Glycan binding profile of a fucolectin-related protein (FRP) encoded by the SP2159 gene of *Streptococcus pneumoniae*

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Abstract

The recombinant fucolectin-related protein (FRP) of unknown function, encoded by the SP2159 gene of *Streptococcus pneumoniae*, was expressed in *E. coli*. In this study, its glycan-recognition epitopes and their binding potencies were examined by enzyme-linked lectinosorbent and inhibition assays. The results indicate that FRP reacted strongly with human blood group ABH and L-Fuc α1/2-active glycotopes and in their polyvalent (super) forms. When expressed by mass relative potency, the binding affinities of FRP to poly-L-Fuc α1/2 glycotopes were about $5.0 \times 10^5$ folds higher than that of the mono-L-Fuc α1/2 glycan form. This unique binding property of FRP can be used as a special tool to differentiate complex forms of L-Fuc α1/2 and other forms of glycotopes.

Keywords: Fucolectin-related protein; Glycotopes; Polyvalency; Glycan recognition epitopes

1. Introduction

*Streptococcus pneumoniae* commonly found in human nose and throat is the major organism to cause pneumonia, sepsisemia, meningitis and otitis media [1]. These diseases result in millions of deaths worldwide each year. Virulence of *S. pneumoniae* is associated with capsular polysaccharides, cell wall, and/or membrane components. For example, the encapsulated smooth strain of *S. pneumoniae* has been shown to kill mice in DNA transformation studies that resulted in the discovery of hereditary material [2]. *S. pneumoniae* is known to produce a hemolysin that causes incomplete hemolysis (α-hemolysis) and to express bacterial surface lectins that facilitate adhesion to epithelial cells of the respiratory tract [3].

Through our studies of the blood group A and B-cleaving endo-β-galactosidase (E-ABase) of *Clostridium perfringens*, we have shown that E-ABase shares 34% sequence identity with a fucolectin-related protein (FRP) of unknown function encoded by the SP2159 gene of *S. pneumoniae* ATCC BAA-334 [4,5]. The recombinant FRP encoded by SP2159 gene and expressed in *E. coli* contains 1038 residues of amino acids with a molecular mass of 117.74 kDa [4]. In order to decipher the biological function of this FRP, its binding profile was analyzed using our established enzyme-linked lectinosorbent assay (ELLSA) and inhibition assay [6] using a series of mammalian monomeric glycotopes and their natural polyvalent forms. Our results showed that: (i) FRP bound avidly with human blood group A, B, H, and L-Fuc α1-2-active glycans; (ii) L-Fuc α1-2 related glycotopes and their polyvalent (super) forms are the two essential recognition elements. The high density of L-Fuc α1-2 related glycotopes, expressed as mass relative potency, were about $5.0 \times 10^5$ folds more potent.
than their monomeric \(1\)-Fuc\(\alpha\) \(\rightarrow\) glyctope; (iii) the recognition site of FRP should be a combination of a cavity shape as the major site to hold the most active \(1\)-Fuc\(\alpha\) \(\rightarrow\) 2 residue and a broad groove shape as subsite for adopting the other part of the glyctope for enhancement, such as \(1\)-Fuc\(\alpha\)-1 \(\rightarrow\) 2Gal\(\beta\)-1 \(\rightarrow\) 4(\(1\)-Fuc\(\alpha\)-1 \(\rightarrow\) 3)Glc glyctope (LDFT); (iv) hydrophobicity is important for both the \(\alpha\) and \(\beta\) anomers of \(1\)-Fuc-complex; (v) by comparing the FRP lectin with the other two \(1\)-Fuc\(\alpha\)-1 \(\rightarrow\) 2 specific lectins, *Pseudomonas aeruginosa*-II Lectin (PA-IIIL) [7] and *Anguilla anguilla* agglutinin (AAA) [8], the unique potency of FRP recognition factors are defined. In this report, we have upgraded the classical concept of lectin-glycan interactions from the mono- or oligo-glyctope level to a complex polyvalent (super)-glyctopes level. The unique binding property of FRP can be used as a special tool to distinguish the complex forms of \(1\)-Fuc\(\alpha\) \(\rightarrow\) 2 and other forms of glyctopes.

2. Materials and methods

2.1. Lectin preparation and biotinylation

Isolation of the *Streptococcus pneumoniae* SP2159 gene encoding FRP and the expression of FRP in *E. coli* were based on the procedures described previously [4]. Biotinylation of FRP was carried out according to the method of Duk et al. [6].

2.2. Super glyctopes containing glycoproteins and polysaccharide

\(ABH\) and \(1\)-Fuc\(\alpha\)-1 \(\rightarrow\) 2 blood group active glycoproteins and the sialic acid-containing glycoproteins were prepared from human ovarian cyst fluid [9–13]. The glycan chains of the cyst gps consist of multiple bioactive saccharide branches attached via O-glycosidic linkages at their reducing ends to serine or threonine residues of the polypeptide backbone [11,12].

Blood group precursor gps (P-1) from cyst gps were prepared by mild acid hydrolysis at pH 1.5, 100 °C for 2 h and/or by Smith degradation to remove most of the blood group key sugars (\(1\)-Fuc\(\alpha\)-1 \(\rightarrow\) 2, GalNAc\(\alpha\)-1 \(\rightarrow\) 3 and Gal\(\alpha\)-1 \(\rightarrow\) 3) [14–17].

Mammalian salivary glycoproteins from ovine (OSM), bovine (BSM), porcine (PSM) submandibular/glands and rat sublingual gland (RLS), were purified according to the method of Tettamanti and Pigman [18] and Herp et al. [19]. Their asialo products were prepared by mild acid hydrolysis at pH 2.0, 80 °C for 90 min and dialyzed against water to remove the released sialic acid.

Glycophorin and asialo-glycophorin were provided by Duk et al. [6]. The \(\text{Tn}\)-type glycophorin (GalNAc\(\alpha\)-1-Ser/Thr) was prepared from asialo-glycophorin by periodate oxidation and mild acid hydrolysis (Smith degradation) [6].

*Pneumococcus* type XIV polysaccharide was a gift from the late B. Lindberg [20] through the late E. A. Kabat [14]. The Human \(\alpha\)-acid gp, poly-2,8-N-acetylneuraminic acid capsular polysaccharide from *Escherichia coli* and mannan from *Saccharomyces cerevisiae* were purchased from Sigma (St. Louis, MO, USA).

Fig. 1 shows several FRP active glyctopes of blood active glycoproteins prepared from human ovarian cyst fluid.

2.3. Monosaccharides and oligosaccharides

Monosaccharides and oligosaccharides were purchased from Sigma Chemical Company (St. Louis, MO, USA) and Dextra (Berkshire, UK).

2.4. Lectin-enzyme binding assay on microtiter plate

The lectin-enzyme binding assay was performed according to the procedures of Duk et al. [6]. The volume of each reagent applied to the plate was 50 \(\mu\)l/well, and all incubations were performed at room temperature (20 °C) except for coating at 4 °C. The reagents, if not indicated otherwise, were diluted with Tris-buffered saline (TBS, 0.05 M Tris, 0.15 M NaCl, pH 7.35) in 0.05% Tween 20 (TBS-T buffer). The TBS-T buffer was also used for washing the plate between changing the reagents. For examining the effect of \(\text{Ca}^{2+}\), \(\text{Mg}^{2+}\) and EDTA on the FRP binding, 2 mM of \(\text{Ca}^{2+}\), \(\text{Mg}^{2+}\) and EDTA were added to the 1st testing well and followed by sequential twofold dilution. The well without adding \(\text{Ca}^{2+}\), \(\text{Mg}^{2+}\) or EDTA served as a control.

For inhibition studies, the twofold serially diluted inhibitor was mixed with an equal volume of solutions containing a fixed amount of FRP. The reference lectin inhibitor was also diluted in the same manner. After incubation at room temperature for 1 h, the binding of FRP to the plate coated with an ovarian cyst gp that contained blood group \(H\) \(1\)-Fuc\(\alpha\)-1 \(\rightarrow\) 2Gal and \(1\)-Fuc\(\alpha\)-1 \(\rightarrow\) 3/4GlcNAc glyctopes was determined as described above [6]. The inhibitory activity was estimated from the inhibition curve and is expressed as the amount of inhibitor (ng/well for glycoprotein or nmole/well for small saccharides) giving 50% inhibition of the FRP binding as compared to that without the inhibitor.

3. Results

3.1. FRP-glycan interaction

The binding intensities of FRP with various glycans, according to the interaction profiles obtained, are shown in Fig. 2. Among the glycoproteins tested, FRP reacted best with all human blood group \(A\), \(B\), \(H\) \(1\)-Fuc\(\alpha\)-1 \(\rightarrow\) 2-active glycoproteins. Less than 0.6 ng each of Ovarian Cyst Medon, Ovarian Cyst Beach Phenol Insoluble, or Ovarian Cyst JS Phenol Insoluble was required to interact with 10 ng of FRP lectin to yield an absorbance (A405) value of 1.5 within 4 h. While Ovarian Cyst Medon and Ovarian Cyst JS 1st Smith Degraded were not binding FRP. Other blood group precursor glycoproteins and polysaccharides exhibited very weak inhibition and gave A405 values less than 0.2. These weak inhibitors were both the sialyl- and asialyl-glycoproteins of HOC 350, human \(\alpha\)-acid glycoprotein, human glycophorin, OSM, PSM and asialofetuin. To prove that the different affinities between FRP and glycoproteins were not due to the
variation in the plate adsorption, the binding intensity of each glycoprotein was further examined by an inhibitory assay described in Section 3.2.

### 3.2. Inhibition of FRP-glycan binding by various polyvalent glycotopes (super glycotopes or glycoproteins)

Fig. 3 shows the inhibitory potencies of various inhibitor glycoproteins toward the binding between FRP and Ovarian Cyst JS Phenol Insoluble glycoprotein contained human blood group H 1-Fucα1→2Gal disaccharide. Among the glycotopes tested, the Cyst JS Phenol Insoluble glycoprotein was the most potent inhibitor. It was $5.0 \times 10^5$ times more active than 1-Fuc monosaccharide and $6.3 \times 10^5$ more active than the inhibition by H 1-Fucα1→2Gal disaccharide. The FRP-glycan interaction was also strongly inhibited by Cyst 14 Phenol Insoluble that contained Galβ1→3Galβ1→3Galβ1→3GalNAcα1→Ser/Thr.

#### Fig. 3. Proposed FRP active glycotopes (shaded) of blood active glycoproteins prepared from human ovarian cyst fluid [11, 12].

As shown in Fig. 2 and 3, CystJS phenol insoluble (H, Leα/Leβ) Cyst Mcdon (Aα and Leα), Cyst 14 phenol insoluble (Aα/Leα) and Cyst Beach phenol insoluble (B Leα) are rich in glycotopes (shaded) for FRP binding. The “h” in Aα and B, indicating in active crypto H (1-Fucα1→2).

![Fig. 1. Proposed FRP active glycotopes (shaded) of blood active glycoproteins prepared from human ovarian cyst fluid [11, 12].](image1)

![Fig. 2. The ELLSA of FRP binding affinities with various glycoproteins](image2)
3.3. Inhibition of FRP-glycan binding by mono- and oligosaccharides

In order to further probe glycotopes in FRP molecule, various known mammalian structural units and monosaccharide derivatives were used to test the inhibition toward the binding of FRP and the human blood group \( H \), \( L \)-Fuc\( ^{1/2}Gal \)- and \( L \)-Fuc\( ^{1/3/4}GlcNAc \)-active Ovarian Cyst JS Phenol Insoluble glycoprotein. Table 1 lists the inhibitory potencies as Molar Relative Potencies for various simple glycans.

Among the monosaccharides and their derivatives, \( p \)-nitrophenyl-\( \beta \)-L-Fuc (Table 1 Sample 1) was the most potent inhibitor, which was twice more active than \( p \)-nitrophenyl-\( \alpha \)-L-Fuc (Sample 2) and 69.1 times more active than the free \( L \)-Fuc (Sample 8). The \( \beta \)-anomer of \( L \)-Fuc was preferred over

Table 1
The inhibitory potencies of oligosaccharides and monosaccharides toward the binding of FRP and Ovarian Cyst JS Phenol Insoluble glycoprotein.

| Sample No. | Sample name | 1\_Fuc as 1.0 | 1\_Fuc\( ^{1/2}Gal \) as 1.0 |
|------------|-------------|---------------|-----------------------------|
| 1          | \( p \)-Nitrophenyl-\( \beta \)-Fuc | 69.1           | 91.1                        |
| 2          | \( p \)-Nitrophenyl-\( \alpha \)-Fuc | 34.5           | 46.5                        |
| 3          | Methyl-\( \beta \)-Fuc | 21.9           | 29.2                        |
| 4          | \( L \)-[Fuc\( ^{1/2}Gal \)\( ^{1/3}Gal \)\( ^{1/4}Glc \)] (LDFT, \( Le^c \)) | 5.7            | 8.2                         |
| 5          | Methyl-\( \alpha \)-Fuc | 4.4            | 5.8                         |
| 6          | \( L \)-Fuc | 4.4            | 5.8                         |
| 7          | \( L \)-[Fuc\( ^{1/2}Gal \)\( ^{1/3}Gal \)\( ^{1/4}Glc \)] (LNDFH I, \( Le^b \)) | 1.8            | 2.6                         |
| 8          | \( L \)-Fuc | 1.0            | 1.3                         |
| 9          | \( L \)-[Fuc\( ^{1/2}Gal \)] (\( H \)) | 0.7            | 1.0                         |
| 10         | \( p \)-Ara | 0.09           | 0.12                        |
| 11         | \( p \)-GalNAc | 0             | 0                           |
the α-anomer in both p-nitrophenyl and methyl glycosides (Samples 1 versus 2; 3 versus 5). Moreover, both α- and β-derivative of p-nitrophenyl-1-Fuc were more active than the methyl-α- and β-analogue (Samples 1 and 2 versus 3 and 5). This indicated the presence of probable apolar nature of FRP protein around the vicinity of the saccharide-binding site.

Among the oligosaccharides tested, the glucose containing oligosaccharide 1-1-Fucα1→2-Galβ1→3(1-1-Fucα1→3) Glc (Sample 4 LDFT) was the best inhibitor. It was about three times as active as Fucα1→2Galβ1→3(Fucα1→4) GlcNAcβ1→3Galβ1→4Glc (Sample 7. LNDFH I) and 8 times more active than H disaccharide (1-1-Fucα1→2Gal) (Sample 9).

With the same pentose structure, d-Ara (Sample 10) was 12.5 times less potent than 1-1-Fuc. No inhibition of FRP binding was observed with the following monosaccharides: d-Man (2.7 \times 10^4 \text{ nmol}), 1-1-Rhamnose (1.5 \times 10^4 \text{ nmol}), d-Fuc (2.3 \times 10^4 \text{ nmol}), d-Gal (2.2 \times 10^4 \text{ nmol}), methyl α-1-Rhamnoside (1.4 \times 10^4 \text{ nmol}), d-Glc (2.1 \times 10^4 \text{ nmol}), and d-GalNAc (7.4 \times 10^4 \text{ nmol}).

3.4. Effect of Ca^{2+}, Mg^{2+} and EDTA on the reactivity of FRP in the binding assay

Metal ions such as 2 mM of Ca^{2+} or Mg^{2+} or ion chelator with 2 mM EDTA in a final volume of 50 μl Tris-buffered saline containing 10 ng FRP did not affect the binding activity of FRP-glycan interaction. This indicates that the lectin does not require metal ions for binding.

4. Discussion

Prior to encounter with FRP, we had cloned and studied an endo-β-galactosidase, E-ABase from Cl. Perfringens [4], that could efficiently cleave off the terminal trisaccharide GlcNAcα1-3(Fucα1-2)-Gal from human blood group A glycoproteins and Galα1-3(Fucα1-2)-Gal from group B glycoproteins. The FRP encoded by S. pneumoniae SP2159 gene called our attention because, after our survey of the gene bank, SP2159 might be a related endo-α-β-galactosidase of S. pneumoniae. However, we found that the recombinant FRP did not have any glycosidase activity. Our current analysis on the glycan binding activity of FRP clearly showed that FRP is a 1-1-Fucα1-recognizing lectin that has high affinity toward human blood group A, B, and H determinants and cell surface glycoproteins. This specific affinity of FRP toward numerous cell surface glycans may provide an effective tool for S. pneumoniae to adhere onto the cell surfaces and gain entrances to infect human and other animals.

Over the past few decades, fucose-binding lectins in various living organisms from plants (UEA-I of Ulex europaeus) [21], animals (Anguilla anguilla agglutinin from eel) [8,22], and micro-organisms Pseudomonas aeruginosa PA-III [7,23] and Alueria aurantia AAL [24], have been reported. Owing to their 1-1-Fucα1→ specificity, these lectins are widely used as reagents in hematological and glyco-biological studies [25–28]. The investigations on human blood group determinants [9–11] showed that 1-1-Fucα1→glycophorins in human blood group chemistry are very complex. Factors that can affect a specific blood group determinant to exhibit different binding potencies toward specific lectins are: (i) The density of the 1-1-Fucα1→glycophorins in the saccharide moiety; (ii) the location of the 1-1-Fucα1→glycophorins, whether it is at the terminal or at the penultimate position of the saccharide chain; and (iii) the 1-1-Fucα1→glycophorins linking to the next saccharide, such as Gal, Glc or GlcNac. In order to be used as a tool for biomedical and immunochemical studies, their recognition patterns should be established. There has been no report to compare the reactivity of all known lectins to bind various glycoproteins or oligosaccharides under a set of standard condition. In this study, we analyzed the binding potencies of FRP toward a collection of glycoproteins, oligosaccharides and mono-saccharides under the same conditions as that performed for assaying Anguilla anguilla agglutinin (AAA) [8]. Therefore, we can make a direct comparison of the lectin potencies between FRP and AAA. Although both FRP and AAA lectins recognize 1-1-Fucα1→2Gal-glycophorins, AAA had the highest potency toward the binding of hog gastric mucin #4 and #9 glycoproteins [8] and FRP had the highest affinity toward the binding of ovarian cyst glycoproteins as shown in Fig. 2 (See Fig. 1 for the structures of A/Le^a, B/Le^a and A). Unlike the ovarian cyst glycoproteins, the hog gastric mucins #4 and #9 showed much weaker binding affinity to FRP (Fig. 2). These results suggest that FRP can recognize and interact much stronger than AAA toward the polyvalent form of 1-1-Fucα1→2Gal-glycophorins. This property of FRP can be used as a special reagent to detect the presence of the polyvalent glycoproteins.

Among the oligosaccharide ligands tested for the inhibition of the binding between FRP and ovarian cyst phenol insoluble glycoprotein, the glyco-analogue of the LDFT, Le^a hapten [Fucα1→2Galβ1→4(Fucα1→3) Glc] (Table 1, Sample 4) was the best inhibitor. This oligo-saccharide having poly 1-1-Fucα1→2Gal-glycophorins exhibited 5.7 times higher inhibitory potency (molar relative potency) than the monosaccharide 1-1-Fucα1→ (Table 1, Sample 8) and 8 times higher inhibitory potency than the monomeric 1-1-Fucα1→glycophorins (Table 1, Sample 9). These results suggest that FRP from S. pneumoniae efficiently interacted with and bound to the polyvalent 1-1-Fucα1→glycophorins on the saccharide moieties of mammalian cell surface glycoproteins.

The binding profile of FRP is similar to those of two other 1-1-Fuc specific lectins, Anguilla anguilla agglutinin (AAA) [8,19] and Pseudomonas aeruginosa-II Lectin (PA-III) [7], but the roles of polyvalent enhancement to these lectins are very different [7,8,28]. FRP resembles AAA and PA-III in its affinity toward 1-1-Fucα1→ related blood group active glycoproteins. However, FRP showed unique features that are different from PA-III and AAA lectins: (i) The affinity enhancement by polyvalent glycoproteins for FRP can be up to 5.0 \times 10^5 times over the monomeric 1-1-Fucα1→2Gal, while that for PA-III [7] and AAA [8] showed only 4 and 1.2 \times 10^3 times over
monomeric L-Fucz-1→2Gal, respectively; (ii) The PA-III had an affinity to high density D-Man (Mannan) [7] and demonstrated a dual carbohydrate-recognition system, while FRP showed no interaction with any of the non-L-Fucz-1→ containing glycoproteins (II, T/Tn mammalian structures and N-glycans such as Mannan) [7]. Although the FRP has been listed as a protein of “unknown function”, it displayed a very effective binding activity toward the polyvalent L-Fucz-1→glycotopes.

During the last decade, the L-Fucz-1→ specific lectins, also called F-type lectins, have been studied from various aspects: The crystal complex of AAA with fucose showed that L-Fucz-1→ recognition site formed specific structure “fold” that provided the structural determinants of oligosaccharide specificity involving in innate immunity [29]; identification and bioinformatics analysis of the fucose gene cluster of S. pneumoniae, strains 100993, R6 and type 4 for their promoter systems in DNA [30]; the structural analysis of blood group antigen recognition by S. pneumoniae TIGR4 [31]; the fucose lectin domain analysis of S. mitis 3LEI and S. pneumoniae 2J1S [32]; and the affinities for blood group antigens among microbial F-type lectins from Cyanobium sp. PCC 7001, Myxococcus h anuspus and Leucothrix mucor [33]. Although the bacterium S. pneumoniae ATCC BAA-334 that we used in our study was not a member in the above cited studies, the general observations about the F-type lectins are: (i) The structural fold of F-type lectins from many organisms are similar to AAA. For example, two proteins of S. pneumoniae TIGR4 (Entrez entries SP2159 and NP346573) have domains that are homologous to AAA. In addition, S. pneumoniae Entrez entry NP 346573 has a tandem of three AAA-like recognition domains [29]; (ii) the genomes organization of the fucose operon is conserved in all three publicly available S. pneumoniae genome sequences of strains 100993, R6 and type 4 [30]; (iii) The S. pneumoniae fucose utilization operon includes a gene of SpGH98 glycohydrolase family. One of the three fucose recognition module is likely to be associated with the role of SpGH98 glycohydrolase catalytic activity. The functions of the other two tandem fucose binding modules are still unknown [31]; and (iv) the bacterial fucose recognition domains generally presented as a single copy, with the exception of three sequence clusters in S. mitis, S. pneumoniae and Myxococcus sp. [32]. Since the genome organization of the same species of organism are largely conserved except some minor variations, we can expect the fucose recognition domains of S. pneumoniae ATCC BAA-334, that we used, would be similar to the S. pneumoniae NP346573 whose gene fold had been tested. However, S. pneumoniae is unable to survive in L- or D-fucose as the sole carbon source in a semi-refined medium, the functions of fucose recognition domains of S. pneumoniae remain to be elucidated [30].

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