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IN VITRO TRANSLATION OF RAT LUNG SURFACTANT APOPROTEIN mRNA

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SUMMARY Rat pulmonary surfactant contains apoproteins of molecular weights 38,000, 32,000, 26,000 and 10,000-12,000. The structural and metabolic interrelationships of these proteins are not clear as yet. In order to investigate if they arise from a single or multiple precursor protein(s), we isolated total poly(A) RNA from rat lungs, performed its translation in vitro in the presence of [^{35}S]-methionine and reticulocyte lysate, immunoprecipitated the translation products with anti-rat surfactant antibody, and analyzed them by SDS-polyacrylamide gel electrophoresis and autoradiography. A single translation product of molecular weight 35,000 was detected. Since the antibody used in the immunoprecipitation recognizes the 38,000, 32,000 and 26,000 dalton proteins, it is concluded that at least these three proteins arise from the 35,000 dalton precursor by post-translational modifications.

Pulmonary surfactant isolated from tracheobronchial washings of rat lungs contains proteins of molecular weights 38,000, 32,000, 26,000 and 10,000-12,000 (1-3). These proteins remain bound to surfactant lipids even after extensive density gradient centrifugations used during the isolation and purification of surfactant and are considered to be an integral part of it as apoproteins (4). Alveolar epithelial type II cells, which are involved in synthesis of pulmonary surfactant, are the source of one or more of the apoproteins (1,3). The role of the latter in surfactant function is under active investigation at the present time (5-8).

The 38,000, 32,000 and 26,000 dalton apoproteins are glycoproteins and show considerable charge heterogeneity upon isoelectric focusing (9). Much of the charge heterogeneity is eliminated if terminally-linked sialic acid residues are removed (9). The nature of the remaining heterogeneity remains unknown, and could be due either to other post-translational modifications of a common polypeptide, or possibly, to

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differences in the polypeptide structure. In order to gain further insight into the metabolic and structural interrelationships of the various apoproteins found in pulmonary surfactant, we investigated whether they arise from a single or multiple polypeptide(s). Towards this goal, we performed the translation of lung total poly(A)RNA in a reticulocyte lysate system. The translation products were immunoprecipitated with an anti-surfactant antibody, which recognizes the 38,000, 32,000 and 26,000 dalton apoproteins. The immunoprecipitated products were finally analysed by gel electrophoresis and autoradiography.

MATERIAL AND METHODS

Reagents: [^{35}S]-methionine (specific activity 900 Ci/mmole), [^{14}C]-labeled marker proteins and ENHANCE were obtained from New England Nuclear, Boston, U.S.A. A kit for performance of *in vitro* translation with reticulocyte lysate was purchased from Bethesda Research Laboratories, Bethesda, MD. U.S.A. Pansorbin (Staphylococcus aureus) was supplied by Calbiochem, La Jolla, Ca. U.S.A. Reagents for electrophoretic separation of proteins were purchased from LKB (Rockville, MD. U.S.A.) and BIORAD (Richmond, CA. U.S.A.). Oligo(dT)-cellulose was obtained from P-L Biochemicals, Milwaukee, Wis. U.S.A.

Animals: Adult Sprague Dawley rats were obtained from Zivic Miller, Allison Park, Pa. U.S.A. Male New Zealand rabbits, weighing 2-2.5 kg, were supplied by Edward McCarthy, Conneaut Lake, Pa. U.S.A. Guinea pigs of the Hartley strain, weighing 250 g, were obtained from Hilltop Laboratories, Scottsdale, Pa. U.S.A.

Preparation of anti-rat surfactant antibody: Lungs of adult rats were lavaged with 0.15 M NaCl containing 0.25 mM phenylmethylsulfonyl fluoride (1). Surfactant was isolated and purified from the lavage fluid (10) and the purified pulmonary surfactant was used to raise antisera in rabbits and guinea pigs (1). A gamma globulin fraction was obtained from each antiserum with ammonium sulfate precipitation and each antibody preparation was absorbed with rat serum. The resultant antibody preparations were tested by agar gel immunodiffusion and by an immunoassay for reactivity to surfactant and serum proteins of rat (1,8).

Isolation of lung total RNA: Rats were killed by decapitation and their lungs were rapidly removed, rinsed in cold saline and homogenized in five volumes of "AES buffer" (0.5 M sodium acetate, pH 5.0, 2 mM EDTA and 0.5% sodium dodecyl sulfate) and an equal volume of water-saturated redistilled phenol, and the homogenates were centrifuged at 3,000 g for 30 min. The aqueous phase was decanted, and the interphase was reextracted with additional 2.5 volumes of AES buffer. Following centrifugation, the two aqueous phases were pooled and again extracted with 4 volumes of freshly distilled and water-saturated phenol and 4 volumes of chloroform/isoamyl alcohol (24:1,v/v). After centrifugation at 3,100 g for 30 min., nucleic acids were precipitated at -20 C from the aqueous phase by addition of 1/10 volume of 2 M sodium acetate, pH 5.5, and 2 volumes of absolute ethanol. The precipitate was collected by centrifugation at 16,000 g for 10 min., dissolved in 1 mM EDTA, pH 7.0 at a concentration of 1 mg/ml, and heat denatured for 1 min. at 70 C.

Following rapid cooling to 4 C, KCl and Tris-HCl (pH 7.6) were added to the nucleic acid extract to a final concentration of 0.5 M and 0.01 M, respectively.

Isolation of poly(A)RNA: The samples of total RNA were applied at a flow rate of 0.5 ml/min to 10 g of oligo(dt)-cellulose, packed in a 1.6 x 15 cm column (11). The poly(A)RNA was eluted from the column with 0.01 M Tris-HCl, pH 7.6, and was precipitated at -20 C from the eluates by addition of potassium acetate (pH 5.5) to a concentration of 2%, and 2 volumes of ethanol. Poly(A)RNA was recovered by centrifugation at 25,000 g and -5 C for 20 min, and redissolved in sterile water and stored at -70 C in small aliquote.

Cell free translation of poly(A)RNA: Translation of lung poly(A)RNA was carried out with a reticulocyte lysate translation kit. In a typical experiment, mRNA was translated in 30 µl of the reaction mixture, containing 10 µCi of [^{35}S]-methionine for 60 min at 30 C.

Immunoprecipitation and polyacrylamide gel analysis: Following translation, the reaction mixtures were diluted with a solution (wash solution) containing 0.05 M phosphate, pH 8.0, 0.15 M NaCl, 2 mM benzamidine, 2 mM methionine, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, pH 7.0, 1% bovine serum albumin and 1% Nonidet P-40. The gamma globulin fraction of a nonimmune rabbit serum was added to each reaction mixture. After 3 h incubation at room temperature, an excess of Pansorbin was added, and the incubation was continued for an additional 30 min. Pansorbin was removed by centrifugation, and the supernatants were further incubated with rabbit anti-rat surfactant antibody, first at room temperature for 2 h, and then at 4 C overnight. Pansorbin was again added to the reaction mixture, and the incubation continued for 30 min. The Pansorbin-bound antigen-antibody complex was removed by centrifugation, and was extensively rinsed with the wash solution. The washed Pansorbin pellet was suspended in sample buffer (0.125 M Tris-Hcl, pH 6.8, 2% SDS, 10% glycerol and 50 mM dithiothreitol), and was heated in a boiling water bath for 5 min. Pansorbin was removed by centrifugation, and the supernatant loaded on 10% polyacrylamide slab gel. These gels were prepared and run according to Laemmli (12). The gels were treated with ENHANCE prior to fluorography. The gels were dried and subjected to autoradiography with Kodak XR x-ray film.

Immunoblotting: Proteins separated by SDS-polyacrylamide gel electrophoresis were transferred to nitrocellulose paper (13). Surfactant apoproteins were detected by the immunoperoxidase technique (1,3).

RESULTS AND DISCUSSION

The antisurfactant antibody used in the immunoprecipitation of the translation products recognizes the 38,000, 32,000 and 26,000 dalton apoproteins, as is evident from the immunoblot in Figure 1. No reactivity was observed towards the 10,000-12,000 dalton surfactant apoprotein.

The autoradiogram in Figure 2 shows the translation products formed from the translation of endogenous mRNA (lane 1) and of total poly(A)RNA isolated from rat lungs (lanes 2-4). Of the large number of peptides synthesized from the translation of lung poly(A)RNA (lane 2), one peptide of molecular weight

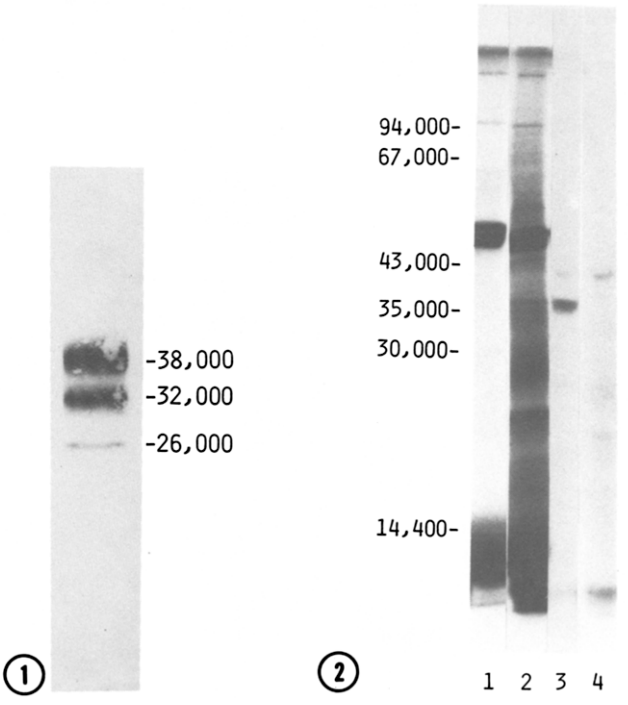

Figure 1. Immunoblot of rat surfactant proteins. After treatment with dithiothreitol, surfactant proteins were separated by SDS-polyacrylamide gel electrophoresis. The proteins were transfered from the gel to nitrocellulose paper. The transblot was subjected to immunoperoxidase staining in the presence of an antibody, which recognizes the 38,000, 32,000 and 26,000 dalton apoproteins. Note the reactivity of only the 38,000, 32,000 and 26,000 dalton proteins. No reactivity was observed with the 10,000-12,000 dalton protein (located near the bottom of the immunoblot).

Figure 2. Autoradiogram of the products synthesized by the translation of endogenous mRNA (lane 1) and total poly(A)RNA isolated from rat lungs (lanes 2-4). The immunoprecipitation of the translation products was performed in the presence of: an anti-rat surfactant antibody (lane 3); and the gamma globulin fraction of a nonimmune rabbit serum (lane 4). Of the large number of peptides synthesized by the translation of lung poly(A)RNA (lane 2), a 35,000 dalton peptide (lane 3) is specifically immuoprecipitated by the antisurfactant antibody.

35,000 is detected in the specimen precipitated by the antisurfactant antibody (lane 3). This peptide is not seen when a gamma globulin fractions of nonimmune rabbit serum was used for the immunoprecipitation (lane 4).

These results show that the 38,000, 32,000 and 26,000 proteins of rat surfactant are derived from a common peptide of molecular weight 35,000.

A number of our earlier observations are compatible with the synthesis of a common precursor for the three surfactant apoproteins, and their post-translational modification. It was earlier reported that the 38,000 and

32,000 dalton apoproteins release peptides of similar sizes after partial proteolysis (2), indicating extensive homology between the two proteins and their origin from a common precursor protein. The relationship of the 26,000 dalton protein to the other two proteins was not clear from the results of partial proteolysis (2). However, the fact that this protein is only detectable in tubular myelin and not in the lamellar bodies indicates that 26,000 dalton protein is derived extracellularly from the 38,000/32,000 dalton proteins (3).

Lynn (14) has suggested that the surfactant apoprotein of molecular weight 36,000 is one of several proteolytic fragments of alveolyn (molecular weight, 250,000), a protein reported to be present in alveolar secretions and term amniotic fluid (15). The present finding of a primary translation product of molecular weight 35,000, and not of 250,000, does not support this suggestion.

We were unable to identify the precursor of the 10,000-12,000 dalton protein. If the 10,000-12,000 dalton protein is structurally related to the 26,000-38,000 dalton proteins, as is suggested from the work of King et al (16), then the 35,000 dalton precursor would be common to all the apoproteins. On the other hand, the 10,000-12,000 dalton apoprotein may be structurally unrelated to the 26,000-38,000 dalton surfactant apoproteins and could be derived from another precursor. The antiserum used in the immunoprecipitations was raised against surfactant containing also the 10,000-12,000 dalton protein. However, we have failed so far (2,3) to detect any reactivity of this antiserum to this protein, as demonstrated also by the immunoblot in Figure 1. The nonreactivity may be due to masking of the antigenic sites by tightly bound surfactant lipids, and, thus, inaccessability of the antibody to the protein. Alternatively, the protein may be a poor immunogen and, as a result, no or only weak antibodies may have been raised against it.

A 35,000 dalton protein was detected in the amniotic fluids of 19 and 20 day pregnant rats by the immunoblot technique, using the anti-rat surfactant antibody (3). It is not yet clear if this protein simply represents an

intermediate in the conversion of the less glycosylated forms of surfactant apoproteins to the more glycosylated forms, or represents the nascent protein, lacking in significant post-translational modifications, due to undeveloped activities of the enzymes involved in the fetal lung. Future work is required to determine if the post-translational modifications are prerequisites for the incorporation of proteins into surfactant and for surfactant secretion as well as to determine the precise nature of the post-translational modifications and the sequence in which they occur.

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