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MD-2 and Der p 2 – a tale of two cousins or distant relatives?

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MD-2, an LPS-binding protein is essential for the recognition of LPS by TLR4. MD-2 belongs to the ML superfamily of lipid-binding proteins. The tertiary structure of mite allergen protein Der p 2 was identified as having the protein fold most compatible with the sequence of MD-2. Comparison of MD-2 and Der p 2 reveals that they have many common biochemical characteristics: they are both rich in β -structure and they are both very stable proteins as they both unfold only above 90°C. In Der p 2, six cysteine residues form three disulfide bridges. We determined one free cysteine residue per recombinant biologically active MD-2 molecule, supporting similar disulfide topology with three disulfides bridges as in Der p 2. MD-2 binds LPS with high affinity; however, only weak binding of LPS was detected with Der p 2. Comparison of electrostatic potentials of the structural model of MD-2 and Der p 2 indicates a region of high positive potential on MD-2 and its absence in Der p 2, which may be the reason for its weak binding of LPS. We suggest that Der p 2 and its homologues probably do not have a role in response to Gram-negative bacteria in insects and that MD-2 family members with their specific role in innate immunity probably evolved from an ML ancestor only in higher vertebrates.

Keywords: MD-2, Der p 2, tertiary structure, ML superfamily, dendrogram

INTRODUCTION

Invasion of bacteria into multicellular organisms activates innate immunity as the first line of defense. Rapid and specific response of the innate immune system is achieved through the recognition of structural motifs common to pathogens or PAMPs (pathogen-associated molecular pattern).¹ Lipopolysaccharide (LPS) from Gram-negative bacteria is one of the most potent stimulants of the innate immune response. The membrane protein Toll-like receptor 4 (TLR4) is required for triggering the inflammatory response to LPS.^{2,3} Glycoprotein MD-2 is physically associated with the extracellular domain of TLR4 on the cell surface and confers responsiveness to

LPS.⁴⁻⁶ MD-2 protein, in contrast to TLR4, binds LPS with high affinity;⁷ however, an increase of its affinity to LPS has been reported in the complex with TLR4.⁸ The first functionally important point mutation of MD-2, Cys95Tyr, has been identified in the LPS non-responder cell line.⁹ This mutation was later shown to impair not only LPS signaling but TLR4 binding as well.¹⁰ We have previously identified a peptide sequence of MD-2 rich in basic and hydrophobic residues, which was able to bind LPS.¹¹ Charge reversal mutations of basic residues in this region impaired LPS signaling.¹² Systematic alanine scanning mutagenesis of murine MD-2 determined the effect of each amino acid residue for binding to TLR4 and signaling of LPS and Taxol.¹³ However, information on tertiary structure would be needed to rationalize the effect of mutations and elucidate the molecular mechanism of LPS recognition.

MD-2 and its homologue MD-1 are members of the ML (MD-2-related lipid recognition) superfamily.¹⁴ Members of this superfamily with known tertiary structure form an immunoglobulin-like, β -rich fold. Tertiary structures of representatives of two ML family proteins have been determined. Der p 2 (a Der f 2 homologue),¹⁵

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GM2-AP,^{16,17} and NPC2¹⁸ contain an apolar lipid-binding pocket between the β -sheets. Binding of hydrophobic ligands into the pocket involves a conformational change and an expansion of the pocket.

Der p 2 is a major dust mite allergen. With Der f 2, it belongs to the group 2 allergens. As much as 80–90% of mite-allergic individuals produce cellular responses to these two allergens.¹⁹ Tertiary structures of Der p 2 have been determined by NMR spectroscopy²⁰ and crystallography.¹⁵ The protein contains three disulfide bonds and two anti-parallel β -pleated sheets, which overlay each other at an angle of approximately 30°. The main difference between the NMR and the crystal structures of Der p 2 is in the distance between the two β -sheets. In the X-ray structure, the β -sheets, which are significantly further apart, create an internal cavity that is occupied by an unidentified hydrophobic ligand.¹⁵ It was suggested that binding of non-polar molecules represents the physiological function of the protein.

We have prepared the tertiary structure model of MD-2, based on homology with Der p 2.²¹ This model was able to put into the structural context most of the properties of MD-2 mutants. We were interested in identifying the biochemical similarities between MD-2 and Der p 2 proteins and consequently if Der p 2 might have a similar role in insects as MD-2 does in vertebrates with respect to recognition of LPS. Our results show that the proteins are very similar in terms of secondary structure and protein stability; however, Der p 2 binds LPS only weakly. Therefore, LPS-recognition has probably evolved later in the evolution of ML proteins and required the addition of specifically positioned basic residues.

MATERIALS AND METHODS

Homology modeling of MD-2 and evolutionary tree construction

Search for human MD-2 homologues in the database of translated sequences was performed using BLAST and PSI-BLAST.²² Alignment of amino acid sequences of the ML superfamily proteins was performed using the Muscle program²³ and a dendrogram was constructed using the Neighbor-joining algorithm. The tertiary structure model of MD-2 based on Der p 2 was prepared using program MODELLER 6v224 as described previously.²¹

Preparation of recombinant proteins

Recombinant MD-2 was prepared in *Escherichia coli* from inclusion bodies as described previously.²¹ Production of recombinant bDer p 2 was carried out in *E. coli* harboring the pETrDer p 2 expression plasmid (a kind

gift from Dr Alisa Smith). Refolding of the protein from solubilized inclusion bodies was performed by dialysis against 20 mM Tris, pH 8.5, 1 mM EDTA buffer. Protein was additionally purified by gel filtration chromatography on Superose 12 (Pharmacia Biotech, Uppsala, Sweden) in 20 mM Tris buffer, pH 8.0 and 100 mM NaCl.

Determination of free cysteine residues

DTNB (5,5'-dithiobis[2-nitro-benzoic acid]; 2.5 mM; Serva, Heidelberg, Germany) was dissolved in 0.1 M Tris buffer, pH 8.0. Freshly prepared DTNB (20 μ l) was added to 180 μ l of protein sample and incubated for 30 min at room temperature. Absorbance of reaction products was measured at 412 nm against a control reaction. Cysteine was used as a calibration standard.

Circular dichroism

Circular dichroic spectra of bMD-2 and bDer p 2 were measured on an Aviv spectropolarimeter 62A (Aviv Associates, NJ, USA) using quartz cuvettes (Hellma Suprasil, Mullheim, Germany) with a path length of 1 mm in the far-UV region (190–260 nm). Mean residue ellipticity θ_{MRW} (deg $\times$ cm² $\times$ decimole⁻¹) was calculated using a mean molar residue weight of 115. For measurements, 0.14 mg/ml of bMD-2 in water and 0.32 mg/ml of bDer p 2 in 10 mM Na-phosphate buffer pH 7.0 were used. Temperature unfolding of the protein was performed by a temperature scan at the heating rate of 1 °/min. Far-UV CD spectra were measured also at 85°C. Cooling and repeated scan indicated that protein unfolding up to 95°C was reversible and caused minimal precipitation of the protein.

Determination of LPS binding

Fluorescence emission spectra were recorded on a Perkin Elmer LS55 (Perkin Elmer, MA, USA) with an excitation wavelength of 290 nm to monitor the fluorescence of single tryptophan residues in each of the proteins. Quartz glass cuvettes (10 mm optical path; Hellma Suprasil, Mullheim, Germany) were used. Binding of LPS (from *Salmonella abortus equi*, a gift from Dr Klaus Brandenburg) to bMD-2 and bDer p 2 was monitored by the fluorescence emission at 348 nm at 25°C. Data are presented as the total fluorescence emission intensity (area under curve; AUC) versus LPS concentration. bDer p 2 was concentrated to 0.6 mM and LPS was added to the protein at a 1:1 molar ratio, which resulted in a formation of the precipitate. In order to extract bDer p 2, the resulting pellet was washed several

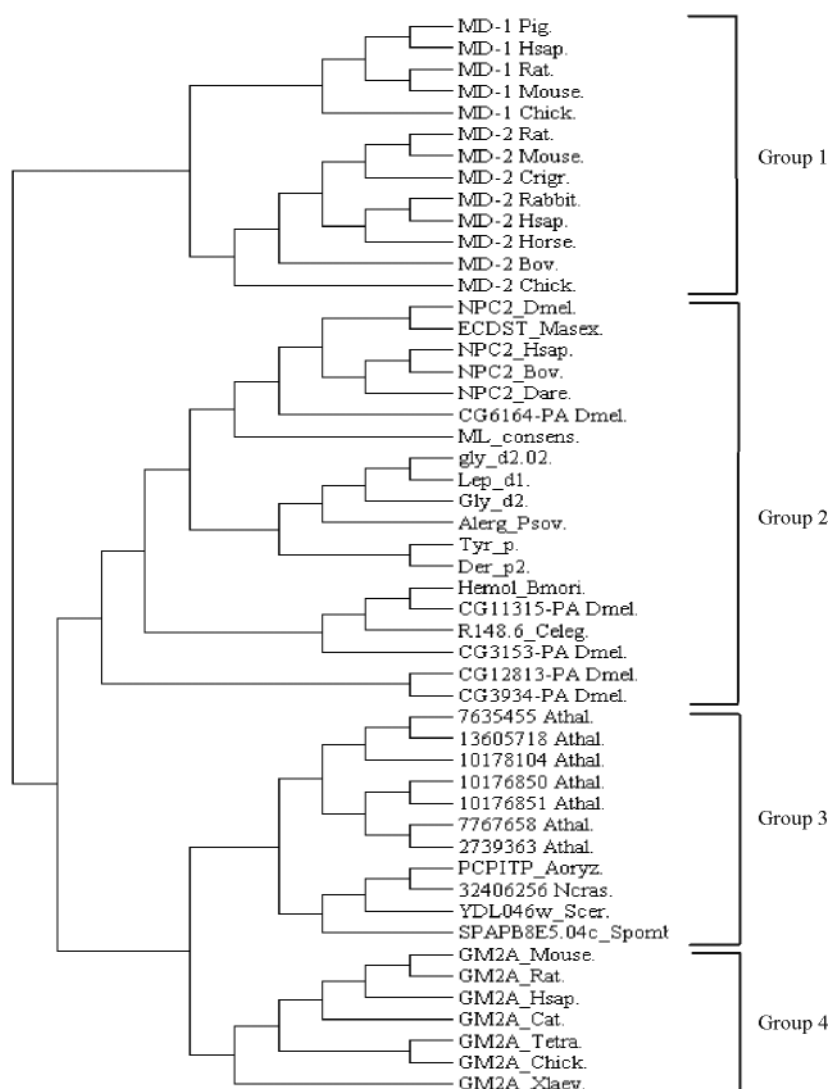


Fig. 1. Cladogram of the amino acid sequences of the members of the ML family. Sequence alignment which was the basis for this alignment was prepared using Muscle multiple sequence alignment. A division of ML proteins into groups as proposed¹⁴ is indicated.

times with milliQ water and subsequently with 0.5 M NaCl. Supernatants were analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

Alignment of ML superfamily members and their clustering into a tree

Alignment of the members of the ML superfamily was performed using the Muscle multiple sequence alignment algorithm (Fig. 1). Der p 2 contains 38% sequence similarity and 16% identity to human MD-2, followed by NPC2 with 26% similar and 12% identical residues. Within the MD family members, the largest divergence was observed in chicken homologues, which are the only known non-mammalian family members. Sequence

identity of chicken MD-2 with other MD-2 variants is between 23–28% in comparison to 50–70% within others. We have identified no MD-2/MD-1 homologue in the available completed Fugu fish genomes.

Secondary structure content and stability of bMD-2 and bDer p 2

Recombinant MD-2 (bMD-2) and Der p 2 (bDer p 2) were prepared in *E. coli* and refolded into a biologically active conformation. bMD-2 was able to confer responsiveness to LPS in cells transfected with TLR4.²¹ Far-UV CD spectra of bMD-2 and bDer p 2 are characteristic for proteins rich in β -structure (Fig. 2), supporting the sequence threading and secondary structure prediction that the proteins have the same type of fold. It has been reported that MD-2 is

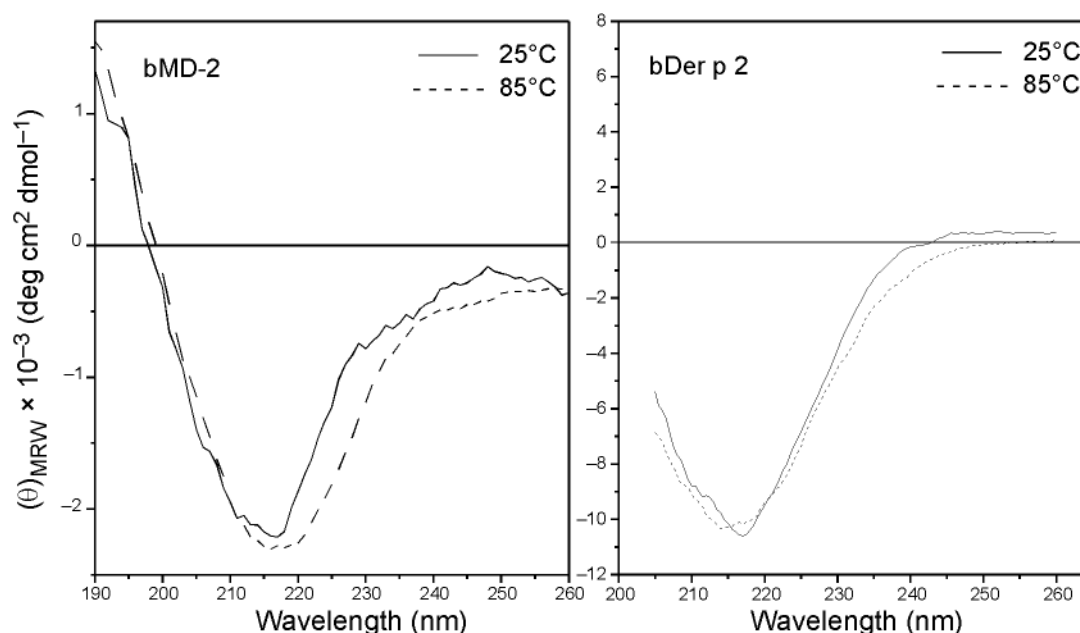


Fig. 2. Circular dichroic spectra of bMD-2 and bDer p 2 at 25°C and 85°C. Far-UV CD spectra of purified bMD-2 (left) and bDer p 2 (right) show the predomination of the β -structure, even at 85°C, indicating a high temperature stability of proteins.

unstable at 37°C and loses its biological activity without the presence of stabilizing LPS ligand.²⁵ Denaturation of MD-2 was suggested to be responsible for this loss of activity rather than proteolytic degradation or formation of aggregates. Der p 2 is a very stable protein and requires temperatures above 100°C for inactivation.²⁶ We were, therefore, interested in the temperature stability of bMD-2. The protein retained its secondary structure even up to 85°C (Fig. 2, left) as was also the case with bDer p 2 (Fig. 2, right). In the presence of reducing agents MD-2 unfolded at lower temperature, indicating the importance of disulfide bridges for the stability of its tertiary structure (not shown). Therefore, in this respect, Der p 2 and MD-2 exhibit similar biochemical properties. Glycosylation of mammalian MD-2 may have some influence on its conformational properties, but we suggest that aggregation rather than unfolding of MD-2 is likely to be the reason for a decline of its biological activity.

Free cysteine residues of MD-2

Similar biochemical properties and sequence similarity provided the rationale for preparation of the model of tertiary structure of MD-2 based on Der p 2. We have determined the content of free cysteine residues of the biologically active recombinant MD-2 at 1.3 ± 0.3 free -SH groups per molecule, which suggests that MD-2 forms three disulfide bridges. Within the alignment of MD-2 homologues along with NPC2 and Der p 2 location

of six of the cysteine residues present within all of them were congruous, suggesting conservation of disulfide-bonding topology. Alanine scanning mutagenesis¹³ and systematic mutation of cysteine residues²⁷ indicated previously that the cysteine residue with minimal effect on its mutation was Cys133, which was in our model present as a free cysteine residue.

Differences in LPS binding to MD-2 and Der p 2

The isoelectric point of most of the ML proteins is acidic, with the exception of the MD-2 family, where the average is pI 7.4. Human MD-2 in particular has several clusters of basic residues on its surface (Fig. 3), resulting in a predicted isoelectric point of 8.52. Several mutations that decreased the electrostatic potential of MD-2 also decreased the LPS signaling activity of MD-2. We have shown, however, that it is the specific position of cationic residues within the structure that is important and not just the indiscriminate net charge effect.²¹ We have determined direct binding of LPS to bMD-2 using fluorescence titration based on the intrinsic fluorescence of MD-2. The maximum of the fluorescence emission of bMD-2 was at 348 nm, indicating high solvent exposure of the single tryptophan residue, which is also in agreement with the tertiary structure model (predicted 24% exposure to solvent). Addition of LPS decreased intrinsic fluorescence implying that binding of LPS affects the chemical environment of the tryptophan residue. From

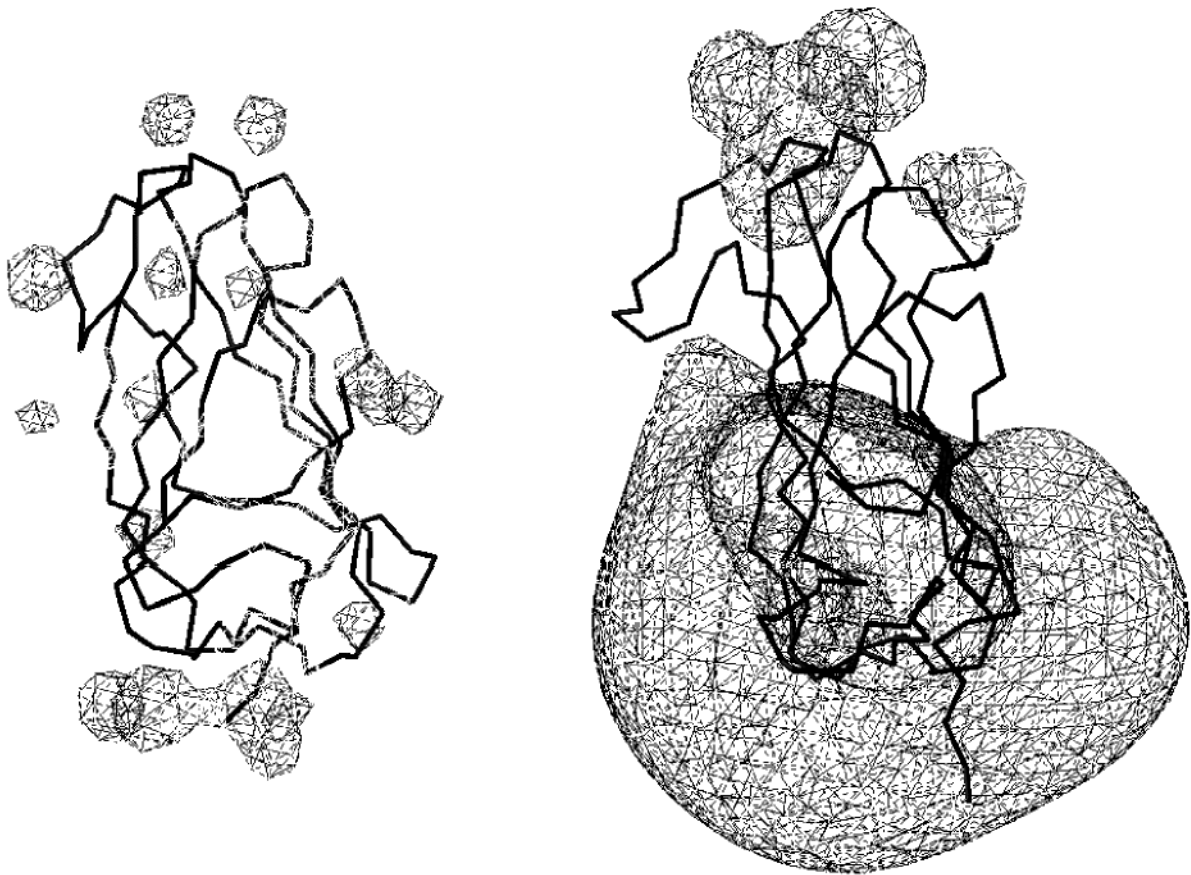


Fig. 3. Comparison of protein folds of Der p 2 and MD-2 and their electrostatic potentials. The polypeptide backbone is shown for Der p 2 (left) and 3-D model of human MD-2 (right). The positive electrostatic potential calculated by DeepView³² <<http://www.expasy.org/spdbv/>> is shown as a mesh.

the fluorescence titration, the stoichiometry of LPS binding was determined to be 1:1 (Fig. 4, bottom), similar to that observed for binding of LOS to MD-2.²⁸ We could not detect direct binding of LPS to bDer p 2 either by measuring the intrinsic tryptophan fluorescence or fluorescently labeled LPS (not shown). Therefore, we measured binding of LPS to Der p 2 and attempted to map the interacting residues using NMR spectroscopy, which requires high sample concentration. Addition of LPS to 0.6 mM Der p 2 caused the formation of a precipitate, indicating interaction with LPS in the 100 μ M range. This complex dissociated at high ionic strength as was shown by solubilizing bDer p 2 from precipitate with 0.5 M NaCl (Fig. 4, top). It has been shown previously that Der f 2, a Der p 2 homologue, is able to bind to the lysate of *E. coli*,²⁹ but it has not been identified which bacterial component might be responsible for this. We, therefore, suggest that LPS may be one of the components that binds to mite allergens, but we can not exclude the existence of any other tightly binding components from bacterial cells. Comparison of Der p 2 and MD-2 model structures show that proteins have significantly different electrostatic potential (Fig. 3). As shown before with MD-2, clusters of basic residues are very important for

LPS binding and signaling,^{12,21} so we believe that the lack of these clusters may determine the weak binding of LPS to Der p 2, but we can not yet rule out any differences in the hydrophobic binding pocket. The crystal structure of Der p 2 contains a hydrophobic ligand buried inside the protein core between the β -sheets, which was absent in the solution structure. We were not able to detect binding of any of the several hydrophobic ligands to Der p 2 with affinities below micromolar concentrations. The only noticeable difference between the sample preparation for solution and crystal structural analysis was that in the crystallization buffer a low concentration of reducing agent (2 mM DTT) was used.¹⁵ DTT might have catalyzed the opening and the closure of the exposed disulphide bonds and thus facilitated the entry of the hydrophobic ligand. In our experiments, the addition of DTT up to 10 mM did not significantly affect the secondary structure of Der p 2; however, the protein was less stable and unfolded above 65°C in the presence of 5 mM DTT (data not shown).

The physiological role of Der p 2 and its homologues in insects is unknown. Based on the 35% identity to a human epididymal gene product (HE1), it was suggested that Der p 2 may play a role in mite reproduction.³⁰ We have identified eight homologues of Der p 2 in the

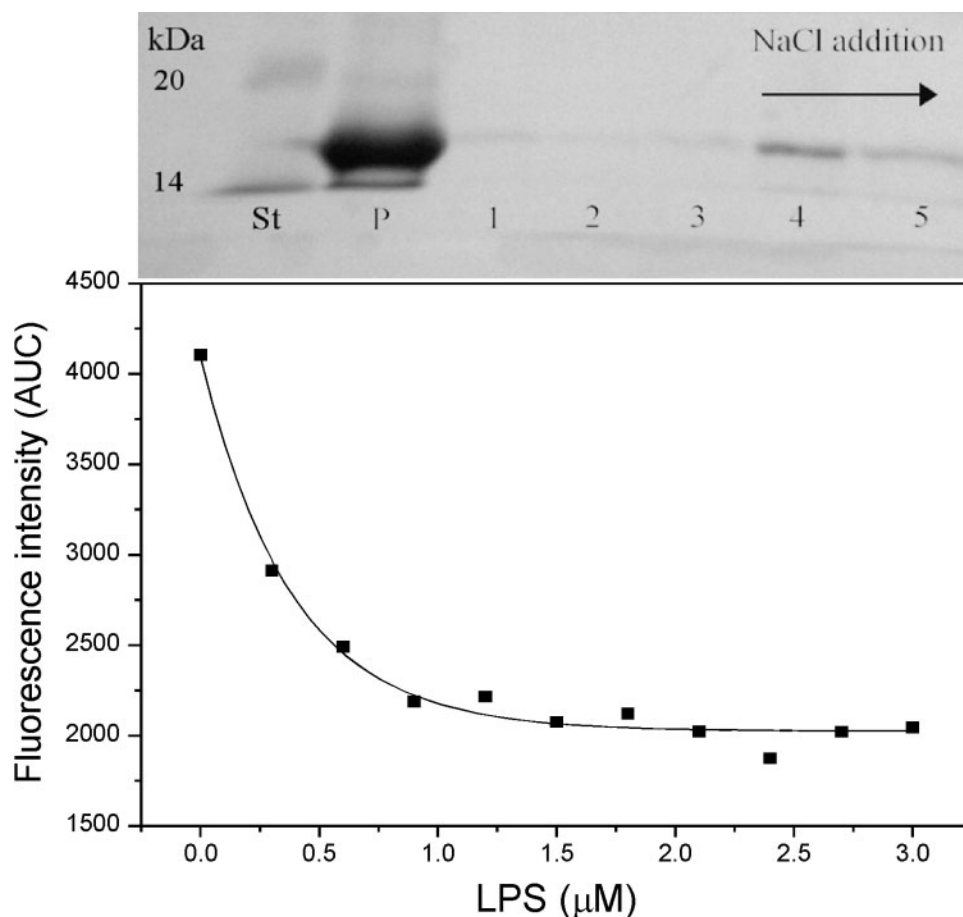


Fig. 4. Binding of LPS to Der p 2 and MD-2. Top: precipitate formed by the addition of LPS to Der p 2 (P), pellet was extracted with water (1–3), followed by 0.5 M NaCl (4–5). St, molecular weight standard proteins. Bottom: intrinsic fluorescence titration of bMD-2 with LPS. Addition of LPS to 1.5 μ M bMD-2 decreased the fluorescence emission. The stoichiometry of LPS binding to MD-2 determined from the curve was 1:1.

genome of *Drosophila*. Two of the protein transcripts (CG11315-PA and CG3153-PA) had been predicted to have basic pI values and might, therefore, have higher affinity for anionic bacterial glycolipids. Analysis of the transcriptional response of the fruit fly to challenge with Gram-negative bacteria indicated that transcription of one of the Der p 2 homologues (CG6164-PA) decreased.³¹ LPS activates the Imd pathway of the immune system in *Drosophila*, while the pathway involving Toll receptor responds to Gram-positive and fungal infection. Our results, therefore, suggest that Der p 2 probably does not have a role in Gram-negative bacterial response in insects and that the MD-2 family evolved from the common ancestor only in higher vertebrates, acquiring as a characteristic the clusters of basic residues.

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