Binding of perlecan to transthyretin *in vitro*

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Transthyretin is one of two specific proteins involved in the transport of thyroid hormones in plasma; it possesses two binding sites for serum retinol-binding protein. In the present study we demonstrate that transthyretin also interacts *in vitro* with [³⁵S]sulphate-labelled material from the medium of HepG2 cells. By using the same strategy as for purifying serum retinol-binding protein, [³⁵S]sulphate-labelled medium was specifically eluted from a transthyretin-affinity column. Ion-exchange chromatography showed that the material was highly polyanionic, and its size and alkali susceptibility suggested that it was a proteoglycan. Structural analyses with chondroitinase ABC lyase and nitrous acid revealed that approx. 20 % was chondroitin

sulphate and 80 % heparan sulphate. Immunoprecipitation showed that the [³⁵S]sulphate-labelled material contained perlecan. Further analysis by binding studies revealed specific and saturable binding of [¹²⁵I]-transthyretin to perlecan-enriched Matrigel. Because inhibition of sulphation by treating HepG2 cells with sodium chlorate increased the affinity of the perlecan for transthyretin, and [³H]heparin was not retained by the transthyretin affinity column, the binding is probably mediated by the core protein and is not a protein–glycosaminoglycan interaction. Because perlecan is released from transthyretin in water, the binding might be due to hydrophobic interactions.

INTRODUCTION

In humans, the major plasma transport protein for thyroid hormones is thyroxine-binding globulin, whereas transthyretin (TTR) seems to have only a minor role [1,2]. In rodents [3], however, TTR is the major thyroxine transport protein. TTR is a homotetramer with subunits of molecular mass 14 550 Da. A channel runs through the centre of the tetramer in which two binding sites for thyroxine are located [4], but only one thyroxine molecule is normally bound to TTR owing to negative cooperativity [5]. In addition to the binding site for thyroxine, the TTR tetramer possesses two binding sites for serum retinol-binding protein (RBP) [6]. Similarly to RBP, TTR is produced mainly by the liver parenchymal cells and at lower level in tissues such as the choroid plexus and retina pigment epithelium cells [7,8].

In liver, TTR and RBP are produced in approximately equal amounts, but owing to faster clearance of RBP there is normally a 2-fold molar excess of TTR in plasma [9]. One probable function of the complexation of RBP and TTR in plasma is to reduce the glomerular filtration of RBP. The role of TTR for the plasma transport of thyroxine and RBP–retinol has recently been studied in transgenic mice homozygous for a null mutant in the TTR locus [10]. Although such mice have decreased their plasma levels of thyroxine and RBP to approx. 5 % of the concentrations in the wild-type animals, they show no signs of hypothyroidism or deficiency in vitamin A.

The mechanism for cellular uptake of RBP-bound retinol is not clear, but several lines of evidence suggest the existence of a specific cell-surface receptor [11–15]. Studies of RBP–receptor interactions have, however, been hampered by the fact that radioactive iodination of RBP changes the binding properties of RBP [16]. To avoid these problems we have used amino acid-labelled RBP produced by HepG2 cells [17] as a ligand in receptor studies. To isolate [³⁵S]methionine-labelled RBP from the medium of HepG2 cells we used TTR-affinity chromatography [18]. In such experiments we observed that high-mole-

cular-mass material co-purified with RBP. This component was demonstrated to be the proteoglycan perlecan. Perlecan is an important constituent of basement membranes and connective tissues and consists of three heparan sulphate chains linked to a large core protein [19].

MATERIALS AND METHODS

Chemicals

Human TTR was purchased from Scigen (Sittingbourne, Kent, U.K.). The different antibodies were obtained from the following sources: rabbit anti-(human RBP) and horseradish peroxidase-conjugated rabbit anti-(human RBP) from DAKO A/S (Glostrup, Denmark); rabbit antiserum was raised against a preparation of rat liver cell-surface heparan sulphate proteoglycan (pan-syndecan) [20], rabbit anti-(human perlecan) antiserum was a gift from Dr. J. Hassell (University of Pittsburgh, Pittsburgh, PA, U.S.A.). As control, rabbit pre-immune syndecan serum was used. Iodobeads were purchased from Pierce Chemical company (Rockford, IL, U.S.A.). Na¹²⁵I, [³⁵S]methionine, [³⁵S]sulphate, [³H]retinol and [³H]heparin were purchased from Dupont–New England Nuclear (Boston, MA, U.S.A.). ¹⁴C-labelled molecular mass markers were purchased from Amersham (Little Chalfont, Bucks., U.K.). Molecular mass standards were obtained from Sigma Chemical Company (Poole, Dorset, U.K.). Matrigel was purchased from Collaborative Biomedical Products/Becton Dickinson Labware (Bedford, MA, U.S.A.).

RBP labelling and quantification

Human RBP was purified and iodinated as described [15].

Binding assay

Matrigel diluted in PBS was coated on 96-well plates for 1 h at 37 °C at a concentration of 9 µg/ml. The wells were washed twice with PBS and used directly in binding assays. Control wells were coated with 1 % (w/v) BSA for 1 h at 37 °C and washed with

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GAG, glycosaminoglycan; RBP, retinol-binding protein; TTR, transthyretin.

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PBS. ^{125}I -TTR and PBS was added and the plates were incubated at 4 °C for 3 h. Unbound ^{125}I -TTR was washed off with PBS; bound material was then released from the wells with 1 % (w/v) SDS and counted in a gamma counter. Experiments were also performed where unlabelled TTR, heparin or anti-perlecan antibodies were included at different concentrations during the binding assay [21].

SDS/PAGE and autoradiography

Fractions from different chromatography steps were separated on SDS/PAGE with 14 % (w/v) pre-cast gels (Novex, San Diego, CA, U.S.A.) and either detected by autoradiography or quantified by an Instant Imager (Packard Instruments, Meriden, CT, U.S.A.).

Cell cultures and metabolic labelling

HepG2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). They were cultured in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, McLean, VA, U.S.A.) supplemented with 10 % (v/v) fetal calf serum (Flow Laboratories), penicillin (100 i.u./ml), streptomycin (0.1 mg/ml), L-glutamine (2 mM) and fungizone (2.5 µg/ml). For metabolic labelling, semi-confluent layers of HepG2 cells were preincubated for 1 h in sulphate-depleted RPMI 1640 medium, DMEM without methionine or DME medium without retinol. The media were changed and cells were incubated overnight with either 100 µCi/ml [^{35}S]sulphate in sulphate-depleted RPMI 1640, 4 h with 100 µCi/ml [^{35}S]methionine in DMEM without methionine, or 4 h with 10 µCi/ml [^3H]retinol in DMEM. In all incubation media unlabelled retinol was added to a final concentration of 2 µM to enhance the secretion of RBP. In some experiments the sulphation of proteoglycans and tyrosine-sulphated proteins residues was inhibited by the addition of sodium chlorate during both the preincubation period and the incubation period [22].

Immunoprecipitation

For immunoprecipitation we used rabbit antiserum raised against human perlecan, rat pan-syndecan antiserum recognizing syndecans 1–4 and a rabbit pre-immune serum. [^{35}S]Sulphate-labelled HepG2 medium, pooled fractions from the TTR-affinity column and Triton X-100 [1 % (w/v) in PBS]-solubilized HepG2 cells were used as the starting material. The cell lysate was also homogenized by aspiration through a 19-gauge needle. The pre-immune serum and the two antisera (final dilution 1:200) were added to aliquots of the three fractions and incubated at 4 °C for 2 h followed by incubation with 5 % Protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden). Immunoprecipitated fractions were washed three times in Tris-buffered saline, pH 7.4, containing 0.05 % Triton X-100 (v/v), 1 % (w/v) BSA and 5 mM MgSO_4 , and three times with the same buffer without BSA. After the final washing step 40 µl of sample buffer was added, the samples were boiled and then subjected to SDS/PAGE and autoradiography.

TTR-affinity chromatography

RBP is known to be eluted from a TTR-affinity column at low ionic strength [18], and we used this strategy for both preparative and analytical studies. For preparative purposes we used a column of CNBr-activated Sepharose 4B (Pharmacia Biotech) coupled with human TTR as described by the manufacturer. Conditioned medium from HepG2 cells was dialysed against PBS at 4 °C, applied to the column, recirculated overnight and

washed with five times the column volume (10 ml) of PBS containing 0.1 % ovalbumin. The bound material was eluted in water, adjusted to pH 10 with ammonia, containing 0.1 % ovalbumin. For analytical studies of TTR binding a Pharmacia *N*-hydroxysuccinimide-activated Hi-Trap affinity column on an FPLC system was used. TTR was coupled to the column in accordance with the manufacturer's procedure. After the material was applied the column was washed with five times the column volume of PBS containing 0.05 % ovalbumin and finally eluted with water (pH 10)/0.05 % ovalbumin. Non-specific binding was measured with an *N*-hydroxysuccinimide-activated column coupled with BSA.

Gel chromatography

Fractions obtained by TTR-affinity chromatography were further analysed by Superose 12 gel chromatography with an FPLC system (Pharmacia Biotech). PBS with 0.05 % ovalbumin was used as running buffer; fractions of 0.5 ml were collected and analysed for radioactivity in a Packard scintillation counter. Molecular mass markers (aprotinin, cytochrome *c*, carbonic anhydrase and albumin) were used to calibrate the column.

Alternatively, ^{35}S -labelled material was subjected to Sepharose CL-6B (Pharmacia Biotech) gel chromatography. The column was run in 0.05 M Tris/HCl, pH 8.0, containing 8 M urea and 0.15 M NaCl. Markers for the void and total volumes of the gel were Dextran Blue and 2,4-dinitrophenylalanine (Sigma Chemical Co., St. Louis, MO, U.S.A.) respectively.

Ion exchange chromatography

^{35}S -labelled material from conditioned medium of HepG2 cells with high affinity for TTR (eluted with water) was pooled and applied to a 2 ml column of DEAE-Sepharose (Pharmacia Biotech). The column was run in 0.05 M Tris/HCl, pH 8.0, containing 0.15 M NaCl. After the labelled material had been applied the ion-exchange column was washed extensively with the same buffer, and material binding to the column was eluted with a gradient of NaCl from 0.15 to 1.5 M in 0.05 M Tris/HCl, pH 8.0. Fractions of 2 ml were collected and the amount of radiolabelled material was measured in a scintillation counter after the addition of scintillation cocktail. In some of the experiments [^3H]heparin was included as internal standard.

Identification of glycosaminoglycans (GAGs)

^{35}S -labelled material with affinity for TTR was subjected to treatment nitrous acid [23], known to depolymerize heparin and heparan sulphate-type GAGs. The reaction products were analysed by Sepharose CL-6B gel chromatography. Furthermore parallel samples were treated with chondroitinase ABC lyase (EC 4.2.2.4) (Seikagaku America, Rockville, MD, U.S.A.) as previously described [24], then analysed by Sepharose CL-6B gel chromatography.

To determine whether the ^{35}S -labelled samples were proteoglycans they were subjected to alkali treatment (0.5 M NaOH) for 20 h at room temperature. This treatment results in the liberation of intact ^{35}S -GAG chains from the proteoglycan molecule. Both untreated and alkali-treated samples were analysed by Sepharose-CL-6B gel chromatography.

RESULTS

Co-elution of several proteins from a TTR-affinity column

Medium from [^{35}S]methionine-labelled HepG2 cells was applied to a TTR-affinity column and washed with PBS; bound material

Figure 1 SDS-PAGE of [^{35}S]methionine-labelled eluate from a TTR-affinity column

[^{35}S]Methionine-labelled pooled eluate from the TTR-affinity column was analysed by SDS/PAGE and detected by autoradiography or an Instant Imager (Packard). The positions of molecular mass standards (numbers indicate molecular masses in kDa) are indicated at the left. The molecular masses reflecting the positions of the bands were calculated with an Instant Imager. The bottom arrow indicates the migration position of RBP as determined by using [^{125}I]-labelled plasma purified human RBP.

Figure 2 SDS/PAGE of [^{35}S]methionine-labelled eluate from a TTR-affinity column separated by Superose 12 gel filtration

[^{35}S]Methionine-labelled pooled eluate from TTR-affinity chromatography was separated by Superose 12 gel filtration, analysed by SDS/PAGE and detected by autoradiography. Lanes are given numbers reflecting the pooled fractions analysed. V_0 corresponds to fraction 17, BSA (66 kDa) to fraction 28 and aprotinin (6.5 kDa) to fraction 38. The arrow indicates the migration position of RBP as determined by using [^{125}I]-labelled plasma purified human RBP. The positions of molecular mass standards (numbers indicate molecular masses in kDa) are indicated at the left.

was then eluted with water adjusted to pH 10. The eluate from the TTR column was pooled and analysed by SDS/PAGE under denaturing conditions. The autoradiogram in Figure 1 shows that in addition to RBP, four other bands appear with molecular masses of approx. 35, 75, 110 and more than 200 kDa. The RBP band was calculated to represent approx. 10% of the [^{35}S]methionine-labelled protein. In contrast, more than 90% of the protein was RBP when serum or plasma was subjected to TTR-affinity chromatography (results not shown). The pooled TTR eluate isolated from HepG2 medium was also analysed by Superose 12 gel filtration under non-denaturing conditions. These experiments confirmed the results from SDS/PAGE. Only approx. 10% of the total radioactivity could be detected in the

RBP peak, the elution position of which was determined by using [^{125}I]-labelled RBP purified from human plasma.

Proteins released from the TTR-affinity column

Medium from [^{35}S]methionine-labelled HepG2 cells was applied to the TTR-affinity column, the column was washed with PBS and bound material was eluted with water, pH 10. The first eight fractions of the water-eluted material were analysed by SDS/PAGE under reducing conditions and the relative amounts of radioactivity in the different bands were determined by using an Instant Imager. Similar proportions of RBP (between 7.9% and 10.4%) and other proteins were detected in all the fractions (results not shown). These results demonstrate that all the proteins that bound to the TTR column were eluted with the same pattern. This result does not distinguish between the released proteins' binding to TTR as multimolecular complexes or their binding individually and independently to TTR.

RBP is not complexed with other proteins when released from the TTR column

To test whether these proteins were eluted from the TTR column as complexes or monomers, [^{35}S]methionine-labelled pooled eluate from the TTR-affinity chromatography was separated by Superose 12 gel filtration with PBS, pH 7.4. The different molecular size fractions were analysed by SDS/PAGE. As shown in Figure 2, RBP was present only in gel-filtration fractions 32–35, whereas the other proteins were present mainly in the earlier fractions. Fractions 32–35 correspond to proteins with a molecular mass of approx. 15–25 kDa. [^{125}I]RBP was shown to be eluted in the same fractions in parallel experiments. Most of the radioactivity was found in high-molecular-mass fractions (i.e. more than 200 kDa). In agreement with this result, immunoreactive RBP was only detected in the low-molecular-mass fractions (results not shown). Thus the results indicate that RBP is eluted from the TTR column as a single protein and not complexed with other proteins.

Proteins co-purifying with RBP can be labelled with [^{35}S]sulphate

The high-molecular-mass component in the fluorogram (Figure 1) and the earlier reported association between TTR and proteoglycans in amyloidosis [25] suggested to us that the high-molecular-mass proteins (Figure 1 and 2) could be a proteoglycan. We therefore incubated HepG2 cells with [^{35}S]sulphate and applied the labelled medium to a TTR-affinity column. Of the radioactivity applied, 4% bound to the TTR column and was eluted with distilled water (compared with 9% of the radioactivity for [^{35}S]methionine-labelled medium). Approx. 1.5% of [^{35}S]sulphate-labelled medium was non-specifically bound to the column as determined by using an albumin-affinity column. When the combined fractions containing the radioactivity released from the TTR column were subjected to a second run of affinity chromatography, 24% of the radioactivity was bound and eluted with water. In contrast, only 1.3% of the initially non-binding fraction became bound upon reapplication and was released with distilled water. From these experiments we conclude that a [^{35}S]sulphate-labelled component secreted from HepG2 cells is bound to the TTR column and can be eluted from the column under conditions normally used to release RBP.

Aliquots of the medium from the [^{35}S]sulphate-labelled cells, the flow-through and the water-eluted fractions were then

Figure 3 SDS/PAGE of medium from [^{35}S]sulphate-labelled HepG2 cells

Medium from [^{35}S]sulphate-labelled HepG2 cells was subjected to preparative TTR-affinity chromatography. The pooled eluate (lane 1), the flow-through (lane 2) and the culture medium (lane 3) were analysed by SDS/PAGE under reducing conditions. The positions of molecular mass standards (numbers indicate molecular masses in kDa) are indicated at the left.

Figure 4 Superose 12 gel filtration of [^{35}S]sulphate-labelled eluate from TTR-affinity chromatography

Medium from [^{35}S]sulphate-labelled HepG2 cells was subjected to TTR-affinity chromatography; the pooled eluate was then subjected to Superose 12 gel filtration in PBS (pH 7.4). The arrows corresponds to V_0 (fraction 17, Blue Dextran), BSA (fraction 28; 66 kDa) and aprotinin (fraction 38; 6.5 kDa).

analysed by SDS/PAGE under denaturing conditions. The results presented in Figure 3 show that the main [^{35}S]sulphate-labelled component in the water-eluted material (lane 1) had a molecular mass of more than 200 kDa. Two fainter bands corresponding to approx. 35 and 75 kDa were also observed. Although the content of these two fainter bands varied between different incubations, the main band in the TTR-bound material was consistently found at the position corresponding to a molecular mass of more than 200 kDa. The flow-through (lane 2) or the medium (lane 3) also contained a broad band with a molecular mass of approx. 45–60 kDa that did not seem to bind to the TTR column (lane 1).

When the [^{35}S]sulphate-labelled eluate of the TTR column was analysed by Superose 12 gel filtration (Figure 4), almost all of the radioactivity appeared in fractions corresponding to a molecular mass of more than 200 kDa. Thus both the autoradiogram and the chromatogram showed that the [^{35}S]sulphate-labelled material with affinity for TTR is a high-molecular-mass component.

Figure 5 DEAE ion-exchange chromatography of [^{35}S]sulphate-labelled material with affinity for TTR

Medium from [^{35}S]sulphate-labelled HepG2 cells was subjected to TTR-affinity chromatography and the pooled eluate was analysed by DEAE-Sephacel ion-exchange chromatography. Material binding to the column was eluted with a NaCl gradient from 150 to 1500 mM. The arrow indicates the peak elution position of [^3H]heparin (750 mM NaCl).

Interestingly, we did not detect radioactivity in the fractions corresponding to the elution position of RBP, either in the autoradiogram (Figure 3) or in the chromatogram (Figure 4), suggesting that RBP secreted from HepG2 cells is not sulphated, in contrast with what has been reported for RBP in plasma [26].

The high-molecular-mass component is a proteoglycan

To investigate whether the [^{35}S]sulphate-labelled high-molecular-mass material was a proteoglycan it was subjected to further analyses. First, as proteoglycans bind tightly to a DEAE ion-exchange column owing to their negative charges, the [^{35}S]sulphate-labelled TTR-binding material from HepG2 medium was chromatographed on a DEAE-Sephacel column. The result (Figure 5) shows that 90 % of the [^{35}S]sulphate-labelled material was eluted from the column at a NaCl concentration of approx. 0.7 M. In parallel experiments it was demonstrated that [^3H]heparin was released from the column at a salt concentration of approx. 0.8 M (Figure 5). The elution profile of the [^{35}S]sulphate-labelled material shows clearly that the high-molecular-mass component co-eluting with RBP from the TTR column is polyanionic.

It is well established that treatment with alkali will release intact GAG chains from proteoglycan molecules. [^{35}S]Sulphate-labelled TTR-binding material was therefore subjected to Sepharose CL-6B gel filtration before and after alkali treatment. The peak K_{av} value increased from 0.37 for untreated material to 0.52 for the alkali-treated species (results not shown). A K_{av} value of 0.52 corresponds to a molecular mass of the GAG chains of approx. 20 kDa (the column was calibrated as in [27]), which is in agreement with the reported size of heparan sulphate chains synthesized by normal liver [28]. This experiment and the ion-exchange chromatography data clearly indicate that the high-molecular-mass [^{35}S]sulphate-labelled material contains proteoglycans.

Characterization of the proteoglycans binding to TTR

Proteoglycans have traditionally been classified on the basis of the composition of their GAG chains. The [^{35}S]sulphated TTR-

Figure 6 Sepharose CL-6B gel filtration

Upper panel: [^{35}S]sulphate-labelled pooled eluate from the TTR-affinity column was subjected to HNO_2 treatment. The reaction products were analysed by Sepharose CL-6B gel filtration and compared with an untreated control. Lower panel: aliquots of the same material were subjected to chondroitinase ABC lyase treatment. The reaction products were analysed by Sepharose CL-6B gel filtration.

binding material was treated either with nitrous acid or chondroitinase ABC lyase for further characterization. The former treatment specifically depolymerizes heparin and heparan sulphate chains; the latter digests chondroitin/dermatan sulphate chains. The results presented in Figure 6 show that most of the [^{35}S]sulphate-labelled material was depolymerized with nitrous acid, whereas only approx. 20% was degraded with chondroitinase ABC lyase. Accordingly, most of the material is heparan sulphate and a minor fraction contains chondroitin/dermatan sulphate.

Identification of the proteoglycan binding to TTR

HepG2 cells have been shown to express mRNA for syndecans 1, 2 and 4 [29,30]. Another candidate is perlecan, the major heparan sulphate proteoglycan of basement membranes, shown to be secreted from cultured human liver parenchymal cells [31]. [^{35}S]Sulphate-labelled cell lysate, conditioned medium and eluate from the TTR column were subjected to immunoprecipitation with a rat pan-syndecan antiserum, recognizing syndecans 1–4, and a human perlecan antiserum. Precipitated material was detected by autoradiography after SDS/PAGE. The results given

Figure 7 Immunoprecipitation of [^{35}S]sulphate-labelled material from HepG2 cells

[^{35}S]Sulphate-labelled cell lysate, medium and eluate from the TTR-affinity column were immunoprecipitated with antiserum to rat pan-syndecan, human perlecan and a rabbit preimmune serum as control. The precipitated material was analysed by SDS/PAGE and autoradiography.

Table 1 Binding of ^{125}I -TTR to Matrigel in the presence or absence of excess unlabelled TTR

Matrigel was immobilized on tissue-culture plates and the binding of ^{125}I -TTR was measured in the absence or presence of excess unlabelled TTR: 60 000–70 000 c.p.m. (2.9×10^9 c.p.m./nmol) was added to each well in a total volume of 100 μl . The values presented are means $\pm$ S.D. for triplicates. Background binding (tissue culture wells coated with albumin) is subtracted (18 ± 3 c.p.m. per well).

Unlabelled TTR ($\mu\text{g/ml}$)	Binding of ^{125}I -TTR (c.p.m. per well)	Inhibition (%)
0	372 ± 15	0
0.2	329 ± 93	11.6
2	385 ± 71	0
20	234 ± 23	37.1
200	95 ± 8	74.5
2000	37 ± 6	90.0

in Figure 7 show that the cell lysate contained both syndecans and perlecan, suggesting that HepG2 cells produce both types of proteoglycans. In the medium significant amounts of perlecan were detected. Some syndecan was also seen, but the level was below that of the pre-immune serum. Most importantly, the TTR eluate was shown to contain only perlecan. By using an Instant Imager the fraction of the radioactivity precipitated by the perlecan antiserum was calculated to be 9.2-fold more for the eluate than for the medium, showing that perlecan was enriched by TTR-affinity chromatography.

^{125}I -TTR shows specific and saturable binding to perlecan-enriched Matrigel

The immunoprecipitation experiments identified perlecan as one proteoglycan interacting with TTR. To analyse further this interaction we studied the binding of ^{125}I -TTR to Matrigel enriched in perlecan. Matrigel was immobilized on tissue culture wells, and binding of ^{125}I -TTR was measured by the absence or presence of unlabelled TTR. The results given in Table 1 show that ^{125}I -TTR bound to immobilized Matrigel, and the binding decreased with increasing concentrations of unlabelled TTR. In contrast, ^{125}I -TTR did not bind to tissue-culture wells coated with BSA. Adding excess unlabelled heparin affected the binding only modestly (Table 2). This is in agreement with the observation that [^3H]heparin was not retained by the TTR-affinity column (results not shown), and suggests that the interaction is not mediated through the GAG chains of perlecan. Adding increasing amounts of anti-perlecan antiserum to the tissue culture wells

Table 2 Binding of ¹²⁵I-TTR to Matrigel in the presence or absence of excess unlabelled heparin

Matrigel was immobilized on tissue-culture plates and the binding of ¹²⁵I-TTR was measured in the absence or presence of excess unlabelled heparin. For other details see Table 1.

Heparin (mg/ml)	Binding of ¹²⁵ I-TTR (c.p.m. per well)	Inhibition (%)
0	235 ± 27	0
0.1	180 ± 27	23.4
0.001	183 ± 4	22.1

Table 3 Binding of ¹²⁵I-TTR to Matrigel in the presence or absence of excess unlabelled anti-perlecan antibody

Matrigel was immobilized on tissue-culture plates and the binding of ¹²⁵I-TTR was measured in the absence or presence of excess unlabelled anti-perlecan antibody. For other details see Table 1.

Anti-perlecan antiserum (dilution)	Binding of ¹²⁵ I-TTR (c.p.m. per well)	Inhibition (%)
None	235 ± 27	0
1:10	93 ± 44	60.4
1:100	138 ± 15	41.3
1:1000	167 ± 34	28.9
1:10000	241 ± 46	0

decreased the binding of ¹²⁵I-TTR to the Matrigel by up to 60 % (Table 3). Thus the immobilization of ¹²⁵I-TTR to Matrigel is to a large extent mediated by binding to perlecan in the matrix.

Chlorate treatment and the binding of [³⁵S]-proteoglycans for TTR

Perlecan is composed of five different structural domains with the GAG chains attached to the N-terminal domain [32]. The results presented indicate that the binding to TTR is not a protein–GAG interaction. To investigate further the possible importance of the GAG chains for the binding to TTR, HepG2 cells were incubated with sodium chlorate, an inhibitor of sulphation [22]. HepG2 cells were incubated with [³⁵S]sulphate in the absence or presence of 50 mM sodium chlorate, and both medium fractions were subjected to TTR-affinity chromatography. In the medium from chlorate-treated cells the peak of the radioactivity was eluted after 12 min, in contrast with 6 min for the control medium (Figure 8, upper panel). The same difference in elution profile was observed when medium from HepG2 cells labelled with [³⁵S]methionine was subjected to the same procedure (Figure 8, lower panel). The recovery from the TTR column after chlorate treatment was decreased by 69 % for [³⁵S]sulphate-labelled material and 30 % for [³⁵S]methionine-labelled material. The results clearly indicate that the binding to TTR is not mediated by sulphated GAG chains, and moreover that the sulphation of the GAG chains in some way might reduce the affinity of the perlecan protein for TTR.

RBP secreted from HepG2 cells does not bind to the perlecan–TTR complex

The results given in Figure 2 show that RBP eluted from the TTR column is not complexed with perlecan. However, to analyse whether RBP secreted from HepG2 cells has the ability to form a trimeric complex (proteoglycan–TTR–RBP) with TTR present, HepG2 cells were incubated with [³H]retinol to label

Figure 8 Effects of treatment with sodium chlorate on affinity for TTR

Upper panel: medium from control and sodium chlorate-treated HepG2 cells labelled with [³⁵S]sulphate were applied to a TTR-affinity column. The results presented represent one of three experiments with similar elution profiles. Lower panel: medium from control and sodium chlorate-treated HepG2 cells labelled with [³⁵S]methionine were applied to a TTR-affinity column. The results presented represent one of three experiments with similar results.

Figure 9 Superose 12 gel filtration of [³H]retinol-labelled eluate from TTR-affinity chromatography

Medium from [³H]retinol-labelled HepG2 cells was subjected to TTR-affinity chromatography. The pooled eluate was analysed by Superose 12 gel-filtration chromatography. An aliquot of the pooled eluate was preincubated with human TTR (100 µg/ml) before Superose 12 gel-filtration chromatography. Fractions correspond to the same molecular masses as described in Figure 4. The elution positions of RBP and RBP–TTR are based on control runs with ¹²⁵I-labelled RBP, with and without TTR.

RBP-[³H]retinol specifically. After labelling, the medium was subjected to TTR-affinity chromatography and the water-eluted material was analysed further by Superose 12 gel chromato-

graphy (Figure 9). Approx. 75 % of the radioactivity migrated in fractions corresponding to free holo-RBP. When human TTR was added to a final concentration of 100 µg/ml to a parallel sample of the pooled eluate from the TTR column, the free holo-RBP peak was diminished and most of the radioactivity was found in the fractions corresponding to the RBP-TTR peak. However, the [³H]retinol present in fractions corresponding to a molecular mass above 200 kDa (fraction 24 or earlier) did not increase. These results therefore suggest that retinol-RBP is not secreted as a trimolecular complex of perlecan-TTR-RBP.

DISCUSSION

The results presented show that perlecan secreted from HepG2 cells interacts with TTR. RBP is typically purified by TTR-affinity chromatography and is normally eluted from such columns in water. By using the same procedure as for RBP isolation, [³⁵S]sulphate-labelled material was eluted from a TTR-affinity column. Superose 12 gel chromatography revealed that the peak of the material had a high molecular mass (more than 200 kDa).

Further analyses showed that almost all of the TTR-binding material also bound to an ion-exchange column and contained mostly heparan sulphate and a small fraction of chondroitin sulphate. By immunoprecipitation we demonstrated that the [³⁵S]sulphate-labelled medium from HepG2 cells contained syndecans and perlecan. The eluate from the TTR-column, however, contained only perlecan, thus showing a selective interaction between perlecan and TTR. This specificity was also demonstrated in binding studies, where ¹²⁵I-labelled TTR was immobilized on perlecan-containing Matrigel. Competition studies revealed that up to 90 % of the binding was inhibited by adding increasing amounts of unlabelled TTR. Moreover, adding anti-perlecan antibodies inhibited the binding by up to 60 %, suggesting that a major part of the binding to Matrigel is mediated by the perlecan component.

Perlecan is an important constituent of basement membranes [19] and consists of three heparan sulphate chains linked to a large core protein of molecular mass more than 200 kDa, but it has also been detected in tissues without GAG chains [33]. The deduced size of the observed [³⁵S]sulphate-labelled material with affinity for TTR is in agreement with the reported molecular sizes of perlecan [34]. Our observation that HepG2 cells secreted perlecan is supported by studies showing that cultured hepatocytes produce perlecan [19].

Our analysis revealed that 20 % of the GAG chains from the material binding TTR were chondroitin/dermatan sulphate. The eluted material might contain distinct heparan sulphate proteoglycans and chondroitin sulphate proteoglycans, or hybrids thereof. However, we could not detect syndecan, the most obvious candidate for such a hybrid, in the eluate from the TTR column. It has, however, been reported that perlecan from the Engelbreth-Holm-Swarm tumour matrix is not only present as a heparan sulphate proteoglycan but also as a hybrid molecule with dermatan sulphate chains or only with chondroitin sulphate chains [35].

Perlecan was eluted from the TTR-affinity column in water, which is a conventional method for RBP purification. The binding of RBP to TTR is proposed to depend on hydrophobic interactions [36]. The conditions used to release perlecan from the TTR column suggest that the interaction between these two molecules is also hydrophobic in nature and is not mediated by a protein-GAG interaction. This conclusion is further supported by the binding studies showing that heparin inhibited the binding of ¹²⁵I-TTR to Matrigel to a minor extent and that [³H]heparin

was not retained by the TTR-affinity column. TTR-affinity chromatography of medium from HepG2 cells exposed to sodium chlorate, a well-known inhibitor of GAG and protein tyrosine sulphation, gave a retarded release from the column compared with control material. This effect of chlorate also suggests that the binding to TTR is not a protein-GAG interaction, and moreover that the sulphated GAG chains decrease the affinity of perlecan for TTR. These experiments do not rule out the possibility that N-acetylated domains, which increase in number on treatment with sodium chlorate, might contribute to the increased binding to TTR. However, the observations that a normally sulphated proteoglycan bound TTR, and that it was eluted with water, argue against any contribution from the GAG chains in this interaction. Perlecan is a large proteoglycan with five distinct structural domains, several of which might participate in protein-protein interactions [32,34], e.g. the epidermal growth factor-like domain and the low-density-lipoprotein receptor-like region.

Interestingly, both perlecan and TTR are associated with amyloidosis, which is a heterogeneous disease characterized by depositions of insoluble amyloid fibrils in different tissues. The amyloidogenic property of TTR has been linked to mutations in the TTR gene [37]. Westermark [38] demonstrated that non-mutated TTR is also involved in the most common form of amyloidosis, senile systemic amyloidosis. Perlecan has been identified as the common proteoglycan component of several types of amyloidosis [39], including those where the liver is the major organ affected [40]. Moreover, heparan sulphate has been detected in amyloid deposits in patients with familial amyloid cardiomyopathy with TTR mutations [25].

Recent results suggest that it is the free retinol-RBP that interacts with the cell-surface receptor, and not the TTR-bound retinol-RBP. Experimental evidence for this comes from mutational analyses of RBP showing that the binding sites for TTR and the plasma membrane RBP receptor are overlapping but not identical [41,42], and results from a system *in vitro* show that retinol is liberated from RBP in the presence of TTR at two-fifths of the rate [43]. Any substance that is able to sequester TTR from RBP will thus liberate retinol-RBP from the retinol-RBP-TTR complex and therefore present the proper ligand, retinol-RBP, to the RBP plasma membrane receptor. Our results presented here suggest that perlecan might have such an effect, because we could not detect a trimolecular complex of RBP-TTR-perlecan.

In summary, the present study demonstrates that perlecan might interact with TTR via hydrophobic interactions. This interaction might affect the uptake of retinol-RBP in cells and might explain the tendency of TTR to co-localize with perlecan in amyloidotic deposits. Thus the reported interaction between TTR and perlecan might have implications for both retinol metabolism and TTR-associated amyloidosis.

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