Glypican-3 is a binding protein on the HepG2 cell surface for tissue factor pathway inhibitor

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Tissue factor pathway inhibitor (TFPI) is a primary regulator of the initiation of blood coagulation. TFPI is internalized and degraded by HepG2 cells through the low-density-lipoprotein receptor-related protein (LRP) but also binds another molecule present on the cell surface at approx. 10-fold the abundance of LRP [Warszawsky, Broze and Schwartz (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6664–6668]. When HepG2 cells are washed with heparin or dextran sulphate, a substance that binds TFPI is removed from the cell surface and can be detected in a slot-blot assay. Preincubation with trypsin destroys the reactivity of the TFPI-binding component in the slot-blot assay, suggesting that it is a protein. In addition, when the sulphation of glycosaminoglycans (GAGs) is prevented by growing the HepG2 cells in the presence of 30 mM sodium chlorate, TFPI binding is

unaffected, whereas the binding of bovine lipoprotein lipase, a protein known to associate with cell-surface GAGs, falls to 50 % of control levels. Dextran sulphate washes of HepG2 cells grown in sodium chlorate have an equal reactivity in slot-blot experiments to that of non-treated cells, suggesting that GAGs are not totally responsible for the binding activity observed. By using the slot blot to follow binding activity and conventional protein purification techniques, a protein species that migrates at 40 kDa after reduction was identified in the HepG2 cell wash. The binding of this protein to TFPI was confirmed with immobilized TFPI. Amino acid sequence analysis identified this protein species as a proteolytic fragment of glypican-3 (also called OCI-5), a member of the glypican family of glycosylphosphatidylinositol-anchored proteoglycans.

INTRODUCTION

The initiation of the coagulation cascade is regulated by tissue factor pathway inhibitor (TFPI), a trivalent Kunitz-type proteinase inhibitor. The second Kunitz domain of TFPI is a potent direct inhibitor of factor Xa; once factor Xa has been bound, the first Kunitz domain rapidly inhibits the factor VIIa/tissue factor complex, effectively blocking tissue factor-initiated coagulation [1]. The third Kunitz domain does not have an identified inhibitory function. In addition to the three Kunitz-type inhibitory domains, TFPI has a highly basic C-terminal region that is required for the optimal inhibition of factor Xa by the second Kunitz domain [2,3] and is the region of TFPI that is required for high-affinity binding to heparin [4]. A substantial portion of TFPI circulating in plasma is associated with lipoproteins. This lipoprotein-bound TFPI is variably C-terminally truncated and is therefore not as effective an anticoagulant in one-stage clotting assays as the full-length protein [5]. Full-length TFPI seems to be associated with the endothelium and is released into the circulating blood after the infusion of heparin, which increases the plasma TFPI concentration 2–4-fold [6,7]. Individuals with abetalipoproteinaemia possess greatly decreased circulating TFPI concentrations but normal amounts of heparin-releasable TFPI. Because these patients are not at increased risk of thrombosis, the endothelial bound TFPI is probably the most important physiological reservoir of TFPI [8].

When TFPI is injected into animals, it is rapidly removed from the circulation, predominantly by the liver [9–11]. HepG2 hepatoma cells internalize and degrade TFPI via the low-density-lipoprotein receptor-related protein (LRP). However, in cell surface binding studies, approx. 10 times more TFPI is bound to

unknown species distinct from LRP [12]. Here we report the identification of glypican-3, a member of the glypican family of glycosylphosphatidylinositol (GPI)-anchored proteoglycans, as a TFPI-binding protein present on the HepG2 cell surface.

MATERIALS AND METHODS

Reagents

Dextran sulphate (average molecular mass 5000 Da), protamine sulphate, dichloroisocoumarin, poly-(L-lysine), dithiothreitol, bovine trypsin and phosphatidylinositol-specific phospholipase C were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pig intestinal heparin was obtained from Rugby Laboratories (Rockville Center, NY, U.S.A.). Sodium chlorate was obtained from Fluka Chemical Corp. (Ronkonoma, NY, U.S.A.). Restriction enzymes were obtained from New England Biolabs (Beverly, MA, U.S.A.).

Proteins

Recombinant full-length human TFPI produced in *Escherichia coli* was a gift from the Monsanto Co. (Chesterfield, MO, U.S.A.). TFPI-160, an altered form of TFPI truncated after Gly-160, was produced in *E. coli* and purified as described previously [11]. The protein contains the entire first two Kunitz domains of TFPI but lacks the third Kunitz domain and the basic C-terminal region. Bovine lipoprotein lipase was a gift from Dr. Ira Goldberg (Columbia University, New York, NY, U.S.A.). Recombinant human full-length glutathione S-transferase 39 kDa protein was produced in and purified from *E. coli* as described previously

Abbreviations used: GAG, glycosaminoglycan; GPI, glycosylphosphatidylinositol; LRP, low-density-lipoprotein receptor-related protein; TFPI, tissue factor pathway inhibitor.

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[13]. The murine monoclonal 2H8 anti-TFPI antibody, which recognizes an epitope within the first Kunitz domain of TFPI, was produced as described previously [5].

Radiolabelling

TFPI was iodinated with Na¹²⁵I (New England Nuclear, Boston, MA, U.S.A.) with Iodobeads® in accordance with instructions provided by the manufacturer (Pierce, Rockford, IL, U.S.A.). cDNA primers were labelled with dAT³²P (New England Nuclear, Boston, MA, U.S.A.) by random primer labelling.

Cell binding studies

The HepG2 cells [grown in Dulbecco's modified Eagle's medium containing 5 % (v/v) fetal bovine serum] were seeded into 24-well plates approx. 2 days before use at approx. 90 % confluence. For assay, the cells were chilled on ice and washed twice with ice-cold Dulbecco's modified Eagle's medium containing 3 % (w/v) BSA (assay buffer). ¹²⁵I-TFPI was added to 5 nM final concentration in assay buffer. The 5 nM concentration was selected because it is well below the estimated 15 nM K_d for the binding of TFPI to hepatoma cells [12]. In some experiments various concentrations of heparin were added to the assay buffer. In another series of experiments the cells were rinsed with various concentrations of heparin in PBS (200 µl). The heparin rinses were removed and saved for use in the slot-blot assay (see below). The cells were then washed three times with assay buffer to remove the remaining heparin before the ¹²⁵I-TFPI was added. In competition binding studies, the competing unlabelled ligand was added to 500 nM final concentration. After incubation for 2 h at 4 °C, the cells were washed three times with ice-cold assay buffer and then lysed with 1 M NaOH. The radioactivity of the cell lysates was determined with a gamma counter.

Slot-blot assay

Samples (100 µl) were blotted on nitrocellulose with a minifold II slot-blot system (Schleicher and Schuell, Keene, NH, U.S.A.). After blotting, the nitrocellulose was incubated on a rotary shaker in 3 % (w/v) non-fat dried milk, reconstituted in 50 mM Tris/100 mM NaCl (pH 7.6) to block non-specific protein-binding sites. TFPI or TFPI-160 was added to a final concentration of 20 nM and incubated for 2 h at 23 °C. The nitrocellulose was washed three times in the above buffer and then incubated for 2 h with 2 µg/ml monoclonal 2H8 anti-TFPI antibody. After being washed, the nitrocellulose was incubated for 1 h with anti-(mouse IgG)-horseradish peroxidase conjugate (Sigma) and then reacted with hydrogen peroxide and diaminobenzidine (Sigma) to develop colour.

Trypsin treatment of the dextran sulphate cell wash

Bovine trypsin at 50 µg/ml final concentration was incubated with the dextran sulphate cell wash for 30 min at 37 °C. After incubation, dichloroisocoumarin was added to 50 µM final concentration and the sample was blotted on nitrocellulose. To rule out the presence of a contaminating substance that blocked binding, trypsin inactivated by incubation for 15 min at 23 °C with dichloroisocoumarin was used instead of active trypsin in some experiments.

Purification of the TFPI-binding protein

By using the slot-blot assay to monitor binding activity, a 40 kDa protein, as assessed by reducing SDS/PAGE, was purified from

dextran sulphate washes of HepG2 cells grown in serum-free media [14]. The starting material was obtained by rinsing 2 litre roller bottles twice a week with 15–20 ml of 100 µg/ml dextran sulphate followed by filtration through a 0.2 µm filter to remove cellular debris. The purification was performed with three chromatography steps. Dextran sulphate cell wash (2–5 litres) was applied to a 3 cm × 27 cm (190 ml) fast Q anion-exchange column at approx. 100 ml/h. The column was then washed with approx. 2 litres of 20 mM Tris/100 mM NaCl/5 mM EDTA (pH 7.8). The TFPI-binding component was eluted at approx. 1.0 M NaCl in a 0.1–2 M NaCl gradient (400 ml total gradient volume). Fractions (2.5 ml) demonstrating a positive reaction in the slot-blot assay were pooled and concentrated with an Amicon (Beverly, MA, U.S.A.) concentration system with a YM10 membrane, to approx. 0.5 ml. The 0.5 ml sample was applied to an FPLC® (Pharmacia, Uppsala, Sweden) Superose 6 gel-filtration column. The TFPI-binding component was eluted at a molecular mass of approx. 300 kDa in 20 mM Tris/100 mM NaCl (pH 7.8) in a volume of approx. 3 ml. This material was applied directly to an FPLC® mono Q anion-exchange column. The column was washed with 25 mM sodium acetate buffer, pH 5.0, and the TFPI-binding component was eluted at approx. 1.0 M NaCl in a 0.1–2 M NaCl gradient (40 ml) total gradient volume.

Reports indicating that members of the glypican family are shed into culture media [15–17] suggested that the 40 kDa band could also be present in conditioned media at levels not detectable with the slot-blot assay. In our laboratory we had available material from 50 litres of HepG2-conditioned media that had been precipitated with CdCl₂ and applied to a bovine factor Xa affinity column to purify TFPI [18]. The flowthrough solution was applied to the fast Q column. The peak that was eluted at approx. 1.0 M NaCl contained a 40 kDa band that was submitted for amino acid sequence analysis after SDS/PAGE purification (see below).

PAGE

SDS/PAGE was performed on samples boiled for 3 min after treatment with 1 % SDS and 50 mM dithiothreitol. A Tris/glycine buffer system was used with 7, 10 or 20 % (w/v) gels. The samples submitted for amino acid sequence analysis were transferred to a PVDF membrane (Millipore, Bedford, MA, U.S.A.) and proteins were revealed by staining with Coomassie Blue R-250. Other samples were revealed directly with the Bio-Rad (Hercules, CA, U.S.A.) silver stain plus kit.

Amino acid sequence analysis

Automated gas-phase Edman degradation was performed by the Washington University Protein Chemistry Laboratory and Midwest Analytical (St. Louis, MO, U.S.A.).

Immobilized TFPI

Because variable truncation of the C-terminal region of TFPI occurs readily, the following procedure was developed to ensure that an affinity column containing full-length TFPI was produced. The monoclonal 2H8 anti-TFPI antibody was covalently linked to Affigel® (Bio-Rad) as previously described [5]. A 0.5 ml anti-TFPI affinity column was prepared in a disposable pipette tip [19] and equilibrated in PBS. The column was charged with full-length TFPI (40 µg) and washed with 10 column volumes of PBS. Immediately after washing, glypican-3 was loaded on the column. After a wash (10 column volumes) with PBS, bound protein was eluted with PBS containing 1 M NaCl. In control

experiments, the immobilized antibody was not charged with TFPI.

Glypican-3 cloning and Northern blot analysis

Oligonucleotides were synthesized on the basis of the coding regions of the glypican-3 (MXR-7) sequence in Genbank from the regions corresponding to the two amino acid sequences obtained (see results). These primers were used as a PCR primer pair (forward primer, 5'-AATGCTGCGGTTTTCCAAGAG-GCC-3'; backward primer, 5'-AAATACTTTTCAGGTCGC-GTC-3') to amplify an approx. 300 bp fragment from a placental cDNA library (provided by Dr. Evan Sadler, Washington University, St. Louis, MO, U.S.A.). This fragment was further subcloned into a TA cloning vector (Invitrogen, San Diego, CA, U.S.A.) and shown to represent a fragment of glypican-3 cDNA by sequencing with the forward primer. It was then labelled with [³²P]dATP and used to screen the placental cDNA library. Two of 22 positive clones were purified and subjected to restriction enzyme analysis (*Apa*I, *Bgl*I, *Dra*III, *Hind*III, *Pst*I, *Sac*I, *Sal*I, *Sma*I and *Xho*I) that produced cleavage patterns predicted by the Genbank glypican-3 cDNA sequence. One, containing an insert of 2.2 kb, was subcloned into pBluescript II KS (Stratagene, La Jolla, CA, U.S.A.) and further analysed by sequencing approx. 100 bases from both the 5' and 3' ends. This 2.2 kb clone was labelled with [³²P]dATP and used as a probe for a multiple-tissue Northern blot (Clontech, Palo Alto, CA, U.S.A.) performed in accordance with instructions provided by the manufacturer.

RESULTS

TFPI binding to HepG2 cells in the presence of heparin

In HepG2 cell-surface binding studies, increasing concentrations of heparin progressively prevented TFPI binding (Figure 1A),

suggesting that the heparin is either blocking the interaction between TFPI and its binding component or removing the binding component from the cell surface. To test this second possibility, the cells were rinsed with PBS containing the indicated concentrations of heparin and the binding study was repeated after removal of the heparin (Figure 1B). Although the effect was not as pronounced as when heparin was present during the binding assay, rinsing with increasing amounts of heparin also progressively decreased the amount of binding observed. Thus it seems that heparin was both blocking the interaction of TFPI with the binding component (at relatively low concentrations) and removing the binding component from the cell surface (at high concentrations). A slot-blot assay, designed to detect a TFPI-binding component(s), confirmed that the heparin cell washes contained a substance that bound TFPI; the amount of binding activity observed correlated with the amount of heparin in the cell wash (Figure 1, inset).

Characterization of the TFPI-binding component in the cell washes

The slot-blot assay was performed on cell washes obtained with several different buffers. Heparin and dextran sulphate removed the binding component from the cell surface much more effectively than 100 µg/ml protamine sulphate, 100 µg/ml poly-(L-lysine), 2 M NaCl, 100 mM glycine (pH 3.25), 100 mM 3-(cyclohexylamino)propane-1-sulphonic acid (pH 10), 0.2 unit/ml phosphatidylinositol-specific phospholipase C or Tris/saline solutions containing 0.1 % NP40, Tween-20 or Triton X-100 (results not shown). Dextran sulphate (average molecular mass 5000 Da) at 100 µg/ml was routinely chosen to wash the cells. Treating the dextran sulphate cell wash with either trypsin or 1 % (w/v) SDS destroyed the reactivity of the TFPI-binding component in the

Figure 1 ¹²⁵I-TFPI binding to HepG2 cells with increasing amounts of heparin in the binding buffer (A), and ¹²⁵I-TFPI binding to HepG2 cells after rinsing with buffer containing the indicated amounts of heparin and then washing to remove the heparin (B)

Inset: slot-blot assay of the heparin rinses, demonstrating that increasing reactivity in the slot-blot assay correlates with decreasing TFPI binding in the cell-binding assay. The data reported are the averages of triplicate experiments. DS5000 indicates experiments performed with 100 µg/ml dextran sulphate (average molecular mass 5000 Da). Abbreviation: %CPM, data standardized by defining the binding of the ligand (TFPI) in the absence of any binding inhibitors as 100% and reporting the amount of cell surface binding in other experiments as the percentage of the radioactivity (c.p.m.) bound when compared with this standard.

Table 1 Binding of TFPI and bovine lipoprotein lipase to untreated HepG2 cells and HepG2 cells grown in the presence of 30 mM sodium chlorate

Results were standardized by defining the binding of the ligand (TFPI) in the absence of any binding inhibitors as 100% and reporting the amount of cell surface binding in other experiments as the percentage of the radioactivity bound when compared with this standard, and are means $\pm$ S.D. The binding of TFPI and lipoprotein lipase (LPL) were standardized separately.

	Binding (%)	
	Untreated cells	Chlorate-treated cells
TFPI	100 $\pm$ 5	107 $\pm$ 28
Bovine LPL	100 $\pm$ 10	51 $\pm$ 13

slot-blot assay, suggesting that it is a protein (results not shown). When the trypsin activity was inhibited by reacting it with dichloroisocoumarin before mixing it with the cell wash, the slot-blot assay result was not affected, excluding the possibility that a contaminant in the trypsin was blocking the reaction (results not shown). Boiling the cell wash in 50 mM dithiothreitol, however, did not affect the reactions observed in this assay (results not shown).

The slot-blot assay was tested to ensure that it was specific for TFPI-binding component(s). Importantly, TFPI-160, a form of TFPI containing the first two Kunitz domains but truncated after Gly-160, did not have binding activity (results not shown). This is consistent with our previous studies demonstrating that TFPI-160 does not bind specifically to the HepG2 cell surface [11] and indicates that the positive reaction observed when full-length TFPI was used was not an artifact. As demonstrated in Figure 1 (inset), washes of the cells with PBS alone did not bind TFPI. Additionally, the heparin and dextran sulphate-containing buffers and the fresh and conditioned cell culture media did not bind TFPI (results not shown).

Studies with HepG2 cells grown in 30 mM sodium chlorate

TFPI binds to heparin-agarose; heparin-releasable TFPI is hypothesized to be released from glycosaminoglycan (GAG)-binding sites on the endothelium [20]. To investigate the role of GAGs in the binding of TFPI to the HepG2 cells, binding and slot-blot experiments were performed with cells grown in 30 mM sodium chlorate, an inhibitor of sulphate adenylyltransferase, the enzyme responsible for the sulphation of cellular proteoglycans [21]. TFPI bound equally well to chlorate-treated and control HepG2 cells (Table 1). Bovine lipoprotein lipase, a protein known to associate with the cell surface via GAGs [22,23], bound to cells grown in sodium chlorate at only 50% of control levels (Table 1). In competition experiments, both a 500-fold excess of unlabelled TFPI and 1 i.u./ml heparin prevented more than 90% of the 125 I-TFPI binding to the chlorate-treated and untreated cells. However, a large molar excess of the 39 kDa protein, a competitive inhibitor of all known LRP ligands, did not affect TFPI binding, indicating that the binding observed is not mediated by the LRP (results not shown). Additionally, the DS 5000 wash from chlorate-treated HepG2 cells bound TFPI in the slot-blot assay (results not shown).

Purification of the TFPI-binding protein

A TFPI-binding protein was purified with a combination of ion-exchange and gel-filtration chromatographies as described in the

Figure 2 Purification of TFPI-binding protein

Upper panel: the A_{280} chromatographic elution profile from the mono Q column, the final purification step. Material was applied to the column as described in the Materials and methods section and eluted with the indicated 0.1–2.0 M NaCl gradient (broken line). The material eluted in the broad peak indicated by the bracket demonstrated a TFPI-binding component in the slot-blot assay and was pooled for SDS/PAGE analysis. Lower panel: reduced SDS/PAGE of the pooled mono Q fractions. The protein was revealed by silver staining. Lane 1, molecular mass standards (97.4, 66.2, 45, 31, 21.5 and 14.4 kDa, with the origin at the top); lane 2, glypican-3.

Materials and methods section. In the final purification step, the protein was eluted from the mono Q column as a broad peak at approx. 1.0 M NaCl (Figure 2, upper panel). When this peak was pooled and analysed by reducing SDS/PAGE, a 40 kDa band was present (Figure 2, lower panel). If proteinase inhibitors were used throughout the purification procedure or the SDS/PAGE was performed under non-reducing conditions, the 40 kDa band was not observed (results not shown). As the slot-blot assay produced only qualitative results, the yield of the TFPI-binding component during the purification procedure could not be determined accurately.

TFPI-affinity chromatography

Fresh TFPI-affinity columns were prepared immediately before use by binding TFPI to a monoclonal 2H8 anti-TFPI affinity column. In control experiments, in which TFPI was not bound to the column, the TFPI-binding component containing the disulphide-linked 40 kDa fragment was eluted with the void volume fraction (Figure 3A). When TFPI was bound to the column, the 40 kDa fragment was concentrated on the column

Figure 3 Reducing SDS/PAGE of TFPI-affinity column fractions of glypican-3

Protein bands were revealed by silver staining. The arrow indicates the 40 kDa band. The positions of molecular mass standards are indicated at the left of each panel. (A) The control experiment where TFPI was not bound to the anti-TFPI column; (B) the TFPI-affinity column where TFPI was bound to the anti-TFPI column. Lane 1, column charge; lane 2, column flowthrough; lane 3, 1 M NaCl eluate. The approx. 32 kDa band in lane 3 of (B) is recombinant TFPI that was also eluted from the column by 1 M NaCl.

and could be eluted with 1 M NaCl (Figure 3B). On the basis of experiments with ^{125}I -labelled preparations, more than 70 % of the radioactivity was eluted from the TFPI-primed column with 1 M NaCl (results not shown). The same amount of protein was loaded on the column in the experiments shown in Figures 3(A) and 3(B). The different staining intensities of the 40 kDa band and the other artifact bands in the two gels is attributed to gel-to-gel variability in the silver-staining technique. The band at approx. 66 kDa is an artifact related to dithiothreitol. The other bands, present at equal amounts in all lanes of the gel, are probably artifacts and do not represent specific TFPI-binding proteins.

Identification of the TFPI-binding protein

The 40 kDa bands purified from both the dextran sulphate washes and the concentrated conditioned media of the HepG2 cells (see the Materials and methods section) were subjected to N-terminal amino acid sequence analysis after cyanogen bromide cleavage because initial experiments suggested that the N-terminus was blocked. Cyanogen bromide treatment produced four bands (13.5, 12.5, 7.9 and 5.8 kDa) on SDS/PAGE. Because a larger amount of protein was purified from the 50 litres of concentrated conditioned media, it provided more complete amino acid sequence data. The 13.5 and 12.5 kDa fragments did not produce sequence data. For the material derived from the conditioned media, the sequences obtained from the 7.9 and 5.8 kDa fragments were PGLPXSALDINEXXRXAARDLK-VF and LXFLIIXNAAVFQEXEIVXRHA respectively. The corresponding sequences obtained from the material derived from the dextran sulphate washes were PXLXDXAXDINXQ and FLIXQNAAVFQ. The Genbank database revealed that these sequences correspond to two regions within the N-terminal 40 kDa portion of glypican-3, a member of the glypican family of GPI-anchored proteoglycans [24].

Figure 4 Glypican-3 Northern blot analysis of adult human tissue

Upper panel: a transcript of approx. 2.4 kb was most highly expressed in placenta, ovary and small intestine. Smaller amounts of expression are seen in the pancreas, colon, testes, prostate and thymus. Lower panel: blot rehybridized with a β -actin probe. Abbreviation: PBL, lane with poly(A)⁺ RNA from peripheral blood leucocytes.

Cloning of the human glypican-3 gene and tissue distribution of the mRNA

A full-length cDNA clone of the human glypican-3 gene was obtained from a human placental cDNA library and used to probe a human multiple-tissue Northern blot (Clontech, Palo Alto, CA, U.S.A.). The placenta, small intestine and ovary express the gene most strongly, whereas the pancreas, colon, testes, prostate and thymus express it weakly. Virtually no expression is detected in the heart, lung, liver and kidney (Figure 4). The lack of expression in these highly vascularized organs is consistent with its lack of expression in mouse endothelial cell lines [16] and our inability to clone the cDNA from either a human liver or a human endothelial cDNA library.

DISCUSSION

TFPI is internalized and degraded by HepG2 cells through a pathway that requires association with the LRP, but cell-surface binding studies indicate that it also binds to separate cell-surface sites approx. 10-fold more abundant than the LRP. We have identified glypican-3, a member of the glypican family of proteoglycans, as a TFPI-binding protein on the HepG2 cell surface. The defining characteristics of this proteoglycan family include a core protein molecular mass of approx. 60 kDa, a GPI membrane anchor, the presence of Ser-Gly GAG attachment sites and a highly conserved pattern of 14 cysteine residues. The family members are 20–40 % identical at the amino acid level and include glypican [15], glypican-3 [25], cerebroglycan [26] and K-glypican [16]. The interspecies amino acid identity between the human, mouse and rat is typically greater than 90 %. The strong evolutionary conservation of the amino acid sequence of these

proteoglycans suggests that the protein core has an important biological role [26]. Unfortunately, it also makes it difficult to produce high-affinity antibodies against these proteins [27] and prevented characterization of the relative contribution of glypican-3 to the binding of TFPI to the HepG2 cell surface.

Glypican-3 cDNA (originally termed OCI-5) was first sequenced by Filmus et al. [25], who identified it as a transcript that accumulates differentially during rat intestinal development. The rat and human proteins are 94% identical at the amino acid level. Glypican-3 has two Ser-Gly segments located adjacent to stretches of acidic residues near the C-terminus of the protein that are potential GAG attachment sites. Following this region is a stretch of hydrophobic residues that represents a potential GPI attachment site. Filmus et al. [27] have confirmed that the glypican-3 protein produced in COS-1 cells contains GAGs and a GPI anchor.

There are several reports indicating that the proteins in the glypican family are shed from the cell membrane into the culture media [15–17]. Indeed, we were able to identify glypican-3 in concentrated HepG2 cell-conditioned media. However, washing the cells with dextran sulphate or heparin releases significantly more TFPI-binding protein, as assessed by the slot-blot assay, than is found in the culture media and provides an enriched source of protein for purification [28]. It is likely that shedding of the protein from the cell surface is an artifact of cell culture where either a phospholipase or proteinase secreted into the culture media cleaves the protein from its anchor. After cleavage the protein remains non-covalently attached to the cell surface but can be efficiently removed by heparin, dextran sulphate or other carbohydrates [29]. The isolation of a TFPI-binding protein that probably contains a GPI anchor *in vivo* is consistent with the results of Sevinsky et al. [30] that suggest that TFPI is bound to a GPI-anchored protein on the surface of a tumour necrosis factor α -stimulated, transformed, human umbilical vein endothelial cell line.

The evidence that glypican-3 actually binds TFPI is in two parts: (1) purified glypican-3, as determined by a single 40 kDa band on a silver stained gel (Figure 2), is strongly reactive in the slot-blot assay; (2) the TFPI-affinity column bound and concentrated the 40 kDa band (Figure 3). During the purification, the 40 kDa band was observed only when proteinase inhibitors were not used and SDS/PAGE was performed under reducing conditions. Apparently the protein is susceptible to a proteolytic cleavage that produces fragments linked by a disulphide bond(s). The 40 kDa fragment, which is from the N-terminal portion of the protein as determined by amino acid sequence data and thus does not have GAG residues, readily migrates into the separating gel and has been observed by others [27]. The purification and electrophoretic characteristics of the glypican-3 protein are very similar to those reported for K-glypican, which contains a 32 kDa proteolytic fragment observed only under reducing conditions when isolated in the absence of proteinase inhibitors [16].

The experiments performed with 30 mM sodium chlorate to prevent the sulphation of the HepG2 cell-surface GAGs indicate that sulphated GAGs are not required for TFPI binding to the cell surface nor to the dextran sulphate cell wash in the slot-blot assay. In contrast, the binding of bovine lipoprotein lipase, a protein known to associate with the cell-surface GAGs [22,23], fell to 50% of control levels in the chlorate-treated cells. Thus the binding interactions between GAGs and these two proteins apparently occur through different mechanisms. Iversen et al. [31] have shown that treatment of human umbilical vein endothelial cells with sodium chlorate, heparinase or chondroitinase ABC does not alter TFPI binding and concluded that TFPI

binding does not involve GAGs. These results suggest that the highly conserved amino acid portion of glypican-3 might have a role in TFPI binding; however, further studies are necessary to confirm this hypothesis.

There are several potential physiological roles for the glypican-3/TFPI interaction. Ho et al. [32] have shown that when complexed with factor Xa, TFPI is internalized and degraded by a cell-surface receptor on HepG2 cells and mouse embryonic fibroblasts that is neither LRP nor tissue factor. Determination of the role of glypican-3 in this process will require the development of high-affinity anti-(glypican-3) antibodies or a mouse fibroblast cell line deficient in glypican-3. Pilia et al. [17] recently reported that the glypican-3 gene is disrupted in patients with the Simpson–Golabi–Behmel overgrowth syndrome. These patients have a variety of tissue defects and are at high risk for the development of Wilm's tumour or neuroblastoma in early childhood. Although glypican-3 is expressed at high levels in the placenta (Figure 4) as well as fetal lung, liver and kidney [17], all highly vascularized tissues, the importance of any interaction between TFPI and glypican-3 during development remains to be determined.

We could not detect the expression of glypican-3 mRNA in adult lung, liver, heart or kidney, nor could the cDNA be isolated from a human endothelial or liver cDNA library. Because glypican-3 is differentially expressed during development [25], the lack of glypican-3 expression in mature non-transformed liver cells is not inconsistent with its presence in the transformed HepG2 hepatoma cell line, which probably expresses many embryonic antigens. The lack of glypican-3 expression in endothelial cells suggests that glypican-3 is not responsible for binding TFPI to the endothelial cell surface in adults. However, a TFPI-binding protein is present in dextran sulphate washes of human microvascular endothelial cells (A. Mast and G. Broze, unpublished work) and studies to identify this protein are currently under way.

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