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Structure of a TLR4-interacting SPA4 peptide

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Abstract

We have recently identified a Toll-like receptor (TLR4)-interacting SPA4 peptide encoding amino acids: GDFRYS^DGTPVNYTNWYRGE, a shorter region of human surfactant protein-A (SP-A). The SPA4 peptide suppressed lipopolysaccharide-induced inflammation (*JPET* 2011, *Innate Immun* 2013). In this report, we examined the structure of synthetic SPA4 peptide in solution by circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy. The CD analysis revealed that the SPA4 peptide is composed of ~35% beta sheet and <5% alpha helix. We used solution NMR to solve the structure of the SPA4 peptide. We calculated NMR structures using Nuclear Overhauser Enhancement (NOE) distance restraints. The superposition of the low energy structures indicated that the central 6–14 amino acids “SDGTPVNYT” of the 20mer SPA4 peptide form a turn, and amino acids on either side (GDFRY and NWYRGE) conform to flexible arms. Furthermore, thermal denaturation experiments demonstrated the structural flexibility of the peptide. The NMR structures of the SPA4 peptide align well with the homologous region within the available structure of rat SP-A and a computationally-docked model of SP-A-TLR4-MD2 protein complex. Together, our results support the structural adaptability of SPA4 peptide for binding to TLR4.

Keywords

Surfactant protein-A-derived peptide; structure; Toll-like receptor-4

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Authors' contributions: Karla Rodgers performed and analyzed the CD results. Asokan Anbanandam obtained the NMR profile of SPA4 peptide at the Biomolecular NMR Core lab at the University of Kansas, Lawrence, KS. Shanjana Awasthi designed and coordinated the study, analyzed the results using the PyMOL and Chimera programs, and compiled the manuscript. All of the authors contributed to their respective sections within the manuscript.

Introduction

Lung surfactant is composed of lipids and proteins, and lines the alveolar epithelium (1). Among four surfactant proteins (SPs), SP-A is the major protein and is critical for maintenance of pulmonary host defense. SP-A modulates pulmonary host defense against a variety of pathogens and stress ligands (2). There is sufficient evidence in the literature in support of modulation of host defense through interaction of SP-A with multiple cell surface receptors or directly with pathogens and stress ligands (3-5). Thus, it is easy to conceive that the structural orientation of the triad of host cell receptor, SP-A, and pathogenic ligands could be important for host defense mechanisms. Our research has focused on the TLR4-interacting regions of SP-A for modulation of host immune responses against Gram-negative bacteria (unpublished results) or the Gram-negative bacterial ligand lipopolysaccharide (LPS; (6, 7). Using computational molecular modelling and docking, and functional screening of a small peptide library derived from the C-terminal region of SP-A, we have identified a 20-mer lead peptide (SPA4, amino acids: GDFRYSDGTPVNYTNWYRGE) that binds to TLR4 and suppresses LPS-TLR4-induced inflammation in cell systems and in a mouse model (6-9). In this study, we questioned whether synthetic SPA4 peptide derived from the TLR4-interacting region of the human SP-A can conform to secondary structure, and how it aligns with the homologous region within the available rat SP-A (10), or a computationally-docked model of SP-A-TLR4-MD2 protein complex (7).

Earlier, we published the *in silico* model of SPA4 peptide using the *de novo* prediction program PEPFOLD (8, 11). We found that the most energetically favorable model consisted of a U-shaped peptide structure flanked by predominantly extended beta strands (8). Many complementary approaches, which can help determine the structure of peptides in solution, are available. In the present study, we employed circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy to experimentally determine the structural and physicochemical characteristics and folding pattern of the SPA4 peptide. The results of NMR, thermal denaturation, and CD analyses demonstrate the structural plasticity of the largely hydrophilic SPA4 peptide. The PEPFOLD predictions and NMR structures showed two arms of the peptide around a turn of amino acids, "SDGTPVNYT". The alignment of SPA4 peptide supports the similarity between the structure of SPA4 peptide and a homologous region within the available structure of rat SP-A, and within the computer model of the SP-A-TLR4-MD2 complex. Together, the results of this study suggest the structural adaptability of SPA4 peptide for binding to TLR4.

Materials and Methods

Peptide synthesis and purification

The SPA4 peptide (amino acids GDFRYSDGTPVNYTNWYRGE) was synthesized by solid-phase peptide synthesis method using N-9-fluorenylmethoxycarbonyl (Fmoc) chemistry and purified by reversed-phase high-performance liquid chromatography (RP-HPLC) at Genscript (Piscataway, NJ). The molecular weight of different batch preparations of SPA4 peptide was confirmed by mass spectroscopy. The SPA4 peptide preparations were at 95% purity. The peptide was suspended in endotoxin-free water at a concentration of 2 mg/ml. The stock solution of the peptide was maintained at -20°C.

Circular dichroism (CD) spectroscopy

Samples containing 0.14 μM peptide were prepared by diluting the stock solution with appropriate amounts of endotoxin-free water or 25-75% methanol in water. Spectra were recorded on a Jasco J-715 CD spectropolarimeter with a PTC-348WI Peltier temperature controller (Jasco Corp, Tokyo, Japan). The spectral parameters were as follows: 250-200 nm wavelength range, 0.1 cm cuvette path length, constant temperature set at 20°C, and 3 accumulations per spectrum. Raw CD data were normalized to mean residue ellipticities using the molar concentration of the peptide and the number of residues in the SPA4 peptide. Secondary structural content was calculated from the CD spectra using three separate algorithms, CONTIN, SELCON3, and CDSSTR (12), as previously described (13).

The thermal stability of the peptide in endotoxin-free water was determined by measuring the CD signal at 220 nm as a function of temperature. The temperature was raised from 20°C to 50°C at a rate of 15°C per h. The denaturation midpoint (T_m) refers to the temperature at the midpoint of the transition between folded and unfolded states, and was calculated for the SPA4 peptide, as described previously (14).

Nuclear magnetic resonance (NMR) spectroscopy

One- and two-dimensional solution NMR spectra of SPA4 peptide were taken in 100% D_2O (pH 3.78) at 7°C using a 600 MHz NMR Bruker Avance III spectrometer equipped with a triple resonance TXI probe. The temperature of 7°C was chosen to minimize the conformational fluctuations. Standard pulse programs available within the Bruker suite of experiments were used with optimization of pulse lengths and power levels. Two-dimensional NMR spectra were recorded in the phase-sensitive mode using states-time proportional phase incrementation (TPPI) for quadrature detection in the t_1 dimension (15). Solvent suppression for 2D-NOESY and TOCSY experiments was achieved using the excitation sculpting method with gradients (16).

TOCSY and NOESY spectra were referenced to the water proton signal at 280 K. TOCSY data were recorded using an MLEV-17 mixing scheme (17). Mixing times of 50 and 80 ms were used. NOESY experiments were carried out with mixing times of 200 and 400 ms. Both TOCSY and NOESY NMR data were used in spin system identification and proton chemical shift assignments. Two-dimensional data were collected with 2048 $\times$ 512 complex data points with eight scans per increment and a spectral width of 10 ppm in each dimension. All data were processed using NMRPipe (18), and analyzed with Sparky programs (19).

To establish whether the isolated SPA4 peptide adopts any secondary or tertiary fold in solution, we examined the SPA4 peptide using homonuclear solution NMR spectroscopy. Severe overlap in the backbone and side chain proton chemical shifts of these residues presented a significant challenge to spin system identification, resonance assignment, and conformational analysis by NMR, with data taken using a 600-MHz NMR spectrometer. To overcome these challenges, we used total correlation spectroscopy (TOCSY) and Nuclear Overhauser Enhancement (NOE) Spectroscopy (NOESY) spectra of SPA4 to identify each spin system and to make sequence-specific assignments. Sequential connectivity between

the alpha proton (H α) of any given residue (i) and the amide proton (HN) of the following residue (i+1) were used to establish residue-specific assignments.

Structure calculations and physicochemical features

NMR-based models of SPA4 were calculated by means of simulated annealing, using NOE-based distance restraints. The NOE-based distance restraints were classified as strong, medium, and weak, corresponding to upper limits of 2.8, 3.4, and 5.0 Å, respectively. The torsion angle dynamics protocol of CNS (Ver. 1.2) was used to calculate 100 structures. The final set of 25 lowest energy structures was used for further analyses. None of the 25 structures showed an NOE violation greater than 0.5 Å. Structures were analyzed using PROCHECK-NMR.

The superposition, surface features, hydrophilicity and distance calculations for individual NMR structures were obtained using the PyMOL Molecular Graphics System (Schrödinger, OR) and the Chimera (University of California, San Francisco, CA) program (20).

Alignment of the lowest energy NMR structure of SPA4 peptide onto the available structure of rat SP-A and the computationally-docked model of SP-A-TLR4-MD2 complex

From the 25 NMR structures of SPA4 peptide, we selected the lowest energy NMR structure of SPA4 peptide and aligned it with the homologous region extracted from the available structure of rat SP-A (PDB ID: 1R13) (10). The structure of human SP-A is still unknown. The Research Collaboratory for Structural Bioinformatics (RCSB) PDB protein comparison tool was used for the alignment. Smith-Waterman superposition was used for alignment algorithm and assessment of percent identity, similarity, and root-mean-square-distance (RMSD)(21).

Next, we aligned the lowest energy NMR structure of the SPA4 peptide to the computationally-docked model of SP-A-TLR4-MD2 complex using the PyMOL program (7). The distances were noted between the closest amino acids of SPA4 peptide (D, N, Y, T, R, G) and TLR4 (E, Q, E, K, F) at the interface (7).

Results

SPA4 peptide conforms to a “U shaped” structure

The SPA4 peptide within the available X-ray structure of the carbohydrate recognition domain and neck region of rat SP-A contains a beta strand and a turn (10). *In silico* folding of the SPA4 peptide by PEPFOLD program also suggested the beta structure and a turn (8). We have now experimentally determined the structure and physicochemical features of synthetic SPA4 peptide in solution.

The CD spectrum of SPA4 peptide in water showed a major negative peak at 216 nm. This is indicative of beta-structure. Using three different secondary structure calculation programs on this spectrum, the results indicate that the peptide contains ~35% beta sheets and <5% alpha-helix (Fig. 1). Interestingly, the spectrum contains a slightly positive peak at 230 nm. This has been observed with beta-sheet-rich proteins that contain aromatic residues. We also obtained the CD spectrum at increasing concentrations of methanol in water (from

25-75%). The CD spectrum of SPA4 peptide in 75% methanol in water is shown in Figure 1. The results show increased intensities of the negative peak at 220 nm, indicative of an increase in alpha-helical structure with increasing concentrations of organic solvent. We propose that this represents folding of portions of the random coil structure to adopt an alpha-helical structure. Overall, these results indicate plasticity in the structure of SPA4, which may be advantageous in adopting a structure suitable for binding to TLR4.

The thermal denaturation of the SPA4 peptide was evaluated by monitoring the CD signal at 220 nm upon increasing the temperature from 20°C to 50°C. The midpoint of transition, T_m , was measured as 32°C (Fig. 2). Changes in the CD spectra observed during thermal denaturation of the SPA4 peptide were not reversible under the conditions used here, which may be due to an irreversible aggregation of the peptide at higher temperatures.

To establish whether isolated SPA4 peptide adopts any secondary or tertiary fold in solution, we examined SPA4 peptide using homonuclear solution NMR spectroscopy. Severe overlap in the backbone and side chain proton chemical shifts of these residues presented a significant challenge to spin system identification, resonance assignment, and conformational analysis by NMR, with data taken using a 600-MHz NMR spectrometer. To overcome these challenges, we used total correlation spectroscopy (TOCSY, Fig. 3A) and Nuclear Overhauser Enhancement (NOE) Spectroscopy (NOESY) spectra of SPA4 to identify each spin system and to make sequence-specific assignments (Table 1). The fingerprint region of the NOESY spectrum of SPA4 is shown in Fig. 3B. Sequential connectivity between the alpha proton (H_α) of any given residue (i) and the amide proton (H_N) of the following residue (i+1) were used to establish residue-specific assignments.

The solution NMR structure of peptide in 100% D_2O was calculated using a simulated annealing protocol given in CNS and using NOE distance restraints. The 25 lowest energy structures are shown in aligned or in individual format in Figs. 4 and 5. The NMR structures were analyzed by PyMOL and Chimera programs for superposition and surface and hydrophilicity characteristics, respectively. The NMR structures revealed that the “SDGTPVNYT” residues form a turn, giving a “U” shape to the peptide. The rest of the amino acids “GDFRY” and “NWYRGE”, form the two flexible arms on both sides. The distance between the first (G) and last residues (E) of the two flexible arms ranged from 5.1 to 16.9 Å (Fig. 5). Further analysis by Chimera program revealed that the SPA4 peptide is largely hydrophilic with two small hydrophobic patches (shown in orange in Fig. 6).

The structure of SPA4 peptide is similar to the homologous region of SP-A

The amino acid sequence: GDFRYSDGTPVNYTNWYRGE for the SPA4 peptide is derived from human SP-A. The homologous amino acids within an available structure of neck and C-terminal regions of rat SP-A (PDB ID: 1R13) are GDFHYLDGASVSYTNWYPGE. Although there are minor differences in the amino acids of SPA4 peptide and the available structure of SP-A, all of the 25 lowest energy NMR structures of SPA4 peptide aligned well with the homologous region extracted from the available structure of SP-A (Fig. 7). When the lowest energy NMR structure of SPA4 peptide was aligned with the homologous region extracted from available structure of rat

SP-A, the Smith-Waterman superposition approach predicted the percent identity, similarity, and RMSD of aligned structures as 70%, 75%, and 6.33, respectively (21).

Similarly, the lowest energy NMR structure of the SPA4 peptide aligned well with the homologous region of SP-A within the computationally-docked model of SP-A-TLR4-MD2 complex (Figs. 8A, B). The distances ranged from 3-10 Å between the predicted amino acids (D, N, Y, T, R, G) of SPA4 peptide and (E, Q, E, K, F) of TLR4 (7) that were close to each other (Fig. 8C). The *in silico* analysis results indicate that the structural features of SPA4 peptide are similar to the homologous region of SP-A, and are attuned to its binding to TLR4.

Discussion

The combinatorial approaches of computational docking and functional screening of a peptide library led to the identification of the TLR4-interacting SPA4 peptide. In our previously published model of the neck-C-terminal region of rat SP-A docked to the crystal structure of extracellular TLR4-MD2 protein, we predicted that the SPA4 peptide region is in close contact with TLR4 (7). Correspondingly, we found that the synthetic SPA4 peptide encoding the human SP-A sequences binds to TLR4 protein in complex with MD2, and suppresses an inflammatory response against LPS (7). Based on our initial predictions from the computationally-docked model of the SP-A-TLR4-MD2 complex (7), we hypothesized that the structural characteristics of SPA4 peptide are important for its function.

Per the recently published reports, activation of TLR4 signaling occurs in multiple steps. The LPS binding protein catalyzes the transfer of LPS to CD14, then LPS transfers from CD14 to MD2, which is followed by LPS interaction with TLR4 (22, 23). Although the molecular mechanism of SPA4 peptide binding to TLR4 is unknown, our results demonstrate that the SPA4 peptide does not bind to or sequester the TLR4 ligand, LPS (24). Interestingly, our published results also revealed that the SPA4 peptide suppresses the LPS-induced inflammatory response when administered therapeutically to LPS-challenged cells or mice and by modulating MYD88-dependent signaling (8). It is expected that the TLR4 would have been activated in these models by LPS at the time when SPA4 peptide was administered (7, 8). As we progress towards investigating the comprehensive mechanism of SPA4 peptide activity, here we report the solved NMR structure and physicochemical features of synthetic SPA4 peptide, and its comparison with the homologous region within the available structure of rat SP-A, and the computationally-docked model of SP-A-TLR4-MD2 complex.

We used conventional CD analysis and NMR spectroscopy to determine the structure of SPA4 peptide. The turn at the “SDGTPVNYT” sequence creates a “U” shaped structure. The results of CD analysis confirm the plasticity of the SPA4 peptide. We also observed that the peptide unfolds in water as the temperature is increased, but it does not fold back when the temperature is brought down. Overall, the results of NMR and CD analysis indicate that the peptide is flexible, and forms a turn at the center when in solution. The SPA4 peptide is largely hydrophilic, except for small hydrophobic patches at two separate places. These results correspond with its hydropathy index and partition co-efficient, as published earlier

(8). It is expected that the hydrophilicity of the SPA4 peptide will prevent the transfer of SPA4 peptide through the lipid-rich membrane. These results indirectly support that the SPA4 peptide will most likely bind to the activated TLR4 on the extracellular surface of immune cells without being integrated into the lipid bilayer of the cell membrane.

The “U” shaped structure of the SPA4 peptide aligns well with the homologous region within the available structure of rat SP-A (PDB: 1R13), and is similar to what was predicted earlier by the PEPFOLD program (8). Further, we aligned the lowest energy NMR structure of SPA4 peptide structure with the computationally-docked model of SP-A-TLR4-MD2 complex. The alignment of SPA4 peptide with the homologous region of SP-A, and the relatively short distances between the closest amino acid residues of the SPA4 peptide and TLR4 (Fig. 8), point towards the structural adaptability of SPA4 peptide for binding to TLR4. Our results indicate that the structure of the synthetic SPA4 peptide is quite identical to the homologous region within the SP-A protein. Although the structure-activity relationship of the SPA4 peptide and TLR4 needs to be studied further, our results show that SP-A and the SPA4 peptide bind to TLR4 and suppress an inflammatory response (6-8). The structural similarity between the SPA4 peptide and the homologous region within SP-A correspond to their immunomodulatory function through their interaction with TLR4.

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Figure 1. Representative CD spectrum of SPA4 peptide suspended in water or 75% methanol in water. The spectra recorded at 20°C are in units of mean residue ellipticity versus wavelength.

Figure 2.

Assessment of thermal stability of the SPA4 peptide. Thermal denaturation of the SPA4 peptide in water was determined by changes in the CD signal intensity at a wavelength of 220 nm. T_m refers to the midpoint of the transition between folded and unfolded states, and was calculated as described in Materials and Methods.

Figure 3.

All of the spin systems were assigned using 80 ms mixing TOCSY (A). Sequence-specific assignments were made using 200 ms and 400 ms mixing NOESY. 200 ms mixing NOESY is shown in (B).

Figure 4. Superposition of 25 low energy NMR structures of SPA4 peptide. The structures were superposed by aligning the C-alpha carbons of the amino acid residues “SDGTPVNYT” (in blue). The superposition was performed using the PyMOL molecular graphics program.

Figure 5.

The distance in Å between the first- G and last- E residues in the individual 25 NMR structures of SPA4 peptide was assessed using the PyMOL molecular graphics program. NMR structures are shown as a ribbon diagram. The structural alignment is similar to those shown in Figure 4.

Figure 6.

Hydrophobic domains within the SPA4 peptide. Orientation is rotated 180 degrees relative to the standard rotation along the vertical axis. The color code is: blue, polar regions; white, in between; orange, hydrophobic regions. The Chimera program was used to obtain information about hydrophobic or hydrophilic regions within the NMR structures of the SPA4 peptide.

Figure 7.

Alignment of the region homologous to SPA4 peptide within the available structure of rat SP-A (PDB ID: 1R13; shown in black) with individual NMR structures. The alignment of SP-A region homologous to SPA4 peptide with (A) all structures superposed and (B) individual 25 low energy NMR structures of SPA4 peptide in solution. NMR structures are shown as ribbon diagrams.

Figure 8.

In silico analysis of SPA4 peptide structure aligned to homologous regions within SP-A in a computationally-docked model of the SP-A-TLR4-MD2 complex. Part of the computationally-docked model of the SP-A-TLR4-MD2 complex is taken from Ref. (7), and is reproduced with permission from the American Society for Pharmacology and Experimental Therapeutics. (A) Alignment of the lowest energy NMR structure of SPA4 peptide (in red) with SP-A (in green) within a computationally-docked model of SP-A-TLR4-MD2 complex. (B) Previously predicted amino acids of the homologous region of rat SP-A (D, S, Y, T, P, G, green sticks) and TLR4 (E, Q, E, K, F, violet sticks). The amino acids (D, N, Y, T, R, G) of the SPA4 peptide are derived from human SP-A, and are shown as red sticks within the computationally-docked model. (C) The distances between the closest amino acids of SPA4 peptide (D, N, Y, T, R, G) and TLR4 (E, Q, E, K, F) were measured using the PyMOL program. The regions surrounding the interface have been made transparent for better viewing. The distances between the intermolecular residues are shown in Å units in yellow.

Table 1
Chemical shift assignment table for SPA4 peptide

#	3-Letter Name	1-Letter Code	NH (ppm)	HA (ppm)	Side chain 1H (ppm)
1	GLY	G	-----	3.71,3.71	
2	ASP	D	8.67	4.65	2.63,2.74
3	PHE	F	8.40	4.50	2.92,2.92
4	ARG	R	8.16	4.20	1.52,1.52,1.34,1.34, 3.01
5	TYR	Y	8.27	4.49	2.84,2.98
6	SER	S	8.36	4.35	3.75,3.77
7	ASP	D	8.52	4.64	2.85,2.85
8	GLY	G	8.37	3.86,3.98	
9	THR	T	8.06	4.50	4.05,1.17
10	PRO	P		4.32	1.91,2.15,1.76,1.84,3.54,3.76
11	VAL	V	8.24	3.87	1.81,0.66,0.77
12	ASN	N	8.40	4.61	2.64,2.72
13	TYR	Y	8.27	4.42	2.84,2.98
14	THR	T	8.11	4.13	4.00,1.02
15	ASN	N	8.31	4.60	2.64,2.71
16	TRP	W	7.99	4.50	3.11,3.11
17	TYR	Y	7.73	4.28	2.72,2.74
18	ARG	R	8.05	4.03	1.72,1.58,1.45, 3.03
19	GLY	G	7.74	3.77,3.85	
20	GLU	E	7.98	4.17	1.95,1.71,2.28