FIVE SPECIFIC surfactant proteins (SP), designated SP-A, SP-B, SP-C, and SP-D (14), have been identified within pulmonary surfactant (19). SP-A, the most abundant, is expressed in a subset of bronchiolar epithelial cells of the lung in all species studied (3, 10, 11, 28). In addition, a number of studies have also shown that SP-A is localized to the alveolar type II epithelial cells in the lung in all species studied (3, 10, 11, 28). Although initial studies appeared to show SP-A mRNA in human bronchiolar epithelial cells (17). SP-A (21) may be more important than its role in surfactant metabolism (12).

Studies at both the protein and mRNA level have shown that SP-A is localized to the alveolar type II epithelial cells of the lung in all species studied (3, 10, 11, 28). In addition, a number of studies have also shown that SP-A is expressed in a subset of bronchiolar epithelial cells of the lung in all species studied (3, 10, 11, 28). Although initial studies appeared to show SP-A mRNA in human bronchiolar epithelial cells (17).

Synthesis of SP and surfactant phospholipids are developmentally regulated for the preparation for pulmonary function after birth (16). Insufficient development of the pulmonary surfactant system is one of the major pathogenic determinants of respiratory distress syndrome (RDS) found in premature neonates (4). To facilitate a better understanding of lung development and RDS in preterm babies, we developed a guinea pig model of prematurity (9). Lung development in the guinea pig is more advanced than in rodents, and these animals can survive premature delivery (8, 9). Using this model, we reported that, during the final 25% of gestation, there is a steady increase in pulmonary phospholipid concentration. Coincidently, the relative contribution of disaturated phospholipid species, the major active lipid of surfactant, is also increased (8). To more fully address the changes in SP-A gene expression in this model, we have now cloned a full-length guinea pig SP-A cDNA. Unexpectedly, initial studies performed with this probe in adolescent animals have revealed that the cellular distribution of SP-A mRNA is unique to the guinea pig, with expression being found only in the alveolar epithelial cells and not in cells of the bronchiolar epithelium.

MATERIALS AND METHODS

Preparation of guinea pig tissue samples. Tissues were collected from 60-day-old Hartley strain guinea pigs obtained from our own colony. Under anesthesia, animals were exsanguinated by abdominal aorta section, and the pulmonary circulation was perfused free of blood with 50 ml of sterilized 0.9% sodium chloride solution via the heart. The lungs were
then removed, washed immediately with ice-cold saline, dried with tissue paper, and snap-frozen in liquid nitrogen for subsequent RNA isolation. Heart, liver, spleen, and kidney tissues were also collected and prepared in a similar manner. When lung tissue was prepared for in situ hybridization, 10 ml of 4% formaldehyde in phosphate-buffered saline (pH 7.0) were infused slowly by syringe through the trachea into the lung tissue until the lung was fully expanded. The tissue was fixed in 4% formaldehyde for 24 h at room temperature and was embedded in paraffin.

Isolation of guinea pig SP-A cDNA clones. Poly(A)+ RNA was isolated from a newborn guinea pig lung by the FastTract system (Invitrogen, De Schelp, NL), and 5 µg were used to generate a directionally cloned oligo(dT) primed cDNA library...
in λZap (Stratagene, Cambridge, UK) according to the manufacturer’s instructions. The library was amplified one time, and random amplification of plaques suggested that the average insert size was 1.3 kb. Four hundred thousand plaque-forming units (pfu) were screened with a 32P-labeled, full-length human SP-A cDNA probe (26). Membranes were prehybridized with QuickHyb solution (Stratagene) for 1 h at 68°C, hybridized for 2 h at the same temperature, and washed in a final solution of 0.1 3 SSC (1 3 SSC = 150 mM sodium chloride and 15 mM sodium citrate) and 0.1% sodium dodecyl sulfate at 65°C for 30 min. Plaques that survived a secondary screen were recovered by excision rescue, and the phagmids were used directly for further analysis. Plasmid DNA was sequenced with the fmol polymerase chain reaction (PCR) sequencing kit (Promega, Southampton, UK), using specific external and internal primers. Compilation and computer analysis of DNA sequences were performed with the PC/Gene databases.

Fig. 2. Alignments of guinea pig SP-A with other SP-A proteins. Alignment of guinea pig SP-A (GP) protein with human SP-A1 (HU1), human SP-A2 (HU2), rat (RT), mouse (MO), rabbit (RB), baboon SP-A1 (B1), baboon SP-A2 (B2), and dog (DO) proteins. Asterisk (*) in guinea pig sequence indicates a gap, and minus (−) indicates conservation of the amino acid. Numbers refer to the number of the amino acids.
spleen tissues of adolescent guinea pigs as described previously (30). Characterization of tissue-specific expression of SP-A mRNA before in situ hybridization was performed by Northern blotting. Total RNA samples (10 µg/lane) were electrophoresed in 1% agarose denaturing gels, transferred to hybridization transfer membranes (Hybond-N; Amersham, Amersham, UK), and fixed with ultraviolet light. The blots were hybridized with a random primed 32P-labeled SP-A cDNA probe for 2 h at 65°C using QuickHyb solution (Stratogene) and were subjected to stringent washing as described previously (30). The X-ray films were exposed to the membranes at –70°C. To confirm the existence of total RNA in all lanes, the blot was stripped and subsequently was rehybridized with 32P-labeled guinea pig catalase probe, as previously described (30).

In situ hybridization. For in situ hybridization studies, a portion of the guinea pig SP-A cDNA corresponding to part of the 3′-untranslated region (nucleotides 1168–1524) was amplified by PCR using the following primers: forward primer 5′-GTC CCA TCA AGA TGT AG-3′; reverse primer 5′-AGG CCA TGG TT GGC TGG-3′. The 356-bp insert was directly cloned into pCRII (Invitrogen) and was sequenced to determine the orientation of the insert. The SP-B probe was generated from a full-length guinea pig cDNA (C. D. Bingle, S. Gowan, and H. T. Yuan, unpublished observations), and the resulting transcripts were subjected to a limited alkaline hydrolysis. Sense and antisense UTP-digoxigenin-labeled riboprobes were prepared using linearized plasmid cDNA as template, the appropriate RNA polymerase, and the conditions recommended in the Dig RNA labeling kit (Boehringer Mannheim, Sussex, UK). In situ hybridization was performed as described previously (1) with minor modifications using an Omnislide programmable in situ system (Hybaid, London, UK). Paraffin-embedded tissue sections (6 µm) were dewaxed by treatment with xylene, treated with proteinase K (250 µg/ml, 37°C for 40 min), and postfixed in 4% paraformaldehyde. The sections were then covered with 50 µl prehybridization mix (50% vol/vol formamide; 5× SSC, 1× Denhardt’s reagent, 0.1 mg/ml heat-denatured salmon sperm DNA, 10% wt/vol dextran sulfate) for 30 min at 50°C, followed by 50 µl of the same mixture containing the digoxigenin-labeled riboprobe. A glass coverslip was applied. Hybridization was allowed to occur at 50°C overnight. After hybridization, the sections were treated with ribonuclease A to remove unbound probe and then were washed with stringent conditions. The hybridized probe was detected by incubating the section with antidigoxigenin antibody conjugated to alkaline phosphatase, followed by the chromogen solution, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate toluidinum. The hybridization transfer membranes that were hybridized with 32P-labeled guinea pig catalase probe, as previously described (30).

RESULTS

Guinea pig SP-A is highly conserved with other species and is expressed specifically within lung tissue. Screening 400,000 pfu of a newborn guinea pig lung cDNA library with a full-length human SP-A cDNA probe resulted in the isolation of 10 individual overlapping cDNA clones. Sequencing and restriction digestion suggested that all were derived from the same mRNA species (results not shown). The longest of these clones was sequenced in both directions and was found to consist of 1,839 bp (Fig. 1) with a single open reading frame from position 55 to 798, coding for a protein of 248 amino acids. As expected, alignment of the nucleotide sequence with those of the two human and baboon cDNAs and the single rabbit, rat, mouse, and dog cDNAs showed that guinea pig SP-A has a high degree of sequence conservation (results not shown). Comparison of the derived amino acid sequence of guinea pig SP-A with those of the two human, two baboon, rabbit, rat, and mouse proteins confirmed the high degree of sequence similarity ranging between 68.8% (baboon SP-A2) and 74.1% (mouse SP-A; Fig. 2). Using the full-length SP-A cDNA as a probe on Northern blots of total RNA isolated from heart, kidney, liver, lung, and spleen, we confirmed that, as expected, guinea pig SP-A is expressed exclusively in lung tissue. Two specific transcripts were detected (Fig. 3, top, lanes 7 and 8) in the lung samples, with the most abundant transcript having the appropriate size for the full-length cDNA. It is unclear if the larger transcript represents a differentially spliced or polyadenylated mRNA species, and we have not detected any clear differential expression of the two transcripts under any conditions (results not shown).

Expression of guinea pig SP-A is confined to the alveolar epithelium. To identify the cellular sites of SP-A expression in guinea pig lung, we performed a series of in situ hybridization studies with sections of lungs from 60-day-old adolescent animals. SP-A mRNA distribution within a representative section of lung tissue is shown in Fig. 4A. SP-A expression is seen exclusively within the presumptive type II cells of the lung parenchyma and is not found in cells of the...
Fig. 4. Expression of SP-A in guinea pig lung is confined to cells of the alveolar epithelium. Tissue sections from adolescent guinea pig lungs were hybridized with antisense guinea pig SP-A (A), antisense guinea pig SP-B (B), or sense guinea pig SP-A (C) riboprobes as described in MATERIALS AND METHODS. Representative views clearly show expression of SP-A mRNA within the alveolar epithelium but not in the bronchiolar epithelial cells (arrowheads in A), whereas SP-B mRNA is found in both alveolar and bronchiolar epithelial cells (B). Sections hybridized with sense SP-A probes were negative for signal (C). Lowercase b in the lumen identifies the bronchiolar region in each view.
bronchiolar epithelium, blood vessels, or pulmonary interstitium (Fig. 4A). Repeated analysis of multiple sections from four individual animals obtained from different matings revealed identical results (results not shown). As this finding is at odds with the expression pattern seen in other species (3, 10, 11, 28) and to ensure that we could detect mRNA hybridization in bronchiolar epithelial cells in similar sections, we hybridized additional sections with a probe to guinea pig SP-B. In direct contrast to the expression pattern seen with SP-A, SP-B mRNA is readily detected in both cellular compartments (Fig. 4B). The specificity of the hybridization reactions is shown by the lack of signal in sections hybridized with a sense SP-A probe (Fig. 4C), and sections hybridized in the absence of probe were also consistently negative.

**DISCUSSION**

When compared with other species, guinea pig SP-A is highly conserved at both the nucleotide and the amino acid levels. The derived amino acid sequence of SP-A contains two distinct domains, the NH2-terminal one-third of the protein is collagen-like, whereas the COOH-terminal two-thirds has the properties of a lectin (6). Additionally, the NH2-terminal region contains a nonconserved signal peptide of 20 amino acids. The collagen-like domain is composed of 24 Gly-X-Y repeats (where Y is frequently proline; 13 from 24) and is interrupted one time between the 13th and 14th Gly-X-Y repeat with an additional proline at the 66th amino acid. SP-A from rat, mouse, rabbit, and dog also have the same number of Gly-Y-X repeats (20). Interestingly, all of these species appear to possess only a single SP-A gene product. In contrast, both in humans and baboons, which express two highly related SP-A genes (15, 20), there are only 23 Gly-X-Y repeats, with the substituted Gly after the 13th triplet being replaced in all four sequences with a Cys. A tentative conclusion from our sequence analysis is that the guinea pig possesses only a single SP-A gene. This conclusion is supported by the observation that all of the cDNAs identified in the library screen had identical sequence over the regions studied. Definitive proof awaits more detailed analysis by Southern blotting and genomic cloning. The NH2-terminal portion of the collagen domain, containing 13 Gly-X-Y repeats, is highly conserved compared with the second region. The four functionally important Cys residues found in the lectin domain (6) are also conserved in all species.

The lack of SP-A mRNA in the bronchiolar epithelium, as shown by the in situ hybridization studies, is unexpected in light of previous observations. It is generally considered that SP-A is expressed in type II cells (3, 10, 11, 17, 28). Studies in a number of species, including humans, rats, mice, rabbits, and baboons, have also shown significant expression of SP-A in the bronchiolar region of the lung (3, 10, 11, 28), although initial studies of SP-A mRNA expression in human bronchiolar epithelial cells proved negative (17). We are confident that our results represent a true lack of expression in these cells for the following reasons. First, in situ hybridization performed using similar tissue sections and a probe to guinea pig SP-B resulted in a marked level of expression within the bronchiolar epithelium. This finding suggests that the lack of signal in the sections hybridized with the SP-A was not due to technical problems associated with the detection of transcripts within this epithelium. Second, we have been unable to detect SP-A signal in multiple sections from different developmental stages (including days 35, 50, 60, and 65 of gestation (normal term = 68 days), term, and 10 days postpartum, results not shown), suggesting that the lack of expression seen is not the result of a temporal difference in SP-A transcript expression in adolescent guinea pig lung compared with the fetal lung but rather that it reflects a true lack of expression of SP-A mRNA within the bronchiolar epithelium. The importance of this difference in expression of SP-A in guinea pig lung compared with other species is unclear. It might be expected that any localized reduction of SP-A protein secretion into the bronchiolar lining fluid would be compensated for by the SP-A synthesized and secreted by the cells of the alveolar epithelium.

The lack of expression of SP-A in the bronchiolar epithelial cells of the guinea pig lung may, however, provide us with a useful model system for the study of the transcriptional regulation of SP genes. The expression of SP genes is regulated by a number of hormones and cytokines (15, 18), and the molecular pathways governing their temporal and spatial expression have begun to be elucidated (7, 27). Recently, a number of studies have implicated the lung and thyroid-enriched homeodomain transcription factor, thyroid transcription factor 1 (TITF-1), acting through binding sites located in the proximal promoter, as a potent activator of SP-A gene expression (2, 27). Studies of the cellular sites of expression of the SP genes and TITF-1 have shown that there is close overlap in expression profiles, with both proteins being expressed in cells of the bronchiolar epithelium as well as parenchymal alveolar type II cells (22, 31), suggesting that TITF-1 expression may be necessary but not sufficient for expression of SP-A. Our preliminary studies have identified TITF-1 expression in both cellular compartments of the guinea pig lung (results not shown), which further supports this contention. Additional studies are required to directly compare the temporal and spatial expression patterns of SP and TITF-1 gene expression within the guinea pig lung.

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Address for reprint requests: C. D. Bingle, Section of Respiratory Medicine, The University of Sheffield Medical School, Sheffield S10 2RX, UK.

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