Volume 282, number 2, 220–224 FEBS 09639 May 1991

ADONIS 001457939100037BE

Antibodies against synthetic amphipathic helical sequences of surfactant protein SP-B detect a conformational change in the native protein

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Received 20 December 1990; revised version received 7 January 1991

Synthetic peptides based on the native human sequence of surfactant protein B have been used to generate polyclonal monospecific antibodies against specific segments of the native SP-B protein. Circular dichroism analysis of the synthetic peptides shows they have a dominant helical content in structure promoting environments and tensiometric measurements indicate these peptides lower surface tension at air–water interfaces implying that they contain amphipathic alpha helical motifs. Antibodies directed against the C-terminal segment of SP-B react with the native protein in the oxidized and reduced state. Antibodies directed against the N-terminal sequence of SP-B react with the native protein only in the reduced state suggesting that this domain has a conformation dependent on disulfide bond formation.

Surfactant peptide; Amphipathic helix; Immunodominance

1. INTRODUCTION

Surfactant protein B is a hydrophobic protein that enhances the adsorption of surfactant lipids to the air–water interface of the lung, thus promoting the formation of a lipid surface film. Although SP-B has a proteolipid-like character because it coisolates with lipid, predictions of its secondary structure based on the primary sequence suggest that the protein has several distinct amphipathic regions [1,2]. Amphipathic synthetic peptides based on native protein sequences have been used to investigate the structural and antigenic properties of native proteins [3,4]. In particular anti-peptide antibodies offer a sensitive approach for the detection of conformational changes in surface accessible sequences of proteins [16]. This approach is especially useful in the study of the native SP-B protein domains because, with the possible exception of disulfide dependent oligomerization, SP-B is not known to undergo post translational modification such as phosphorylation or glycosylation [5]. In this report we describe the binding characteristics of two antibodies raised against synthetic peptides containing surface seeking segments of surfactant protein B.

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2. MATERIALS AND METHODS

2.1. *Synthesis and purification of synthetic peptides of SP-B*

Peptides of SP-B segments (Table I) were prepared by the Merrifield solid phase synthesis method using a BOC strategy at the UCLA Peptide Synthesis Facility, as described previously [1,6]. After cleavage of the peptides from the resin, the purity of the product was estimated by compositional analysis at the UCLA Protein Sequencing Facility. The synthetic peptides were further purified by reverse-phase HPLC and the expected molecular mass obtained by FAB-Mass Spectrometry (City of Hope, Mass Spectrometry Facility, Duarte, CA).

2.2. *Analysis of peptide secondary structure by circular dichroism*

The secondary structure of synthetic peptides was determined from circular dichroism spectra using an AVIV model 60DS spectropolarimeter or a JASCO J600 spectropolarimeter. Samples were suspended in saline buffer (154 mM NaCl, 5 mM potassium phosphate, pH 7.0) and trifluoroethanol (TFE - Aldrich Chemical Co., Milwaukee, WI), TFE/5 mM potassium phosphate buffer pH 7.0, 2.5:7.5, and 1:1, v:v or sodium dodecylsulfate 20 mM (BioRad, Richmond, CA) with 5 mM potassium phosphate, pH 7.0 and spectra were obtained using 0.01 cm, 0.05 cm and 0.1 cm pathlength quartz cells. Peptide sample concentrations were based on quantitative amino acid analysis at the UCLA sequencing facility.

The amount of helix, coil, and β-sheet for the peptides in the various solvents was estimated by fitting the spectra to the reference data of Greenfield and Fasman [7] using a non-restrained least-squares algorithm and by the CD spectral analysis program, CONTIN [8].

2.3. *Surface activity measurements of peptides*

The surface activity of dispersions of the selected amphipathic segments of SP-B in saline solution was determined using a King/Clements surface adsorption device [9]. The sample peptide was injected beneath the hypophase and the change in surface tension was monitored at the air–water interface using a platinum plate hung from a Gould Metrigram 50 force transducer. The time course of adsorp-

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Table I

Sequences from SP-B used for studies

SP-B(1-7)OH

F P I P L P Y
1 7

Trp[14]SP-B(14-25)OH

W I K R I Q A M I P K G
14 25

SP-B(1-25)OH

F P I P L P Y C W L C R A L I K R I Q A M I P K G
1 25

Nleu[65]SP-B(49-66) NH_2

L A E R Y S V I L L D T L L G R N I R L $CONH_2$
49 66

Amino acid sequences of SP-B peptide that were used for studies. Residues are numbered from the N-terminus; $CONH_2$ represents carboxyamidation of the C-terminus; Nl is the non-oxidizable methionine analog, norleucine.

tion of the peptide to the air-water interface was recorded using a Hewlett-Packard 7044B X-Y recorder.

2.4. *Antisera to synthetic peptides*

Antisera used in the study were obtained by hyperimmunizing New Zealand white rabbits with synthetic peptides of SP-B (residues 1-25 and 49-66, Table I) or with native bovine surfactant proteins after Sephadex LH-20 delipidation [10]. Peptides and proteins were injected in Freund's adjuvant without conjugation to other protein to avoid possible alteration of the antigenic properties of the peptide [11].

2.5. *SDS polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulphate (SDS) was conducted by dissolving dried bovine surfactant proteins obtained from Sephadex LH-20 chromatography in SDS sample buffer. Polyacrylamide gel electrophoresis was performed on PhastSystem (Pharmacia, Piscataway, N.J.) using 20% polyacrylamide gels and loaded with 250 ng surfactant protein/μl with or without reducing agents in SDS.

2.6. *Electroblotting and immunostaining*

Electroblotting and immunostaining of the proteins were done according to methods of Burnette [12]) and Phelps et al. [13] modified by using a trans-blot cell (BioRad, Richmond, CA) and polyvinylidene difluoride (PVDF, Millipore, Bedford, MA) membranes with a pore size of 0.45 μm. After SDS-PAGE electrophoresis, the gel was removed from the plastic backing, soaked in transfer buffer, sandwiched between a sheet of PVDF membrane and two sheets of filter paper, assembled in the trans-blot cell, and electroblotted. After transfer, non-specific binding on the PVDF membrane was blocked by 3% gelatin. The membrane was then incubated with each rabbit antisera 1:500 in 1% gelatin for 1 h. The membrane was further washed, incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Cappel, Malvern, PA) diluted at 1 : 1000 for 30 min. The membrane was washed again, and color was developed with 3,3'-diaminobenzidine tetrahydrochloride (Aldrich, Milwaukee, WIS) and 3% hydrogen peroxide for 15 min. The reaction was stopped by deionized water, and the membrane was dried on filter paper. The presence of antigen-antibody complex on the PVDF membranes can be easily identified as a dark brown color.

3. RESULTS AND DISCUSSION

The CD spectra of the SP-B synthetic peptides were measured in normal saline solution containing varying concentrations of the structure promoting solvent TFE and in the interfacial micellar environment of sodium dodecyl sulfate. CD data for the synthetic SP-B segments are summarized in Table II.

In dilute TFE-saline solution (TFE/saline, 2.5:7.5, v:v) the shorter segments of SP-B, SP-B (1-7)OH and

Table II

Summary of conformation analysis for SP-B peptides by curve fitting of circular dichroism spectra. Spectra were recorded from 250 nm to 195 nm, at 0.2 nm intervals using a scan speed of 10 nm/min and were the average of 8 scans. Spectra were smoothed before computer analysis. The peptide concentration was 25 μM.

	Percent helix content[1]		
Peptide	25% TFE[2]	50% TFE[3]	20 mM SDS[4]
SP-B(1-25)OH	40	75	75
Nleu[65]SP-B(49-66)NH_2	65	75	70
Trp[14]SP-B(14-25)OH	20	65	35
SP-B(1-7)OH	10	15	10

[1]Based on poly-Lys reference data [7] using unrestrained least squares fit algorithm [14].
[2]Trifluoroethanol/154 mM NaCl, 5 mM potassium phosphate buffer pH 7.0, 2.5:7.5, v:v.
[3]Trifluoroethanol/154 mM NaCl, 5 mM potassium buffer pH 7.0, 1:1, v:v.
[4]20 mM SDS, 5 mM potassium phosphate buffer pH 7.0.

Trp[14]SP-B (14–25)OH had little secondary structure. In contrast to these shorter segments of the N-terminus of SP-B, the longer segment SP-B (1–25)OH and the C-terminal segment Nleu[65]SP-B(49–66)NH_2 had appreciable helical content in dilute TFE. Fifty-fold dilution of the two longer SP-B synthetic peptide (from 20 μM to 0.4 μM peptide) reduced the helical component to approximately one-half suggesting that these segments have self association properties in this environment.

When the concentration of TFE was increased (TFE/saline, 1:1, v:v) the CD spectra changed (Table II) indicating an enhanced helical component for Trp[14]SP-B(14–25)OH, SP-B(1–25)OH and Nleu[65]SP-B(49–66)NH_2. The shorter segment, SP-B(1–7)OH, showed no appreciable difference in secondary structure in dilute or concentrated TFE solution.

Examination of the peptides in SDS solution indicated that three of the peptides adopt considerable secondary structure in this interfacial environment. SP-B(1–25)OH and Nleu[65]SP-B(49–66)NH_2 in SDS had helical components similar to that in TFE/saline (1:1, v:v) suggesting that these segments express maximal helix content in these structure promoting environments. Trp[14]SP-B(14–25)OH had less helical content in SDS than in TFE/saline (1:1, v:v). This finding may be related to the partition of the peptide between the interface of the SDS micelle and the bulk solution since the CD spectrum of the peptide represents a composite of random and helix conformations. SP-B(1–7)OH had little secondary structure in SDS and TFE were similar. Air oxidation of SP-B(1–25)OH in TFE/normal saline or 20 mM SDS for 24 h (30% disulfide oligomers) did not alter the CD spectra, implying that peptide-peptide disulfide oligomerization does not affect conformation in these solvent systems.

Surface tension measurements of the synthetic peptides in saline solution also provide information regarding possible structure (Fig. 1). SP-B(1–25)OH and Nleu[65]SP-B(49–66)NH_2 showed surface activity resembling that of many types of amphipathic peptides. The shorter segments of the SP-B (1–25)OH had less surface activity than the intact peptide. Trp[14]SP-B(14–25)OH was more effective than SP-B(1–7)OH in lowering surface tension.

In combination with the CD measurements, the surface tension experiments with the peptides suggest that the three segments used in this study contain elements that assume amphipathic helical structures as predicted from the hydrophobic moment plot analysis [1,15] of the human SP-B sequence. Since SP-B(1–25)OH and Nleu[65]SP-B(49–66)NH_2 had the highest surface activity and adopt the highest helical secondary structure, they were selected for studies with antibodies.

Antisera developed against native bovine hydrophobic surfactant proteins identify oligomers of native bovine SP-B protein [10] at 26 and 17 kDa (lanes 1 and 3, Fig. 2), and have some reactivity with

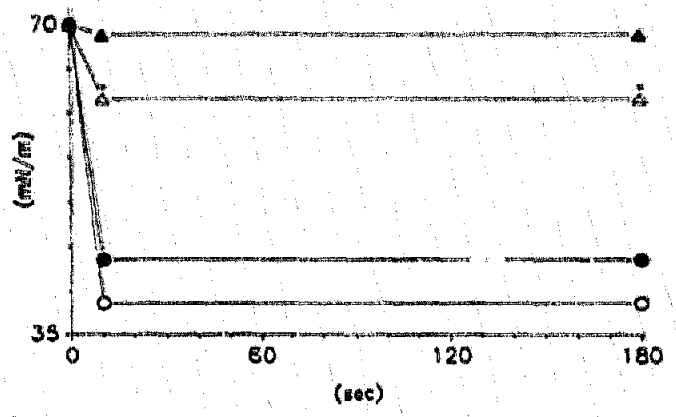

Fig. 1. Surface tension changes of SP-B peptides at the air-water interface, after injection of synthetic peptides in saline dispersions. x axis, time in seconds, y axis, surface tension in mN/m. Hypophase, 154 mM NaCl, pH 5.3, 37°C. Peptide concentration 1 μM. (▲) SP-B 1–7. (△) SP-B 14–25. (●) SP-B 1–25. (○) SP-B 49–66. Mean ± standard deviation.

monomers at 8 kDa (lanes 1–4) with or without delipidation.

When synthetic peptides were used as antigen, the antisera against the N-terminal SP-B peptide reacted with

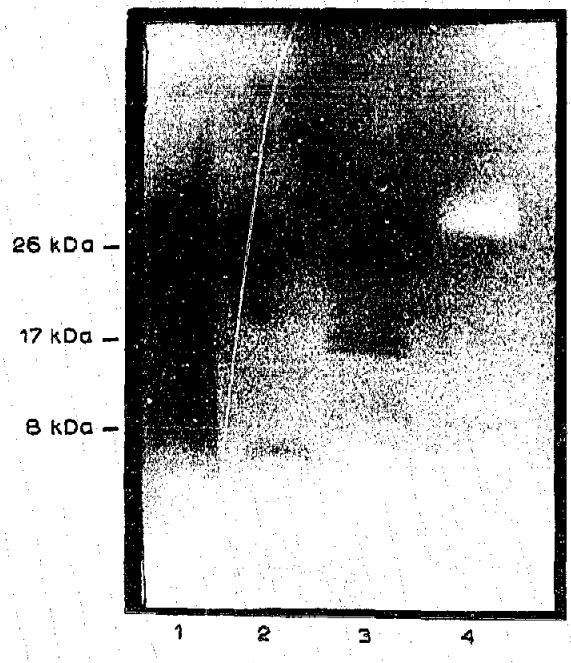

Fig. 2. Immunostaining of bovine surfactant with antisera directed against bovine hydrophobic surfactant proteins. Each lane contains 250 ng of proteins. Lane 1: non-reduced low molecular weight hydrophobic surfactant proteins after Sephadex LH-20 chromatography delipidation. The 8, 17 and 26 kDa bands are identified with heavy background. Lane 2: same materials as lane 1 but reduced. Only a faint 8 kDa band is identified. Lane 3: non-reduced bovine surfactant from bronchoalveolar lavage, the 8, 17 (doublet) and 26 kDa bands are seen. The wavy looking line at the end of the lane is due to lipid. Lane 4: same materials as lane 3 but reduced. Only 8 kDa band is identified. The heavily negatively stained 35 kDa (not labeled) region is due to surfactant protein A.

both the oxidized and reduced synthetic peptide SP-B(1-25)OH. These results complement the CD measurements of the N-terminal peptide that indicate there is no detectable change in helical content in this segment in the oxidized and reduced forms.

When the antibody directed against the N-terminus of SP-B (1-25)OH is used for immunostaining, only monomeric SP-B is identified at 8 kDa for both delipidated and undelipidated materials (lanes 2 and 4, Fig. 3). The oxidized oligomeric native SP-B does not react with antibody raised against SP-B(1-25)OH.

If the antisera directed against the C-terminus segment (Nleu^{65}SP-B(49-66)-NH_2) are used, different results are observed (Fig. 4). Antibodies against the synthetic C-terminal segment identify native SP-B oligomers at 26 kDa (lanes 1 and 3) and 17 kDa (lanes 1 and 2), and native SP-B monomers (lanes 1-4), before and after delipidation. These immunoreactivities are summarized in Table III.

These observations suggest some conformational dependency on disulfide bond formation. The antisera against the C-terminal segment of SP-B react with native SP-B in both the oxidized and reduced state. This finding indicates the C-terminal segment (residues 49-66) of native SP-B is accessible before and after disulfide bond formation and oligomerization. In contrast, the antisera developed against the N-terminal sequence of SP-B only react with the native monomers in the reduced state suggesting that this domain has undergone conformational changes after disulfide bond formation and oligomerization. These results suggest that C-terminal segments of native SP-B are less constrained hence more mobile than other sections of the polypeptide chain, and are surface-oriented as is typical of C-terminal domains in many proteins [17,18].

Antisera raised against native hydrophobic surfactant proteins identify SP-B oligomers, namely 26 kDa bands, much better than the monomers at 8 kDa (Fig. 2). The C-terminal synthetic segment antisera identify monomeric 8 kDa SP-B better than oligomers (Fig. 4), especially on undelipidated material (lane 3 of Fig. 4). This observation suggests that delipidated material used to raised antisera may be predominantly oligomeric, or oligomers are immunodominant, thus the polyclonal antibodies raised mostly identify oligomeric forms.

In conclusion, the amphipathic synthetic peptides of SP-B used in this study appear to be useful in the production of antibodies against native surfactant protein B. The N-terminal and C-terminal segment specificity of the antibodies toward the native protein in the oxidized and reduced state allows determination of monomeric and oligomeric states of the protein. Such specificity should provide detection of different SP-B conformations in the development of clinically relevant protocols.

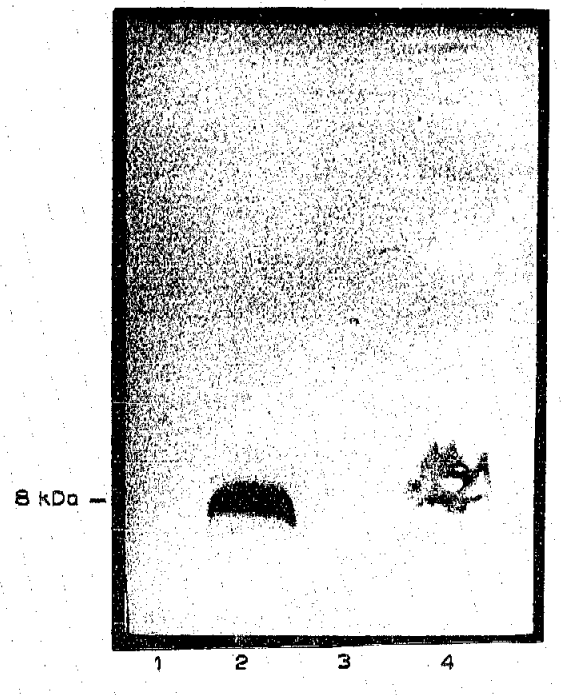

Fig. 3. Immunostaining of a similar gel as Fig. 2, using antisera against synthetic SP-B(1-25)OH. Only 8 kDa bands in lanes 2 and 4 are identified indicating antibody binding only with reduced, monomeric SP-B.

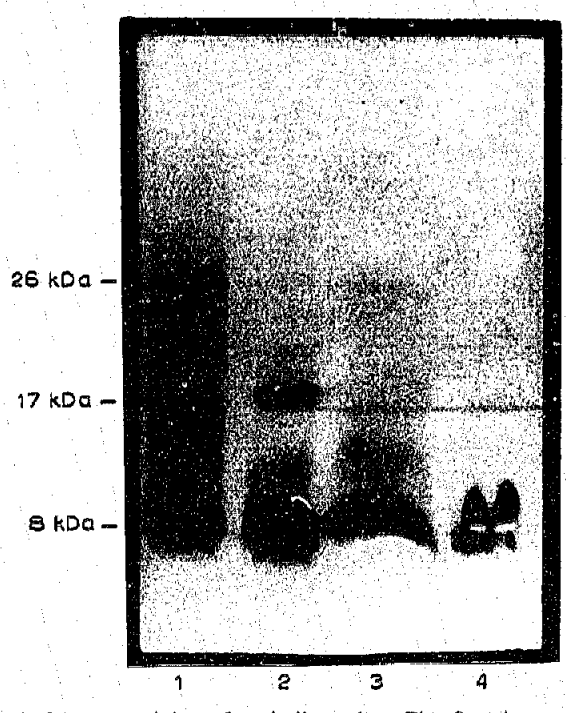

Fig. 4. Immunostaining of a similar gel as Fig. 2, using antisera against Nleu^{65}SP-B(49-66)NH_2. Lane 1: the 8, 17 and 26 kDa bands are all identified. Lane 2: the 8 and 17 kDa bands are seen. The presence of 17 kDa band may be due to incomplete reduction. Lane 3: the 8 kDa and a very faint 26 kDa bands are seen. Lane 4: a wavy 8 kDa band is identified. Thus antibody against the segment near the carboxyl terminus of SP-B binds in a similar fashion to antibody raised against native bovine SP-B, yet favor monomers more than oligomers.

Table III

Summary of immunoreactivities* between native SP-B and three antibodies

Lane	Antigen	Antibodies		
		Native	N-terminus	C-terminus
1	Extracted SP-B oxidized	8, 17, 26	-	8, 17, 26
2	Extracted SP-B reduced	8	8	8, 17
3	Bovine surfactant oxidized	8, 26	-	8, 26
4	Bovine surfactant reduced	8	8	8

* numbers refer to molecular weight in kDa of bands identified with immunostaining

Acknowledgments: The authors would like to thank Dr. James McKnight and Dr. Lila Gierasch for their helpful suggestions with CD analysis. We thank Dr. Shiuan Chen for use of the CD spectrometer at the Department of Immunology, Beckman Research Institute of the City of Hope National Medical Center. Dr. Janice Young and Dr. Richard Stevens have our continuing gratitude for their excellent peptide synthesis. This work was supported in part by grants from the California American Lung Association, NIH HL 40666, Ross Laboratories, and Research Centers in Minority Institutions Award RR03026 of the National Center for Research Resources of NIH.

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