Sialomucin complex in the rat respiratory tract: a model for its role in epithelial protection

Richard R. McNEER, Daming HUANG, Nevis L. FREGIEN and Kermit L. CARRAWAY[1]

Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, FL 33101, U.S.A.

The pulmonary epithelium has a multitude of specialized functions, which depend on regulated growth and differentiation of several cell types. One such function is the synthesis and secretion of mucins, which offer the epithelium protection from and a means for removal of noxious environmental factors. Sialomucin complex (SMC) is a heterodimeric glycoprotein consisting of a mucin subunit (ASGP-1, ascites sialoglycoprotein-1) and a transmembrane protein (ASGP-2) with two epidermal-growth-factor-like domains. SMC was originally discovered in a highly metastatic rat mammary adenocarcinoma and has been implicated in metastasis and in the protection of the tumour cells from natural killer cells. It can also act as a ligand for the receptor tyrosine kinase 185^{neu}, suggesting that it is bifunctional as well as heterodimeric. SMC is expressed on the epithelium of rat conducting airways, with the highest levels occurring in the proximal trachea and progressively decreasing into the bronchioles. Airway SMC consists of two forms: a soluble form that lacks the C-terminal cytoplasmic and transmembrane domains and accounts for about 70% of the total, and a membrane-associated form that has the C-terminal domains. Immunocytochemical analyses show that SMC is predominantly present on the apical surfaces of the airway epithelium, but not in goblet cells. Soluble form can be removed from the trachea by rinsing, suggesting that a fraction of the protein is adsorbed to the apical surface. Based on these results, we propose a protective mechanism in which membrane and soluble forms of SMC are produced by airway luminal epithelial cells to provide a cell-associated epithelial glycoprotein barrier that also serves as an interface with flowing mucus. In support of this mechanism, we demonstrated secretion of soluble SMC by primary cultures of tracheal epithelial cells. This model suggests that SMC is a critical element in the protective barrier of the airway epithelium.

INTRODUCTION

The respiratory airway is in constant contact with noxious environmental agents, such as smoke, bacteria and viruses, but it is partially protected by a viscous layer of mucus that coats its surface epithelium. This mucus, which flows proximally and is eventually swallowed or expectorated, contains proteoglycans [1] and respiratory mucins [2–4] as its primary macromolecular constituents. Abnormal mucin production has been implicated in certain respiratory disease processes, including cystic fibrosis and chronic bronchitis [5,6]. Characterization of mucins has revealed that they are high-molecular-mass, long, rod-like molecules [7] with multiple tandem repeats rich in serine and/or threonine, which are heavily *O*-glycosylated [8]. Two types of mucins have been isolated and characterized, soluble and membrane [9–11]. The latter type was first isolated from ascites tumour cells and proposed to protect the tumour cells from recognition by the immune system [9,12]. The most-studied membrane mucin, MUC1, was originally recognized as a constituent of milk-fat-globule membranes [13–15]. It was the first mucin to be cloned and sequenced [16,17] and was subsequently shown to be present at apical surfaces of a number of epithelia [10], potentially providing a direct barrier to microbial invasion and other noxious agents [8,11,18]. These considerations raise the question of whether a membrane mucin may participate in protection of the airway epithelium. MUC1 is present in the airway, but little is known of its distribution, localization and role [19].

A second well-characterized membrane mucin, called sialomucin complex (SMC), was originally isolated and characterized as a heterodimeric glycoprotein complex from highly metastatic 13762 rat mammary adenocarcinoma ascites cells [20], in which the mucin subunit ASGP-1 (ascites sialoglycoprotein-1) is the major detectable glycoprotein [21]. Compared with MUC1, ASGP-1 has chemical characteristics more typical of a secreted mucin; a high molecular mass (> 500 kDa) and extensive *O*-linked oligosaccharides [21,22] in a region of 12 tandem repeats rich in serines and threonines [23]. However, ASGP-1 in the ascites cells is exclusively present in a complex with a transmembrane subunit, ASGP-2 [20]. In this respect it resembles the membrane mucin MUC1, which is also found as a heterodimeric complex containing a transmembrane subunit [24]. It has been proposed that SMC [9], like other membrane mucins, has a protective function at cell surfaces [12,25]. But SMC is unique among mucins described to date in that the ASGP-2 subunit possesses two epidermal-growth-factor-like domains which contain all of the consensus residues found in epidermal-growth-factor-like sequences of proteins known to possess growth-factor activity [26]. Moreover, ASGP-2 has been shown to act as a ligand for the receptor tyrosine kinase ErbB-2, when both are expressed in the same cell [27]. Thus, SMC may be bifunctional as well as heterodimeric.

Recently, the presence of SMC has been demonstrated in a number of normal epithelial tissues in which it is expressed in both non-membrane and transmembrane forms [28]. Among its potential biological roles, SMC is suggested to be important in intestinal development [28] and to serve as an anti-implantation factor in the uterus (R. McNeer, unpublished work). Previously, we have reported the expression of SMC in the airway [28,29].

Abbreviations used: SMC, sialomucin complex; ASGP, ascites sialoglycoprotein; mAb, monoclonal antibody; TE, tracheal epithelial; MEM, Dulbecco's modified Eagle's medium; RIPA, radioimmunoprecipitation assay.

[1] Author to whom correspondence should be addressed, at the Department of Cell Biology and Anatomy (R-124), University of Miami School of Medicine, Miami, FL 33101, U.S.A.

However, the results of the two studies, using PCR and immunoblotting, respectively, were somewhat variable, possibly depending on the site of the tissue that was analyzed. In the present study we analyzed SMC quantitatively in different regions of the airway to resolve the discrepancies. SMC is abundantly expressed in the upper airway, decreasing in a proximal to distal progression. It is present in both membrane and non-membrane forms, predominantly at the apical surface of airway lumen, indicating the possibility of a dual protective function of the epithelium as a mucin. Consistent with this role, both soluble and membrane mucins are produced by primary tracheal epithelial (TE) cells in culture.

EXPERIMENTAL

Antibodies

Polyclonal antisera against whole ASGP-2 and its C-terminal cytoplasmic domain have been described [28,30]. Monoclonal antibodies (mAbs) to ASGP-1 and ASGP-2 were produced as previously described [28].

Preparation of airway tissues

The following tissues were collected from adult Fischer 344 female rats: (i) lung not including the primary bronchus; (ii) bronchi with the lower half of the trachea; and (iii) upper half of the trachea up to but not including the cricoid cartilage. The tissues were snap-frozen in liquid N_2 and pulverized with a mortar and pestle. The frozen powder was stored at -80 °C until needed. Powdered tissues pooled from five rats were solubilized in RIPA (radioimmunoprecipitation assay) buffer (150 mM NaCl/1 % Nonidet P-40/0.5 % deoxycholate/0.1 % SDS/50 mM Tris/HCl, pH 8.0) with proteinase inhibitors (0.5 mM EDTA/1 mM PMSF/1 kiu (kallikrein-inhibitory units)/ml aprotinin/1 mM leupeptin/1 mM pepstatin) and homogenized with a probe sonicator. The lysate was centrifuged at 2000 ***g***, and the supernatant (2S) was used in immunoblot and immunoprecipitation analyses. Alternatively, powdered tissue was homogenized in a hypotonic lysis buffer containing Dulbecco's PBS without calcium with 4.8 % sucrose and proteinase inhibitors. Supernatant was collected after a 2000 ***g*** centrifugation and used for subcellular fractionation. For tracheal-rinse experiments, the trachea was promptly removed and briefly rinsed in $1 \times$ PBS. A needle (16 gauge) was carefully inserted in the proximal opening and 2 ml of $1 \times$ PBS with proteinase inhibitors was forced through the lumen and collected. This procedure was repeated four times on two tracheae, each time reusing the same 2 ml of solution.

Immunoprecipitation

For immunoprecipitation the RIPA-solublized 2S fraction (0.5–1 ml) was added to protein A–agarose with bound antiserum [28]. The samples were rotated overnight at 4 °C, centrifuged six times (15–45 min) in RIPA buffer, boiled in SDS-PAGE sample buffer to release bound protein, and analyzed by anti-ASGP-2 immunoblotting.

Subcellular fractionation

For cellular fractionation the detergent-free 2000 ***g*** supernatants were fractionated by consecutive centrifugations at 12000 ***g*** for 30 min and 100000 ***g*** for 90 min. The pellets from each centrifugation (12P and 100P) were diluted to the volume from which they were pelleted, and the pellets and the supernatant from the 100000 ***g*** centrifugation (100S) were analyzed by anti-ASGP-2 immunoblotting or by immunoprecipitation. In the latter case, bound protein was then analyzed by SDS-PAGE and anti-ASGP-2 immunoblotting.

Co-affinity purification of ASGP-1 and ASGP-2

Affinity columns were prepared using the Immunopure® Protein A IgG Orientation Kit (Pierce, Rockford, IL, U.S.A.) with anti-ASGP-2 or anti-C-pep polyclonal antisera and goat anti-mouse IgG1 (heavy-chain-specific) agarose (Sigma, St. Louis, MI, U.S.A.) or with anti-ASGP-2 mAb 13C4, following the supplied protocols. Seven female rats were killed, and their tracheae were removed and processed to a 15 ml fraction of RIPA-solubilized 2S supernatant (see above). The sample was first fractionated on the anti-C-pep column and eluted with acid (0.1 M glycine, pH 2.8) to obtain the membrane form of SMC. The flowthrough, containing the soluble form, was further purified on the anti-ASGP-2 column and eluted with acid. Triton X-100 was added to both eluents to a final concentration of 0.05 %. The eluents were neutralized with 1 M Tris (pH 9.5) and dialysed against water. Both products were then further purified on the 13C4 mAb column and eluted with acid (0.1 M glycine, pH 2.4), and Triton X-100 was added to a final concentration of 0.05 %. The products were neutralized (see above) and dialysed against water. Before concentration, 60 mg of SDS was added to each sample. A Speed Vac SC110 (Savant, Holbrook, NY, U.S.A.) was used to obtain pellets of each sample, which were then resuspended in 30 μl of sample buffer and analyzed by SDS-PAGE.

Immunoperoxidase staining

Adult respiratory tissue sections (9 μm) were prepared for immunocytochemistry, stained with mAb 4F12 and counterstained with haematoxylin and eosin as previously reported [28].

Primary rat tracheal epithelial-cell cultures

Excised rat trachea was rinsed with cold calcium-free Dulbecco's modified Eagle's medium (MEM) from the laryngeal end to the bronchial end, followed by the slow injection of 0.5 % protease type XIV (Sigma) in MEM into the lumen. After overnight digestion at 4 °C the lumen was flushed with 50 ml of cold MEM to remove the released cells. The cell suspension was centrifuged for 10 minutes at 150 ***g*** and washed once with MEM. Primary TE cell cultures were obtained by plating $1–2 \times 10^5$ dispersed cells into each well of six-well plates and incubating in MEM with or without 10 % fetal bovine serum at 37 °C with 5 % CO_2 in air. The cells and conditioned medium of primary TE cultures were harvested 48 h after plating. The cell pellets were washed twice with PBS and lysed in 1 ml RIPA buffer (20 mM Tris (pH 7.4)/1 % Triton X-100/0.1 % SDS/0.5 % sodium deoxycholate with protease inhibitors). Lysates were cleared by a 15 min centrifugation at 12000 ***g***. The cleared lysates and conditioned medium were immunoprecipitated with anti-ASGP2 polyclonal Ab. The immunoprecipitates were washed six times with RIPA buffer and analyzed by 6 % SDS-PAGE followed by immunoblotting with anti-ASGP2 mAb 4F12.

RESULTS

Expression of SMC in rat respiratory tissues

SMC has recently been shown to be expressed in a number of epithelial tissues. Using antibodies against the transmembrane subunit, its expression level was shown to be highest in lactating mammary gland and colon, and it was expressed to a lesser extent in the small intestine and uterus [28]. SMC expression in

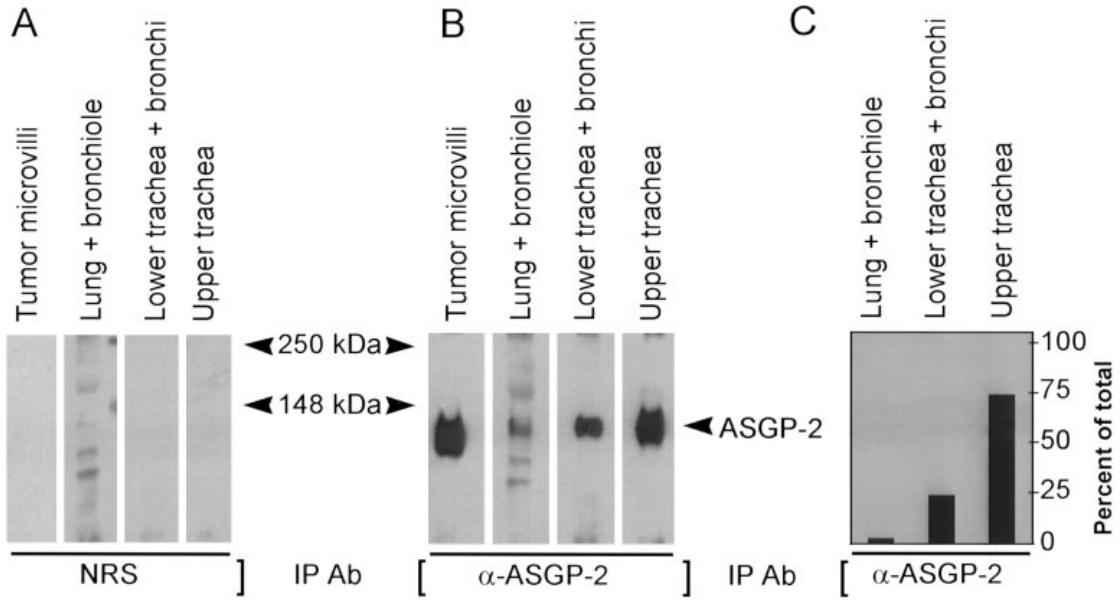

Figure 1 Expression of ASGP-2 (SMC) in rat respiratory tissues

Rat lung with bronchioles, bronchi and lower trachea, and upper trachea homogenates were removed from three Fischer rats, frozen in liquid N_2 and pulverized. RIPA buffer (4 ml) with proteinase inhibitors was added to the powdered samples, and the solutions were briefly sonicated. After a 2000 ***g*** spin, the supernates (2S) and a sample of Triton-solubilized tumour SMC were immunoprecipitated with normal rabbit serum (NRS) (A) or anti-ASGP-2 (B), and the imunoprecipitates were subjected to SDS-PAGE analysis and immunoblotting with 4F12 mAb (A, B). A lighter exposure of the blot used in B was analyzed by densitometry and quantified (C). The values are depicted as percentages of the total ASGP-2 protein detected from the respiratory tissues. IP, immunoprecipitation.

the proximal airway passages of the respiratory system is comparable to levels in the uterus and small intestine, but SMC cannot be detected by direct immunoblotting in the lung and distal airways (data not shown). Therefore, to increase the sensitivity of detection in the distal airways, immunoprecipitations were performed. The respiratory tract was divided into three sections: the lungs, the bronchi plus lower half of the trachea and the upper half of the trachea up to the cricoid cartilage. Polyclonal anti-ASGP-2 was used to immunoprecipitate ASGP-2 (SMC) from a 2S-supernate fraction of lysates of these tissues and from a Triton X-100-soluble fraction of 13762 ascites tumour microvilli that contains SMC [31]. Normal rabbit serum was used as a negative control for the immunoprecipitations. Immunoprecipitates were analyzed by immunoblotting with mAb anti-ASGP-2 4F12 (Figure 1). ASGP-2 (SMC) is expressed in all three fractions, with highest expression occurring in the upper trachea, and lower levels occurring in distal airways (Figure 1B). No ASGP-2 (SMC) was detected in normal-rabbit-serum immunoprecipitates (Figure 1A). To estimate the relative expression levels of ASGP-2 (SMC) in these tissues, the blot used in Figure 1(B) was exposed for a shorter time and subjected to densitometric analysis (Figure 1C). Clearly, most (about 75%) of the ASGP-2 (SMC) protein is found in the upper trachea, with expression diminishing in the lower airways.

Characterization of SMC forms in rat respiratory tissues

The function of SMC in particular tissues is undoubtedly dictated by the form(s) expressed. Both membrane and non-membrane

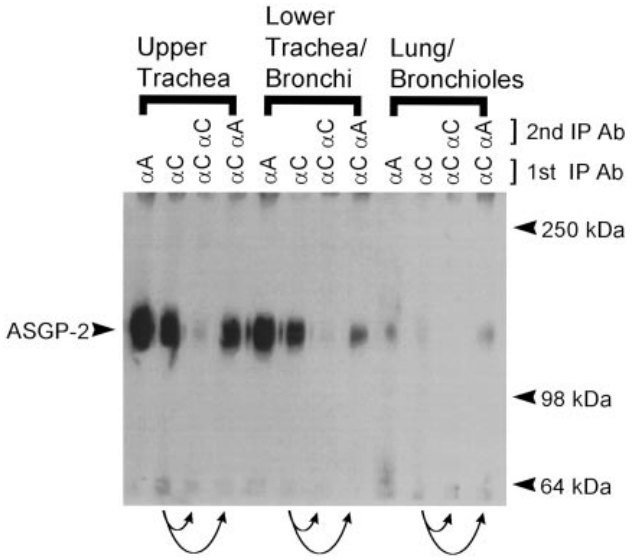

Figure 2 Detection of forms of ASGP-2 (SMC) by serial immunoprecipitation analysis of respiratory tissues

RIPA-solubilized samples of rat lung with bronchioles, bronchi plus lower trachea, and upper trachea were immunoprecipitated with either polyclonal anti-ASGP-2 (αA) or mAb anti-C-pep (αC). The supernatants from the anti-C-pep immunoprecipitates were divided into two equal aliquots and immunoprecipitated with anti-C-pep (αCαC) or anti-ASGP-2 (αCαA). These immunoprecipitates were subjected to SDS-PAGE analysis and immunoblotting with 4F12 mAb. IP, immunoprecipitation.

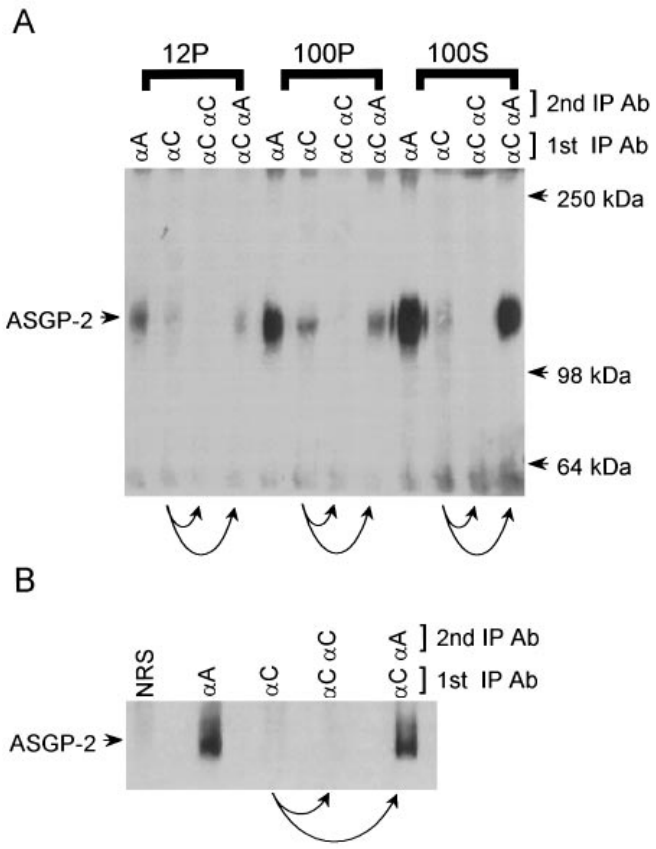

Figure 3 Fractionation and serial immunoprecipitation analysis of ASGP-2 (SMC) protein in trachea

The tracheae from three rats were removed, frozen in liquid N_2 and pulverized. Detergent-free buffer (PBS with proteinase inhibitors added) was added to the powder, and the mixture was briefly sonicated and fractionated to give 12P, 100P and 100S fractions, which were immunoprecipitated with anti-ASGP-2 (αA) or anti-C-pep (αC). The supernatants from the anti-C-pep immunoprecipitates were divided into two equal aliquots and immunoprecipitated with anti-C-pep (αCαC) or anti-ASGP-2 (αCαA). These immunoprecipitates were subjected to SDS-PAGE analysis and immunoblotting with 4F12 mAb (A). Tracheal rinses were performed briefly with $1 \times$ PBS, as described under Experimental. The rinse was subjected to serial immunoprecipitation with anti-ASGP-2 and anti-C-pep as described above, and pellets were analyzed by SDS-PAGE and immunoblotting with 4F12 mAb (B).

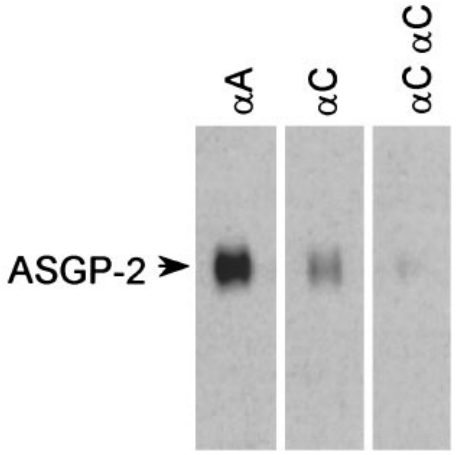

Figure 4 Analysis of different forms of ASGP-2 (SMC) protein in trachea

RIPA/PBS with proteinase inhibitors was used to solubilize frozen powder from three rat tracheae. After brief sonication, the solution was spun at 2000 ***g*** and the supernatant was immunoprecipitated with anti-ASGP-2 or anti-C-pep. The entire supernatant from the anti-C-pep immunoprecipitation was immunoprecipitated with anti-C-pep again. The resulting pellets (αA, αC and αCαC) were subjected to SDS-PAGE analysis and immunoblotting with 4F12 mAb.

forms of SMC have been observed in lactating mammary gland, but only the non-membrane form was present in the colon [28]. To analyze the form(s) of SMC in the rat respiratory system, the tissues were subjected to serial immunoprecipitation analysis (Figure 2) with anti-ASGP-2 and anti-C-pep polyclonal antisera, which recognize the C-terminal cytoplasmic domain of ASGP-2 (absent from the non-membrane form). In the upper trachea, lower trachea plus bronchi, and lung/bronchioles, ASGP-2 (SMC) was detected in both anti-ASGP-2 and anti-C-pep immunoprecipitates (αA and αC), indicating the presence of both membrane and non-membrane forms. To confirm the presence of the non-membrane form, the supernate from the anti-C-pep immunoprecipitation was divided and re-precipitated with the two antibodies. Significant ASGP-2 (SMC) was found only with polyclonal anti-ASGP-2 in the second immunoprecipitation (αCαA). These results show clearly that both forms of ASGP-2 are present in the respiratory tract, one which possesses the C-pep sequence and another which does not. In previous studies of other tissues the latter form has been shown to be soluble and secreted [28].

To determine whether the lack of the cytoplasmic domain correlates with tracheal ASGP-2 (SMC) behaviour, detergent-free trachea 2S fraction was fractionated by differential centrifugation into 12P, 100P and 100S fractions, which were subjected to immunoprecipitation analysis after solubilization in RIPA buffer (Figure 3). The αA, αC and αCαC bands for each fraction were quantified by densitometry (data not shown). Most of the ASGP-2 (SMC) immunoprecipitated with anti-ASGP-2 was found in the 100S fraction, and most of the ASGP-2 (SMC) immunoprecipitated with anti-C-pep was in the 100P fraction, confirming that the transmembrane form of ASGP-2 (SMC), which contains the cytoplasmic peptide sequence, is associated with the tracheal membrane fraction. In a related experiment, tracheae were rinsed with $1 \times$ PBS, and the rinse was subjected to serial immunoprecipitation analysis with anti-ASGP-2 and anti-C-pep (Figure 3B). Densitometric analysis showed no significant amounts of ASGP-2 (SMC) in anti-C-pep immunoprecipitates of tracheal rinses (data not shown). Thus, tracheal rinses contain exclusively non-membrane ASGP-2 (SMC) protein. The fact that most of the ASGP-2 (SMC) fractionates to the soluble fraction from whole trachea, implies that the non-membrane form must make up the majority of total ASGP-2 (SMC).

To estimate the relative amounts of each form of ASGP-2 (SMC) in respiratory trachea, RIPA-solubilized trachea 2S fraction was immunoprecipitated with anti-ASGP-2 or anti-C-pep. The entire supernatant from the anti-C-pep immunoprecipitate was collected and immunoprecipitated again with anti-C-pep. The immunoprecipitates (αA, αC and αC-αC) were then solubilized and subjected to SDS-PAGE and immunoblot analysis (Figure 4). According to this analysis, about 30% of ASGP-2 (SMC) in the trachea is in the form of a membrane-bound protein, whereas the other 70% is the non-membrane form.

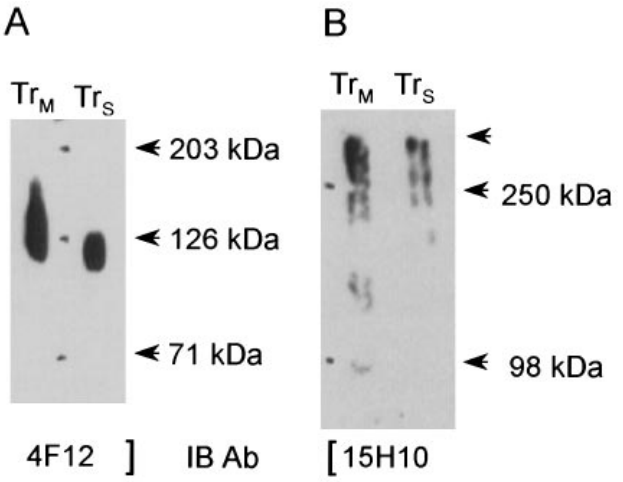

Figure 5 Co-affinity purification of ASGP-1 and ASGP-2 from trachea homogenate

Trachea homogenate was sequentially purified on anti-C-pep and anti-ASGP-2 affinity columns and analyzed by SDS-PAGE and immunoblotting with anti-ASGP-2 mAb 4F12 (A). The products were further purified on an anti-ASGP-2 (13C4 mAb) affinity column. The membrane and non-membrane forms were then analyzed by SDS-PAGE and immunoblotting with anti-ASGP-1 mAb 15H10 (B). The arrowhead shows the stacking/resolving gel interface in B.

Co-affinity purification of ASGP-1 and ASGP-2 from the rat airway

In the experiments described above, SMC was analyzed in airway samples using anti-ASGP-2 antibodies. However, in the airway a major concern is the protective role of SMC as a mucin. In all other systems examined, including rat mammary ascites cells, colon and lactating mammary gland [28], ASGP-2 has been found as a complex with the mucin subunit ASGP-1. To confirm that both components are present in the soluble and membrane forms of the SMC in the airway, trachea homogenate was fractionated by sequential immunoaffinity chromatography. First, RIPA-solubilized homogenate was run over an anti-C-pep affinity column to obtain the membrane form. The flowthrough was passed over an anti-ASGP-2 affinity column to obtain the non-membrane ASGP-2. Both eluents were analyzed by SDS-PAGE and immunoblotting with anti-ASGP-2 mAb 4F12 (Figure 5A). Each sample contained ASGP-2 with an apparent size of about 126 kDa, although the membrane form appeared to run slightly higher, as shown in Figure 5A. The affinity-purified forms were further purified over an anti-ASGP-2 mAb 13C4 affinity column. The eluted samples were analyzed by SDS-PAGE and immunoblotted with anti-ASGP-1 mAb 15H10 (Figure 5B). Both samples were positive for a high-molecular-mass protein (Figure 5B) that migrates as a smear at the interface of the resolving gel, as does ascites-tumour-cell ASGP-1 (data not shown). Considering the nature of the purification and the specificity of mAb 15H10 [28], these results indicate that ASGP-1 is present in a complex with both forms of ASGP-2 in the rat airway.

Localization of SMC in rat airway

To determine where ASGP-2 (SMC) is localized in rat respiratory tissues, cross-sections of rat airway were analyzed by immunoperoxidase labelling. Adjacent sections of each tissue were labelled with or without 4F12 mAb (Figures 6A and 6B). In trachea, which possesses solely conducting airway epithelium,

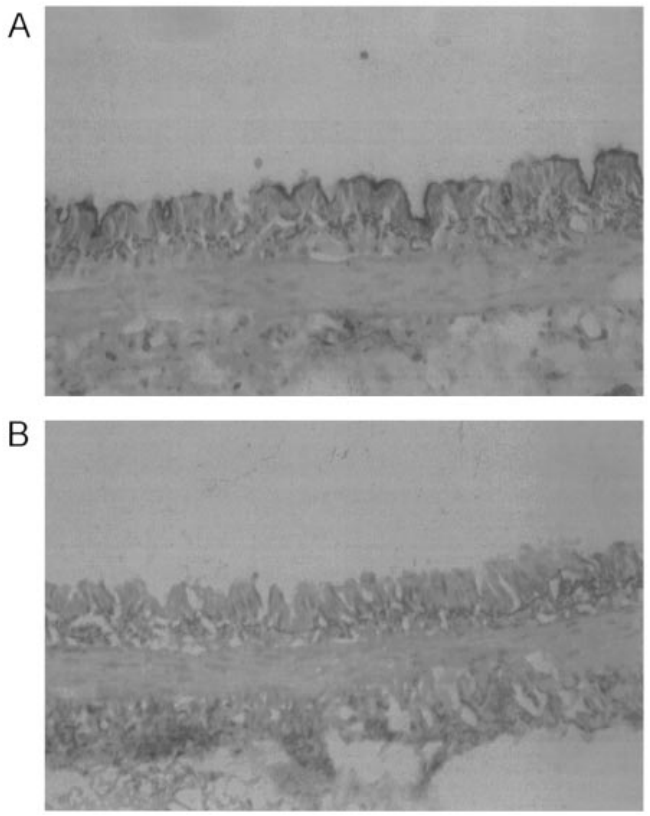

Figure 6 Immunoperoxidase localization of SMC in rat respiratory tissues

Consecutive sections (9 μm) of rat trachea were analyzed by immunoperoxidase labelling with (A) and without (B) anti-ASGP-2 4F12 mAb. Sections were counterstained with haematoxylin and eosin.

4F12 mAb labelling is localized to the apical surface of the airway lumen (Figure 6A). Significantly, all of the cells of the luminal cell surface are labelled, and there is no indication of SMC-containing secretory granules, such as those observed in the Paneth and goblet cells in the intestine ([28], R. McNeer and E. Rossi, unpublished work). No labelling is evident without primary antibody (Figure 6B). The bronchioles and alveoli of the lung were also analyzed (data not shown) but were negative for 4F12 staining. Hence, ASGP-2 (SMC) appears to be absent or poorly expressed on respiratory epithelium and bronchioles, but present on the epithelium of the more proximal conducting airway.

As described above, a substantial fraction of the SMC of airway tissue is the non-membrane form. Since SMC appears to be localized to the apical surface of the epithelium, the soluble form might be adsorbed to this surface and easily removed by washing. Therefore, tracheae were removed from adult rats, and the lumens were promptly rinsed with $1 \times$ PBS. The rinsed tracheae were homogenized and centrifuged to yield a 2S fraction. Both this fraction and the rinse were immunoprecipitated with anti-ASGP-2. Although a majority of ASGP-2 (SMC) remained in the rinsed trachea (data not shown), a significant amount of ASGP-2 (SMC) was immunoprecipitated from the tracheal-rinse sample (Figure 3C). These data confirm that SMC is present in the rat trachea as a soluble form as well as the transmembrane form found in the ascites tumour and suggest that the apical

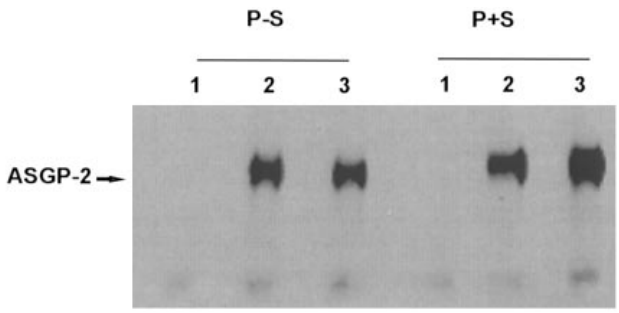

Figure 7 Detection of the expression of ASGP-2 (SMC) in primary tracheal epithelial cell-culture by immunoprecipitation

The conditioned media (lanes 2) from the primary TE culture on plastic with serum (P + S) and without serum (P — S) were immunoprecipitated with anti-ASGP2 polyclonal Ab or normal rabbit serum (lanes 1). The cell lysates (lanes 3) were immunoprecipitated with anti-ASGP2 polyclonal Ab. The immunoprecipitates were immunoblotted with anti-ASGP2 mAb 4F12.

staining of the epithelium (Figure 6) might represent both membrane and adsorbed soluble forms of the SMC.

Expression of soluble SMC by tracheal epithelial-cell cultures

The results above suggest a model in which both membrane and non-membrane forms of SMC are produced by epithelial cells of the luminal surface. To test this model, we established primary tracheal epithelial cell cultures and analyzed them for the production of soluble SMC (Figure 7). Both medium and cell-associated forms of SMC were found in the cultures, suggesting that the primary cell cultures are recapitulating the behaviour of the luminal cells.

DISCUSSION

Recent studies suggest that human respiratory tissues produce one of the most complex mixtures of mucins of all epithelia, including MUC1–MUC7 [19,32]. Characterization of respiratory secretions by biochemical and biophysical methods have shown that their predominant components are high-molecular-mass, disulphide-crosslinked mucins [3], which are the products of the *MUC5AC* and *MUC5B* genes [33,34]. However, these studies concentrate solely on secreted products and do not consider possible roles for the membrane mucins. MUC1 is the only transmembrane mucin previously shown to be present in the airway, and it is expressed apically on the airway epithelial cells [19,35]. Little is known about the function(s) of MUC1 in the respiratory tract, but experiments *in vitro* suggest that it influences cell–matrix and cell–cell interactions [25,36,37]. The primary function of MUC1 may be to contribute to formation of a membrane-associated barrier. Formation of this barrier depends on normal tissue architecture. When disrupted or not yet differentiated, epithelia have been shown to be less effective in preventing, among other things, virus-mediated gene transfer [38,39]. It has been proposed that the anti-adhesive properties of

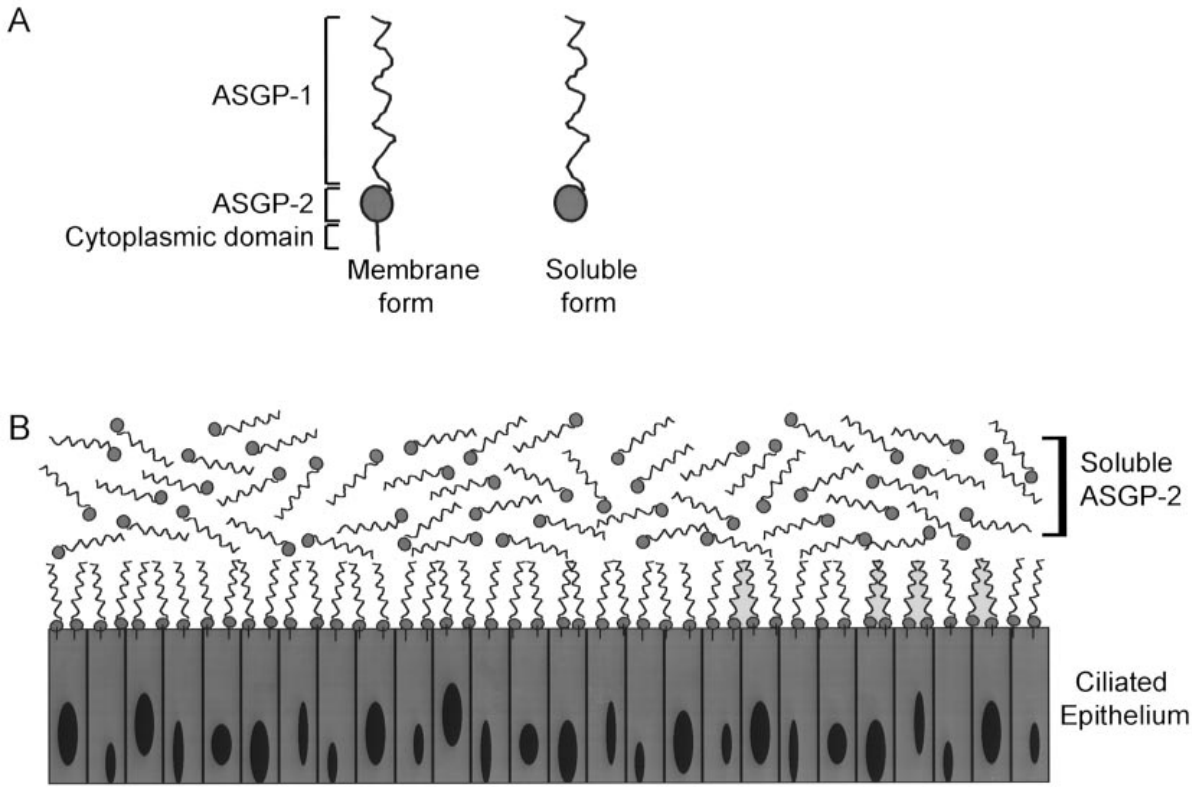

Figure 8 Model of SMC in the airway

Based on evidence reported here, the following model is proposed. The membrane form (A) of SMC is found apically expressed on the luminal airway epithelial cells (B). The nonmembrane form of SMC, which lacks the cytoplasmic domain (A), is found in the periciliary fluid which overlays the luminal surface of the trachea between the mucus gel (not shown) and the apical membrane with its attached cilia (not shown). The figure is meant to be representational, not to scale. ASGP-1 is $\approx$ 500 nm long [48]. Tracheal cilia are $\approx$ 6 μm long, which is also the approximated depth of the ciliary fluid layer and the distance from the epithelial cell surfaces to the mucin gel layer (see [42] for a further discussion and details).

MUC1 contribute to the formation of the polarized epithelium [40]. Thus, MUC1 may also be critical to keeping respiratory epithelial cells correctly oriented so that proper epithelial architecture can be attained and/or sustained.

Our results indicate that ASGP-1/ASGP-2 SMC can now be added to the group of respiratory mucins as the second transmembrane mucin. Since SMC exhibits similar properties to MUC1 as a membrane mucin, it can be expected to contribute to some of the same functions ascribed to MUC1. However, SMC has two additional properties which may play an important role in the epithelium of the respiratory tract. First, SMC is expressed as both transmembrane and non-membrane forms in the respiratory tract. Membrane SMC extending from the apical surface is expected to provide a protective or anti-adhesive function, as proposed for MUC1 and other membrane mucins for many types of epithelia [9,40]. However, a substantial fraction of the SMC (70%) associated with the trachea is the non-membrane form. This observation suggests a role for SMC in the epithelial protection provided by the mucus gel layer that lines the pulmonary epithelium [41]. The fluid layer covering the tracheal epithelium is customarily divided into two layers: the periciliary fluid layer (sol phase) and the mucus layer (gel phase) [42]. We suggest that soluble SMC is an important component of the periciliary fluid layer. Several observations are consistent with this model (see Figure 8). (i) SMC does not form crosslinked multimers [20], so it would not be expected to be incorporated into the mucin gel. (ii) By immunocytochemistry SMC is found at the luminal surface of the epithelium in a glycocalyx-like layer. (iii) The soluble form of SMC is loosely associated with the luminal surface of the trachea and can be removed by gentle washing. (iv) SMC was not observed in the submucosal glands or goblet cells of the trachea involved in the production of the gel-forming mucins. In contrast, SMC is readily observed in secretory granules of goblet cells in the colon. Therefore, we suggest that both forms of SMC are constitutively expressed by the luminal epithelial cells and transported to their apical cell surfaces where the transmembrane form is associated with the extracellular face of the plasma membrane and the soluble form is released into the pericellular fluid of the tracheal lumen. (v) Soluble SMC is produced by primary cultures of tracheal-surface epithelial cells [43]. Interestingly, previous reports have described a protease-releasable cell-surface mucin from these cells [44], which might be the transmembrane form of SMC. In this model the soluble SMC may aid in controlling the level and consistency of the pericellular fluid to facilitate optimal ciliary beating to move the mucus gel over the tracheal surface [42].

Second, SMC is unique among all mucins in that it has two epidermal-growth-factor-like domains in its ASGP-2 subunit. ASGP-2 and the receptor tyrosine kinase ErbB-2 are capable of being co-immunoprecipitated from 13762 ascites cells, in which they are both expressed [45], and from insect cells when they are co-expressed (K. L. Carraway III, E. Rossi and D. Huang, unpublished work). So far no other ligand for ErbB-2 has been described. Moreover, ASGP-2 appears to act as a nonclassical ligand via an intracrine, intramembrane mechanism. Thus, its role may be different from that of soluble receptor ligands. Most epithelial tissues (including the respiratory airways) express ErbB-2 [46,47]. These observations suggest a potential role for SMC in proliferative, repair and/or differentiative processes of the upper airway, which is particularly accessible and vulnerable to insult and injury.

We thank S. Price-Schiavi for assistance with the preparation of some of the figures and G. Conner and A. Wanner for their comments on the manuscript. This research was supported in part by Grant CA 52498 from the National Institutes of Health, a Career Investigator Award from the American Lung Association of Florida, a grant from Amgen, Inc., and by the Sylvester Comprehensive Cancer Center (Grant CA 14395).

REFERENCES

1 Bhaskar, K. R., O'Sullivan, D. D., Seltzer, J., Rossing, T. H., Drazen, J. M. and Reid, L. M. (1985) Exp. Lung Res. **9**, 289–308
2 Lamblin, G., Aubert, J. P., Perini, J. M., Klein, A., Porchet, N., Degand, P. and Roussel, P. (1992) Eur. Respir. J. **6**, 247–258
3 Sheehan, J. K., Thornton, D. J., Somerville, M. and Carlstedt, I. (1991) Am. Rev. Respir. Dis. **144**, S4–S9
4 Thornton, D. J., Devine, P. L., Hanski, C., Howard, M. and Sheehan, J. K. (1994) Am. J. Respir. Crit. Care Med. **150**, 823–832
5 Collins, F. S. (1992) Science **256**, 774–779
6 Jany, B. and Basbaum, C. B. (1991) Am. Rev. Respir. Dis. **144**, S38–S41
7 Jentoft, N. (1990) Trends Biochem. Sci. **15**, 291–294
8 Carraway, K. L. and Hull, S. R. (1991) Glycobiology **1**, 131–138
9 Carraway, K. L., Fregien, N., Carraway, C. A. C. and Carraway, III., K. L. (1992) J. Cell Sci. **103**, 299–307
10 Gendler, S. J. and Spicer, A. P. (1995) Annu. Rev. Physiol. **57**, 607–634
11 Carraway, K. L. and Fregien, N. (1995) Trends Glycosci. Glycotechnol. **7**, 31–44
12 Codington, J. F. and Frim, D. M. (1983) Biomembranes **11**, 207–258
13 Snow, L. D., Colton, D. G. and Carraway, K. L. (1977) Arch. Biochem. Biophys. **179**, 690–697
14 Ceriani, R. L., Peterson, J. A., Lee, J. Y., Moncada, R. and Blank, E. W. (1983) Somatic Cell Genet. **9**, 415–427
15 Patton, S., Gendler, S. J. and Spicer, A. P. (1995) Biochim. Biophys. Acta **1241**, 407–424
16 Gendler, S. J., Burchell, J. M., Duhig, T., Lamport, D., White, R., Parker, M. and Taylor-Papadimitriou, J. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 6060–6064
17 Siddiqui, J., Abe, M., Hayes, D., Shani, E., Yunis, E. and Kufe, D. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 2320–2323
18 Rose, M. C. (1992) Am. J. Physiol. **263**, L413–L429
19 Pemberton, L., Taylor-Papadimitriou, J. and Gendler, S. (1992) Biochem. Biophys. Res. Commun. **185**, 167–175
20 Sherblom, A. P. and Carraway, K. L. (1980) J. Biol. Chem. **255**, 12051–12059
21 Sherblom, A. P., Buck, R. L. and Carraway, K. L. (1980) J. Biol. Chem. **255**, 783–790
22 Hull, S. R., Laine, R. A., Kaizu, T., Rodriquez, I. and Carraway, K. L. (1984) J. Biol. Chem. **259**, 4866–4877
23 Wu, K., Fregien, N. and Carraway, K. L. (1994) J. Biol. Chem. **269**, 11950–11955
24 Ligtenberg, M. J. L., Kruijshaar, L., Buijs, F., van Meijer, M., Litvinov, S. V. and Hilkens, J. (1992) J. Biol. Chem. **267**, 6171–6177
25 Ligtenberg, M. J. L., Buijs, F., Vos, H. L. and Hilkens, J. (1992) Cancer Res. **52**, 2318–2324
26 Sheng, Z., Wu, K., Carraway, K. L. and Fregien, N. (1992) J. Biol. Chem. **267**, 16341–16346
27 Carraway, K. L., Carraway, C. A. C. and Carraway, III., K. L. (1997) J. Mammary Gland Biol. Neoplasia **2**, 187–198
28 Rossi, E. A., McNeer, R., Price-Schiavi, S. A., Komatsu, M., Van den Brande, J. M. H., Thompson, J. F., Carraway, C. A. C., Fregien, N. L. and Carraway, K. L. (1996) J. Biol. Chem. **271**, 33476–33485
29 Wu, K., Salas, P. J. I., Yee, L., Fregien, N. and Carraway, K. L. (1994) Oncogene **9**, 3139–3147
30 Sheng, Z., Hull, S. R. and Carraway, K. L. (1990) J. Biol. Chem. **265**, 8505–8510
31 Hull, S. R., Sheng, Z., Vanderpuye, O., David, C. and Carraway, K. L. (1990) Biochem. J. **265**, 121–129
32 Audie, J. P., Porchet, N., Copin, M. C., Gosselin, B. and Aubert, J. P. (1993) J. Histochem. Cytochem. **41**, 1479–1485
33 Thornton, D. J., Carlstedt, I., Howard, M., Devine, P. L., Price, M. R. and Sheehan, J. K. (1996) Biochem. J. **310**, 967–975
34 Thornton, D. J., Howard, M., Khan, N. and Sheehan, J. K. (1997) J. Biol. Chem. **272**, 9561–9566
35 Gendler, S. J., Lancaster, C. A., Taylor-Papadimitriou, J., Duhig, T., Peat, N., Burchell, J., Pemberton, L., Lalani, E.-N. and Wilson, D. (1990) J. Biol. Chem. **265**, 15286–15293
36 Wesseling, J., van der Valk, S. W., Vos, H. L., Sonnenberg, A. and Hilkens, J. (1995) J. Cell Biol. **129**, 255–265
37 Wesseling, J., van der Valk, S. W. and Hilkens, J. (1996) Mol. Biol. Cell **7**, 565–577
38 Goldman, M. J. and Wilson, J. M. (1995) J. Virol. **69**, 5951–5958
39 Pickles, R. J., Barker, P. M., Ye, H. and Boucher, R. C. (1996) Hum. Gene Ther. **7**, 921–931
40 Hilkens, J., Ligtenberg, M. J. L., Vos, H. L. and Litvinov, S. V. (1992) Trends Biochem. Sci. **17**, 359–363

41 Litt, M. (1984) Ciba Found. Symp. **109**, 196–211
42 Wanner, A., Salathe, M. and O'Riordan, T. G. (1996) Am. J. Respir. Crit. Care Med. **154**, 1868–1902
43 Kim, K. C., Nassiri, J. and Brody, J. S. (1989) Am. J. Respir. Cell Mol. Biol. **1**, 137–143
44 Kim, K. C., Wasano, K., Niles, R. M., Schuster, J. E., Stone, P. J. and Brody, J. S. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 9304–9308
45 Carraway, C. A. C., Carvajal, M. E., Li, Y. and Carraway, K. L. (1993) J. Biol. Chem. **268**, 5582–5587
46 Kokai, Y., Cohen, J. A., Drebin, J. A. and Greene, M. I. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 8498–8501
47 Press, M. F., Cordon-Cardo, C. and Slamon, D. J. (1990) Oncogene **5**, 953–962
48 Komatsu, M., Carraway, C. A. C., Fregien, N. L. and Carraway, K. L. J. Biol. Chem., in the press

Received 4 September 1997/20 October 1997; accepted 4 November 1997