The Major Lung Surfactant Protein, SP 28–36, Is a Calcium-dependent, Carbohydrate-binding Protein*

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SP 28–36, a major protein of pulmonary surfactant, has striking amino acid sequence homology with soluble mannose-binding proteins isolated from rat liver and contains residues common to the carbohydrate-binding domains of other mammalian lectins. We have used carbohydrate-affinity chromatography to investigate carbohydrate-binding properties of SP 28–36 isolated from canine and human (alveolar proteinosis patients) lung lavage. SP 28–36 binds to immobilized D-mannose, L-fucose, D-galactose, and D-glucose. The protein binds only weakly to N-acetyl-D-galactosamine and N-acetyl-D-glucosamine. Binding is Ca2+-dependent. The threshold Ca2+ concentration is 0.6 mM and maximal binding occurs with 1 mM Ca2+. Bound protein is quantitatively recovered by elution with 2 mM EDTA. Ba2+, Sr2+, and Mn2+, but not Mg2+, can substitute for Ca2+. Unlike some other mammalian lectins, SP 28–36 binds to carbohydrate at pH 5.0. Recombinant human SP 28–36 isolated from the media of Chinese hamster ovary cells, transfected with a DNA construct encoding SP 28–36, has similar carbohydrate-binding activity to the native proteins. Mannose affinity chromatography of the culture medium of Chinese hamster ovary cells results in an efficient purification of the secreted recombinant human SP 28–36.

EXPERIMENTAL PROCEDURES

Isolation of SP 28–36—Pulmonary surfactant was isolated by bronchoalveolar lavage of adult dogs (8) and patients with alveolar proteinosis (12). The surfactant in water (1.5 mg of protein/ml) was extracted in 1-butanol (1.50, v/v) at room temperature (13). The precipitated butanol mixture was centrifuged twice at 10,000 × g, for 20 min. The precipitated protein was dried under nitrogen and washed twice in 20 mM octyl-β-D-glucopyranoside, 100 mM NaCl, 10 mM Hepes (pH 7.4). The proteins which were insoluble in this buffer were suspended in 5 mM Hepes, pH 7.4, and dialyzed against the same buffer for 48 h. The insoluble material was removed by centrifugation at 100,000 × g, for 30 min and the supernatant, containing the purified SP 28–36, was stored in small aliquotes at −20°C. Detailed procedures for the expression and isolation of human recombinant SP 28–36 will be published elsewhere.

Analyses—Sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis was performed according to Laemmli (14). Dithiothreitol (50 mM) was added to each of the samples. Protein determinations were carried out according to Böhlen et al. (15), using bovine serum albumin as a standard.

Reduction and Alkylation of SP 28–36—Purified SP 28–36 (0.5 mg) was incubated for 30 min at 37°C in 3 ml of 5 mM Tris-Cl, pH 7.4, 50 mM dithiothreitol. Iodoacetamide (1 M, in ethanol) was added to the mixture to a final concentration of 100 mM. The mixture was kept for 1 h at room temperature in the dark, dialyzed against 20 mM Tris-Cl, pH 7.4, and finally dialyzed against 5 mM Hepes, pH 7.4.

Digestion of SP 28–36 with Collagenase—Collagenase (Worthington) was purified according to Bicak and Harper (16). The collagenase-resistant fragment of SP 28–36 was generated as follows. SP 28–36 (250 μg) was incubated for 18 h at 37°C in the presence of purified collagenase in 50 mM Tris-Cl (pH 7.4) and 2 mM CaCl2 (total volume, 1 ml). The mixture was dialyzed against 5 mM Hepes (pH 7.4) and analyzed by SDS-polyacrylamide gel electrophoresis.

Carbohydrate-binding Assay—Affinity chromatography was used to assess the carbohydrate-binding properties of SP 28–36. The assay was carried out at 4°C. To prevent aggregation of the protein at this temperature, low ionic strength buffers were used that contained 0.1% (v/v) Triton X-100. Purified SP 28–36 (30 μg) in 5 mM Hepes, pH 7.4, 1 mM CaCl2, 0.1% (v/v) Triton X-100 (total volume, 1 ml), was applied to columns containing a 1-ml gel of immobilized monosaccharides (Selectins, Pierce Chemical Co.). After loading, the columns were eluted with 5 ml of 5 mM Hepes, pH 7.4, 1 mM CaCl2.
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0.1% (v/v) Triton X-100 and subsequently with 5 ml of the same buffer containing 2 mM EDTA instead of 1 mM CaCl₂. Fractions of 1 ml were collected. Aliquots of the fractions were taken for SDS-polyacrylamide gel electrophoresis and for protein determinations. To express binding, the amount of protein which appears in the EDTA wash was calculated as a percentage of the total protein recovered.

RESULTS AND DISCUSSION

SP 28–36, isolated from canine and human lung lavage, binds to a number of immobilized monosaccharides under the conditions tested. Binding requires Ca²⁺ (1 mM) and the proteins are eluted from the affinity columns in the presence of 2 mM EDTA. Examples of gels on column fractions are shown in Fig. 1, and the results of numerous column runs are summarized in Table I. Both proteins were retained nearly quantitatively by immobilized mannose and fucose. Protein bound to the mannose column could be eluted by a 20 mM but not 10 mM mannose solution in the presence of 1 mM Ca²⁺. Canine SP 28–36 consistently bound only partially to immobilized glucose and galactose. When either the glucose-bound or unbound protein was reapplied to immobilized glucose, the same distribution of binding was found, suggesting a reversible equilibrium in binding ability. Delipidated SP 28–36 in aqueous buffers at physiological ionic strength is a large oligomeric protein of unknown structure (7). We found that canine and human SP 28–36 was precipitated by 1 mM Ca²⁺ when the NaCl concentration was increased beyond 20 mM at 4 °C even in the presence of Triton X-100. Even at the low ionic strength used in this study, it is likely that variable oligomerization of the protein occurs in the presence of Ca²⁺. The extent of protein aggregation may differ between species (17). Variable aggregation may influence protein binding to the immobilized sugars. Both human and canine SP 28–36 bound poorly to immobilized N-acetyl-sugars. SP 28–36 did not bind to the column matrix with or without Ca²⁺. Although these results suggest different affinities for the various sugars, it should be emphasized that the column assay cannot be used to quantify affinities. The use of low ionic strength buffers in this study may also have affected relative binding potencies. Further studies will be required to establish the relative specificity of carbohydrate binding to various sugars at physiological ionic strength.

The binding of SP 28–36 to immobilized monosaccharides is Ca²⁺-dependent, just as for some other mammalian lectins (18–22). The threshold Ca²⁺ concentration for binding is 0.6 mM and maximal binding occurs with 1 mM Ca²⁺ (Fig. 2). Sr²⁺, Mn²⁺ and Ba²⁺ could substitute for Ca²⁺, although Mn²⁺, and particularly, Ba²⁺, seem to be less effective (Fig. 2). Mg²⁺, in concentrations up to 2 mM, could not substitute for Ca²⁺. Unlike the membrane-bound hepatic receptors (18–20) and the soluble membrane-binding proteins (22), SP 28–36 retains its Ca²⁺-dependent binding activity at pH 5. Partial proteolysis of rat (23) and chicken (24) hepatic lectin and collagenase treatment of soluble mannose-binding protein from rat liver (11) yield COOH-terminal fragments that are able to bind to carbohydrate-affinity columns. This suggests that the mem-

**Table I**

| Monosaccharide       | Amount of SP 28–36 bound to immobilized monosaccharide* |
|----------------------|---------------------------------------------------------|
|                      | Canine SP 28–36 | Human SP 28–36 |
| D-Mannose            | 85 ± 12 (12)    | 95 ± 10 (10)   |
| L-Fucose             | 87 ± 9 (5)      | 100 (3)        |
| D-Glucose            | 59 ± 13 (8)     | 100 (2)        |
| D-Galactose          | 50 ± 20 (5)     | 100 (2)        |
| N-Acetyl-D-galactosamine | 22 ± 12 (7)  | 7 (2)          |
| N-Acetyl-D-glucoasamine | 8 ± 6 (7)   | 2 (2)          |

*Expressed as the percentage of total recovered protein. 94 ± 8% of applied protein was recovered.

**Fig. 2.** Binding of human SP 28–36 to immobilized mannose as a function of the divalent cation concentration. SP 28–36 was applied to the affinity columns in 1 ml of 5 mM Hepes, pH 7.4, 0.1% (v/v) Triton X-100, and different concentrations of divalent cations (as chlorides). The columns were eluted with 5 ml of the same buffer and were subsequently eluted with 5 ml of 5 mM Hepes, pH 7.4, 0.1% (v/v) Triton X-100, and 2 mM EDTA. The amount of bound protein that eluted in the presence of 2 mM EDTA was expressed as the percentage of the total recovered protein.
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Effects of reduction and heat treatment of SP 28–36 on the ability to bind to immobilized D-mannose

| Treatment          | Amount of SP 28–36 bound to immobilized D-mannose |
|--------------------|---------------------------------------------------|
|                    | Canine SP 28–36 | Human SP 28–36 |
| Reduction          | %               | %               |
| Heat treatment     |                 |                 |
| 40 °C, 10 min      | 95              | ND              |
| 45 °C, 10 min      | 95              | ND              |
| 50 °C, 10 min      | 5               | ND              |
| 90 °C, 10 min      | 8               | 16              |

* ND, not determined.

Experiments were carried out as described under "Experimental Procedures." Proteins are not required for carbohydrate binding. Cysteine secreted by Chinese hamster ovary cells transfected with the gene for human media proteins are not bound to immobilized mannose in the presence of Ca²⁺. Cysteine residues and residues located near cysteine residues in the carbohydrate-recognition domains are highly conserved, suggesting that disulfide bond formation may be necessary to produce an active binding domain in all of these proteins. As shown in Table II, reduction of SP 28–36 results in the complete loss of binding activity. This is consistent with the suggestion that the homologous COOH-terminal portions of the human and dog SP 28–36 and the hepatic lectins are folded into similar disulfide-bonded domains.

In spite of the similarities in the structures of SP 28–36 and the mannose-binding proteins, we were not able to demonstrate carbohydrate binding of either the human or canine collagenase-resistant fragment. We have previously reported the NH₂-terminal amino acid of this fragment as Gly-78 in the dog. The failure to bind the collagenase-resistant fragment to the monosaccharide columns may be the result of nonspecific cleavage or other modifications within the binding domain as a result of the collagenase treatment but it is interesting that the binding capacity of SP 28–36 could also be destroyed by heat treatment for 10 min at 50 °C but not at 45 °C (Table II). These observations suggest that, although the sequence homology with other lectins indicates that the carbohydrate-binding domain is probably in the COOH-terminal part of SP 28–36, the oligomeric structure, as determined in part by the collagen-like domain of SP 28–36, may greatly influence binding affinity.

Recombinant human SP 28–36 has recently been expressed in Chinese hamster ovary cells. SP 28–36, produced by these cells, is undistinguishable from the native protein with respect to various chemical, physical, and biological properties. The carbohydrate-binding properties are similar to the native proteins. We have also tested the ability of recombinant human SP 28–36 to bind to carbohydrates. Mannose affinity chromatography of the media of transfected Chinese hamster ovary cells results in an efficient purification of SP 28–36 as shown in Fig. 3. No protein of this molecular weight is obtained by affinity chromatography of media obtained from untransfected cells (data not shown).

The carbohydrate-binding property of SP 28–36 may be involved in surfactant metabolism and alveolar defense. SP 28–36 promotes uptake of surfactant lipids and inhibits surfactant secretion by type II cells (10, 26). These events may be mediated by recognition of cell surface carbohydrates. The observation that ferritin-labeled lectins attach to type II cell membranes and quickly appear in lamellar bodies (27) is consistent with this hypothesis. Surfactant may have several important functions in the pulmonary defense system (28). One of these possible functions is precoating of bacteria to facilitate phagocytosis by alveolar macrophages. SP 28–36 may recognize polysaccharides on the bacterial surface and thus initiate coating and phagocytosis of bacteria.

This study shows that properties of proteins may sometimes be predicted from sequence homologies with other proteins. SP 28–36 shares a common carbohydrate-recognition domain with other proteins and was found to bind to immobilized monosaccharides in a Ca²⁺-dependent fashion. In order to elucidate the biological significance of this finding, other assays will be needed to determine the specific binding affinities of SP 28–36.

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REFERENCES

1. King, R. J. (1985) Annu. Rev. Physiol. 47, 775–788
2. Phelps, D. S., and Taeusch, H. W. (1985) Comp. Biochem. Physiol. 82, 441–446
3. Benson, B., Hawgood, S., Schilling, J., Clements, J., Damm, D., Cordell, B., and White, R. T. (1986) Proc Natl. Acad. Sci. U. S. A. 82, 6379–6383
4. R. T. White, B. J. Benson, D. Buckley, J. Schilling, and D. Damm, manuscript in preparation.
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4. White, R. T., Damm, D., Miller, J., Spratt, K., Schilling, J., Hawgood, S., Benson, B., and Cordell, B. (1986) Nature 317, 361–363
5. Ross, G. F., Meuth, J., Ohning, B., Kim, Y., and Whitsett, J. A. (1986) Biochim. Biophys. Acta 870, 267–278
6. King, R. J., and MacBeth, M. C. (1981) Biochim. Biophys. Acta 647, 159–168
7. King, R. J., Carmichael, M. C., and Horowitz, P. M. (1983) J. Biol. Chem. 258, 10672–10680
8. Hawgood, S., Benson, B. J., and Hamilton, R. L. (1985) Biochemistry 24, 184–190
9. Hawgood, S., Benson, B. J., Schilling, J., Damm, D., Clements, J. A., and White, R. T. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 66–70
10. Wright, J. R., Wager, R. E., Hawgood, S., Dobbs, L., and Clements, J. A. (1987) J. Biol. Chem. 262, 2888–2894
11. Drickamer, K., Dordal, M. S., and Reynolds, L. (1986) J. Biol. Chem. 261, 6878–6887
12. Phelps, D. S., Taeusch, H. W., Benson, B., and Hawgood, S. (1984) Biochim. Biophys. Acta 791, 226–238
13. Sigrist, H., Sigrist-Nelson, K., and Gitler, C. (1977) Biochem. Biophys. Res. Commun. 74, 178–184
14. Laemmli, U. K. (1970) Nature 227, 680–685
15. Bohlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213–220
16. Birsak, T. A., and Harper, E. (1985) Anal. Biochem. 145, 286–291
17. Ross, G. F., Ohning, B. L., Tannenbaum, D., and Whitsett, J. A. (1987) Biochim. Biophys. Acta 911, 294–305
18. Ashwell, G., and Harford, J. (1982) Annu. Rev. Biochem. 51, 531–554
19. Lehman, M. A., Haltiwanger, R. S., and Hill, R. L. (1986) J. Biol. Chem. 261, 7426–7432
20. Haltiwanger, R. S., and Hill, R. L. (1986) J. Biol. Chem. 261, 7440–7444
21. Summerfield, J. A., and Taylor, M. E. (1986) Biochim. Biophys. Acta 883, 197–206
22. Mizuno, Y., Kozutsumi, Y., Kawasaki, T., and Yamasuna, I. (1981) J. Biol. Chem. 256, 4247–4252
23. Hauch, E. C., Holland, E. C. Carrera, G. M., and Drickamer, K. (1986) J. Biol. Chem. 261, 4940–4947
24. Chiacchia, K. B., and Drickamer, K. (1984) J. Biol. Chem. 259, 15440–15446
25. Brodsky-Doyle, B., Leonard, K. R., and Reid, K. B. M. (1976) Biochem. J. 159, 273–286
26. Dobbs, L. G., Wright, J. R., Hawgood, S., Gonzalez, R. F., Venstrom, K., and Nellenbogen, J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1010–1014
27. Williams, M. C. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6383–6387
28. Jarstrand, C. (1984) Pulmonary Surfactant (Robertson, B., Van Golde, L. M. G., and Batenburg, J. J., eds) pp. 187–201, Elsevier, Amsterdam