Contribution of Phenylalanine 335 to Ligand Recognition by Human Surfactant Protein D
RING INTERACTIONS WITH SP-D LIGANDS*

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Surfactant protein D (SP-D) is an innate immune effector that contributes to antimicrobial host defense and immune regulation. Interactions of SP-D with microorganisms and organic antigens involve binding of glycoconjugates to the C-type lectin carbohydrate recognition domain (CRD). A trimeric fusion protein encoding the human neck+CRD bound to the aromatic glycoside p-nitrophenyl-α-D-maltoside with nearly a log-fold higher affinity than maltose, the prototypical competitor. Maltotriose, which has the same linkage pattern as the maltoside, bound with intermediate affinity. Site-directed substitution of leucine for phenylalanine 335 (Phe-335) decreased affinities for the maltoside and maltotriose without significantly altering the affinity for maltose or glucose, and substitution of tyrosine or tryptophan for leucine restored preferential binding to maltotriose and the maltoside. A mutant with alanine at this position failed to bind to mannan or maltose-substituted solid supports. Crystallographic analysis of the human neck+CRD complexed with maltotriose or p-nitrophenyl-maltoside showed stacking of the terminal glucose or nitrophenyl ring with the aromatic ring of Phe-335. Our studies indicate that Phe-335, which is evolutionarily conserved in all known SP-Ds, plays important, if not critical, roles in SP-D function.

MATERIALS AND METHODS

The p-nitrophenyl-α-D-maltoside (number 487542) was from EMD Biosciences (La Jolla, CA). The p-nitrophenyl-β-D-maltoside (N1884), p-nitrophenyl-α-D-glucopyranoside (N1377), p-nitrophenyl-α-D-galactopyranoside (N0877), maltotriose (M8378), and maltotetraose (Dp4, Supelco) were from Sigma-Aldrich. Trypsin-TPCK (T-8802) and all simple sugars were also from Sigma.

Expression, Purification, and Biochemical Characterization of Trimeric Neck+CRDs—The design, expression, and characterization of N-terminal-tagged, trimeric human NCRD fusion proteins has been previously described (9). For these studies, we performed site-directed substitutions at position 335 within the CRD, as defined by numbering of the full-length protein: alanine (F335A), leucine (F335L), tyrosine (F335Y), and tryptophan (F335W) (Fig. 1, A–D) (9) and introduced site-directed substitutions at position 335. We also examined the crystal structures of purified human trimeric neck+CRD complexed with the p-nitrophenyl-maltoside and maltotriose.

Surfactant Protein D (SP-D) is a collagenous C-type lectin (collectin) that contributes to antimicrobial host defense and immune regulation in the lung and certain extra-pulmonary tissues (1–4). Like many other effectors of innate immunity, SP-D is a pattern recognition molecule with a variety of potential ligands, including microorganisms, organic particulate antigens, and apoptotic cells. However, there is also evidence that SP-D contributes to pulmonary surfactant homeostasis, in part by regulating the organization and epithelial uptake of surfactant lipids (5).

SP-D is a member of a family of collectins, or collagenous C-type lectins (6). In mammals, this family also includes surfactant protein A (SP-A) and serum mannose binding lectin. Like most other collectins, SP-D is assembled as multimeric complexes of trimeric subunits, or as trimers (7). Each trimer includes an N-terminal cross-linking and collagen domain; a trimeric neck domain; and a C-terminal trimeric array of C-type carbohydrate recognition domains (CRDs).

Crystallographic studies of trimeric human SP-D neck+CRD domains have shown that maltose, a preferred saccharide ligand, binds to calcium via the vicinal 3- and 4-OH groups of the non-reducing glucose, previously designated calcium ion 1 and glucose 1 (Glc1), respectively (8). These interactions are further stabilized by hydrogen bonding of Glc1 to amino acid side chains that also coordinate with calcium ion 1.

The present studies were prompted by the initial observation that p-nitrophenyl-α-D-maltoside (p-NP-maltoside) is a potent inhibitor of SP-D binding to mannan. We hypothesized that there were interactions of the aromatic substrate with a hydrophobic or aromatic group in proximity to calcium ion 1. Preliminary modeling focused our attention on phenylalanine 335 (Phe-335) (Fig. 1A). To test our hypothesis, we used a functional, trimeric neck+CRD (NCRD) fusion protein containing the neck+CRD domains of human SP-D (Fig. 1, B–D) (9) and introduced site-directed substitutions at position 335. We also examined the crystal structures of purified human trimeric neck+CRD complexed with the p-nitrophenyl-maltoside and maltotriose.

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‡ The abbreviations used are: SP-D, surfactant protein D; CRD, carbohydrate recognition domain; Glc, glucose; NCRD, human neck+CRD; NP, nitrophenyl; TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone.
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The concentration of competitor (mM) required for 50% inhibition of binding (I50) was calculated. Protease protection assays of F335A were performed using trypsin-TPCK and modifications of published procedures (10). Following digestion in the absence and presence of various concentrations of calcium, the NCGRD fusion proteins showing His tag (6X His), S-tag, enterokinase cleavage site (EK), and the neck + CRD domains with indicated site of mutations. The approximate position of Phe-335 (arrow) in relation to the carbohydrate binding groove. C, sequence of the wild-type NCRD showing the approximate position of Phe-335 within the CRD. The neck peptide sequence is quantified by densitometry. The percent of cleavage relative to controls is shown under "Results," Table 2.

RESULTS

Human NCGRDs Recognize the Apolar Substituent of an Aromatic Malto-side—Hydropobic or apolar interactions are known to contribute to the interactions of various carbohydrate-binding proteins, including some C-type lectins (17). Studies of plant and bacterial lectins have often employed carbohydrates with hydrophobic substituents such as aromatic glycosides (e.g. p-nitrophenyl-glycosides) (18–20). Because maltose is the most potent simple competitor of SP-D binding to mannan, nearly a log-fold more potent than the prototypical p-nitrophenyl-maltoside. The aromatic maltoside was a potent competitive inhibitor of calcium-dependent human NCGRD binding to mannan, nearly a log-fold more potent than the prototypical SP-D competitor, maltose, an α-1,4-linked, glucose disaccharide (Fig. 2, Table 1). To assess specificity and the mechanism of binding, we examined other available p-nitrophenyl derivatives. The effects of the p-NP sub-
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TABLE 1
Inhibition of human NCRD by saccharides and aromatic glycosides

| Human NCRD competitor | I_{50} (mean ± S.E.) | Number of independent assays |
|------------------------|---------------------|-----------------------------|
| Glucose                | 3.5 ± 0.34          | 12                          |
| Maltose                | 2.3 ± 0.13          | 19                          |
| Maltotriose            | 0.94 ± 0.08         | 11                          |
| Maltotetraose          | 1.8 ± 0.20          | 4                           |
| p-Nitrophenyl-α-D-maltoside | 0.32 ± 0.01      | 12                          |
| p-Nitrophenyl-α-D-glucoside | 3.0 ± 0.26         | 4                           |

FIGURE 2. A: Percent control binding of human NCRD in the presence of competing sugars. B: Bar graph showing mean inhibition of NCRD binding to mannan by the indicated sugars.

The molar inhibitory potencies of glucose (Glc1), maltose (Glc1-Glc2), and maltotriose (Glc1-Glc2-Glc3) strongly suggested secondary ligand interactions involving both Glc2 and Glc3. However, there was no further increase in binding affinity upon addition of a fourth α-linked substituent. Maltotetraose was slightly more potent than maltose, but less effective than maltotriose (Table 1).

Rationale for Site-directed Substitution of Phe-335—The above findings initially appeared inconsistent with published crystallographic data of human SP-D NCRD complexed with maltose (8). As visualized in that study, the disaccharide enters the binding groove perpendicular to the binding face of a trimeric CRD, with Glc2 directed away from the binding surface. Collectively, our observations suggested a non-conventional binding orientation for Glc1 of maltotriose and/or torsional effects on the terminal sugars of the bound ligand. Both of these possibilities would likely require the participation of other residues in the vicinity of the primary carbohydrate binding site. Because other classes of carbohydrate-binding proteins utilize aromatic residues to mediate or fine-tune carbohydrate binding, our attention soon focused on Phe-335 of the mature protein, a residue that is surface exposed and near the calcium ion at the carbohydrate binding interface (see below).

Site-directed Substitution of Alanine for Phe-335 Abrogates Binding to Mannan—C-type lectins tolerate many different residues at the position corresponding to Phe-335, including alanine (21). Accordingly, we reasoned that a non-conservative substitution of alanine for Phe-335 should be minimally disruptive to conformation, while providing information about the role of the aromatic side chain. The mutant (F335A) was isolated in high yield from inclusion bodies, and the chromatographic properties were indistinguishable from the wild-type protein. In addition, the purified protein co-migrated on SDS-PAGE with the wild-type human NCRD in the absence and presence of dithiothreitol, consistent with the formation of normal intrachain disulfide bonds (data not shown). Nevertheless, two separate preparations of the alanine mutant showed no detectable interaction with mannan in solid phase binding assays (e.g. Fig. 3A). Furthermore, the protein did not bind to maltosyl-bovine serum albumin or to maltosyl-agarose using routine conditions of saccharide affinity chromatography (data not shown).

Because the ring of Phe-335 appears to “stack” with the side chain of Asn-41, a conserved residue of the C-type lectin motif that coordinates with calcium ion 1, we considered potential effects of the mutation on calcium binding affinity. However, saccharide binding was not “rescued” by calcium concentrations as high as 50 mM (data not shown), a strategy that has been used to restore binding activity of C-type lectin mutants with reduced calcium binding affinity (23). In addition, there was no significant difference in the susceptibility of the wild-type protein and F335A to proteolytic cleavage in the presence or absence of calcium (10, 24). The enterokinase-liberated wild-type and mutant neck+CRD domains were similarly resistant to cleavage by trypsin (1:10 ratio).
w/w) for periods of up to 2 h at 37 °C in the presence of physiological concentrations of calcium. However, both proteins were readily degraded in the absence of calcium, and the calcium concentration associated with 50% degradation (Ca50) was slightly lower for F335A than the wild-type protein, 0.2 ± 0.1 mM (n = 2 independent experiments) versus 0.7 ± 0.1 mM (n = 4), respectively. These calculated Ca50s are very similar to the minimum calcium concentration required for binding of the wild-type fusion protein to mannan (0.2 mM) (9).

Substitution of Leucine at Position 335 Preferentially Decreases the Affinity for Maltotriose and p-NP-maltoside—We next performed a more conservative substitution by replacing Phe-335 with leucine, a non-aromatic but hydrophobic amino acid. The purified protein (F335L) appeared indistinguishable from the wild-type protein and F335A on SDS-PAGE (Fig. 1D). However, in contrast with F335A, F335L showed specific, dose-dependent binding to mannan, similar to the wild-type protein (Fig. 3A). The apparent Kd for the mutant was (2.7 ± 0.8) × 10^{-7} M (n = 2) as compared with (1.8 ± 0.6) × 10^{-7} M (n = 3) for the wild-type NCRD. In addition, binding was efficiently inhibited by the usual competitors, including maltose (Fig. 3B). However, the inhibitory activities of maltotriose and p-nitrophenyl-maltoside were preferentially reduced as compared with the parent molecule, with molar affinities similar to glucose (Figs. 3B and 4B, below). The binding affinities for glucose, maltose, and other tested sugars were unchanged or slightly decreased as compared with the wild-type protein (Fig. 3B, data not shown). Comparable results were obtained for two independent preparations of mutant protein.

An Aromatic or Indole Ring Is Required for High Affinity Binding to Maltotriose—Given that F335L was functional as a lectin but showed no preferential interaction with maltotriose or p-NP-maltoside, we hypothesized that the ring structure is critical for stabilizing interac-
tions with these ligands. Accordingly, we substituted tyrosine or tryptophan for Phe-335, using the F335L DNA as a template. Both mutants were isolated as trimers and were indistinguishable from the wild-type fusion protein on SDS-PAGE (Fig. 1D). Both mutants (designated F335Y or F335W, respectively) showed specific, dose-dependent binding to mannan (data not shown). Although binding of F335W was comparable with F335L and the wild-type protein, binding of F335Y was increased in three independent experiments, with a minimum apparent $K_d$ of $3.1 \times 10^{-8} \text{ M}$.

F335Y and F335W also resembled the wild-type protein in their relative preference for maltotriose over maltose (Fig. 4). Interestingly, the $I_{50}$ of maltotriose was even lower for F335W than those observed for the wild-type protein or other mutants. There were no significant effects of the mutations on the inhibitory activity of other examined saccharide competitors.

Crystal Structure of the Human NCRD Complexed with p-NP-maltotriose and Maltotriose—To further define the binding mechanism, the crystal structure of the enterokinase-cleaved human trimeric NCRD was determined as complexes with maltotriose and $p$-nitrophenyl-$\alpha$-D-maltoside (Figs. 5–8, and Table 2). As shown in Fig. 7, Glc3 of maltotriose is aligned over but slightly offset with respect to the ring of Phe-335, such that C5 is situated nearly over the centroid of the phenylalanine ring, separated by a distance of $\sim 3.7 \text{ Å}$. Notably, the plane of the Glc3 ring is deflected $\sim 90^\circ$ relative to Glc1 (Fig. 7, left). The same binding orientation was observed for $p$-NP-$\alpha$-D-maltoside. Glc1 and Glc2 essentially superimposed on those of bound maltotriose, and the nitrophenyl ring was positioned, with a slight tilt, over Phe-335 at a separation of $\sim 3.8 \text{ Å}$ (Fig. 7, right).

Both ring structures showed other interactions with the CRD. There is a hydrogen bond from the side chain oxygen of Thr-336 to the C1-OH of Glc3, the latter being preferentially selected as the $\beta$-anomer. A similar bond is formed between Thr-336 and one of the nitro-oxygens of the maltoside. Another hydrogen bond is apparent in subunits A and B from the main chain amide of Asn-337 to the second nitro-oxygen of the maltoside.

As compared with the previously published maltose structure, Glc2 is deflected toward Phe-335, and there is an associated tilting of Glc 1 (Fig.

FIGURE 6. Structure of a single CRD with maltotriose. Left, surface representation shaded from cyan to red with increasing hydrophobicity and looking toward the binding surface. Right, maltotriose, at the same scale and in the same orientation, shown as a magenta stick model aligned with residues in CRD that are within 6 Å of the ligand. The three rings of maltotriose (Glc1-Glc3) are labeled G1-G3 in magenta.

FIGURE 7. Complexes of the wild-type human NCRD with $p$-NP-$\alpha$-D-maltoside and maltotriose. Left, model and associated $2F_o - F_c$ electron density (contoured at 1.5 $\sigma$) of maltotriose and nearby residues of CRD. The rings of maltotriose are labeled Glc1–3. Right, superimposition of the structures of the same regions of complexes of $p$-NP-$\alpha$-D-maltoside and maltotriose (cyan).
8). As compared with ligands that do not utilize Phe-335, there is also an associated local deformation of the polypeptide backbone, with a deflection of Thr-336 toward the aromatic ring (data not shown). Thus, stacking of the carbohydrate ring of Glc3 with the aromatic ring of Phe-335 appears to alter the overall binding orientation of the trisaccharide and locally perturb protein conformation.

In each complex, the side chain of Arg-343 is adjacent to the C6-OH of both Glc1 and Glc2. However, the orientation of the Arg-343 side chain appears constrained by hydrogen bonds and other interactions among Arg-343, Arg-349, and Glu-333 such that the angle for any direct hydrogen bond from Arg-343 to the first or second sugar ring of the ligand is unfavorable. Nevertheless, it seems likely that the ligand interaction is stabilized by the proximity of the sugar C6-OH groups to the positive charge on the Arg-343 guanidyl group.

**DISCUSSION**

The mutagenesis and crystallographic data indicate that the aromatic ring of Phe-335 provides a secondary stabilizing interaction that renders maltotriose more effective as a competitor than the prototypical saccharide ligand, maltose. In addition, the structure of the side chain at position 335 influences the solid phase interactions of SP-D with mannan, a complex, branched fungal polysaccharide.

Phe-335 is evolutionarily conserved among all known SP-D CRDs and is located near calcium ion 1 and the carbohydrate binding groove (Fig. 1) (22). Although this position is poorly conserved among C-type lectins, other collectins show aromatic or hydrophobic residues in this position, suggesting some shared functional roles. Aromatic residues or ring interactions are known to stabilize a variety of protein-carbohydrate interactions (25), and there is precedence for this among other C-type lectins, albeit primarily with respect to monosaccharide recognition (26).

We speculate that the higher binding affinity for the maltotriose analog p-NP-maltoside results from the greater stability of the parallel displaced pi-stacking of planar aromatic rings, as compared with stacking of the aromatic ring against Glc3. However, there are also the contributions of hydrogen bonds involving Thr-336 and Asn-337. These residues probably cooperate with Phe-335, creating an extended saccharide recognition site at the ligand binding interface. Thr-336 is also found in mouse and rat SP-D, whereas Asn-337 is conserved in all known SP-Ds, suggesting a conserved binding motif. Interestingly, the substitution of tryptophan for phenylalanine significantly increased the affinity for

### TABLE 2

Data collection and refinement statistics

| Data set                  | p-Nitrophenyl-α-D-maltoside | Maltotriose |
|---------------------------|-----------------------------|-------------|
| Space group               | P2₁                         | P2₁         |
| Cell dimensions           |                             |             |
| a = 55.43, b = 106.4, c = 55.08, beta = 92.65 | a = 55.73, b = 108.93, c = 55.84, beta = 91.13 |
| Wavelength (Å)            | 1.1                         | 1.1         |
| Resolution range (Å)      | 50-1.9                      | 50-1.85     |
| Measured reflections      | 805322                      | 630158      |
| Unique reflections        | 50929                       | 56776       |
| Completeness (%) overall  | 99.8 (99.8)                 | 99.9 (99.8) |
| (I/σI) (final shell)      | 18.8 (5.6)                  | 20.3 (4.8)  |
| Rmerge (%) (final shell)  | 0.07 (0.28)                 | 0.05 (0.30) |

### Refinement

|                  | p-Nitrophenyl-α-D-maltoside | Maltotriose |
|------------------|-----------------------------|-------------|
| Rcryst           | 0.214                       | 0.221       |
| Rfree            | 0.238                       | 0.247       |

### Atoms/Asymmetric unit

|                  | p-Nitrophenyl-α-D-maltoside | Maltotriose |
|------------------|-----------------------------|-------------|
| Protein          | 3462                        | 3462        |
| Water            | 259                         | 277         |
| Ions             | 9                           | 9           |
| Ligand           | 96                          | 102         |
| Total            | 3826                        | 3850        |

### Average B (Å²)

|                  | p-Nitrophenyl-α-D-maltoside | Maltotriose |
|------------------|-----------------------------|-------------|
| Main chain       | 27.7                        | 22.7        |
| Side chain       | 29.9                        | 25.2        |
| Ligand           | 36.2                        | 32.0        |
| All atoms        | 29.3                        | 24.5        |

### R.m.s.d. †

|                  | p-Nitrophenyl-α-D-maltoside | Maltotriose |
|------------------|-----------------------------|-------------|
| Bonds (Å)        | 0.006                       | 0.006       |
| Angles (°)       | 1.137                       | 1.105       |

† Root mean square deviation.
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maltotriose as compared with the maltoside, possibly as a result of the greater planar area of the indole ring.

Maltotetraose showed a lower molar inhibitory potency than maltotriose. We were unable to find information relating to the preferred solution conformation of this tetrasaccharide. However, the findings suggest that interactions with Phe-335 could preferentially stabilize binding to terminal sugars or specific branched structures in complex polysaccharides.

In the previously published maltose complex, both rings of maltose were only visualized in a single subunit (8). However, in the present study, all three subunits were occupied by maltotriose or the maltoside, and all parts of both ligands were observed. This difference can be attributed to stabilization of the complexes by secondary interactions involving Glc3 or the nitrophenyl group. Nevertheless, our observations are consistent with the findings of Shrive et al. (8) with respect to the mechanism and general orientation of Glc1 binding.

Our findings are also consistent with the previous suggestion that Arg-343, which is on the N-terminal ridge of the SP-D groove, can interact with Glc2 of maltose (22). At least within the context of maltotriose and the maltoside, Arg-343 is positioned to potentially interact with the C6-OH of Glc1 and/or Glc2. This provides a possible explanation for the greater affinity of human SP-D for maltose as compared with glucose. However, the extent of these interactions depends on the orientation for the greater affinity of human SP-D for maltose as compared with glucose. Within a “hydrophobic patch” previously suggested to play a role in the planar surface of the aromatic ring.

Because F335L showed essentially normal binding to mannan and was quantitatively bound to maltosyl-agarose, stacking interactions with an aromatic ring at residue 335 are not required for binding to solid phase ligands. On the other hand, F335Y showed enhanced binding to mannan, possibly as a result of additional hydrogen bonding interactions. In addition, the findings with F335A suggest that a bulky hydrophobic residue is required at position 335 for binding to at least some solid phase carbohydrates. Although we did not detect any unintended structural perturbations in F335A or effects on calcium-dependent resistant proteolysis, additional characterization of the mutant is in progress.

The other lung collectin, surfactant protein A (SP-A), has a conserved tyrosine (Tyr-208) at the same position as Phe-335. This residue is within a “hydrophobic patch” previously suggested to play a role in ligand binding (27). The planes of the flanking aromatic rings of Tyr-164 and Tyr-194 in SP-A are oriented relatively perpendicular to and above the plane of the ring of Tyr-208. Interestingly, these residues are replaced by alanine in SP-D, thereby increasing the potential accessibility of the planar surface of the aromatic ring.

In summary, our studies demonstrate that Phe-335 participates in an extended carbohydrate binding at the ligand binding interface. We suggest that the aromatic ring stabilizes binding of the CRDs near surfaces by stacking with carbohydrate rings or hydrophobic moieties in the same or different molecules. These findings have important implications for the interactions of SP-D with surfactant and with polysaccharides and other glycoconjugates associated with microbial cell walls and organic particulate antigens.

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