

Lipopolysaccharide binding of the mite allergen Der f 2

Saori Ichikawa¹, Toshiro Takai², Tomoe Yashiki¹, Seizo Takahashi¹, Ko Okumura², Hideoki Ogawa², Daisuke Kohda³ and Hideki Hatanaka^{3,4*}

¹Department of Material and Biological Sciences, Faculty of Science, Japan Women's University, 2-8-1, Mejirodai, Bunkyo-ku, Tokyo 112-8681, Japan

²Atopy (Allergy) Research Center, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

³Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

⁴Graduate School of Systems Life Sciences, 6-10-1 Hakozaiki, Higashi-ku, Fukuoka 812-8581, Japan

Lipid-binding properties and/or involvement with host defense are often found in allergen proteins, implying that these intrinsic biological functions likely contribute to the allergenicity of allergens. The group 2 major mite allergens, Der f 2 and Der p 2, show structural homology with MD-2, the lipopolysaccharide (LPS)-binding component of the Toll-like receptor (TLR) 4 signalling complex. Elucidation of the ligand-binding properties of group 2 mite allergens and identification of interaction sites by structural studies are important to explore the relationship between allergenicity and biological function. Here, we report a ligand-fishing approach in which His-tagged Der f 2 was incubated with sonicated stable isotope-labelled *Escherichia coli* as a potential ligand source, followed by isolation of Der f 2-bound material by a HisTrap column and NMR analysis. We found that Der f 2 binds to LPS with a nanomolar affinity and, using fluorescence and gel filtration assays that LPS binds to Der f 2 in a molar ratio of 1 : 1. We mapped the LPS-binding interface of Der f 2 by NMR perturbation studies, which suggested that LPS binds Der f 2 between the two large β -sheets, similar to its binding to MD-2, the LPS-binding component of the innate immunity receptor TLR4.

Introduction

‘What makes an allergen an allergen?’ This question was asked by Aas (1978) 30 years ago, and has fascinated immunologists to the present day. Sources of allergens, such as pollen and house-dust mites, include many kinds of proteins, but only a few proteins from each source are strong allergens.

Der f 2, the group 2 major allergen of the house-dust mite *Dermatophagoides farinae*, is a protein whose biological function was unknown until we determined its tertiary structure (Ichikawa *et al.* 1998). As suggested by the structure, we found that Der f 2 binds bacterial surfaces, from which we inferred that Der f 2 is involved in the innate antibacterial defense system in mites. And a survey of the literature led us to the idea that many other major allergens are components of host defense systems.

Our hypothesis concerning Der f 2 function was reinforced when Der f 2 was reported to be distantly

related to the protein MD-2, which binds lipopolysaccharide (LPS) and activates innate immunity through its docking partner Toll-like receptor 4 (TLR4) (Inohara & Núñez 2002). Meanwhile, Seong & Matzinger (2004) proposed the HYPPO hypothesis which suggests that solvent-exposed hydrophobic portions of molecules are the danger signals that stimulate immunity, and they further suggested that many allergens are lipid binding and play a role in host defense of their source organism.

Der f 2 is a single-domain protein composed of an immunoglobulin-fold β -sandwich (Ichikawa *et al.* 1998). However, a hydrophobic cavity that shows promise for ligand-binding lies between the two large β -sheets. We previously proposed that these two β -sheets make clamshell-like motions to accommodate lipid molecules (Ichikawa *et al.* 2005). Members of the ML superfamily, to which Der f 2 and MD-2 belong, have different separations and angles between the β -sheets, and the structure of these molecules ranges from a β -sandwich to the β -cup structure of the GM2-activator protein, which is not a sandwich at all (Wright *et al.* 2000). Both ‘closed’ and

Communicated by: Toshio Hakoshima

*Correspondence: hideki@dbcls.jp

DOI: 10.1111/j.1365-2443.2009.01334.x

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Genes to Cells (2009) **1**

somewhat 'open' structures were observed for the Der f 2 molecule. The separation and angle between the sheets in the Der f 2 NMR structure described in our previous report (Ichikawa *et al.* 2005), and in a crystal structure (Suzuki *et al.* 2006), are much narrower than those described in the first reported Der f 2 crystal structure (Johannessen *et al.* 2005).

Here, we report ligand fishing for Der f 2, and show that Der f 2 binds to LPS with nanomolar affinity and with a molar ratio of LPS : Der f 2 binding of 1 : 1. We also mapped the LPS-binding interface of Der f 2, which suggests that Der f 2 binds LPS via the hydrophobic space between the β -sheets. We further discuss our results in relation to the recent remarkable paper (Trompette *et al.* 2009) which reported that Der p 2, the group 2 allergen from *Dermatophagoides pteronissinus* that shows 88% amino acid identity to Der f 2, mimics MD-2 in binding to human TLR4 and in transducing LPS signals.

Results

Fishing for a Der f 2 ligand in sonicated *Escherichia coli*

To investigate if a ligand for Der f 2 exists in *E. coli*, uniformly $^{13}\text{C}/^{15}\text{N}$ -labelled *E. coli* JM109 was sonicated and incubated with or without nonlabelled His-tagged Der f 2. When the sonicates was incubated without Der f 2, they got through a HisTrap column, and nothing was eluted with imidazole. In the presence of Der f 2, some material was trapped by a HisTrap column and eluted by increasing imidazole concentration. To obtain structural information on ligands from *E. coli* JM109, the eluted fractions were analysed by NMR. The ^1H - ^{13}C heteronuclear single-quantum coherence (HSQC) spectrum of Der f 2-containing fractions displayed an intense resonance pattern indicative of saccharides and aliphatics (Fig. 1a). Three signals characteristic of anomeric protons were also observed. Partial assignments of the sugar moieties were possible by a combination of 2D and 3D NMR methods designed for proteins, starting from the *N*-acetyl groups. The ^1H and ^{13}C chemical shift assignments agreed very well with those previously reported for the cyclic enterobacterial common antigen (ECA_{CYC}) from *Yersinia pestis* (Vinogradov *et al.* 1994), which is comprised of the trisaccharide repeat unit 4-acetamido-4,6-dideoxy-D-galactose (Fuc4NAc), *N*-acetyl-D-mannosaminuronic acid (ManNAcA) and *N*-acetyl-D-glucosamine (GlcNAc). The ^1H - ^{13}C HSQC spectrum also

displayed many methyl and methylene signals in the aliphatic region. However, no NOE signals were detected between protons from ECA and these aliphatic protons.

We then further investigated Der f 2-associated material by gel filtration chromatography (Fig. 1b), followed by NMR analysis (Fig. 1c). The apparent molecular weights of the peaks of gel filtration chromatography, as calculated from the elution volumes, were 43 and 15 kDa. The ^1H - ^{13}C HSQC spectrum of the peak at 43 kDa was composed of ECA signals only and did not include lipid-like signals (data not shown), implying that this gel-filtration peak represents ECA_{CYC} associated with Der f 2. As ECA_{CYC} has been recently reported to be a common contaminant which often fails to be separated from bacterially-expressed protein preparations (Erbel *et al.* 2004), we infer it bound to Der f 2 nonspecifically. On the other hand, the ^1H - ^{13}C HSQC spectrum of the peak at 15 kDa showed resonances characteristic of aliphatic groups and saccharides (Fig. 1c), indicating that some glycolipid from *E. coli* JM109 is bound to Der f 2.

The glycolipid that copurified with Der f 2 could not be identified based on the spectrum. However, considering that Der f 2 binds the *E. coli* surface (Ichikawa *et al.* 1998), LPS was the most likely candidate. The observed chemical shifts and broadness of the methyl and methylene signals of the bound glycolipid matched very well with those of a part of LPS that is bound to CD14 (Albright *et al.* 2009). This similarity suggests that the glycolipid binds to Der f 2 with its acyl chains inserted into a hydrophobic cavity of Der f 2, reminiscent of the LPS-binding pocket of CD14 (Kim *et al.* 2005). This concept of a compact docking mode, coupled with the fact that JM109 is a K12-derived strain that expresses low-molecular-weight rough-type LPS (Hancock *et al.* 1994), might explain why the elution volume of Der f 2 in gel filtration chromatography was unchanged by glycolipid binding (Fig. 1b). These data led us to consider the possibility that the Der f 2 ligand in *E. coli* JM109 might be LPS.

Der f 2 binds to LPS

To determine if Der f 2 can bind LPS, we carried out several types of binding assays using LPS purified from *E. coli* strain O111:B4. First, a fluorescence assay was done. This assay was based on measurement of the fluorescence of the only endogenous tryptophan residue of Der f 2, Trp-92. As this tryptophan residue is located at the entrance of the cavity between the

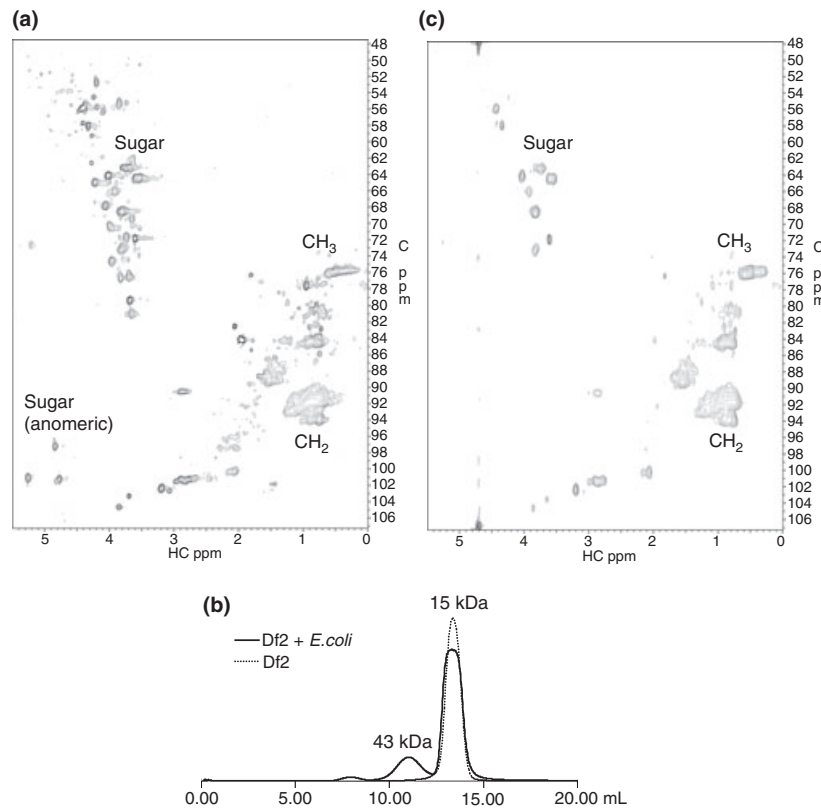


Figure 1 Analysis of a Der f 2 ligand from sonicated $^{13}\text{C}/^{15}\text{N}$ -labelled *Escherichia coli*. (a) The ^1H - ^{13}C HSQC spectrum of His-tagged Der f 2-containing material eluted from a HisTrap HP column. For NMR analysis, the samples shown in Fig. 1a,c were dissolved in 20 mM sodium phosphate (pH 7.5), lyophilized and dissolved in 99.96% D_2O . The NMR signals, except for the top left saccharide-like peaks, were aliased to reduce the ^{13}C sweeping width into 60 ppm. NMR signals were assigned taking into account the natural abundance of the ^{13}C background of Der f 2. (b) Gel filtration chromatography of His-tagged Der f 2 incubated with or without sonicated *E. coli* and eluted from a HisTrap HP column. The data represent absorbance at 220 nm. The apparent molecular weights estimated from the elution volumes are shown. (c) The ^1H - ^{13}C HSQC spectrum of the fractionated sample eluted at a position of 15 kDa in gel filtration chromatography (B, Der f 2 + *E. coli*). Contaminating ECA_{CYC} was eliminated.

two β -sheets, and its side-chain is oriented towards the inside of the molecule, LPS binding would be expected to affect its fluorescence. Intrinsic fluorescence of this tryptophan shows emission maxima at 324 nm, indicating that Trp-92 is likely buried and in a 'nonpolar' environment (Vivian & Callis 2001). This result is in agreement with the 'closed' tertiary structure (Ichikawa *et al.* 2005; Suzuki *et al.* 2006), where the indole ring of Trp-92 is covered by the Ile-54 side-chain from the opposite β -sheet. As shown in Fig. 2, Der f 2 fluorescence was quenched following stoichiometric addition of LPS, suggesting that LPS binding affects the chemical environment of the tryptophan residue. The initial slope of Fig. 2 suggests equimolar binding of Der f 2 and LPS. The dissociation constant, K_d , for LPS binding to Der f 2

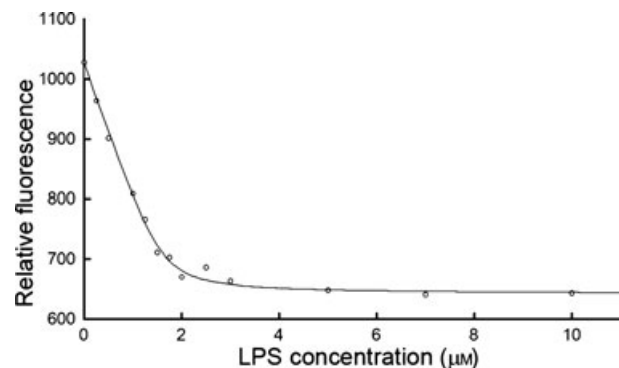


Figure 2 Fluorescence assay of Der f 2-LPS binding. Fluorescence data were obtained with the indicated total concentrations of LPS. Each circle represents the average of three individual data, with background subtracted.

was estimated to be $6 \pm 2 \times 10^{-8}$ M by a nonlinear iterative fitting procedure using xcrvfit, developed by R. Boyko and B. Sykes (University of Alberta, Canada).

We next assessed the LPS-binding state of Der f 2 by gel filtration chromatography, using LPS from the smooth *E. coli* strain O111:B4, whose molecular weight is much higher than that of the rough strain JM109 that was used for the fishing experiments. Figure 3a shows that incubation with sonicated LPS alters the elution volume of Der f 2. Elution of a peak at a position corresponding to a molecular weight of 22 kDa suggests that the molar ratio of LPS binding to Der f 2 is 1 : 1, considering the equimolar binding indicated by the fluorescence assay. LPS alone forms large aggregates in aqueous solution. Therefore, the highest molecular weight peak eluted from the column represents Der f 2 that is embedded in large LPS aggregates, and the peak at 80 kDa likely represents small aggregates of Der f 2 and LPS. In contrast to its effect on Der f 2, LPS had little

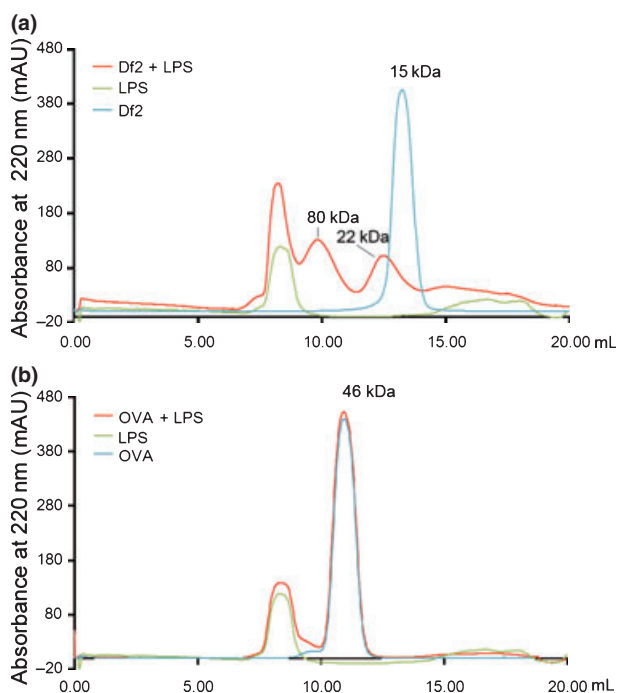


Figure 3 Gel filtration analysis of lipopolysaccharide (LPS) binding to Der f 2. (a) Der f 2, incubated in the absence or presence of LPS, was analysed by gel filtration chromatography as described in Experimental procedures. LPS alone was also incubated in the same buffer and subjected to gel filtration chromatography. (b) OVA, used as a negative control, was treated in the same manner as in (a).

effect on the elution volume or peak profile of ovalbumin (OVA) that was used as a negative control (Fig. 3b).

To confirm the interaction between Der f 2 and LPS, we determined whether Der f 2 and LPS could be cross-linked by the chemical cross-linker BS³. BS³ is a homobifunctional, water-soluble cross-linker that contains an amine-reactive *N*-hydroxysulfosuccinimide (NHS) ester at each end of an 8-carbon spacer arm. Cross-linking of Der f 2 alone yielded intermolecular, homodimeric and homotrimeric cross-linked bands as assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining of the gel (Fig. 4a lane 1 and Fig. 4b lane 2), as well as low-molecular-weight ladder-like bands considered to be intramolecular cross-linked products, which were not observed without BS³ (Fig. 4b lane 1). We next assessed cross-linking of Der f 2 and LPS, which revealed a new band that migrated with an apparent molecular weight of 32 kDa. As the amount of LPS was increased, the intensity of the 32 kDa band was enhanced (Fig. 4a, lanes 2–4). The observed ladder-like bands in the high-molecular-weight range were considered to be due to heterogeneity of LPS. No bands were

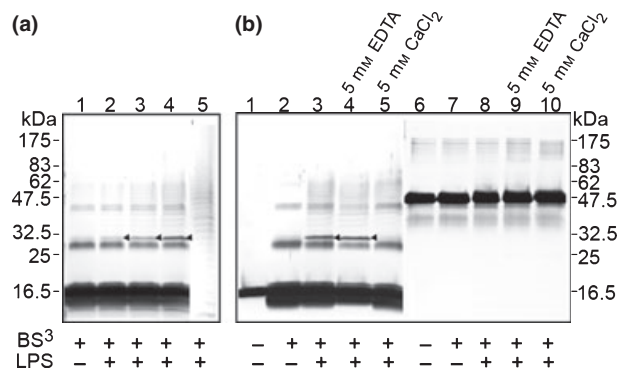


Figure 4 Cross-linking of Der f 2 and lipopolysaccharide (LPS) with BS³. (a) 10 µM of Der f 2, incubated for 30 min at 37 °C with increasing concentrations of LPS, was subjected to cross-linking with BS³ and the products were analysed by SDS-PAGE followed by silver staining. Lanes 1–4 contained Der f 2 and the following concentrations of LPS: lane 1, no LPS; lane 2, 10 µM LPS; lane 3, 20 µM LPS; lane 4, 40 µM LPS. Lane 5 contained 40 µM LPS alone. (b) Lanes 3–5, cross-linking of 10 µM Der f 2 with 40 µM LPS in the presence or absence of 5 mM EDTA or 5 mM Ca²⁺. Der f 2 in the absence of cross-linking (lane 1) or cross-linked in the absence of LPS (lane 2) is also shown. Lanes 6–10, Der f 2 was replaced by the negative control OVA and treated as described for lanes 1–5 respectively. Cross-linked products are indicated by triangles.

observed when LPS alone was treated with BS³ (Fig. 4a lane 5), indicating that the new cross-linked species at 32 kDa reflects association of Der f 2 with LPS. NHS esters of BS³ have reactivity toward primary amines. Der f 2 contains 14 lysine residues whose primary amines in the side-chains are available as targets for BS³. The above data indicates that Der f 2 and LPS were in close enough proximity to allow cross-linking, and we assume that some lysine residues in Der f 2 and ethanolamine in LPS were cross-linked with BS³.

To assess if the binding of Der f 2 to LPS is influenced by calcium, we performed similar cross-linking assays in the presence of Ca²⁺ or EDTA in the binding buffers. As illustrated in Fig. 4b lane 5, the Der f 2–LPS 32 kDa band was not detected in the presence of Ca²⁺, indicating that cross-linking between Der f 2

and LPS was inhibited by Ca²⁺. This result suggests that electrostatic interactions of Ca²⁺ with the phosphate groups of LPS may mask these groups and interfere with the binding site. The negative control OVA was not cross-linked with LPS either in the absence or presence of Ca²⁺ (Fig. 4b, lanes 6–10).

Mapping of the LPS-binding site by NMR

As mentioned in the Introduction, Der f 2 has a hydrophobic cavity, whose size can be changed by clamshell-like motions of the two β -sheets. To identify the LPS interaction sites of Der f 2, we employed the NMR chemical shift perturbation method (Craik & Wilce 1997). Figure 5a represents the magnitude of the combined ¹H and ¹⁵N chemical shift differences of backbone amides between free and

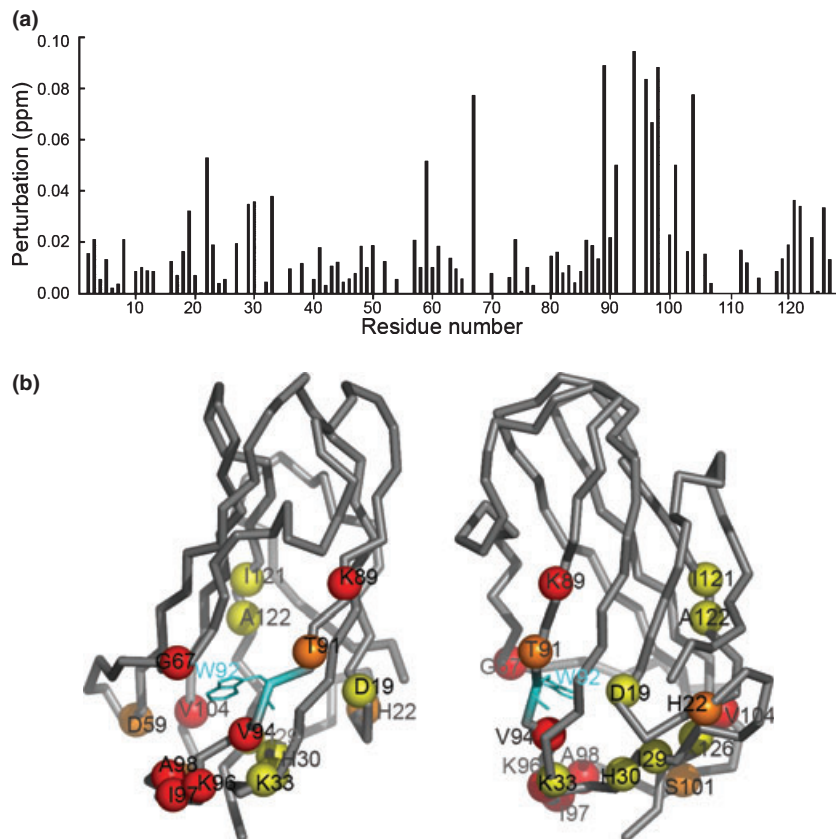


Figure 5 Mapping the LPS-binding site in Der f 2 by chemical shift perturbations. (a) Combined chemical shift perturbations that were calculated according to the empirical equation $\Delta\delta = (\Delta\delta_{\text{HN}}^2 + 0.2^2 \times \Delta\delta_{\text{N}}^2)^{1/2}$, where $\Delta\delta_{\text{HN}}$ and $\Delta\delta_{\text{N}}$ are the chemical shift changes of ¹H and ¹⁵N respectively. Perturbations of 37 residues were not determined due to being prolines, peak overlaps or ambiguous assignments. (b) Backbone nitrogen atoms with significant chemical shift perturbations are mapped on the C α carbon trace of Der f 2 (Johannessen *et al.* 2005): red, $\Delta\delta > 0.06$; orange, $0.05 < \Delta\delta \leq 0.06$; yellow, $0.03 < \Delta\delta \leq 0.05$. Trp-92 is indicated by cyan sticks.

LPS-bound forms of Der f 2, and Fig. 5b maps significantly perturbed residues on the somewhat 'open' structure of Der f 2 (Johannessen *et al.* 2005).

Most residues with perturbations larger than 0.06 ppm upon LPS binding were concentrated on the rim of a β -sheet which forms the entrance to the hydrophobic cavity. In particular, the residues Lys-89, Val-94, Lys-96, and Ala-98 all showed perturbation greater than 0.08 ppm. Although Trp-92 and Asn-93 were promising candidate residues for perturbation in the light of the observed change in fluorescence of Trp-92, the signals of these residues were not sufficiently resolved in the HSQC spectra to determine if they were perturbed or not. Large chemical shift changes (>0.05 ppm) were also observed for the residues His-22, Asp-59, Ser-101 and Val-104 and significant changes (>0.03 ppm) were observed for the residues Asp-19, Ile-29, His-30, Lys-33, Ile-121, Ala-122 and Lys-126. These residues are located around the hydrophobic cavity between the two β -sheets, as indicated in the bottom half of Fig. 5b. There is a cluster of basic residues in Der f 2, which suggests the binding of negatively charged ligands in the vicinity of the entrance to the hydrophobic cavity. Interestingly, the residues Lys-96, Lys-33, and Lys-126 that are located in this basic cluster were also significantly perturbed upon LPS binding.

Discussion

Binding of LPS between the two β -sheets of Der f 2

We have shown that Der f 2 binds LPS (Fig. 2), and our results suggest that this binding shares many biochemical characteristics with that of MD-2, which binds LPS between two β -sheets (Ohto *et al.* 2007; Park *et al.* 2009). First, the dissociation constant of the LPS–Der f 2 complex was estimated as 6×10^{-8} M (Fig. 2), which is comparable to that reported for binding of LPS to MD-2 (65 nM) (Viriyakosol *et al.* 2001), although different values have been reported for MD-2 by other groups (Mancek-Keber & Jerala 2006; Shin *et al.* 2007). Second, our fluorescence and gel filtration experiments indicate that LPS–Der f 2 complex is an equimolar complex (Fig. 2) with an apparent molecular weight of 22 kDa (Fig. 3a), similar to the one-to-one complex of LPS–MD-2. Third, the fluorescence of Trp-92 at the entrance to the hydrophobic cavity between the two β -sheets of Der f 2 (Fig. 5b) was modulated by LPS addition, indicating that Trp-92

contacts with the bound LPS. Fourth, the observed chemical shifts and the broadness of the methyl and methylene signals of the Der f 2-bound ligand (Fig. 1c) indicates that these groups are confined within a proteinous environment with some conformational and/or environmental variety.

Based on the combined data, we interpret the results of the chemical shift perturbation experiment (Fig. 5) to indicate that Der f 2 binds LPS via the internal hydrophobic space between the two β -sheets in a similar manner to MD-2. All significant perturbations (spheres in Fig. 5b) within Der f 2 occur on the four segments 89–104, 59–67, 121–126 and 19–33. The first two segments, which include all perturbations larger than 0.04 ppm (red and orange) with the exception of His-22, form the entrance to the intersheet space, and probably function to attach the phosphorylated β -1,6-linked D-glucosamine disaccharide of LPS. The other two segments are adjacent to the segment 89–104, surround the internal space, and possibly make contacts with the acyl chains linked to the disaccharide of LPS.

Role of Der f 2 clamshell-like motions in LPS binding

Lipopolysaccharides binding between two β -sheets requires clamshell-like motions of the β -sheets as we previously proposed (Ichikawa *et al.* 2005), as the unliganded 'closed' structure (Ichikawa *et al.* 2005; Suzuki *et al.* 2006) has only a small space. The somewhat 'open' structure (Johannessen *et al.* 2005) shown in Fig. 5b has a large hydrophobic cavity. However, lipid A, the lipid portion of LPS that is responsible for its binding to MD-2, and lipid IVa, a precursor of lipid A biosynthesis, have several acyl chains, which form a large, thick hydrophobic slab. This slab is more bulky than the pocket formed by the 'open' structure of group 2 allergens, which appears to be capable of accommodating only string-like compounds such as polyethylene glycol (Johannessen *et al.* 2005) or a pair of fatty acids (Derewenda *et al.* 2002). Therefore, in order for Der f 2 to accommodate LPS, the space between the two β -sheets needs to be more open, in a manner similar to MD-2, than this previously described 'open' structure, which could be termed 'intermediately open'.

This becomes clear when this 'intermediately open' structure of Der f 2 is superimposed on the structure of ligand-bound MD-2 (Park *et al.* 2009) (Fig. 6). The superimpositions shown in Fig. 6a,c indicate that the space between the two β -sheets of

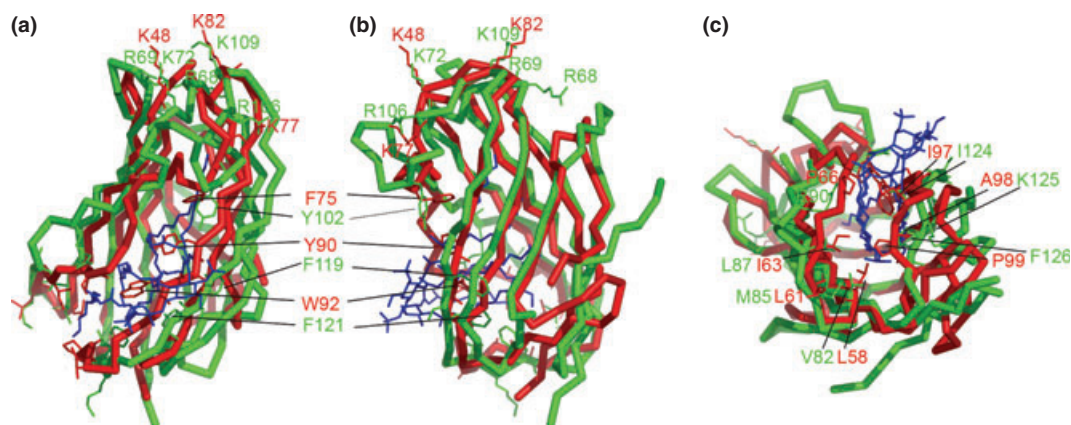


Figure 6 Structural comparison of Der f 2 and MD-2 complex. (a) Superposition of the C α traces of Der f 2 (red) (Johannessen *et al.* 2005) and MD-2 complexed with LPS (green) in TLR4/MD-2/LPS dimer (Park *et al.* 2009). The lipid A portion of LPS (blue) is also shown as thin sticks. The core oligosaccharide portion of LPS is omitted for clarity. The side-chains of some residues that are conserved between MD-2 and Der f 2 are labelled and are also indicated as thin sticks. (b) The view rotated 90° around the y-axis. (c) The view that is related to (a) by 90° x rotation followed by 45° z rotation.

the 'intermediately open' Der f 2 structure (red) is narrower than that of MD-2 structure (green), and that the aromatic rings of Tyr-90 and Trp-92 of Der f 2 occupy the space of lipid A (blue) complexed with MD-2. Therefore, to fully open up the cavity of Der f 2 to the size of the MD-2 internal space, Der f 2 needs to open up the four segments described above, within which all the significant chemical shift perturbations occurred, and to reorient the two aromatic side-chains. These requirements are consistent with the results of the NMR and fluorescence experiments of the present study.

LPS binding to Der f 2 and MD-2

The lipid A portion of LPS has five to seven acyl chains, which means that its hydrophobic part is more bulky than its tetra-acylated analogue, lipid IVa. Nevertheless, MD-2 complexed with LPS (Park *et al.* 2009) shows only a localized structural difference from MD-2 complexed with lipid IVa (Ohto *et al.* 2007). LPS binds to MD-2 with the phosphorylated glucosamine backbone in the orientation opposite to lipid IVa and ~5 Å further from MD-2 (Park *et al.* 2009). Thus there is some variability in the regions of MD-2 which bind to acyl chains and phosphates. This versatility in MD-2 binding complicates the following discussions concerning Der f 2–LPS binding.

The residues Phe-119 and Phe-121 of MD-2 were reported to be required for LPS binding (Tsuneyoshi *et al.* 2005). The aromatic rings of these residues (Fig. 6) are oriented towards the bottom of the

hydrophobic pocket, and together they form a flat hydrophobic wall that, with some versatility, guides the bulky slab of LPS acyl chains. The residue Ser-120, which lies between the two aromatic residues, plays a role in hydrogen bonding to the ligands. As the corresponding residues in Der f 2 (Tyr-90, Thr-91 and Trp-92) are contained in the region that is significantly perturbed upon LPS binding, it is presumed that these Der f 2 residues interact with lipid A in a similar manner.

MD-2 is a basic protein, and each edge of the hydrophobic pocket entrance has a cluster of basic residues. These two basic clusters of MD-2 are important for LPS binding as has been reported in a previous mutagenesis study (Gruber *et al.* 2004). In contrast, Der f 2 is a neutral protein that contains many basic and acidic residues but has a cluster of basic residues at only one edge of the pocket entrance, although this cluster is large. In MD-2, the basic clusters at the pocket entrance are believed to function mainly in the attraction of negatively charged LPS, rather than in direct binding to the phosphate groups of LPS (Ohto *et al.* 2007), and this appears to be the case also with the basic cluster in Der f 2.

Similar to MD-2, the internal surface of the cavity in Der f 2 is almost exclusively hydrophobic. However, the distribution of aromatic residues in Der f 2 is distinct from that in MD-2. Thus, aromatic residues in the cavity of MD-2 are distributed over the entire cavity. In contrast, in Der f 2 the aromatic residues are mainly clustered on two strands, and small hydrophobic residues are distributed within the rest of the

cavity in place of the aromatic residues found in MD-2. Hence, it is conceivable that the conformation of a lipid moiety confined in the pocket of Der f 2 would be different from that in MD-2.

Possible interaction interface of Der f 2 with TLR4

Recently Trompette *et al.* (2009) reported that Der p 2 mimics MD-2 by facilitating LPS signalling through binding not only with LPS but also with TLR4, which they proposed as the origin of its strong allergenicity. As Der f 2 shows 88% amino acid identity to Der p 2 and similar allergenicity, it is highly conceivable that Der f 2 also binds to TLR4 in the same manner, although we have no direct evidence yet. Interestingly, Der f 2 presents a triangle of basic residues composed of Lys-48, Lys-77 and Lys-82 (see top of Fig. 6a,b), which occupy exactly the same position as Lys-72, Arg-106 and Lys-109 of MD-2, of which the last two residues are key residues for TLR4 binding (Kim *et al.* 2007). Therefore Der f 2 is likely to dock to TLR4 using the same surface as MD-2.

The Phe-75 residue of Der f 2 is located at the same position as Tyr-102 of MD-2 (Fig. 6), which simultaneously interacts with the side-chain of Arg-264 of TLR4 and with a fatty acid of LPS (Kim *et al.* 2007). In the Der p 2 molecule, this position is occupied by tyrosine, and mutation of this residue to alanine was reported to ablate its functional mimicry of MD-2 (Trompette *et al.* 2009). However, this residue is often substituted by phenylalanine, histidine or valine in other group 2 mite allergens, suggesting that tyrosine at this position is not essential for the ability of group 2 mite allergens to mimic MD-2. The Tyr-102 of MD-2 is located on the TLR4-docking bulge (visible in Fig. 6b, left) that is formed by the loop created by a disulfide bridge between Cys-95 and Cys-105. However, this bulge is much smaller in group 2 allergens (between Cys-73 and Cys-78 in Der f 2), and this difference might affect docking of allergens to TLR4.

The crystal structure of a dimer of TLR4-MD-2-LPS was determined very recently (Park *et al.* 2009). This structure indicated that heterotrimer dimerization leading to LPS signalling occurs via the bottom face of MD-2 in Fig. 6a,b; that is, through a phosphate group and an acyl chain from LPS, and residues Val-82, Met-85, Leu-87, Arg-90, Ile-124, Lys-125 and Phe-126 from MD-2. These MD-2 residues correspond to the Der f 2 residues Leu-58, Leu-61, Ile-63, Pro-66, Ile-97, Ala-98 and Pro-99 respectively.

In spite of the fact that there is little sequence similarity between the two molecules, these residues show a surprising conservation with the exception of the two positive charges. The high conservation of these amino acids, together with the existence of the basic residues Lys-96, Lys-100, Arg-31 and Arg-128 adjacent to the segments 97–99, make it very likely that the LPS–Der f 2 complex also has an interface capable of docking to the second TLR4 molecule. To our further surprise, all these residues reside in a small area which shows large chemical shift perturbations upon LPS binding. As MD-2 undergoes a localized structural change in the same area upon LPS binding, the chemical shift perturbations we observed in Der f 2 might be a result of the same type of restructuring. This putative interface of Der f 2 for TLR4 dimerization supports the autoadjuvant property proposed for the origin of its strong allergenicity (Trompette *et al.* 2009), although it remains mystery why such an interface in Der f 2 arose in mites.

This study showed that Der f 2 binds LPS with nanomolar affinity as a 1 : 1 complex. Based on the results of the combined experiments, we conclude that LPS binds Der f 2 with its acyl chains inserted between the two large β -sheets, in a similar manner to MD-2. The putative structure of the Der f 2–LPS complex has two surface areas that show promise as TLR4 dimerization sites, which provide a possible explanation for the allergenicity of Der f 2.

Experimental procedures

Preparation of recombinant Der f 2 proteins

Escherichia coli BL21 (DE3) cells harboring a vector for expression of recombinant Der f 2 (Takai *et al.* 2005) with the amino acid sequence of the clone 1/Der f 2.0101 were cultured in M9 minimum medium in the presence of [^{13}C]glucose and [^{15}N]ammonium chloride. Labelled recombinant Der f 2 was expressed and purified as previously described (Nakazawa *et al.* 2005; Takai *et al.* 2005).

His-tagged recombinant Der f 2 was prepared as follows: the cDNA for Der f 2 (390 base pairs) was subcloned into the pET28a vector (Novagen, Madison, WI, USA) which resulted in insertion of a 6 $\times$ His tag at the N terminus. His-tagged Der f 2 was then expressed in BL21 (DE3) *E. coli*. For the preparation of nonlabelled His-tagged Der f 2 the *E. coli* were cultivated in LB medium containing 10 $\mu\text{g}/\text{mL}$ kanamycin and 34 $\mu\text{g}/\text{mL}$ chloramphenicol at 30 °C. For preparation of ^{15}N -labelled His-tagged Der f 2 the *E. coli* were cultured in M9 minimum medium in the presence of [^{15}N] ammonium chloride. Cells were harvested by centrifugation at 7000 $\times g$ for 15 min, suspended in Tris buffer (50 mM Tris, pH 9.0), sonicated, solubilized with 8 M urea containing Tris buffer,

and dialysed against Tris buffer for 12 h to obtain the correctly refolded molecules. The dialysate containing refolded Der f 2 was applied to, and purified from a HisTrap HP column (GE Healthcare, Uppsala, Sweden). His-tagged Der f 2 was eluted by increasing the imidazole concentration from 70 to 500 mM. The Der f 2-containing fractions were further purified by gel filtration chromatography on a Superdex75 column (GE Healthcare) and confirmed by SDS-PAGE.

Sonicated *E. coli* and Der f 2 binding

The *E. coli* JM109 was grown to late log phase in 200 mL of M9 minimal medium containing [^{13}C]glucose and [^{15}N]ammonium chloride at 37 °C. The cells were harvested by centrifugation and suspended in 2 mL of 20 mM sodium phosphate buffer, pH 7.5, 150 mM NaCl. The suspension was then completely sonicated on ice for 15 min and immediately added to the purified His-tagged Der f 2 at a protein concentration of 0.2 mg/mL. The mixture was then incubated at 25 °C for 30 min with gentle shaking, followed by centrifugation at $16\,128 \times g$ for 5 min at 4 °C. The supernatant solution was loaded onto a HisTrap HP column (GE Healthcare) pre-equilibrated with 10 volumes of buffer (20 mM sodium phosphate, 20 mM imidazole, 500 mM NaCl, pH 7.5). After washing the column with 10 volumes of the same buffer, His-tagged Der f 2 and associated material was eluted by increasing the imidazole concentration from 70 to 500 mM. Fractions containing Der f 2-associated material were determined by subjecting an aliquot to gel electrophoresis using 15–25% SDS-PAGE gels and then dialysed into 20 mM sodium phosphate, pH 7.5, 150 mM NaCl. It was concentrated in an Amicon Centrprep YM-10 filter and then loaded onto a Superdex75 column (GE Healthcare) equilibrated in 50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.5. Isolated fractions were desalted through PD-10 columns (GE Healthcare) and then concentrated in Amicon Centricon YM-10 filters for NMR spectroscopy.

NMR spectroscopy

NMR spectra were acquired at 25 °C on a Bruker Avance600 or 700 NMR spectrometer equipped with a triple resonance pulse field gradient probe. All the NMR data were processed with NMRPipe (Delaglio *et al.* 1995).

For ligand-fishing experiments, purified sample was lyophilized and dissolved in 400 μL of either 99.96% D_2O or 20 mM sodium phosphate (pH 7.5) containing 10% D_2O . Carbon and nitrogen chemical shift assignments were based on 2D ^1H - ^{13}C and ^1H - ^{15}N HSQC spectra and the following 3D pulse sequences: HNCO, HNCA, CBCA(CO)NH, HNCACB, HNCAHA, HBHA(CO)NH, ^{15}N -NOESY, HCCH-TOCSY. Although these standard 3D experiments are generally used to assign protein backbone and side-chain atoms, they were also useful to assign sugar atoms starting from amides in *N*-acetyl groups. Then the partial chemical shift assignments were

compared with ^1H and ^{13}C chemical shifts in SUGABASE (van Kuik *et al.* 1992) to determine the chemical identity.

For chemical shift perturbation experiments, purified ^{15}N -labelled Der f 2 was dissolved at a final concentration of 0.3 mM in 20 mM sodium phosphate buffer (pH 7.0), containing 10% D_2O . LPS binding site was investigated by 2D ^1H - ^{15}N HSQC spectra. ^{15}N -labelled Der f 2 and unlabelled LPS (L3024; Sigma, St Louis, MO, USA) were combined at a molar ratio of 1 : 1. The calculation of Der f 2 and LPS molarity was based on the assumption that the molecular weights of smooth LPS and Der f 2 are 10 000 and 14 000 respectively.

Analysis of Der f 2-LPS binding by gel filtration

Escherichia coli LPS (L3024; Sigma) was sonicated for 5 min and incubated with 10 μM Der f 2 in 50 mM sodium phosphate buffer (pH 7.0) including 150 mM NaCl, at 37 °C for 30 min. The molar ratio of LPS to Der f 2 was maintained at 10 : 1. LPS binding was assessed by the shift in retention time of Der f 2 during gel filtration chromatography on a superdex75 column (GE Healthcare). As a negative control, OVA (EndoGrade; Profos AG, Regensburg, Germany) was treated in the same manner as Der f 2.

Fluorescence assay of Der f 2-LPS binding

Lipopolysaccharide (L3024, Sigma), prepared at a range of different concentrations in binding buffer (20 mM HEPES, 150 mM NaCl, pH 7.0), was added to Der f 2 and incubated for 30 min at 37 °C. The final concentration of Der f 2 in the incubation mixtures was 1.6 μM . Fluorescence measurements were performed at 37 °C on a Spectra MAX GEMINI EM (Molecular Devices, Sunnyvale, CA, USA). The intrinsic fluorescence of Der f 2 was measured at an excitation wavelength of 290 nm, and the emission was assayed at 324 nm.

Chemical cross-linking and SDS-PAGE analysis

Lipopolysaccharide (L3024; Sigma), prepared at a range of concentrations in binding buffer (20 mM HEPES, 150 mM NaCl, pH 7.0), was added to Der f 2 and incubated for 30 min at 37 °C. Then this solution containing 10 μM Der f 2 was incubated for 30 min at room temperature with freshly prepared 200 μM bis(sulphosuccinimidyl)suberate (BS^3) (Pierce, Rockford, IL, USA), dissolved in the same HEPES buffer. In some experiments 5 mM CaCl_2 or 5 mM EDTA was added to the binding buffer. The reactions were stopped by the addition of 20 μL of 2 $\times$ SDS-PAGE loading buffer [125 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β -mercaptoethanol and 0.001% (w/v) bromophenol blue] followed by incubation at room temperature for 15 min. A 15- μL aliquot of each solution was then subjected to SDS-PAGE and stained with silver stain. The negative control OVA (EndoGrade; Profos AG) was treated in the same manner.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Received: 23 April 2009

Accepted: 8 June 2009