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Residue specific partitioning of KL_4 into phospholipid bilayers

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ABSTRACT

KL_4, which has demonstrated success in the treatment of respiratory distress, is a synthetic helical, amphipathic peptide mimetic of lung surfactant protein B. The unusual periodicity of charged residues within KL_4 and its relatively high hydrophobicity distinguish it from canonical amphipathic helical peptides. Here we utilized site specific spin labeling of both lipids and the peptide coupled with EPR spectroscopy to discern the effects of KL_4 on lipid dynamics, the residue specific dynamics of hydrophobic regions within KL_4, and the partitioning depths of specific KL_4 residues into the DPPC/POPG and POPC/POPG lipid bilayers under physiologically relevant conditions. KL_4 induces alterations in acyl chain dynamics in a lipid-dependent manner, with the peptide partitioning more deeply into DPPC-rich bilayers. Combined with an earlier NMR study of changes in lipid dynamics on addition of KL_4 (V.C. Antharam et al., 2009), we are able to distinguish how KL_4 affects both collective bilayer motions and intramolecular acyl chain dynamics in a lipid-dependent manner. EPR power saturation results for spin labeled lipids demonstrate that KL_4 also alters the accessibility profiles of paramagnetic colliders in a lipid-dependent manner. Measurements of dynamics and depth parameters for individual spin-labeled residues within KL_4 are consistent with a model where the peptide partitions deeply into the lipid bilayers but lies parallel to the bilayer interface in both lipid environments; the depth of partitioning is dependent on the degree of lipid acyl chain saturation within the bilayer.

1. Introduction

Pulmonary surfactant (PS) is a vital, lipid-rich extracellular fluid that lowers surface tension and provides immunoprotection within the alveoli. PS is comprised of a mixture of phospholipids and proteins, with the major lipid component being 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). Low concentrations of hydrophobic proteins within PS are critical to its unique physical properties and lipid trafficking functions including the following: facilitating lung expansion at ambient pressure, preventing alveolar collapse, and significantly reducing surface tension in a highly dynamic, organized process [1–3]. Inadequate PS production in premature infants is the leading cause of acute respiratory distress syndrome (ARDS), [4–6] where typical treatment is the administration of exogenous, animal-derived PS via the airways [7,8]. The reliance on these biologically sourced surfactants is due to the difficulty in recombinant expression and purification of the highly hydrophobic surfactant proteins critical to PS function, particularly surfactant protein B (SP-B). Failure to produce functional SP-B leads to respiratory failure and eventual death [9].

Synthetic, peptide-based lung surfactant replacement therapies for RDS therapy have shown promise and can potentially reduce the cost and immunologic risks associated with exogenous animal-derived PS. Functional studies have shown that much of the action of SP-B can be recaptured using peptides 20–25 amino acids in length based on the N- and C-termini of SP-B [10–12]. KL_4, is a 21-mer peptide mimetic of the C-terminus of SP-B, of sequence KLLLLKLLLLKLLLLKLLLLK, and it was designed based on the charge distribution and hydrophobicity of the native protein sequence [13]. KL_4 has demonstrated efficacy for treating ARDS when formulated with palmitic acid and lipids commonly found in PS [14,15]. This formulation, termed Lucinactant, was recently approved for the prevention of RDS in high risk premature infants [16]. Although KL_4 is a promising replacement of SP-B in the treatment for RDS, its mechanism of action remains controversial. The increased effectiveness of KL_4 when compared to other clinically available formulations [17,18] and its greatly simplified amino acid sequence suggest that an understanding of its structural properties and its effects on the molecular and biophysical properties of the lipids could yield fundamental

Abbreviations: PS, pulmonary surfactant; SP-B, surfactant protein B; MLV, multilamellar vesicle; ARDS, acute respiratory distress syndrome; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; *n*-doxyl-PSPC, 1-palmitoyl-2-stearoyl-(*n*-doxyl)-*sn*-glycero-3-phosphocholine; P/L, peptide/lipid molar ratio; CD, circular dichroism; FTIR, Fourier transform infrared spectroscopy; SDSL, site-directed spin label; CW-EPR, continuous wave electron paramagnetic resonance; ΔH_{pp}, peak-to-peak linewidth of central resonance line; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; NiAA, nickel (II) acetylacetonate; NiEDDA, nickel (II) ethylenediamine-N,N′-diacetic acid; TEMPO-PC, 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phospho(tempo)choline; IAP, iodoacetamido-PROXYL spin label

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insights into peptide/lipid interactions for the rational design of novel synthetic mimetics.

Low resolution structural characterization of KL_4 in lipid environments via CD and FTIR has consistently indicated the formation of a helical peptide conformation in lipid suspensions; although some variations in helicity with lipid composition have been reported [19,20]. Experimental characterizations of the orientation of KL_4 have yielded inconsistent, conflicting results. In early FTIR work using DPPC/DPPG (7:3) bilayers, KL_4 was found to be helical and spanning the bilayer in a transmembrane orientation [21]. Later FTIR work, using either DPPC or DPPC/DPPG (7:3) mixtures, indicated that KL_4 lies along the surface of the lipids as a mixture of β-sheet and α-helix at the air/water interface [22]. However, these lower-resolution assays monitored amide I stretching frequencies, which can be solvent dependent [23], and assumed that KL_4 adopts classical β-sheet or α-helix secondary structures. A more recent transcription–translation assay found KL_4 capable of traversing lipid bilayers, suggesting a transmembrane orientation [24]. However, this transcription–translation assay relied on the integration of a DNA sequence encoding KL_4 into the *Escherichia coli* inner membrane protein leader peptidase (Lep) gene, translation of the protein at ER-derived microsomal membranes, and assaying for glycosylation using proteinase K to determine whether the peptide traverses the membrane. This type of host–guest experiment may influence the secondary structure of KL_4 in the lipid bilayers, and KL_4 is known to alter lipid dynamics and trafficking, which may affect the overall integrity of the microsomal membranes.

A possible explanation for these mixed results may lie in a detailed investigation of the exact helical nature of KL_4. Interestingly, the occurrence of lysines at every *fifth* residue in KL_4 prevents a simple amphipathic helical wheel prediction for the structure and orientation of KL_4 in a lipid environment; nevertheless, this lysine pattern was found to convey maximal surface activity [25]. A helical wheel projection of the KL_4 sequence assuming a canonical α-helical conformation reveals a side chain distribution that is at odds with partitioning of an amphipathic peptide within the plane of lipid bilayers in a membrane environment (Fig. 1A). Specifically, the canonical α-helical pitch does not produce a significant hydrophobic moment nor impart amphipathic characteristics to the peptide. The KL_4 sequence also does not contain a long enough hydrophobic segment to traverse the membrane, i.e. a transmembrane orientation of the peptide would bury 2–3 charged lysine side chains in the hydrophobic interior of the membrane. Previously, we have characterized the structure of KL_4 in POPC/POPG (3:1) lipid bilayers via solid state NMR spectroscopy and found that it adopts a structure that is helical yet more amphipathic than would be predicted by an α-helical conformation [20]. As depicted in Fig. 1B and C, structural models derived from NMR data of KL_4 in two different lipid environments exhibit a helical pitch of >4 residues/turn, in contrast to a canonical α-helix with 3.6 residues/ turn.

In order to more directly probe the insertion depth of KL_4 at the residue level, to characterize the effects of KL_4 on lipid dynamics on a nanosecond rather than microsecond timescale, and to determine the effects of KL_4 on solvent penetration into the lipid bilayers, we utilized site-directed spin labeling (SDSL) and EPR spectroscopy to examine the interaction of KL_4 with DPPC/POPG and POPC/POPG lipid vesicles. The use of power saturation experiments to generate a more quantitative, residue-specific partitioning profile for membrane-associated proteins is well-established [26], and yields information that is highly complimentary to our previous NMR studies [27,28]. By analyzing EPR line shapes and paramagnetic accessibility in a site-specific manner, we are developing a comprehensive model of the membrane bound orientation of KL_4, thus providing structural insights into its mechanism of action in restoring PS function and directing future rational development of more active SP-B peptidomimetics.

2. Materials & methods

2.1. Materials

POPC, DPPC, POPG, n-doxyl-PSPC (where $n = 5$, 7 and 12) and TEMPO-PC were purchased as chloroform solutions from Avanti Polar Lipids (Alabaster, AL) and quantified by phosphate analysis (Bioassay Systems, Hayward, CA). Iodoacetamido-PROXYL spin label (IAP) was purchased from Sigma (St. Louis, MO) and used as received. Unless otherwise stated, all other reagents were purchased from Fisher Scientific (Hampton, NH) and used as received.

2.2. Synthesis of KL_4

KL_4, (KLLLLKLLLLKLLLLKLLLLK) was synthesized via solid-phase peptide synthesis (ICBR Facility, UF), purified by RP-HPLC, and verified by mass spectrometry (m/z = 2469). Peptide was dissolved in methanol and analyzed by amino acid analysis for concentration (Molecular Structure Facility, UC Davis). Eight cysteine variants of KL_4, in which individual leucines were replaced by cysteine were also synthesized via solid-phase peptide synthesis (SPPS), purified, and mass verified (m/z = 2459).

2.3. Spin-labeling of KL_4 cysteine mutants

Cysteine containing KL_4 was dissolved in a minimal amount of King's reagent [29] and ether precipitated to ensure that the cysteine was reduced prior to spin labeling. The peptide was then dissolved at a concentration of ~0.1 mM in MeOH and KOH was added to adjust the pH to

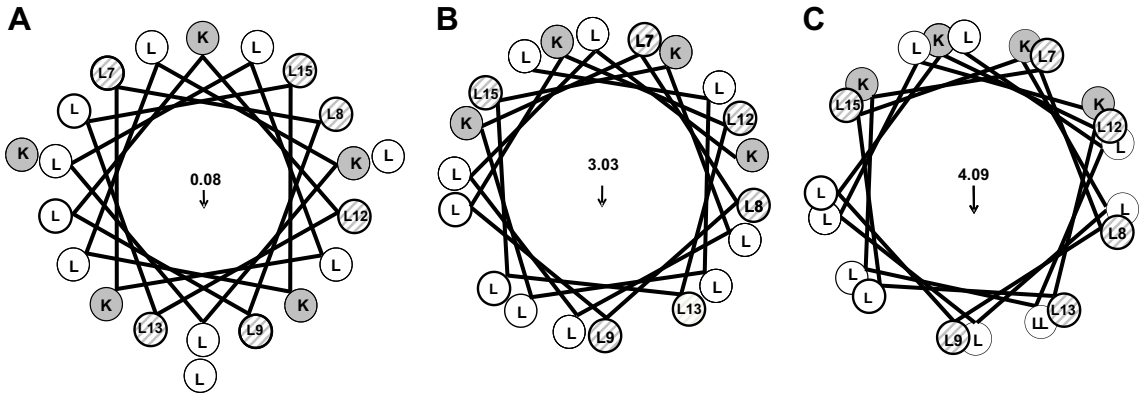

Fig. 1. Helical wheel plots of KL_4 with varying pitch. Wheels generated (A) assuming a canonical α-helix (3.6 residues/turn); (B) using the average torsion angles determined in POPC/POPG vesicles (4.1 residues/turn) [20]; and (C) using the average torsion angles suggested by preliminary NMR experiments with DPPC/POPG vesicles (4.6 residues/turn). Arrows indicate the net hydrophobic moments resulting from the distribution of charged lysine sidechains on the helix surface. The first lysine is not shown in (B) and (C) as NMR data indicate that the N-terminus is less structured relative to the rest of the helix. Leucines successively substituted with cysteine for SDSL-EPR experiments in this work are shown in gray crosshatch.