— ARTICLES —

Prolonged Survival in Hereditary Surfactant Protein B (SP-B) Deficiency Associated with a Novel Splicing Mutation

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ABSTRACT

Hereditary surfactant protein B (SP-B) deficiency has been lethal in the first year of life without lung transplantation. We tested the hypothesis that SP-B gene mutations may result in milder phenotypes by investigating the mechanisms for lung disease in two children with less severe symptoms than have been previously observed in SP-B deficiency. Immunostaining patterns for pulmonary surfactant proteins were consistent with SP-B deficiency in both children. DNA sequence analysis indicated that both children were homozygous for a mutation in exon 5 that created an alternative splice site. Reverse transcriptase PCR and sequence analysis confirmed use of this splice site, which resulted in a frameshift and a premature termination codon in exon 7. The predominant reverse transcriptase PCR product, however, lacked exon 7, which restored the reading frame but would not allow translation of the exons that encode mature SP-B. Western blot analysis detected reduced amounts of mature SP-B as well as an aberrant SP-B proprotein that corresponded to the size expected from translation of the abnormal transcript. We conclude that a novel splicing mutation was the cause of lung disease in these children and that hereditary SP-B deficiency can be the cause of lung disease in older children. (*Pediatr Res* 48: 275–282, 2000)

Abbreviations
SP-B, surfactant protein B
proSP-B, SP-B proprotein
SP-C, surfactant protein C
proSP-C, SP-C proprotein
RG-PCR, restriction site generating PCR
LB, lamellar body
TM, tubular myelin
Sat PC, saturated phosphatidylcholine

Pulmonary surfactant is a complex of lipids and proteins essential for normal lung function. SP-B facilitates spreading of surfactant lipids, formation of TM, and metabolism of surfactant components (1). The absence of SP-B in mice genetically engineered to be homozygous for SP-B null alleles and in human infants with an inherited deficiency of SP-B results in lethal respiratory distress (2, 3). Hereditary SP-B deficiency is an autosomal recessive disorder that typically presents in neonates shortly after birth with unrelenting respiratory distress and a chest radiographic appearance similar to respiratory distress syndrome. The gene for SP-B has 11 exons spanning 9.5 kb on the short arm of chromosome 2 (4, 5). Translation of SP-B mRNA yields a preproprotein (proSP-B) that is processed to a 79-amino acid mature SP-B protein

encoded by exons 6 and 7 (6). The most common mutation causing SP-B deficiency is an insertion in exon 4 (121ins2) that results in a frameshift and a premature codon for the termination of translation and is associated with a marked decrease in SP-B mRNA and the complete absence of proSP-B and mature SP-B proteins (3). Although a child who was a compound heterozygote for SP-B gene mutations resulting in a partial deficiency of SP-B has been reported, there has been little variation in the phenotype of SP-B deficiency (7). With aggressive respiratory support, SP-B–deficient infants have survived outside the neonatal period, but, without lung transplantation, the disease has been lethal in the first year of life in all children identified to date (8). We tested the hypothesis that mutations in the SP-B gene may result in milder lung disease by investigating the mechanisms for lung disease in two unrelated children of French-Canadian descent. The first was a full-term infant who was oxygen but not ventilator dependent at 1 mo of age, and the second was an ambulatory 6-y-old with interstitial lung disease and a lifelong oxygen requirement.

METHODS

Case Histories

Patient 1. Patient 1 was a 3.5-kg male infant born at 40 wk gestation *via* cesarean section because of fetal distress who developed respiratory distress at 8 h of age. Chest radiographic appearance was consistent with respiratory distress syndrome. He developed a spontaneous pneumothorax at 18 h of age that required thoracostomy drainage and conventional mechanical ventilation. He did not receive surfactant replacement. He was extubated but remained tachypneic and required supplemental oxygen. Dexamethasone did not improve his respiratory symptoms. There was no family history of neonatal respiratory distress, and the parents were not related. Because of persistent respiratory symptoms, he underwent an open lung biopsy and had blood drawn to evaluate for surfactant protein deficiency at 5 wk of life. While he remained extubated, his respiratory function progressively declined, and he underwent bilateral lung transplantation at 4 mo of age.

Patient 2. Patient 2 was a full-term female born after an uncomplicated pregnancy who developed respiratory distress shortly after birth but did not require mechanical ventilation. She had a persistent oxygen requirement prompting an open lung biopsy at 2 mo of age, with histopathologic changes that included interstitial fibrosis and alveolar proteinosis, although no clinical diagnosis was established. Her respiratory symptoms persisted despite diuretic and steroid therapy. She was discharged at 6 mo of life with home oxygen therapy. Her course has been complicated by the progressive development of pulmonary hypertension and right ventricular hypertrophy. A trial of cyclophosphamide was not effective in treating her lung disease. She required a brief period of mechanical ventilation at 2 y of age as a result of pneumonia. She has now been stable for several years with a persistent oxygen requirement. She is ambulatory and attends school 5 d a week. There was no family history of neonatal lung disease, and the parents were unrelated.

A protocol for the evaluation of infants with unexplained neonatal respiratory disease was approved by the Institutional Review Board of the respective institutions. Informed consent was obtained before obtaining blood for genetic studies.

Antisera, immunohistochemistry, and protein analysis. Antisera specific for mature SP-B (28031), full-length proSP-B (55522), the amino terminal portion of proSP-B (55019), the carboxy terminal portion of proSP-B (96189), full-length proSP-C (76983), and the amino terminal portion of proSP-C (68514) were generated in rabbits as previously described (3). Immunostaining of formalin-fixed paraffin-embedded lung biopsy specimens for surfactant proteins was performed with and without antigen retrieval using methods that have been previously described (9). A Vectastain ABC peroxidase elite rabbit IgG kit was used to detect the antigen-antibody complexes (Vector Laboratories, Inc., Burlingame, CA, U.S.A.). Lung homogenates were prepared from sections of right lower and left lower lobe from patient 1 that were obtained at the time of transplant and snap-frozen in liquid nitrogen. Homogenates were separated by SDS-PAGE, transferred to nitrocellulose paper, and immunoreacted for surfactant proteins (3). Proteins were detected by enhanced chemiluminescence using a horseradish peroxidase conjugated secondary antibody (Amersham Life Sciences, Arlington Heights, IL, U.S.A.) with exposure to Kodak x-ray film. The relative amounts of SP-B detected by Western blot analysis were quantified by densitometric analysis using a commercially available imager and software package (Gel-Doc 2000 and Quantity One, Bio-Rad, Hercules, CA, U.S.A.) under exposure conditions that provided a linear response to the amount of protein loaded.

Electron microscopy. Tissue for electron microscopy was fixed in 3% glutaraldehyde and postfixed in 1% osmium tetroxide (OsO_4) in 0.1 M sodium cacodylate buffer. Tissue cubes were stained with 3% uranyl acetate, dehydrated in increasing concentrations of alcohol, and embedded in spurr plastic. Thin sections were mounted on plain copper grids and stained with uranyl acetate and lead citrate.

DNA analysis. Genomic DNA was isolated from peripheral blood (Puregene, Gentra Systems, Minneapolis, MN, U.S.A.). Coding regions and flanking intron sequences of the SP-B gene were amplified by PCR as previously described (9). PCR products were sequenced using SP-B specific primers end labeled with ^{33}P-dATP using T_4 polynucleotide kinase (New England Biolabs, Beverly, MA, U.S.A.) and thermocycle sequencing (Thermosequenase, Amersham Life Sciences, Cleveland, OH, U.S.A.). Sequencing reaction products were separated on 6% polyacrylamide gels and visualized by autoradiography after exposure to Kodak x-ray film. Patient SP-B genomic sequences were compared with published SP-B gene sequence (4).

RG-PCR. An antisense primer (AGGTTTGGGCAGGG-GGTCTGAC*G*T; nucleotides 2480–2503) was designed with a single base pair difference (A$\rightarrow$ *G*) from the wild-type sequence so that an *Rsa*I restriction site was introduced in amplimers derived from mutant alleles. This primer was used with a sense primer and conditions that have been previously described to amplify 193 bp of the SP-B gene (10). PCR products were digested with *Rsa*I (New England Biolabs,

Beverly, MA, U.S.A.), and the restriction fragments were separated on 3:1 Nuseive (FMC Bioproducts, Rockland, ME, U.S.A.) gels and visualized using ethidium bromide under UV light. DNA from 50 anonymous control individuals without lung disease was analyzed for the presence of the mutant allele.

RNA analysis. RNA was prepared from lung tissue by an acid phenol extraction method (Trizol, BRL Life Technologies, Gaithersburg, MD, U.S.A.). SP-B cDNA fragments were generated using reverse transcriptase PCR (RT-PCR) and subcloned into a plasmid vector (TA cloning vector, Invitrogen, San Diego, CA, U.S.A.) as previously described (11). Automated sequencing of cDNA fragments was performed by the DNA analysis facility of the Johns Hopkins University School of Medicine, and this sequence was compared with the known SP-B cDNA sequence (12). Sequence analysis software (Gene Runner version 3.05, Hastings, NY, U.S.A.) was used to calculate the expected molecular weight of a protein produced by translation of aberrant transcripts.

Two antisense primers corresponding to sequences in exons 6 and 8 were designed to amplify transcripts lacking exon 7 (13). These primers incorporated the last eight nucleotides of exon 6 and either the first 15 nucleotides of exon 8 [GCG-GCAGCCATTCTCCTTGGGAA (antisense nucleotides 2506–2513/4703–4717)] or the 13th–28th nucleotides of exon 8 [CTCCTGTCGGCGACCCTTGGGAA (antisense nucleotides 2506–2513/4715–4728)] to account for the use of a known cryptic splice site in exon 8. These primers were used in separate PCR reactions with a common sense primer (TTC-CTGGAGCAGGAGTGC, nucleotides 1471–1488) for 35 cycles (95°C × 20 s, 64°C × 20 s, 72°C × 45 s) followed by extension for 10 min at 72°C. PCR products were separated on 2% agarose gels containing ethidium bromide and visualized under UV light.

Northern blot analysis of total RNA was performed by hybridization with SP-B cDNA according to methods that have been previously described (3). The cDNA probes were labeled using a random primer labeling system, and hybridized probes were detected by chemiluminescence (Gene Images, Amersham Life Sciences, Arlington Heights, IL, U.S.A.) after exposure to Kodak x-ray film.

Phospholipid analysis. Frozen lung tissue was homogenized in 0.9% NaCl. Aliquots of lung tissue were extracted with chloroform-methanol (2:1), and Sat PC was recovered by neutral alumina column chromatography after exposure to osmium tetroxide and was quantified by phosphorus assay (14, 15). Total protein in lung homogenates was measured with the method of Lowry *et al.* (16). For measurements of phospholipid composition, extracted lipid from lung tissue was used for two-dimensional thin-layer chromatography. The spots were visualized with iodine vapor, scraped, and assayed for phosphorus content (17). Explanted lung tissue from infants who were homozygous for the 121ins2 mutation and unused donor lung tissue were used as controls.

RESULTS

Histopathology and electron microscopy. Histologic examination of lung tissue from both patients revealed variable alveolar septal widening with increased cellularity and type II pneumocyte hyperplasia. The open lung biopsy of patient 2 and the explanted lungs of patient 1 had alveolar spaces filled with granular eosinophilic material admixed with foamy macrophages and desquamated pneumocytes. Alveolar eosinophilic material was nominal in the open lung biopsy from patient 1. Electron microscopy of the explanted lung tissue from patient 1 (Fig. 1A) revealed numerous apically polarized cytoplasmic structures resembling LB in hyperplastic type II pneumocytes. A few of the LB-like structures had compact evenly spaced lamella; however, most contained noncompact irregularly spaced membranous whorls in contrast with the well-organized LB seen in non-SP-B–deficient lung tissue (Fig. 1B). LB-like structures were often grouped together in clusters of two or more and occasionally with multivesicular structures. Alveolar spaces contained LB-like structures, small multivesicular structures, and amorphous material. Focally, ribbon-like material coated the surface of pneumocytes, and there were rare
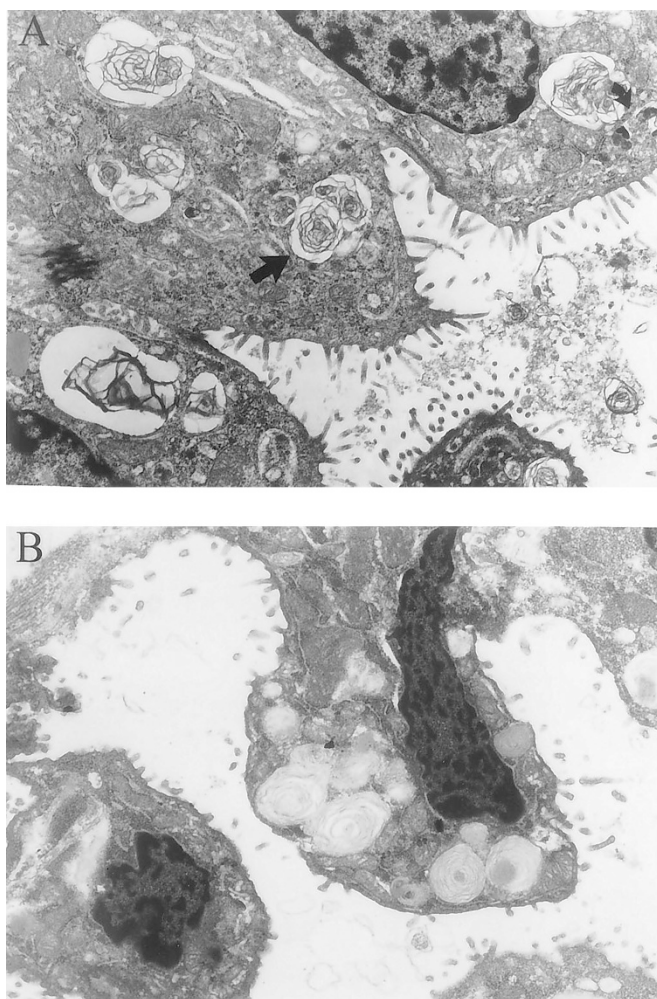

Figure 1. Electron microscopy. (*A*) Explanted tissue from patient 1. Hyperplastic type II pneumocytes contain numerous apically polarized LB-like structures (*large arrow*); multivesicular structures are also present (*small arrow*). The alveolar space contains LB-like structures, small multivesicular structures, and amorphous material. (*B*) Control tissue from a 3-mo-old child with pulmonary hypoplasia secondary to congenital diaphragmatic hernia. A type II pneumocyte contains well-organized LB, some with a dense core matrix. There is minimal alveolar material.

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haphazardly arranged structures suggestive of abnormally formed TM; however, definite TM was not identified.

Immunohistochemistry. Immunohistochemical analysis was performed to assess surfactant protein expression and distribution. SP-B staining was markedly reduced compared with controls and varied between the patients. Using a 1:2000 dilution of antisera specific for mature SP-B, staining was readily detected in control lung tissue (Fig. 2*A*), whereas no staining was detected in lung tissue from patient 1 (not shown), and only very weak variable staining was detected in patient 2 (Fig. 2*B*). Using a 1:1000 dilution of the same antisera, no staining was detected in lung tissue from patient 1 (Fig. 2*C*), although staining was now readily detected in tissue from patient 2 (Fig. 2*D*). Similarly, staining for proSP-B was not detected in patient 1, but it was readily detected in lung tissue from patient 2 (not shown). Antigen retrieval techniques resulted in detection of both mature SP-B and proSP-B staining in patient 1 (not shown) and enhanced staining for mature SP-B and proSP-B in patient 2 (not shown). In both patients, proSP-B staining was restricted to type II pneumocytes (not shown), whereas focal staining for mature SP-B was observed in the material in the alveolar lumen as well as in type II cells. ProSP-C staining was intense in the alveolar luminal material and in type II cells from both patients (Fig. 2, *E* and *F*).
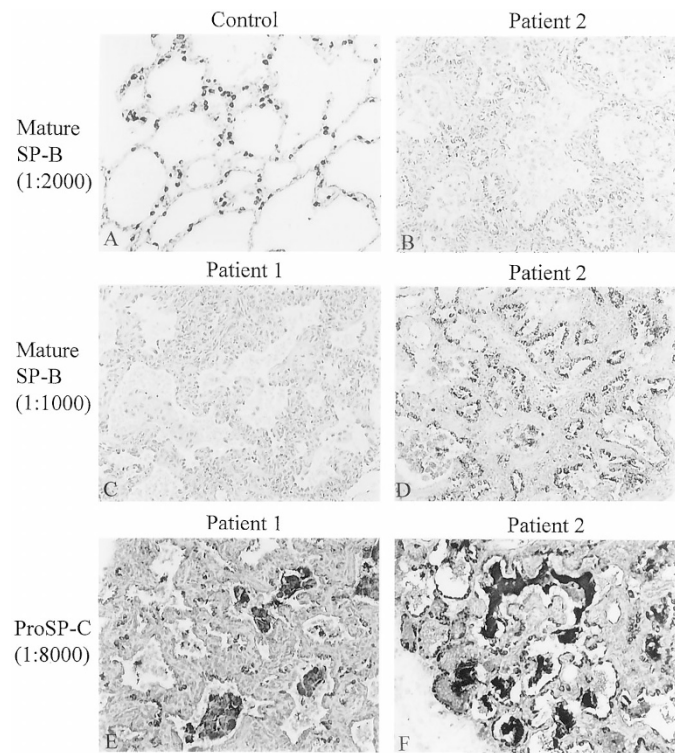

Figure 2. Immunohistochemical analysis of lung biopsy specimens for surfactant proteins. *Panels A and B* were reacted with antisera specific for mature SP-B (28031, 1:2000 dilution). (*A*) Control, positive for SP-B; (*B*) Patient 2, weakly positive for SP-B. *Panels C and D* were reacted with antisera specific for mature SP-B (28031, 1:1000 dilution). (*C*) Patient 1, negative for SP-B; (*D*) Patient 2, positive for SP-B. *Panels E and F* were reacted with antisera specific for proSP-C (68514). ProSP-C staining was intense in the alveolar luminal material and in type II cells from both patients. All panels are shown at ×230 magnification.

DNA analysis. A G→ T transversion at genomic nucleotide 2479 (cDNA nucleotide 479) was identified in both patients and was the only deviation from the published SP-B coding sequences. This substitution is in the wobble position of codon 155 in exon 5 and would not be predicted to alter the amino acid sequence, although it would create an alternate donor splice site (AGGGATGT→ AG*GTATGT*). Utilization of this splice site would delete 119 bases of exon 5 from the mRNA and result in a frameshift with the introduction of a premature termination codon in exon 7.

RG-PCR was used to screen genomic DNA for c.479G>T alleles. Amplimers from both patients were completely digested on restriction analysis, suggesting that these patients were homozygous for c.479G>T alleles (Fig. 3). The c.479G>T allele was not present on restriction analysis of 100 chromosomes from control individuals without lung disease.

RNA analysis. SP-B mRNA was markedly reduced on northern analysis of RNA from patient 1 (not shown). RT-PCR was used to determine whether low levels of SP-B mRNA were present and whether such transcripts were appropriately spliced. Three different SP-B cDNA were amplified and sequenced (Fig. 4). Transcripts corresponding to both the normal SP-B mRNA sequence and those that used the newly created splice site and had 119 bases of exon 5 sequence deleted were identified. However, the major RT-PCR product was approximately 300 bases smaller than that generated from control lung mRNA. Sequence analysis demonstrated that this cDNA resulted from use of the novel splice site and lacked 119 bases of exon 5 sequence as well as the entire sequence from exon 7 (184 bp). This deletion eliminated the premature termination codon and restored the reading frame.

To discover whether skipping of exon 7 was selective for this mutation, RT-PCR was used to amplify transcripts lacking exon 7. Two different antisense primers were constructed to amplify transcripts using the normal exon 8 acceptor splice site as well as those using a known cryptic splice site excluding the first 12 bases of exon 8 (11, 18). Transcripts lacking exon 7 were identified in RNA from normal tissue (Fig. 5). When these primers were used with a different sense primer and genomic DNA as a template to amplify a fragment of compa-
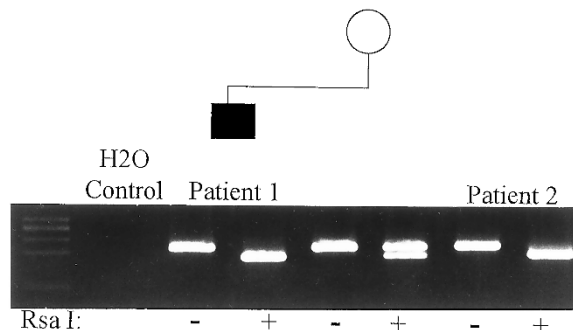

Figure 3. RG-PCR and restriction analysis. *Rsa*I digestion of 193 bp amplimers from c.479G>T alleles yields fragments of 169 and 24 bp, whereas amplimers from wild-type alleles are not digested. Amplimers from patient 1 and 2 were completely digested, consistent with these children being homozygous for the c.479G>T mutation. After *Rsa*I digestion of amplimers from patient 1's mother, the presence of bands at both 193 and 169 bp indicates that she had the c.479G>T mutation on one allele.
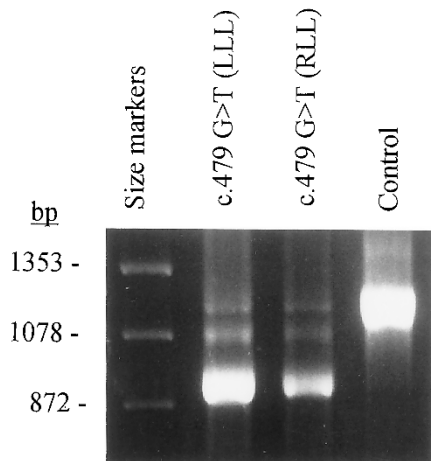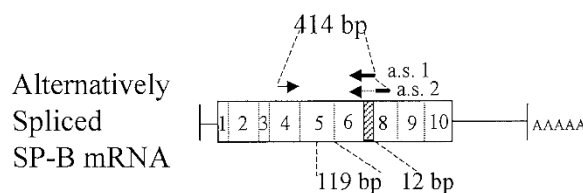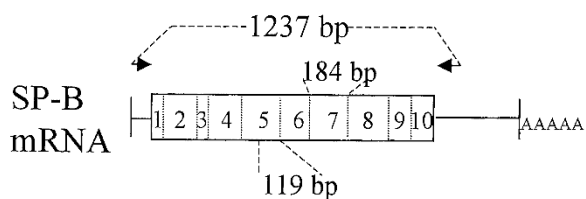
Figure 4. RT-PCR amplification of SP-B transcripts. An RT-PCR product of the predicted size (1237 bp) was observed in control tissue (*lane 4*). The predominant product in tissues from patient 1 (*lanes 2* and *3*) was approximately 300 bp shorter, corresponding to the size predicted from the deletions of part of exon 5 and all of exon 7. Less intense products were also observed in the patient samples, corresponding to the sizes of the normal transcript and those with a deletion of part of exon 5.
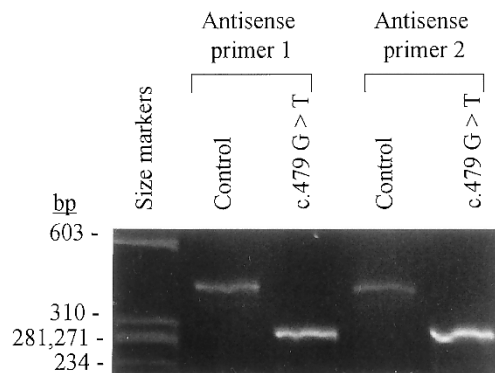

Figure 5. RT-PCR using primers specific for transcripts lacking exon 7. RT-PCR was performed using antisense primers corresponding to part of exon 6 and either the beginning of SP-B exon 8 (antisense primer 1/a.s. 1) or the 13th–28th nucleotides of exon 8 (antisense primer 2/a.s. 2), accounting for the use of a cryptic splice site (*shaded area*) in exon 8. RT-PCR products of the predicted size (414 bp) were observed in control tissues (*lanes 2* and *4*), indicating the presence of transcripts lacking exon 7. RT-PCR products amplified from RNA isolated from patient 1 (*lanes 3* and *5*) were shorter by the amount predicted to result from use of the new splice site in exon 5.

rable length, no products were observed, indicating that the 8 bp of these primers corresponding to exon 6 were not sufficient for PCR amplification under the same conditions (not shown).

Protein analysis. ProSP-B of $M_r = 40,000$ D and a processing intermediate of $M_r = 26,000$ D were readily detected in control lung tissues using antisera directed against proSP-B but were not observed in lung tissue from patient 1. However, these antisera detected an approximately 30,000-D protein that was present in an amount roughly comparable to proSP-B in control tissues (Fig. 6, upper panel). The size of this protein corresponded to the size predicted from translation of transcripts with deletion of part of exon 5 and all of exon 7. Similar results were obtained with antisera specific to the entire proSP-B protein and the amino terminal regions of proSP-B (not shown). Mature SP-B was detected in homogenates of lung tissue from patient 1 (Fig. 6, lower panel); the amount of mature SP-B detected was variable but was generally reduced compared with controls. By densitometric analysis, the amount of SP-B was approximately 12 to 16% of that observed in normal lung tissue. Aberrant SP-C peptides were abundant in all lung homogenate samples from patient 1 (Fig. 6, lower panel).

Phospholipid analysis. Surfactant phospholipid contents and compositions were examined in lung tissues from patient 1, two infants homozygous for 121ins2 mutation, and normal lung (Table 1). All samples were run in triplicate with similar results. The Sat PC to protein ratio was markedly increased in lung tissue from patient 1 when compared with control lung

tissue, from patients with the 121ins2 mutation, and previously reported values (19). Phosphatidylglycerol content in lung tissue from patient 1 was lower than in control tissue, although the reduction was not as dramatic as that seen in infants homozygous for the 121ins2 mutation (7).

DISCUSSION

Hereditary SP-B deficiency has been uniformly lethal in infancy without lung transplantation, with the majority of the infants dying in the first month of life (20). Partial SP-B deficiency has been reported in a child with chronic lung disease who was a compound heterozygote for two different SP-B gene mutations (121ins2/R236C) (7). This child, however, remained hospitalized his entire life, was ventilator dependent, and died at 9 mo of age while awaiting lung transplantation. In the present study, we report two SP-B–deficient children with less severe clinical symptoms than have been previously observed, including an ambulatory 6-y-old who has survived into childhood without the need for ventilatory assistance or lung transplantation. Both children shared a novel splicing mutation in their SP-B genes.

Approximately 15% of the point mutations that have been identified as responsible for human genetic disease result in defective RNA splicing (21). The c.479G>T mutation creates a donor splice site with an almost identical consensus value score as the donor splice site normally used for exon 5 (89.5 *versus* 89.3), suggesting that they should be equally attractive targets for splicing based on splice site sequence (22). Muta-
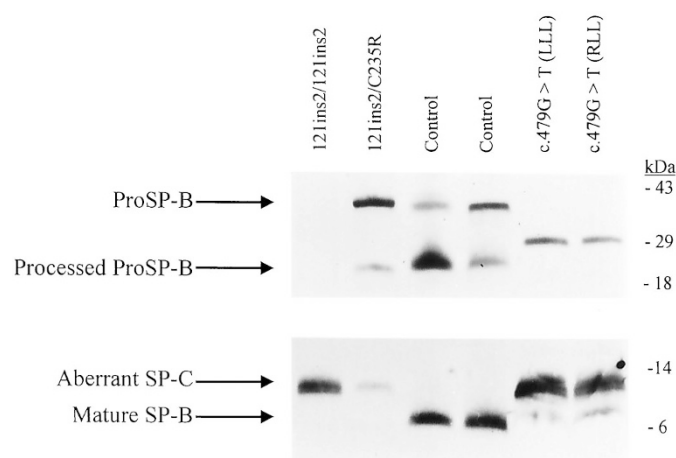
Figure 6. Western blot analysis of lung homogenates. Migration of molecular weight standards are indicated on the right. *Upper panel*, Western blot analysis using antisera directed against the carboxy terminal region of proSP-B. Proteins of M_r = 40 and 26 kD, corresponding to proSP-B and a processing intermediate, were present in control tissues (*lanes 3* and *4*) but not in tissues from an infant homozygous for the 121ins2 mutation (*lane 1*) or from patient 1 who was homozygous for the c.479G>T mutation (*lanes 5* and *6*). A 30-kD protein was observed only in lung tissue from the infant homozygous for the c.479G>T mutation (*lanes 5* and *6*). *Lower panel*, Analysis using antisera directed against proSP-C and mature SP-B. Mature SP-B and abundant aberrant proSP-C peptides were both detected in lung tissue from patient 1 (*lanes 5* and *6*). Aberrant proSP-C peptides but not mature SP-B were detected in lung from an infant homozygous for the 121ins2 mutation (*lane 1*) and in lung tissue from an infant who was a compound heterozygote for SP-B gene mutations (*lane 2*). Mature SP-B but not aberrant proSP-C peptides were detected in control lung tissue (*lanes 3* and *4*).

Table 1. *Surfactant phospholipid content and composition*

	121ins2	121ins2	c.479G > T	Normal
Sat PC (μmol/mg protein)	164	66	845	31
Surfactant composition (% phospholipid)				
Phosphatidylcholine	63.3	57.6	69.6	54.1
Phosphatidylglycerol	2.6	2.6	7.7	16.0
Phosphatidylinositol	5.2	5.4	4.6	3.0
Phosphatidylethanolamine	7.5	14.2	3.5	9.4
Phosphatidylserine	1.8	2.3	1.0	3.0
Lysophosphatidylcholine	8.0	8.3	2.7	11.3
Sphingomyelin	3.7	1.2	3.6	1.6
Lyso-bis-phoshatidic acid	4.1	1.9	4.2	4.7
Unknown	7.0	6.6	3.1	—

tions that alter splice sites have resulted in moderate phenotypes or variable penetrance through the preferential use of splice sites, a phenomenon that has been observed with several human genetic diseases including cystic fibrosis, leukocyte adhesion deficiency, and Sandhoff disease (23–25). Variable use of the normal splice site may explain the decreased severity of lung disease in these children. The presence of normally spliced transcript in RNA from patient 1 and the detection of low levels of SP-B in both lung tissue and alveolar luminal material from these children support this hypothesis. The severity of disease associated with this mutation was variable, as one child progressed to lung transplantation in infancy whereas the other has survived with medical management alone. The milder phenotype in the older child correlated with more readily detectable mature SP-B staining on immunohis-

tochemical analysis, an observation that could result from different frequencies of splice site use between these patients. Alternatively, factors other than genotype such as gender or differential expression of other genes impacting on pulmonary homeostasis may have influenced the phenotypes in these children.

Although the complete absence of SP-B in both mice and humans is lethal, the level of SP-B expression required for normal pulmonary function is not clear (2, 3). Mice genetically engineered to be heterozygous for an SP-B null allele do not have obvious respiratory symptoms and survive to adulthood (2). However, these mice have a 50% reduction in SP-B in lung tissue that is associated with changes in pulmonary mechanics, decreased compliance and mild air trapping, and an increased susceptibility to hyperoxic lung injury (17, 26, 27). Pulmonary function testing in nine human adult carriers for the 121ins2 mutation was normal, although it is not known whether this genotype increases susceptibility to pulmonary injury in humans (28). Reduction of SP-B levels to an estimated 6% of the adult level in one infant resulted in severe newborn respiratory distress and was lethal in the first year of life despite aggressive respiratory support (7). Lung homogenates from patient 1 contained approximately 12 to 16% of adult SP-B levels; however, this child still had a pulmonary pathology and abnormal SP-C metabolism characteristically seen with SP-B deficiency. Although this child did not require aggressive ventilatory support, he did require moderate supplemental oxygen that in combination with decreased SP-B levels may have incited additional pulmonary injury. Although frozen lung tissue was not available from patient 2 to directly assess SP-B levels, immunohistochemical staining of this child's lung tissue was more intense than that seen in tissue from patient 1 when the same dilution of SP-B–specific antisera was used. We speculate that the level of SP-B production in this child more closely approximated the level required for adequate pulmonary function, allowing her survival into childhood.

Use of the alternative splice site would result in a frameshift and the introduction of a premature termination codon in exon 7; however, the majority of the transcripts also lacked exon 7. Skipping of exon 7 has been observed in infants homozygous for the 121ins2 mutation but has not been reported in normal lung tissue or in tissues from patients with other lung diseases (11). Premature termination codons can induce skipping of the exons in which they are contained (29–31). In this study, transcripts lacking exon 7 were demonstrated in RNA from control tissues, indicating that skipping of this exon is not the consequence of the mutation. The skipping of exon 7 in transcripts using the novel splice site would restore the reading frame, and an aberrant proSP-B protein corresponding to the size predicted from this aberrant transcript was identified, indicating that these transcripts were translated (Fig. 7).

ProSP-B has structural homology to prosaposin and includes three tandemly repeated saposin motifs, each consisting of a characteristic combination of cysteine residues paired as disulfides and accompanying hydrophobic regions (32). The saposins are glycoproteins that activate lysosomal hydrolases and are derived from proteolytic cleavage of prosaposin, a common precursor protein (33). Mature SP-B corresponds to the middle
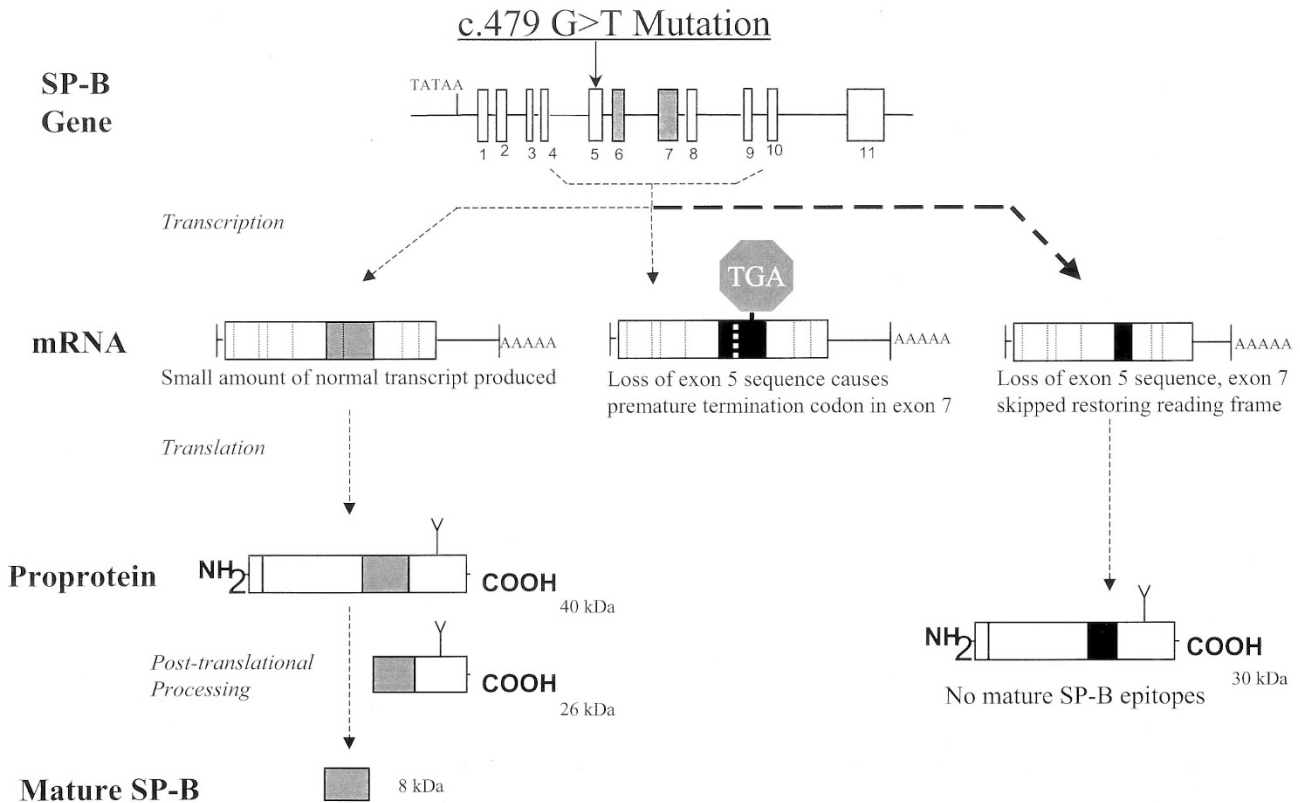
Figure 7. SP-B mRNA and protein production from c.479G>T alleles. Utilization of the normal exon 5 donor splice site allows for production of normal SP-B transcripts and mature SP-B protein. Use of the splice site created by the c.479G>T mutation deletes 119 bases of exon 5, produces a frameshift, and introduces a premature termination codon in exon 7. However, transcripts that are also lacking exon 7, resulting in the loss of the premature termination codon, have a restored reading frame and may lead to the production of an aberrant SP-B propeptide. The proSP-B amino and carboxy terminal regions are in frame in these transcripts, and these epitopes remain immunoreactive in the aberrant protein.

saposin domain. The aberrant proSP-B protein derived from the alternatively spliced transcripts caused by the c.479G>T mutation would maintain the amino and carboxy terminal saposin domains connected by a 30-amino acid peptide that is unrelated to mature SP-B due to the frameshift and absence of exon 7. *In vitro* expression of SP-B constructs containing the proSP-B amino terminal domain has suggested the importance of this region for proper trafficking of the proprotein to distal cellular compartments (34, 35). Genetically engineered mice that expressed only SP-B constructs that lacked sequences encoding the proSP-B carboxy terminal domains produced mature SP-B but had perturbed proSP-C processing and abnormally large LB, suggesting a role for the carboxy terminal region in LB biosynthesis and SP-C proprotein processing (36). However, SP-C metabolism was abnormal in the children we describe despite the presence of the aberrant protein, suggesting that the carboxy terminal region of the abnormal peptide was nonfunctional or that the reduced level of mature SP-B protein was also important in altered proSP-C processing. The aberrant proSP-B protein was present in amounts comparable to proSP-B in controls, even though total SP-B mRNA was reduced compared with controls, suggesting that the abnormal protein may not have been metabolized normally. Electron microscopy revealed that the aberrant protein did permit formation of LB-like structures, in contrast with that seen with the 121ins2 mutation that results in markedly decreased numbers of cytoplasmic LB and loss of apical polar-

ization of surfactant structures (37). The phosphatidylglycerol content in lung tissue from patient 1 was intermediate between homozygous 121ins2 and normal levels, consistent with a partial defect in SP-B function; however, the mechanisms for the increased Sat PC content in this tissue are not clear and may be related to the presence of the abnormal protein (7, 38). It is possible that the milder lung disease in these children may have been related to some preserved function of the abnormal proprotein in these children, although severe lung disease has been observed in children whose mutations allowed for proSP-B production (9).

We identified a novel SP-B gene-splicing mutation as the cause of lung disease in two unrelated children of French-Canadian descent. Given the association with decreased and possibly variable severity of lung disease, the contribution to morbidity and mortality attributed to this mutation needs to be examined in this population. The mechanism for decreased severity of lung disease in these children remains unclear but may be related to the production of low levels of normal SP-B transcript or partial function of an aberrant proSP-B protein. We demonstrate that hereditary SP-B deficiency need not be fatal in infancy and can be the cause of lung disease in older children. These findings suggest that SP-B deficiency may be an etiology for a broader range of lung disease than previously suspected and that abnormal SP-B expression should be considered as a potential etiology in older individuals with interstitial lung disease.

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