Structural Determinants of Ricinus communis Agglutinin and Toxin Specificity for Oligosaccharides*

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Structural determinants of oligosaccharides affecting interaction with the saccharide-binding sites of two lectins, the agglutinin of $M_r = 120,000$ and toxin of $M_r = 60,000$ (RCA$_1$ and RCA$_{11}$, respectively), isolated from Ricinus communis beans have been examined by performing binding studies with iodinated glycopeptides and their degradation products. Association constants can be determined by Scatchard plot analysis of the saturation curves obtained. Interaction with complex, $N$-acytelyglucosamine-containing glycopeptides and with glycopeptides bearing Gal$eta$1,3GalNAc moieties is directed predominantly at the $\beta$-linked galactose residues for both RCA$_1$ and RCA$_{11}$. The presence of sialic acid substituents on these galactose residues markedly reduces the association constants of both RCA$_1$ and RCA$_{11}$ for complex glycopeptides and completely abolishes binding of glycopeptides bearing Gal$eta$1,3GalNAc oligosaccharides. RCA$_1$ and RCA$_{11}$ yield indistinguishable association constants for binding of trichubranched, complex oligosaccharides with 3 galactose residues, whereas RCA$_1$ binds dibranched structures and their degradation products with association constant values some 2- to 3-fold higher than those obtained with RCA$_{11}$. In contrast, the association constants obtained for RCA$_{11}$ binding of glycopeptides bearing 1, 2, or 4 Gal$eta$1,3GalNAc disaccharides are all some 2.5- to 10-fold higher than those obtained with RCA$_1$. The ability of RCA$_1$ to bind to Gal$eta$1,3GalNAc disaccharides appears to be markedly influenced by the relative locations of the disaccharides along the peptide backbone. The ability of either RCA$_1$ or RCA$_{11}$ covalently linked to Sepharose 4B to bind iodinated glycopeptides correlates with association constant values greater than $5 \times 10^8$ M$^{-1}$.

Plant lectins are carbohydrate-binding proteins which have proved to be useful reagents for probing structural features of cell surface glycoproteins and for isolating glycoproteins and glycopeptides by affinity chromatography (1). The common castor bean (Ricinus communis) contains two proteins, RCA$_1$ and RCA$_{11}$. RCA$_1$ which bind carbohydrate (2). RCA$_1$ is a hemagglutinin with a molecular weight of 120,000 consisting of two A chains ($M_r = 29,500$) and two B chains ($M_r = 37,000$), while RCA$_{11}$ is a highly toxic, nonagglutinating protein with a molecular weight of 60,000 consisting of one A chain ($M_r = 29,500$) and one B chain ($M_r = 34,000$) (2). The polypeptides of these two lectins are closely related as demonstrated by the extensive cross-reactivity of antibodies directed at either the A or B chains, the similarity of the amino acid compositions of the respective A and B chains (2), and the recent report that the sequence of the first 19 amino acids of the A chains (2) and 17 of the first 18 residues of the B chains of RCA$_1$ and RCA$_{11}$ were identical (3). RCA$_1$ and RCA$_{11}$ do, however, display differences in their specificity for binding both simple sugars (2, 4, 5) and oligosaccharides (6). Information about the structural features of oligosaccharides affecting binding by RCA$_1$ and RCA$_{11}$ has remained incomplete and is of an indirect nature. This study is a detailed examination of the specificity of RCA$_1$ and RCA$_{11}$ for both complex, $N$-acytelyglucosamine-containing oligosaccharides and $N$-acytelyglactosamine-containing oligosaccharides utilizing a number of iodinated glycopeptides of both types in a precipitation assay which permits Scatchard plot analyses of the saturation curves obtained.

EXPERIMENTAL PROCEDURES

Materials—Sephadex G-10 and Sepharose 4B were from Pharmacia, Bio-Gel P-150 from Bio-Rad, type A-E glass fiber filters from Gelman, enzyme grade ammonium sulfate from Schwarz/Mann, 3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester from Calbiochem, and Na$_2$HPO$_4$ (900 mCi/mmol, carrier-free) from Amersham. All chemicals were of the highest purity available commercially. The preparation and characterization of IgG, IgAl, and fetuin glycopeptides have been described previously (7–11). Glycopeptides of IgG, IgAl and fetuin glycopeptides were prepared from fetuin as described by Spiro and Bhoyroo (12). The isolation of neuraminidase from Clostridium perfringens and $\alpha$-galactosidase, $\beta$ $N$-acytelyglucosaminidase, endo-$\beta$-$N$-acytelyglucosaminidase, and endo-$\alpha$-$N$-acytelygalactosaminidase from Streptococcus pneumoniae were carried out according to published procedures (13, 14). The conditions for degradation of iodinated glycopeptides with these enzymes have been described (8).

Preparation of RCA$_1$ and RCA$_{11}$—RCA$_1$ and RCA$_{11}$ were prepared by affinity chromatography on p-aminoethyl-$\beta$-thiogalactosyl succinimidylhydrazide polycrylamide (Bio-Gel P-150, 5.7 $\mu$mol of galactose per ml) (15) and subsequently separated by gel filtration chromatography on Sephacel G-100 as described by Nicolaus et al. (2). The lectins were judged to be homogeneous by sodium dodecyl sulfate polyacrylamide gel electrophoresis performed according to Laemmli (16) and consisted of A and B chains and with apparent molecular weights similar to those previously reported (2, 3).

Binding Assay—The binding assays were performed as described previously (8). Concentrations of RCA$_1$ and RCA$_{11}$ were established by absorption at 280 nm using extinction coefficients ($e_{280}$) of 11.7 for RCA$_1$ and 11.8 for RCA$_{11}$ (5) and confirmed by the Lowry protein assay (17). The concentrations of both lectins were subsequently expressed as moles of binding site based on the presence of one...
binding site per 60,000 molecular weight A and B chain complex (5). The 50-μl incubation reactions contained 5 mM sodium phosphate buffer, pH 7.2, 0.2 mM NaCl and 0.1% bovine serum albumin, the iodinated glycopeptide, and dilutions of RCA1 or RCAII ranging from 8 × 10^{-5} M to 8 × 10^{-7} M based on the binding site concentration. Precipitation with ammonium sulfate and collection on glass fiber filters was done as described previously (8). The saturation curves were analyzed using the method of Scatchard (18).

Iodination of Glycopeptides—Iodination and characterization of glycopeptides using (125)I-3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester prepared by the method of Bolton and Hunter (21) has been described (8).

Removal of Terminal Galactose Residues from Galβ1,3GalNAc-containing Glycopeptides—Preparation of O-glycosidically linked oligosaccharides with terminal N-acetylgalactosamine presented some difficulty as none of the β-galactosidase preparations available to us in a highly purified form would release the β1,3-linked galactose residues (10, 12). It was, therefore, necessary to accomplish this by performing a Smith periodate degradation as described by Spiro and Bhoyroo (12) for the fetuin glycopeptides Fet. C-1 and Fet. B-4, which were subsequently iodinated as described above. In the case of GP-I isolated from IgA, the product following Smith periodate degradation could not be iodinated in this manner. To overcome this difficulty the intact glycopeptide was derivatized with the 3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester prior to carrying out the Smith periodate degradation. The final product was iodinated directly using the chloramine-T method of Hunter (20) or 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (21).

RESULTS AND DISCUSSION

General Features of the Binding Assay for RCA1 and RCAII—The addition of increasing amounts of RCA1 or RCAII to fixed amounts of iodinated glycopeptide yields saturation curves such as those illustrated in Fig. 1. The presence of a 200-fold excess of unlabelled glycopeptide reduces the counts bound to that observed in the absence of added lectin. Subsequent to binding of iodinated glycopeptides by either RCA1 or RCAII, the addition of a 200-fold excess of unlabelled glycopeptide results in complete displacement of the iodinated glycopeptide in less than 1 min at room temperature, indicating that the binding is reversible and equilibrates rapidly. The iodinated glycopeptide-lectin complex is stable for at least 1 h at 4°C following precipitation with ammonium sulfate. All binding studies were performed a minimum of three times for each structure examined, and the association constants reported in Figs. 2 and 3 are the means from three or more determinations.

Specificity for Complex Oligosaccharides with 1, 2, or 3 Galβ1,4GlcNAc Moieties—The association constants obtained for RCA1 and RCAII binding of two series of complex oligosaccharides and their degradation products are shown in Fig. 2. Binding by both lectines is directed at the β1,4-linked galactose residues of these complex oligosaccharides as evidenced by the lack of detectable binding following removal of all galactose residues (Fig. 2, Structures F1 and G-C). The presence of a greater number of Galβ1,4GlcNAc moieties (1, 2, and 3 in Structures G-B1, G-B2, and F1, respectively) results in a progressive increase in the association constants for both RCA1 and RCAII, however, the relative increase is considerably greater for RCAII than RCA1. Consequently, the association constants for RCAII binding of structures with 1 or 2 terminal galactose residues are some 2- to 2.5-fold lower than those for RCA1, whereas the values are nearly identical for a structure such as F1 with 3 terminal galactose residues.

Both RCA1 and RCAII recognize and bind NANAα2,6Galβ1,4GlcNAc moieties, i.e. penultimate galactose residues (see Structures G-A and G-A2 in Fig. 2). On the other hand, the presence of α2,6-linked sialic acid substituents reduces the association constants some 2- to 4-fold for both RCA1 and RCAII binding of sialylated as compared to nonsialylated glycopeptides (compare G-A and G-A2 with G-B1 and G-B2, respectively, in Fig. 2). The influence of α2,3, as opposed to α2,6-linked sialic acid substituents on the penultimate galactose residues can be evaluated by comparison of binding of Structure F1 with G-A and G-A2. RCA1 binding of fetuin glycopeptide F1 appears to be directed predominantly, if not exclusively, at the NANAα2,6Galβ1,4GlcNAc unit since the association constant for F1 is nearly the same as that for G-A2. In contrast, the progressively greater association constants for RCAII binding of G-A2, G-A, and F1 suggest that both 3- and 6-substituted galactose residues are recognized and involved in binding by RCAII. This specificity for RCAII binding was also indicated by the work of Drysdale et al. using simple haptens (4). They found that the hemagglutinating activity of Ricinus communis could be inhibited only by sugars with the 3-galacto conformation on carbon 2, carbon 3, and carbon 4, suggesting that galactose residues bearing a substituent at position 6 would be recognized whereas those with a substituent at position 2, 3, or 4 would not.

Thus both RCA1 and RCAII are able to bind sialylated and non-sialylated galactose residues. The greater binding affinity of RCAII for the sialylated structures is consistent with the specificity of HIV for RCAII (22). This is the first example of a functionally important difference between RCA1 and RCAII binding of specific oligosaccharide structures.
nonsialylated complex oligosaccharides. The association constants for RCAI and RCAII binding of the tribranched oligosaccharide isolated from fetuin are virtually indistinguishable, whereas RCAI and RCAII differ considerably in their binding of structures such as those of the G series with either 1 or 2 galactose residues. Generally speaking, the association constants for RCAI binding of the G series of glycopeptides in Fig. 2 were some 2- to 3-fold greater than those for RCAII; however, arrangement in order of decreasing association constant values yields the same sequence for both RCAI and RCAII.

Specificity for Glycopeptides Bearing Gal\beta1,3GalNAc Moieties—The association constants for RCAI and RCAII binding of glycopeptides bearing Gal\beta1,3GalNAc moieties are presented in Fig. 3. In contrast to binding of complex oligosaccharides, the values obtained for RCAII binding are all considerably lower than those obtained with complex oligosaccharides. Removal of the terminal galactose residue results in complete abolition of RCAI binding except in the case of GP-I with 4 remaining terminal N-acetylgalactosamine residues where a low but reproducible value of $4.4 \times 10^6$ M$^{-1}$ was obtained. Thus RCAI interaction with O-glycosidically linked Gal\beta1,3GalNAc units is almost exclusively directed at the galactose residue. Binding of Gal\beta1,3GalNAc moieties by RCAII also involves predominantly the $\beta1,3$-linked galactose residues as evidenced by the marked decrease in the association constants following removal of the terminal galactose from Fet. C-1 and GP-I (1 and 4 units, respectively). The markedly greater association constant for RCAII binding of GP-I as compared to desialyzed Fet. C-1 and Fet. B-4, and intact GP-I indicates a progressive increase when 1, 2, or 4 Gal\beta1,3GalNAc units are present; however, the values are considerably lower than those obtained with complex oligosaccharides. Removal of the terminal galactose residue results in complete abolition of RCAI binding except in the case of GP-I with 4 remaining terminal N-acetylgalactosamine residues where a low but reproducible value of $4.4 \times 10^6$ M$^{-1}$ was obtained. Thus RCAI interaction with O-glycosidically linked Gal\beta1,3GalNAc units is almost exclusively directed at the galactose residue. Binding of Gal\beta1,3GalNAc moieties by RCAII also involves predominantly the $\beta1,3$-linked galactose residues as evidenced by the marked decrease in the association constants following removal of the terminal galactose from Fet. C-1 and GP-I (1 and 4 units, respectively). The markedly greater association constant for RCAII binding of GP-I as compared to desialyzed Fet. C-1 and Fet. B-4, and the unexpectedly high value of $8.9 \times 10^6$ M$^{-1}$ for Fet. B 4 following removal of the galactose residues as compared to Fet. C-1 and GP-I following removal of the galactose residues, indicates that the location of the Gal\beta1,3GalNAc units along the peptide backbone is also of critical importance for binding. That it is indeed the N-acetylgalactosamine which is involved in recognition by RCAII in the latter cases is demonstrated by the loss of binding following removal of the Gal\beta1,3GalNAc

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**Fig. 2.** RCAI and RCAII binding of complex, N-acetylglucosamine-containing glycopeptides. Linkages are only indicated for the parent structures F1 and G-A. The association constants below each structure are the mean of three or more determinations. N.B. indicates no detectable binding was observed.

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**Fig. 3.** The association constants for RCAI and RCAII binding of the G series of glycopeptides in Fig. 2 were some 2- to 3-fold greater than those for RCAII; however, arrangement in order of decreasing association constant values yields the same sequence for both RCAI and RCAII. The values obtained for RCAII binding are all considerably lower than those obtained with complex oligosaccharides. Removal of the terminal galactose residue results in complete abolition of RCAI binding except in the case of GP-I with 4 remaining terminal N-acetylgalactosamine residues where a low but reproducible value of $4.4 \times 10^6$ M$^{-1}$ was obtained. Thus RCAI interaction with O-glycosidically linked Gal\beta1,3GalNAc units is almost exclusively directed at the galactose residue. Binding of Gal\beta1,3GalNAc moieties by RCAII also involves predominantly the $\beta1,3$-linked galactose residues as evidenced by the marked decrease in the association constants following removal of the terminal galactose from Fet. C-1 and GP-I (1 and 4 units, respectively). The markedly greater association constant for RCAII binding of GP-I as compared to desialyzed Fet. C-1 and Fet. B-4, and the unexpectedly high value of $8.9 \times 10^6$ M$^{-1}$ for Fet. B 4 following removal of the galactose residues as compared to Fet. C-1 and GP-I following removal of the galactose residues, indicates that the location of the Gal\beta1,3GalNAc units along the peptide backbone is also of critical importance for binding. That it is indeed the N-acetylgalactosamine which is involved in recognition by RCAII in the latter cases is demonstrated by the loss of binding following removal of the Gal\beta1,3GalNAc
**RCA₁ and RCA₁₁ Specificity**

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<tr>
<th>Glycopeptide Structure</th>
<th>RCA₁</th>
<th>RCA₁₁</th>
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<tr>
<td>GalNAc(ProProGlyAla)</td>
<td>N.B.</td>
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<tr>
<td>GalNAc(ProProGlyAla)</td>
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**FIG. 3. RCA₁ and RCA₁₁ binding of glycopeptides bearing Galβ1,3GalNAc moieties.** Linkages are only indicated for the parent structures. The association constants below each structure are the means of three or more determinations. N.B. indicates no detectable binding was observed.

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unit(s) with endo-α-N-acetylgalactosaminidase. Prior removal of the galactose residues from Fet. C-1 and Fet. B-4 with periodate resulted in complete resistance to endo-α-N-acetylgalactosaminidase and no alteration in the association constant following treatment with the endo-α-N-acetylgalactosaminidase, thus confirming the effectiveness of the periodate degradation, since endo-α-N-acetylgalactosaminidase will not remove terminal N-acetylgalactosamine residues.

**CONCLUSIONS**

Examination of the association constants obtained by performing binding studies with well characterized, iodinated glycopeptides and lectins such as RCA₁ and RCA₁₁ yields considerable and detailed information about the features of oligosaccharide structure which affect binding. Work by Ølsvik et al. (6, 22) has demonstrated that it is the B chains of RCA₁ and RCA₁₁ which are responsible for saccharide binding. Recent evidence has indicated that the B chains of RCA₁ and RCA₁₁ are not only closely related in terms of saccharide-binding specificity and antigenicity but also in terms of their primary sequences (3). This observation has led Cawley et al. (3) to suggest that RCA₁ may be a precursor form of RCA₁₁. In light of the interesting features of these two lectins and their use as probes for the study and isolation of oligosaccharides present on membranes, detailed information about their specificity for oligosaccharides should prove of considerable value.

In general, the results which we have presented are in agreement with the findings of others using simple haptens (1, 2, 4, 5). It is clear from this study that RCA₁ and RCA₁₁ differ in their specificities for both complex, N-acetylglucosamine-containing oligosaccharides and Galβ1,3GalNAc-containing glycopeptides. Thus RCA₁ will bind complex oligosaccharides of the G series with greater association constants than RCA₁₁, whereas the converse is true for glycopeptides bearing Galβ1,3GalNAc units. The basis for the markedly
greater association constant for RCAII binding of GP-I is unclear but may be related to the tertiary structure of the glycopeptide unit. That this may be an important factor is suggested by the small decrease in the association constant for Fet B-4 following removal of the galactose residues, whereas Fet C-1 and GP-I display a 10- to 15-fold decrease in their association constants following galactose removal. It should also be noted that even though RCAII can bind glycopeptides with only terminal N-acetylgalactosamine, while RCAI cannot, the association constant is considerably higher for two of the glycopeptides in the presence of the β-linked galactose, indicating RCAII interacts predominantly with galactose residues.

The association constants we have obtained for the binding of iodinated glycopeptides by RCAI and RCAII fall within the range of values (8 x 10⁶ M⁻¹ to 4.2 x 10⁸ M⁻¹) reported by Sandvig et al. (23) for iodinated RCAI and RCAII binding to erythrocytes and HeLa cells. The lower values reported by other workers were attributed by Sandvig et al. (23) to damage of the lectin during iodination with chloramine-T. The higher association constant values seen with erythrocytes may reflect differences in the methods of analysis, the markedly greater association constants for oligosaccharides with a single galactose residue could also reflect weak interactions of the saccharide-binding site with portions of the oligosaccharides other than the β-linked galactose residues. Differences in the association constants for RCAI and RCAII binding of G-B2 (Fig. 2) and asialofetuin B4 (Fig. 2) demonstrate that the affinity for the lectin-Sepharose columns to retain glycopeptides generally correlates with association constants greater than about 5 x 10⁶ M⁻¹.

REFERENCES

Structural determinants of Ricinus communis agglutinin and toxin specificity for oligosaccharides.
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