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Characteristics of Human Surfactant-Associated Glyoproteins A

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ABSTRACT. Surfactant-associated glycoprotein A [molecular weight (Mr) = 34,000, isoelectric point (pI) 4.6–5.0] and its sulfhydryl dependent oligomers were purified and partially characterized from surfactant obtained from human alveolar lavage. Two major forms of the protein were identified by silver stain and immunoblot analysis of surfactant using human surfactant-associated glycoprotein A antisera: glycoprotein A_2, Mr = 34,000 and glycoprotein A_1, Mr = 28,000. The larger form was reduced to Mr = 28,000 by treatment with endogylcosidase F, indicating the presence of complex N-linked oligosaccharide on the molecule. Charge heterogeneity was decreased and the isoelectric point increased by treatment with neuroaminidase, supporting the presence of sialic acid. Homology between the proteins Mr = 34,000 and 28,000 was confirmed by analysis of two-dimensional tryptic and chymotryptic peptides of ^{125}I-iodo-glycoproteins A_1 and A_2 which were identical. The protein was very rich in glycine and its amino acid composition was similar to that of glycoprotein A previously reported for the dog and rat. Treatment of glycoproteins A with bacterial collagenase resulted in the generation of highly glycosylated peptides Mr = 20,000–22,000, pI 4.6–5.0, which no longer formed sulfhydryl-dependent oligomers, supporting the presence of significant collagen-like region in the molecule. In the absence of reducing agents, glycoprotein A from surfactant was present as sulfhydryl-dependent dimers and larger oligomers. Higher molecular weight aggregates of glycoproteins A were also present in lavage material even after sulfhydryl reduction. Glycoproteins A were identified in surfactant from amniotic fluid, normal adult lung lavage, human cadaver lung lavage, and material obtained from lung lavage from a patient with alveolar proteinosis. Alveolar proteinosis proteins contained larger amounts of the higher molecular weight aggregates and smaller molecular weight proteolytic fragments of glycoproteins A than material obtained from other sources. Peptide mapping of the ^{125}I-iodinated aggregates, approximately Mr = 50,000, 70,000, 100,000, and greater were identical to glycoproteins A (Mr = 34,000 and 28,000) from normal human lung lavage. A smaller immunoreactive form (Mr = 20,000) shared several peptides but lacked others, supporting its origin as a proteolytic fragment of glycoproteins A_1 or A_2. Human glycoprotein A_2 is a complex N-linked glycoprotein likely representing the glycosylated form of a polypeptide precursor Mr = 28,000. (*Pediatr Res* 19: 501–508, 1985)

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Abbreviations

pI, isoelectric point
Mr, molecular weight
PMSF, phenylmethylsulfonylfluoride
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
2-D-IEF-PAGE, two-dimensional IEF PAGE
IEF-SDS-PAGE, isoelectric focusing SDS PAGE

Pulmonary surfactant-associated proteins have been identified in lung lavage material from a variety of species including the human (1). Although there is controversy regarding the physiologic role of the surfactant-associated proteins, the protein components of pulmonary surfactant appear to alter its physicochemical properties (2–5). Canine glycoprotein A binds to phospholipids and enhances the spreading of lipid monolayers and adsorption to the air liquid interface (2). Addition of surfactant-associated proteins to phospholipids produces a preparation with properties similar to natural lung surfactant. Recent surfactant preparations for treatment of respiratory distress syndrome in premature infants have included lung proteins prepared from bovine lung or from human amniotic fluid, respectively (6–9).

Although a number of surfactant-associated proteins have been reported, the major forms apparently somewhat similar in all species are glycoproteins A Mr = 30,000–40,000 first described by King and Clements (10). These proteins migrate as complex acidic proteins, pI 4.2–5.0, Mr = 30,000–40,000 with a minor component migrating at approximately Mr = 26,000 in the rat and dog (11–13). Similar glycoprotein has been identified in lung lavage fluid obtained from patients with alveolar proteinosis (14). Bhattacharyya and Lynn (14) previously characterized surfactant-associated glycoproteins and provided data that the molecules migrating with Mr = 30,000–40,000 are fragments of a larger Mr = 80,000 dalton polypeptide and have recently described complex N-linked carbohydrates on these molecules (15). Several distinct proteins reactive with antisera generated against glycoprotein A have been identified with characteristics of glycoproteins A in lung lavage fluid (16). Identity and relationships among the various molecular weight forms described of the surfactant-associated proteins remain somewhat unclear. Shelly *et al.* (17) have characterized glycoprotein from human cadaver lung lavage, demonstrating similar proteins with molecular weight approximately 400,000 which upon sulfhydryl reduction produced peptides of 34,000 daltons. In that study, antisera prepared against this human protein selectively stained human alveolar type II cells and the alveolar lining of human lung. Despite progress with the characterization of surfactant-associated proteins, the identity and relationships among the surfac-

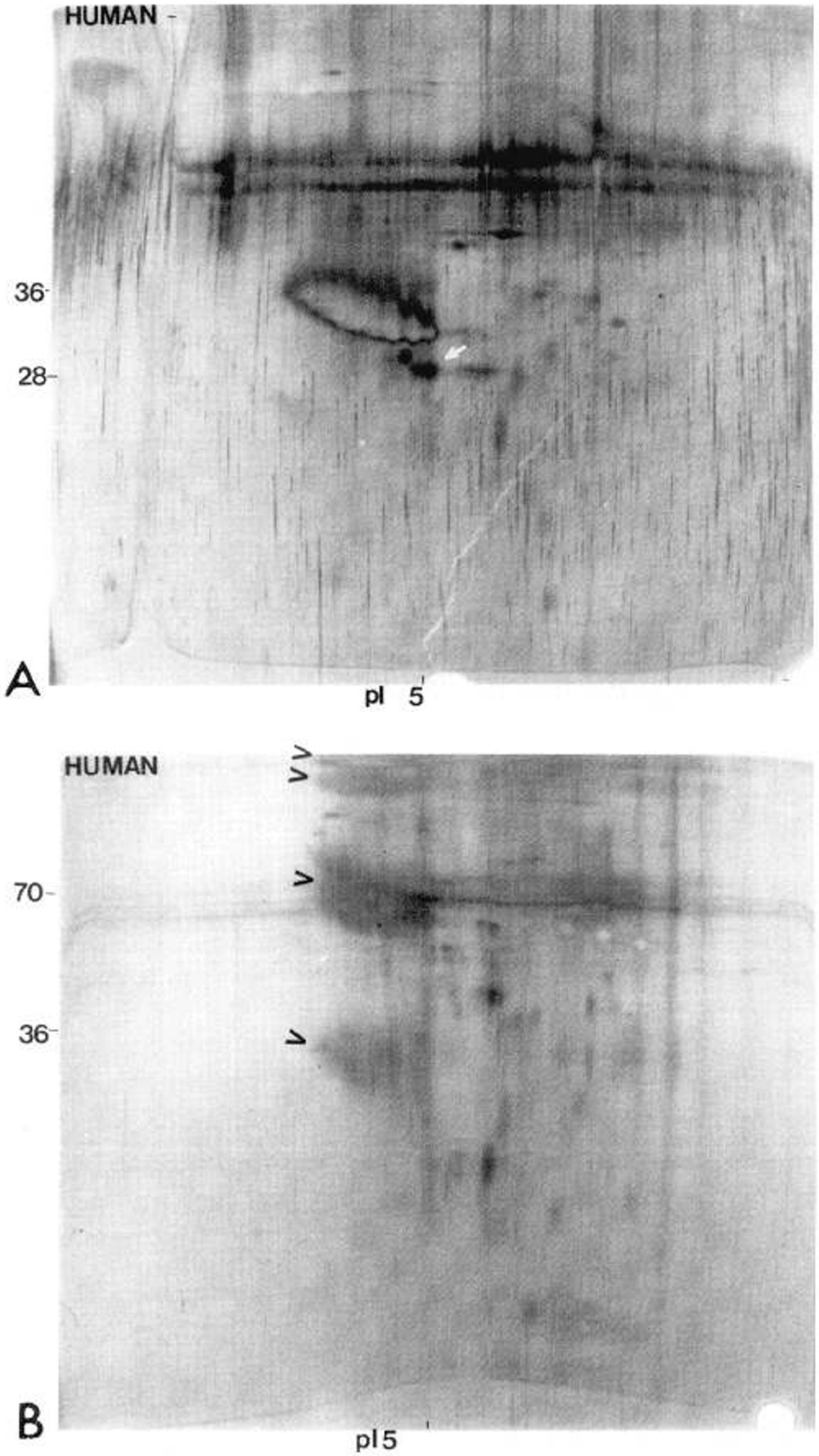

Fig. 1. *A*, silver stain analysis of 2-D-IEF-polyacrylamide gel electrophoresis of surfactant-assoicated glycoproteins. Surfactant obtained from lung lavage material, approximately 15 μg protein, was separated by isoelectric focusing in the horizontal plane (pH range 4–6) followed by electrophoresis in 10% polyacrylamide in the vertical plane in the presence of 1 mM β-mercaptoethanol. Glycoproteins A were stained characteristically salmon pink and the major form migrated with Mr approximately equal 36,000, pI 4.6–5. Molecular weights are noted to the *left* $\times 10^{-3}$. *B*, represents identical experiment in the absence of sulfhydryl reduction.

tant-associated proteins from normal human surfactant and the larger molecular weight forms found in human alveolar proteinosis material remain unclear.

METHODS

Samples of human surfactant were obtained under protocols approved by the Humn Research Committee of the University of Cincinnati College of Medicine and Children's Hospital, Cincinnati, OH. Selected bronchial lavage from adult volunteers was used to obtain adult human surfactant under an approved protocol. Surfactant from lung lavage was collected in 0.9% sodium chloride and placed in ice. The sample was filtered through two layers of cheese cloth and pelleted at 1000 × *g* for 5 min at 4° C. Supernatant was removed and the centrifugation step repeated. Supernatant was then centrifuged 40,000 × *g* for 20 min and the dense pellet collected in 0.9% sodium chloride, 1 mM PMSF (phenylmethylsulfonylfluoride) and stored at −80° C. Amniotic fluid samples were obtained at elective cesarian section at term. Surfactant was prepared from amniotic fluid as described above. Lung lavage samples were also obtained at autopsy by lung lavage with 0.9% sodium chloride, surfactant prepared as described above.

Antisera against amniotic fluid surfactant was prepared in New Zealand rabbits by injection of partially purified lavage material in Freund's adjuvant. Antisera were extensively adsorbed with human red blood cells and human serum-Sepharose affinity column and produced an antisera selectively reactive for glycoprotein A. Monospecific antisera were generated using human glycoprotein A_2 (Mr = 34,000) electroeluted from 2-D-IEF-PAGE as described by Hunkapiller *et al.* (18) as the immunogen. Silver-stain analysis of this immunogen was entirely homogeneous for glycoprotein A_2. This antisera cross-reacts with glycoprotein A from the dog and the rat as assessed by immunoblots of the surfactant after IEF-SDS-PAGE.

Analysis of protein was carried out by immunoblot, silver, and Coomassie staining. Purified lavage surfactant was pelleted, resuspended in buffer containing 2% SDS and β-mercapthoethanol as described by Laemmli (19), for one-dimensional SDS-PAGE or in the buffer used by Anderson and Anderson (20), for 2-D-IEF-PAGE as described by Garrison and Johnson (21). Isoelectric points were determined from the focusing gels by excision of 1-cm pieces which were placed in 2 ml distilled degassed water for determination of pH. Unfixed gels were used for immunoblot analysis and proteins transferred electrophoretically to nitrocellulose and treated with antiglycoprotein 1:100 or 1:300 dilution of antisera followed by horseradish peroxidase-conjugated goat antirabbit IgG. Color was developed as described by Towbin *et al.* (22) except that 4-chloro-napthol was used as a substrate. Gelatin (3%) was included in the incubation to reduce nonspecific background color (23). Molecular weight markers included unlabeled and ^{14}C-labeled myosin (220,000) phosphorylase B (93,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,000) obtained from Amersham, Arlington Heights, IL. Silver staining was performed by a slight modification of the procedure of Sammons *et al.* (24) in which 5% acetic acid was used to control background staining following a 5–10 min Na_2CO_3 color development step. Figures are generally representative of at least three separate experiments.

Peptide mapping of glycoprotein(s) A. Glycoproteins A_1 and A_2 were lightly stained with Coomassie brilliant blue and excised from the 2-D-IEF-SDS-PAGE gels of reduced alveolar lavage surfactant. Stain was removed by shaking the gels in methanol

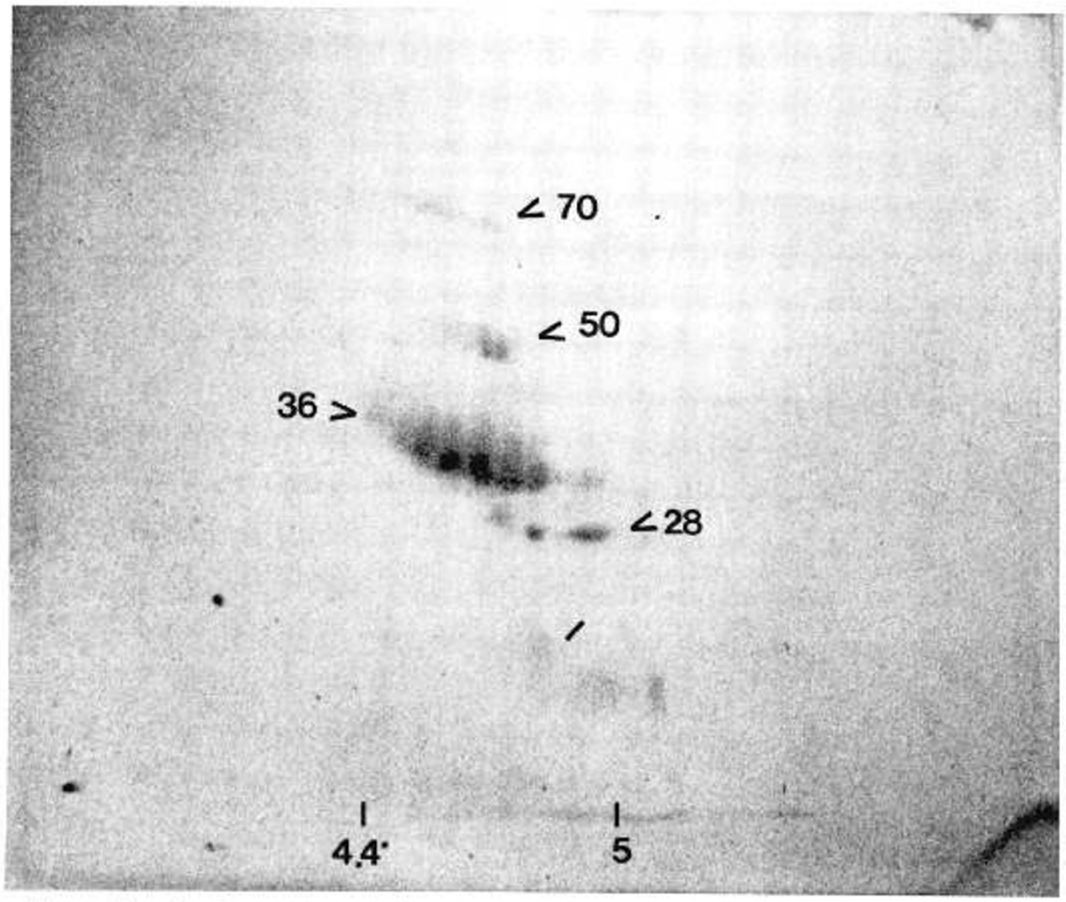

Fig. 2. Immunoblot analysis of surfactant-associated proteins separated by 2-D-IEF-polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol. Surfactant obtained from human alveolar lavage was subjected to electrophoresis and immunoblotted as described in "Methods." Immunoreactivity was assessed with the antihuman glycoprotein A antisera. Molecular weights are $\times 10^{-3}$.

prior to peptide mapping as described by Elder and Alexander (25). Proteins were iodinated with ^{125}I-iodine (17.4 mCi/mg) by the chloromine T method dialyzed against 10% methanol, lyopholized and incubated with chymotrypsin (50 μg/ml), and trypsin (50 μg/ml) in 50 mM ammonium acetate, pH 8.0 for 16 h at room temperature (26). ^{125}I-iodopeptides were isolated by electrophoresis and thin-layer chromotography as described by Gibson (27), and subjected to autoradiography. Peptide maps of normal lung lavage and alveolar proteinosis samples were obtained in a similar manner.

Treatment of surfactant glycoproteins with endoglycosidase F, collagenase, and neuraminidase. Endo-β-N-acetylglucosaminidase F (endoglycosidase F) from *Flavobacterium menisigoseptiicum* was purchased from New England Nuclear, Boston, MA. Neuroaminidase was purchased from Calbiochemical, LaJolla, CA. Lung lavage material was delipidated by the chloroform-methanol extraction as described by Folch and Less (28). Extracted lavage proteins were solubilized in 0.5 ml of 0.1 M of Tris-Cl pH 8.2 containing 1% SDS, 1% β-mercapthoethanol, and boiled for 2 min. Reduced sulfhydryl bonds were alkylated by addition of 0.15 ml of 1 M iodoacetamide in 0.1 M Tris-Cl, pH 8.2. Following incubation at room temperature for 30 min,

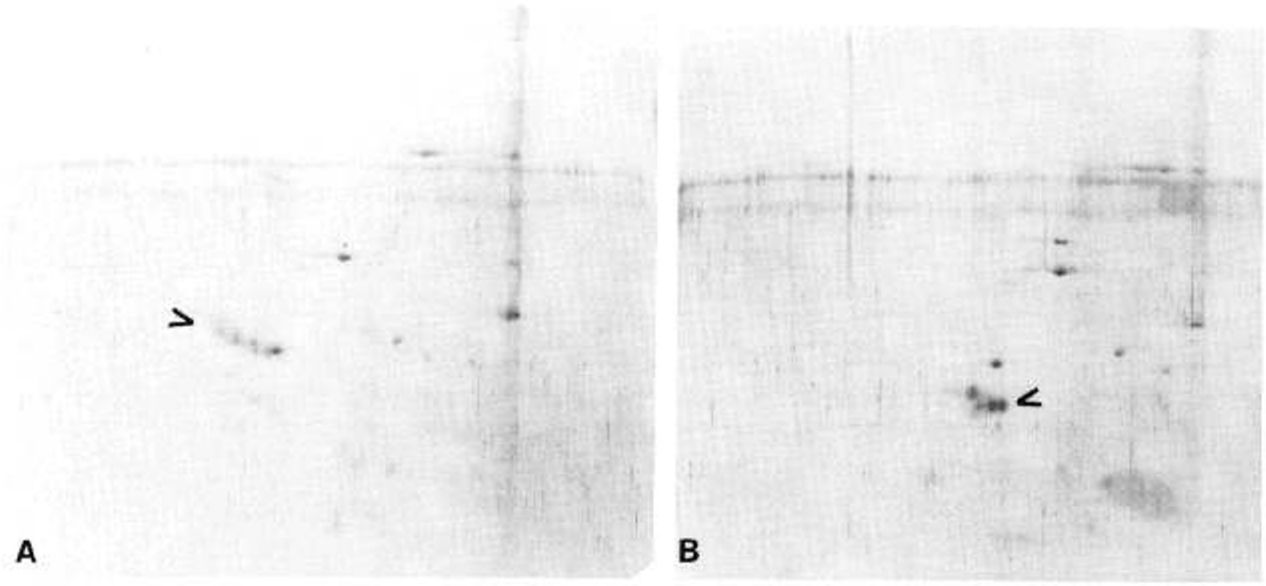

Fig. 3. *A* and *B*, digestion of glycoproteins A_2 with endoglycosidase F. Human surfactant proteins were delipidated, reduced, and aklylated as described in "Methods." Proteins samples (approximately 300 μg/ml) were suspended resuspended in 50 mM sodium phosphate, pH 6.2, containing 10 mM EDTA and 0.1% NP-40. Samples were incubated at 37° C in the absence (*A*) or presence of 2 U/ml endoglycosidase F (*B*). Lyophilized digests were prepared for one- or two-dimensional analysis. Proteins were then stained by silver staining as described in "Methods.".

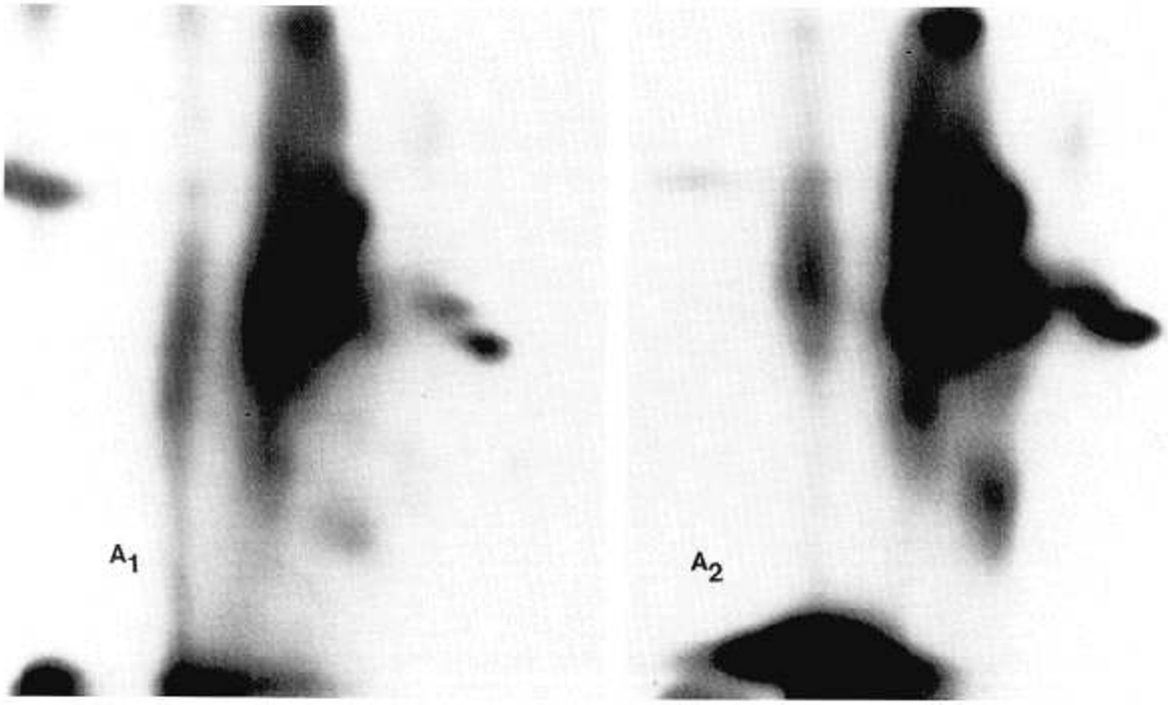

Fig. 4. Chymotryptic-tryptic peptide maps of glycoproteins A_1 and A_2 from human surfactant. Single protein spots from Mr = 34,000 and 28,000 were excised, iodinated, and subjected to chymotryptic-tryptic peptide analysis as described in "Methods." Samples were then subjected to autoradiography. Peptide maps of glycoproteins A_2 and A_2 were identical.

protein was precipitated by addition of cold acetone to 90% of total volume. The sample was chilled on ice for 10 min and centrifuged at 20,000 × *g* for 25 min. The dried protein pellets were then treated essentially as described by Elder and Alexander (25). Reduced and alkylated protein was resuspended in 5 mM sodium phosphate, pH 6.2, 10 mM disodium EDTA, 0.1% NP-40, giving an approximate protein concentration of 300 μg/ml. Indicated samples were treated with endoglycosidase F (2 U/ml) or neuraminidase (0.1 U/ml) for 16 h at 37° C. Samples were also treated as above with bacterial collagenase (50 U/ml) obtained from Advance Biofactures, Lynbrook, NY, and subjected to IEF-SDS-PAGE. Composition of purified glycoprotein A_2 (34,000) was obtained using the Beckman 6300 amino acid analyzer.

Fig. 5. Purified glycoproteins A after separation by isoelectric focusing and sizing. Human lung lavage surfactant was subjected to preparative isoelectric focusing and sizing as described in "Methods." Purified protein was subjected to SDS-PAGE (13%). Only glycoprotein A was detectable in the final purified material by one- or two-dimensional SDS-PAGE. Higher molecular weight staining are artifacts related to the silver stain and are observed in empty lanes to each side of the sample.

RESULTS

Glycoprotein A, Mr = 34,000, pI 4.6–5.0 was the major protein component of surfactant material whether obtained from normal adult lung lavage, human cadaver lung lavage, or amniotic fluid (latter not shown) (Fig. 1*A*). This protein migrated with marked charge and size heterogeneity, pI 4.6–5.0 on isoelectric focusing gels of the reduced material. In the absence of sulfhydryl reduction larger oligomeric forms of the protein were observed with formation of dimers at approximately 65,000–70,000 and larger oligomers, Figure 1B. Immunoblot analysis, using monospecific or antisurfactant antibody identified Mr = 34,000 and a immunoreactive protein Mr = 28,000, pI 5.0, with silver staining characteristics (salmon-pink staining) similar to that of the larger forms (Fig. 2). We have called these glycoprotein A_1 (Mr = 28,000) and glycoprotein A_2 (Mr = 34,000). Smaller amounts of immunoreactive material were observed Mr = 50,000, 65,000–70,000, and greater in the reduced samples. Treatment of the larger form (Mr = 34,000) with endoglycosidase F resulted in reduction of most of the material to Mr = 28,000. Several more intensely stained protein spots with increased isoelectric points (5.0) were observed (Fig. 3). Charge heterogeneity was decreased and the isoelectric point increased by treatment with neuraminidase supporting the presence of sialic acid in the Mr = 34,000 protein, not shown. Reduction of Mr = 34,000 to Mr = 28,000 by endoglycosidase F supported the likelihood that these proteins shared a common amino acid sequence. This was confirmed by two-dimensional peptide mapping of tryptic-chymotryptic peptides of the ^{125}I-iodoglycoproteins (Fig. 4*A* and *B*). Two-dimensional peptide maps of the proteins Mr = 34,000 and 28,000 were indistinguishable from one another. Further analysis of the amino acid composition of the protein required its purification which was accomplished by 2D-IEF-SDS-PAGE and electroelution (18).

Purification of glycoprotein A_1 and A_2 was accomplished utilizing a isoelectric focusing followed by separation of proteins by size, taking advantage of the acidic isoelectric point of glycoproteins A (Fig. 5). A variety of purification schemes including DEAE-cellulose, CM-cellulose, and lectin-affinity chromatography were attempted; however, an initial separation by isoelectric focusing provided the best purification and became the method of choice. With the purified human glycoprotein A_2 (Fig. 5) amino acid composition data were obtained and are listed in Table 1. The protein was found to be rich in glycine and had characteristics similar to that previously described for rat and dog glycoproteins A (11). There are also similarities between the glycoprotein composition presently reported and that of alveolar

Table 1. *Partial amino acid composition of human glycoprotein A**

Human glycoprotein A_2 (34,000 dalton)	
ASX	30
THR	10
SER	23
GLX	35
PRO	12
GLY	45
ALA	16
CYS	
VAL	14
MET	4
ILE	10
LEU	20
TYR	9
PHE	14
HIS	4
LYS	11
ARG	12

* Numbers represent the residues detected; CYS was not preserved by this analysis.

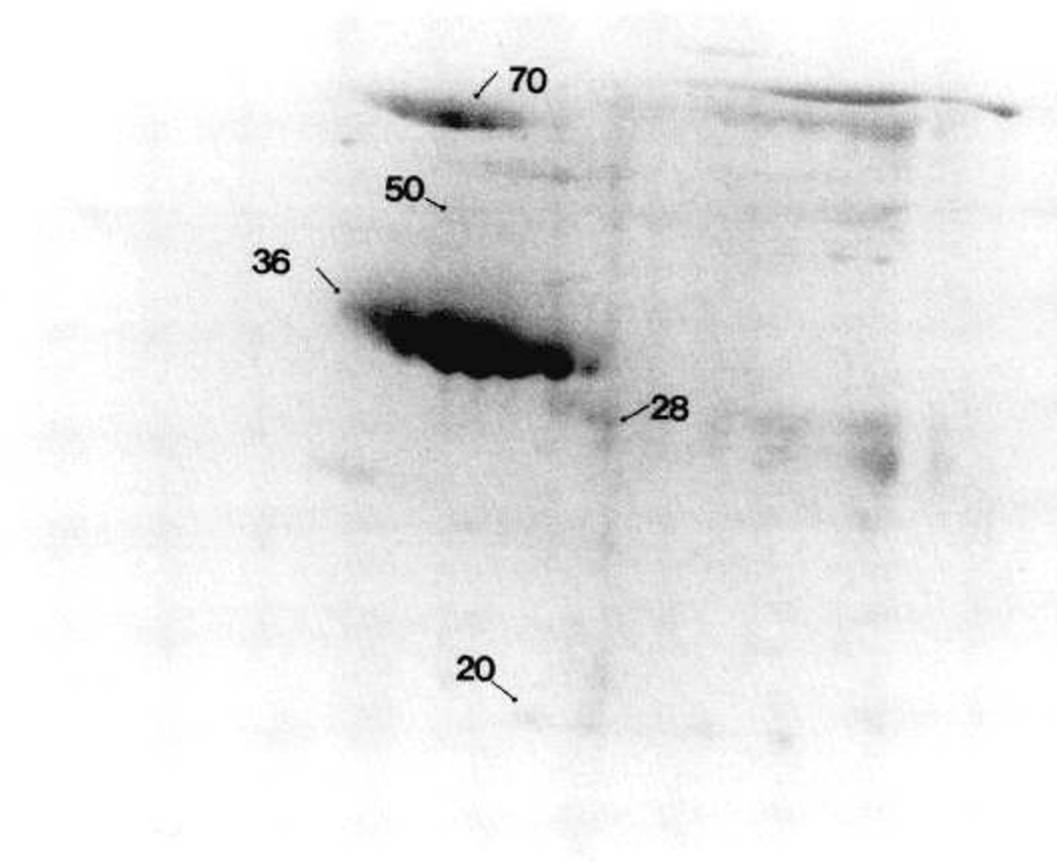

Fig. 6. Coomassie brilliant blue staining of alveolar proteinosis proteins subjected to 2-D-IEF-polyacrylamide gel electrophoresis. Lavage from an alveolar proteinosis patient, approximately 100 μg, was applied to the 2-D-IEF-polyacrylamide gel electrophoresis as described in "Methods." Approximate molecular weights are indicated $\times 10^{-3}$. The distinct bands utilized for peptide mapping are indicated by the *arrows*. Larger aggregates are visible in gels but not adequately seen after photography.

glycoprotein Mr= 36,000 previously described by Bhattacharyyra and Lynn (14) who previously demonstrated a high glycine content and collagenase-like sequences in the alveolar proteinosis glycoprotein. Because of these similarities in size and composition, comparisons of the protein from human alveolar proteinosis lavage and the glycoprotein A from normal human lavage were made.

Glycoprotein A and the major alveolar proteinosis protein comigrated in 2-D-IEF-SDS-PAGE. Higher molecular weight aggregates were relatively more abundant in the alveolar proteinosis sample (Fig. 6). Similar higher molecular weight forms were detectable by immunoblot analysis of normal lung lavage material (Fig. 2). Bacterial collagenase which is selective for gly (X,Y) gly sequences, selectively reduced human glycoprotein A_2 to glycoproteins approximately Mr = 20,000 which retained the charge heterogeneity observed in the glycosylated form of the molecule (not shown). The smaller fragment(s) generated by collagenase could not be detected even with high percentage (15%) one-dimensional SDS-polyacrylamide gels (using silver staining). In order to more fully characterize relationships among the human glycoproteins A and the human alveolar proteinosis proteins, peptide mapping of the various forms of the alveolar proteinosis proteins was compared with that from glycoproteins A_1 and A_2 (Fig. 7). Peptide maps of the major alveolar proteinosis protein Mr = 34,000 were identical to those obtained from glycoproteins A_1 and A_2 from normal human lavage. The larger molecular weight forms of the proteinosis samples, Mr = 50,000, 68,000–70,000, 100,000, and greater were also identical to the peptide maps of glycoproteins A_1 and A_2. No new peptides were identified in any of the larger molecular weight proteins, suggesting that they result from aggregation of the 34,000 or 28,000 dalton precursors or their fragments. Smaller immunoreactive proteins were also observed in alveolar proteinosis samples, Mr = 20,000, pI 4.8. Peptide maps of this smaller protein were similar to those of glycoproteins A_1 and A_2; however, they lacked several major tryptic peptide fragments. The absence of any new proteolytic fragments generated from the higher molecular weight forms of alveolar proteinosis protein strongly supports their identity as aggregates of either the 34,000 and 28,000 precursors or their fragments.

DISCUSSION

The present study partially characterizes glycoproteins A from surfactant from human lung lavage, amniotic fluid, and alveolar proteinosis material. Human surfactant associated glycoprotein A_2, Mr = 34,000 after sulfhydryl reduction was detected in all of these preparations and appears to be formed by N-linked glycosylation of proteins Mr = 28,000. The proteins were present as sulfhydryl-dependent oligomers composed of glycoproteins A in the absence of sulfhydryl reduction. Higher molecular weight aggregates and proteolytic fragments of glycoproteins A account for the various forms present in alveolar proteinosis material. Human glycoproteins A are rich in glycine and contain a significant portion of collagen-like amino acid sequence which is also required for the observed sulfhydryl-dependent oligomerization.

Glycoproteins A were identified as major protein components of surfactant and alveolar proteinosis samples; the proteins were virtually identical from these sources as assessed by 2-D-IEF-PAGE and peptide mapping. Reduction of glycoprotein A_2 Mr = 34,000 to A_1 (Mr = 28,000) by endoglycosidase F, shared immunoreactivity and the observation that tryptic peptide maps of these proteins were identical, strongly supports the concept that glycoprotein A_2 is a glycosylated form of the polypeptide

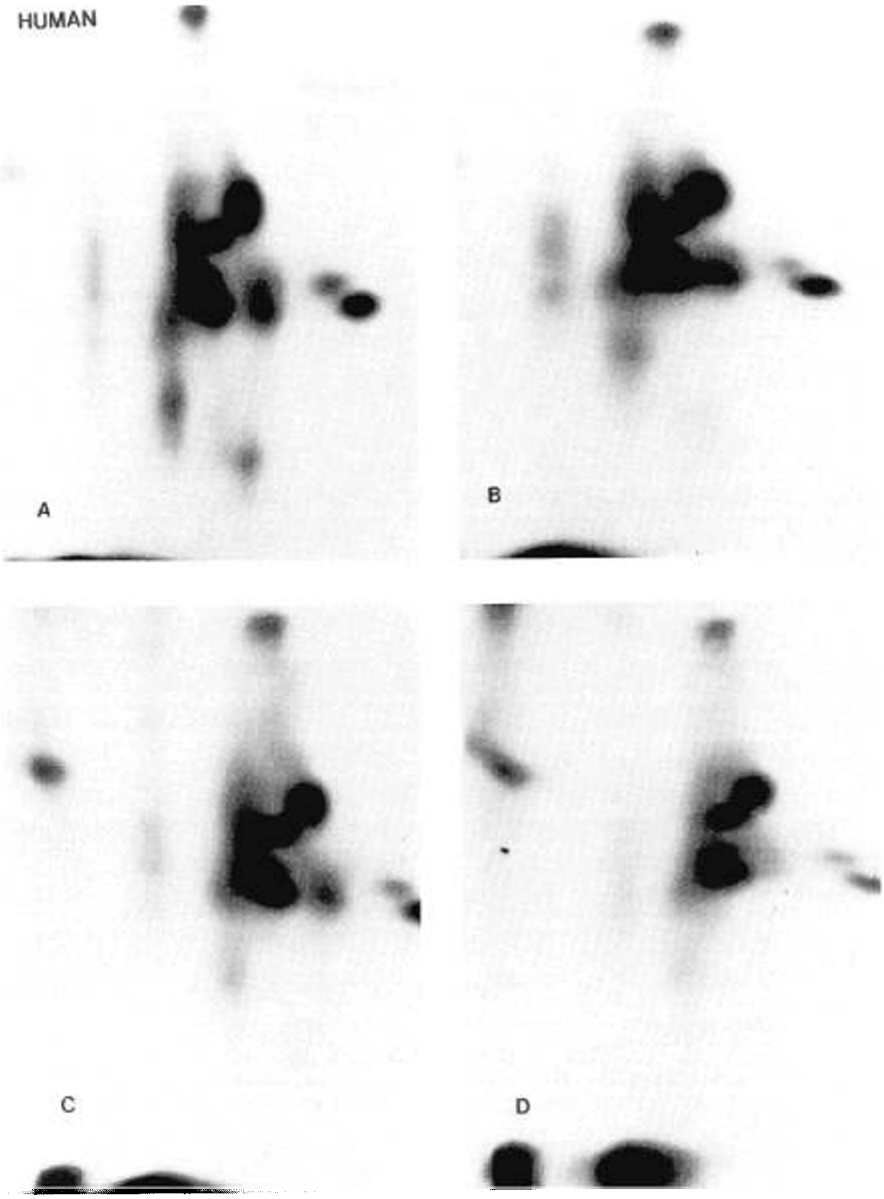

Fig. 7. Chymotryptic-tryptic peptide maps of alveolar proteinosis proteins. Proteins as indicated from Figure 6 were excised, iodinated, and subjected to chymotryptic-tryptic mapping analysis as described in "Methods." Iodinated peptides were then subjected to autoradiography for approximately 24 h. Peptide maps of the proteins were identical to those seen in Figure 4 from normal lung lavage glycoproteins A. *A*, peptide map of Mr = 36,000; *B*, 65,000–70,000; *C*, Mr = 50,000; *D*, Mr = 20,000. Larger forms, Mr = 100,000 and greater were also identical (not shown).

precursor Mr = 28,000. Endoglycosidase F, specific for N-linked, mannose-containing complex-oligosaccharides, decreased molecular weight and increased the isoelectric point of Mr = 34,000 to 28,000. In agreement with other studies with rat and rabbit glycoprotein A, treatment with neuraminidase reduced the charge heterogeneity and increased the isoelectric point of Mr = 34,000 supporting the presence of sialic acid which is commonly associated with such complex oligosaccharides (12, 29). The oligosaccharide components presently observed are consistent with the recent data by Bhattacharyya *et al.* (15) who demonstrated N-linked mannose-rich oligosaccharides in proteins Mr = 36,000 from alveolar proteinosis samples.

The characteristics of migration of human glycoproteins A are similar to those previously described for this protein from the dog, rabbit, human, and rat (11–13, 16, 30). Peptide maps of the human glycoprotein A were also similar to but not identical to those obtained by our laboratory from rat and canine glycoprotein A (13; unpublished observations). Characteristics and compositions are also similar to those reported for glycoprotein A in the dog and rat (11, 12). The partial amino acid composition data presently reported are quite similar, but distinct from that reported by Bhattacharyya and Lynn (15) for the alveolar proteinosis glycoprotein, Mr = 36,000. Our present amino acid composition data, demonstrating high glycine content and the observation that a highly purified bacterial collagenase selectively reduced glycoprotein A_2 to peptides of Mr = 20,000–23,000 are consistent with the presence of a significant collagen-like amino acid sequence(s) in the molecule. Charge heterogeneity of the collagenase-resistant region suggests that much if not all of the glycosylation and sialic acid is present in a noncollagenous region of the molecule. The collagenase-resistant glycoprotein fragments no longer formed sulfhydryl-dependent aggregates suggesting that the collagen-like domain is required for the sulfhydryl-dependent oligomerization of the molecule. On the basis of composition and sequence information, Bhattacharyya and Lynn (15) suggested that the glycoprotein Mr = 36,000 was a fragment of larger glycoproteins Mr = 80,000 and 62,000 present in the alveolar proteinosis. In the present study, a number of larger proteins were readily identifiable as major components of the alveolar proteinosis material. However, peptide mapping of the

acidic proteins supports their origin as aggregates of glycoproteins A_1 and A_2 or their fragments. Larger molecular weight proteins contain no peptides which were not identifiable as major peptides in glycoprotein A_1 or A_2. Smaller forms of the molecules contained most but not all of the characteristic tryptic peptides seen in glycoprotein A_1 and A_2 suggesting their origin results from proteolysis of glycoproteins A.

The concept that glycoprotein A_1 (Mr = 28,000) is a precursor polypeptide to the other forms is further supported by recent studies of glycoprotein A_1 and A_2 synthesized by rat type II epithelial cells by this laboratory (13). Pulse-chase experiments performed with ^{35}S-methionine labeled type II epithelial cells in primary culture, resulted in immunoprecipitable proteins Mr = 26,000 which was observed early during labeling and proceeded to form the larger forms of glycoproteins A with continued incubation. Rat glycoprotein A_1 comigrated with this early immunoprecipitable form and comigrated with the primary translation product of rat poly A^+ mRNA immunoprecipitated with the antiglycoprotein A antisera (30). These rat studies and the present work with human surfactant strongly support the identity of glycoprotein A_1 as the precursor for the various glycosylated and oligomeric forms of glycoproteins A identified in normal surfactant as well as those present in greater abundance in alveolar proteinosis proteins.

Purification of the human glycoproteins A_1 and A_2 has been accomplished by isoelectric focusing, molecular sizing, and electroelution utilizing the acidic isoelectric point of the molecules as a major purification step. The present procedure is fast and can readily be adapted to a preparative level under nondenaturing conditions. Such purification procedure therefore will likely make readily available significant quantities of undenatured glycoproteins A for further analysis of its structure and function.

Surfactant contains a variety of serum and nonserum proteins which may contribute to surfactant function. Glycoproteins A are a major surfactant specific protein and is synthesized by the type II epithelial cell lining the alveolar lining of the lung. Glycoproteins A are clearly developmentally regulated appearing only in late gestation in amniotic fluid and lung tissues (31). The physiological function of surfactant-associated glycoproteins A and the importance of its remarkable developmental expression by the respiratory epithelium remain to be more fully clarified.

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