Recombinant human SP-A1 and SP-A2 proteins have different carbohydrate-binding characteristics

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Oberley, Rebecca E., and Jeanne M. Snyder. Recombinant human SP-A1 and SP-A2 proteins have different carbohydrate-binding characteristics. *Am J Physiol Lung Cell Mol Physiol* 284: L871–L881, 2003. First published December 27, 2002; 10.1152/ajplung.00241.2002.—Surfactant protein (SP)-A is a member of the collectin family of proteins and plays a role in innate host defense of the lung. SP-A binds to the carbohydrates of lung pathogens via its calcium-dependant carbohydrate-binding domain. Native human alveolar SP-A consists of two distinct gene products: SP-A1 and SP-A2; however, only SP-A2 is expressed in the submucosal glands of the conducting airways. The function of the isolated SP-A2 protein is unknown. We hypothesized that SP-A1 and SP-A2 might have different carbohydrate-binding properties. In this study, we characterized the carbohydrate-binding specificities of native human alveolar SP-A and recombinant human SP-A1 and SP-A2 in the presence of either 1 or 5 mM Ca$^{2+}$. We found that all of the SP-A proteins bind carbohydrates but with different affinities. All of the SP-A proteins bind to fucose with the greatest affinity. SP-A2 binds with a higher affinity to a wider variety of sugars than SP-A1 at either 1 or 5 mM Ca$^{2+}$. These findings are suggestive that SP-A2 may interact with a greater variety of pathogens than native SP-A.

PULMONARY SURFACTANT IS SECRETED by alveolar type II cells and reduces surface tension at the alveolar air-lung interface (33). The most abundant protein in surfactant is surfactant protein A (SP-A) (5). SP-A has been shown to be involved in innate host defense mechanisms in the lung, i.e., by binding to pathogens and acting as an opsonin and by stimulating phagocytosis by alveolar macrophages (5, 6, 26). SP-A is a member of the collectin family of proteins that contain both a type IV-like collagen domain and a calcium-dependent, carbohydrate-binding domain (5, 6, 24). It is believed that the carbohydrate-binding domain of SP-A interacts with lung pathogens by binding to their surface carbohydrates (5, 6, 9).

Native human alveolar SP-A is composed of two different proteins: SP-A1 and SP-A2 (30). It is thought that two SP-A1 molecules combine with one SP-A2 molecule to form a heterotrimer; six of the SP-A heterotrimers then bind together to form the “flower bouquet” structure of native SP-A (24). SP-A1 and SP-A2 are very similar proteins; only seven amino acids differ between the most common SP-A1 and SP-A2 alleles (25). Although the sequences for these two proteins are very similar, some functional differences have been observed between SP-A1 and SP-A2. SP-A2 protein has been shown to cause a twofold greater increase in TNF-α production by THP-1 cells than SP-A1 protein (31). In addition, SP-A2 stimulates the production of IL-8 by THP-1 cells to a greater degree than SP-A1 (32). Recent studies also show that the two human SP-A proteins are expressed in different parts of the lung. Both SP-A1 and SP-A2 are expressed in alveolar type II cells (12, 34). In contrast, SP-A2 has been shown to be expressed without any accompanying SP-A1 in the submucosal glands of the conducting airways (14, 28).

SP-A knockout mice have an increased susceptibility to lung infection and a decreased ability to phagocytose pathogens into macrophages (17–19). SP-A has been shown to act directly on macrophages to enhance phagocytosis by binding to receptors present on the macrophage plasma membrane (4, 6). SP-A binds to sugars on the surface of bacteria and acts as an opsonin for pathogens and enhances their phagocytosis by macrophages (5, 6, 26). Many lung pathogens display a complex of polysaccharides on their outside coat, i.e., *Streptococcus pneumoniae* has mannose sugars on surface glycoconjugates (13). *Pseudomonas aeruginosa*, another common lung pathogen, contains N-acetylgalactosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) in coat polysaccharides (29).

SP-A requires calcium to bind to carbohydrates present on the outside of bacteria. In most sites in the human body, extracellular calcium levels are 1–2 mM (1, 21). However, calcium levels may be much higher in the alveolar fluid lining the lung alveolus, i.e., 3.5–6 mM (7, 11). Therefore, in the present study, we examined the effects of calcium at two concentrations, 1 or 5 mM, on the carbohydrate-binding specificities of native human SP-A and recombinant human SP-A1 and SP-A2.

MATERIALS AND METHODS

Isolation of native human SP-A. The 600-g pellet of human alveolar proteinosis lavage material (1 ml; a kind gift from...
Dr. Scott Ferguson, Department of Internal Medicine, University of Iowa) was delipidated by mixing the material with isopropyl ether (12 ml) and 1-butanol (8 ml) for 30 min. The aqueous phase was collected after centrifugation of the mixture (18,000 g for 30 min) and then precipitated with 100% ethanol (40 ml) at 20°C for 3 h. The precipitated material was centrifuged at 18,000 g for 30 min at 4°C, and the resulting pellet was resuspended in 1 ml of 20 mM KH2PO4. The resuspended material was then eluted over an Affi-gel Blue column (1 ml; Bio-Rad, Hercules, CA), which was subsequently washed with 20 mM KH2PO4. The Affi-gel Blue column binds serum proteins while SP-A protein passes through the column. The column flow-through was collected and then dialyzed against deionized water for 48 h at 4°C. We determined the protein concentration in the sample by a Bradford assay (3). The purity of the purified SP-A protein was characterized by PAGE followed by staining with Coomassie blue.

**SP-9 insect cell maintenance.** SF-9 cells, obtained from the American Type Culture Collection (Rockville, MD), were grown as a nonadherent monolayer in T-75 flasks and purchased from EY Laboratories and Sigma-Aldrich (St. Louis, MO). Carbohydrate affinity columns (1 ml) were prepared with 10 ml of 250 mM NaH2PO4, pH 7.4, an aliquot of the media (30 ml) and 1-ml fractions were collected from the EDTA elution, and these fractions were subjected to Bradford analysis to determine which fractions contained protein. The fractions containing protein were then dialyzed against deionized water for 2 days, and the water was changed frequently. The dialyzed protein was analyzed by PAGE and then stained with Coomassie blue to determine the purity of the recombinant SP-A protein recovered.

**Carbohydrate-binding assay.** Fucose, mannose, GalNAc, and GlcNAc carbohydrates attached to Sepharose resin were purchased from EY Laboratories and Sigma-Aldrich (St. Louis, MO). Carbohydrate affinity columns (1 ml) were washed with 1 or 5 mM Ca2+ buffer and then native human SP-A (3 μg), purified SP-A1 and SP-A2 (3 μg), or 1 ml of insect cell media that contained recombinant human SP-A1 or SP-A2 (~3 μg) was loaded onto the columns. After the sample had entered the resin, the columns were washed with either 1 or 5 mM Ca2+-containing buffer (4 ml) and 1-ml fractions were collected. The columns were then washed with 2 mM EDTA buffer (4 ml), and 1-ml fractions were collected. Western blot analysis for SP-A protein in the collected fractions was performed as described above. As a control, native SP-A and recombinant SP-A1 and SP-A2 were also purified over a Sepharose column without any attached carbohydrates. SP-A requires calcium to bind carbohydrates; thus any SP-A protein recovered in the calcium wash was unable to bind to the carbohydrate tested. When calcium is chelated by EDTA, the SP-A protein can no longer bind to carbohydrates and is released from the affinity column. Thus the SP-A protein recovered in the EDTA wash reflects SP-A protein that is able to bind to the carbohydrate being tested.

**N-glycosidase F treatment.** Native SP-A or recombinant SP-A1 or SP-A2 (5 μg) was incubated with 10 μl of 250 mM NaHPO4, pH 7.5; then the mixture was brought to 50 μl with deionized water and vortexed. N-glycosidase F enzyme (2 μl) (Calbiochem, La Jolla, CA) was added, and the reaction mixture incubated at 37°C for 24 h. N-glycosidase F-treated and nontreated native SP-A (1 μg) were purified over a Sepharose column (1 ml), washed with 5 mM Ca2+-buffer (4 ml), and then eluted with 2 mM EDTA buffer (4 ml). One-milliliter fractions were collected from both the Ca2+ and EDTA washes, and a Western blot for SP-A was performed to determine which fractions contained the SP-A protein.

**Native gels.** A Tris-HCl gel (7.5%) was pre-electrophoresed at 40 mA for 1 h at 4°C in pre-electrophoresis buffer (190 mM Tris and 1 mM EDTA). The gel was left in pre-electrophoresis buffer overnight at 4°C. Samples were diluted 1:1 in sample loading buffer [50% stacking gel buffer (0.5 M Tris and 8 mM EDTA, pH 6.8), 50% glycerol, 5% bromphenol blue] and loaded into freshly washed sample wells. The gel was electrophoresed for 3 h at 40 mA at 4°C in pre-electrophoresis buffer. The gel was then placed in electrophoresis buffer (50 mM Tris, 300 mM glycine, and 1.8 mM EDTA) and electrophoresed for an additional 1–2 h at 4°C. The gel was then placed in a solution that contained 1% SDS, 1× transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol), and 20% glycerol for 1 h at room temperature to charge the native
proteins. The charged proteins were then transferred to nitrocellulose at 36 V overnight at 4°C and then at 100 V for 1 h at 4°C. The blot was blocked in 7% nonfat dry milk for 2 h at room temperature, and the membrane was probed for SP-A protein by Western blot analysis as described above.

Statistics. All data are derived from at least three independent experiments. Western blots were quantified by densitometry, and the densitometric units corresponding to the total immunoreactive SP-A protein obtained from the calcium wash and the EDTA elution fractions were determined. These densitometric data were then used to calculate the percentage of the total SP-A protein recovered in the flow-through (calcium wash) and that bound to the column (EDTA wash). The data were analyzed by one-way analysis of variance followed by a Student-Newman-Keuls test (35).

RESULTS

To characterize native and recombinant human SP-A proteins, we electrophoresed native SP-A and recombinant SP-A1 and SP-A2 under nondenaturing conditions (Fig. 1A). Native SP-A migrated differently from the recombinant SP-A1 and SP-A2 proteins, a difference that could be the result of different charges and/or molecular weights (Fig. 1A). The recombinant SP-A proteins have the identical amino acid sequence as the SP-A proteins present in the native alveolar human SP-A; however, the recombinant SP-A proteins migrate at a slightly lower molecular weight on a denaturing gel compared with the native SP-A protein (Fig. 1B). It is known that insect cells do not glycosylate proteins in the same manner as mammalian cells (22). Insect cells synthesize simple O- and N-linked sugars but are unable to add the terminal sialic acid residues found on many mammalian carbohydrate modifications (22). Therefore, we hypothesized that the difference in molecular weight observed between the native and recombinant SP-A proteins might be the result of differences in glycosylation. To address this question, we treated the native and recombinant SP-A proteins with N-glycosidase F to remove N-linked oligosaccharides. The digested SP-A proteins all migrated at a slightly lower molecular weight than the corresponding untreated protein (Fig. 1B). In addition, all three SP-A proteins migrated at essentially the same molecular weight after digestion (Fig. 1B). Native SP-A and the native SP-A treated with glycosidase also had an additional higher-molecular-weight band (presumably a dimer), which the recombinant SP-A proteins lacked.

Native SP-A is thought to form a flower bouquet-type structure consisting of six heterotrimers of SP-A1 and
SP-A2. Thus the results shown in Fig. 1A are suggestive that the recombinant human SP-A proteins may not be able to form the same type of high-molecular-weight multimers as native SP-A (30). To determine the multimeric profiles of the various SP-A proteins, we electrophoresed the proteins in the presence of SDS but absence of β-mercaptoethanol (Fig. 1C). Recombinant SP-A1 and SP-A2 were able to form aggregates with equal propensity and similar migration patterns. The proportion of recombinant SP-A protein multimers migrating at very high molecular weights was not as great as for the native SP-A protein (Fig. 1C, lanes 1–3). Deglycosylation of native and recombinant SP-A did not affect the multimerization of these proteins (Fig. 1C, lanes 4–6). Together, these data are suggestive that the recombinant SP-A proteins may not form the large flower bouquet structure characteristic of native SP-A.

The direct effects of calcium concentration on the multimerization of SP-A were also evaluated. We re-suspended native SP-A (3 μg) in 1 or 5 mM Ca2+ and subjected to PAGE analysis while omitting β-mercaptoethanol. The multimerization of the SP-A was unaffected by calcium concentration (Fig. 1C, last two lanes). We also analyzed the effects of calcium on SP-A aggregation using absorbance in a spectrophotometer at 360 nm (27). Native SP-A (3 μg/ml) was diluted in 1 or 5 mM Ca2+ in water, and the absorbance was measured at both calcium concentrations. There was no difference in the absorbance of the two SP-A solutions (data not shown). However, when SP-A concentrations were increased to 30 μg/ml, SP-A aggregation was observed at the 5 mM calcium concentration. At 1 mM calcium, the mean absorbance was 0.036 ± 0.008, and at 5 mM calcium the mean absorbance was 0.078 ± 0.004. We found no difference in SP-A aggregation at SP-A concentrations of either 3 or 30 μg/ml in tissue culture media when the calcium concentration was increased to 5 mM. These findings are in agreement with results previously obtained by Ruano et al. (27). The SP-A in media condition more accurately reflects the in vivo condition, and thus we conclude that 5 mM calcium does not cause SP-A to aggregate under physiological conditions.

**Effect of calcium on recombinant SP-A1 and SP-A2 carbohydrate binding.** To evaluate the effects of calcium on the carbohydrate binding properties of native human SP-A, recombinant human SP-A1, and recombinant human SP-A2, we purified the different SP-A proteins using columns of affinity resins conjugated to four different carbohydrates, i.e., mannose, fucose, GlcNAc, and GalNAc, in the presence of either 1 or 5 mM calcium. SP-A1 binds to fucose with relatively high affinity in the presence of either 1 or 5 mM Ca2+ (Fig. 2, A and C). In contrast, SP-A1 bound relatively weakly to mannose in the presence of 1 mM Ca2+ (Fig. 2, A and B). When calcium levels were increased to 5 mM, there was a significant increase in the ability of SP-A1 to bind to mannose (Fig. 2, A and B). SP-A2 was able to bind to fucose with high affinity at both the 1 and 5 mM Ca2+ concentrations (Fig. 2, D and F). SP-A2 binds mannose weakly in the presence of 1 mM Ca2+. When calcium levels were increased to 5 mM, SP-A2 was able to bind mannose with greater affinity; however, the difference was not statistically significant (Fig. 2, D and E).

SP-A1 and SP-A2 binding to GalNAc and GlcNAc was also evaluated (Fig. 3). SP-A1 did not bind GalNAc at 1 mM Ca2+; however, it did bind at 5 mM Ca2+ (Fig. 3, A and B). SP-A1 bound weakly to GlcNAc in the presence of either 1 or 5 mM Ca2+ (Fig. 3, A and C). SP-A2 was able to weakly bind to both GlcNAc and GalNAc in the presence of 1 mM Ca2+ (Fig. 3, D–F). At 5 mM Ca2+, SP-A2 bound GalNAc with a significantly higher affinity (Fig. 3, D and E). The binding of SP-A2 to GlcNAc at 5 mM Ca2+ was higher but not significantly different from the binding observed at 1 mM Ca2+ (Fig. 3, D and F).

As shown in Fig. 4, human SP-A1 and SP-A2 bind to fucose with greater affinity than to any of the other sugars, at both 1 and 5 mM Ca2+ concentrations. There was no statistically significant difference between the levels of SP-A1 and SP-A2 binding for any of the carbohydrates at 1 mM Ca2+ (Fig. 4A). However, at 5 mM Ca2+, SP-A2 binds GalNAc and GlcNAc significantly better than SP-A1 (Fig. 4B). Purified SP-A1 and SP-A2 proteins (3 μg) were also passed over the four carbohydrate affinity columns in the presence of either 1 or 5 mM Ca2+. The results obtained using the purified recombinant SP-A proteins were similar to the results obtained using media containing the recombinant SP-A1 or SP-A2 (data not shown).

**Effect of calcium on native SP-A carbohydrate binding.** Native human SP-A also binds to fucose with the highest affinity (Fig. 5). It binds to mannose with a weaker affinity at 1 mM Ca2+ than at 5 mM Ca2+ (Fig. 5). At 1 mM Ca2+, native SP-A was unable to bind to either GalNAc or GlcNAc (Fig. 5). However, when calcium levels were increased to 5 mM, native SP-A appeared to be able to bind to all of the tested carbohydrates with the same, relatively high affinity (Fig. 5).

**Effect of calcium on native SP-A and recombinant SP-A1 and SP-A2 binding to Sepharose.** The resins used in the carbohydrate-binding affinity columns consist of Sepharose conjugated to the different carbohydrates. Therefore, as a control, we evaluated the binding of the various SP-A proteins to unmodified Sepharose resin. Figure 6A shows that SP-A1 did not bind Sepharose in the presence of either 1 mM Ca2+ or 5 mM Ca2+. Likewise, SP-A2 also did not bind Sepharose in the presence of either 1 or 5 mM Ca2+ concentrations (Fig. 6B). Native SP-A did not bind appreciably to the Sepharose in the presence of 1 mM Ca2+; however, in the presence of 5 mM Ca2+, the native SP-A binds to the Sepharose with relatively high affinity (Fig. 6C).

**N-glycosidase F-treated native SP-A carbohydrate binding.** We hypothesized that the difference in binding to Sepharose observed between the native and recombinant SP-A proteins might be the result of differences in glycosylation. Glycosidase-treated native SP-A was purified on a Sepharose column in the presence of 5 mM Ca2+ to determine whether the presence
or absence of glycosylation affects the ability of the native SP-A to bind to carbohydrates. The data in Fig. 6D show that the majority of the glycosidase-treated native SP-A (~75%) binds to Sepharose in the presence of 5 mM Ca²⁺. A: representative immunoblots for SP-A1 protein (~3 μg) purified on a mannose or fucose affinity column in the presence of 1 or 5 mM Ca²⁺. B: quantitative data from 3 experiments in which SP-A1 was passed over a mannose affinity column in the presence of 1 or 5 mM Ca²⁺. There was a significant increase in the binding of SP-A1 to mannose at 5 mM Ca²⁺ compared with 1 mM Ca²⁺ (*P < 0.05). C: quantitative data from 3 experiments in which SP-A1 was passed over a fucose affinity column in the presence of 1 or 5 mM Ca²⁺. SP-A1 binds to fucose with a greater affinity than mannose. There was no effect of calcium concentration on SP-A1 binding to fucose. D: representative immunoblots for SP-A2 protein (~3 μg) that was purified on a mannose or fucose affinity column in the presence of 1 or 5 mM Ca²⁺. E: quantitative data from 3 experiments in which SP-A2 was passed over a mannose affinity column in the presence of 1 or 5 mM Ca²⁺. F: quantitative data from 3 independent experiments in which SP-A2 was passed over a fucose column in the presence of 1 or 5 mM Ca²⁺ levels. SP-A2 binds to fucose with a higher affinity than to mannose. There was no effect of calcium on the binding of SP-A2 to either mannose or fucose.

**Effect of SP-A multimerization on carbohydrate binding.** We have shown that the recombinant SP-A proteins and native SP-A protein have different multimeric structures; i.e., native SP-A is able to form higher-molecular-weight aggregates than the recombinant SP-A proteins. Therefore, we examined the ability of SP-A multimers to bind carbohydrates. Native SP-A protein and recombinant SP-A1 and SP-A2 were purified on the four carbohydrate affinity columns, and it was determined that the higher-molecular-weight multimer forms of the SP-A proteins also bind to the carbohydrate resins. For example, Fig. 7 shows recombinant SP-A1 and SP-A2 and native SP-A purified over a mannose column in the presence of 1 mM calcium. The SP-A1 and SP-A2 proteins do not bind to mannose with high affinity at 1 mM Ca²⁺; however, the small amount of protein that does bind is almost all the higher-molecular-weight forms (Fig. 7). Only ~3% of the total SP-A1 and SP-A2 protein recovered in the
calcium wash was the higher-molecular-weight multimers. In sharp contrast, ∼80% of the bound SP-A1 and ∼30% of the bound SP-A2 protein (i.e., the EDTA wash) was composed of high-molecular-weight multimers. There appears to be more SP-A2 lower-molecular-weight multimers binding to mannose compared with SP-A1. The significance of this is unknown. Also, almost all of the native SP-A protein that bound was higher-molecular-weight multimers (∼95%). These experiments suggest that higher-molecular-weight multimers of SP-A bind to carbohydrates with higher affinity.

DISCUSSION

In the present study, we found that native human SP-A and recombinant human SP-A1 and SP-A2 have different carbohydrate-binding affinities and that
Several investigators have demonstrated that SP-A interacts with bacteria through its carbohydrate-binding domain to facilitate the phagocytosis of bacteria into macrophages (8, 15). SP-A has been shown to interact directly with macrophages as well; however, SP-A is thought to bind to a protein receptor on macrophages and apparently does not bind to the sugars present on macrophage cell surfaces (4). There is very little known about the biological function of SP-A1 vs. SP-A2. Both proteins are expressed in alveolar type II cells, and native alveolar SP-A is thought to consist of both proteins (14, 28). Because the SP-A2 protein alone is expressed in the submucosal glands of the conducting airways, we hypothesized that this form of the SP-A protein may also be involved in host defense mechanisms (14). We found that both SP-A1 and SP-A2 proteins were able to bind carbohydrates. This observation is suggestive that both of these proteins are involved in innate host defense mechanisms. However, we also found that SP-A2 tends to bind to a wider variety of carbohydrates with a higher affinity than the isolated SP-A1 protein. In fact, at 5 mM Ca$^{2+}$, SP-A2 was able to bind to GalNAc and GlcNAc in significantly higher proportion than SP-A1. We believe that this is an important finding because both of these sugars are present on the surface of a wide variety of bacteria. Pseudomonas aeruginosa, many oral Streptococcus bacteria, Helicobacter pylori, and Escherichia coli all contain GlcNAc, GalNAc, or both carbohydrates in their coat polysaccharides (10, 20, 23, 29). There is only one amino acid difference in the carbohydrate-binding domains between the SP-A2 allele 1A1 and SP-A1 allele 6A2. At amino acid number 223, the SP-A1 allele has a glutamine and the SP-A2 allele has a lysine. Lysine is a positively charged amino acid, whereas glutamine is a neutrally charged amino acid. This amino acid change may account for the observed differences in the carbohydrate-binding specificities of the two proteins. We also believe that the 5 mM Ca$^{2+}$ concentration more accurately reflects the natural environment in which the SP-A proteins reside, because the alveolar lining of the lung has calcium concentrations ranging from 3.5 to 6 mM (7, 11). Therefore, our data support the theory that the SP-A2 protein found alone in the submucosal glands of the conducting airways may be involved in the innate host defense of the lung.

Another interesting finding in our study is that all three SP-A proteins bind fucose with the highest affinity. Recombinant SP-A2 protein tends to bind to all four carbohydrates that were tested, i.e., mannose, fucose, GalNAc, and GlcNAc, with a slightly higher affinity than recombinant SP-A1, at either 1 or 5 mM Ca$^{2+}$. At 5 mM Ca$^{2+}$, SP-A2 binds to GalNAc and GlcNAc with significantly higher affinity than SP-A1 protein. Native SP-A, at 1 mM Ca$^{2+}$, displayed a carbohydrate-binding specificity that is a mixture of both SP-A1 and SP-A2 carbohydrate-binding characteristics. At 5 mM Ca$^{2+}$, native SP-A binds to Sepharose, but neither the recombinant SP-A1 nor SP-A2 proteins were able to bind to this resin. Therefore, the native SP-A carbohydrate-binding data are not interpretable at 5 mM calcium.

These studies might help us understand the biological function of SP-A and provide insights into the role of different SP-A isoforms in innate host defense.
Fig. 5. Human native SP-A binding to mannose, fucose, GalNAc, and GlcNAc affinity columns in the presence of 1 or 5 mM Ca\(^{2+}\). A: representative immunoblots for native SP-A (~3 μg) purified on a mannose or fucose affinity column in the presence of 1 or 5 mM Ca\(^{2+}\). B: quantitative data from 3 native SP-A-mannose experiments. There was a significant increase in native SP-A binding to mannose at 5 mM Ca\(^{2+}\) compared with 1 mM Ca\(^{2+}\) (*P < 0.05). C: quantitative data from 3 native SP-A-fucose experiments. Native SP-A binds to fucose with a greater affinity than mannose. There was no effect of calcium concentration on native SP-A binding to fucose. D: representative immunoblots for native SP-A (~3 μg) purified on a GalNAc or GlcNAc affinity columns in the presence of 1 or 5 mM Ca\(^{2+}\). E: quantitative data from 3 native SP-A-GalNAc experiments. There was a significant increase in native SP-A binding to GalNAc at 5 mM Ca\(^{2+}\) compared with 1 mM Ca\(^{2+}\) (*P < 0.05). F: quantitative data from 3 native SP-A-GlcNAc experiments. There was a significant increase in native SP-A binding to GlcNAc at 5 mM Ca\(^{2+}\) compared with 1 mM Ca\(^{2+}\) (*P < 0.05). The arrows in A and D indicate the monomer and dimer forms characteristic of native SP-A. The monomer form of native SP-A has a molecular weight ~35,000.
We also characterized the recombinant SP-A1 and SP-A2 proteins made by SF-9 insect cells. The insect cells produced recombinant human SP-A1 and SP-A2 proteins that were recognized by antibodies directed against native human SP-A. The recombinant SP-A proteins are underglycosylated compared with the native SP-A protein; however, we showed that glycosylation does not affect the ability of the recombinant SP-A to bind carbohydrates. Likewise, when the native SP-A was treated with endoglycosidase to remove N-linked carbohydrates, the protein remained biologically active and could still bind to carbohydrates. Therefore, even though the insect cells do not glycosylate proteins in a manner identical to human cells, the recombinant SP-A proteins produced by insect cells are biologically active, at least with respect to carbohydrate-binding properties.

A major difference between the native human SP-A protein and recombinant human SP-A1 and SP-A2 proteins appears to be their structural conformation. Native SP-A and recombinant SP-A1 and SP-A2 were electrophoresed under nonreducing conditions, and the native SP-A protein migrated differently from the recombinant SP-A protein. The native SP-A protein did not migrate into the gel as far as the recombinant SP-A1 and SP-A2 proteins, indicating that the native SP-A protein may form larger structures than the recombinant SP-A proteins. To evaluate the multimerization state of the native and recombinant SP-A, we electrophoresed the proteins in the presence of SDS but absence of β-mercaptoethanol. This allowed the disulfide bonds in the SP-A proteins to remain intact, and, therefore, we could observe the multimerization of the human SP-A proteins on a polyacrylamide gel. The majority of the native SP-A protein forms very high-

![Fig. 6. Recombinant human SP-A1 or SP-A2 and native human SP-A binding to Sepharose in the presence of 1 or 5 mM Ca2+. A: representative immunoblot for SP-A1 protein (−3 µg) purified on a Sepharose column in the presence of 1 or 5 mM Ca2+. B: representative immunoblots for SP-A2 (−3 µg) purified on a Sepharose column in the presence of 1 or 5 mM Ca2+. C: representative immunoblots for native SP-A (−3 µg) purified on a Sepharose column in the presence of 1 or 5 mM Ca2+. The experiments were repeated 2 times with similar results. D: glycosidase-treated (top) or untreated human native SP-A (bottom) binding to a Sepharose column in the presence of 5 mM Ca2+. This experiment was repeated 2 times. The two arrows at left indicate the monomer and dimer forms characteristic of native SP-A.](http://ajplung.physiology.org/)

![Fig. 7. SP-A multimers binding to carbohydrates. Recombinant SP-A1 or SP-A2 or native SP-A were purified on a mannose column in the presence of 1 mM Ca2+. The various SP-A proteins were electrophoresed in SDS-containing electrophoresis buffer with no β-mercaptoethanol added so that the higher-molecular-weight SP-A multimers could be observed. Around 80% of the bound SP-A1 protein (i.e., eluted with EDTA) was composed of higher-molecular-weight multimers, and ∼30% of the bound SP-A2 protein was composed of higher-molecular-weight multimers. However, only 3% of the unbound SP-A1 and SP-A2 protein was higher-molecular-weight multimers (>131,000). The native SP-A protein was mostly composed of higher-molecular-weight multimers, and ∼95% of the bound SP-A protein was composed of these high-molecular-weight multimers.](http://ajplung.physiology.org/)
molecular-weight multimers, which probably represent the flower bouquet structure (six trimers, molecular wt ~63,000). The recombinant SP-A1 and SP-A2 proteins also form multimers; however, the majority of the recombinant SP-A proteins form a monomer, dimer, or trimer structure. We also showed that these higher-molecular-weight SP-A multimers bind with a high affinity to the carbohydrate affinity columns. Therefore, differences in the carbohydrate-binding characteristics of the native and recombinant SP-A proteins may be the result of the multimeric structure of the SP-A protein. A difference in structure between native and recombinant SP-A1 and SP-A2 proteins has also been observed when using Chinese hamster ovary cells to produce the recombinant human SP-A proteins (30). Voss et al. (30) showed that SP-A1 and SP-A2 recombinant proteins form smaller-sized multimers than native human SP-A.

In conclusion, we have shown that human recombinant SP-A1 and SP-A2 and native human SP-A have different carbohydrate-binding specificities. We have also shown their carbohydrate-binding characteristics change when calcium concentrations are increased from 1 to 5 mM. These results are suggestive that the biological properties of SP-A may be altered by changes in environmental calcium concentrations. In addition, our results are indicative that SP-A2, because it binds to carbohydrates with specificities and affinities different from either SP-A1 or native SP-A, may have a different biological function from the native, alveolar SP-A.

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