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Alterations of the C-terminal end do not affect *in vitro* or *in vivo* activity of surfactant protein C analogs

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ABSTRACT

The secondary structure, orientation and hydrogen/deuterium exchange of SP-C33, a surfactant protein C analog, in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine/egg phosphatidylglycerol (8:2, wt./wt.) bilayers, was studied by attenuated total reflection Fourier transform infrared spectroscopy. This showed a transmembrane α-helix, in which about 55% of the amide hydrogens do not exchange for up to 20 h. Moreover, C-terminally modified SP-C33, either truncated after position 30, or having the methionine at position 31 exchanged for either lysine or isoleucine, showed the same secondary structure and orientation. The different peptides, suspended in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol 68:31 (wt./wt.), were tested for surfactant activity *in vitro* in a captive bubble surfactometer and *in vivo* in an animal model of respiratory distress syndrome using premature rabbit fetuses. All preparations showed similar surface activity in the captive bubble surfactometer. Also, in the rabbit model, all preparations performed equally well and significantly better than non-treated controls, both regarding tidal volumes and lung gas volumes. Thus, truncation or residue replacements in the C-terminal part of SP-C33 do not seem to affect membrane association or surfactant activity.

1. Introduction

The complex lipid/protein mixture of pulmonary surfactant reduces the surface tension at the alveolar air–liquid interface to near-zero values and thereby prevents the alveoli from collapsing at exhalation. Lack of active surfactant is associated with respiratory distress syndrome (RDS) which is common in premature babies. Surfactant consists of several components with different functional properties [1,2]. The main component, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), is responsible for surface tension reduction but is dependent on more fluid phospholipids and the hydrophobic surfactant specific proteins (SP-) SP-B and SP-C to rapidly adsorb to the air/liquid interface [3,4]. Both SP-B and SP-C are required for establishment of alveolar patency at end-expiration [5].

SP-C contains 35 residues with a random coil N-terminal segment and a middle/C-terminal transmembrane α-helix which is rich in valines. In spite of its rather simple structure, chemical synthesis of SP-C is complicated by the propensity of valine to form β-sheets instead of α-helices [6]. SP-C therefore is a metastable protein that tends to unfold and turn into β-sheets [7,8]. Different approaches have been taken to obtain SP-C analogs and we have previously designed the SP-C analog, SP-C33, that has been shown to have surfactant properties similar to native SP-C [9]. Differences of this peptide compared to human SP-C includes removal of the two N-terminal amino acid residues, replacement of the palmitoyl-Cys residues at positions 5 and 6 (positions 3 and 4 in SP-C33) with Ser, replacement of Leu 14 with Lys, and replacement of the poly-valine part (positions 15–21 and 23–28) with poly-leucine.

The mechanism of SP-C action is not known, and several different hypotheses have been discussed. It has been shown that SP-C disorders the acyl chains of a phospholipid bilayer while the head groups are stabilized, possibly due to its mobility gradient across the bilayer [10,11]. The mobility gradient is suggested to derive from interactions of a Lys–Arg pair in the N-terminal end of the transmembrane α-helix with phospholipid head-groups and lack of residues in the C-terminal end that are able to interact with surrounding phospholipids. In the "squeeze-out" hypothesis, SP-C is assumed to contribute to the process of removal of non-DPPC lipids from the air/liquid interface upon overcompression [1]. SP-C is also thought to facilitate the reinsertion of surface active

Abbreviations: CBS, captive bubble surfactometer; DPPC, 1,2-dipalmitoyl-*sn*-glycero-phosphocholine; ATR-FTIR, attenuated total reflection Fourier transform infrared spectroscopy; LGV, lung gas volume; PEEP, positive end expiratory pressure; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; RDS, respiratory distress syndrome; SP, surfactant protein

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molecules into the interface from surface-associated reservoirs during alveolar expansion [12].

In this study, we have examined the secondary structure, orientation, and dynamics of SP-C33 and how modifications of the C-terminal end of SP-C33 affect the activity of a synthetic surfactant preparation. Three different SP-C33 variants were studied. SP-C30 is a truncated variant that is predicted to be too short to fully span a phospholipid bilayer and thereby may obtain increased mobility. SP-C33M31K, in which the methionine at position 31 has been exchanged with a lysine, may anchor the C-terminal part of the α-helix in the bilayer and thereby reduce its mobility gradient. SP-C33M31I, where the methionine at position 31 was substituted with an isoleucine, is not expected to affect mobility of the peptide.

2. Materials and methods

2.1. Lipids and peptides

DPPC and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were obtained from Sigma Chemical (St. Louis, MO, USA).

SP-C33 (IPSSPVHLKRLKLLLLLLLLILLLILGALLMGL) was provided by Chiesi Farmaceutici S.p.A (Parma, Italy).

SP-C33 M31K (IPSSPVHLKRLKLLLLLLLLILLLILGALLKGL), SP-C33 M31I (IPSSPVHLKRLKLLLLLLLLILLLILGALLIGL), and SP-C30 (IPSSPVHLKRLKLLLLLLLLILLLILGALL) were obtained from Thermo Electron GmbH.

2.2. Surfactant preparations

DPPC/POPG (68:31, wt./wt.) was dissolved in chloroform/methanol (1:1, vol./vol.) and mixed with either of the four SP-C analogs. The peptide content in the preparations was 2% in relation to the phospholipid weight. The solvents were evaporated under nitrogen and the preparations were resuspended in normal saline to a final phospholipid concentration of 80 mg/ml by slow rotation at 37 °C. The preparations were stored at −20 °C. Prior to examination of *in vitro* surface activity, all solutions were diluted with saline to a final concentration of 10 mg phospholipids per milliliter.

For attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy of the SP-C analogs in DPPC/PG (8:2, wt./wt.), lipid/peptide mixtures in chloroform/methanol (lipid/peptide mass ratio, 100:10 or 100:20) were evaporated under nitrogen and dried under vacuum for 3 h. The lipid/peptide films were then suspended in 5 mM HEPES buffer, pH 7.4. Lipid vesicles containing the peptides were separated from unbound constituents by centrifugation in a 5%–40% (wt./vol.) continuous sucrose gradient. The sucrose gradient was fractioned and the proteoliposomes were detected by turbidity measurement at 410 nm. To eliminate sucrose, the vesicles were pelleted twice by ultracentrifugation and resuspended in 5 mM HEPES buffer, pH 7.4.

2.3. Structure determination and hydrogen/deuterium (H/D) exchange measurements

Structure and orientation of SP-C analogs in phospholipid membrane and H/D exchange for SP-C33 were obtained by ATR-FTIR spectroscopy [13]. ATR-FTIR spectra were recorded at 20 °C on a Bruker IF-55 spectrometer (Ettlingen, Germany), equipped with a liquid nitrogen cooled mercury cadmium telluride detector. The internal reflection element was a germanium crystal (50×20×2 mm; ACM, Villiers St. Frédéric, France) with an aperture angle of 45° yielding 25 internal reflections. The samples were spread on the ATR element by slowly evaporating the solvent under nitrogen. The spectra were recorded at a nominal resolution of 2 cm^{-1} with 256 accumulations to improve the signal/noise ratio. The secondary structure was determined from the shape of the deuterated amide I band [14]. Briefly, Fourier self-deconvolution was applied to detect the different secondary structure components. To quantify the percentage of each resulting band, a least square iterative curve-fitting was performed to fit Lorentzian line shapes to the initial spectrum between 1700 and 1600 cm^{-1}. The percentage of each secondary structure was determined from the Lorentzian band areas in the respective frequency region.

The orientation of SP-C analogs in a lipid bilayer was determined from the orientation of the peptide bond C=O group as described previously [14]. Additional spectra with parallel and perpendicular polarized light with respect to the incidence plane were recorded. To determine the α-helix orientation, a 27° deviation angle between the α-helix axis and the C=O transition dipole moment was taken into account. The mean angle between the helix axis and a normal to the ATR plate was calculated from the dichroic ratio. The dichroic ratio corresponding to the transition dipole moment of the $\gamma_w(CH_2)$ band at 1200 cm^{-1} was used to characterize the lipid acyl chain orientation.

H/D exchange of accessible amide protons was obtained by flushing the sample on the ATR element with D_2O saturated nitrogen at room temperature. The decay of the NH-associated amide II band area (1520–1580 cm^{-1}) to lipid ν(C=O) band area was used to monitor the H/D exchange in the amide group [14].

2.4. Circular dichroism (CD) spectroscopy

SP-C33 was dissolved in either methanol or in sodium dodecyl sulfate (SDS) micelles in sodium phosphate buffer, pH 6.0, to a final concentration of 25 μM. CD spectra from 250 to 190 nm were recorded in 1.0 mm path length quartz cuvettes using a J-810 spectropolarimeter (Jasco, Tokyo, Japan). The scan speed was 50 nm/min, response time was 2 s, and with an acquisition interval of 0.5 nm and band width of 1 nm. The residual molar ellipticity (θ) is expressed in $kdeg \times cm^2 \times dmol^{-1}$. The α-helical content was calculated with the following equations: $(\theta_{208} - 4000/-37000) \times 100$, $(\theta_{222} - 3000/-39000) \times 100$ [15].

2.5. In vitro surface activity measurements

Surface tension was measured in triplicates in an *in vitro* simulation of the alveolus by a captive bubble surfactometer (CBS) [16]. In the CBS, surfactant and an air-bubble representing the alveolus are present in an air-tight enclosed chamber. To evaluate surface activity under dynamic circumstances, the chamber is compressed and surface tension can be calculated by studying the shape and height/width ratio of the bubble.

In the experiment, 2 μl of the surfactant preparation (10 mg/ml) was inserted into the sucrose-filled test chamber. After insertion, an air-bubble was created and surface tension was measured during 5 min of adsorption. In the following quasi-static cycling experiments, the bubble was compressed stepwise from the initial volume until a surface tension less than 5 mN/m was reached, alternatively to a maximum area compression of 50%, and then expanded during five cycles. For each run, minimum and maximum surface tensions as well as rate of compression were registered and a median value was calculated.

2.6. In vivo experiments

To assess the surfactant activity of the different preparations *in vivo*, preterm newborn rabbits obtained at a gestational age of 27 days (term, 31 days) were used. The animals, randomly allocated to different treatment groups, were anesthetized directly after birth, tracheotomized, and then received one of the surfactant preparations via a tracheal cannula. Surfactant, 80 mg/ml, was administered at a dose of 2.5 ml/kg and non-treated animals served as negative controls. The animals were kept in pletysmograph boxes at 37 °C and ventilated in parallel with 100% oxygen at a frequency of 40 Hz and an inspiration/expiration ratio of 1:1. The experiments were

performed with a positive end-expiratory pressure (PEEP) of 3–4 cm H_2O. To open up the lungs, peak inspiratory pressure (PIP) was first set at 35 cm H_2O for 1 min. Then, pressure was lowered to 28 cm H_2O for 2 min and further on to 23 cm H_2O for 2 min, 18 cm H_2O for 15 min, 13 cm H_2O for 5 min, and 10 cm H_2O for 5 min. Finally, pressure was raised again to 18 cm H_2O for 5 min, after which the lungs were ventilated for additional 5 min with nitrogen to minimize volume loss due to diffusion. Tidal volumes were recorded every 5 min during the experiment. At the end of the experiment, the tracheal cannula was clamped at end expiration, the trachea was ligated, and the lungs were excised and weighed. Lung gas volumes were determined by water displacement technique [5,17].

2.7. Statistical analysis

Statistical differences were analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison test using GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA).

2.8. Ethical approval

Animal experiments were approved by the local ethical committee for animal research, Stockholms Norra Djurförsöksetiska Nämnd (205/04, 316/06).

3. Results

3.1. SP-C analogs secondary structure and orientation in a phospholipid membrane

The structure of SP-C analogs reconstituted in a DPPC/PG (8:2, wt./wt.) environment was determined by ATR-FTIR. Analysis of the IR-spectrum in the spectral region associated with secondary structure shows that SP-C33 mainly adopts an α-helical structure, characterized by an amide I band (1700–1600 cm^{-1}) maximum at 1657 cm^{-1} (Fig. 1A). Quantification of the different secondary structure components by deconvolution and curve-fitting of the amide I band demonstrates that more than 90% of the polypeptide chain forms an α-helical structure. Upon reconstitution in a lipid bilayer, the SP-C33M31K, SP-C33M31I, and SP-C30 peptides adopt a similar α-helical structure as illustrated by the superimposition of their respective amide I bands (Fig. 1B).

SP-C33 dissolved in methanol or SDS micelles was analyzed by CD spectroscopy. The resulting spectra (Fig. 2) showed double minima at 208 and 222 nm, typical features for α-helical proteins. Estimation of the α-helix contents from the residual molar ellipticities at 208 and 222 nm yields about 75% helix for SP-C33 in SDS micelles and more than 90% in methanol.

To determine the orientation of SP-C analogs inserted in a lipid bilayer, spectra were recorded with two orthogonal polarizations of the incident light, parallel (//) and perpendicular (⊥) with respect to the incident plane as illustrated for SP-C33M31I in Fig. 3 as an example. The difference spectrum (//−⊥) reports an important positive deviation in the amide I band region (Fig. 3C). From the dichroic ratios of this band, the calculated maximum tilts of the long helix axis with respect to a normal to the crystal surface were 22°, 18°, 18°, and 17° for SP-C33, SP-C33M31K, SP-C33M31I, and SP-C30, respectively. From the dichroic ratios of the $\gamma_w(CH_2)$ band at 1200 cm^{-1}, we have determined that the maximum tilt between the lipid hydrocarbon chains and a normal to the germanium surface was 25 °. These data demonstrate that the four SP-C analogs adopt a dominantly transmembrane orientation in a lipid bilayer.

3.2. H/D exchange of SP-C33 in DPPC/PG

H/D exchange kinetics of SP-C33 reconstituted in a DPPC/PG (8:2, wt./wt.) lipid bilayer was recorded using ATR-FTIR where the sample was flushed with D_2O at room temperature. After 12 h of deuteration, 55% of the peptide bond protons had not undergone exchange (Fig. 4). This implies that about half of the helical structure of SP-C33 is rigid enough to resist exchange of amide hydrogens. No modification of the α-helical structure was observed during the kinetics, indicating that the secondary structure of SP-C33 is stable.

3.3. In vitro surface activity of SP-C33 and variants thereof

For comparison of the *in vitro* surface activity of the different peptides in DPPC/POPG (68:31, wt./wt.), the surface tension during dynamic conditions was evaluated in the CBS. After the initial adsorption, quasi-static expansion was performed in which all preparations showed similar values with no significant differences regarding maximum and minimum surface tensions after five pulsation cycles (Table 1). The medians of the different synthetic preparations showed γ_{min} around 1–2 mN/m and γ_{max} between 22–27 mN/m. In addition, the degree of compression required to reach 5 mN/m was similar (22%–32%) with no significant differences between tested preparations.

3.4. In vivo activity of SP-C33 and variants thereof

In line with the *in vitro* results, all peptide-containing preparations behaved similarly in a rabbit model of neonatal RDS. The rabbits were

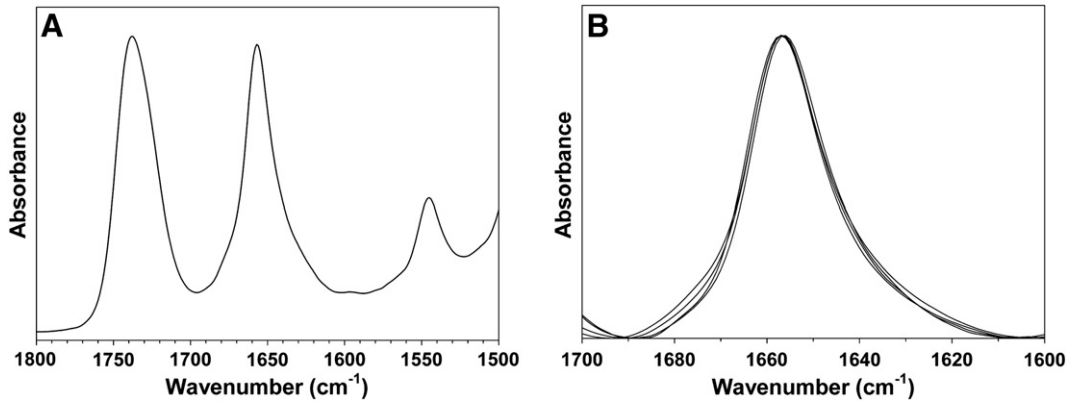

Fig. 1. (A) ATR-FTIR spectrum in the 1800–1500 cm^{-1} region for SP-C33 in DPPC/PG (8:2, wt./wt.) lipid environment. The amide I band (1700–1600 cm^{-1}) presents the characteristic shape of an α-helical peptide with a maximum at 1657 cm^{-1}. (B) Superimposition of SP-C33, SP-C33M31K, SP-C33M31I, and SP-C30 amide I bands. The four peptides were reconstituted in DPPC/PG (8:2, wt./wt.) lipid environment. The samples in panels A and B were deuterated during 20 min and the contributions of lateral side chains were subtracted.

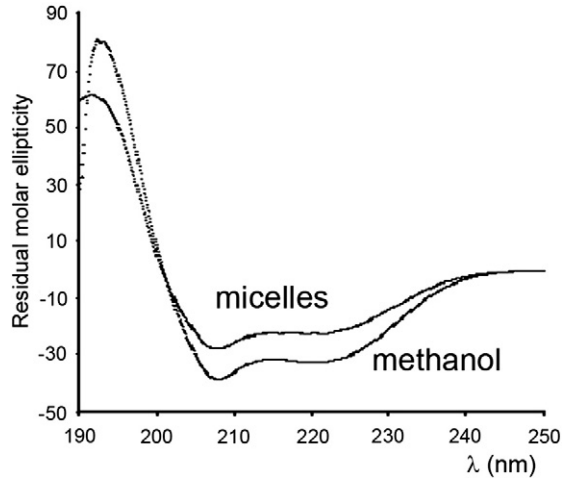

Fig. 2. CD spectra of SP-C33 dissolved in SDS micelles or methanol. The residual molar ellipticity (θ) is expressed in kdeg × cm^2 × dmol^{-1}.

ventilated with a standardized sequence of insufflations pressure, and throughout experiments, tidal volumes were very similar in all groups that received surfactant. After 35 min of ventilation, tidal volumes around 12–15 ml/kg were observed (Fig. 5). Likewise, the lung gas volumes measured after the experiments were similar for all surfactant-treated groups (Fig. 6). For tidal volumes, throughout the whole experiment, as well as lung gas volumes, all the surfactants containing peptides were significantly better than non-treated controls but with no significant differences between the tested surfactant preparations.

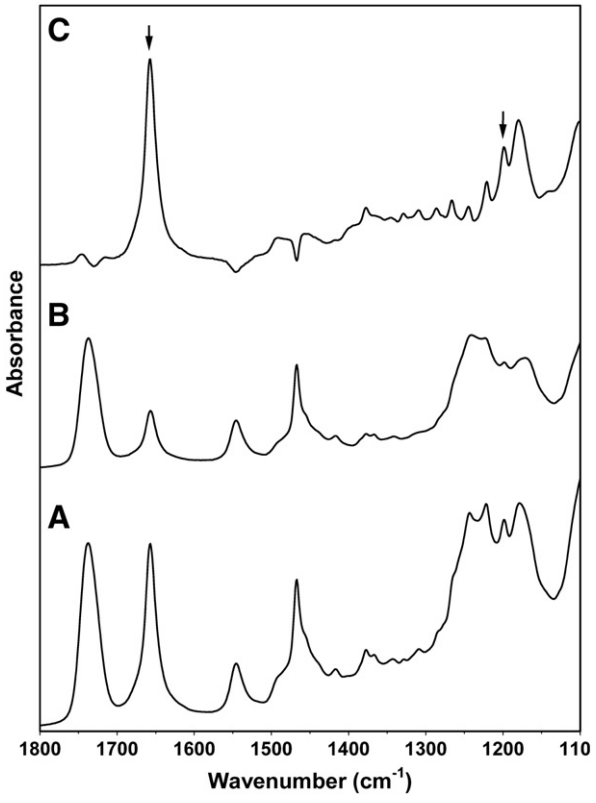

Fig. 3. ATR-FTIR polarized spectra of SP-C33M31I reconstituted in DPPC/PG (8:2, wt./wt.) lipid environment. The spectra were recorded with parallel (A) and perpendicular (B) polarized light with respect to the incident plane. (C) The dichroic spectrum obtained by subtracting (A–B) spectra is expanded twofold in the ordinate direction. The arrows indicate the protein amide I and the phospholipid $\gamma_w(CH_2)$ bands.

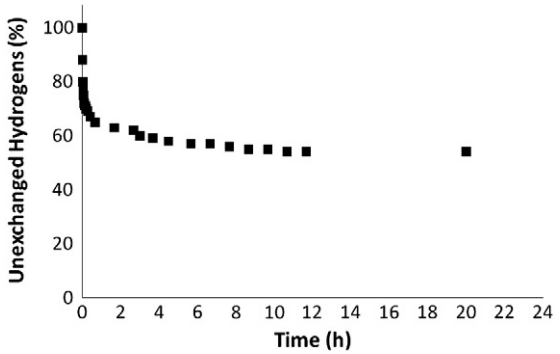

Fig. 4. Hydrogen/deuterium exchange kinetics evaluated using ATR-FTIR for SP-C33 reconstituted in DPPC/PG (8:2, wt./wt.). Evolution of the proportion of unexchanged amide bonds as a function of deuteration time.

4. Discussion

Investigation of the secondary structure of the SP-C analog SP-C33 using FTIR and CD spectroscopy reveals that the peptide adopts an α-helical structure when inserted in a DPPC/PG lipid environment, in SDS micelles and in methanol. The results obtained show that 75%–90% of the peptide adopts helical conformation. Previous FTIR and CD studies of native SP-C indicate an α-helical content of 60%–80% [14,18] while the predecessors to SP-C33, SP-C(Leu) and SP-C(LKS), show an α-helical content of 84% [19] and 75% [20], respectively. This indicates that the overall helical contents of native SP-C and analogs thereof determined by CD and FTIR spectroscopy are similar and in good agreement with the α-helical content (about 75%) deduced from the NMR structure of native SP-C dissolved in chloroform/methanol [21]. We have demonstrated that the three C-terminally modified variants of SP-C33 adopt the same conformation as SP-C33 which is consistent with the fact that all so far analyzed SP-C variants with a poly-leucine sequence or other synthetic analogs SP-C(CC) and SP-C (1–21) have very similar helical contents [22].

The orientation of SP-C in lipid membranes have been studied comprehensively [11,14,18], and in a lipid membrane, native SP-C has been shown to adopt an orientation where the α-helix is inserted parallel to the lipid acyl chains. This transmembrane location is assumed to be an important part of its surfactant activity and is therefore desirable to include in an analog. By recording FTIR-spectra with parallel and perpendicular light with respect to the incidence plane, the transmembrane orientation of SP-C analogs when inserted in DPPC/PG (8:2, wt./wt.) was confirmed.

In contrast to the propensity of native SP-C to transform from α-helix to β-sheet in organic solvents, it largely retains its helical structure when inserted in phospholipids. This is in line with the observation that membrane embedded valine-rich helix domains are protected in a hydrophobic environment. Using H/D exchange, membrane proteins with higher abundance of helix-stabilizing leucines showed less exchange of amide hydrogen atoms than those

Table 1
In vitro surface activity of suspensions (10 mg/ml) containing SP-C analogs (2% wt./wt.) in DPPC/POPG (68:31, wt./wt.) measured in the captive bubble surfactometer.

Peptide in DPPC/POPG (68:31) $n = 3$ for all preparations	Compression needed to reach 5 mN/m (area, %)	γ_{min} (mN/m)	γ_{max} (mN/m)
SP-C33	32 (25–33)	1.1 (1.0–1.6)	27 (26–27)
SP-C30	26 (25–27)	1.8 (1.0–2.7)	22 (18–27)
SP-C33 M31K	32 (20–33)	2.3 (1.9–4.4)	24 (18–25)
SP-C33 M31I	22 (15–33)	0.9 (0.7–1.1)	26 (25–28)

Compression is measured as the difference in bubble area between maximum surface tension and when surface tension less or equal to 5 mN/m is reached. Presented values for minimum and maximum surface tensions are from the fifth cycle of quasi-static dynamics. Values are given as median (range).

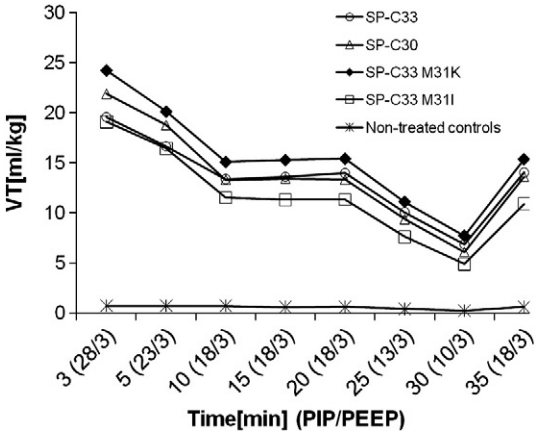

Fig. 5. Tidal volumes (V_T) in preterm newborn rabbits treated with SP-C analogs in DPPC/POPG (68:31, wt./wt.) (80 mg/ml, 2.5 ml/kg, $n=8$ (SP-C33, SP-C30, SP-C33 M31K) or $n=7$ (SP-C33 M31I)) or non-treated controls ($n=7$). Values are given as median. Statistical significance: $p<0.001$ for non-treated controls *vs.* all other groups at all time points.

containing the helix-destabilizing residues valine and isoleucine [23]. This would suggest that SP-C33, with a poly-leucine helix, is less flexible than native SP-C, with a poly-valine helix. In contrast, our results show a faster H/D exchange with about 60% unexchanged hydrogens for SP-C33 after 4 h compared to 70%–90% for native dipalmitoylated SP-C [14,18]. However, after 20 h, the H/D exchange was rather similar for both SP-C33 and native SP-C with about 55%–60% unexchanged hydrogens. These results suggest that, while the presence of a few helix-destabilizing Val or Ile increases flexibility, a long poly-valine helix like in native SP-C is exceedingly stable. This is in line with the observation that the SP-C helix is stabilized by a high activation barrier to unfolding and that, once helix unfolding occurs, it is not reversible. The presence of peptide bound fatty acyl chains has a stabilizing effect on conformationally dynamic helices in general [24] and the palmitoyl groups in SP-C stabilize the helix [25]. It is possible that the palmitoyl groups linked to SP-C are important for preventing unfolding of the metastable α-helix *in vivo*. The helix of SP-C33, in contrast, is able to unfold and refold and is consequently not dependent on covalently linked fatty acyl chains for stabilization. In line with this supposition, palmitoylation of SP-C33 does not result in alterations of its function [9].

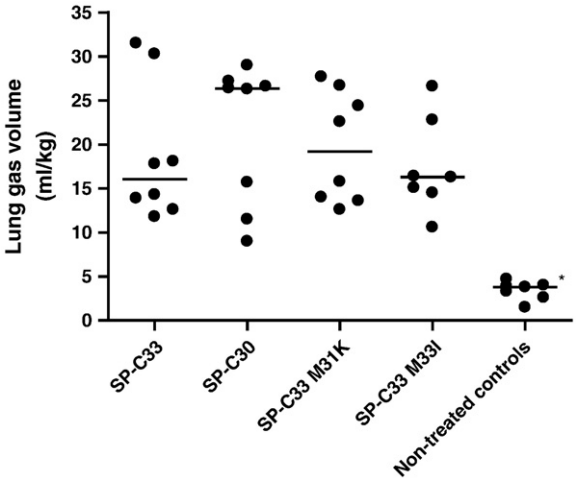

Fig. 6. Lung gas volumes after 35 min of ventilation in preterm newborn rabbits treated with SP-C analogs in DPPC/POPG (68:31, wt./wt.) (80 mg/ml, 2.5 ml/kg) or non-treated controls. Levels of statistical significance: $^*p<0.001$ vs. all other groups. The lines indicate median values.

The effects of modifications of the C-terminal end of SP-C on surface activity were investigated using variants of SP-C33. From the CBS *in vitro* study, no significant differences in surface activity due to the alterations were noticed. All preparations were equally active with surface tension shifting from ~25 mN/m to ~1 mN/m at a compression rate of 25%–30%. There were also no significant differences found *in vivo*; tidal volumes and lung gas volumes, reflecting the functional residual capacities, were all approximately the same, despite the exact nature of the C-terminal part. These findings can be related to reports on other surfactant protein analogs. It seems that the presence of an α-helical structure is of greater importance than the primary structure since exchange of the whole helical sequence of SP-C with that from bacteriorhodopsin does not influence the surface activity *in vitro* [22]. In the same study, it was shown that the length of the helix was of importance since truncation of the native SP-C sequence at residues 12, 17, or 21 noticeably decreased surface activities, while in our study, SP-C30 showed no variance compared to the longer peptides. This implies that an α-helical peptide that is able to span at least the nonpolar parts of a phospholipid membrane is necessary for activity of a synthetic surfactant. The importance of the helical length for surface activity has also been shown in a study using peptoid SP-C mimics. Here, it was shown that a helical length of approximately 28 Å was superior in comparison with peptoids containing shorter helices [26].

Earlier studies show surfactant activity of SP-C33 to be similar to the activity of native SP-C. Both *in vivo* and *in vitro* data of the modified peptides in this study, in comparison to SP-C33, thus suggest that all the studied analogs are similarly efficient regarding surfactant activity as native SP-C and could therefore be used as a functional analog in a synthetic surfactant.

Acknowledgments

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