An alternatively spliced surfactant protein B mRNA in normal human lung: disease implication

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We identified an alternatively-spliced surfactant protein B (SP-B) mRNA from normal human lung with a 12 nt deletion at the beginning of exon 8. This deletion causes a loss of four amino acids in the SP-B precursor protein. Sequence comparison of the 3′ splice sites reveals only one difference in the frequency of U/C in the 11 predominantly-pyrimidine nucleotide tract, 73% for the normal and 45% for the alternatively-spliced SP-B mRNA (77–99% for the consensus sequence). Analysis of SP-B mRNA in lung indicates that the abundance of the alternatively-spliced form is very low and varies among individuals. Although the relative abundance of the deletion form of SP-B mRNA remains constant among normal lungs, it is found with relatively higher abundance in the lungs of some individuals with diseases such as congenital alveolar proteinosis, respiratory distress syndrome, bronchopulmonary dysplasia, alveolar capillary dysplasia and hypophosphatasia. This observation points to the possibility that the alternative splicing is a potential regulatory mechanism of SP-B and may play a role in the pathogenesis of disease under certain circumstances.

Key words: bronchopulmonary dysplasia, congenital alveolar proteinosis, lung disease, respiratory distress syndrome, surfactant protein B expression.

INTRODUCTION

Human pulmonary surfactant protein B (SP-B) is essential for normal lung function [1–4]. Observations made from work with the SP-B knockout mouse [5] and the human congenital alveolar proteinosis (CAP) model [6,7] clearly indicate that life can not be supported in the absence of SP-B. Moreover, the heterozygous (+/−) SP-B mice have half the amount of SP-B compared with the homozygous (+/+) mice and exhibit small physiological abnormalities [8]. These include decreased compliance and increased air trapping. It is currently unknown whether these abnormalities are enhanced under certain pathogenic conditions.

The human SP-B gene consists of 11 exons [9] and is located on the short arm of chromosome 2 [10]. The entire coding region, from exon 1 to exon 10, encodes a 42 kDa precursor protein [11]. After post-translational processing the mature protein, 79 amino acids encoded by exons 6 and 7, is only 8 kDa [1]. SP-B mature protein is a hydrophobic peptide and is found in broncheoalveolar lavage. Genetic mutations of human SP-B have been associated with CAP [6,7]. SP-B allele association studies indicate that certain SP-B alleles are associated with human lung disease such as respiratory distress syndrome (RDS) [12], acute respiratory distress syndrome [13,14] and chronic obstructive pulmonary disease [15].

We have recently identified a CAP pedigree [16] where no known genetic mutation is detected and no intact SP-B mRNA is observed, leading to the hypothesis that aberrant mRNA splicing is involved in the mRNA deficiency observed in this pedigree. Moreover, splice-recognition-site mutations have been found to be a novel mechanism in the pathogenesis of disease [17,18]. It has been estimated that approx. 15% of disease-causing point mutations affect pre-mRNA splicing [19]. Recently, an alternatively-spliced SP-B mRNA was identified from mouse, rat, and rabbit, but not from human. This novel SP-B mRNA isoform was shown to contain a 69 nt deletion at the beginning of exon 7 [20]. In the present study, we describe an alternatively-spliced SP-B mRNA in human lung, which has a 12 nt deletion at the beginning of exon 8, and its abundance appears to increase in some disease lungs but not in normal lungs.

MATERIALS AND METHODS

Tissue acquisition

Normal human lung tissue was non-tumour lung adjacent to tumour in lobectomies from lung carcinomas. This tissue was deemed 'normal' on macroscopic examination by a pathologist [21]. CAP, alveolar capillary dysplasia (ACD), hypophosphatasia (HPP), and four RDS lung tissue were from lung biopsies from patients. Bronchopulmonary dysplasia (BPD) and two RDS lungs were from lung autopsies. These tissues were obtained with the approval of the Institutional Review Board on human investigations (J. F. and D. E. D.).

RNA isolation

Caesium chloride cushion method

Frozen human lung tissue was crushed to a powder and homogenized in buffer containing 4 M guanidinium thiocyanate. The homogenate was passed repeatedly through a needle to shear the DNA. Total RNA was isolated by centrifugation

Abbreviations used: ACD, alveolar capillary dysplasia; BPD, bronchopulmonary dysplasia; CAP, congenital alveolar proteinosis; HPP, hypophosphatasia; RDS, respiratory distress syndrome; RT, reverse transcription; SP-B, surfactant protein B.

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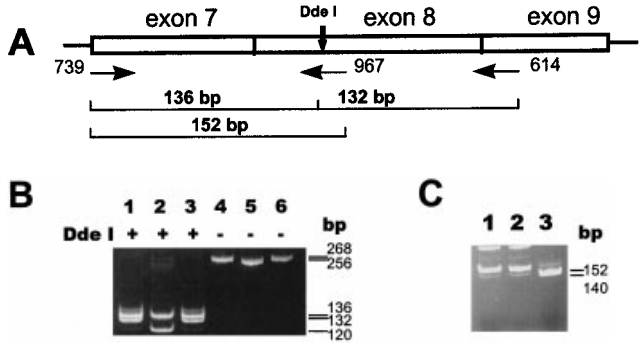

Figure 1 RT-PCR analysis of human SP-B mRNA

RT-RCR was carried out as described in the Materials and Methods section. The RT primer was poly(dT). PCR products or *Dde*I digested PCR products were separated on an 8% polyacrylamide gel and stained with ethidium bromide. The templates for PCR were adult lung RT cDNA, and phSPB-d, SP-B cDNA with the 12 nt deletion. (**A**) The locations of the *Dde*I recognition site and the PCR primers on SP-B cDNA. The predicted sizes of the PCR products and the *Dde*I fragments are indicated. (**B**) PCR products amplified with primer pair 739 and 614, and the corresponding fragments following *Dde*I digestion. Lanes 1, 3, 4 and 6, cDNA from normal lung mRNA from two individuals; lanes 2 and 5, phSPB-d cDNA clone. Size markers are shown to the right of the gel. (**C**) PCR products amplified with 739 and 967. Lanes 1 and 2, cDNA from normal lung mRNA from two individuals; lane 3, phSPB-d cDNA clone. Size markers are shown to the right of the gel.

through a caesium chloride cushion as described previously [21,22].

RNAzol B method

The powdered human lung tissue (200 mg) was homogenized with 2 ml of RNAzol B (Biotecx Laboratories, Inc., Friendswood, TX, U.S.A.) in a glass-Teflon homogenizer. After adding 0.2 ml of chloroform, the homogenate was shaken vigorously for 15 s and kept on ice for another 5 min, followed by centrifugation at 12000 ***g*** at 4 °C for 15 min. The colourless aqueous upper phase was collected and an equal volume of isopropanol was added to precipitate the total RNA. The RNA pellet was dissolved in RNase free water.

Reverse transcription (RT)-PCR

Reverse transcription was performed according to Karinch and Floros [21]. Briefly, 100 ng of RNA was incubated with 15 ng of poly(dT) primer at 70 °C for 10 min and cooled on ice. The RNA was reverse transcribed at 46 °C for 1 h in 25 μl of reaction mixture containing 1 mM of each dNTP, 100 mM dithiothreitol, 0.5 μl of RNAsin (Promega), 1 × 1st strand buffer and 150 units of MMLV-reverse transcriptase (Gibco BRL, Gaithersberg, MD, U.S.A.). The reaction was terminated by heating at 95 °C for 5 min.

The PCR protocol was as described previously [7]. One microlitre of the RT reaction was used as the cDNA template for PCR amplification. The PCR primers used in this study were primer 739, 967 and 614. Primer 739 was a sense primer located in exon 7, primer 967 was an antisense primer located in exon 8, and primer 614 was an antisense primer located in exon 9 (Figure 1A). The sequences are: primer 739: 5′TACTCCGTCATCCTGCTCGA3′, primer 967: 5′ATGCAGAGGTGGCACTCAGAG3′, and primer 614: 5′GCTGCTCCACAAATTGCTTG3′ [9,11]. The PCR reaction was in a 50 μl volume containing 1 μl of RT reaction, 1 μl of each primer (100 ng), 4 μl of dNTP (1.25 mM of each dNTP), 2.5 μl of 10 × buffer 1 and 2.5 μl of 10 × buffer 2 (Boehringer Mannheim), and 1 unit of AmpliTaq (Perkin–Elmer). The PCR profile consisted of a denaturation step at 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min; and a final extension step at 72 °C for 4 min. Five microlitres of PCR products, with or without *Dde*I digestion, were electrophoresed on an 8% (w/v) polyacylamide gel and stained with ethidium bromide. The intensity of stained DNA bands was quantified by densitometry.

DNA cloning and sequencing

RT-PCR products with primers 739 and 967 were separated on an 8% polyacrylamide gel. The gel slice containing the 152 and 140 bp bands (Figure 1C) were cut out and re-amplified with primers 739 and 967. The re-amplified PCR products were separated on a 2% (w/v) agarose gel. The two fragments were purified and cloned into T-vector (Promega). The plasmids were purified using a QIAGEN plasmid mini Kit and sequenced with the appropriate primers on an ABI Prism 377 DNA sequencer (Perkin–Elmer), using Big Dye terminator chemistry under standard conditions.

RESULTS AND DISCUSSION

An alternatively-spliced SP-B mRNA from human lung tissue

Recently, we found one SP-B cDNA clone, phSPB-d, with a deletion in exon 8. This clone is from the same cDNA library [23] as the clone 17. Clone 17 is a full length SP-B cDNA, and its sequence was published in 1987 [11]. To rule out the possibility that the deletion is caused by cDNA cloning, DNA subcloning or bacterial recombination, we analysed the deleted region of SP-B mRNA from lung tissue by RT-PCR. As indicated in Figure 1(A), the sense PCR primer 739 is located in exon 7 and antisense primer 614 is located in exon 9. The PCR product is a 268 bp fragment that consists of the entire exon 8 and parts of exon 7 and exon 9. Compared with phSPB-d (Figure 1B, lane 5), the PCR product from lung tissue is slightly longer (Figure 1B, lanes 4 and 6). This difference is much clearer when the PCR products are digested with *Dde*I. Lung RT-PCR products digested with *Dde*I result in two fragments of 136 and 132 bp (Figure 1B, lanes 1 and 3), while the phSPB-d plasmid DNA when digested with *Dde*I gives two fragments of 136 and 120 bp (Figure 1B, lane 2). Interestingly, there is a low intensity band, at the same position as the products of phSPB-d, in the human normal lung tissue (Figure 1B, lanes 1 and 3). To confirm this finding, antisense primer 967 and sense primer 739 (Figure 1A) are used to amplify, from human lung RNA, a 152 bp sequence flanking the 12 bp deletion. As expected, two fragments are amplified from normal lung tissue with a predominant band from the full length mRNA and a faint band from the alternatively-spliced smaller sized mRNA (Figure 1C, lanes 1 and 2). The smaller fragment is the same size as that observed when the phSPB-d cDNA is used as template (Figure 1C, lane 3).

From these data we conclude that the phSPB-d cDNA clone is from an alternatively-spliced human lung SP-B mRNA, and that this form of SP-B mRNA exists in human lung tissue at a detectable level.

Sequence comparison of two 3′ splice sites at the intron 7/exon 8 junction

The two PCR fragments amplified with primer pair 739/967 from normal human lung tissue, as shown in Figure 1(C), were isolated and further re-amplified with 739/967. The re-amplified PCR products were cloned and sequenced. The sequencing results together with sequences of the clone phSPB-d are shown

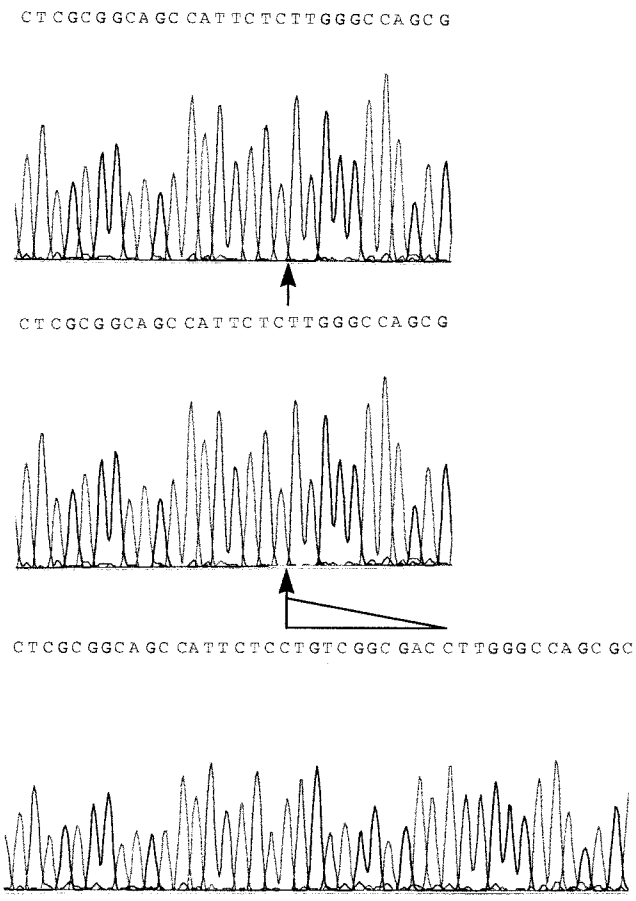

Figure 2 Sequence analysis of two RT-PCR fragments from normal lung SP-B mRNA and from the SP-B cDNA clone, phSPB-d

Top panel: SP-B cDNA clone phSPB-d. Middle panel: alternatively-spliced cDNA sequence (short form of SP-B mRNA). Bottom panel: normally-spliced SP-B cDNA (full length SP-B mRNA).

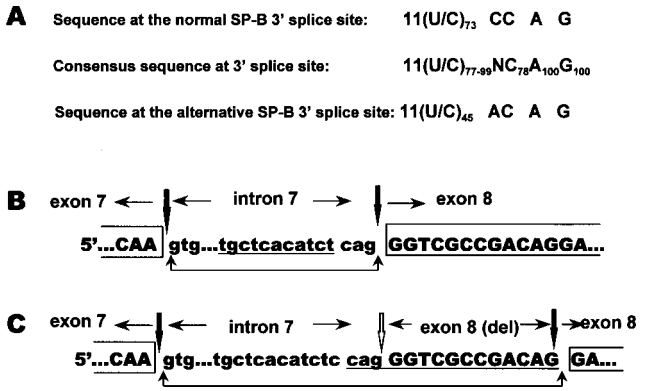

Figure 3 Sequence comparison of exon–intron junctions between the normal and the alternative 3′ splice site in the human *SP-B* gene

(**A**) The consensus sequence and the sequence of the normal and the alternative SP-B 3′ splice site. The subscript numbers indicate the frequency of the various nucleotides at the specified positions. The actual sequences of the normal (**B**) and the alternative (**C**) 3′ splice sites respectively. The 11 predominantly-pyridine nucleotide tract is underlined. The intron 7 in (**B**), before the 12 base deletion, and (**C**), after the deletion, are further noted with bent arrows below each sequence.

in Figure 2. The alternatively-spliced short form of SP-B mRNA has a 12 nt deletion. The deleted sequence is GGTCGCC-GACAG, located at the beginning of exon 8 (Figure 3).

The two 3′ splice sites in the intron 7/exon 8 junction are shown in Figure 3. When compared with the consensus sequences at the 3′ splice site [24], the only difference observed is in the frequency of U/C in the 11 nt pyrimidine-predominant tract. With respect to the remaining sequence, there is no difference among the alternative SP-B splice site, normal SP-B splice site, and the consensus sequence. In the normal splice site, the frequency of U/C in the 11 nt is predominantly a pyrimidine tract (73%), that is very close to 77–99% of the consensus sequence. However, in the alternative 3′ splice site the frequency of U/C is only 45%, much lower than that dictated by the consensus sequence, and may, in part, explain the low content of mRNA observed for the alternatively-spliced SP-B mRNA in human lung tissue (Figures 1B and 1C).

The 12 nt deletion in the alternatively-spliced SP-B mRNA leads to one nucleotide change in codon 286 (AGG to AGA) without change in the encoded amino acid (Arg), and a loss of four amino acids Ser(287)-Pro(288)-Thr(289)-Gly(290). In codon 286, A comes from exon 7 and GG comes from exon 8. PreproSP-B consists of 381 amino acids and the mature SP-B peptide consists of 79 amino acids that is flanked by 200 amino acids at the N-terminus and 102 amino acids at the C-terminus. The processing of SP-B has been studied in human lung explant culture [25]. The changes caused by the 12 nt deletion occur at the C-terminal portion of the preprotein. It has been demonstrated that the C-terminal region is not required for processing or sorting of SP-B *in vitro* [26]. In knockout mice the C-terminus plays a role in SP-C proprotein processing and the maintance of lamellar body size [27]. Whether SP-B has similar functions in human lung *in vivo* is currently unknown. Therefore, the impact of the loss of the four residues on the preproSP-B in health or disease is not clear.

Analysis of the alternatively-spliced SP-B mRNA in different human lung tissues

We analysed the alternatively-spliced short SP-B mRNA in normal lung tissues obtained from 16 adult individuals by RT-PCR. The alternatively-spliced short SP-B mRNA is detected in all adult lung tissues analysed, although the predominant form is the normally spliced full length SP-B mRNA. The ratio of the alternatively-spliced to the normally spliced SP-B mRNA is similar in the lung tissue from all 16 individuals. A portion of these results is shown in Figure 4(A). We then analysed the alternatively-spliced SP-B mRNA in lung tissue from patients with CAP. The two forms of SP-B mRNA are also found in these lung tissues. However, compared with the adult normal lung tissue, the ratio of the alternatively-spliced to the normally spliced SP-B mRNA appears to be slightly higher in these CAP lung tissues. Moreover, among the three CAP lung tissues some differences are observed (compare products in Figure 4B, lane 2 with those in lanes 3 and 4). Therefore, it is possible that quantitative and/or qualitative differences exist between the normal and the alternatively-spliced SP-B mRNA under certain circumstances.

To gain further insight into the presence of the alternatively-spliced SP-B mRNA in lung tissues from other diseases, we analysed the relative abundance of the alternatively-spliced SP-B mRNA in a total of 32 lung tissues from different individuals (15 from control and 17 from diseased lungs) using RT-PCR and

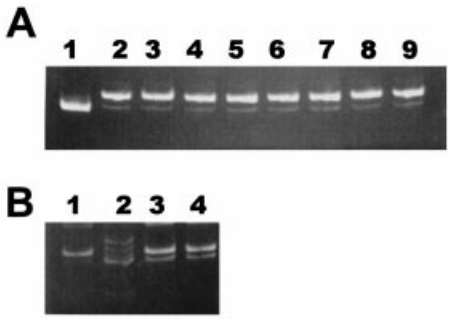

Figure 4 Expression of the deleted short SP-B mRNA in lung tissues from normal and CAP individuals

RT-PCR was carried out as described in the Materials and methods section. PCR primers used were 739 and 967, and the predicted PCR fragments are 152 bp and 140 bp for full length and the alternatively-spliced SP-B mRNA respectively. (**A**) PCR products from the phSPB-d cDNA clone (lane 1) and from the lung tissue of several individuals (lanes 2–9). (**B**) RT-PCR products from normal lung (lane 1) and from the lung of patients with CAP (lanes 2–4).

Table 1 Content of the alternatively-spliced SP-B mRNA in different human lung tissues

The content of the alternatively-spliced SP-B mRNA, determined from the RT-PCR products, is given as a percentage of the total SP-B mRNA.

Description	Number of individuals	Content of the alternatively-spliced SP-B mRNA (%)	Number of individuals with high content (> 25%)
Control	15	13.8–24.6	0
Lung disease			
CAP	5	19.0–61.8	3
RDS	6	15.9–76.7	3
BPD	4	19.7–88.2	3
ACD	1	28.2	1
HPP	1	26.5	1
Total	17	15.9–88.2	11

densitometric quantification of PCR products. The results are shown in Table 1. Because PCR is not designed for accurate quantitative analysis, the abundance of the alternatively-spliced SP-B mRNA is shown as a percentage of the total SP-B mRNA. In the 15 control lungs the alternatively-spliced SP-B content is 13.8–24.6%. In the 17 lungs with disease, CAP (5), RDS (6), BPD (4), ACD (1) and HPP (1), the content of the alternatively-spliced SP-B mRNA ranges from 15.9% (similar to normal) to as high as 88.2%. Using Fisher's exact t-test, the difference of the alternatively-spliced SP-B mRNA content between the 15 control lungs and the 17 pulmonary disease lungs is significant ($P = 0.0007$). A high content (more than 25%) of the alternatively-spliced SP-B mRNA is only found in a subgroup of patient lungs, three CAP, three RDS, three BPD, one ACD and one HPP. Out of the 17 patient lungs analysed, 65% of them showed a high content (> 25%) of the alternatively-spliced SP-B mRNA compared with 0% observed in the 15 control lungs.

Subsequently we pooled all the disease lungs into one disease group and compared this group with the control lungs by determining odds ratios, after dividing the disease and the control groups into two subgroups, low content (< 25%) and high content (> 25%) of the alternatively-spliced SP-B mRNA. In the disease group, the number of individuals with low content is six and that with high content is 11. According to this subdivision the 'low' subgroup of the control group consists of the 15 individuals and the 'high' subgroup of zero individuals. Following odds ratio analysis we observed a > 25.7-fold $[(14 \div 1)/(6 \div 11) \approx 25.7]$ likelihood of a patient having the high content of the alternatively-spliced SP-B mRNA than a normal individual. The odds ratio calculation is based on the assumption that at least one control individual has high content of the alternatively-spliced SP-B mRNA, otherwise the odds ratio would have been infinity, since 'zero' controls had high content.

Recently, Chi et al. [20] identified a novel alternatively-spliced SP-B mRNA in mouse, rat, and rabbit lung, with an abundance of approx. 30% of the total SP-B mRNA. This SP-B mRNA isoform contains a 69 nt deletion at the beginning of exon 7. This deletion form has not been found in human lung [20]. In the present study, we identified an alternatively-spliced SP-B mRNA in normal human lung tissue, which is formed by a 12 nt deletion at the beginning of exon 8. Of interest, and potential relevance, is our observation of an mRNA abnormality in CAP [16]. In these patients, the abnormal mRNA is split into two parts with a breakpoint between exon 7 and exon 8. Because all these SP-B mRNA aberrations occur in the region of exon 7–exon 8, or between Alu sequence 6 and Alu 8, it is tempting to speculate that Alu 6 and Alu 8 play a role in SP-B mRNA transcription or processing. Alu 6 and Alu 8 are located after exons 6 and 8 respectively, and are only 1600 nt apart and in reverse orientation from one another. With more than 80% of the two Alu sequences being complementary, the two Alu sequences could form a 250 bp double-stranded stem. This in turn would allow the 1600 nt to form a big loop with the 500 nt sequence containing exon 7–intron 7–exon 8 at the top of the loop. This structure can potentially occur in either the unwinded single-sense strand of SP-B DNA or SP-B mRNA precursor molecule. This stem-loop structure, if it exists *in vivo*, may affect transcription and/or processing of SP-B mRNA. This notion may be supported by previous findings of aberrant SP-B mRNA in CAP [6]. Genetic studies have clearly demonstrated that Alu repeats play an important role in genetic mutations such as duplication [28], deletion [29,30] and genetic recombination [31,32]. The mechanism whereby Alu repeats may alter mRNA processing has not yet been clarified. Genetic mutations at splice sites have been shown to contribute to human disease [33–36]. Approx. 15% of disease-causing point mutations result in aberrant mRNA splicing [18,19].

In summary, we have identified a novel alternatively-spliced SP-B mRNA in human lung. Although alternative splicing of human SP-B is a normal process in the lung, the regulation of this process seems to be altered in pulmonary disease. Its actual role in health and disease is currently unknown. However, given its location, along with the specific characteristic sequences present in this particular region, under certain circumstances this novel mRNA probably plays a role in the pathogenesis of disease.

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