Molecular characterization of the rat Kupffer cell glycoprotein receptor

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Received on December 19, 2002; revised on March 10, 2003; accepted on March 12, 2003

The Kupffer cell receptor for glycoproteins has been reported to have a role in clearance of galactose- and fucose-terminated glycoproteins from circulation. Although the gene and a cDNA encoding the receptor have been described, there has been little study of the receptor protein. To address some questions about possible ligands and functions for this receptor, fragments representing portions of the extracellular domain have been expressed and characterized. The extracellular domain consists of a trimer stabilized by an extended coiled-coil of α-helices. The receptor displays monosaccharide-binding characteristics similar to the hepatic asialoglycoprotein receptor, but with somewhat less selectivity. The two best monosaccharide ligands are GalNAc and galactose. α-Methyl fucoside is a particularly poor ligand. Analysis of Kupffer cell receptor binding to glycoproteins and oligosaccharides released from them reveals highest affinity for desialylated, complex N-linked glycans. The best glycoprotein ligands contain multiple highly branched oligosaccharides. A human ortholog of the rat receptor gene does not encode a full-length protein and is not expressed in liver. These characteristics suggest that the receptor may have functions parallel to those of the hepatocyte asialoglycoprotein receptor in some (but not all) mammalian species.

Key words: clearance/glycoprotein/Kupffer cell/lectin/receptor

Introduction

Oligosaccharides attached to serum glycoproteins can serve as recognition tags that lead to clearance of the proteins from circulation (Drickamer and Taylor, 1998). The asialoglycoprotein receptor found on hepatocytes was the first such clearance system to be described (Ashwell and Harford, 1982). This receptor can mediate uptake of desialylated glycoproteins that bear terminal galactose residues, but it binds with even higher affinity to terminal GalNAc residues. It is the prototype for the C-type lectin family, in which a C-type carbohydrate-recognition domain (CRD) mediates Ca2+-dependent binding to oligosaccharides. Another member of the family, the mannose receptor of macrophages and hepatic endothelial cells, recognizes two distinct classes of glycoprotein ligands (Taylor, 2001). High-mannose oligosaccharides on lysosomal enzymes, tissue plasminogen activator, and other products of tissue damage are recognized through a series of C-type CRDs that bind GlcNAc and fucose as well as mannose. Pituitary hormones that display terminal GalNAc sulfate structures bind to a ricin-like CRD in the mannose receptor (Fiete et al., 1998).

The existence of an additional hepatic system for recognition of glycoproteins was postulated based on the uptake of synthetic neoglycoproteins with exposed fucose residues (Lehrman et al., 1986b). A novel receptor, designated the Kupffer cell receptor (KCR), was extracted from rat liver; it was suggested that this protein serves as a fucose receptor (Haltiwanger et al., 1986; Lehrman et al., 1986a; Lehrman and Hill, 1986). The KCR was shown to be present on the surface of Kupffer cells and is a type II transmembrane protein, consisting of an N-terminal cytoplasmic tail, a transmembrane region, a long neck region, and a C-terminal C-type CRD (Hoyle and Hill, 1988).

Based on their ligand-binding properties, C-type CRDs can be divided into two groups: those that bind mannose, GlcNAc, and fucose and those that bind galactose and GalNAc (Drickamer, 1999; Weis et al., 1998). CRDs in these two groups differ in a few key residues around the ligand-binding site. Ligand binding to the C-type CRDs involves interaction of two adjacent hydroxyl groups with Ca2+. Thus the primary determinant of binding selectivity is usually the orientation of the 3- and 4-hydroxyl groups of the sugar, although fucose presents a special case because it is an L sugar. In general, C-type lectins that bind fucose well do not bind galactose well and vice versa. The KCR, having been described as a fucose receptor, might appear to be a member of the former group, but studies on the purified protein suggest that it actually binds fucose relatively poorly compared to galactose (Lehrman et al., 1986a). Sequence alignments also indicate that the KCR would be predicted to be a galactose/GalNAc-binding protein. Because most of the previous work on the ligand-binding properties of the KCR was carried out on protein purified from liver, the possibility of contamination by the mannose receptor or the asialoglycoprotein receptor has been a persistent concern in the interpretation of these studies. Nevertheless, the fact that the receptor was purified from rat liver by affinity chromatography on immobilized fucose suggested that it must have significant fucose-binding character.

The aims of this article were to produce a recombinant extracellular fragment of the KCR free from any other
lectins and to facilitate a more definitive analysis of its sugar-binding properties and its structural organization. Overlapping binding specificities of the KCR and the asialoglycoprotein receptor and the absence of a functional gene for the KCR in humans have important implications for the role of multiple hepatic lectins in clearance of glycoproteins and particles from circulation.

Results

Expression and purification of KCR fragments

The coding regions for the entire extracellular domain of the KCR and the CRD were amplified from a rat liver cDNA library (Figure 1). These sequences were fused in frame with the bacterial ompA signal sequence to facilitate expression of active protein by secretion into the periplasm of Escherichia coli. Protein expressed in the presence of Ca²⁺ was released from the bacteria by sonication and passed over galactose-Sepharose columns (Figure 2), which were washed with Ca²⁺-containing buffer and eluted in the presence of ethylenediamine tetra-acetic acid (EDTA). For the extracellular domain, the eluate contained two predominant protein bands. N-terminal sequencing of the bands revealed that the larger polypeptide (KCR-A) makes up residues 70–551 of the receptor, in keeping with the structure of the expression vector. In contrast, the predominant, lower-molecular-weight form (KCR-B) consists of two fragments that differ by three amino acids starting from residues 245 and 248. These fragments appear to have resulted from proteolytic cleavage in the bacterial expression system. The CRD-only construct yielded a single fragment, designated KCR-C.

Although all of the fragments could be purified on immobilized galactose, the longer KCR-A and KCR-B fragments bound well to columns containing immobilized fucose, whereas the CRD interacted only weakly and eluted from the column in the presence of Ca²⁺ (Figure 3). Similar results were obtained with resins bearing other immobilized sugars, including mannose and N-GlcNAc, and a further experiment with underivatized Sepharose also revealed the same elution profile (data not shown). These results indicate that galactose is a relatively high-affinity ligand for the

Fig. 1. Sequence of the rat KCR and human homolog. The transmembrane domain is underscored. In the neck region, residues that form the heptad repeat of hydrophobic amino acids are shaded. Positions of introns in the genes are indicated by downward triangles. The human pseudogene (ENSEMBL identifier ENSG00000152672) is also shown, along with portions of the CRD from the major subunit of the rat asialoglycoprotein receptor. Residues that form key interactions with saccharide ligands are marked with diamonds. An asterisk denotes a histidine residue that mediates pH sensitivity of binding. Angle brackets indicate N-terminal.

Fig. 2. Purification of fragments of the extracellular domain of the KCR. (A) SDS–PAGE following affinity chromatography on galactose-Sepharose. Molecular weights in kDa are indicated. (B) Further purification of KCR-A and KCR-B by anion exchange chromatography on a MonoQ column. (C) SDS–PAGE of fractions from the MonoQ column. Gels were stained with Coomassie blue.
receptor and that binding to the galactose component of agarose (rather than the attached sugars) is responsible for interaction of the receptor with the other resins.

**Structural analysis of KCR fragments**

Anion-exchange chromatography was used to resolve fragments KCR-A and KCR-B for further structural studies (Figure 2). Equilibrium analytical ultracentrifugation yielded molecular weights of 156 kDa for KCR-A and 103 kDa for KCR-B (Figure 4). The values compare closely to the predicted values of 161 and 104 kDa for trimeric fragments. In contrast, the KCR-C fragment sediments as a monomer, with a measured molecular weight of 19.4 kDa, compared to 17.1 kDa predicted from the sequence. The trimeric nature of the KCR-A and KCR-B fragments was corroborated by chemical cross-linking studies. These experiments reveal the formation of discrete dimers and trimers of both KCR-A and KCR-B with increasing cross-linking reagent in both presence and absence of Ca²⁺, whereas the KCR-C fragment remains monomeric (Figure 5). These results demonstrate that the KCR exists as a trimer stabilized by the neck region.

The neck domain of the KCR consists of heptad repeats that are typically found in coiled-coils of α-helices. The structure of the neck domain was investigated by determining the circular dichroism spectra of the KCR-B and KCR-C fragments from the extracellular domain. The difference between these spectra, representing the C-terminal half of the neck region, is completely α-helical in character (Figure 6), with a mean residue ellipticity of $-28 \times 10^3$ deg-cm²/dmol at 222 nm. Insufficient amounts of the KCR-A fragment were available for a similar experiment. However, the experimental results combined with the sequence pattern shown in Figure 1 indicate that the overall structure of the extracellular portion of the KCR can be visualized as an extended, trimeric coiled-coil stalk terminated with a cluster of CRDs.

**Analysis of KCR binding to monosaccharides**

Because the KCR-B polypeptide is produced in greatest abundance and retains the trimeric organization of the receptor, it was used for analysis of the sugar-binding properties of the KCR. The KCR-B fragment was immobilized in polystyrene wells, and mono- and oligosaccharide ligands were tested for their ability to compete with binding of a neoglycoprotein reporter ligand, $^{125}$I-galactose–bovine serum albumin ($^{125}$I-galactose-BSA). The relative affinities of the ligands were characterized by the ratio of their inhibition constants compared to the inhibition constant for galactose (Table I). The results demonstrate that ligands with highest affinity for the KCR have axial 4-hydroxyl groups. The receptor displays a sixfold higher affinity for GalNAc compared to galactose. Preferential binding to GalNAc has also been observed for the asialoglycoprotein receptor, although in that case the affinity enhancement is approximately 60-fold (Iobst and Drickamer, 1996).

Although the disposition of the 3- and 4-hydroxyl groups is usually the primary determinant of binding to C-type CRDs, other modes of binding have been observed. For free sugars, weak interactions with the 1- and 2-hydroxyl groups have been observed (Ng et al., 1996). In the case of the KCR, the fact that α-methyl mannose competes as effectively as mannose indicates that the anomeric hydroxyl group of mannose does not contribute to binding. The complete absence of competition by GlcNAc suggests that a large substituent at the 2 position interferes with binding...
and might suggest that mannose binds through the 2- and 3-hydroxyl groups.

Of particular interest is the ability of the receptor to interact with fucose and related sugars. Fucose competes only threefold less effectively than galactose. Binding of fucose to the asialoglycoprotein receptor is 15-fold weaker than binding of galactose (Iobst and Drickamer, 1994), so fucose competes for binding to the KCR relatively well.

Interactions with glycoproteins and oligosaccharides

To assess the types of endogenous glycans that might serve as ligands for the KCR, the trimeric KCR-B fragment was radiolabeled and used to probe blots of glycoproteins that bear different types of N- and O-linked glycans (Figure 7). The major glycoproteins detected in this way are desialylated α1-acid glycoprotein and fetuin. The absence of reactivity with the native proteins before treatment with neuraminidase is consistent with the critical role of terminal galactose residues in ligand binding. However, asialotransferrin and asialofibrinogen show little or no reactivity. The glycans attached to α1-acid glycoprotein and fetuin are predominantly tetra- and triantennary complex structures, and those attached to transferrin and fibrinogen are mostly biantennary (Green et al., 1988; Shiyan and Bovin, 1997). There are five glycosylation sites in α1-acid glycoprotein, three in fetuin, and one in transferrin. Thus the different reactivities of these glycoproteins could reflect differences in the structures of the attached glycans or their number and spacing.

The possibility that highly branched structures with three or four terminal galactose residues are the highest affinity ligands for the KCR was tested by comparing binding of the KCR-B fragment to neoglycolipids prepared from purified, galactose-terminated bi-, tri-, and tetraantennary oligosaccharides (Figure 8). Binding to all three structures was detected, but the degree of reactivity increased only slightly for the larger structures. Because α1-acid glycoprotein contains fucose residues on some of the outer arms, the desialylated protein displays Lewis structures (Shiyan and

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>$K_{i,sugar}/K_{i,galactose}$</th>
</tr>
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<tbody>
<tr>
<td>Galactose</td>
<td>1</td>
</tr>
<tr>
<td>GalNAc</td>
<td>$0.17 \pm 0.02$</td>
</tr>
<tr>
<td>α-Methyl galactoside</td>
<td>$2.5 \pm 0.1$</td>
</tr>
<tr>
<td>2-Deoxygalactose</td>
<td>$1.2 \pm 0.1$</td>
</tr>
<tr>
<td>Fucose</td>
<td>$3.1 \pm 0.2$</td>
</tr>
<tr>
<td>α-Methyl fucoside</td>
<td>$26 \pm 5$</td>
</tr>
<tr>
<td>β-Methyl fucoside</td>
<td>$4.4 \pm 0.2$</td>
</tr>
<tr>
<td>Mannose</td>
<td>$15 \pm 1$</td>
</tr>
<tr>
<td>α-Methyl mannoside</td>
<td>$14 \pm 1$</td>
</tr>
<tr>
<td>Glucose</td>
<td>$9 \pm 1$</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>No inhibition</td>
</tr>
</tbody>
</table>

The KCR-B fragment was immobilized on polystyrene wells and probed with 125I-labeled galactose-BSA. Apparent affinities for different competing monosaccharides are compared by presenting the ratio of the inhibition constant for each sugar compared to the inhibition constant for galactose.

However, there is a marked difference in the affinity of the KCR for the methyl glycosides of fucose. The very weak binding of α-methyl fucoside suggests that most mammalian fucose-containing oligosaccharides would not be good ligands for the KCR.
To determine if these groups might bind with particularly high affinity to the KCR, neoglycolipids made from glycans released from α1-acid glycoprotein were probed with KCR-B, but no enhanced reactivity was observed (Figure 8). Binding to pentasaccharides terminating in Lewisx was also tested, but only weak reactivity was observed (data not shown). Competition studies with immobilized KCR confirmed that there is no substantial increase in affinity for more branched oligosaccharides. For example, no competition was observed with bi- and triantennary oligosaccharides at concentrations in excess of 50 μM. This result contrasts with previous studies on the asialoglycoprotein receptor in which the ability of oligosaccharides to compete for binding increased roughly three orders of magnitude for each additional branch in going from mono- to bi- to triantennary structures (Lee et al., 1983). Testing of the Lewisx trisaccharide in the competition assay also failed to reveal inhibition at concentrations up to 1 mM, which was the highest that could be achieved with the amounts of oligosaccharide available.

Considered together, the results with purified glycoproteins and oligosaccharides suggest that the presence of multiple oligosaccharides on a single glycoprotein, rather than specific glycan structures, is the most important factor in high-affinity binding to certain glycoproteins. The ability of the rat KCR to react with endogenous rat glycoproteins was further tested by probing rat serum glycoproteins resolved on sodium dodecyl sulfate (SDS)–polyacrylamide gels (Figure 9). The labeled KCR-B fragment interacts with several bands in neuraminidase-treated serum but not in the untreated sample. The reactive bands correspond to major serum proteins that shift in molecular weight following neuraminidase treatment. Thus desialylation of several major serum components would make them good ligands for the KCR. Similar patterns of reactivity were obtained for rat and human serum.

**Discussion**

The use of recombinant extracellular domain of the KCR has provided, for the first time, sufficient material to allow physical characterization of the receptor. Although the presence of 14-amino-acid repeating sequences in the neck region of the receptor was previously noted (Hoyle and Hill, 1988, 1991), the potential to form a coiled-coil of α helices was not emphasized. The current results provide...
glycoprotein receptor and the mannose receptor, they also contamination from other liver lectins, such as the asialo-sugar-binding specificity of this receptor. For the monosac- been analyzed.

A high specificity ratio for galactose over mannose is associated with precise positioning of a specificity-determining tryptophan residue in the asialoglycoprotein receptor. The tryptophan residue is held in position by an adjacent glycine-rich loop (Iobst and Drickamer, 1994; Kolatkar and Weis, 1996). Although a tryptophan residue is found at an equivalent position in the KCR, the adjacent sequence differs significantly from the glycine-rich loop of the asialoglycoprotein receptor (Iobst and Drickamer, 1994). These results suggest that the binding site does not exclude man nosel and related sugars very effectively.

Perhaps the most surprising result from the monosaccharide inhibition studies is the very poor interaction with methyl fucosides. The fact that both α- and β-methyl fucosides inhibit less effectively than free fucose suggests that some of the apparent affinity for fucose results from interaction involving the 1- and 2-hydroxyl groups in the way that other free sugars have been shown to interact with C-type CRDs (Ng et al., 1996). Fucose in mammalian glycans is found almost exclusively in α linkage, so the very poor inhibition by α-methyl fucose suggests that such glycans would be poor ligands for the receptor. Although this result is consistent with previous studies with neoglycoproteins (Lehrman et al., 1986a), the monosaccharide inhibition studies reported herein provide a quantitative comparison. The neoglycolipid binding and inhibition studies employing Lewis\textsuperscript{a} structures are consistent with the primary binding site on glycoproteins, such as asialo α\textsubscript{b}Gc glycoprotein, being terminal galactose residues with little if any contribution by fucose.

Previous studies have documented the ability of the KCR to mediate clearance of glycoprotein from circulation, and the loss of ligand-binding activity observed at acidic pH is consistent with a role in receptor-mediated endocytosis (Lehrman et al., 1986a,b). The pH dependence of ligand binding is similar to that of the asialoglycoprotein receptor, which correlates with the presence of a key histidine residue that mediates loss of binding activity to the asialoglycoprotein receptor at low pH (Figure 1) (Feinberg et al., 2000; Wragg and Drickamer, 1999).

The extended neck of the receptor and the preference for galactose-containing ligands are consistent with the suggestion that the KCR is the galactose particle receptor, which participates in binding and internalization of ligands over 12 nm in size (Kuiper et al., 1994). However, the KCR clearly has high affinity for endogenous glycoproteins from which sialic acid is removed, suggesting that it might have a function parallel to the asialoglycoprotein receptor in clearance of desialylated glycoproteins. This possibility has important implications for understanding the phenotype of asialoglycoprotein receptor–deficient mice. Such mice fail to show rapid clearance of asialoglycoproteins from circulation, but no long-term accumulation of desialylated serum glycoproteins is observed. A slower uptake of such glycoproteins mediated by the KCR might provide enough residual clearance capacity to keep circulating levels of asialoglycoproteins low while not being evident in short-term clearance studies.

The observed binding to thyrotropin suggests that the KCR may also bind terminal GalNAc residues on the hormones, which have become exposed due to loss of sulfate. Thus, the KCR could serve as a back-up for clearance of hormones that usually bear the 4-sulfo-GalNAc marker, which leads to endocytosis by the mannose receptor (Fiete et al., 1998). The preferential binding to GalNAc is consistent with previous studies in which adhesion to glycolipids was measured because it was observed that the best binding is obtained with a glycolipid bearing a terminal GalNAcβ1-3Gal structure (Tiemeyer et al., 1992).

Analysis of the human genome sequence reveals a partial gene closely related to the gene for the KCR in rats and mice encoded on chromosome 2, region 2p13.3, which is near to the gene for another C-type lectin, langerin (Figure 1). The fragment consists of three exons encoding a CRD and one exon encoding a sequence homologous to the C-terminal portion of the neck of the rat KCR. Extensive searches of the region 3’ to the gene fragment fail to detect any additional exons, suggesting that this is a pseudogene. No cDNAs have been reported for this gene in any of the expressed sequence tag libraries. Attempts to amplify a cDNA from a liver library using primers that lie within the portion of the gene that is present fail to yield any detectable amplification product, providing further evidence that this gene is not functional (Fadden and Drickamer, unpublished data). Thus there appears to be no human ortholog of the KCR. The blotting studies using desialylated rat and human serum show that similar ligands are present in both species, so it seems unlikely that the KCR interacts with a set of ligands found uniquely in rodents or specifically absent from humans. If the KCR recognizes ligands that are also recognized by the
asialoglycoprotein receptor, its role may have become redundant.

Materials and methods

Materials

The rat liver cDNA library and Taq (Advantage 2) polymerase were obtained from Clontech (Basingstoke, Hampshire, UK). Custom oligonucleotides were purchased from Invitrogen (Carlsbad, CA). Other reagents for molecular biology as well as Clostridium perfringens neuraminidase were supplied by New England Biolabs (Beverly, MA). The Mono-Q anion exchange column and radioisotopes were purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Sugar affinity columns were prepared by the divinyl sulfone method (Fornstedt and Paroth, 1975). Polystyrene assay plates were a product of Dynex Laboratories (Middlesex, UK). Monosaccharides, glycoproteins, and materials for preparation of neoglycolipids were obtained from Sigma Chemical (St. Louis, MO). Oligosaccharides were purchased from Glyko (Novato, CA), and neoglycoproteins were from E-Y Laboratories (San Mateo, CA).

Cloning of the extracellular domain of the KCR

The cDNA sequence encoding the extracellular domain of the KCR was amplified using the primers 5'-aaggccgacctggactgccaggttctccagctgatcatgcag-3' (forward) and 5'-tggcggctcagtgtctgttcgctgccagaca-3' (reverse). The CRD-encoding region was amplified with the alternative forward primer 5'-aaggccgacctggactgccaggttctccagctgatcatgcag-3' (reverse). These primers include restriction sites for FseI and NorI. Following denaturation at 95°C for 1 min, 40 cycles of 95°C for 30 s and 68°C for 1 min were executed. Fragments were digested with FseI and NorI and inserted into a pNIIIIIompA2 expression vector (Grayeb et al., 1984) modified to contain the restriction sites FseI and NorI downstream of the ompA signal sequence. In each case, a mistake-free clone was generated using restriction fragments from two different clones. The resulting plasmids were used to transform E. coli strain JA221. To avoid mutations due to possible toxicity of the extracellular domain, the FseI site was introduced in a way that interrupts the reading frame. The correct reading frame was then generated by digesting with FseI followed by trimming of the 3' extensions with T4 DNA polymerase. The sequence of all plasmids was confirmed by DNA sequencing.

Expression and purification of soluble KCR fragments

Protein expression was carried out in LB medium containing 50 µg/ml ampicillin. An overnight culture (200 ml) was diluted into 6 L of medium and incubated at 30°C with shaking until an A_595 of 0.8 was reached. Following addition of 10 µg/L of isopropyl-β-thiogalactoside and CaCl_2 to a final concentration of 100 mM, cultures were incubated for a further 20 h at 30°C with shaking. Cells were harvested by centrifugation at 4000 × g for 15 min at 4°C. The cell pellet was suspended in 300 ml loading buffer (25 mM Tris-Cl, pH 7.8, 125 mM NaCl, 25 mM CaCl_2) and lysed by sonication (6 × 30-s bursts). Debris was removed by centrifugation at 10,000 × g for 15 min at 4°C and then 137,000 × g for 45 min at 4°C in a Beckman (Palo Alto, CA) 45Ti rotor. After clarification, the clarified extract was loaded onto a 5-ml column of galactose-Sepharose equilibrated with loading buffer. The column was washed with five aliquots of 2-ml loading buffer and eluted with five aliquots of 2-ml elution buffer (25 mM Tris-Cl, pH 7.8, 125 mM NaCl, 2.5 mM EDTA). Fractions were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE).

Protein from the affinity column was dialyzed against 50 mM Tris-Cl, pH 7.8, containing 50 mM NaCl and loaded onto a 1-ml Mono-Q column equilibrated with 50 mM Tris-Cl, pH 7.8, and eluted with a gradient from 0 to 500 mM NaCl over 20 min at a flow rate of 1 ml/min. Absorbance was measured at 280 nm, and 1-ml fractions were collected and analyzed by SDS–PAGE.

Analytical methods

Analytical ultracentrifugation was carried out in a Beckman XL-A analytical ultracentrifuge using an An60Ti rotor. Proteins were dialyzed against 10 mM Tris-Cl, pH 7.8, 150 mM NaCl, 5 mM CaCl_2. Experiments were performed at 6500, 8000 and 48,000 rpm at 20°C. Data were collected at either 233 nm or 280 nm, with the wavelength adjusted so that the initial absorbance was approximately 0.5. A baseline scan at 360 nm was used to correct for optical imperfections. The equilibrium distributions from different loading concentrations were analyzed simultaneously using the software supplied with the centrifuge.

Proteins were sequenced on a Beckman LF-3000 sequencer following blotting onto polyvinylidene difluoride membranes (Matsudaira, 1987). Circular dichroism spectra were measured on a Jasco J600 spectropolarimeter (Great Dunmow, Cambridgeshire, UK) using 200-µl samples in a 1-mm quartz cuvette at room temperature. Five scans were carried out on each sample from with a bandwidth of 2 nm and a scan rate of 20 nm/min. Protein concentrations were determined by alkaline ninhydrin assay (Hirs, 1967).

Protein cross-linking

The KCR fragments purified by anion exchange chromatography were dialyzed against 100 mM sodium HEPES, pH 7.5, 150 mM NaCl. CaCl_2 was added to some samples to a final concentration of 10 mM. Aliquots (25 µl) of each sample were treated with bis(sulfosuccinimidyl) suberate for 60 min at room temperature. Samples were diluted 1:1 with double-strength gel sample buffer, heated to 100°C for 5 min, and run on SDS–polyacrylamide gels.

Solid phase binding assay

Wells of microtiter plates were coated by incubating with 50 µl KCR-B fragment in loading buffer overnight at 4°C. Wells were washed twice with loading buffer and blocked with 5% BSA in loading buffer for 2 h at 4°C. The wells were washed twice again and incubated with 125I-galactose-BSA and competing sugar in loading buffer containing 5% BSA. After incubation at 4°C for 2 h, the wells were washed three times, dried, and counted in a γ-counter. Experiments were performed in duplicate and the data
averaged. A nonlinear least squares fitting program (SigmaPlot, Jandel Scientific, San Rafael, CA) was used to fit the data to the following equation:

Fraction of maximal binding = \( K_i / (K_i + \text{inhibitor}) \)

where \( K_i \) is the inhibitor concentration at which inhibition is half maximal.

**Glycoprotein and neoglycolipid binding assays**

The KCR-B fragment was dialyzed into 25 mM sodium-HEPES, pH 7.5, 100 mM NaCl, 25 mM CaCl\(_2\). Approximately 100 μg protein in 200 μl was reacted with 0.1 μCi of Bolton-Hunter reagent (Bolton and Hunter, 1973) for 10 min at room temperature. The iodinated protein was mixed with 1 ml loading buffer, repurified on a 1-ml column of galactose-Sepharose, and diluted into 50 ml loading buffer containing 2% bovine hemoglobin.

Serum was dialyzed against water and diluted eightfold prior to digestion with neuraminidase. Aliquots (40 μl) were treated with 50 U neuraminidase for 3 h at 37°C using buffer supplied by the manufacturer. Following addition of an equal volume of double-strength sample buffer, samples were heated to 100°C for 5 min. Aliquots containing the equivalent of 0.25 μl serum were resolved on 17.5% SDS–polyacrylamide gels and blotted onto nitrocellulose. The membrane was blocked with 2% hemoglobin in loading buffer for 60 min at room temperature and incubated with the radio-Iodinated KCR-B fragment for 90 min at room temperature, followed by four 5-min washes with cold loading buffer. Radioactivity was detected using a phosphorimager from Molecular Dynamics (Chesham, Buckinghamshire, UK).

Neoglycolipids were prepared by conjugation of oligosaccharides to phosphatidylethanolamine dipalmitate in the presence of sodium cyanoborohydride (Mizuochi et al., 1989). High performance thin-layer chromatograms of the neoglycolipids were developed with chloroform: methanol:water (105:100:28, v/v). After fixation for 30 s in 1 mg/ml poly(isobutylmethacrylate) in hexane, chromatograms were blocked and incubated with iodinated KCR-B following the same procedures as for the glycoprotein gel blots. Oligosaccharides were released from α1-acid glycoprotein by hydrazinolysis (Patel and Parekh, 1994). Sialic acid was released by treatment with 50 mM H\(_2\)SO\(_4\) for 60 min at 80°C. Published procedures were used for repurification of released oligosaccharides on Dowex 50 resin and of neoglycolipids on C8 cartridges (Feizi et al., 1994).

**Acknowledgments**

We thank Brian Matthews for performing the hydrazinolysis, Maureen Taylor for comments on the manuscript, and Russell Wallis for help with the interpretation of the ultracentrifugation experiments. This work was supported by grant no. 041845 from the Wellcome Trust. The analytical ultracentrifugation facility is supported by the Wellcome Trust and the Biotechnology and Biological Sciences Research Council.

**Abbreviations**

BSA, bovine serum albumin; CRD, carbohydrate-recognition domain; EDTA, ethylenediamine tetra-acetic acid; KCR, Kupffer cell receptor; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

**References**


