Pure & Appl. Chem., Vol. 63, No. 4, pp. 499–506, 1991.
Printed in Great Britain.

ADONIS 2069222091000637

Topography of binding sites of animal lectins: ligands' view

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Abstract - Synthetic and semisynthetic carbohydrates were used to probe sugar combining sites of animal lectins. For example, using derivatives of monosaccharides, it was determined that the 3-OH and 4-OH groups are absolutely essential and the 6-OH group was dispensable for both mammalian and chicken hepatic lectins. When the glycosyl units are clustered into a di- or tri-branched structure, the binding affinity increased dramatically. This phenomenon is less pronounced when the lectin is "solubilized" with detergent. Rat and chicken hepatic lectins appear to have a similar geometric dimension of binding sites. The optimal binding appears to require terminal sugar residues to be separated by ca. 1.5–2.5 nm. More exact probing of rat hepatic lectin topography was performed with three derivatives of a tri-branched glycopeptides specifically derivatized with a photoactivatable group at the different terminally exposed Gal residues. The results of photoaffinity labelling showed that the rat hepatic lectin binds the tribranched oligosaccharides with a specific steric orientation.

INTRODUCTION

Animal lectins can be classified into two types: i) C-type--those requiring calcium for binding; ii) S-type--those requiring SH-reagents for binding (ref. 1). More C-type lectins are known than S-type lectins at this juncture, and there are C-type lectins specific for Gal(Note a)/GalNAc, GlcNAc, Man, L-Fuc, and Man-6-phosphate. Many of the C-type lectins are membrane-bound, made of multi-subunits, and show binding specificity that discriminates branched structures of oligosaccharides (ref. 2-4).

To understand the exact mechanism of the carbohydrate-protein interaction, it is desirable to use synthetic or semisynthetic compounds, because the naturally derived oligosaccharides have limited variations. For example, the fact that the hepatic lectins recognize only the terminal sugar residues in glycoproteins can be readily shown by means of neoglycoproteins containing only monosaccharide derivatives, but this concept can not be established easily if one uses only naturally occurring oligosaccharides and their derivatives. For most of the C-type animal lectins, it is difficult to obtain soluble fragment amenable to the conventional protein techniques or to obtain crystals for X-ray crystallography. Thus, at the present, probing of binding sites must rely heavily on modification of the structures of the carbohydrate ligands.

HEPATIC LECTINS

1. Mammalian hepatic lectins

That mammalian hepatic lectins recognize only the terminal sugar residues (Gal) in desialylated serum type glycoproteins was unambiguously proven by the use of neoglycoproteins containing only monosaccharide derivatives

Note a: Unless otherwise specified, all sugars are of D-configuration and in pyranose form.

(ref. 3, 4). Bovine serum albumin, BSA (Note b), modified only with thioglycosides of Gal or GalNAc showed binding potency equal or superior to the naturally derived compounds (ref. 5, 6). Structural requirements for binding were initially determined using a number of neoglycoproteins modified with Gal derivatives, variations being on the hydroxyl groups of the parent sugar. For example, it was readily established that the 6-OH was dispensable for binding because Fuc-BSA was bound as well as Gal-BSA. Similarly, because GalNAc-BSA was bound more tightly than Gal-BSA, the 2-NAc group clearly enhances the binding.

It became apparent, however, that the binding of a sugar residue by the mammalian hepatic lectins requires more than one functional group of the sugar residue. To examine contribution of each of the functional groups on a target sugar residue, synthetic derivatives of GalN, in which one or more functional groups are modified, were used (ref. 7, 8). Binding potency of these compounds is measured by inhibition assay, rather than direct binding because many of them are not readily radiolabelled. In the inhibition assay, binding of a radiolabelled reference compound (e.g., ^{125}I-asialoorosomucoid for mammalian hepatic lectin) is competed with an inhibitor which are serially diluted to generate an inhibition curve, from which binding potency can be judged by the inhibitor concentration required for 50% inbhibition of the labelled ligand. TABLE 1 shows that bulky groups (benzoyl or phthalimido) on 2-amino group are detrimental to binding.

TABLE 1. Effect of N-Acyl group of GalN on binding potency (ref. 7).

Aglycon	N-Acyl Group	Relative Potency[a]
OCH_2CH_2OH	$COCH_3$	1
OCH_2CH_2OH	Propanoyl	1
OCH_2CH_2OH	Benzoyl	0.25
OCH_2CH_2OH	Phthaloyl	0.025
$O(CH_2)_6NH_2$	$COCH_3$	3.3
$O(CH_2)_6NH_2$	$COCF_3$	1

a. Binding to rat hepatocytes.

Similarly, the essentiality of the hydroxyl groups at different carbons was tested by methylating the OH-group or replacing it with a fluorine atom or a hydrogen atom. Clearly, the OH-groups at C-3 and C-4 are quite important in binding, but 6-OH group is not required in binding by this lectin.

TABLE 2. Effect of modification of OH-groups in GalNAc (ref. 7,8).

Aglycon	C-3	C-4	C-6	Relative potency[a]
Allyl α	OH	OH	OH	1
Allyl α	OH	OH	OMe	2.5
Allyl α	OMe	OH	OH	0.003
Allyl α	OH	OMe	OMe	0.007
Allyl β	OH	OH	OH	1.7
Allyl β	OH	F	OH	<0.2
Allyl β	H	OH	OH	<0.001
Allyl β	OH	H	OH	<0.001

a. Binding by isolated rat hepatocytes.

Note b: Abbreviations used are: BSA,. bovine serum albumin; nOe, nuclear Öberhauser effect; HSEA, hard-sphere exo-anomeric effect; RHL, rat hepatic lectin; CRD, carbohydrate-recongnition domain.

TABLE 3. Inter-Gal distance of Gal(1,4)-Tri and Gal(1,3)-Tri (ref. 10).

Inter-Gal distance (nm)				
	Gal(1,4)-Tri		Gal(1,3)-Tri	
Conforma -tion	g-t[a]	g-g[b]	g-t	g-g
A	2.5	2.4	2.4	2.4
B	3.1	2.8	2.9	2.7
C	1.5	1.6	1.9	1.9

a. gauche-trans orientation of the H5-C5 and C6-O bonds of 6-linked Man.
b. gauche-gauche orientation of the H5-C5 and C6-O bonds of 6-linked Man.

Although hepatic lectins require only the terminal sugar residues in the ligands they bind, it was shown that as the number of sugar residues increased linearly, the inhibitory potency increased nearly logarithmically (ref. 2-4). It is also noted that branched oligosaccharides are bound much more tightly than the monosaccharides or single chain oligosaccharides. This is termed "cluster effect", and is conceptualized as simultaneous binding of more than one sugar residues by the mammalian lectins. The first experimental test of this concept was with a series of relatively simple cluster glycosides based on tris(hydroxymethyl)aminomethane (ref. 9). A combination of nOe measurement and HSEA modeling revealed that the best desialylated tri-branched glycopeptides, in its minimum energy conformation structures, orient Gal residues in such a way as to have separation of 1.5 nm -- 2.5 nm between the C-4's (Fig. 1, Table 3, ref. 10).

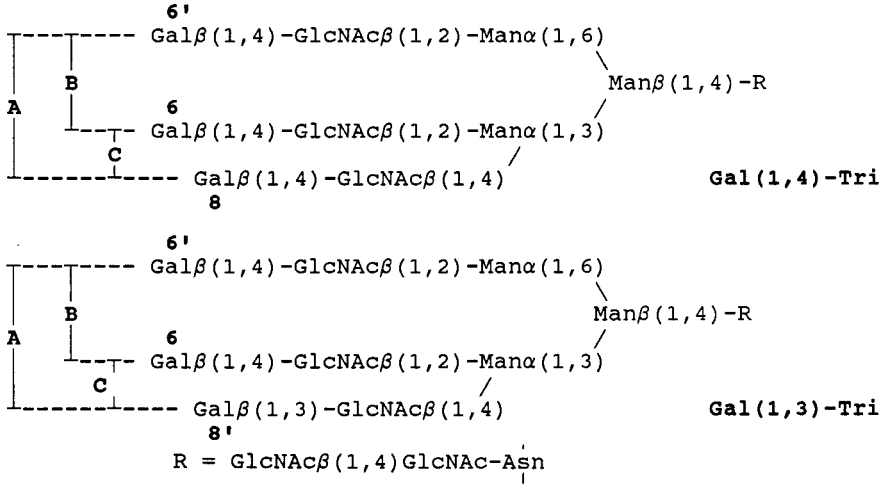

Fig. 1. Gal(1,4)-Tri and Gal(1,3)-Tri structures from bovine fetuin. The bold-faced numerals by the Gal residues are used to distinguish these residues.

The rather stringent requirement of the placement of the Gal residues for optimal binding was demonstrated by binding of a variant tri-branched oligosaccharide from bovine fetuin (Fig. 1), in which one of the terminal Gal-GlcNAc segments is linked β1-3 [Gal(1,3)-Tri] rather than β1-4 [Gal(1,4)-Tri]. Gal(1,3)-Tri was bound more than 100-fold weaker than Gal(1,4)-Tri (ref. 11), and it is interpreted as the difference in the inter-Gal distance between these tri-branched oligosaccharides, as shown in Fig. 1 and TABLE 3. The importance of the Gal-GlcNAc branch in question will be demonstrated again in the results of photoaffinity labelling of rat hepatocytes.

Based on these results, it became obvious that the simplistic cluster glycosides based on tris(hydroxymethyl)aminomethane would not provide such inter-Gal distances. By extending the linking arm between the terminal Gal and the tris(hydroxymethyl)aminomethane, binding affinity improved considerably. Later, branched structures consisting of amino acids and 6-aminohexyl glycosides were devised and they were shown to have similar

TABLE 4. Binding affinity of GalNAc-containing cluster glycosides.

Cluster glycosides	Concentration for 50% Inhibition (nM)	
	Soluble receptor	Isolated hepatocytes
GalNAcAH	5000	4000
$NAcY(GalNAcAH)_1$	2400	10000
$NAcYDG_2(GalNAcAH)_2$	30	3
$YEE(GalNAcAH)_2$	50	3
$YEE(GalNAcAH)_3$	4	0.2

pattern of binding enhancement with increasing number of branches (ref. 12, 13), as found in the natural branched oligosaccharides. Bi- and tri-branched cluster glycosides (Fig. 2) are quite potent, I_{50} values being in the nM range (TABLE 4).

Determination of the binding stoichiometry of the subunits of rat hepatic lectin was possible only with small and tight-binding ligands such as $YEE(GalNAcAH)_2$ and $YEE(GalNAcAH)_3$, which also can be radiolabelled with iodine. The results of stoichiometry determination with the Hummel-Dreyer method revealed that each subunits can bind two GalNAc residues (ref. 14).

Another approach to the topographic mapping of the mammalian hepatic lectins is to perform photoaffinity labelling. Photoactivatable groups were introduced into the C-6 of the terminal Gal residues of bovine fetuin tribranched glycopeptides by the scheme depicted in Fig. 3 (ref. 15, 16). Non-equivalence of the subunits by such labelling is demonstrated by photoaffinity as well as other labelling techniques such as GalNAc-modified lactoperoxidase-catalyzed iodination with or without digitonin. Probing the

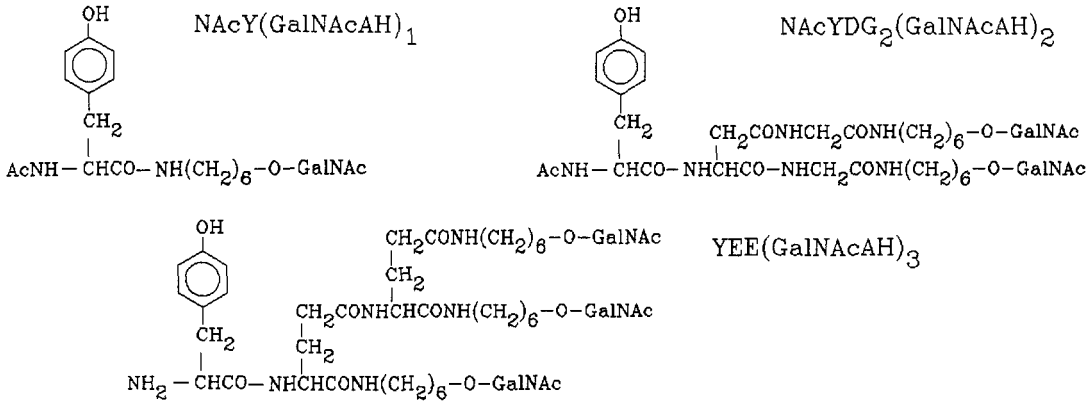

Fig. 2. Some GalNAc-containing synthetic cluster ligands.

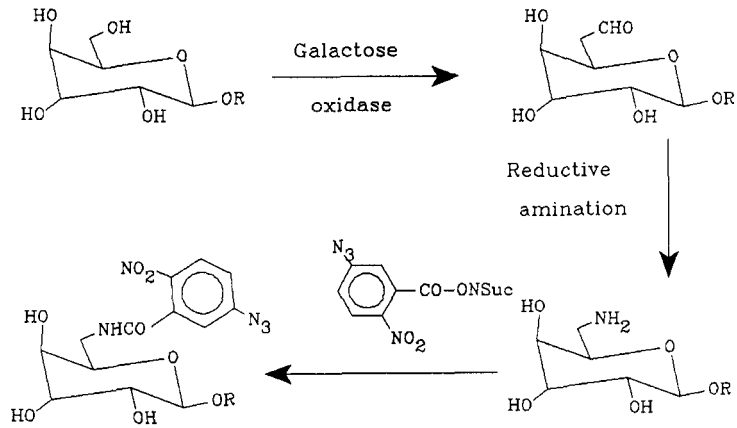

Fig. 3. Modification of galactosyl residues to acquire photoactivatable group (ref. 15, 16).

stereospecificity of ligand binding was achieved by preparing three different photoactivatable ligands from Gal(1,4)-Tri glycopeptide from bovine fetuin, in which each of the three Gal residue is specifically derivatized with 2-nitro-5-azido-benzoyl group (ref. 16, 17).

The results revealed that when the photoactivatable group is on the Gal residue **8**, only the RHL-2/3 subunits (the minor subunits, 50 and 60 kDa, respectively) are labelled, whereas when the photoactivatable group is on either Gal residues **6** or **6'**, the labelling is on the RHL-1 subunit (the major subunit, 43 kDa). If the three Galβ(1,4)GlcNAc groups of the ligand bound indiscriminately to the three Gal-combining sites of the lectin, random labelling of subunits would have occurred by each isomeric photoaffinity probe. The totally stereospecific labelling pattern observed indicates that the receptor subunits are organized into a unique geometric alignment, and the underlying core saccharides of the ligand orients the Galβ(1,4)-residues correctly to provide a precise fit. The unique behavior of Gal residue **8** in the binding is also reflected in the fact that Gal(1,3)-Tri (Fig. 2) is bound ca. 100-fold weaker than Gal(1,4)-Tri (ref. 11).

2. Chicken hepatic lectin

Chicken hepatic lectin, with respect to the protein construct, has a considerable homology with the mammalian hepatic lectin, as shown in Fig. 4 below (ref. 1). As to the amino acid sequence, there is ca. 40% homology in the carbohydrate-recognizing domains (CRD), which are located on the C-terminal side. Both lectins appear to form stable hexamers in Triton-solubilized state. Both lectins have a small binding site, the area of recognition being limited to the terminal monosaccharide region.

In addition, the chicken and mammalian hepatic lectins share some similarities in the binding characteristics at the monosaccharide level (ref. 18, 19). They are: (1) Large aglycons are permitted; (2) C-2 equatorial NAc-group enhances the binding, and the effects of the different N-acyl groups are also similar (TABLE 5); (3) Large substituents are allowed at C-6, and 6-OH is dispensable; and (4) Both 3-OH and 4-OH are required (ref. 20). The fact that large substituents are allowed at both C-1 and C-6 suggests that the binding site of both lectins is most likely a trough rather than a pocket.

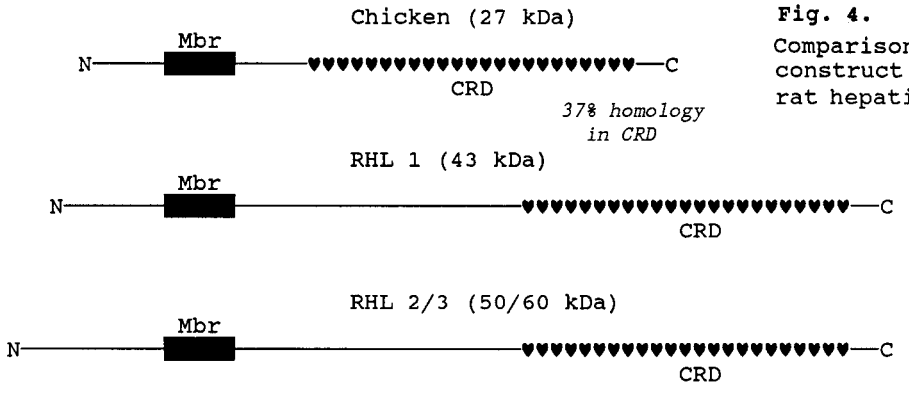

Fig. 4.
Comparison of molecular construct of chicken and rat hepatic lectins.

TABLE 5. Effect of N-Acyl group of GlcN on binding potency (ref. 19).

Aglycon	N-Acyl Group	Relative Potency[a]
Allyl	None	ca. 0.005
Allyl	COCHO	0.56
Allyl	$COCH_3$	1.00
Allyl	Propanoyl	0.71
Allyl	Butanoyl	0.43
Allyl	Isobutanoyl	0.37
Allyl	Benzoyl	0.20
Allyl	Phthaloyl	<0.33

a. Binding by chicken hepatocytes.

However, there are a number of differences between them (TABLE 2 and TABLE 6). They are (1) Chicken lectin requires an equatorial 4-OH, while the mammalian lectin requires axial 4-OH; (2) The chicken lectin prefers a negatively charged aglycon (e.g., 1-O-phosphate); (3) A negative group at C-6 (e.g., 6-O-phosphate or uronic acid) can be tolerated by the chicken lectin, but not by the mammalian lectin.

Like mammalian hepatic lectin, the chicken hepatic lectin also shows higher binding affinities towards branched structures. This is demonstrated by the fact that amino-acid based synthetic cluster glycosides of GlcNAc analogous to those shown in Fig. 2 are bound by the chicken hepatic lectin with as great an avidity as the corresponding cluster glycosides of GalNAc are bound by the mammalian hepatic lectins (ref. 13, 18). Moreover, the stringent requirement for optimal placement of GlcNAc residues is clearly shown by the di-branched compounds differing in the linker length and hence the inter-GlcNAc distance (TABLE 7). NAcYD(G-GlcNAcAH)$_2$ appears to have the optimal arrangement of the sugar residues compared to others without glycine [NAcYD(GlcNAcAH)$_2$] or with an additional glycine [NAcYD(G-G-GlcNAcAH)$_2$]. The maximal sugar separation of ca. 3 nm in this type of cluster glycoside was also the optimal separation for the mammalian hepatic lectin.

By using molecular models, we have determined the inter-GlcNAc distances in a desialylated, degalactosylated tri-branched structure shown in Fig. 5 (ref. 18). The inter-GlcNAc distances are not too different between the different energy minima conformations resulting from two possible torsion angles along the $\alpha(1,6)$-glycosidic bond (TABLE 8). As expected, the inter-GlcNAc distances are shorter than the inter-Gal distances in the Gal-terminated structure shown in Fig. 1.

TABLE 6. Effect of modification of OH-groups in GlcNAc (ref. 19)

Aglycon	C-3	C-4	C-6	I_{50} (mM)[a]
Allyl β	OH	OH	OH	0.18
Allyl β	H	OH	OH	20
Allyl β	OH	H	OH	N.I.
TFAAH[b] β	OH	OH	H	0.28
TFAAH β	OH	OH	None	1.0
Allyl α	OH	OH	OH	0.34
Allyl α	OH	F	COOH	1.1
Allyl α	OH	OH	COOMe	1.1
UDP	OH	OH	OH	0.027

a. Binding by isolated chicken hepatocytes.
b. $CF_3CONH(CH_2)_6$-O-

TABLE 7. Binding of synthetic cluster glycosides of GlcNAc by chicken hepatocytes (ref. 18).

Compounds	I_{50} (μM)	Inter-GlcNAc distance[a]
NAcYD(GlcNAcAH)$_1$	500	-
NAcYD(GlcNAcAH)$_2$	15	2.7
NAcYD(G-GlcNAcAH)$_2$	9	3.3
NAcYD(G-G-GlcNAcAH)$_2$	28	4.0
YEE(GlcNAcAH)$_3$	0.17	2.7 3.3, 3.4

a. In nm. The numbers are those of maximum separation.

Since the results shown in TABLE 7 suggest that the inter-binding site distances of the chicken hepatic lectin is similar to those of the mammalian hepatic lectin, it is not surprising that the truncated tri-branched structure with exposed GlcNAc (Fig. 5) had an I_{50} value (12 μM) much higher than the corresponding value for the binding by mammalian hepatocytes of Gal-terminated parent glycopeptide (ca. 10 nM). There may exist in nature some oligosaccharide structures in which GlcNAc residues are placed in positions further out than shown in Fig. 5. In reality, however, the "endogenous" ligands for the chicken hepatic lectin are yet to be identified.

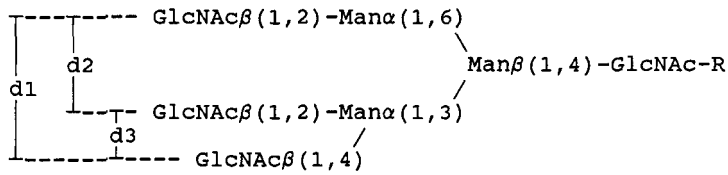

Fig. 5. Diagram of tri-branched GlcNAc-exposed oligosaccharide.

TABLE 8. Inter-GlcNAc distances in the desialylated, degalactosylated tri-branched glycopeptide (ref. 18).

	Inter GlcNAc distance (nm)			
minima	d1	d2	d3	relative energy[d]
1[a]	1.6	1.8	0.8	0
2[b]	1.5	1.5	0.8	1.6
3[c]	1.5	1.7	0.8	4.2

a. $\omega = -10°$, $\phi_6 = -60°$, and $\psi_6 = 190°$.
b. $\omega = 180°$, $\phi_6 = -50°$, and $\psi_6 = 120°$.
c. $\omega = 180°$, $\phi_6 = -50°$, and $\psi_6 = 190°$.
d. kcal/mol.

MACROPHAGE LECTIN

Rabbit alveolar macrophages contain a very active mannose-specific lectin (ref. 20, 21), which also belongs to the C-type. The characteristics of this lectin are in many ways similar to the above-mentioned hepatic lectins. It requires calcium for binding, and it shows a distinctive cluster effect as described above.

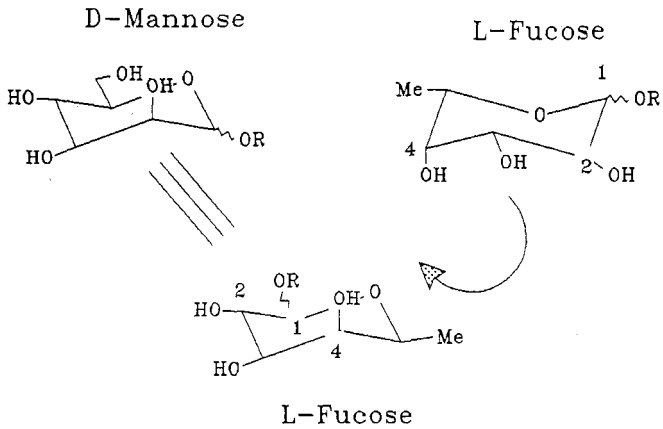

Fig. 6. Illustration of stereochemical similarities of mannose and L-fucose. The numerals denote the carbon numbers of L-fucose.

An intriguing steric requirement for this lectin is that it binds L-fucose-ligands as well as mannose-ligands. Although this seems confusing, but when the structures of Man and L-Fuc are compared, it becomes clear why the same lectin binds both of these apparently different sugars. Fig. 6 shows the similarities of these two sugars at the carbons 2, 3, and 4. It also shows that the unimportance of the exact nature of the C-1 and the C-6 substituents in these sugars for binding activity. Since this lectin binds L-Fuc-BSA as well as Man-BSA, it also attests to the general crevice-like construct of the binding trough frequently found in the C-type lectins, in which exist open space both at the C1 and C6 sides of the trough.

HUMAN SPLEEN GALAPTIN

Human spleen galaptin, though similar to the mammalian hepatic lectins in its ability to bind galactose, belongs to the S-type lectin (ref. 1), and does not require calcium for binding. The characteristics of this lectin which distinguish from the mammalian hepatic lectin are that it can be obtained in

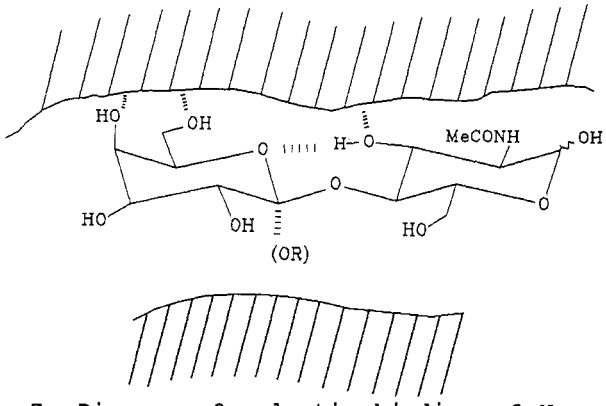

Fig. 7. Diagram of galaptin binding of N-acetyl-lactosamine. The (OR) indicates an alternative bulky α-aglycon which may hydrophobically interacts with the lectin molecule.

soluble form (i.e., not membrane-bound) and it recognizes more than the terminal sugar residue. There are many similar lectins from different tissues of different animals. The common structural element of their target sugar seems to be N-acetyl-lactosamine (Galβ1-4GlcNAc, LacNAc), as shown in Fig 7 (ref. 22).

The 4- and 6-OH groups of the Gal terminal is considered essential, but the 2-OH and 3-OH can be substituted by other sugars including sialic acids. Thus galaptin can bind internal N-acetyl-lactosamine unit. The loss of 3-OH of GlcNAc residue causes ca. 50-fold decrease in affinity, although the conformation of the disaccharide as determined by nOe measurement is similar to LacNAc. This suggests that this OH-group may play a role in hydrogen-bonding to the lectin molecule. The use of synthetic ligands also point out that the lectin can interact with an axial aglycon by hydrophobic attraction.

Acknowledgement

The authors are grateful for the National Institutes of Health Research Grant DK 09970 and the National Institutes of Health Fellowship GM 13013 Bi-4 (KR).

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