Imparting Exquisite Specificity to Peanut Agglutinin for the Tumor-associated Thomsen-Friedenreich Antigen by Redesign of Its Combining Site*

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Lectins from legumes constitute one of the most thoroughly studied families of proteins, yet the absence of a rigorous framework to explain their carbohydrate binding specificities appears to have prevented a rational approach to alter their ligand binding activity. Studies reported here deal with the redesign of the recognition propensity of peanut agglutinin (PNA), an important member of the family. PNA is extensively used as a tool for recognition of the tumor-associated Thomsen-Friedenreich antigen (T-antigen; Galβ1-3GalNAc) on the surfaces of malignant cells and immature thymocytes. PNA also recognizes N-acetyllactosamine (LacNAc; Galβ1-4GlcNAc), which is present at the termini of several cell-surface glycoproteins. The crystal structure of the PNA-lactose complex revealed, in addition to the expected interactions with the residues constituting the binding site, the presence of leucine 212 at a position close enough to be in steric contact with the acetamido group on LacNAc. We report here two leucine mutants, one to asparagine (L212N) and the other to alanine (L212A), that exhibit distinct preference for T-antigen and N-acetyllactosamine, respectively. Carbohydrate binding studies reveal that mutant L212N does not recognize LacNAc at high concentrations, thus making it an exquisitely specific cell-surface marker compared with its wild-type counterpart.

Recognition of a variety of cell-surface carbohydrates by lectins has been widely implicated in important biological processes such as protein targeting to cellular compartments, homing of lymphocytes, host-pathogen interactions, and fertilization (1–4). The ability of plant lectins to detect subtle variations in carbohydrate structures found on cell-surface glycoproteins, it contributes to the residual but significant binding of PNA at the cell surface (24). Several other T-antigen-binding lectins (22, 23, 25) and antibodies (26) currently used exhibit, at best, equivalent or poorer specificity for it, thus necessitating an approach to improvise its specificity by structure-based engineering.

EXPERIMENTAL PROCEDURES

Materials—Restriction and modifying enzymes were purchased from Amersham Corp. and New England Biolabs Inc. [35S]dATP was from Amersham Corp. Horseradish peroxidase conjugated to goat anti-rabbit IgG antibody was procured from Bangalore Genei (Bangalore, India). Fetuin, neuraminidase, and other chemicals were purchased from Sigma. The Escherichia coli strains used for expression and mutagenesis were NM522, TG1, and CJ 236.

Site-directed Mutagenesis—The mutants were prepared by site-directed mutagenesis of the expression plasmid of PNA (27) using the method of Kunkel et al. (28). The synthetic digonucleotides 5′-ACGAC-CCCAATTGAGGCGGA-3′ and 5′-ATCGAGACCAGCCCGAGGAGCGGAGGC-3′, complementary to the sense strand with mismatches (shown underlined) at the nucleotides corresponding to Leu-212 of PNA, were used to generate mutants L212N and L212A, respectively. The changes result in the concomitant loss of a Styl site in both mutants and the appearance of a new Nael site in the L212A mutant. Mutations were confirmed by complete sequencing of the clones using the Sequenase DNA sequencing kit (Version 2.0) from U. S. Biochemical Corp. Standard recombinant DNA techniques (29) were utilized if not otherwise indicated.

Purification of Wild-type and Mutant PNAs—Wild-type and mutant

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The atomic coordinates (code 2PEL) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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1 The abbreviations used are: PNA, peanut agglutinin; Lac, lactose (Galβ1-4Glc); LacNAc, N-acetyllactosamine (Galβ1-4GlcNAc); [35S]dATP, [35S]deoxyadenosinetriphosphateS.
PNAs were expressed as described previously (27), except that purification of guanidine-solubilized protein under denaturing conditions using the His.Tag affinity on Ni\(^{2+}\)-nitrilotriacetic acid resin preceded refolding. The lectin thus purified appeared as a single peak corresponding to the molecular mass of tetrameric PNA when subjected to gel filtration.

Hemagglutination Assays—Hemagglutination and inhibition procedures were as described previously (27). The minimum concentration of the lectin that gave complete hemagglutination was used to compare the lectin activity, and the minimum concentrations of the saccharide required for complete hemagglutination inhibition by 3 \(\mu\)g/ml lectin for the sugars were compared.

PNA-Asialofetuin Binding Assays—The binding assay was as described earlier for the 14-kDa \(\beta\)-galactoside-binding human lectin (30). The Costar serocluster enzyme-linked immunosorbent assay plate was coated with asialofetuin (20 \(\mu\)g/well) overnight at 4°C. After blocking the wells with 1% bovine serum albumin, 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 antibody and goat anti-rabbit IgG conjugated to horse-radish peroxidase as the secondary antibody. Each step was followed by three washes with Tris-buffered saline, and after the final wash, the plates were developed using tetramethyl benzidine/H\(_2\)O\(_2\) as substrate and read at 450 nm on a BIOTEK EL 311 enzyme-linked immunosorbent assay reader. The concentration of sugar required for 50% inhibition (IC\(_{50}\)) was calculated, taking the quantity of lectin bound in the absence of inhibitor as 100%. The IC\(_{50}\) values reported below and in Table II are averages of three independent experiments.

RESULTS AND DISCUSSION

Molecular Recognition of Carbohydrate by PNA—The crystal structure determination of PNA by us (31) revealed that the four identical subunits have the same jelly-roll tertiary structure, but that they associate in the most unusual fashion, giving rise to an open quaternary structure unknown in any tetrameric protein thus far. The complex of the lectin with lactose, now available at a high resolution (32), shows that the interaction is mediated through nine hydrogen bonds (Fig. 1) with amino acid residues in the four loops constituting the carbohydrate-binding site of legume lectins. These include Asp-83, Gly-104, and Asn-127, which are highly conserved in legume lectins, as well as Asp-80, Ser-211, and Gly-213, which are unique to PNA. In addition, the conserved Tyr-125 stacks against the terminal galactose ring. Yet another invariant residue is Ala-82, the peptide bond succeeding which adopts a cis-conformation in all legume lectins. This is known to be crucial for properly orienting Asp-83, which is a key binding site residue. Even a conservative replacement at most of these
positions drastically affects the sugar binding of PNA. In the absence of the x-ray structures of PNA complexed with either LacNAc or T-antigen, we analyzed the PNA-lactose complex to identify those residues around the binding pocket that do not make direct contact with lactose, but could bind to LacNAc or T-antigen. One such residue is Leu-212, the side chain of which extends at the brim of the variable edge of the binding pocket in PNA. The backbone atoms of the loop carrying Leu-212 are close to the glucopyranoside ring of the lactose in the structure. The 2'-OH of this ring, the position at which Lac differs from LacNAc by the absence of bulky N-acetamido group, does not make any contact with the lectin, but is 4.1–4.4 Å from the atoms of the Leu-212 side chain. This implies that the leucyl side chain would be in close proximity to the acetamido group on binding to LacNAc. The accommodation of this large group on binding to LacNAc would therefore necessitate conformational rearrangement of the loop containing Leu-212. This could involve displacement of the loop away from the GlcNAc ring of LacNAc, facilitated by the conformational flexibility of two consecutive glycines after Leu-212 with the consequent restructuring of the hydrogen bonds. The possible sacrifice of the interaction between the backbone amide of Gly-213 and the 3'-OH in the process could in effect or partly explain the observed lower affinity of PNA for LacNAc in comparison with that for Lac (34). The additional hydrophobic stabilization that might accrue due to interactions between the acetamido group and the leucyl side chain might not be large enough to compensate for the disruption referred to above. The conformation of the nonreducing sugar residue in LacNAc (or Lac) and T-antigen is identical. However, due to differences in their glycosidic linkages (β1-4 in LacNAc compared with β1-3 in T-antigen), the orientation of the reducing sugar residue with respect to the nonreducing one is not the same in these disaccharides (Fig. 2). Consequently, the C-2 substituent of the reducing sugar residue of LacNAc occupies a position equivalent to that of the hydroxymethyl (6'-OH) group in the corresponding sugar residue of T-antigen. Thus, T-antigen would require neither a large conformational readjustment of the binding site nor a hydrophobic environment in this region. The higher affinity of T-antigen, known to be facilitated by the presence of an acetamido group, is perhaps determined by additional interactions with a different region of the binding pocket.

The initial analysis of the combining site in the PNA-lactose complex clearly implicated Leu-212 as a residue with significant proclivity to discriminate among the disaccharides binding to PNA. Any alteration in Leu-212 would seem to influence only the substituents at the 2'-OH of Lac or the 6'-OH of T-antigen. A smaller aliphatic amino acid instead of leucine would seem to prefer the binding of sugars with the bulkier acetamido group in the above positions, whereas a longer hydrophilic residue in the same place would prohibit substitution by large nonpolar groups. Therefore, we decided to characterize the two mutants generated by replacing Leu-212 with alanine and asparagine in bacterially produced PNA (27). Alanine was an obvious choice for exploring the steric role of the longer leucyl side chain, and asparagine, being a hydrophilic residue closest in size to leucine, was ideal for studying the influence of the hydrophobic environment.

**Lectin Activity of Mutant PNAs—** Upon SDS-polyacrylamide gel electrophoresis, the purified L212A and L212N mutants showed a single band that comigrated with recombinant PNA. They exhibited similar hemagglutinating activity compared with wild-type PNA, as shown in Table I. The mutations did

![Table I](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1067093/figure/t01.jpg)

| MHC a | Gal | GaIN | Lac | LacNAc | T-antigen |
|-------|-----|------|-----|--------|-----------|
| Wild-type | 0.30 | 20 | 12.5 | 6.25 | 25.0 | 0.62 |
| L212N  | 0.40 | 20 | 12.5 | 3.125 | NI at 100 | 0.62 |
| L212A  | 0.25 | 20 | 12.5 | 6.25 | 3.125 | NI at 10 |

* MHC, minimum hemagglutination concentration; MIC, minimum inhibitory concentration; NI, no inhibition.

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2 V. Sharma and A. Surolia, unpublished observations.
Redesigning PNA Specificity

Table II

Inhibition of PNA binding to asialofetuin by saccharides

| IC50          | Wild-type | L212N | L212A | Wild-type/mutant ratio |
|---------------|-----------|-------|-------|------------------------|
|              | mM        |       |       |                        |
| Gal           | 3.84 ± 0.42 | 3.70 ± 0.38 | 3.80 ± 0.54 | 1.04                   |
| Galβ1–4Glc    | 1.37 ± 0.18 | 0.42 ± 0.05 | 1.44 ± 0.15 | 3.26                   |
| Galβ1–4GlcNAc | 5.66 ± 0.49 | N.I. at 80 | 0.70 ± 0.12 | 8.08                   |
| Galβ1–3GlcNAc | 0.10 ± 0.01 | 0.09 ± 0.01 | 20 (35%)b   | 1.11                   |

a N.I., no inhibition.
b 35% inhibition.

not affect the affinity of the lectin for sialylated T-antigen or galactose, which was evident from their ability to agglutinate only desialylated rabbit erythrocytes, which have the terminal galactose residues exposed on the surface. Hemagglutination inhibition experiments confirmed that the affinity of lectins for monosaccharides like galactose and galactosamine is impervious to the alterations in Leu-212, whereas it varies for the disaccharides lactose and T-antigen (Table I). This is expected as Leu-212 is too far from the primary binding site to influence the binding. The hemagglutination inhibition experiments indicated an improved potency of lactose to inhibit L212N, while LacNAc and T-antigen were inactive toward L212N and L212A, respectively. Both mutants reacted with the polyclonal anti-PNA antibody and bound to immobilized asialofetuin, as did wild-type PNA. The carbohydrate specificity and binding affinities were therefore further examined by assaying the binding of the lectins to asialofetuin in the presence of varying amounts of sugar inhibitors using a solid-phase microtiter plate assay (Fig. 3). The data were normalized with respect to the values obtained in the absence of sugar. All the sugars that gave complete inhibition exhibited a sigmoidal curve for the percentage binding as a function of the log of the sugar concentration. The concentration at which 50% inhibition was achieved (IC50) was used to compare the binding affinities of the different sugars.

Carbohydrate Specificity of L212N and L212A—The results of the asialofetuin binding assay (Table II) confirmed that the mutations of Leu-212 do not alter the affinity for monosaccharide ligands such as galactose. Most interestingly, mutant L212N does not bind LacNAc at concentrations as high as 80 mM, i.e. even at 20 times the IC50 of wild-type PNA. The presence of the asparaginyl side chain, however, seems to improve the binding to lactose, which then had a 3-fold lower IC50.

T-antigen, which binds wild-type PNA with an IC50 of ~0.1 mM, exhibits a comparable affinity (IC50 = 0.09 mM) for L212N. The increase in the binding constant for lactose suggests improved association with the lectin, probably mediated by additional interaction between the asparaginyl side chain and the 2'-OH of the glucopyranoside ring. Following the same rationale, non-binding of LacNAc is apparently a result of disruption of the favorable interaction between its acetamido group and the hydrophobic side chain of leucine by the asparagine residue at the equivalent position. The other mutant, L212A, shows pronounced affinity for LacNAc, with the IC50 value improving by 8-fold in contrast to that for lactose, which is not altered appreciably. Thus, the replacement of leucine by an amino acid with a shorter aliphatic chain (alanine) facilitates the binding of LacNAc, while its substitution by a polar residue of nearly equivalent size (asparagine) compromises the binding. Surprisingly, however, L212A interacts very poorly with T-antigen, and we failed to observe >35% inhibition even at concentrations as high as 20 mM. Considering that wild-type PNA has an IC50 of 0.1 mM, the mutation L212A leads to >100-fold deterioration of the binding affinity of the lectin for the T-hapten. This underlines the subtle differences in the binding of lactose and T-antigen with PNA. Moreover, both mutants did not interact with either GalNAc (100 mM) or sialylated T-antigen (NeuAc2–3Galβ1–3GalNAc and NeuAc2–3Galβ1–3NeuAc2–6GalNAc, at 5 mM each). Since fetuin contains both N- and O-linked chains, specificities of the mutant and wild-type PNAS were also examined by their reactivities toward asialo-orosomucoid (α-acid glycoprotein), which contains only N-linked glycan. An equimolar concentration of asialo-orosomucoid exhibits about 48, 0–5, and 157% activity compared with fetuin for wild-type PNA, L212N, and L212A, respectively. This is qualitatively consistent with the ligand inhibition data, further confirming the enhancement in the anti-T-antigen specificity of L212N and that of L212A for N-acetyllactosamine. Thus, it appears that Leu-212 is a residue at a position critical for discriminating the carbohydrate recognition in PNA. The two mutants L212N and L212A exhibit differential and almost complementary specificity for the two PNA ligands (Fig. 4). The L212N mutant shows no binding to LacNAc and displays exquisite T-antigen specificity, while L212A interacts extremely poorly with T-antigen and recognizes LacNAc with greater efficacy.

In conclusion, we have been able to modify the specificity of PNA for LacNAc and T-antigen by rational site-directed mutagenesis. Our data show that, by subtle manipulations in the more hypervariable loop (7) of the binding site of a legume lectin, one could achieve meaningful alterations in specificity and that residues other than the ones that make primary contact with the saccharide are important in influencing the lectin specificity. Owing to their ability to differentiate between complex carbohydrates based on subtle variations, plant lectins
are extensively used as probes for cell-surface receptor sites. It is for this reason that the plant lectins, although first used as tools in the beginning of the century, continue to play a central role in immunology, cancer biology, etc., and their importance has not been affected by the advent of newer technologies. Our results give us grounds to believe that engineering lectins to further improve specificities would lead to the development of better tools for carbohydrate recognition.

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