Isolation of the SO₄-4-GalNAcβ1,4GlcNAcβ1,2Manα-specific Receptor from Rat Liver*

(Received for publication, March 7, 1997)

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Glycoproteins, such as the glycoprotein hormone lutropin (LH), bear oligosaccharides terminating with the sequence SO₄-4GalNAcβ1,4GlcNAcβ1,2Manα (S4GGnM) and are rapidly removed from the circulation by a receptor present in hepatic endothelial cells and Kupffer cells. Rapid removal from the circulation is essential for attaining maximal hormone activity in vivo. We have isolated a protein from rat liver which has the properties expected for the S4GGnM-specific receptor (S4GGnM-R). The S4GGnM-R is closely related to the macrophage mannose receptor (Man-R) both antigenically and structurally. At least 12 peptides prepared from the S4GGnM-R have amino acid sequences that are identical to those of the Man-R. Nonetheless, the ligand binding properties of the S4GGnM-R and the Man-R differ in a number of respects. The S4GGnM-R binds to immobilized LH but not to immobilized mannose, whereas the Man-R binds to immobilized mannose but not to immobilized LH. When analyzed using a binding assay that precipitates receptor ligand complexes with polyeylene glycol, the S4GGnM-R is able to bind S4GGnM-bovine serum albumin (S4GGnM-BSA) conjugates whereas the Man-R is not. In contrast both the S4GGnM-R and the Man-R are able to bind Man-BSA. Monosaccharides that inhibit binding of Man-BSA by the Man-R enhance binding by the S4GGnM-R. Oligosaccharides terminating with S4GGnM and those terminating with Man are bound at independent sites on the S4GGnM-R. The S4GGnM-R present in hepatic endothelial cells may account for clearance of glycoproteins bearing oligosaccharides terminating with either mannose, fucose, or N-acetylgalcosamine.

Asn-linked oligosaccharides present on the glycoprotein hormones lutropin (LH) 1 and thyrotropin (TSH) terminate with the sequence SO₄-4GalNAcβ1,4GlcNAcβ1,2Manα (S4GGnM), whereas those on folliculopin and chorionic gonadotropin (CG) terminate with the sequence Sia₂,3/6Galβ1,4GlcNAcβ1,2Manα (1−5). We have proposed that the sulfated oligosaccharides present on LH and TSH are critical for the expression of full biologic function by these hormones (6−9). Terminal GalNAc-4SO₄ does not influence binding to or activation of the LH/CG receptor itself (10) but does have a marked impact on the circulatory half-life of LH following secretion (7, 11) due to recognition of the sulfated oligosaccharides by a receptor expressed at the surface of hepatic endothelial cells and Kupffer cells (11, 12). The rapid removal of LH from the circulation in conjunction with its release from granules in response to gonadotropin releasing hormone accounts for the episodic rise and fall in hormone levels seen in the circulation. Since the LH/CG receptor is a G-protein-coupled receptor, which rapidly becomes refractory to further stimulation following ligand binding (13−15), episodic stimulation may provide for maximal activation during the preovulatory surge in circulating LH levels. TSH shows similar properties with respect to half-life and receptor activation (16−19).

Glycoproteins bound by the S4GGnM-specific receptor are subsequently transported to lysosomes and degraded. There are roughly 600,000 S4GGnM-specific binding sites at the cell surface of hepatic endothelial cells, which bind LH through its sulfated oligosaccharides with an apparent $K_d$ of $2.7 \times 10^{-7}$ M. Binding is pH-dependent, requiring a pH > 5.0, but does not require Ca²⁺ (12). The location of the sulfate in the 4-position is critical since glycanconjugates bearing oligosaccharides terminating with the sequence SO₄-3GalNAcβ1,4GlcNAcβ1,2Manα (S3GGnM) are not bound by hepatic endothelial cells. We have now identified and isolated a glycoprotein from rat liver that has the properties expected for the receptor, which mediates removal of LH from the circulation on the basis of its sulfated oligosaccharides.

**MATERIALS AND METHODS**

**Analytical Procedures**

Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad) or ISS Protein-Gold (Integrated Separation Systems). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed according to Laemmli (20). Following separation by SDS-PAGE on 5% or 7.5% acrylamide gels, proteins were transferred electrophoretically to polyvinylidene difluoride membranes using CAPS buffer as described by Matsudaira (21) for amino-terminal sequence determination, peptide mapping, and detection with specific antisera. Proteins detected by antisera were developed using 125I-F(ab)₂ goat anti-rabbit IgG.

**Affinity Columns**

Wheat Germ Agglutinin (WGA)-Sepharose—WGA (Sigma) was dissolved in 100 mM NaHCO₃, 100 mM GlcNAc, 200 mM NaCl, pH 8.4, at a concentration of 2.0 mg/ml and added to cyanogen bromide-activated Sepharose 4B (Sigma) at a ratio of 300 μg/mg. Following separation by SDS-PAGE on 5% or 7.5% acrylamide gels, proteins were transferred electrophoretically to polyvinylidene difluoride membranes using CAPS buffer as described by Matsudaira (21) for amino-terminal sequence determination, peptide mapping, and detection with specific antisera. Proteins detected by antisera were developed using 125I-F(ab)₂ goat anti-rabbit IgG.

*This work was supported by National Institutes of Health Grant R01-CA21923. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: LH, lutropin; TSH, thyrotropin; CG, chorionic gonadotropin; PEG, polyeylene glycol; BSA, bovine serum albumin; S4GGnM, SO₄-4GalNAcβ1,4GlcNAcβ1,2Manα (S4GGnM); S4GGnM-specific receptor; S3GGnM, SO₄-3GalNAcβ1,4GlcNAcβ1,2Manα; Man-R, macrophage mannose-specific receptor; PAGE, polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; Cys-R, cysteine-rich; Fn-II, fibronectin type II; CRD, carbohydrate recognition domain; ASGP-R, asialoglycoprotein receptor; Bf, bovine lutropin; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

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m NaCl, 2.0 mM CaCl$_2$, pH 7.8, containing 0.2% NaN$_3$.

**bLH-Sepharose**—bLH-Sepharose was prepared in the same fashion with the following modifications. bLH was dissolved in 100 mM NaHCO$_3$, 200 mM NaCl, pH 8.4, at a concentration of 3.8 mg/ml and added to cyanogen bromide-activated Sepharose 4B at a ratio of 6.5 mg/ml of gel. The coupling efficiency was approximately 95% at room temperature. Remaining active sites were quenched by incubation at 4 °C overnight in 1.0 M ethanolamine, pH 8.3. bLH-Sepharose was stored in 20 mM Tris HCl, 0.15 mM NaCl, 2.0 mM CaCl$_2$, pH 7.8, containing 0.2% NaN$_3$.

**Radiolabeling**

SO$_4$-4GalNAc$_β$1,4GlcNAc$_β$1,2Man$_α$(CH$_2$)$_8$COO-bovine serum albumin (S4GGnM-BSA), Mannose-bovine serum albumin (Man-BSA), or purified S4GGnM receptor, 5–10 μg, were dissolved in 50 μl of 20 mM Tris-HCl, 0.15 mM NaCl, pH 7.4, and incubated for 15 min on ice with a single IODOBEAD (Pierce) and 2.5 μCi of $^{125}$I (Amersham, IMS30). Labeled proteins were separated from reaction products by gel filtration on Sephades G-10 (Pharmacia Biotech Inc.) in 20 mM Tris-HCl, 0.15 mM NaCl, pH 7.8, containing 1 mg/ml bovine serum albumin. The fractions containing $^{125}$I-labeled product were pooled and stored at −20 °C for no longer than 2 months. F(ab')2 goat anti-rabbit IgG (250 μg) was iodinated in the same fashion using 500 μCi of $^{125}$I in 500 μl of 20 mM NaPO$_4$, 0.15 mM NaCl, pH 7.5, and incubated 1 h at room temperature.

**Binding Assays**

Binding assays (total volume of 150 μl) contained 3–5 ng of S4GGnM receptor $^{125}$I-S4GGnM-BSA (2–3 × 10$^8$ dpm), and 90 μg of hyaluronic acid and/or 90 μg of fucoidin in 20 mM Tris-HCl, 0.15 mM NaCl, 2 mM CaCl$_2$, 1% (w/v) Triton X-100, 20 mM pH 7.8. Hyaluronic acid is a weak inhibitor of S4GGnM-BSA binding, whereas fucoidin is a potent inhibitor (12). Incubations were performed in a 10 × 75-mm glass tube at room temperature for 30 min. The reactions were terminated by adding 1.5 ml of 20 mM Tris-HCl, 10% (w/v) PEG 8000 (Sigma) in 20 mM Tris-HCl, 0.15 mM NaCl, 2 mM CaCl$_2$, and mixing. After 30 min on ice, precipitated $^{125}$I-S4GGnM-BSA:S4GGnM receptor complexes were collected by vacuum filtration on Whatman GF/C filter discs, which had been soaked in 20 mM Tris-HCl, 0.15 mM NaCl, 2 mM CaCl$_2$, 5 mg/ml bovine serum albumin. The filters were washed twice with 1.5 ml of ice-cold 20 mM Tris-HCl, 2 mM CaCl$_2$, 10% (w/v) PEG 8000, and the amount of $^{125}$I determined by counting the filter in a γ-counter. In the absence of added S4GGnM receptor, <5% of the added $^{125}$I-S4GGnM-BSA was captured on the filter. One unit of activity is defined as the amount of S4GGnM receptor that is able to precipitate 1 ng of S4GGnM-BSA in the presence of hyaluronic acid above that precipitated in the presence of both hyaluronic acid and fucoidin.

**S4GGnM Receptor Isolation**

**Step 1: Homogenate**—Harlan Sprague Dawley rats, 150–200 g each, were anesthetized and heparinized and their livers perfused with ice-cold 20 mM P0$_4$, 0.15 mM NaCl, pH 7.5, through the portal vein. Each liver was suspended in 20 ml of 0.25 mM EDTA, 0.02% NaN$_3$ (w/v) brought to pH 7.8 with solid NaHCO$_3$ and containing 50 units/ml aprotinin. The suspension was homogenized with three 20-s bursts of a Polytron homogenizer (Brinkman) at a setting of 8.

**Step 2: Triton X-100 Solubilization**—Sufficient 25% (w/v) Triton X-100 in 20 mM Tris-HCl, 0.15 mM NaCl, 2 mM CaCl$_2$, 10% (w/v) PEG 8000, and 0.05% NaN$_3$ (w/v) brought to pH 7.8 with solid NaHCO$_3$ and containing 50 units/ml aprotinin. The suspension was homogenized with four 15-s bursts of a Polytron homogenizer (Brinkman) at a setting of 8.

**Step 3: Triton X-100 100 Solubilization of PEG Precipitate**—The Triton extract was passed through cheesecloth to remove connective tissue and sedimented at 7100 × g for 20 min.

**Step 4: Triton X-100 Solubilization of PEG Precipitate**—The Triton extract was resuspended by vigorous stirring for 30 min in 20 mM Tris-HCl, 0.2 mM NaCl, 0.05 mM NaN$_3$, 3 mM CaCl$_2$, 1% (w/v) Triton X-100, pH 7.8, 200 mM NaCl.

**Step 5: WGA-Sepharose**—Solubilized proteins from the PEG precipitate were incubated with WGA-Sepharose (1–2 ml of WGA-Sepharose/liver) overnight at 4 °C. Bound glycoproteins were subsequently eluted using the same buffer containing 300 mM GlcNAc.

**Step 6: bLH-Sepharose**—The bLH-Sepharose eluate was incubated with 0.5 ml of bLH-Sepharose/liver overnight at 4 °C with rotation. Bound proteins were removed by washing on a sintered glass funnel with 20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% Triton X-100. The bLH-Sepharose was then eluted with 50 mM sodium acetate, pH 4.4, 1% Triton X-100. The eluate was immediately adjusted to pH 7.0 by addition of 1.0 M Tris base and the volume reduced using a Centriprep-10 (Amicon).

**Step 7: Mannan-Sepharose or Mannose-Sepharose**—The bLH-Sepharose eluate was incubated with either mannan-Sepharose (0.05 mM/liver) or mannose-Sepharose (0.05 mM/liver) and the unbound fraction taken. Bound proteins were eluted from the mannan-Sepharose and mannose-Sepharose by successive incubation with 50 mM galactose in 20 mM Tris, pH 7.5, and 0.5% Pefabloc SC (Boehringer Mannheim) adjusted to pH 7.5 and 200 mM mannose and 5 mM EDTA in the same buffer.

**Preparation of Rabbit Antisera to the S4GGnM Receptor**

Polyclonal antisera to the S4GGnM receptor were raised in New Zealand White rabbits by immunization with the 180-kDa protein band isolated from an SDS-polyacrylamide gel. The protein band was purified, and, after extracting the gel pieces with 95% ethanol to reduce the SDS content, the gel was lyophilized. The dried gel was pulverized using a mortar/pestle and then emulsified with saline by passing through a series of successively smaller needles ranging from 18 to 23 gauge. The emulsified gel containing 5–10 μg of protein was added to TDM-emulsion (RIB) and injected intramuscularly into the hind leg and subsequently at four separate sites. The rabbit was boosted in the same manner 3 weeks later and sera obtained 8–10 days later. The rabbit was subsequently boosted with antigen as required to maintain the titer of the antisera.

**Radiomunnoassay for S4GGnM Receptor**

The affinity-purified S4GGnM receptor was labeled with $^{125}$I as described above. An antibody saturation curve was established using a constant amount of radiolabeled receptor (5–10 ng, 3 × 10$^5$ cpm) and increasing amounts of antisera. Following an overnight incubation at 4 °C, protein A-Sepharose-antigen-antibody complexes were washed twice with 20 mM phosphate-buffered saline, pH 7.5, containing 0.1% BSA (w/v) and counted in a γ-counter. Standard inhibition curves were constructed using a constant amount of radiolabeled receptor, sufficient antisera to precipitate 50–70% of the $^{125}$I-S4GGnM-R, and increasing amounts of unlabeled receptor that had been quantitated by amino acid analysis. The amount of receptor in “unknown” samples was then determined by comparison to the standard inhibition curve.

**RESULTS**

**Purification**—We previously identified a receptor in rat liver that can account for the rapid removal of native LH bearing Asn-linked oligosaccharides terminating with the sequence S4GGnM from the circulation (12). The S4GGnM-R is located predominantly in hepatic endothelial cells and Kupffer cells and displays a high degree of specificity, recognizing S4GGnM-BSA but not S3GGnM-BSA. Fucoidin, a sulfated polysaccharide, inhibits binding of S4GGnM-BSA and LH by the receptor, whereas other sulfated and anionic polysaccharides do not inhibit binding or require much higher concentrations to do so. Glycoproteins bound to the S4GGnM-R are internalized, transported to lysosomes, and degraded. Binding is pH-dependent, requiring a pH above 5–6, and is not dependent on divalent cations even though divalent cations do enhance binding.

We established conditions that allowed us to purify and solubilize a binding activity with the properties we had described for the S4GGnM-R. Parenchymal cells (hepatocytes) and endothelial/Kupffer cells, prepared by collagenase perfusion as described previously (12, 22), were disrupted by Dounce homogenization, and the nuclei and unbroken cells were collected by centrifugation. Soluble and total membrane fractions were then pelleted.
BSA in the presence of increasing amounts of Triton X-100 as indicated.

Membranes prepared from rat liver were incubated with 125I-S4GGnM-BSA in the presence of increasing amounts of Triton X-100 and assayed for S4GGnM-BSA binding using the PEG precipitation assay. The amount of receptor protein present was determined by a RIA in which unlabeled S4GGnM-R was used to inhibit binding of 125I-S4GGnM-R by a rabbit antibody raised to the purified S4GGnM-R.

Fractions were brought to a final concentration of 1% (w/v) 125I-S4GGnM-BSA and the PEG precipitation assay. The amount of receptor binding of S4GGnM-R that had been eluted from bLH-Sepharose was bound by immobilized mannan-Sepharose or mannose-Sepharose. Neither the S4GGnM-R-specific binding activity nor the protein with an Mr of 180,000 was bound by immobilized mannan or mannose, whereas the other proteins in the eluate from bLH-Sepharose were bound to either mannan-Sepharose or mannose, whereas the other proteins in the eluate from bLH-Sepharose were bound to either mannan-Sepharose or mannose-Sepharose and removed. The mannose-Sepharose unbound fraction was homogeneous and consisted of a single band, migrating with an Mr of 180,000 when analyzed by SDS-PAGE, a major band was found to be present, which had an Mr of 180,000 (Fig. 2A). Additional proteins with apparent molecular weights of 75–80,000, 60,000, 43,000, and 35,000 were also present. Following electrophoretic transfer to Immobilon-P, a ligand blot was performed using 125I-S4GGnM-BSA, which demonstrated that the only protein with an Mr of 180,000 was reactive (Fig. 2B).

A NH2-terminal sequence of LK(Y/S)QYQFLIYNE was obtained for the protein with an Mr of 180,000, suggesting it was closely related to the murine macrophage mannose receptor (Man-R), which has an NH2-terminal sequence of LLDAQFLIYNE (23).

TABLE I

| Step | Protein Binding | Total binding units/rat liver | Total receptor by RIA |
|------|-----------------|------------------------------|-----------------------|
| 1. Homogenate | ND* | | |
| 2. Triton X-100 | 355 | 62 | 100 | 33.3 | 100 |
| 3. PEG Pellet | 180 | 50 | 81 | 13.2 | 40 |
| 4. Triton X-100 | ND* | 30 | 48 | | |
| 5. WGA-Sepharose bound | 6.4 | 6.3 | 10 | 9.9 | 30 |
| 6. bLH-Sepharose bound | 0.05–0.2 | 1.15 | 1.9 | 6.7 | 20 |
| 7. Man-Sepharose unbound | 0.05–0.2 | 1.08 | 1.7 | | |

a ND, the amount of protein determined to be in the homogenate and the PEG pellet resuspended in Triton X-100 was not considered accurate and is not included.

Solubilization of the S4GGnM receptor by Triton X-100.

Membranes prepared from rat liver were incubated with 125I-S4GGnM-BSA in the presence of increasing amounts of Triton X-100 as indicated. 125I-S4GGnM-BSA-S4GGnM-R complexes were collected either by sedimentation at 190,000 × g for 5 min in an Airfuge in the absence of added PEG (−PEG) or by filtration of GFC glass fiber filters following addition of PEG (+PEG). Precipitation with PEG was not performed for the 0.01% Triton concentration. Nonspecific binding was determined by performing incubations in the presence of 100 μg/ml fucoidin and has been subtracted.

Fractions were brought to a final concentration of 1% (w/v) Triton X-100 prior to incubation with WGA-Sepharose. The S4GGnM-R bound to WGA-Sepharose and was eluted with 300 mM GlcNAc. The WGA-Sepharose eluate containing the S4GGnM-R was incubated with bLH-Sepharose. S4GGnM-R that bound to bLH-Sepharose was eluted by reducing the pH to 4.0 with acetic buffer. When this material was examined by SDS-PAGE, a major band was found to be present, which had an Mr of 180,000 (Fig. 2A). Additional proteins with apparent molecular weights of 75–80,000, 60,000, 43,000, and 35,000 were also present. Following electrophoretic transfer to Immobilon-P, a ligand blot was performed using 125I-S4GGnM-BSA, which demonstrated that only the protein with an Mr of 180,000 was reactive (Fig. 2B). An NH2-terminal sequence of LK(Y/S)QYQFLIYNE was obtained for the protein with an Mr of 180,000, suggesting it was closely related to the murine macrophage mannose receptor (Man-R), which has an NH2-terminal sequence of LLDAQFLIYNE (23).

SO4-4-GalNAcβ1,4GlcNAcβ1,2Manα-specific Receptor Isolation

We examined the ability of Triton X-100 to solubilize the binding activity from membranes. Membranes were incubated with 125I-S4GGnM-BSA in the presence of increasing amounts of Triton X-100 (Fig. 1). Membranes were either collected by sedimentation in an Airfuge at 190,000 × g (Beckman) in the absence of added PEG or by filtration on GF/C glass fiber filters following addition of PEG (+PEG). Solubilization of the S4GGnM receptor by Triton X-100 was determined that: 1) 3-fold more 125I-S4GGnM-BSA is bound at pH 7.5 than at pH 5.0 or below; 2) EDTA does not abolish binding of S4GGnM-BSA but does reduce it to 62% of that seen in the presence of 4 mM Ca2+; and 3) fucoidin is a significantly more potent inhibitor of binding than other sulfated or anionic polysaccharides such as hyaluronic acid, heparin, chondroitin sulfate, and dextran sulfate. Thus, a binding activity with the properties expected for the S4GGnM-R could be detected in rat liver membranes and solubilized with Triton X-100. We therefore developed the isolation scheme summarized in Table I.

The S4GGnM-R was solubilized using 10% Triton X-100, concentrated by precipitation with PEG 8000, and solubilized in 10% Triton X-100 prior to incubation with WGA-Sepharose. The S4GGnM-R bound to WGA-Sepharose and was eluted with 300 mM GlcNAc. The WGA-Sepharose eluate containing the S4GGnM-R was incubated with bLH-Sepharose. S4GGnM-R that bound to bLH-Sepharose was eluted by reducing the pH to 4.0 with acetic buffer. When this material was examined by SDS-PAGE, a major band was found to be present, which had an Mr of 180,000 (Fig. 2A). Additional proteins with apparent molecular weights of 75–80,000, 60,000, 43,000, and 35,000 were also present. Following electrophoretic transfer to Immobilon-P, a ligand blot was performed using 125I-S4GGnM-BSA, which demonstrated that only the protein with an Mr of 180,000 was reactive (Fig. 2B). An NH2-terminal sequence of LK(Y/S)QYQFLIYNE was obtained for the protein with an Mr of 180,000, suggesting it was closely related to the murine macrophage mannose receptor (Man-R), which has an NH2-terminal sequence of LLDAQFLIYNE (23).
The S4GGnM-R and the Man-R Provide Comparable Peptide Maps—The S4GGnM-R (200 μg) and the Man-R (150 μg) were subjected to electrophoretic separation on 5% polyacrylamide gels and electrophoretically transferred to Immobilon (Millipore) in CAPS buffer. After staining with Ponceau Red, the regions containing the transferred protein were excised for analysis. Peptides were released by digestion with LysC or trypsin in the presence of reduced Triton X-100 and separated by reverse phase chromatography. The separations are shown in Fig. 3 for peptides released by LysC digestion of the S4GGnM-R and the Man-R. The profiles were nearly identical. Only peaks 57 and 75 of the S4GGnM-R were not also present in the Man-R. Peaks 45, 54, and 91 of the S4GGnM-R appeared to be identical to peaks 37, 54, and 84 of the Man-R, respectively. Peaks that appeared to be identical and peaks that appeared to differ between the S4GGnM-R and the Man-R were analyzed. The results of these analyses are summarized in Table II.

Sequence was obtained for 12 peptides from three different S4GGnM-R preparations. Nine of the peptide sequences obtained from the S4GGnM-R were identical to peptide sequences predicted to be present in the macrophage Man-R (23). The sequences obtained originate from a number of different regions and encompass the entire 1385 amino acids of the Man-R extracellular domain (Table II). As expected from the similarity of the peptide maps, the predominant peptides in peaks 45, 54, and 91 of the S4GGnM-R were identical to those of the Man-R, which had no equivalent in the Man-R peptide profile, were sequenced that could not be identified in the Man-R sequence. These were minor components, however, and were identified within the asialoglycoprotein receptor (ASGP-R) sequence. Since small amounts of terminal β1,4-linked GalNAc are present on bLH and would be recognized by the ASGP-R, the presence of low levels of ASGP-R in the final preparation of the S4GGnM-R would not be unexpected. We did not, however, detect ASGP-R in the S4GGnM-R preparation by Western blot analysis using ASGP-R-specific antisera.

The peptide sequence analyses summarized in Table II, in conjunction with the nearly identical peptide maps obtained for the S4GGnM-R and the Man-R in Fig. 3, support the conclusion.
that the S4GGnM-R from liver is closely related to the macrophage Man-R isolated from rat lung. Furthermore, the S4GGnM-R and the Man-R both react with antisera raised to terminal S4GGnM and Man. The S4GGnM-R and the Man-R represent the major forms of these receptors in liver and lung, respectively, since significant amounts of Man-R cannot be isolated from liver by affinity chromatography on mannose-Sepharose, but did not bind to bLH-Sepharose, which contains multiple oligosaccharides terminating with terminal mannose such as mannan-Sepharose, Man-BSA.

The inability of the Man-R to precipitate S4GGnM-BSA is consistent with its inability to bind to immobilized bLH, which bears multiple Asn-linked oligosaccharides terminating with the sequence S4GGnM (3). The ability of the S4GGnM-R to precipitate soluble Man-BSA was not expected since the S4GGnM-R is not able to bind to immobilized ligands containing terminal mannose such as mannose-Sepharose, Man-BSA-Sepharose, and Man-BSA-Sepharose.

The S4GGnM-R and the Man-R both react with Man-BSA using the PEG precipitation assay (Fig. 6B). The inability of the Man-R to precipitate S4GGnM-BSA is consistent with its inability to bind to immobilized bLH, which bears multiple Asn-linked oligosaccharides terminating with the sequence S4GGnM (3). The ability of the S4GGnM-R to precipitate soluble Man-BSA was not expected since the S4GGnM-R is not able to bind to immobilized ligands containing terminal mannose such as mannose-Sepharose, Man-Sepharose, and Man-BSA-Sepharose.

The S4GGnM-R and the Man-R both react with Man-BSA.
when examined by ligand blotting with 125I-Man-BSA following SDS-PAGE (Fig. 7). When equal units of Man-BSA-specific binding activity, as measured by the PEG precipitation assay, were examined by ligand blotting with 125I-Man-BSA, the S4GGnM-R in lane 3 was more intensely labeled than the Man-R in lane 4. This suggested that even though 5–6-fold more S4GGnM-R than Man-R was required to precipitate the same amount of soluble Man-BSA (compare panels A and B of Fig. 6), this difference in binding capacity was not retained in the ligand blot following SDS-PAGE. In support of this conclusion, we found that equal amounts of the S4GGnM-R and Man-R reacted with equal intensity when examined by ligand blotting with 125I-Man-BSA following SDS-PAGE. This suggests that binding in the soluble assay may reflect binding to a different site or in a different manner than when the same receptor is probed with ligand following SDS-PAGE. In light of the structural similarities between the S4GGnM-R and the Man-R, it is notable that they bind Man-BSA with equal intensity following SDS-PAGE even though they show marked differences in Man-BSA binding in their native state.

A remarkable feature of the macrophage Man-R is its ability to bind ligands with terminal Man, GlcNAc, Glc, and Fuc (28, 29). These same monosaccharides can be utilized as inhibitors of binding by the Man-R. We therefore compared inhibition of Man-BSA binding by monosaccharides for both the S4GGnM-R and the Man-R. As reported by others (28, 30, 31), we found binding of Man-BSA by the Man-R in the PEG precipitation assay is inhibited by Man, Fuc, Glc, and GlcNAc, whereas Gal inhibits weakly and GalNAc not at all (Fig. 8). In contrast, low concentrations of Man enhance Man-BSA binding by the S4GGnM-R. Concentrations of Man as high as 200 mM are not inhibitory, although they do reduce binding as compared with Man concentrations ranging from 25–100 mM (Fig. 8). Fuc, Glc, and GlcNAc have similar effects whereas GalNAc is without effect (Fig. 8). Gal, which is a poor inhibitor of binding by the Man-R, enhances binding by the S4GGnM-R at a concentration of 200 mM but not at 50 mM. Thus, the same monosaccharides affect binding by the S4GGnM-R and the Man-R; however, they enhance binding by the S4GGnM-R and inhibit binding by the Man-R. Even though the S4GGnM-R and the Man-R both are able to bind Man-BSA, the properties of this binding reaction for the native receptors differ dramatically with respect to the effect of monosaccharides and the amount of receptor required to precipitate a given amount of Man-BSA.

Man-BSA and S4GGnM-BSA Bind to the S4GGnM-R Independently—We next determined if there was any relationship between the mannose- and S4GGnM-specific binding sites of the S4GGnM-R. Like the Man-R, we found that the S4GGnM-R is able to bind Fuc-BSA as well as Man-BSA (Fig. 9). Addition of excess Man-BSA inhibited binding of 125I-Man-BSA and 125I-Fuc-BSA by the S4GGnM-R. Man-BSA had no effect on the binding of 125I-S4GGnM-BSA by the S4GGnM-R (Fig. 9). Fuc-BSA was also able to inhibit binding of both 125I-Man-BSA and 125I-Fuc-BSA by the S4GGnM-R, suggesting Man-BASA and Fuc-BSA compete for the same sites on the receptor. In contrast, excess S4GGnM-BSA inhibits binding of 125I-S4GGnM-BSA by the S4GGnM-R but not binding of either 125I-Man-BSA or 125I-Fuc-BSA (Fig. 9). The addition of either 40 mM mannose or fucose enhanced binding of Fuc-BSA and to an even greater extent than Man-BSA. At a concentration of 200 mM, binding of Man-BSA and Fuc-BSA were reduced as compared with that seen in the presence of 40 mM monosaccharide but not to the levels seen in the complete absence of added monosaccharides. Neither mannose nor fucose had any effect on S4GGnM-BSA.
binding at either concentration (Fig. 9). Thus S4GGnM-BSA appears to bind to a site on the S4GGnM-R that is distinct from and independent of the Man/Fuc-specific binding site.

Kinetics of Man-BSA and S4GGnM-BSA Binding—The kinetics of binding of S4GGnM-BSA and Man-BSA in the presence and absence of GlcNAc were assessed for both the S4GGnM-R and the Man-R. The saturation curves obtained were analyzed using the LIGAND program (32) as summarized in Table III. At saturation, 0.06 mol of S4GGnM-BSA/mol of S4GGnM-R was bound with an apparent $K_d$ of $3.0 \times 10^{-9}$ M. The presence of 50 mM GlcNAc had no impact on the kinetics of S4GGnM-BSA binding. Man-BSA was bound by the S4GGnM-R with an apparent $K_d$ of $3.9 \times 10^{-9}$ M and a mole ratio of 0.11 at saturation. In the presence of 50 mM GlcNAc, the apparent $K_d$ for binding of Man-BSA by the S4GGnM-R was reduced to $1.7 \times 10^{-8}$ M while the mole ratio at saturation increased to 1.06. The apparent $K_d$ for binding of Man-BSA by the Man-R from lung was $3.1 \times 10^{-9}$ M with a mole ratio of 0.77 at saturation. As with the S4GGnM-R, 50 mM GlcNAc reduced the apparent $K_d$ to $1.5 \times 10^{-8}$ M and increased the mole ratio to 1.05 at saturation.

Thus, even though the S4GGnM-R is able to bind both S4GGnM-BSA and Man-BSA, the kinetics seen for binding of Man-BSA differ from those seen for binding of Man-BSA by the Man-R. Since both the apparent $K_d$ and the $B_{\max}$ for binding of Man-BSA by the S4GGnM-R are influenced by addition of monosaccharides such as GlcNAc, the effects of monosaccharides on binding by the S4GGnM-R must be considered complex and will require more detailed analysis to be understood. It would appear, however, that the S4GGnM-R is capable of binding and internalizing ligands with terminal GlcNAc, Man, or Fuc as well as those with terminal S4GGnM. Thus, it is not clear at present if the receptor responsible for clearance of glycoproteins bearing oligomannose type oligosaccharides or neoglycoproteins such as Man-BSA from the blood (33) is the S4GGnM-R or the Man-R.

DISCUSSION

We have isolated a protein that has the properties predicted for the S4GGnM-R in hepatic endothelial cells and Kupffer cells. The S4GGnM-R mediates the rapid clearance of glycoproteins bearing oligosaccharides with the terminal sequence S4GGnM, for example LH and TSH, from the circulation (12). We have proposed that this function is critical for the expression of hormone biologic activity and the clearance of glycoproteins not bound by the receptor. Thus, even though the S4GGnM-R is able to bind both S4GGnM-BSA and Man-BSA, the kinetics seen for binding of Man-BSA differ from those seen for binding of Man-BSA by the Man-R. Since both the apparent $K_d$ and the $B_{\max}$ for binding of Man-BSA by the S4GGnM-R are influenced by addition of monosaccharides such as GlcNAc, the effects of monosaccharides on binding by the S4GGnM-R must be considered complex and will require more detailed analysis to be understood. It would appear, however, that the S4GGnM-R is capable of binding and internalizing ligands with terminal GlcNAc, Man, or Fuc as well as those with terminal S4GGnM. Thus, it is not clear at present if the receptor responsible for clearance of glycoproteins bearing oligomannose type oligosaccharides or neoglycoproteins such as Man-BSA from the blood (33) is the S4GGnM-R or the Man-R.

We have isolated a protein that has the properties predicted for the S4GGnM-R in hepatic endothelial cells and Kupffer cells. The S4GGnM-R mediates the rapid clearance of glycoproteins bearing oligosaccharides with the terminal sequence S4GGnM, for example LH and TSH, from the circulation (12). We have proposed that this function is critical for the expression of hormone biologic activity in vivo (6–9, 34). Peptide maps, amino acid sequence of multiple peptides, and immune cross-reactivity indicate the S4GGnM-R is closely related to the previously characterized macrophage Man-R (23, 35). The Man-R is one member of a family of structurally related membrane proteins, which includes DEC-205 (36), the phospholipase A$_2$ receptor (37, 38), and a novel type C lectin expressed in fetal but not adult liver (39). Each consists of a signal sequence that is cleaved, a cysteine-rich (Cys-R) domain, a domain with fibronectin type II repeats (FN-II), 8–10 type C carbohydrate recognition domains (CRD) separated by “stalks,” a transmembrane domain, and a cytosolic domain. The S4GGnM-R peptides for which sequence was obtained (Table II) represent sequences that are identical to portions of the Cys-R domain, the FN-II domain, CRD 2, CRD 3, CRD 5, CRD 8, and Stalk 2 of the macrophage Man-R. If we assume that this similarity in structure is retained throughout the extracellular domain of the S4GGnM-R, it will consist of a Cys-R domain, a FN-II domain, 8 CRDs, 9 stalks, a transmembrane domain, and
a cytosolic domain.

How closely related are the S4GGnM-R and the Man-R and what is the structural basis for the differences in their ligand specificities? The data we have obtained indicates that the structural relationship between the S4GGnM-R and the Man-R is extensive. Possible mechanisms that could result in such closely related receptors include: 1) the existence of two genes encoding closely related proteins, 2) a post-transcriptional alteration in the mRNA sequence producing different forms of the receptor, and 3) a post-translational modification which alters the specificity of the receptor. Any differences in the structure of the S4GGnM-R and the Man-R must account for the differences in the ability to bind ligands terminating with S4GGnM and for differences in the characteristics for binding of ligands containing terminal mannose or fucose.

The macrophage Man-R displays multiple specificities, being able to bind carbohydrate moieties terminating with Man, GlcNAc, or Fuc (28–30). CRDs 4–8 together account for the high affinity binding of ligands such as Man-BSA and mannan. CRD 4 plays a predominant role in binding and is the only CRD that is able to bind mannose containing ligands in the absence of other CRDs (28). Binding of Man-BSA and Fuc-BSA by the S4GGnM-R, like binding by the Man-R, is Ca2+- and pH-dependent. Furthermore, the same monosaccharides have an impact on binding by both receptors; however, in the case of the Man-R these monosaccharides are strictly inhibitory, whereas for the S4GGnM-R they have a complex effect resulting in enhanced rather than reduced binding. This suggests that one or more of the CRDs that mediate Man-BSA binding by the Man-R are altered in the S4GGnM-R. Based on peptide sequences from the S4GGnM-R the Cys-R region, FN-II region, CRD 2, and CRD 3 are present and likely have the same sequence as in the Man-R. The functional significance of the Cys-R region and FN-II region is not known for the Man-R or other members of this family, nor is there evidence of ligand binding by CRDs 1–3 of the Man-R (28). Should the S4GGnM-R prove to have the same overall structure as the Man-R throughout its extracellular domain, there would be an ample number of regions, which could potentially account for the independent binding of ligands terminating with S4GGnM and those terminating with either mannose or fucose.

The S4GGnM-R may be the first example of a carbohydrate-specific receptor that can bind unrelated oligosaccharide structures at independent sites. Since binding of S4GGnM-BSA does not require Ca2+, it is likely that the structural motif which accounts for binding of GalNAc-4-SO4-containing ligands differs from the Ca2+-dependent CRDs, which are characteristic of the Man binding sites. If distinct regions account for S4GGnM- and Man-specific binding by the S4GGnM-R, it would seem likely that structural differences between the S4GGnM-R and the Man-R would have to involve more than one region. An intriguing feature of the S4GGnM-R is that the same peptide bearing different carbohydrate moieties could be bound by the same receptor at different sites and with differing kinetics. This could result in differing kinetics of clearance from the circulation. For example, LH bearing high mannose type structures may be cleared more rapidly than LH bearing oligosaccharides terminating with S4GGnM. Should this be the case it would suggest that the precise rate of clearance as determined by the structure of the oligosaccharide is indeed critical for maintaining biologic activity in vivo.

A number of studies have suggested that a receptor with the same properties as the Man-R isolated from lung and placenta is present in liver endothelial cells and Kupffer cells (30, 40–42). The relationship of the hepatic receptor to the macrophage Man-R present in alveolar macrophages and other tissues may have to be re-evaluated in light of the current findings. It is not surprising that it has been difficult to purify the Man-R from liver using affinity chromatography on immobilized ligands containing terminal mannose, procedures that are effective for isolation of the Man-R from lung (24), macrophage lines (26), and placenta (25), in light of the properties of the S4GGnM-R. It now seems likely that the S4GGnM-R we have isolated from liver for a major fraction of binding and internalization of ligands containing terminal Man, Fuc, or GlcNAc by hepatic endothelial cells and Kupffer cells (41–43). The characteristics of binding and internalization of Man-BSA by the S4GGnM-R are likely to differ from those encountered with the Man-R of alveolar macrophages.

Many issues remain to be addressed. How does the S4GGnM-R differ from the Man-R structurally? What region accounts for binding of S4GGnM? Is the region accounting for S4GGnM binding structurally related to the Ca2+-dependent C type lectin motif, or does it represent a new binding motif? Is expression of the S4GGnM-R, like that of the Man-R, highly regulated? Is expression regulated by estrogen? Is the S4GGnM-R expressed in other cells and in other tissues? These are some of the issues we will address in our future studies. The answers promise to reveal new insights about the biologic significance of oligosaccharides terminating with S4GGnM for the glycoprotein hormones as well as other glycoproteins, which are continually being added to the list family of glycoproteins bearing S4GGnM structures.

Acknowledgments—We thank William S. Lane and colleagues at the Harvard Microchemistry Facility for their helpful suggestions while performing the peptide maps and sequence analyses. We also thank Dr. P. D. Stahl, Washington University, St. Louis, MO for providing us with antibody raised to the Man-R and Dr. R. L. Hill, Duke University, University Medical Center, Durham, NC for providing samples of Fuc-BSA and antibody raised to the Man-R.

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*J. Biol. Chem.* 1997, 272:14629-14637. doi: 10.1074/jbc.272.23.14629

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