melting-point agarose gel and developed as in (A). Lane 1 represents untreated protein sample.

Discussion

We have used AP-tagging and ACE to measure the binding affinity of glypican

molecules—Type I collagen, bFGF, and laminin-1—are shown in Figure 4. The ACE gels for CHO-derived glypican-AP also show, in addition to the expected ACE patterns, a dark, non-shifting, horizontal band across all nine lanes near the top (Figure 4, arrow). The low mobility of that band suggests that it represents non-

against various extracellular molecules. AP-tagging was used to specifically label glypican as to allow for its detection during ACE. Before interpreting the ACE results, it should be noted that the DEAE-purified glypican fraction also contains other moderately to highly charged proteins, including endogenous PGs that, in theory, can bind the ligands and interfere with the binding assays. However, since the cell lines only express small amounts of endogenous PG, and since the APtag-2 vector selectively amplifies the expression of the glypican-AP fusion protein, the ratio of glypican-AP to endogenous PG is likely large

BACK NEXT

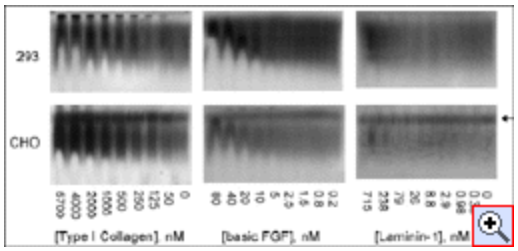

Figure 4
ACE electrophoretograms for the interactions between 293- and CHO-derived glypican-AP fusion proteins and 3 extracellular ligands: Type I collagen, bFGF, and laminin-1. The top gels are for 293-derived glypican-AP, and the bottom gels for CHO-derived glypican-AP. The ligand concentrations for each binding assay are indicated at the bottom of the gels. The gels for CHO-derived glypican-AP also show, in addition to the expected ACE patterns, a relatively dark, non-shifting horizontal band near the top (arrow). The presence of this band is probably due to degraded products of the fusion protein and is accounted for as described in Materials and Methods. Images of the gels were taken with a digital camera using 1D Image Analysis Software (Kodak Digital Science).

enough to make the interference due to endogenous PG insignificant. It should also be noted that the purified glypican-AP contains HS chains of varying length and degree of sulfation so that the K obtained represents an average value of a population of glypican molecules with the same core protein, but with different glycanation patterns.

Among the ligands examined, bFGF yields the highest binding affinity against glypican, which is consistent with previous findings that HSPGs are necessary for receptor binding and mitogenic activity of bFGF (Aviezer et al. 1994). The affinities determined for the binding between 293-derived glypican and collagen are similar to those obtained for the binding of low-molecular weight heparin (K=315±83 nM) and syndecan (derived from normal murine mammary gland epithelial cells, K= 164±7 nM) against the same ligand (San Antonio et al. 1993). Glypican, syndecan, and heparin share a common structural feature, namely the presence of sulfated polysaccharides; the similarity in the binding affinities thus suggests that glypican may have a similar collagen-binding determinant as heparin or syndecan within the sugar chains. The K value obtained for laminin-1, on the other hand, is substantially lower than that obtained for the binding between laminin-1 and glypican (obtained in the rat brain; Herndon and Lander 1992). Brain-derived glypican tends to have longer,

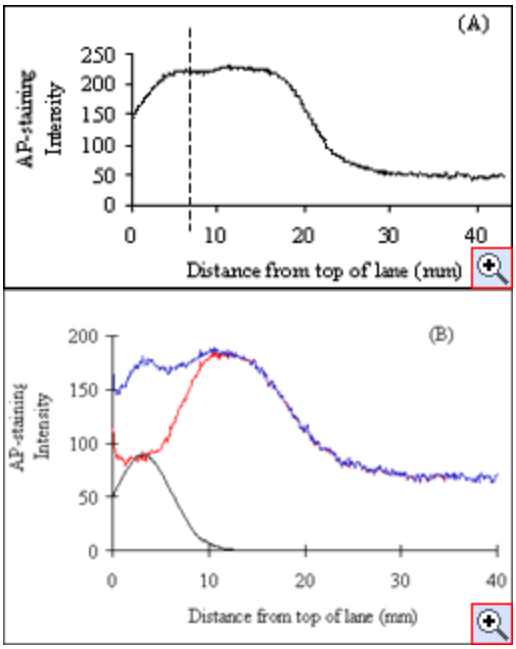

Figure 5
Determination of ACE retardation coefficients (R's). To determine the retardation coefficients (Figure 2) we need to measure the mobility of the PG front, taken to be the distance from the top of each lane to the "midpoint" of the PG band. To determine the midpoint of the PG band, relative AP-staining intensity along a 2 mm wide vertical band in the middle of the lane is measured as a function of the distance from the top of each lane. A typical plot of AP-staining intensity versus migration distance is shown in (A). The staining intensity ranges from 0 (lightest) to 255 (darkest). The midpoint of the band is taken to be the point, or line, that divides the area under the intensity curve into equal halves (shown as the dashed line), after subtracting the background intensity level (about 50 in this case). In some cases, significant amounts of non-binding AP-positive degraded products were present (Figure 4, arrow), which register as a secondary peak on the intensity curve (B, top curve). The degraded products are accounted for by subtracting from the original intensity values the intensity curve due to the degraded product alone (bottom curve) which yields a more accurate intensity curve (middle curve). The midpoint of the corrected curve is then determined as described.

Binding heterogeneity in the interaction of glypican and its ligands±as shown by highly extended bands on the ACE electrophoretograms±is lower for bFGF than for collagen (and possibly laminin). Further, the low R± values for collagen strongly suggest the presence of a glypican

more heavily sulfated HS chains, suggesting that 1) glypican's binding affinity against laminin-1 depends primarily on its GAG chains rather than its core protein and 2) that the nature of the GAG chains (for example, their length and degree of sulfation) is more important for binding against laminin than against bFGF.

subpopulation in the PG sample that does not bind collagen. Yet no such subpopulation is seen with bFGF. Apparently, there exists a glypican subpopulation that 1) does not bind, or binds very weakly to, collagen and 2) binds bFGF with moderate to high affinities. Comparison of K_d values obtained for the binding of glypican-AP from 293 and CHO cells against the three ligands shows that glypican from the two cell lines binds bFGF or laminin-1 with similar affinities, yet binds collagen with significantly different affinities (Figure 7).

BACK NEXT

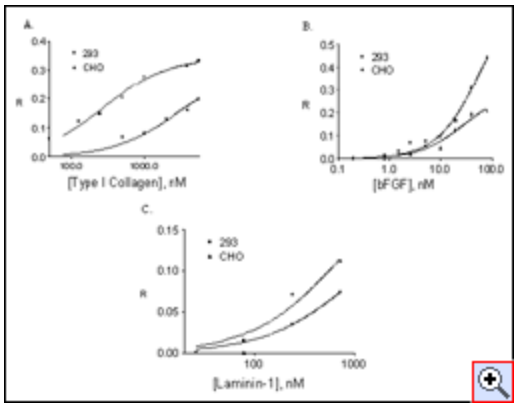

Figure 6
Calculation of dissociation constants (K_d) for the binding between 293- and CHO-derived glypican-AP fusion proteins against Type I collagen (A), bFGF (B), and laminin-1 (C). For each of the ACE electrophoretograms in Figure 4, retardation coefficients R's were determined and plotted against ligand concentrations as described in Materials and Methods. For each plot a K_d value was calculated. The results are shown in Figure 7.

Taken together, these results suggest that the HS moieties on glypican are able to mediate selective binding against extracellular molecules. The fact that glypican produced from different cell lines bind some extracellular molecules with similar binding affinities and other molecules with significantly different affinities suggest that HS-mediated binding may depend on subtle differences in glycanation patterns, consistent with previous findings (Sanderson et al. 1994). An alternative explanation is that binding against bFGF depends primarily on the core protein, which is the same across cell lines; binding against collagen depends primarily on the glycanation patterns, which likely differ across cell lines. This interpretation, however, is not likely to be correct since the biological activity of bFGF is found to be greatly influenced by direct binding to heparin and HS (Ishihara et al. 1994).

Using AP-tagging and ACE to measure the binding affinities of glypican-AP fusion proteins derived from two different cell lines against three common extracellular ligands, we have found that differences exist in glycanation patterns for the two mammalian cell lines—293 and CHO (inferred from cell-type dependent differences in binding affinities against collagen)—and these differences affect the binding of glypican against some, but not all, ligands. Similarities across cell types in the binding affinities of glypican against bFGF and laminin-1 suggest that some features in the glycanation pattern of the glypican molecule are preserved across the cell types examined. On the other hand, cell-type dependent differences in the binding affinity of glypican against collagen suggest that glypican has at least some differences in glycanation patterns in different cell lines. These differences may not simply reflect gross discrepancies in chain length or charge. J. D. San Antonio et al. (1993) found that differences in the binding affinities of subpopulations of low-molecular weight heparin against extracellular matrix molecules are likely to be the result of more subtle structural features of heparin, such as specific sugar chain composition and structure rather than chain length or charge. Further studies are necessary to determine the differences in glycanation patterns across cell types that affect PG binding and the significance of these differences *in vivo*.

Acknowledgements

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BACK NEXT

Page 6

Huy Pham - Determination of Functionally Relevant Cell-Type... [1] [2] [3] [4] [5] [6] [7]

Back to Journal 1998 Index

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BACK

Page 7

Huy Pham - Determination of Functionally Relevant Cell-Type... [1] [2] [3] [4] [5] [6] [7]

Back to Journal 1998 Index