Molecular basis of recognition by Gal/GalNAc specific legume lectins: influence of Glu 129 on the specificity of peanut agglutinin (PNA) towards C2-substituents of galactose

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Received on February 4, 1998; revised on March 17, 1998; accepted on March 17, 1998

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The ability to discriminate between galactose and N-acetylgalactosamine, observed in some lectins, is crucial for their biological activity as well as their usefulness as tools in biology and medicine. However, the molecular basis of differential binding of lectins to these two sugars is poorly understood. Peanut agglutinin (PNA) is one of the few galactose-specific legume lectins which does not bind N-acetylgalactosamine at all and is, therefore, ideal for the study of the basis of specificity towards C-2 substituted derivatives of galactopyranosides. Examination of the three-dimensional structure of PNA in complex with lactose revealed the presence of both a longer loop and bulkier residues in the region surrounding the C-2 hydroxyl of the galactopyranose ring, which can sterically prevent the accommodation of a bulky substituent in this position. One such residue, is a glutamic acid at position 129 which protrudes into the binding site and perhaps directly obstructs any substitution at the C-2 position. Two mutants in bacterially expressed PNA were therefore constructed. These were E129D and E129A, in which Glu129 was replaced by Asp and Ala, respectively. The specificity of the mutants for galactose, galactosamine, and N-acetylgalactosamine was examined through observing the inhibition of hemagglutination and binding of the lectin to immobilized asialofetuin. The results showed that the affinity of E129A and E129D for C-2-substituted derivatives of the galactose varies. The mutant E129D showed significant binding towards C-2-substituted derivatives of the galactose in complex with lactose revealed the presence of both a longer loop and bulkier residues in the region surrounding the C-2 hydroxyl of the galactopyranose ring, which can sterically prevent the accommodation of a bulky substituent in this position. One such residue, is a glutamic acid at position 129 (Glu129), appears to offer major hindrance to the binding of GalNAc. The present study reports two Glu129 mutants, one to aspartate (E129D) and the other to alanine (E129A), that exhibit variable affinities for GalNAc. Most lectins can tolerate greater variation at the anomeric (C-1) and C-2 positions of their monosaccharide inhibitors than at the C-3 and C-4 hydroxyls (Goldstein and Hayes, 1978; Sharon and Lis, 1989). Lectins from Erythrina corallodendron (coral tree lectin, ECorL) and Glycine max (soybean agglutinin, SBA), for example, bind both galactose and N-acetylgalactosamine (Sharon and Lis, 1990; Wu and Sugi, 1991). The tumor-associated Thomsen-Friedenreich binding agglutinin from peanut (Arachis hypogaea), however, does not associate with either GalNAc or other galactose derivatives with bulky C-2 substituents (Lotan et al., 1975; Pereira et al., 1976; Reisner et al., 1979; Young et al., 1984; Swamy et al., 1991). An understanding of the molecular basis of the specificity of peanut agglutinin (PNA) for galactose and its poor tolerance of variability at the C-2 position is, therefore, of considerable interest both for elucidating the mechanism of specific carbohydrate recognition as well as engineering novel binding activities in legume lectins. It has been proposed that the interaction of acetamido group, as well as dansylamido or other bulky hydrophobic C-2 substituents, on galactose, is mediated by a few residues which constitute a hydrophobic patch within GalNAc-specific lectins. The variance at equivalent positions in PNA has, therefore, been hypothesized to be responsible for the lack of GalNAc binding by it. These differences include the absence of two conserved aromatic residues (Tyr108 and Trp135 in ECorL) in PNA (Figure 1). Mutagenesis studies on Erythrina corallodendron have shown that the replacement of the dipeptide Pro134-Trp135 of ECorL with a tetrapeptide Ser128-Glu-Tyr-Asn131 present in PNA reduced its affinity for GalNAc and other dansyl sugars (Arango et al., 1993). However, this reduction in the binding could be either due to the absence of conserved Trp or the presence of other residues in the mutated region. It was therefore imperative to look into the role of additional factors in determining the basis for the galactose-specificity of PNA. The crystal structure of the PNA-lactose complex (Banerjee et al., 1994, 1996), has revealed interesting features that account for its lack of tolerance towards C-2 substituted derivatives. Among these, a glutamic acid residue at position 129 (Glu129), appears to offer major hindrance to the binding of GalNAc. The present study reports two Glu129 mutants, one to aspartate (E129D) and the other to alanine (E129A), that exhibit variable affinities towards different galactose derivatives.

Results and discussion

Molecular recognition by Gal/GalNAc binding lectins and the role of Glu 129 in PNA

The carbohydrate binding site of Gal/GalNAc binding lectins consists of residues from four sequentially distinct regions which have been designated as loops A, B, C, and D (Sharma and Surolia, 1997). In these lectins, the primary interactions of
Fig. 1. Stereo view of the combining sites of ECorL (A) and PNA (B). The carbohydrate binding site in both the lectins is constituted by residues from four loops. The stacking interactions of lactose with an aromatic residue (F131 or Y125) as well as hydrogen bonds (dotted lines) with Asp, Gly, and Asn residues (D89, G107, and N133 in ECorL; D83, G104, and N127) are conserved. Two water molecules make hydrogen bonds between the C-2 hydroxyl of galactose and NH of Gly 104 and O$_{\varepsilon}$ of Glu 129. The side chain of Glu 129 of PNA along with the bound water (B) occupy the space corresponding to a hydrophobic pocket which accommodates bulky C-2 substituents in ECorL (A). This figure is generated using the program MOLSCRIPT (Kraulis et al., 1991).

galactose orient the pyranose ring such that the C2-OH faces loop C, which exhibits considerable variation in size and sequence (Table I). The size of this loop varies by up to two residues and influences the binding in such a way that lectins with a shorter loop (SBA and WBA I) recognize GalNAc with higher affinities than those with longer loops (DBL and LBL). PNA has a long loop C which partially explains its inability to bind GalNAc (Sharma and Surolia, 1997). However, while the affinity for GalNAc decreases in the order: SBA > WBAI > DBL > ECorL > LBL >>> PNA (Table I), the affinity for Gal is not exactly reverse but follows the order: PNA = ECorL > WBAI > SBA >> DBL or LBL (see references for Table I). This implies that the specificity for GalNAc is determined by factors other than the ones that determine the affinity for this sugar. Thermodynamic parameters for both Gal and GalNAc, now known for three lectins: SBA, WBA I, and ECorL, suggest that the ratio of association constants \(K_a(GalNAc)/K_a(Gal)\) for the two sugars is 25, 6, and 0.9, respectively (Hammarström et al., 1977; Khan et al., 1986; Surolia et al., 1996). This variation corresponds to the size of the polar residue (Ser in SBA, Thr in WBA I, and Gln in ECorL) preceding the conserved Trp in loop C (Table I). Thus, the cavity that accommodates the C-2 substituents in GalNAc binding lectins arises out of both shortening of loop C as well as the presence of amino acids with smaller side chains (Sharma and Surolia, 1997).
Conserved residues Asp 83, Gly 104, and Asn 127 as well as Asp 80, Ser 211, and Gly 213, which are unique to PNA, are involved in making nine hydrogen bonds with lactose (Table II; Bannerjee et al., 1996). We analyzed the PNA–lactose complex to identify those residues around the binding pocket which do not make direct contact with the terminal galactose, but could bind to GalNAc or other C-2 substituted galactopyranosides. One such residue is Glu 129, the side-chain of which, extends into the binding pocket with the carboxylate group located at 43–45 nm from the C-2 hydroxyl of galactopyranoside ring in the crystal structure (Figure 1B). The space between glutamyl side-chain and the C-2 hydroxyl is occupied by a water molecule which makes hydrogen bonds to both. It appears that the 1.5- to 1.7-fold higher affinity of PNA toward galactosamine as compared to galactose could be attributed to improved water-mediated hydrogen bonding of the Glu 129 to the amino group of C-2 position of the former. However, a bulkier substitution at the same site would lead to displacement of the bound water molecule as well as steric hindrance with the long Glu 129 side chain. The glutamyl side chain, in effect, would sterically prevent the binding of GalNAc such that local conformational readjustments may not be sufficient to allow accommodation of the bulky N-acetamido group. The decreased length of the loop as well as the absence of any residue with an equally extended side chain in other Gal/GalNAc binding legume lectins, therefore, seems to be responsible for their inability to differentiate between various galactosyl derivatives. The replacement of E129 with the shorter aspartic acid, would slightly reduce the steric hindrance without significantly altering the polar environment in the region whereas asparatic acid, would slightly reduce the steric hindrance without significantly altering the polar environment in the region whereas a smaller aliphatic residue, like alanine, would create a larger non-polar void perhaps capable of accommodating galactose derivatives with bulkier substituents at the C-2 position. We, therefore, characterized carbohydrate recognition by the two mutants E129D and E129A.

**Construction of Glu 129 mutants and their lectin activity**

The E129A and E129D mutant constructs generated were both associated with the loss of a RsaI site (GTAC) in the wt sequence. This was checked for, in the initial screening of the clones. The wild type pBSH-PN (Sharma and Surolia, 1994) construct contains an additional RsaI site in the vector sequence giving rise to two fragments of 2209 bp and 1408 bp length, upon enzymatic cleavage. In contrast, the plasmids carrying the mutations E129D and E129A have only one site for RsaI in the vector portion of the constructs and are, consequently, just linearized upon restriction digestion. Sequencing of E129A and E129D confirmed the introduction of the respective mutations.

### Table I. Correlation between the sequence of loop C and affinity of some Gal/GalNAc specific lectins

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Species</th>
<th>Loop C sequence</th>
<th>Monosaccharide specificity</th>
<th>Relative affinity for GalNAc</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBA</td>
<td>Glycine max</td>
<td>VEFDTFRANW</td>
<td>GalNAc &gt; &gt; Gal (25)</td>
<td>40</td>
<td>Hammarström et al., 1977; De Boeck et al., 1984</td>
</tr>
<tr>
<td>WBA I</td>
<td>Phaseolus lunatus</td>
<td>VEFDTFRANW</td>
<td>GalNAc &gt; &gt; Gal (6.0)</td>
<td>17</td>
<td>Khan et al., 1986; Schwarz et al., 1991</td>
</tr>
<tr>
<td>EcorL</td>
<td>Dolichos biflorus</td>
<td>VEFDTFRANW</td>
<td>GalNAc = Gal (0.9)</td>
<td>3</td>
<td>Surolia et al., 1996</td>
</tr>
<tr>
<td>LBL</td>
<td>Arachis hypogea</td>
<td>VEFDTYNSEYYNPP</td>
<td>Gal &gt; &gt; GalNAc (0)</td>
<td>0</td>
<td>Young et al., 1984</td>
</tr>
</tbody>
</table>

*The sequences of loop C (Val120-Pro133 in PNA) for different Gal/GalNAc-specific legume lectins have been aligned such that conserved residues (underlined) flank the binding-site residues. The length of this loop varies by two residues.

**The preference of the lectin between N-acetylgalactosamine and galactose. The values in parentheses represent the ratio of the association constants (K_a(GalNAc)/K_a(Gal)) for the two sugars exhibited by the lectins for which detailed thermodynamic studies have been done.

**Relative affinities of the different lectins towards GalNAc as determined by the ratio of their reported association constants with that of lime bean lectin (LBL).**

### Table II. Hydrogen bonds between monosaccharides and different Gal/GalNAc binding legume lectins

<table>
<thead>
<tr>
<th>Sugar residue</th>
<th>EcorL</th>
<th>PNA</th>
<th>GS IV</th>
<th>SBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>O2</td>
<td>—</td>
<td>H_2O..NH Gly^104</td>
<td>H_2O..O_2 Asp^137</td>
<td>—</td>
</tr>
<tr>
<td>O3</td>
<td>O_2 Asp^89</td>
<td>O_2 Asp^83</td>
<td>O_2 Asp^89</td>
<td>O_2 Asp^88</td>
</tr>
<tr>
<td>O4</td>
<td>O_2 Asp^89</td>
<td>O_2 Asp^83</td>
<td>O_2 Asp^89</td>
<td>O_2 Asp^88</td>
</tr>
<tr>
<td>O5</td>
<td>—</td>
<td>O_2 Asp^83</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>O6</td>
<td>—</td>
<td>O_2 Asp^83</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Na</td>
<td>7</td>
<td>9</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

*N represents the total number of hydrogen bonds with the galactosyl ring.
Table III. Hemagglutination assay of Glu 129 mutants

<table>
<thead>
<tr>
<th>Lectin</th>
<th>MHC (µg/ml)</th>
<th>Galactose</th>
<th>Galactosamine</th>
<th>GalNAc</th>
<th>Lactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.30</td>
<td>20</td>
<td>12.5</td>
<td>N.I.</td>
<td>6.25</td>
</tr>
<tr>
<td>E129D</td>
<td>0.40</td>
<td>20</td>
<td>40</td>
<td>20</td>
<td>6.25</td>
</tr>
<tr>
<td>E129A</td>
<td>0.50</td>
<td>20</td>
<td>40</td>
<td>&gt;100</td>
<td>50</td>
</tr>
</tbody>
</table>

Hemagglutinating activity of wild type and mutant PNAs using desialylated rabbit RBCs.

Purified E129D and E129A mutant proteins when subjected to SDS–PAGE, displayed a single band similar to that observed for rPNA and their tetrameric nature was found to be unaltered as determined by gel-filtration (data not shown). They also exhibited hemagglutinating activities that were similar to that observed for wild-type PNA (wtPNA) (Table III). Hemagglutination inhibition experiments showed that both of these Glu 129 mutants are still specific towards galactopyranosyl ligands and do not bind to mannose or glucose. In other words, an axial orientation of the C4 hydroxyl group as in galactose, remains obligatory for the binding activity of these mutants just as observed for their wild type or recombinant counterparts. In both cases, galactose appeared to be a 2-fold poorer inhibitor than wtPNA. The inhibitory potency of lactose and galactosamine was 2- to 4-fold lower in relation to E129D and was reduced yet further with respect to E129A (Table III). GalNAc inhibited the mutant E129D as effectively as galactose, in contrast to the situation observed for wtPNA and E129A, both of which failed to recognize this sugar. Hemagglutination inhibition experiments indicated improved potency of GalNAc to inhibit E129D while it was inactive against both E129A and wtPNA. The saccharide with a still bulkier substituent at C-2 of galactose, i.e., 2'-fucosyllactose, bound neither the mutants nor the wt PNA (data not shown). This indicates that the E129D mutation allows the creation of space which is just enough for, and complementary to, the C-2 acetamido group of GalNAc.

Carbohydrate specificity of E129D and E129A

The Glu 129 mutants interacted with the polyclonal anti-PNA antibody and bound to immobilized asialofetuin like wtPNA. The carbohydrate specificity and binding affinities were, therefore, further examined by assaying the binding of the lectins to asialofetuin in the presence of varying concentrations of sugar inhibitors using a solid-phase microtiter plate assay. The results of the asialofetuin binding assay (Table IV) confirmed that the E129A and E129D mutations do not alter interactions at the C-3 and C-4 hydroxyls of the galactosides, as both mutants did not bind either mannose or glucose (data not shown). Most interestingly, E129D protein displayed a pronounced affinity for GalNAc which was on par with its affinity for Gal. This was in contrast to the behavior of wtPNA and E129A, both of which do not bind the former sugar even at concentrations as high as 100 µM. In general, however, both E129D and E129A exhibited weaker binding for most sugars indicating that Glu 129 has a distinct role in the recognition of galactosides by PNA. This role appears to be only partially or poorly accomplished by replacements with Asp or Ala, respectively. The presence of an asparatyl side-chain reduces the binding of most sugars by 2- to 4-fold, whereas an alanyl group in the same position affects the interaction much more drastically (Figure 2). The affinity toward galactosamine reduces to 30% and 6% of the level observed for wtPNA, when the glutamyl residue is replaced by the shorter Asp and the nonpolar Ala side-chains, respectively. This suggests that Glu 129 is involved in polar interaction(s) with the amino group of the sugar, which are perhaps mediated by the bound water molecule. Thus, while the asparatyl side chain in E129D could substantially maintain such polar contacts, the alanyl side chain in E129A may be leading toward their complete disruption. This could also explain the better affinity of this sugar relative to galactose toward wtPNA.

Table IV. Inhibition of the binding of Glu 129 mutant proteins to asialofetuin by various sugars

<table>
<thead>
<tr>
<th>Sugar used</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>Gal</td>
<td>3.84 ± 0.42</td>
</tr>
<tr>
<td>GalN</td>
<td>2.80 ± 0.25</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N.I.</td>
</tr>
<tr>
<td>Galβ1–4Glc</td>
<td>1.37 ± 0.18</td>
</tr>
<tr>
<td>Galβ1–3GalNAc</td>
<td>0.10 ± 0.01</td>
</tr>
</tbody>
</table>

Competition assays were done using fixed amounts (250 ng and 500 ng) of the lectin per well. Fucet–1–2Galβ1–4Glc was inactive even at 20 nM concentration in all of the cases.

These results also suggest that the improved binding of GalNAc by E129D is not only on account of the apparent removal of steric hindrance but, may also be facilitated by a favorable interaction between the acetamido group and the asparatyl side-chain. This polar contact, probably mediated through the acetamido group of GalNAc, is lost in E129A in which an alanine...
residue is at the equivalent position. Thus, while short contacts with the side chains of Glu 129 are substantially responsible for nonbinding of PNA to GalNAc, removal of the steric hindrance is not sufficient in itself to bring about exclusive GalNAc-specificity in legume lectins.

In summary, this study explores the role of the Glu 129 of PNA in the differential recognition of galactosides varying in their C-2 substituents, by this protein. Our results implicate this glutamic acid residue as a polar interacting partner to galactosamine and a steric obstructor for the binding of GalNAc. We have been able to successfully engineer GalNAc binding into PNA, which normally fails to recognize this sugar. In conjunction with earlier reports on the modification of the binding site of ECorL (Arango et al., 1993), this study suggests that both steric factors and bonding interactions determine the specificity of the Gal/GalNAc binding lectins towards the C-2 substituents. This work also provides a rational basis for the engineering of binding to other C-2 substituted galactosides through the incorporation of changes which could provide additional interactions with that substituent group.

Materials and methods

Materials

Restriction and modifying enzymes were purchased from Amer sham and New England Biolabs. [35 S]dATP was obtained from Amersham. Horseradish peroxidase conjugated to goat anti-rabbit IgG antibody was procured from Bangalore Genei, India. Fetuin, neuraminidase, and other chemicals were purchased from Sigma. The E. coli strains used for expression and mutagenesis were NM522, TG1, and CJ236.

Site-directed mutagenesis

Mutants were prepared by site-directed mutagenesis on an expression plasmid of PNA (Sharma and Surolia, 1994) using Kunkel’s method (Kunkel et al., 1987). Synthetic oligonucleotides 5′GGATCGTTGTAGTCACTGGGGA3′ and 5′GGATCTGTGTATGCTGGGAA–TAGGT3′ complementary to the sense strand with mismatches (shown underlined) at the nucleotides corresponding to Glu 129 of PNA, were used to generate the mutants E129D and E129A, respectively. The mutational changes are accompanied by the concomitant loss of a Rsal site in both the mutants. Mutations were confirmed by complete sequencing of the clones using the Sequenase (ver. 2.0) DNA sequencing kit. Standard recombinant DNA techniques (Sambrook et al., 1989) were utilized unless otherwise indicated.

Purification of wild-type and mutant PNAs

Wild-type and mutant PNAs were expressed as described previously (Sharma and Surolia, 1994; Sharma et al., 1996). Purification of guanidine-solubilized protein under denaturing conditions using the His affinity tag on Ni2+ -NTA resin, was performed prior to refolding. Lectins thus purified appeared as single peaks corresponding to the position of the PNA tetramer when subjected to gel filtration.

Hemagglutination assays

Hemagglutination and inhibition assays were performed as described previously (Sharma and Surolia, 1994). MHC (minimum concentration of lectin giving complete hemagglutination) was used to compare lectin activities and MIC (minimum concentration of sugar required for complete hemagglutination inhibition by 3 µg/ml of lectin) for the sugars were compared.

PNA- asialofetuin binding assays

Binding assay was as described earlier for the 14 kDa β-galactoside-binding human lectin (Hirabayashi and Kasai, 1991) and PNA mutants (Sharma et al., 1996). The Costar serocluster EIA plate was coated with asialofetuin (20 µg/well) overnight at 4°C. After blocking the wells with 1% BSA, the lectins (500 ng/well) were allowed to interact for 1 h at room temperature in the presence of varying concentrations of different carbohydrate inhibitors. The bound lectin was quantified by a double antibody method using anti-PNA IgG as the primary and goat anti-rabbit IgG conjugated to horseradish peroxidase (Bangalore Genei, India) as the secondary antibody. Each step was followed by three washes with TBS and after the final wash the plates were developed using TMB/H2O2 as substrate and read at 450 nm on BIOTEK EL 311 ELISA reader. The concentration of sugar required for 50% inhibition (IC50) was calculated, taking the quantity of lectin bound in the absence of inhibitor as 100%. The values of IC50 reported in the text and in Table IV are averages of three independent experiments.

Acknowledgments

This work has been supported by a grant from the Department of Biotechnology, Government of India to A.S. by the University Grants Commission (P.A. is a research associate in the above grant). V.S. has been a Senior Research Fellow supported by University Grants Commission, India.

Abbreviations

DBL, Dolichos biflorus lectin; ECorL, Erythrina corallodendron lectin; Gal, galactose; GalNAc, N-Acetyl galactosamine; LBL, lima bean (Phaseolus lunatus) lectin; MHC, minimum concentration of the lectin that gives complete hemagglutination; MIC, minimum concentration of the saccharide required for complete hemagglutination inhibition; N.L., no inhibition; PNA, peanut agglutinin; SBA, soybean (Glycine max) agglutinin; WBA I, winged bean (Psophocarpus tetragonolobus) basic agglutinin; T-antigen, Thomsen-Friedenrich antigen (Galβ1–3GalNAc); Tn-antigen, GalNAcβ1–O-Ser/Thr; TMB, tetramethyl benzidine; rPNA, recombinant PNA; wtPNA, wild type PNA. Mutant proteins are designated by giving the wild type amino acid and its replacement (one letter code), for instance, E129D is PNA in which Glu 129 is replaced by aspartic acid.

References


