Post-translational modification of the major human surfactant-associated proteins

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The major protein in human pulmonary surfactant is a sialoglycoprotein of 32–36 kDa (PSP-A) that has been shown by translation of lung mRNA *in vitro* to be derived from precursor molecules of 29–31 kDa [Floros, Phelps & Jaesch (1985) J. Biol. Chem. 260, 495–500]. We show here that two-dimensional gel patterns of PSP-A similar to that of the primary translation products are obtained by incorporation of [³⁵S]methionine in the presence of tunicamycin or by *N*-glycanase digestion of the 32–36 kDa group. Additional gel patterns are also observed in which the isoelectric-point heterogeneity is similar to that of either tunicamycin-treated tissue or primary translation products, but with higher molecular masses. The gel patterns showing higher-molecular-mass components are obtained when terminal sialic acid addition is prevented by the incubation of lung tissue with monensin or when terminal sialic acids are digested from the fully processed protein with neuraminidase. The 32–36 kDa forms have been shown to contain [¹⁴C]mannose. Pulse-chase experiments indicate that the acidic isoforms in the protein group arise from basic isoforms that are detectable within 10 min.

INTRODUCTION

The principal protein associated with human pulmonary surfactant is a 32–36 kDa sialoglycoprotein. This protein, which is commonly called 'surfactant apoprotein A' or 'surfactant-associated protein A', has been characterized by several laboratories using material isolated from a variety of species (King & Martin, 1980; Sueishi & Benson, 1981; Ng *et al.*, 1983; Phelps *et al.*, 1984; Katyal & Singh, 1984; Whitsett *et al.*, 1985; Phelps & Taeusch, 1985). Pulmonary surfactant-associated protein A (PSP-A) is thought to be involved in the assembly of pulmonary surfactant and in the rapid adsorption of surfactant to the air/liquid interface (King *et al.*, 1983; Hawgood *et al.*, 1985). Furthermore, levels of this protein in amniotic fluid reflect the degree of foetal-lung maturity (King *et al.*, 1975; Shelley *et al.*, 1982; Katyal & Singh, 1983; Chida *et al.*, 1985).

Recently, our laboratory and others have described the synthesis of PSP-A by human lung tissue and identified the primary translation products of this protein (Floros *et al.*, 1985; Whitsett *et al.*, 1985). In the present study we have extended these observations by examining the time course of synthesis and post-translational processing of PSP-A in human lung tissue. We have also correlated various steps in the well-described *N*-linked-oligosaccharide processing pathway with distinctive two-dimensional gel-electrophoretic patterns. This has been achieved in part by using various metabolic inhibitors and enzymes. We have also used different radiolabelled precursor molecules in conjunction with both continuous-label and pulse-chase-labelling protocols to examine the post-translational processing of PSP-A.

METHODS AND MATERIALS

Human lung tissue was obtained from the grossly normal margins of resected lung tumours and immediately

placed in cold culture medium. The tissue was minced in cold MEM (Gibco) and four to eight 1 mm³ pieces were then transferred to fresh medium and incubated at 37 °C in an atmosphere of CO₂/air (1:19). In order to deplete endogenous precursor levels, the tissue was incubated in either methionine-free or glucose-free medium for 2 h before the addition of radiolabelled precursors. In experiments with tunicamycin or monensin, the inhibitors were present during the pre-incubation. Tunicamycin (Calbiochem) was used at a final concentration of 2 µg/ml and monensin (Sigma) at a final concentration of 1 µM. For continuous-labelling studies, [³⁵S]methionine (sp. radioactivity 1200 Ci/mmol; Amersham International) labelling was performed for periods ranging from 30 min to 24 h. The four to eight tissue pieces were transferred from the depleted medium to 400 µl of medium containing [³⁵S]methionine at a concentration of 500 µCi/ml. Both the pulse-chase and continuous-label experiments involved incubation of the tissue pieces in 100 µl of methionine-free medium containing 2 mCi of [³⁵S]methionine/ml for a 10 min labelling period. In the pulse-chase experiments the labelling period was followed by rinsing the tissue in fresh MEM and continued incubation in 1 ml of 'chase' medium containing a 50-fold excess of unlabelled methionine. Labelling with [¹⁴C]mannose (Amersham International; sp. radioactivity 300 mCi/mmol) was done in 400 µl of glucose-free MEM at a [¹⁴C]mannose concentration of 100 µCi/ml. Incubations with [¹⁴C]glucosamine (Amersham International; sp. radioactivity 52 mCi/mmol) and [¹⁴C]-fucose (Amersham International; sp. radioactivity 55 mCi/mmol) were also performed in glucose-free medium at concentrations of 10 µCi/ml and 20 µCi/ml respectively. Neuraminidase Type X (*Clostridium perfringens*) was obtained from Sigma. Immunoprecipitated PSP-A from four to eight pieces of tissue was digested with 3 units of neuraminidase in 500 µl of 0.1 M-sodium acetate, pH 5.0, at 37 °C for 18 h. *N*-Glycanase

Abbreviations used: PSP-A, pulmonary surfactant-associated protein A; MEM, minimum essential medium.

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Fig. 1. Comparison of PSP-A labelled with [^{35}S]methionine and [^{14}C]mannose

Lung tissue was labelled for 6 h with [^{35}S]methionine (a) and [^{14}C]mannose (b). The tissue was then lysed and processed as usual. (a) Represents 3-day fluorographic exposure and (b) a 10-week exposure. Molecular masses (in kDa) of two marker proteins are given on the left (carbonic anhydrase, 29 kDa, and glyceraldehyde-3-phosphate dehydrogenase, 36 kDa), and acidic and basic ends of the isoelectric-focusing (I.e.f.) dimension are indicated.

(peptide:*N*-glycosidase F; Genzyme, Boston, MA, U.S.A.) digestions were performed on immunoprecipitated proteins from four to eight pieces of tissue. The protein mixture was suspended in 50 mM-sodium phosphate (pH 8.7)/10 mM-EDTA/0.5% SDS/1.0% Nonidet P40/0.1 M-2-mercaptoethanol and was placed in boiling water for 3 min to denature the proteins. Then the mixture was cooled and 1.5 units of *N*-glycanase were added. Digestions were at 30 °C for 18 h. The protein mixtures were then freeze-dried and subjected to electrophoresis.

Sample preparation and electrophoresis

After the desired labelling period, the tissue was removed from the medium, blotted dry and immediately frozen. Sample preparation, which we described in detail previously (Floros *et al.*, 1985), consisted of sonication of tissue fragments after the addition of 0.1 mM-phenylmethanesulphonyl fluoride, immunoprecipitation with a rabbit polyclonal antiserum to the surfactant-associated proteins, followed by two-dimensional gel electrophoresis (O'Farrell, 1975) and fluorography. Two-dimensional gel electrophoresis was required because of the marked heterogeneity of the protein. The antiserum was raised in a New Zealand White rabbit against the 35 kDa protein group (PSP-A) from alveolar-proteinosis lavage. The protein was purified by one-dimensional SDS/polyacrylamide-gel electrophoresis and electroelution. The specificity of this antiserum appears to be nearly identical with that of the antiserum we have previously characterized (Phelps *et al.*, 1984; Floros *et al.*, 1985).

Fig. 2. Treatment of lung tissue with enzymes and inhibitors

All panels show immunoprecipitated PSP-A. (a) Shows PSP-A from monensin-treated tissue. In (b), PSP-A was digested with neuraminidase. In (c), PSP-A is shown after [^{35}S]methionine labelling of tunicamycin-treated tissue (Floros *et al.*, 1985). (d) Shows PSP-A that was digested with *N*-glycanase after immunoprecipitation from [^{35}S]methionine-labelled lung tissue. The arrowhead in each panel indicates the same protein spot. The fluorographs were developed after a 3-day exposure period. The molecular mass of carbonic anhydrase (29 kDa) is indicated.

RESULTS

Sugar labelling

In order to demonstrate directly the incorporation of carbohydrate into PSP-A, lung tissue was labelled with [^{14}C]mannose for 6 h. Before labelling with [^{14}C]mannose the tissue was incubated for 2 h in glucose-free MEM in order to deplete the endogenous sugar pool and then transferred to fresh glucose-free MEM containing 100 μCi of [^{14}C]mannose/ml. The immunoprecipitated glycoproteins resulting from this incubation are shown in Fig. 1(b). Fig. 1(a) depicts a parallel incubation of tissue from the same lung with [^{35}S]methionine. The two patterns are nearly identical, although they differ in intensity. This observation directly confirms that the forms of PSP-A with M_r values of 32 000 or more are indeed glycosylated. However, the autoradiograph from the [^{14}C]mannose experiment (Fig. 1b) resulted from a 10-week exposure period versus a 3-day exposure period for the autoradiograph of the [^{35}S]methionine-labelled proteins (Fig. 1a). Similar results were also obtained with [^{14}C]fucose and [^{14}C]glucosamine (results not shown). In subsequent studies, [^{35}S]methionine-labelled proteins were examined.

Treatment with monensin and glycosidases

We used the carboxylic ionophore monensin to inhibit the processing of the *N*-glycosylated protein, including the addition of sialic acid by the Golgi apparatus (Tartakoff, 1983). Fig. 2(a) shows the immunoprecipitated proteins from monensin-treated tissue after 6 h of labelling with [^{35}S]methionine. A similar pattern was obtained when PSP-A is incubated with neuraminidase (Fig. 2b), removing terminal sialic acid residues. In the case of monensin, about five isoforms are present at about 32–34 kDa. After neuraminidase treatment, only four of the five isoforms are seen at 32–34 kDa, and increased amounts of 29 kDa forms are seen (cf. Figs. 2b and 2a). The reasons for this change are unclear, but could result from the presence of small amounts of other glycosidases in the neuraminidase preparation. The more acidic

Fig. 3. PSP-A after different labelling periods

Human lung tissue was incubated in the presence of [^{35}S]methionine for (a) 10 min, (b) 30 min, (c) 1 h, (d) 2 h, (e) 4 h and (f) 24 h. Tissue lysates were immunoprecipitated, subjected to electrophoresis and then autoradiography. The fluorographs were exposed for 3 days. Markers are as for Fig. 1.

isoforms of the 35 kDa (PSP-A) protein group, which presumably arise from the addition of sialic acid residues to the glycoprotein (Fig. 1), are absent in both cases (Figs. 2a and 2b).

Fig. 2(c) depicts the pattern of PSP-A when [^{35}S]methionine labelling is done in the presence of tunicamycin. In this case, PSP-A consists of five isoforms identical, with respect to their isoelectric points, with those seen in monensin-treated tissue, but with molecular masses about 3 kDa lower. We then compared PSP-A in tunicamycin-treated tissue to fully processed PSP-A after deglycosylation with *N*-glycanase (Fig. 2d) (Tarentino *et al.*, 1985). The tunicamycin results have been described in detail elsewhere (Floros *et al.*, 1985). An arrowhead has been used in Figs. 2(a)–2(d) to indicate the same 29 kDa isoform in each gel as a point of reference. The isoforms of the PSP-A treated with *N*-glycanase appear to be compressed slightly. This may result from either the conversion of asparagine into aspartic acid during hydrolysis (Technical Bulletin, Genzyme) or from the denaturation of the proteins before *N*-glycanase digestion. The isoelectric points of the spots in these patterns are similar in all four panels. The difference in molecular mass between PSP-A from the monensin-treated tissue (Fig. 2a) and that from tunicamycin-treated tissue (Fig. 2c) reflects the addition of asparagine-linked oligosaccharide to the protein. The pattern seen in Figs. 2(c) and 2(d) is similar to that obtained after the immunoprecipitation with PSP-A antiserum of proteins synthesized in a cell-free translation system in the presence of lung mRNA (Floros *et al.*, 1985).

Continuous-labelling experiments

We then examined the time course of PSP-A synthesis

by studying the two-dimensional electrophoretic characteristics of PSP-A. In this series of experiments, identical amounts of lung tissue were incubated with [^{35}S]methionine for 10 min, 30 min, 1 h, 2 h, 4 h and 24 h (Figs. 3a–3f respectively). In Fig. 3(a), which shows the immunoprecipitated protein resulting from a 10 min labelling period, a faint group of spots can be seen at 29 and 31 kDa (arrow). These spots correspond to the position of the non-glycosylated forms of PSP-A (Figs. 2c and 2d). A second set of spots, which is heavily labelled, has the same isoelectric points, but molecular masses that are about 3 kDa greater. These spots correspond to those seen in the monensin-treated tissue (Fig. 2a). The latter forms are visible as soon as PSP-A is detectable, since *N*-linked glycosylation is a co-translational event (Hubbard & Ivatt, 1981).

After a 30 min labelling period (Fig. 3b), a pattern similar to that in Fig. 3(a) is seen, although more radioactivity has been incorporated, particularly into the 32–34 kDa group. At 1 h (Fig. 3c), further incorporation of [^{35}S]methionine into the same protein spots that were visible in the first 10 min of labelling is seen. In addition, some higher-molecular-mass, more acidic, forms can also be seen. These more acidic forms are part of the charge train composing the secreted PSP-A (35 kDa) complex and become increasingly prominent after 2 and 4 h labelling periods (Figs. 3d and 3e). At 24 h (Fig. 3f), in addition to the acidic charge train, a significant amount of labelling is seen at the more basic end of the PSP-A complex. These basic forms range in molecular mass from 29 to 36 kDa and could be incompletely processed forms of PSP-A. It is also possible that these forms occur because of disruptions of normal processing resulting from the prolonged incubation period. In other experiments incubations were limited to 6 h.

In Fig. 4, PSP-A secreted by tissue after 4, 6 and 24 h of labelling are shown. In all of these cases, the same pattern of protein spots is seen, although there is a time-dependent increase in [^{35}S]methionine incorporation.

Pulse-chase experiments

The experiment represented by Fig. 5 employed a 10 min labelling period followed by 'chase' periods in MEM with a 50-fold excess of unlabelled methionine for 30 min, 1 h, 2 h, 4 h and 6 h. This protocol permits the protein molecules synthesized during the pulse period to be monitored without the continuous addition of newly synthesized labelled proteins to the pool. The 10 min pulse sample (Fig. 5a) is virtually identical with that seen in continuous-label experiments (Fig. 3a). After a 30 min chase period, further incorporation of [^{35}S]methionine into these spots is seen. By 1 h (Fig. 5c), considerable radioactivity is present in the acidic portion of the charge train.

After chase periods of 2, 4 and 6 h (Figs. 5d–5f respectively), there is a progressive shift of [^{35}S]methionine from the basic side of the PSP-A complex to the acidic side. These results demonstrate the precursor-product relationship of the basic to acidic isoforms in the 35 kDa (PSP-A) group. This shift could not be detected in the continuous-labelling experiments because the disappearance of the basic precursor forms (Figs. 3a and 5a) due to their conversion into more acidic isoforms (Figs. 5d–5f) was hidden by the constant synthesis of new precursor molecules.

Fig. 4. Comparison of secreted PSP-A after various periods

Lung tissue was labelled with [^{35}S]methionine for 4, 6 and 24 h and then the incubation medium was immunoprecipitated. (a), (b) and (c) depict the secreted proteins obtained respectively from these incubations. Fluorographic exposures were for 3 days. Markers are as for Fig. 1.

DISCUSSION

In a number of reports, post-translational modifications and processing of various glycoproteins have been studied. Many of these studies have examined the oligosaccharides and the changes they undergo as the protein is routed through the Golgi apparatus to its ultimate location within the cell or to extracellular destinations (Strous & Lodish, 1980; Dunphy *et al.*, 1981; Ledger *et al.*, 1983; Bartalena & Robbins, 1984; Brown & Simoni, 1984; Lodish & Kong, 1984; Yeo *et al.*, 1985). Important tools for these studies have been various glycosidases with well-defined substrate specificities, such as neuraminidase, endoglycosidase H and endoglycosidase F. The development of inhibitors such as tunicamycin, monensin and deoxynojirimycin, which can interfere specifically with various glycoprotein processing steps, has also aided greatly in the examination of post-translational processing. In many cases the effects of the enzymes have been assessed by examining changes in electrophoretic mobility on one-dimensional SDS/polyacrylamide gels.

We have used two-dimensional gel electrophoresis to analyse the steps involved in the post-translational modification of the major human surfactant-associated protein group (PSP-A) in conjunction with inhibitors, enzymes and metabolic labelling. This approach was

Fig. 5. Pulse-chase labelling of PSP-A

Human lung tissue was labelled with [^{35}S]methionine for 10 min (a) and then incubated in medium containing a 50-fold excess of unlabelled methionine for (b) 30 min, (c) 1 h, (d) 2 h, (e) 4 h and (f) 6 h. The tissue was then prepared, immunoprecipitated and subjected to electrophoresis as described in the text. The fluorographs were exposed for 3 days. Markers are as for Fig. 1.

necessary because of the isoelectric-point heterogeneity of both the precursors and the fully processed forms of the protein. (Phelps *et al.*, 1984; Floros *et al.*, 1985). We have been able to demonstrate directly the incorporation of radiolabelled sugars into surfactant-associated protein A (PSP-A) in human lung tissue by incubating the tissue with [^{14}C]mannose, [^{14}C]fucose and [^{14}C]glucosamine. The incorporation of [^{14}C]mannose into PSP-A (Fig. 1b) gives a pattern that is virtually identical with the one that results from [^{35}S]methionine labelling (Fig. 1a). This observation confirms that the forms of PSP-A with molecular masses of 33 kDa or more are indeed glycosylated. However, the fluorograph from the [^{14}C]mannose-labelled protein resulted from a 10-week exposure, and that from the ^{35}S -methionine labelled protein from 3 days. Labelling with [^{35}S]methionine, therefore, provides a far more realistic experimental approach, so we have used this amino acid for most of our studies correlating gel patterns with glycoprotein-processing steps.

Evidence that PSP-A was *N*-glycosylated came initially from the analysis of sugars cleaved by sequential enzyme treatment of the 35 kDa (PSP-A) protein in alveolar-proteinosis material (Bhattacharyya & Lynn, 1977). More recently the structure of the oligosaccharides has been further examined by n.m.r. (Munakata *et al.*, 1982; Bhattacharyya *et al.*, 1984). The demonstration by workers in our laboratory that tunicamycin-treated tissue contained newly synthesized PSP-A identical with that obtained by the translation of lung mRNA *in vitro* confirmed that this glycosylation is indeed an *N*-linked addition (Floros *et al.*, 1985). Further confirmation came

from the observation that endoglycosidase F treatment of the PSP-A group resulted in a two-dimensional gel pattern identical with that obtained from both translation *in vitro* and tunicamycin treatment (Whitsett *et al.*, 1985). In the present study we have used *N*-glycanase, which will cleave *N*-linked oligosaccharides, regardless of structure (Tarentino *et al.*, 1985). The gel pattern obtained after *N*-glycanase digestion (Fig. 2d) was similar to that resulting from tunicamycin treatment (Fig. 2c). We have also shown that, even after only 10 min of [³⁵S]methionine labelling (Figs. 3a and 5a), most of the immunoprecipitated PSP-A was of molecular mass 32–34 kDa, with the same isoelectric points as the non-glycosylated (Figs. 2c and 2d) forms. The rapid appearance of these forms rather than the 29 and 31 kDa primary translation products (Floros *et al.*, 1985) is to be expected with *N*-linked glycosylation, since it is a co-translational event (Hubbard & Ivatt, 1981).

The nature of the more acidic isoforms in the 35 kDa charge train was subsequently examined. Monensin, a carboxylic ionophore, has been shown to halt the progression of protein through the Golgi apparatus, presumably by interfering with the budding-off of vesicles from cisternae in the *cis* or medial Golgi (Tartakoff, 1983). This action prevents the protein from reaching the enzymes involved in terminal-sugar addition. When the human lung tissue was incubated with monensin, the resulting forms of PSP-A had isoelectric points identical with those of the unglycosylated proteins after tunicamycin treatment or translation *in vitro*, but molecular masses of 32–34 kDa. These forms correspond to those seen within 10 min of labelling (Fig. 3a).

These results complement previous studies from our laboratory and others (Ng *et al.*, 1983; Katyal & Singh, 1984; Phelps *et al.*, 1984) in which the 35 kDa protein group (PSP-A) from bronchoalveolar lavage was digested with neuraminidase, removing terminal sialic acid residues. Treatment of newly synthesized PSP-A with neuraminidase resulted in a gel pattern (Fig. 2b) similar to that seen in the monensin experiments, when terminal glycosylation is prevented (Fig. 2a). As the duration of the labelling or 'chase' periods increased, more of the acidic isoforms appear in the tissue. These forms were not present until after more than 30 min of labelling or 'chase' time. This time course is consistent with the kinetics of oligosaccharide processing and terminal-sugar addition in the Golgi apparatus (Hubbard & Ivatt, 1981). The increase in the acidic isoforms is mirrored in the pulse-chase experiments by a definite decrease in the more basic forms (Fig. 5). The two-dimensional gel patterns of secreted PSP-A were virtually identical at 4 and 24 h of labelling (Fig. 4). The PSP-A found secreted into the medium and in normal bronchoalveolar lavage is composed largely of the acidic isoforms. These experiments, taken together, indicate that, in lung tissue, there is a conversion of basic isoforms into acidic isoforms of PSP-A resulting from the addition of terminal sugars, including sialic acid, in the Golgi apparatus.

In conclusion, by using two-dimensional gel electrophoresis in conjunction with various metabolic labelling schemes, we have described some of the events in the post-translational processing of the major surfactant-associated glycoprotein (PSP-A). We have been able to demonstrate changes in the gel pattern accompanying

both *N*-linked glycosylation of the protein and terminal sialic acid addition to the glycoprotein, by using enzymes and metabolic inhibitors. It appears that most of the isoforms in the mature, secreted, PSP-A group are generated from the basic isoforms by the addition of terminal sugars, including sialic acid, to the *N*-linked oligosaccharides.

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