Multiple interactions between pituitary hormones and the mannose receptor

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The macrophage mannose receptor, which has a well-documented role in the innate immune system, has an additional function in the clearance of pituitary hormones. Clearance is mediated by the recognition of sulphated terminal *N*-acetyl-galactosamine residues (SO₄-4GalNAc) on the hormones. Previous studies with an SO₄-4GalNAc-containing neoglycoprotein suggest that the SO₄-4GalNAc-binding site is localized to the N-terminal cysteine-rich domain of the receptor, distinct from the mannose/*N*-acetylglucosamine/fucose-specific C-type carbohydrate-recognition domains (CRDs). The present study characterizes the binding of natural pituitary hormone ligands to a soluble portion of the mannose receptor consisting of the whole extracellular domain and to a truncated form containing the eight CRDs but lacking the N-terminal cysteine-rich domain and

the fibronectin type II repeat. Both forms of the receptor show high-affinity saturable binding of lutropin and thyrotropin. Binding to the full-length receptor is dependent on pH and ionic strength and is inhibited effectively by SO₄-4GalNAc but only partly by mannose. In contrast, binding to the truncated form of the receptor, which is also dependent on pH and ionic strength, is inhibited by mannose but not by SO₄-4GalNAc. The results are consistent with the presence of an SO₄-4GalNAc-specific binding site in the cysteine-rich domain of the mannose receptor but indicate that interactions between other sugars on the hormones and the CRDs are also important in hormone binding.

Key words: *N*-acetylgalactosamine sulphate, C-type lectin, lutropin, thyrotropin.

INTRODUCTION

The mannose receptor of macrophages and hepatic endothelial cells has a well-documented role in the clearance of pathogenic micro-organisms and harmful glycoproteins [1,2]. Glycoconjugates terminating in mannose, fucose or GlcNAc are recognized in a Ca²⁺-dependent manner by several of the eight C-type carbohydrate-recognition domains (CRDs) in the extracellular region of the receptor [3–5]. Recent studies have suggested a further function for the mannose receptor in the clearance of pituitary hormones such as lutropin (LH) and thyrotropin (TSH) [6,7]. These hormones must be removed from the blood after acting on their target cells, to prevent desensitization of the target cell receptors. Clearance of TSH and LH is mediated by a receptor on liver endothelial cells that recognizes unusual oligosaccharides terminating in sulphated *N*-acetylgalactosamine (SO₄-4GalNAc) that are present on the hormones [8]. Isolation and characterization of the SO₄-4GalNAc-specific receptor from rat liver showed that it is identical with the mannose receptor [6].

The finding that the mannose receptor is able to recognize SO₄-4GalNAc-terminated oligosaccharides was surprising because structure–function analysis of the mannose-specific CRDs of the receptor and other C-type lectins suggest that these do not bind galactose-containing ligands [2]. However, the mannose receptor is a multidomain protein and only some of its domains are needed for the recognition of mannose-terminated glycoconjugates. The N-terminal cysteine-rich domain, the fibronectin type II repeat and the first three C-type CRDs seem to have no role in binding mannose-terminated glycoconjugates, with CRDs 4–8 being sufficient to match the affinity of the whole receptor for such ligands [3,4]. Studies with a neoglycoprotein, consist-

ing of BSA conjugated to the trisaccharide SO₄-4GalNAcβ1,4GlcNAcβ1,2Manα, suggest that the mannose-binding and SO₄-4GalNAc-binding sites of the mannose receptor are distinct, with the SO₄-4GalNAc-binding site being localized to the N-terminal cysteine-rich domain [9]. No previous ligand-binding activity has been associated with the cysteine-rich domain, which is unique to the mannose receptor and three other receptors in the mannose receptor family, and shows no sequence similarity to other carbohydrate-binding proteins [10].

Although SO₄-4GalNAc-containing neoglycoproteins are useful as defined ligands in characterizing a binding site for SO₄-4GalNAc, they do not accurately reproduce the structure of a naturally occurring glycoprotein. The pituitary hormones LH and TSH each contain three sites for the attachment of N-linked oligosaccharides, which are often occupied by biantennary oligosaccharides terminating in SO₄-4GalNAcβ1,4GlcNAcβ1,2Manα [11,12]. However, hybrid oligosaccharides in which one arm terminates in SO₄-4GalNAcβ1,4GlcNAcβ1,2Manα whereas the other has terminal mannose are also found on these hormones. In addition, the structures often have fucose residues linked to the core GlcNAc [11,12]. Thus the possibility that pituitary hormone binding to the mannose receptor involves the specific binding of mannose, GlcNAc and/or fucose as well as of SO₄-4GalNAc needs to be considered.

This study describes experiments undertaken to characterize the binding of natural pituitary hormones to the mannose receptor. The results are consistent with the major SO₄-4GalNAc-binding site being localized to the cysteine-rich domain but indicate that additional interactions occur between the hormone oligosaccharides and other domains of the mannose receptor.

Abbreviations used: CRD, carbohydrate-recognition domain; LH, lutropin; Man-BSA, mannose-conjugated BSA; MMR1–8, macrophage mannose receptor containing only the eight CRDs without the N-terminal cysteine-rich domain and the fibronectin type II repeat; MMR-S, extracellular domain of the macrophage mannose receptor; TSH, thyrotropin.

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EXPERIMENTAL

Materials

Mannose₃₁-BSA was purchased from EY Laboratories. SO₄-4GalNAc was obtained from Dextra laboratories; other monosaccharides and bovine TSH were from Sigma. Na¹²⁵I was obtained from Amersham. Restriction enzymes and other DNA modification enzymes were from New England Biolabs. Nucleoside-free minimal essential medium, dialysed foetal calf serum and methotrexate were from Gibco Life Technologies. Bovine pituitaries were obtained from PelFreeze Biologicals.

Preparation and characterization of glycoprotein hormone ligands

LH was purified from bovine pituitaries by the method of Papkoff and Gan [13]. Oligosaccharides found on TSH and LH preparations were characterized by matrix-assisted laser desorption ionization time-of-flight MS after release from the proteins with peptide:N-glycosidase F and treatment with neuraminidase [14]. 2,5-Dihydroxybenzoic acid was used as the matrix and both positive-ion and negative-ion spectra were recorded on a Perceptive Biosystems Voyager Elite Mass Spectrometer. All structures previously identified on bovine LH and TSH were found [12]. No species with masses corresponding to high-mannose type oligosaccharides were seen (results not shown).

Expression and purification of mannose receptor

Two secreted forms of the mannose receptor were expressed in Chinese hamster ovary cells (see Figure 1). The human mannose receptor cDNA was modified to code for each of the two secreted versions of the receptor. Standard recombinant DNA techniques were used in the construction of plasmids. The region of the cDNA coding for the membrane anchor and the cytoplasmic tail was removed by cutting at the *Bsp*HI site at position 4210, followed by the addition of an *Xba*I linker to make a stop codon. The resulting cDNA coded for the entire extracellular domain of the receptor (MMR-S), ending at residue 1349. Further modification was necessary to create a version of the receptor containing only the eight CRDs without the N-terminal cysteine-rich domain and the fibronectin type II repeat (MMR1–8). The region of the cDNA coding for the signal sequence, the cysteine-rich domain and the fibronectin type II repeat was removed by cutting at the *Hind*III site at position 754, resulting in DNA coding for a receptor starting at residue 200 at the beginning of CRD-1 and ending at residue 1349 at the end of CRD-8. The portion of the cDNA coding for the eight CRDs was fused to codons specifying the dog preproinsulin signal sequence [4]. The preproinsulin signal sequence has previously been shown to direct the efficient secretion of other truncated versions of the mannose receptor [4,5]. Modified cDNA species were inserted into the vector pED between the *Xba*I and *Eco*RI sites following the region encoding the adenovirus major late promoter and preceding the region encoding the dihydrofolate reductase gene [15].

The resulting plasmids were transfected into DXB11 Chinese hamster ovary cells, which are deficient in dihydrofolate reductase, by using the calcium phosphate method. Transfectants were selected by growth in minimal essential α medium without nucleosides and supplemented with 10% (v/v) dialysed foetal calf serum. Amplification of protein expression was achieved by passaging cells into medium containing increasing concentrations (up to 0.5 μ M) of methotrexate, over several weeks. For protein production, cells were grown to confluence in medium containing 0.5 μ M methotrexate and the medium was collected. Additional

batches of medium were harvested every 3 days. Tris/HCl, pH 7.8, CaCl₂ and NaCl were added to the medium to give final concentrations of 20 mM, 10 mM and 0.5 M respectively. After centrifugation at 15300 *g* in a Beckman JA14 rotor for 15 min, the medium was loaded on a column of mannose-Sepharose [16] equilibrated in 20 mM Tris/HCl (pH 7.8)/10 mM CaCl₂/0.5 M NaCl (loading buffer). The column was washed with loading buffer before elution with 20 mM Tris/HCl (pH 7.8)/0.5 M NaCl/2 mM EDTA.

Solid-phase binding assays

Ligand binding assays with mannose receptor immobilized on polystyrene wells were performed in accordance with published procedures, except that the composition of buffers used was varied to test effects of ionic strength, pH and the presence of Ca²⁺ [4]. Test ligands were ¹²⁵I-Man₃₁-BSA (BSA conjugated with 31 mol of mannose/mol), ¹²⁵I-LH and ¹²⁵I-TSH, all iodinated by using the chloramine-T procedure [17]. For competition assays, values for *K_i*, corresponding to the concentration of monosaccharide that gave 50% inhibition of test ligand binding, were calculated with a non-linear least-squares-fitting program (SigmaPlot; Jandel Scientific). Iodinated reporter ligands were used at a concentration of 1 μ g/ml. For direct binding assays, radioiodinated ligand was diluted with non-radioactive ligand and serial 1:1 dilutions were prepared for incubation in the mannose-receptor-coated wells. Data were fitted to the following equation for saturable binding superimposed on a linearly increasing background of non-specific binding:

$$\text{Ligand bound} = \text{Bkg} + \text{Max}[L]/(K_d + [L]) + \text{Slope}[L] \quad (1)$$

where Bkg is the background binding, Max is the saturation level for specific binding, Slope is the linear increase in non-specific binding, [L] is the ligand concentration and *K_d* is the concentration of ligand at which half-maximal specific saturable binding is achieved. *K_d* values derived from experimental data were converted to molarities by taking 73 kDa as the molecular mass of Man₃₁-BSA and 28 kDa as the molecular mass of TSH and LH [18].

RESULTS

Pituitary hormones bind to mannose receptor lacking the cysteine-rich domain

To determine the relative contributions of the C-type CRDs and the cysteine-rich domain to the binding of pituitary hormones by the mannose receptor, it was necessary to produce appropriate forms of the receptor. Two secreted fragments of the mannose receptor, MMR-S and MMR1–8, were produced by over-expression in Chinese hamster ovary cells (Figure 1). Both expressed portions of the receptor were secreted efficiently into the medium and could be purified by affinity chromatography on mannose-Sepharose.

A solid-phase binding assay previously developed to study ligand binding to smaller expressed portions of the mannose receptor was used to study the binding of pituitary hormones to MMR-S and MMR1–8 immobilized on microtitre plates. Both MMR-S and MMR1–8 bound mannose-conjugated BSA (Man-BSA) in a saturable manner, with similar *K_d* values (Figure 2, top panel). *K_d* values derived from three independent assays expressed as means $\pm$ S.D. were 56 ± 7 nM for MMR-S and 45 ± 27 nM for MMR1–8. These results confirm earlier studies showing that the N-terminal cysteine-rich domain and the fibronectin type II repeat are not required for the binding of mannose-terminated glycoconjugates [4]. TSH and LH also showed saturable binding

Figure 1 Expression of soluble forms of the mannose receptor

Two portions of the mannose receptor expressed in Chinese hamster ovary cells are shown diagrammatically. The structure of the intact mannose receptor is summarized in the top panel. The tinted box represents the mannose receptor signal sequence and the filled box represents the transmembrane region. The open box represents the dog preproinsulin signal sequence. Amino acids present in each construct are indicated by the residue numbers from the intact receptor. Y and I shapes represent potential or actual attachment sites for sugar to asparagine and threonine respectively.

to both MMR-S and MMR1-8 (Figure 2, middle and bottom panels respectively). No significant difference could be detected between the strength of binding of MMR-S and that to MMR1-8, but LH bound more strongly to MMR-S than to MMR1-8. K_d values derived from three independent assays expressed as means $\pm$ S.D. for MMR-S were $K_d^{LH} = 180 \pm 80$ nM and $K_d^{TSH} = 500 \pm 250$ nM, and for MMR1-8 were $K_d^{LH} = 750 \pm 310$ nM and $K_d^{TSH} = 570 \pm 50$ nM. The K_d values obtained for hormone binding to each portion of the receptor were similar to the value obtained for the binding of LH to isolated hepatic endothelial cells [8]. These results are not inconsistent with the SO_4 -4GalNAc binding site being localized to the cysteine-rich domain of the mannose receptor [9] but they indicate that the CRDs of the receptor can also interact with pituitary hormones.

Inhibition studies showed that both unlabelled TSH and unlabelled LH were able to compete for the binding of ^{125}I -TSH to MMR-S or MMR1-8, indicating that TSH and LH bound at identical or overlapping sites on each of the receptor fragments (Figure 3). K_i values obtained from these assays were similar to those obtained in the direct binding assays, giving an independent measure of the strength of binding of LH and TSH to each fragment of the receptor. However, in this assay no significant difference could be detected between the strength of binding of either LH or TSH to MMR-S and that to MMR1-8.

An analysis of the sulphated oligosaccharides on the TSH and LH preparations (Figure 4) showed that a high proportion (40% for TSH, 60% for LH) were hybrid structures with one or two terminal mannose residues as well as a terminal SO_4 -4GalNAc residue. In addition, high proportions of the oligosaccharides (49% for TSH, 37% for LH) were core-fucosylated, particularly structures S1 and S2, which did not have any terminal mannose residues. It is therefore possible that binding of pituitary hormones to MMR1-8 could be accounted for by the binding of terminal mannose residues of hybrid oligo-

Figure 2 Binding of pituitary hormones to the mannose receptor

Results from direct binding assays are shown fitted to eqn. (1). All buffers used contained 25 mM Tris/HCl, pH 7.8, and 10 mM $CaCl_2$.

saccharides and/or core fucose residues. Therefore additional experiments to characterize the nature of pituitary hormone binding to MMR-S and MMR1-8 were undertaken.

Binding of pituitary hormones is dependent on ionic strength

Initial binding assays performed under the conditions of high salt concentration normally used for assaying the binding of Man-BSA by the mannose receptor showed only weak binding of TSH and LH to both MMR-S and MMR1-8, suggesting that the binding of glycoprotein hormones to the receptor was dependent on ionic strength. Figure 5 shows the effects of NaCl on the binding of ligands to both soluble forms of the receptor. The binding of both ^{125}I -TSH and ^{125}I -LH to each portion of the receptor was inhibited by NaCl, although increasing [NaCl] had less effect on the binding of hormone to MMR1-8 than to MMR-S. In contrast, NaCl up to 2.5 M had little effect on the binding of ^{125}I -Man-BSA to either MMR-S or MMR1-8.

The effect of NaCl on the affinity of binding of TSH and LH is shown in Table 1. Each ligand bound considerably less tightly to each portion of the receptor in the presence of 1.25 M NaCl than in the absence of NaCl; intermediate results were seen in the

Figure 3 Inhibition of TSH binding by LH

Top panel: inhibition of ^{125}I -TSH binding to MMR-S and MMR1-8 by LH. Lower panel: inhibition of ^{125}I -TSH binding to MMR-S and MMR1-8 by unlabelled TSH. All buffers used contained 25 mM Tris/HCl, pH 7.8, and 10 mM CaCl_2 . K_i values (means $\pm$ S.D.) determined from three independent experiments are as follows: MMR-S $K_i^{\text{LH}} = 470 \pm 330$ nM, $K_i^{\text{TSH}} = 250 \pm 90$ nM; MMR1-8 $K_i^{\text{LH}} = 1.06 \pm 0.09$ μM , $K_i^{\text{TSH}} = 400 \pm 30$ nM.

presence of 0.15 M NaCl. As expected from the inhibition studies, increasing the NaCl concentration did not affect the strength of binding of Man-BSA to either form of the receptor (results not shown).

The inhibition of the binding of pituitary hormones to MMR-S by NaCl is consistent with an electrostatic interaction between the cysteine-rich domain and the sulphate group of the terminal SO_4 -4GalNAc residues on the hormones. However, electrostatic interactions also seem to be involved in the binding of hormones to MMR1-8. The fact that the binding of hormones, unlike the binding of Man-BSA, to MMR1-8 is affected by increased ionic strength suggests that the interaction between the CRDs and the hormones is not simply due to terminal mannose residues on hybrid oligosaccharides binding to the receptor by the same mechanism that mannose residues on Man-BSA are bound.

Binding of pituitary hormones can be inhibited by SO_4 -4GalNAc or mannose

To gain information about the relative specificities of the hormone-binding sites on MMR-S and MMR1-8, inhibition experiments with monosaccharides were performed. Examples of inhibition curves for both MMR-S and MMR1-8 with ^{125}I -TSH, ^{125}I -LH or ^{125}I -Man-BSA as reporter ligand are shown in Figure

Figure 4 Oligosaccharide structures found on LH and TSH

Negative-ion matrix-assisted laser desorption ionization mass spectra of oligosaccharides released from LH and TSH gave masses consistent with structures S-1(A) to S-1(N) previously identified by Green and Baenziger [12]. The approximate amounts of each type of oligosaccharide are shown, expressed as a percentage of the total pool of sulphated oligosaccharides. Figures in parentheses indicate the percentage of structures with core fucose residues. Positive-ion spectra gave masses consistent with the same structures without sulphate residues, as well as a small percentage of unidentified structures, none of which had masses corresponding to high-mannose-type oligosaccharides (results not shown). Symbols: ●, Gal; ○, Man; ■, GlcNAc; □, GalNAc.

6; inhibition constants derived from these curves are shown in Table 2.

The binding of both hormones to MMR-S was strongly inhibited by SO_4 -4GalNAc but not by GalNAc, whereas SO_4 -4GalNAc had only a weak inhibitory effect on the binding of hormones to MMR1-8. These results are consistent with a specific SO_4 -4GalNAc-binding site being localized to the cysteine-rich domain [9]. SO_4 -6Gal was 5–15-fold less effective as an inhibitor of hormone binding to MMR-S than SO_4 -4GalNAc, indicating specificity for the sugar portion of the ligand and not just the sulphate group. However, PO_4 -6Gal was approximately 10-fold weaker as an inhibitor of hormone binding to MMR-S than SO_4 -6Gal, showing that there was also specificity for the sulphate group. Interestingly, in assays employing buffers containing the sulphonyl compounds Mes and 2-(*N*-cyclohexylamino)ethane-sulphonic acid, the binding of hormone to MMR-S was completely inhibited, presumably through competition for the SO_4 -4GalNAc-binding site.

The binding of hormones to MMR-S was weakly inhibited by mannose, but this inhibition was significant when compared with the complete lack of inhibition by GalNAc. This result suggests that although the binding of SO_4 -4GalNAc residues might be the major interaction between hormone oligosaccharides and the mannose receptor, terminal mannose residues of hybrid oligosaccharides or core fucose residues could also have a role in hormone binding.

Figure 5 Inhibition of binding of pituitary hormones by NaCl

^{125}I -TSH ($\circ$), ^{125}I -LH ($\bullet$) or ^{125}I -Man-BSA ($\blacksquare$) at $1\ \mu\text{g/ml}$ was incubated in receptor-coated wells with increasing concentrations of NaCl in 25 mM Tris/HCl (pH 7.8)/10 mM CaCl_2 .

Table 1 Effect of ionic strength on the affinity of hormone binding

Affinity constants were determined by using the direct binding assay. All buffers contained 25 mM Tris/HCl, pH 7.8, and 10 mM CaCl_2 .

NaCl concentration (M)	MMR-S		MMR1-8	
	$K_d^{\text{LH}}\ (\mu\text{M})$	$K_d^{\text{TSH}}\ (\mu\text{M})$	$K_d^{\text{LH}}\ (\mu\text{M})$	$K_d^{\text{TSH}}\ (\mu\text{M})$
0	0.39	0.14	1.4	0.43
0.15	1.4	0.43	2.8	0.93
1.25	7.6	26	5	1.6

Mannose was an effective inhibitor of binding of both TSH and LH to MMR1-8, indicating that terminal mannose residues of hybrid oligosaccharides or core fucose residues are likely to be involved in this interaction. Fucose also inhibited the binding of TSH and LH to MMR1-8 (results not shown). Because fucose and mannose are known to interact at the same sites [4], these results cannot distinguish between interactions of the receptor with terminal mannose residues or core fucose residues. However, the inhibition of hormone binding but not Man-BSA binding to MMR1-8 by PO_4 -6Gal and SO_4 -6Gal indicates that hormones do not bind by the same mechanism as Man-BSA. Inhibition by these charged sugars, like inhibition by NaCl, indicates that there is an electrostatic component in the binding of pituitary hormones to the mannose receptor CRDs.

Dependence of binding of pituitary hormones on Ca^{2+}

The binding of mannose/GlcNAc/fucose-terminated glycoconjugates to the mannose receptor is absolutely dependent on Ca^{2+} [19,20] but previous experiments have indicated that the binding of SO_4 -4GalNAc might not be strictly Ca^{2+} -dependent. Binding of the neoglycoprotein SO_4 -4GalNAc β 1,4GlcNAc β 1,2Man α -BSA to isolated liver cells is not decreased significantly in the presence of EDTA, but binding of this ligand to isolated mannose receptor is decreased by EDTA to approx.

60% of the level in the presence of Ca^{2+} [7,8]. These results are interpreted as indicating that the binding of SO_4 -4GalNAc is not dependent on Ca^{2+} but is enhanced in the presence of Ca^{2+} [7].

The Ca^{2+} dependence of pituitary hormone binding to the mannose receptor has not previously been analysed but it might be expected to have some dependence on Ca^{2+} if terminal mannose residues and core fucose residues of the hormones were involved in binding. However, quantitative analysis of the importance of Ca^{2+} to pituitary hormone binding proved difficult because variable results were produced. TSH is able to bind to both MMR-S and MMR1-8 in the presence of 10 mM EDTA, in contrast with Man-BSA, which does not show saturable binding to either fragment of the receptor in the presence of EDTA (Figure 7). These results indicate that the binding of TSH to the mannose receptor, like the binding of SO_4 -4GalNAc β 1,4GlcNAc β 1,2Man α -BSA, was not absolutely dependent on Ca^{2+} . When the results are compared with those obtained in the presence of 10 mM CaCl_2 , the binding of TSH to MMR1-8 was always enhanced in the presence of CaCl_2 , indicating that a component of the binding was Ca^{2+} dependent. Results for MMR-S were more variable: some assays showed little difference between binding of TSH in the presence of Ca^{2+} or EDTA, whereas others showed a slight enhancement of binding in the presence of Ca^{2+} . Ca^{2+} could enhance the binding of pituitary hormones to MMR-S either by allowing the in-

Figure 6 Inhibition of binding of pituitary hormones by monosaccharides

The competition binding assay was performed with ^{125}I -TSH, ^{125}I -LH or ^{125}I -Man-BSA as reporter ligand. Buffers used contained 25 mM Tris/HCl, pH 7.8, and 10 mM CaCl_2 except for inhibition experiments with $\text{PO}_4\text{-6Gal}$, for which the CaCl_2 concentration was decreased to 1 mM. Each curve is representative of at least two separate experiments. Symbols: $\square$, GalNAc; $\bullet$, Man; $\circ$, $\text{SO}_4\text{-6Gal}$; $\blacksquare$, $\text{SO}_4\text{-4GalNAc}$; $\triangle$, $\text{PO}_4\text{-6Gal}$.

teraction of mannose and fucose residues with the CRDs or by an indirect structural effect on the cysteine-rich domain.

One factor that makes interpretation of these results difficult is that the binding of pituitary hormones is dependent on ionic strength and buffers containing either 10 mM EDTA or 10 mM CaCl_2 have different ionic strengths. The second factor that needs to be taken into account is that the CRDs of the receptor, if not the cysteine-rich domain, require Ca^{2+} to maintain their

folded conformation. Thus the receptor is denatured in the presence of EDTA. This fact might explain why saturable binding of LH to either MMR-S or MMR1-8 could not be quantified in the presence of 10 mM EDTA. In assays with LH in the presence of EDTA, there was a large increase in the amount of non-specific binding, presumably owing to interactions of charged sugars with the denatured receptor (results not shown).

Table 2 Inhibition of LH and TSH binding by monosaccharides

Inhibition constants were determined by using the competition binding assay with either ^{125}I -TSH or ^{125}I -LH as reporter ligand. The values given are derived from curves shown in Figure 6. Abbreviation: n.d., not determined.

Monosaccharide	MMR-S		MMR1-8	
	K_i^{TSH} (mM)	K_i^{LH} (mM)	K_i^{TSH} (mM)	K_i^{LH} (mM)
Man	53	67	1.0	1.5
$\text{SO}_4\text{-4GalNAc}$	0.13	0.18	56	150
$\text{SO}_4\text{-6Gal}$	0.78	2.7	15	41
$\text{PO}_4\text{-6Gal}$	n.d.	31	n.d.	11

Figure 7 Dependence of binding of pituitary hormones on Ca^{2+}

Data from direct binding assays are shown fitted to eqn. (1). Buffers used contained 25 mM Tris/HCl, pH 7.8, and either 10 mM CaCl_2 or 10 mM EDTA.

Dependence of binding of pituitary hormones on pH

The binding of both mannose/GlcNAc/fucose-terminated glycoconjugates and $\text{SO}_4\text{-4GalNAc}/\beta\text{1,4GlcNAc}/\beta\text{1,2Man}\alpha\text{-BSA}$ to the mannose receptor is pH-dependent, reflecting the need for the receptor to release its ligands in the acid environment of the endosomes [8,19]. Because pituitary hormones are endocytosed by the mannose receptor, it is not surprising that the binding of LH and TSH to the receptor was also dependent on pH (Figure 8). The binding of LH and TSH to both MMR1-8 and MMR-

S was maximal at pH 6–7 and decreased sharply below pH 5.5. There was little difference between the abilities of MMR-S and MMR1-8 to release the hormones at low pH.

The pH dependence of the release of Man-BSA from the receptor CRDs has previously been shown to be affected by Ca^{2+} , with ligand-binding being resistant to pH changes at concentrations of Ca^{2+} above 1 mM [20]. The pH-dependent hormone-binding assays shown were performed at 1 mM Ca^{2+} . There were some differences in the curves at 10 mM Ca^{2+} , with

Figure 8 Dependence of binding of pituitary hormones on pH

^{125}I -TSH (●,○) or ^{125}I -LH (■,□) was incubated in receptor-coated wells with 25 mM sodium acetate/1 mM CaCl_2 (pH 4.0–7.0) or 25 mM Tris/HCl/10 mM CaCl_2 (pH 7.0–10.0). At pH 7.0, values obtained with both acetate buffer (●,■) and Tris buffer (○,□) are shown. Washing and blocking buffers contained 25 mM Tris/HCl, pH 7.8, and 1 mM CaCl_2 .

20–30% more of each ligand being retained by both MMR-S and MMR1-8 at pH 5.0 (results not shown). However, changing the pH had little effect on the binding of Man-BSA to either MMR-S or MMR1-8 at 10 mM Ca^{2+} , with maximal binding being maintained down to pH 4.5 (results not shown). Thus Ca^{2+} had a much greater effect on the pH-dependent release of Man-BSA than that of the hormones. These results provide further evidence that hormones do not bind to the CRDs of MMR1-8 by exactly the same mechanism as Man-BSA.

DISCUSSION

The demonstration of high-affinity binding of pituitary hormones to a truncated form of the mannose receptor lacking the cysteine-rich domain and the fibronectin type II repeat indicates that the CRDs of the mannose receptor can interact with the oligosaccharides of the hormones. This result was unexpected because the binding of pituitary hormones was thought to be largely due to the recognition of terminal SO_4 -4GalNAc residues and a binding site for SO_4 -4GalNAc has been localized to the cysteine-rich

domain of the mannose receptor [9]. However, the binding of hormones to the truncated form of the receptor is not inhibited by SO_4 -4GalNAc, indicating that there is no specific interaction of the receptor CRDs with terminal SO_4 -4GalNAc residues of the hormones. Although these experiments cannot rule out the participation of the fibronectin type II repeat in hormone binding, the results are consistent with the cysteine-rich domain containing an SO_4 -4GalNAc-binding site.

The most likely explanation for the binding of hormones to the mannose receptor in the absence of the cysteine-rich domain is that the CRDs interact with terminal mannose residues or core fucose residues. Consistent with this hypothesis are the findings that approx. 50% of the oligosaccharides of TSH and LH contain such residues and that the binding of hormones to the CRDs is inhibited by mannose and fucose. However, other characteristics of the binding, including partial inhibition by NaCl and some charged sugars, indicate that there is also an electrostatic component to the interaction. Hormone binding is also not fully abolished in the absence of Ca^{2+} . Together, these findings suggest that, as well as interaction with mannose or fucose residues, there is a relatively non-specific interaction between charged sugars and the CRDs of the mannose receptor that contributes to hormone binding. There is precedent for the Ca^{2+} -independent binding of charged residues of sulphatides to the CRDs of the selectins [21,22]. However, the physiological relevance of the binding of sulphatides to selectins has not been established.

The finding that hormones can interact with the CRDs of the mannose receptor raises the question of how important interactions with the CRDs are in the binding of hormones to the full-length receptor. Partial inhibition of the binding of hormones to the full-length receptor by mannose indicates that some interaction between mannose and/or fucose residues does occur even when the SO_4 -4GalNAc-binding site is present. However, these interactions seem to be weak in comparison with the binding of SO_4 -4GalNAc.

Many oligosaccharides of the hormones have both terminal mannose residues and terminal SO_4 -4GalNAc residues; others have terminal SO_4 -4GalNAc residues and core fucose residues, and a few have all three types of sugars. Thus, apart from weak non-specific interactions between charged sugars and the CRDs, there are three potential points of interaction between each oligosaccharide and the mannose receptor. Each hormone molecule also has three glycosylation sites, each of which could be occupied by a different oligosaccharide. Therefore the possibility exists that one oligosaccharide could bind through both an SO_4 -4GalNAc residue and a mannose or fucose residue. Another possibility is that more than one oligosaccharide from each hormone molecule could interact with the receptor through different sites. Whether or not these interactions occur must depend on the numbers of terminal mannose or core fucose residues relative to the numbers of terminal SO_4 -4GalNAc residues as well as on the disposition of the oligosaccharides relative to the different binding sites of the receptor. Evidence presented here suggests that the recognition of oligosaccharides at different sites on the receptor does occur in some fraction of the hormone molecules bound. However, structural information on the conformation of both the receptor and the oligosaccharides will be required for a determination of the relative importance of multiple interactions between the two.

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REFERENCES

- 1 Drickamer, K. and Taylor, M. E. (1993) *Annu. Rev. Cell Biol.* **9**, 237–264
- 2 Weis, W. I., Taylor, M. E. and Drickamer, K. (1998) *Immunol. Rev.* **163**, 19–34
- 3 Taylor, M. E., Conary, J. T., Lennarz, M. R., Stahl, P. D. and Drickamer, K. (1990) *J. Biol. Chem.* **265**, 12156–12162
- 4 Taylor, M. E., Bezouska, K. and Drickamer, K. (1992) *J. Biol. Chem.* **267**, 1719–1726
- 5 Taylor, M. E. and Drickamer, K. (1993) *J. Biol. Chem.* **268**, 399–404
- 6 Fiete, D. and Baenziger, J. U. (1997) *J. Biol. Chem.* **272**, 14629–14637
- 7 Fiete, D., Beranek, M. C. and Baenziger, J. U. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 11256–11261
- 8 Fiete, D., Srivastava, V., Hindsgaul, O. and Baenziger, J. U. (1991) *Cell* **67**, 1103–1110
- 9 Fiete, D., Beranek, M. C. and Baenziger, J. U. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 2089–2093
- 10 Taylor, M. E. (1997) *Glycobiology* **7** (3), v–viii
- 11 Green, E. D. and Baenziger, J. U. (1987) *J. Biol. Chem.* **262**, 25–35
- 12 Green, E. D. and Baenziger, J. U. (1987) *J. Biol. Chem.* **262**, 36–44
- 13 Papkoff, H. and Gan, J. (1970) *Arch. Biochem. Biophys.* **136**, 522–528
- 14 Harvey, D. J., Rudd, P. M., Bateman, R. H., Bordoli, R. S., Howes, J. B. and Vickers, R. G. (1994) *Org. Mass Spectrom.* **27**, 753–766
- 15 Kaufman, R. J., Davies, M. V., Wasley, L. C. and Michnick, D. (1991) *Nucleic Acids Res.* **19**, 4485–4490
- 16 Fornstedt, N. and Porath, J. (1975) *FEBS Lett.* **57**, 187–191
- 17 Greenwood, F. C., Hunter, W. M. and Glover, J. S. (1963) *Biochem. J.* **89**, 114–123
- 18 Liao, T.-H. and Pierce, J. G. (1970) *J. Biol. Chem.* **245**, 3275–3281
- 19 Lennarz, M. R., Cole, F. S., Shepherd, V. L., Wileman, T. E. and Stahl, P. D. (1987) *J. Biol. Chem.* **262**, 9942–9944
- 20 Mullin, N. P., Hall, K. T. and Taylor, M. E. (1994) *J. Biol. Chem.* **269**, 28405–284134
- 21 Arruffo, A., Kolanus, W., Walz, G., Fredman, P. and Seed, B. (1991) *Cell* **67**, 35–44
- 22 Needham, L. K. and Schnaar, R. L. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1359–1363

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