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Interaction of Synthetic Glycopeptides Carrying Clusters of *O*-Glycosidic Disaccharide Chains (β-D-Gal(1-3)-α-D-GalNAc) with β-D-Galactose-binding Lectins

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The specificity of several D-galactose-binding lectins including *Agaricus bisporus* (mushroom), *Arachis hypogaea* (peanut), *Bauhinia purpurea* and *Vicia graminea* has been examined by inhibition of hemagglutination using a series of synthetic oligopeptides representing the N-terminal end of glycophorin A from N and M individuals, all carrying one or several disaccharide chains, D-Galβ1-3-D-GalNAcα- (T-hapten).

Peanut lectin was inhibited by T-hapten-carrying glycopeptides, but the presence of a cluster of disaccharide chains had no effect on the lectin specificity. On the contrary, both *Agaricus bisporus* and *Bauhinia purpurea* lectins exhibited an enhanced reactivity with polyglycosylated peptides suggesting that their combining site might include two proximal galactose residues.

All synthetic glycopeptides inhibiting *Vicia graminea* lectin carry a cluster of T-disaccharide chains and the leucine residue at the N-terminal end, and the presence of a Glu residue at position 5 slightly increased the lectin activity. It is concluded that the binding of *Vicia graminea* is dependent upon a specific spatial conformation including a cluster of T-hapten chains in close vicinity of a hydrophobic surface represented by an appropriate N-terminal amino acid residue.

Glycophorin A, the best characterized red cell membrane glycoprotein, is a single polypeptide chain of 31 000 dalton containing 60% by weight of carbohydrate [1]. The entire amino acid sequence of glycophorin A is known [2] and it has been clearly established

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that the blood group MN determinants present on this glycoprotein are carried by N-terminal pentaglycopeptides which differ by amino acid substitutions at positions 1 and 5, ^{1}Ser/^{5}Gly in glycophorin A from M individuals (also denoted A^{M}) and ^{1}Leu/^{5}Glu in glycophorin A from N individuals (also denoted A^{N})[3,4]. This is the only structural difference between the M and N antigens and both A^{M} and A^{N} glycoproteins carry similar sialotetrasaccharides, NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAc, α-linked by an *O*-glycosidic bond to ^{2}Ser, ^{3}Thr and ^{4}Thr residues [5, 6]. Glycophorin A carries 15 of these sialotetrasaccharide chains and only one *N*-glycosidic chain, attached to ^{26}Asn, the structure of which has been recently elucidated [7].

Because of the large proportion of carbohydrate present in glycophorin A, it is a good candidate for lectin binding and indeed many lectins (for instance wheat germ agglutinin, WGA; *Maclura pomifera* agglutinin, MPA; *Lens culinaris* agglutinin, LcA; Peanut agglutinin, PNA; *Ricinus communis* agglutinin, RCA and *Vicia graminea* agglutinin VGA) interact with this cell surface glycoprotein, either before or after neuraminidase treatment of erythrocytes [8-13]. Several D-galactose-binding lectins, including PNA, VGA, ABA (*Agaricus bisporus* agglutinin) and BPA (*Bauhinia purpurea* agglutinin) agglutinate neuraminidase-treated erythrocytes by interacting with the uncovered disaccharide structure, D-Galβ1-3-D-GalNAcα- (T-hapten)[14-19]. However, there are subtle differences of specificity between these lectins since PNA, ABA and BPA required only sugars for binding, whereas VGA recognized a complex determinant which involved both a carbohydrate and a peptide moiety [12, 13, 20, 21].

In order to clarify further the characteristics and size of the epitope for each of these lectins, we have determined by agglutination inhibition tests the relative inhibitory potency of several synthetic glycopeptide-containing T-hapten structures present at the N-terminal end of glycophorin A^{M} and A^{N} [22, 23]. A further aim of this study was to evaluate the effect on the lectin specificity of clustering the carbohydrate chains present on the peptide backbone, and also the influence of the N-terminal amino acid residue.

Materials and Methods

Red Cells

MM and NN erythrocytes from blood group O donors, all typed as ss, were from the Centre National de Transfusion Sanguine, Paris, France.

Lectins

Vicia graminea agglutinin (VGA) was purified as previously described [24] from seeds cultivated under artificial conditions (Phytotron du CNRS, Gif sur Yvette, France) and used at 1 mg/ml in 100 mM Tris-HCl buffer pH 7.5 containing 50 mM NaCl. *Bauhinia purpurea* (BPA) and *Agaricus bisporus* (ABA) agglutinin were affinity purified lectins purchased from E.Y. Laboratories (USA) and used at 2 and 5 mg/ml, respectively, in PBS (phosphate buffered saline, 10 mM phosphate buffer pH 7.2 containing 150 mM NaCl). Affinity purified *Arachis hypogaea* (peanut, PNA) lectin was purchased from Boehringer (W. Germany) and used at 2 mg/ml in 150 mM NaCl.

Neuraminidase Treatment of Erythrocytes

The release of sialic acids from intact red cells was carried out by incubating 0.8 units neuraminidase from *Vibrio cholerae* (Behring, W. Germany) and 1 ml of washed packed red cells suspended in 150 mM NaCl containing 10 mM $CaCl_2$. After incubation for 60 min at 37°C with occasional shaking, the cells were washed 3 times in PBS and resuspended at 1% concentration (v/v) in the same buffer.

Synthetic Oligopeptides

Sixteen glycopeptides containing the T-hapten structure were chemically synthesized as described elsewhere [22, 23]. The pentapeptides and glycopeptides containing *N*-acetyl-D-galactosamine residues were a generous gift from Dr. Pavia (Avignon, France) and were also obtained by chemical synthesis [25, 26].

Inhibition of Hemagglutination

Inhibition tests were carried out as previously described with the synthetic peptides and glycopeptides diluted in PBS [27]. When necessary the pH was re-adjusted to 7.2 to avoid hemolysis. Four hemagglutinating doses of lectin were used throughout and the results expressed as pmole of inhibitory substance/100 μl reaction volume.

Results and Discussion

Peanut Agglutinin

It is well established that the best inhibitor of peanut agglutinin is the disaccharide, β-D-Gal(1-3)-α-D-GalNAc (T-hapten), either free or attached to glycolipid (asialo-G_{M1}-ganglioside) or glycoproteins (asialoglycophorin, antifreeze glycoproteins)[15, 16, 28]. Both T-hapten structures α- or β-linked are potent inhibitors [17] but D-Galβ1-3-*N*-acetyl-D-galactosaminitol has a severely reduced activity [16].

The results presented in Table 1 demonstrate that all synthetic glycopeptides carrying the T-hapten structure inhibit PNA. Glycopeptides **13** and **14** which have no terminal D-galactose are inactive. There is no significant difference between glycopeptides carrying a leucine residue at the N-terminal end and those carrying a serine residue (structures **9, 10, 11**). However, the latter are often slightly more active than the former.

When the inhibitory activity of the glycopeptides is compared on the basis of the molar concentration of galactose no differences are observed. Assuming that all disaccharides located on adjacent amino acid residues can react independently with the lectin, which is not formally proven here, the results suggest that the combining site of the lectin consists of a single oligosaccharide chain and that clusters of T-haptens present in glycopeptides **4, 5, 6, 7, 8, 10** and **11** do not significantly enhance the lectin reactivity.

Table 1. Inhibitory effect of synthetic oligopeptides on agglutinating activity of peanut agglutinin

No.	Inhibitor[1] Structure	Minimum concentration (pmole/100 μl) giving complete inhibition[2] as substance	as galactose
1.	Sêr	33	33
2.	Thr	60	60
3.	Leu-Sêr	40	40
4.	Sêr-Thr	10	20
5.	Thr-Thr	30	60
6.	Leu-Sêr-Thr	15	30
7.	Sêr-Thr-Thr	6	18
8.	Leu-Sêr-Thr-Thr	8	24
9.	Ser-Sêr	16	16
10.	Ser-Sêr-Thr	8	16
11.	Ser-Sêr-Thr-Thr	15	45
12.	Leu-Ser-Thr-Thr-Glu	>200	
13.	Ser-Sêr-Thr-Thr-Gly	> 60	
14.	Thr-Gly	>900	

[1] * = ß-D-Gal(1-3)α-D-GalNAc; ° = α-D-GalNAc

[2] Peanut agglutinin against neuraminidase-treated NN erythrocytes. Results expressed both as concentration of inhibitory substance and concentration of terminal galactose residues.

Agaricus bisporus and Bauhinia purpurea Agglutinins

The purified lectins from *Agaricus bisporus* and *Bauhinia purpurea* are devoid of blood group reactivity. *Agaricus bisporus* lectin is highly inhibited by the T-hapten α-linked to serine or threonine and this linkage is of primary importance since the disaccharide D-Galβ1-3-D-GalNAc is weakly active and D-Galβ1-3-*N*-acetyl-D-galactosaminitol is inactive [14]. These observations and the pattern of precipitation with various glycoproteins differentiate ABA from PNA [29].

Bauhinia purpurea lectin exhibits a broader specificity since it reacts with T-hapten structures α- or β-linked as well as with simple sugars (Gal, GalNAc) either free or linked to glycoconjugates [15, 17, 18, 30]. The results presented in Table 2 are in agreement with the sugar specificity of ABA and BPA lectins discussed above, showing that all glycopeptides carrying a T-hapten are inhibitors. It is shown in addition that glycopeptides **4, 5** and **7** which carry 2 or 3 disaccharide chains are about 3 times better inhibitors than glycopeptides **1** and **2** which carry only one disaccharide chain. The presence of a leucine or a serine residue at the N-terminal end of glycopeptides does not modify the lectin re-

Table 2. Inhibitory effect of synthetic glycopeptides on the agglutinating activity of *Agaricus bisporus* (ABA) and *Bauhinia purpurea* (BPA) agglutinins

	Inhibitor[1]	Minimum concentration giving complete inhibition[2] (pmole/100 μl)			
		ABA		BPA	
No.	Structure	as substance	as galactose	as substance	as galactose
1.	Ser*	70	70	130	130
2.	Thr*	60	60	140	140
4.	Ser*-Thr*	20	40	20	40
5.	Thr*-Thr*	5	10	20	40
7.	Ser*-Thr*-Thr*	6	18	12	36

[1] * = ß-D-Gal(1-3)-α-D-GalNAc

[2] Four hemagglutinating doses of lectin used with human NN erythrocytes treated with neuraminidase. Results expressed both as concentration of inhibitory substance and concentration of terminal galactose residues.

activity (not shown). These results suggest that the combining site of both ABA and BPA might require at least 2 proximal galactose residues for optimal binding.

Vicia graminea Agglutinin

The lectin from *Vicia graminea* (VGA) exhibits a blood group N specificity towards native human erythrocytes but agglutinates strongly both N and M red cells treated with trypsin or neuraminidase. However, inhibition studies have shown that the lectin still recognizes N better than M desialylated glycopeptides derived from the human red cell membrane (for review see ref. 31). The erythrocyte membrane receptors for VGA have been recently characterized as glycophorin A and B on native N red cells and glycophorin B only on native M red cells [12, 13]. The specificity of VGA is rather complex since it involves both a sugar sequence (D-Galβ1-3-D-GalNAcα-) and a peptide moiety [20, 21, 31] and so far no simple sugars alone, including the T-hapten disaccharide, inhibited the lectin.

In order to delineate more precisely the VGA specificity, several synthetic glycopeptides, representing the N-terminal end of glycophorin A from N and M individuals were used in inhibition of agglutination. The results are presented in Table 3, and include inhibition tests carried out with native and neuraminidase-treated N and M red cells. Glycopeptides **1, 2, 4, 5** and **7** which carry one or several T-hapten disaccharides linked to serine or threonine are not inhibitory, supporting the view that the oligosaccharide chain(s) alone are not a sufficient requirement for binding. Similarly, the pentapeptides Leu-Ser-Thr-Thr-Glu and Ser-Ser-Thr-Thr-Gly are not inhibitory at 230 pmole/100 μl con-

Table 3. Inhibitory effect of synthetic glycopeptides on agglutinating activity of *Vicia graminea* lectin

	Inhibitor[1]	Minimum concentration of substance (pmole/100 μl) giving a complete inhibition of 4 hemagglutinating doses[2]			
	Structure	NN(nt)	MM(nt)	NN(t)	MM(t)
1.	Ser*	>1000		>1000	
2.	Thr*	> 970		> 970	
4.	Ser*-Thr*	> 80		> 80	
5.	Thr*-Thr*	> 80		> 80	
7.	Ser*-Thr*-Thr*	> 50		> 50	
3.	Leu-Ser*	160		160	
6.	Leu-Ser*-Thr*	31		62	
8.	Leu-Ser*-Thr*-Thr*	17		34	
15.	Leu-Ser*-Thr*-Thr*-Glu	2	8	10	
9.	Ser-Ser*	> 125	> 125	> 125	> 125
10.	Ser-Ser*-Thr*	> 125	> 125	> 125	> 125
11.	Ser-Ser*-Thr*-Thr*	> 110	> 50	> 110	> 110
16.	Ser-Ser*-Thr*-Thr*-Gly		> 50	> 50	

[1] * = ß-D-Gal(1-3)-α-D-GalNAc

[2] Four hemagglutinating doses of lectin against red cells treated (t) or not treated (nt) with neuraminidase.

centration (not shown) suggesting that the peptide sequence itself, either derived from N or M typical amino-acid sequence, does not represent an adequate determinant for binding.

The best inhibitors of VGA are represented by glycopeptides carrying the T-hapten structure and a terminal leucine residue (structures **3, 6, 8, 15**) whereas those carrying a terminal serine residue are inactive (structures **9, 10, 11, 16**). These results indicate clearly that the determinant specific for VGA includes both carbohydrates and a hydrophobic amino acid. Obviously, the presence of *N*-acetylneuraminic acid is not necessary for the N specificity expressed by VGA. These observations correlate well with previous findings showing that either Edman degradation of N and M erythrocyte glycopeptides or chemical blockage of their terminal amino-group abolishes the VGA activity [31, 32].

Further examination of Table 3 shows that on the basis of galactose concentration, glycopeptide **3** is respectively 3-, 5- or 30-times less inhibitory than glycopeptide **6, 8** or **15** (as seen using native N red cells). The results demonstrate that the highest VGA activity is obtained with glycopeptides carrying a leucine at the N-terminal end together with a cluster of T-hapten disaccharides at position 2, 3 and 4. Such an effect might result from a specific spacial arrangement of the heavily clustered glycopeptides which is properly recognized by VGA. In addition, since glycopeptide **15** had the highest activity, it

is possible that the Glu residue at postion 5 also favours somewhat the best overall structure adapted to VGA.

Our results bring strong experimental support to the proposal [32, 33] that the receptor site for VGA includes a glycopeptide with a specific conformation which is determined by a heavy cluster of T-hapten disaccharides in close vicinity to a hydrophobic area represented by a terminal amino acid with a free amino-group. Interestingly, such a typical determinant (cluster of T-hapten and a valine residue) is also present in the region between residue 10 and 16 of glycophorin A [1, 2] and is uncovered after removal of sialic acids. Such a region in the molecule might represent a potential binding site for VGA to neuraminidase-treated N and M erythrocytes, although the valine is N-protected. Synthesis of glycopeptides corresponding to the 10-17 segment of glycophorin A may clarify this point.

The results presented in this paper have demonstrated the usefulness of synthetic glycopeptides in elucidation of the structural requirement of several D-galactose-binding lectins since most of the structures used have not been obtained so far by chemical or enzymatic fragmentations of biological material. Further investigations using these substances, together with synthesis of other clustered glycopeptides, might provide more information on the combining site of these lectins.

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