Identification and characterization of a novel ABCA3 mutation

Sang-Kyu Park,1,2,3*, Louella Amos,1* Aparna Rao,1 Michael W. Quasney,1,2,3 Yoshihiro Matsumura,1 Nobuya Inagaki,5 and Mary K. Dahmer1,2,3

1Department of Pediatrics, 2Children’s Research Institute, and 3Human and Molecular Genetics Center, Medical College of Wisconsin, Milwaukee, Wisconsin; 4Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, Oregon; and 5Department of Diabetes and Clinical Nutrition, Kyoto University, Kyoto, Japan

Submitted 31 July 2009; accepted in final form 25 October 2009

Park SK, Amos L, Rao A, Quasney MW, Matsumura Y, Inagaki N, Dahmer MK. Identification and characterization of a novel ABCA3 mutation. Physiol Genomics 40: 94–99, 2010. First published October 27, 2009; doi:10.1152/physiolgenomics.00123.2009.—Mutations in the gene coding for ATP-binding cassette protein A3 (ABCA3) are recognized as a genetic cause of lung disease of varying severity. Characterization of a number of mutant ABCA3 proteins has demonstrated that the mutations generally affect intracellular localization or the ability of the protein to hydrolyze ATP. A novel heterozygous mutation that results in the substitution of cysteine for arginine at amino acid 295 in ABCA3 was identified in a premature infant with chronic respiratory insufficiency and abnormal lamellar bodies. Sequencing of DNA performed in study participants demonstrated that this was a mutation and not a common variant. Plasmid vectors containing ABCA3 with the identified novel mutation tagged with green fluorescent protein on the carboxy terminus were generated. The effect of the mutation on protein function was characterized by examining the glycosylation state of the mutant protein in transiently transfected HEK293 cells and by examining ATP hydrolysis activity of the mutant protein with a vanadate-induced nucleotide trapping assay in stably transfected HEK293 cells. The ABCA3 protein containing the R295C mutation undergoes normal glycosylation and intracellular localization but has dramatically reduced ATP hydrolysis activity (12% of wild type). The identification of one copy of this novel mutation in a premature infant with chronic respiratory insufficiency suggests that ABCA3 haploinsufficiency together with lung prematurity may result in more severe, or more prolonged, respiratory failure.

chronic respiratory insufficiency; surfactant; pediatrics; lung disease

SURFACTANT IS ESSENTIAL for normal lung function partly by lowering alveolar surface tension and preventing end-expiratory atelectasis. Surfactant is found in lamellar bodies in type II pneumocytes and is composed of phospholipids and the surfactant-associated proteins SP-A, SP-B, SP-C, and SP-D. Inherited mutations in SP-B and SP-C are associated with surfactant-associated proteins and chronic respiratory insufficiency; surfactant; pediatrics; lung disease

MATERIALS AND METHODS

Enrollment of subjects. Individuals of a Hmong community in Wisconsin eligible for enrollment included 1) healthy unrelated adults ≥18 yr of age of Hmong descent on no medications or 2) parents and relatives of the index case. Subjects were identified through community outreach and the parents of the index case. Subjects who could not speak English were excluded, as were individuals whose immediate family member or first-degree relative was already enrolled. Research personnel obtained written consent from eligible subjects, and a buccal swab was obtained. A unique code was applied to the swab, and no identifiers were obtained by the investigators, with the exception of the parents and relatives of the index case. This study was approved by the Institutional Review Board.

DNA analysis. Buccal swabs were stored at −20°C until extraction. DNA was extracted from buccal swabs with the Epicentre MasterAmp Buccal Swab DNA Extraction kit (MB79015) and stored at −80°C. DNA samples were amplified in the region of the variant with AmpliTag Gold polymerase (Applied Biosystems, Foster City, CA) and the primers 5’-TCACTTGGACAGAAGGGACG-3’ and 5’-AGTAAGACCTGTCGAAATGCAGCAG-3’. The PCR reaction conditions were 96°C for 5 min followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 45 s, followed by 72°C for 10 min. The PCR product (248 bp) was treated with ExoSAP-IT (USB, Cleveland, OH) and sequenced.

* S.-K. Park and L. Amos contributed equally to the project and authorship of the manuscript.
Address for reprint requests and other correspondence: M. K. Dahmer, Div. of Critical Care, Dept. of Pediatrics, Medical College of Wisconsin, 9000 West Wisconsin Ave., MS681, Milwaukee, WI 53201 (e-mail: mdahmer@mcw.edu).

AGTAAGACCTGTCGAAATGCAGCAG-3’

5’-TCACTTGGACAGAAGGGACG-3’

Downloaded from http://physiolgenomics.physiology.org/ by 10.220.22.46 on June 19, 2017
Cell culture. HEK293 cells purchased from American Type Culture Collection (Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 25 mM HEPES, and 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ at 37°C.

DNA construction. The R295C mutant was initially generated from the pEGFPN1-ABCA3-green fluorescent protein (GFP) construct (14) with the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the following primers: forward 5'-AGGCTGAAGGAGTACATGTGCATGATGGGGCTCAGCAG-3' and reverse 5'-CTGCTGAGCCCCATCATGCACTGTACTCTCTTCACGCT-3' (underlines indicate substituted nucleotides). A R295C-GFP construct in a pCAGIpuro vector was generated by inserting the coding region of ABCA3-R295C-GFP into the pCAGIpuro vector. Presence of the mutation in the pEGFN1-ABCA3-R295C-GFP and pCAGIpuro-ABCA3-R295C-GFP constructs was confirmed by sequencing.

Glycosylation of wild-type and mutant ABCA3-GFP proteins. Transient transfections of HEK293 cells with wild-type ABCA3-GFP and ABCA3 mutants L101P-GFP, N568D-GFP, and L982P-GFP (14), as well as the new pEGFPN1 construct for R295C-GFP, were performed with FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN) as previously described (14). Briefly, for each experiment cells were seeded into 100-mm dishes at a density of 3 × 10⁶/dish for the assay and cultured for 1 day, and each plate was then transfected with 6 µg of one of the plasmid vectors listed above. Cells were cultured for an additional 48 h and lysed, and membranes were prepared as described previously (14). Membrane protein (10 µg) was treated with 100 U of peptide N-glycosidase F (PNGase F) or 500 U of endoglycosidase H (Endo H) (New England Biolabs, Beverly, MA) for 30 min at 37°C in a total volume of 20 µl. The samples were then electrophoresed on 5% SDS-polyacrylamide gels, and immunoblot analysis was performed with anti-GFP monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Stable transfection of wild-type and mutant ABCA3-GFP. Clonally selected HEK293 cell lines stably expressing wild-type or various mutant ABCA3-GFP genes were developed as previously described (14) and maintained in DMEM containing 2.5 µg/ml puromycin (Sigma, St. Louis, MO).

Vanadate-induced nucleotide trapping assay of wild-type and mutant ABCA3-GFP proteins. Vanadate-induced nucleotide trapping was performed with 8-azido-[α-32P]ATP purchased from Affinity Labeling Technologies (ALT, Lexington, KY) as described previously (14). Samples were analyzed by SDS-PAGE on 5% polyacrylamide gel, electrotransferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), and quantified with a STORM 860 PhosphorImager system (Amersham Biosciences, Piscataway, NJ). Each experiment was performed on a different passage of stably transfected cell lines.

RESULTS

Identification of a novel R295C mutation. A Hmong male born at 25 wk of gestation (weighing 790 g) was intubated at birth and received surfactant therapy for RDS. He required high-frequency oscillatory ventilation for 2½ mo and conventional mechanical ventilation for 1 mo and was eventually transitioned to noninvasive ventilation. After a 4-mo hospitalization in the neonatal intensive care unit, he was discharged on oxygen therapy with a nasal cannula. Within 4 wk of discharge, he was hospitalized with worsening respiratory failure, increasing oxygen need, and poor weight gain. Chest computerized tomography demonstrated coarse interstitial opacities, cystic changes, and focal hyperinflation, while bronchoscopy revealed normal upper and lower airway anatomy. Despite appropriate medical therapies, the child was hospital-
ized during the majority of his first year of life for refractory respiratory insufficiency and eventually had a tracheostomy at age 1 yr. To determine whether the chronic respiratory insufficiency observed might be due to an inherited disorder resulting in surfactant deficiency, genetic testing (for mutations in the genes for SP-B, SP-C, and ABCA3 by sequencing of exons and splice sites) and a lung biopsy were performed. Lung biopsy demonstrated the presence of abnormal lamellar bodies (Fig. 1; some normal lamellar bodies were also observed) and chronic bronchopulmonary dysplasia (BPD) with persistent fetal pulmonary architecture. DNA sequencing revealed no mutations in the genes coding for SP-B and SP-C; however, the child was heterozygous for a novel variation in the ABCA3 gene (replacement of a C with a T at nucleotide position 883). This change results in the replacement of arginine by cysteine at amino acid position 295. No other mutations in ABCA3 were identified.

Although the R295C variant had not been observed in previously characterized populations, it was unclear whether the R295C variant was a common polymorphism in Hmong individuals or a clinically significant mutation. To determine whether the R295C variant was a polymorphism or a mutation, the frequency of this variant in the Hmong population was examined. DNA from individuals from the child’s family, and from individuals in the Hmong community, was sequenced in the region of the variation. DNA samples from 90 of 91 individuals from the general Hmong population were sequenced successfully. None of these individuals had the R295C variant, indicating that this variation is indeed a mutation and not a polymorphism. Several members of the child’s immediate family, including one of the parents, were heterozygous for the mutation.

**Effects of ABCA3 R295C mutation on function.** The R295C mutation is located in the first ICL (ICL-1) of the protein (Fig. 2A) and is adjacent to the previously reported mutant E292V (2). The R295C mutation resides in a region that is conserved in different members of the ABCA subfamily (Fig. 2B) and across ABCA3 homologs in vertebrates (Fig. 2C). Previous studies (4, 13, 14) have identified mutations that affect ABCA3 function by either altering intracellular localization (type I mutants) or impairing ATP hydrolysis activity (type II mutants).

To examine whether the intracellular localization of the R295C mutant was altered, the glycosylation state of the R295C mutant was characterized. Membranes from HEK293 cells expressing wild-type ABCA3-GFP, the R295C-GFP mutant, or several previously characterized mutants were examined for sensitivity to the glycosidases Endo H and PNGase F. In HEK293 cells, wild-type ABCA3-GFP is mainly localized in lysosomal organelles, mimicking the trafficking of ABCA3 to lamellar bodies in alveolar type II cells (14). Because Endo H only cleaves sugars from high-mannose oligosaccharides, and not from complex oligosaccharides, resistance to Endo H indicates that the protein is in post-Golgi membranes (presumably lamellar body-like organelles). After treatment
also decreased in the N568D mutant as reported previously (14). Quantitation of three independent experiments demonstrated that the degree of trapping in the R295C mutant was dramatically reduced to 12% of that of the wild type (Fig. 4B). These results indicate that the ability of the R295C mutant to hydrolyze ATP is severely impaired.

**DISCUSSION**

The results presented here demonstrate that R295C is a novel mutation that results in severely impaired ATP hydrolysis activity as indicated by the dramatic reduction in vanadate-induced nucleotide trapping. Other mutations in the ABCA3 protein also result in impaired ATP hydrolysis, including E292V, N568D, G1221S, L1580P, and T1114M (13, 14). The E292V mutation is in ICL-1 only three amino acids from the R295C mutation. Clearly, the presence of two mutations that affect ATP hydrolysis in this ICL suggests that the ICL is important for normal ATP hydrolysis activity and normal

To determine whether the R295C mutation affected the ATP hydrolysis activity of the R295C mutant, vanadate-induced nucleotide trapping with photoaffinity labeling of the trapped intermediate (3) was examined. In this assay, ATP hydrolysis with production of a stable intermediate can be assessed based on the intensity of photoaffinity labeling of the ABCA3 protein. As shown in Fig. 4A, the level of vanadate-induced nucleotide trapping in the R295C mutant was greatly reduced compared with that of the wild-type ABCA3 protein. The level of the ABCA3-R295C-GFP mutant protein was comparable to that of wild-type ABCA3-GFP as demonstrated in the anti-GFP immunoblot. Vanadate-induced nucleotide trapping was

with Endo H the wild-type ABCA3 protein is present as a doublet (Fig. 3A, lane 3), with much of the protein being resistant to Endo H, suggesting it is in post-Golgi membranes (Fig. 3A, compare lanes 2 and 3). This observation is consistent with previous reports. The R295C variant demonstrated a level of resistance to Endo H comparable to that of the wild-type protein (Fig. 3A, compare lanes 6 and 7 to lanes 2 and 3), suggesting that the variant protein has undergone normal glycosylation and resides in post-Golgi membranes. As reported previously, the N568D variant shows resistance to Endo H (Fig. 3A, lanes 8 and 9) at a level similar to that of the wild-type protein; however, the L101P and L982P variants (Fig. 3A, lanes 4 and 5 and lanes 10 and 11, respectively) show no Endo H resistance, indicating that these mutants have not left the endoplasmic reticulum (14). As expected, the wild-type and mutant ABCA3 proteins are all sensitive to PNGase F (Fig. 3B), which cleaves both high-mannose and complex oligosaccharide from N-linked glycoproteins.

**Fig. 3.** Glycosylation of wild-type (WT) and mutant ABCA3-green fluorescent protein (GFP) proteins. A: 20 μg of membrane fraction from HEK293 cells transiently transfected with WT ABCA3-GFP (lanes 2 and 3) or with ABCA3-GFP mutants L101P (lanes 4 and 5), R295C (lanes 6 and 7), N568D (lanes 8 and 9), and L982P (lanes 10 and 11) were treated without (+) or with (−) endoglycosidase H (Endo H) and analyzed by 5% SDS-PAGE followed by immunoblotting with anti-GFP antibody. Lane 1, immunoblotting of untransfected HEK293 cells. B: WT ABCA3-GFP (lanes 2 and 3) or ABCA3-GFP mutants L101P (lanes 4 and 5), R295C (lanes 6 and 7), N568D (lanes 8 and 9), and L982P (lanes 10 and 11) were treated without (−) or with (+) peptide N-glycosidase F (PNGase F) and were then analyzed by 5% SDS-PAGE followed by immunoblotting with anti-GFP antibody. Lane 1, immunoblotting of untransfected HEK293 cells. Results from 1 representative experiment from a total of 3 separate experiments are shown.

To determine whether the R295C mutation affected the ATP hydrolysis activity of the R295C mutant, vanadate-induced nucleotide trapping with photoaffinity labeling of the trapped intermediate (3) was examined. In this assay, ATP hydrolysis with production of a stable intermediate can be assessed based on the intensity of photoaffinity labeling of the ABCA3 protein. As shown in Fig. 4A, the level of vanadate-induced nucleotide trapping in the R295C mutant was greatly reduced compared with that of the wild-type ABCA3 protein. The level of the ABCA3-R295C-GFP mutant protein was comparable to that of wild-type ABCA3-GFP as demonstrated in the anti-GFP immunoblot. Vanadate-induced nucleotide trapping was

**Fig. 4.** Vanadate-induced nucleotide trapping in ABCA3-GFP and ABCA3-GFP mutants. A: 20 μg of membrane fraction from untransfected HEK293 cells (lanes 1 and 2), HEK293 cells stably expressing WT ABCA3-GFP (lanes 3 and 4), ABCA3-GFP mutants N568D (lanes 5 and 6), and R295C (lanes 7 and 8) were incubated with 20 μM 8-azido-[α-32P]ATP in the absence (−) or presence (+) of 0.4 mM orthovanadate (Vi) and 3 mM MgCl2 as described under MATERIALS AND METHODS. Photoaffinity-labeled ATP was detected by autoradiography (top) and immunoblotting (IB) using anti-GFP antibody (α-GFP) was used as a loading control (bottom). Results from 1 representative experiment from 3 separate experiments performed are shown. B: radioactivity of photoaffinity-labeled protein bands was measured and quantified (220 kDa of upper band intensities + 220 kDa of lower band intensities) with STORM 860 PhosphorImager. These were normalized to ABCA3-GFP protein from immunoblot (220 kDa of upper band intensities + 220 kDa of lower band intensities), and then radioactivity in the absence of orthovanadate was subtracted from that in the presence of orthovanadate. Data shown are means ± SD for 3 separate experiments (n = 3), 1 of which is shown in A.
functioning of the protein. This conclusion is also supported by the crystal structure of the bacterial ABC protein SAV1866, which suggests that ICLs transmit conformational changes important for function of the protein (7), and by the report of a mutation that reduces ATP hydrolysis activity in an ICL of multidrug resistance protein 1, another human ABC transporter protein (19).

The R295C mutation does not affect glycosylation and intracellular localization of the protein. The trafficking of proteins accompanies the processing of oligosaccharides from high-mannose to complex sugar types, with the presence of complex oligosaccharides indicating that the protein is in post-Golgi membranes. Resistance to Endo H has been demonstrated previously to be associated with localization of the ABCA3 protein to lamellar body-like organelles (14). Normal glycosylation and intracellular localization of the R295C mutant is indicated by the similar levels of sensitivity to Endo H and PNGase F observed for the wild-type ABCA3-GFP protein and the R295C mutant. The observation that a substantial portion of the R295C mutant protein is resistant to Endo H indicates that the mutation does not affect intracellular localization.

Although it is clear that the mutation impairs the function of the ABCA3 protein, the patient in whom this mutation was discovered is heterozygous for the mutation. While it is possible that either a mutation in a regulatory region of the noncoding sequence or an insertion or deletion of one or more exons might be present in the second copy of the ABCA3 gene, it is more likely that the child has one normal copy of the ABCA3 gene and consequently has normal as well as impaired ABCA3 protein. That the mutation has an effect on the cellular level of functional ABCA3 is indicated by the abnormal lamellar bodies observed in the patient’s alveolar epithelial cells, although normal lamellar bodies are also present. Because one of the child’s parents and two siblings also have the mutation and none has a history of severe lung disease, it is likely that there is sufficient functional ABCA3 present for normal lung function under normal conditions. However, the patient was born prematurely and was exposed to a number of stresses that are not seen in term infants, consequently haploinsufficiency (having just 1 functional copy of the gene) may explain the more severe injury and/or prolonged recovery period observed in this patient. Most premature infants require only supplemental oxygen on discharge from the neonatal intensive care unit; rarely do they need tracheostomy for long-term mechanical ventilation as was required for this patient. (By 2.5 yr old this patient had improved enough that a tracheostomy was no longer required.) This patient’s haploinsufficiency may have caused surfactant dysfunction milder than would be expected in an individual homozygous for the mutation, but significant enough to cause chronic respiratory insufficiency in a premature infant. One possibility is that an interaction between the R295C mutation and the patient’s prematurity resulted in the severe BPD observed. As has been suggested for individuals heterozygous for functional SP-B mutations (10), it is possible that for children heterozygous for a functional ABCA3 mutation any environmental or developmental stress that alters ABCA3 expression may result in more severe respiratory stress because of their already reduced level of functional ABCA3. Interestingly, the frequency of individuals heterozygous for the E292V mutation is elevated in a cohort of children with RDS, suggesting that a mutation in this region might impart increased genetic risk for respiratory insufficiency, even in heterozygotes (9).

In conclusion, clinical management of a premature infant with severe BPD and chronic respiratory failure led to the discovery of the novel ABCA3 mutation R295C. This mutation, present in ICL-1, does not affect intracellular localization but severely impairs ATP hydrolysis activity of the ABCA3 mutant protein and is likely responsible for the aberrant lamellar bodies observed on lung biopsy. The identification of one copy of this novel mutation in a premature infant with chronic respiratory insufficiency suggests that ABCA3 haploinsufficiency together with lung prematurity may result in more severe, or more prolonged, respiratory failure. Testing for ABCA3 mutations in infants with refractory respiratory insufficiency or respiratory failure and a history of prematurity may help identify new mutations, clarify the function of ABCA3 and its various domains, and explain observed clinical deterioration despite appropriate medical management.

ACKNOWLEDGMENTS

We thank Daniel Merchant for excellent technical assistance and Dr. James F. Southern for providing histological analysis.

GRANTS

This work was supported, in part, by the Children’s Research Institute at the Medical College of Wisconsin.

DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES

1. Brusch F, Schimanski S, Muhlfeld C, Barlage S, Langmann T, Aslani-
   dis C, Boettridge A, Dada A, Schrotten H, Mildenberger E, Prueet E,
   Ballmann M, Ochs M, Johnen G, Griese M, Schnitz G. Alteration of
   the pulmonary surfactant system in full-term infants with hereditary
2. Bullard JE, Wert SE, Whitsett JA, Dean M, Nogee LM. ABCA3
   mutations associated with pediatric interstitial lung disease. Am J
3. Carrier I, Julien M, Gros P. Analysis of catalytic carboxylate mutants
   E552Q and E1197Q suggests asymmetric ATP hydrolysis by the two
   nucleotide-binding domains of P-glycoprotein. Biochemistry 42:
4. Cheong N, Madesh M, Gonzales LW, Zhao M, Yu K, Ballard PI,
   Shuman H. Functional and trafficking defects in ATP binding cassette
   A3 mutants associated with respiratory distress syndrome. J Biol
   Savani RC, Shuman H. ABCA3 is critical for lamellar body biogenesis
7. Dawson RJ, Locher KP. Structure of a bacterial multidrug ABC trans-
8. Garmny TH, Mosley MA, White FY, Dean M, Hull WM, Whitsett
   JA, Nogee LM, Hannas A. Surfactant composition and function in
   Cole FS, Hannas A. Population and disease-based prevalence of the
   common mutations associated with surfactant deficiency. Pediatr Res


