Expression of the amino acid transporter ATB\(^{0+}\) in lung: possible role in luminal protein removal

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Sloan, Jennifer L., Barbara R. Grubb, and Sela Mager. Expression of the amino acid transporter ATB\(^{0+}\) in lung: possible role in luminal protein removal. Am J Physiol Lung Cell Mol Physiol 284: L39–L49, 2003. First published August 23, 2002; 10.1152/ajplung.00164.2002.—Normal lung function requires transepithelial clearance of luminal proteins; however, little is known about the molecular mechanisms of protein transport. Protein degradation followed by transport of peptides and amino acids may play an important role in this process. We previously cloned and functionally characterized the neutral and cationic amino acid transporter ATB\(^{0+}\) and showed expression in the lung by mRNA analysis. In this study, the tissue distribution, subcellular localization, and function of the transporter in native tissue were investigated. Western blots showed expression of the ATB\(^{0+}\) protein in mouse lung, stomach, colon, testis, blastocysts, and human lung. Immunohistochemistry revealed that ATB\(^{0+}\) is predominantly expressed on the apical membrane of ciliated epithelial cells throughout mouse airways from trachea to bronchioles and in alveolar type I cells. Electrical measurements from mouse trachea preparations showed \(\text{Na}^+\) - and \(\text{Cl}^-\) -dependent, amino acid-induced short-circuit current consistent with the properties of ATB\(^{0+}\). We hypothesize that, by removing amino acids from the airway lumen, the transporter contributes to protein clearance and, by maintaining a low nutrient environment, plays a role in lung defense.

mucociliary clearance; airway surface liquid; acute respiratory distress syndrome; pulmonary alveolar phospholipidosis; glucose transport

THROUGHOUT THE LUNG, proteins and peptides such as mucins, lysozyme, transferrin, defensins, and surfactants are secreted in the airway lumen and alveolar space. The architecture of the lung requires that proteins must be removed from the airway surface (27, 48). The mucociliary escalator transports a portion of the protein up the airways, but most of the protein must eventually traverse the epithelium (9). Several studies have shown that exogenously applied proteins can cross the lung epithelium by endocytosis and paracellular diffusion (6–9, 16, 23, 24, 29, 33); however, the mechanisms of transepithelial transport of endogenous proteins are not known. Transepithelial protein transport from the airway surface may also be mediated by protein degradation followed by transport of peptides and amino acids. Proteases and peptidases are expressed in the lung (4, 26, 28, 40, 44, 51). Although their role in protein degradation has not been studied, amino acid concentrations have been measured in the airway surface liquid (ASL; see Refs. 15 and 41). The presence of proteases and amino acids in the airway lumen suggests that protein degradation and removal of the degradation products by specific transporters is an important mechanism of protein clearance from the airway and alveolar space.

Our laboratory cloned and functionally characterized the human amino acid transporter B\(^{0+}\) (hATB\(^{0+}\), SLC6A14), which is a \(\text{Na}^+\)- and \(\text{Cl}^-\)-dependent broad (B) specificity transporter for neutral (0) and cationic (+) amino acids (B\(^{0+}\); see Ref. 39). The highest hATB\(^{0+}\) mRNA levels were detected in the lung and trachea (39). Previous work demonstrated amino acid transport with characteristics similar to that of ATB\(^{0+}\) at the apical membrane of human bronchial and rat alveolar epithelial cells in culture (10, 22). Therefore, we hypothesized that ATB\(^{0+}\) mediates transport of amino acids from lumen of the airway and alveolar space. In this report, we used antibodies raised against human and mouse ATB\(^{0+}\) to determine the tissue distribution and subcellular localization of the transporter. In addition, measurements of amino acid-induced short-circuit current (\(I_{SC}\)) were performed on excised mouse tracheas mounted in Ussing chambers. These studies suggest that the transporter is expressed on the luminal membrane of the airway and alveolar epithelium. By removing amino acids from the lumen, ATB\(^{0+}\) may contribute to protein clearance and, by maintaining a low nutrient environment, play a role in lung defense.

MATERIALS AND METHODS

Plasmids. The coding region of hATB\(^{0+}\) (GenBank accession no. NM_007231, nucleotides 74–2027) was amplified by PCR and ligated into the EcoR I sites of pcDNA3.1 (Invitrogen, Carlsbad, CA). The coding region of mATB\(^{0+}\) (GenBank Accession no. AF161714, nucleotides 247–2229) was amplified by PCR and ligated into pcDNA3.1/CT-GFP-TOPO, but

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the protein was not fused to the GFP tag (Invitrogen). These constructs will be referred to as pcDNA-hATB0+ and pcDNA-mATB0+, respectively.

**Cell culture.** HEK293 (ATCC CRL-1573) and COS-7 (ATCC CRL-1651) cells were maintained in DMEM containing 4,500 mg/l glucose, 10% FBS, and 100 units penicillin/streptomycin and split every 5–7 days using trypsin-EDTA (GIBCO, Carlsbad, CA). HEK293 or COS-7 cells were seeded in 100-mm dishes (1.5 × 10^6 cells/dish) for membrane preparation. Transfections were performed the following day using Effectene according to the manufacturer’s instructions (Qiagen, Valencia, CA) and harvested at 40–48 h posttransfection.

**Membrane preparation.** HEK293 or COS-7 cells transfected with pcDNA-hATB0+ or pcDNA-mATB0+ were washed with PBS and pelleted by centrifugation at 700 g for 5 min. Hypotonic lysis buffer (20 mM HEPES, pH 7.5, 20 mM NaCl, and 5 mM EDTA containing protease inhibitors 10 μM leupeptin, 10 μM pepstatin A, 4 μM aprotinin, and 30 μg/ml phenylmethylsulfonyl fluoride) was added to the cell pellet. Cells were lysed by four freeze-thaw cycles, and lysate was centrifuged at 100,000 g for 20 min at 4°C in a Beckman TL-A rotor to pellet the membrane fraction. The high-speed pellet was resuspended in the same lysis buffer containing 1% Triton X-100 to solubilize membrane proteins.

Human lung samples were obtained from Scott Randell at the University of North Carolina (UNC) Cystic Fibrosis Pulmonary Research and Treatment Center. The human lung samples originated from patients with varying pathology: emphysema (patient 1); cystic fibrosis (patients 2 and 3); normal (patients 4 and 5). The cartilaginous airways collected were ~1–2 cm in diameter and are designated by the patient number followed by “a” for airway. Distal lung samples were obtained from the most distal 1–2 cm of the human lung and are designated by the patient number followed by “dl” for distal lung. All tissue samples were rinsed in ice-cold PBS, immediately frozen in liquid nitrogen, and stored at −80°C. Mouse organs and human lung were pulverized into fine powder with a mortar and pestle. Tissues were homogenized with an electronic homogenizer (Omni International, Warrenton, VA) for 15 s in hypotonic lysis buffer. Membrane preparation of tissues was described as described for HEK293 cells. The blastocysts were provided by Ann Sutherland at the University of Virginia and were harvested as described. At 24–48 h, the blastocysts were fixed with 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.5% Triton X-100 in PBS for 20 min, and incubated in blocking solution (5% goat serum in PBS) for 1 h at room temperature. Cells were incubated with α-hATB0+ or α-mATB0+ at a dilution of 1:2,000 in blocking solution at 4°C overnight and the following day exposed to secondary antibody (Alexa Fluor 488 conjugated goat anti-rabbit IgG) for 1 h (Molecular Probes, Eugene, OR). Preimmune serum and antibodies blocked with 20 μg/ml of the antigenic peptide for 1 h were used as controls.

Mouse trachea and lungs were removed from C57Bl mice and inflation fixed by perfusing the trachea with a 1:1 ratio of optimum-curtaining temperature (OCT) compound and PBS. Blocks of tissue were frozen in OCT and stored at −80°C. Mouse lung and trachea specimens were cryostat sectioned at a thickness of 10 μm and placed on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Slides were thawed to room temperature and immediately fixed with 2% paraformaldehyde for 20 min. Tissue was permeabilized with 0.5% Triton X-100 in PBS for 20 min. Subsequently, slides were blocked with 5% goat serum in PBS for 1 h. Primary antibodies, protein G column-purified rabbit α-mATB0+ and mouse anti-β-tubulin IV (Biogenex), were incubated with the tissue in blocking solution at dilutions of 1:5,000 and 1:1,000, respectively, at 4°C overnight. In addition, for peptide-blocking experiments, α-mATB0+ was preincubated with 20 μg/ml of the immunizing peptide for 1 h at room temperature. Secondary antibodies, Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated goat anti-mouse IgG, were diluted to 2 μg/ml in blocking solution, and slides were incubated for 1 h at room temperature (Molecular Probes). To stain nuclei, slides were incubated in TO-PRO3 at a 1:1,000 dilution in PBS for 30 min (Molecular Probes).
Images were captured using a Zeiss fluorescent microscope with a x10 objective using MetaMorph software and a Leica TCS-NT confocal microscope with x40 and x100 objectives.

**Ussing chamber experiments.** Ussing chamber experiments were conducted as described previously (13, 14). In short, adult 12-wk-old C57B6 mice were killed by carbon dioxide exposure. Tracheas were excised and mounted on Ussing chambers with a surface area of 0.025 cm². Tissue was bathed in Krebs-Ringer-bicarbonate (KRB) buffer containing (in mM) 140 Na⁺, 120 Cl⁻, 5.2 K⁺, 1.2 Mg²⁺, 1.2 Ca²⁺, 2.4 HPO₄²⁻, 0.4 H₂PO₄⁻, and 25 HCO₃⁻. In Na⁺-free experiments, the Na⁺ in NaCl and NaHCO₃ was replaced with N-methyl-D-glucamine (NMDG). In Cl⁻-free experiments, sodium gluconate and MgSO₄ replaced the Cl⁻ salts. Mannitol and glucose (5 mM) were added to the apical and basolateral chambers, respectively. Indomethacin (1 μM) was added bilaterally at the beginning of each experiment to reduce spontaneous Cl⁻ secretion. Tissues were gassed with 95% O₂-5% CO₂ and maintained at 37°C during the experiments. Amiloride (100 μM), leucine (100 μM), arginine (500 μM), glutamate (100 μM), glucose (5 mM), and UTP (1 μM) were applied sequentially to the apical chamber, and the change in Isc was recorded.

**RESULTS**

To gain insight into the physiological role of ATB₀⁺ in the lung, we examined the transporter tissue distribution and subcellular localization with antibodies generated against both human and mouse ATB₀⁺. Antibody characterization was performed by Western blot analysis of membrane fractions of COS-7 or HEK293 cells expressing pcDNA-hATB₀⁺ or pcDNA-mATB₀⁺. Western blot analysis showed that α-hATB₀⁺ and α-mATB₀⁺ recognized broad bands, ~75–85 kDa, in membrane fractions of transfected HEK293 cells, respectively, which were not detected in the soluble fraction (Fig. 1A), or untransfected HEK293 cells (data not shown). For both antibodies, no bands were detected in the 75- to 85-kDa range with preimmune serum or when antibodies were preincubated with the immunizing peptide (data not shown). The actual molecular mass, ~75–85 kDa, is larger than the predicted molecular mass, ~72 kDa, for both human and mouse ATB₀⁺. There are eight and seven putative extracellular N-glycosylation sites in the primary amino acid sequence of human ATB₀⁺ and mouse ATB₀⁺ (39), respectively, that may contribute to the difference between the predicted and actual molecular mass and to the difference in size between species. Antibodies were also tested for the ability to detect hATB₀⁺ and mATB₀⁺ by immunocytochemistry in COS-7 cells overexpressing the transporters. After respective antibody incubation, immunoreactivity was detected with Alexa Flour 488-conjugated goat anti-rabbit IgG. Both α-hATB₀⁺ and α-mATB₀⁺ showed distinct staining of transfected cells but not untransfected cells (Fig. 1, B and C). Preimmune serum and antibodies preadsorbed to the immunizing peptide showed no immunoreactivity (data not shown). Antibody characterization demonstrated that the antibodies were specific and effective for both Western blotting and immunostaining of ATB₀⁺-overexpressing cells; therefore, tissue distribution and immunolocalization experiments were subsequently initiated.

mRNA analysis showed that hATB₀⁺ is expressed at high levels in the lung, fetal lung, trachea, mammary gland, and salivary gland and at lower levels in the pituitary gland, stomach, colon, uterus, prostate, and testis (39). To determine if the mATB₀⁺ protein had a
similar distribution, we performed Western blot analysis on several mouse tissues. Membrane fractions were prepared from mouse lung, pancreas, small intestine, colon, and testis, and Western blot analysis was performed with α-mATB0⁺. A broad band of ~75–85 kDa, consistent with the size of the recombinant protein, appeared in membrane preparations of mouse lung, stomach, colon, and testis but not in small intestine or pancreas (Fig. 2). mATB0⁺ protein expression was evident throughout all regions of mouse lung: trachea, bronchi, and distal lung (Fig. 2). Importantly, the mATB0⁺ protein was also expressed early in development in mouse blastocysts (Fig. 2) where the B0⁺ amino acid transport system was first described (45).

To determine if the human lung also expressed the ATB0⁺ protein, human lung samples of varying pathology were obtained from the UNC Cystic Fibrosis Pulmonary Research and Treatment Center. Membrane fractions of two different regions of human lung, cartilaginous airway and distal lung, were tested for hATB0⁺ expression by Western blot. An ~75–85 kDa band, similar in molecular mass to the recombinant hATB0⁺ protein, was observed in all human airway and distal lung protein samples (Fig. 3). Interestingly, there was a consistent difference in protein migration on SDS-PAGE between hATB0⁺ protein from airway (Fig. 3, samples 1a, 2a, 4a, and 5a) and distal lung (Fig. 3, samples 1dl, 2dl, 3dl, and 4dl). The distal lung hATB0⁺ appeared as a broader band that extended into a higher molecular mass (Fig. 3). The apparent difference in hATB0⁺ protein size might be attributed to alternative splicing or posttranslational modification.

Northern (39) and Western blot analysis (Figs. 2 and 3) demonstrated that ATB0⁺ is expressed throughout the lung. Measurements of amino acid-induced current in cultured lung epithelial cells (10, 22) suggest the presence of an amino acid transporter with properties similar to that of ATB0⁺ at the apical membrane (39). Experiments were initiated to determine the cellular and subcellular localization of ATB0⁺ in mouse lung. Immunohistochemistry was performed on 10 μm frozen sections of inflation-fixed mouse trachea and whole lung using protein G-purified α-mATB0⁺ (Figs. 4–7). Immunolocalization revealed mATB0⁺ expression in the epithelial cells of the trachea, bronchioles and alveoli (Figs. 4–7), and bronchi (data not shown) of mouse lung. Sections incubated with α-mATB0⁺ preadsorbed with the immunizing peptide were not labeled (Fig. 4B). These data indicate that immunolocalization was specific for ATB0⁺ (Fig. 4). In both upper and lower airways, immunohistochemistry revealed that mATB0⁺ is expressed in the epithelial cells lining the trachea (Fig. 5B), bronchi (data not shown), and bronchioles (Figs. 4 and 6B). To determine the specific cell type expression of mATB0⁺, an antibody against β-tubulin IV, a protein enriched in cilia, was used as a marker for ciliated cells (Figs. 5A and 6A; see Ref. 42). mATB0⁺ colocalized with β-tubulin IV in ciliated columnar epithelial cells of the trachea (Fig. 5C) and ciliated cuboidal cells of the bronchioles (Fig. 6C). In the bronchioles, nonciliated cells showed a low level of immunostaining (Fig. 6B). These data suggest that ATB0⁺ is expressed...
at higher levels in ciliated cells of the airways but may also express in the secretory Clara cells lining the lower airways (Fig. 6). Colocalization with β-tubulin IV also suggests that ATB0\(^{+}\) is expressed at the luminal side of epithelial cells lining the airways (Figs. 5 and 6). In addition to the airways, immunostaining of mouse lung using α-mATB0\(^{+}\) also showed expression of the transporter in the alveolar epithelium (Figs. 4 and 7). There was a continuous strong network of staining throughout the distal lung, and containing with nuclear marker, TO-PRO3, revealed that the majority of cells in the distal lung expressed mATB0\(^{+}\) (Fig. 7). Because alveolar type I cells comprise 95% of the alveolar surface area, these data indicate that ATB0\(^{+}\) is expressed in alveolar type I pneumocytes (Fig. 7A). Because of the squamous nature of alveolar type I cells, it was difficult to determine the subcellular distribution; however, mATB0\(^{+}\) appears to be localized to the membrane of alveolar type I cells (Fig. 7B). Because alveolar type II cells only comprise 5% of the alveolar surface area, it was more difficult to determine if these cells expressed mATB0\(^{+}\). However, based on the morphology and localization of type II cells in a cornering pattern in the alveolar sac, we concluded that type II cells might also express mATB0\(^{+}\) (Fig. 7B).

The amino acid transporter ATB0\(^{+}\) has been functionally characterized in heterologous expression systems (17, 39, 43), and these studies have determined that neutral and cationic amino acids are transported in a Na\(^{+}\)- and Cl\(^{-}\)-dependent manner. Because of its ion-coupled transport mechanism, the process is electrogenic in nature and can be measured using electrophysiological recordings. We and others have demonstrated that application of amino acids induced an inward current in Xenopus oocytes expressing the transporter (39, 43). To detect ATB0\(^{+}\) function in native tissue, we measured \(I_{SC}\) on excised mouse tracheae mounted in Ussing chambers. These experiments were conducted in KRB containing NaCl or with Na\(^{+}\)-free buffer in which gluconate was substituted for Cl\(^{-}\). Amino acids and glucose were applied sequentially to the apical surface of the mouse trachea, and changes in \(I_{SC}\) were quantified. In the presence of amiloride, the first application of arginine (500 μM) and leucine (100 μM) generated changes in \(I_{SC}\) current that were 2.06 ± 0.37 μA/cm\(^2\) (n = 14) and 2.72 ± 0.35 μA/cm\(^2\) (n = 4), respectively (Table 1 and Fig. 8). In contrast, glutamate (100 μM) did not generate current (Fig. 8A). If arginine and leucine induced current by different mechanisms, subsequent application of the other amino acid should result in an additive current. We observed that application of leucine after arginine and vice versa resulted in no additional current (Fig. 8), suggesting the amino acids share a common mechanism and are likely to be carried by the same ion-coupled transporter protein. The properties of the amino acid-induced \(\Delta I_{SC}\) are in agreement with the known function of ATB0\(^{+}\) as a neutral and cationic but not anionic amino acid transporter.

Another property of ATB0\(^{+}\) is that amino acid uptake and transport current depend on the presence of extracellular Na\(^{+}\) and Cl\(^{-}\) (39). In solution lacking Na\(^{+}\), arginine failed to generate a change in \(\Delta I_{SC}\) (Table 1).

However, we observed a response to 1 μM UTP, which stimulates Cl\(^{-}\) secretion (14), suggesting that the preparations were viable (data not shown). Arginine also failed to generate current under Cl\(^{-}\)-free conditions (Table 1), but amiloride-sensitive current was present (data not shown). The Na\(^{+}\) and Cl\(^{-}\) dependence of the arginine-induced \(\Delta I_{SC}\) is consistent with the characteristics of ATB0\(^{+}\) and is likely to represent ATB0\(^{+}\) function, specifically since ATB0\(^{+}\) is the only known Na\(^{+}\)- and Cl\(^{-}\)-dependent cationic (e.g., arginine) transporter.

Transporters for other organic molecules may also be localized to the apical membrane of the airway epithelium. In addition to arginine- and leucine-induced current, we observed a current associated with application of 5 mM glucose to the apical chamber, which was...
2.79 ± 0.31 μA/cm² (Fig. 8 and Table 1). This current was present after application of leucine and arginine, indicating that it is mediated by an independent transporter (Fig. 8). The glucose response was virtually absent (0.13 ± 0.08 μA/cm²) in preparations lacking Na⁺; therefore, glucose is likely to be transported by a Na⁺-coupled transporter (Table 1). However, under Cl⁻-free conditions, the glucose-induced current was similar in magnitude to the glucose response in KRB, in accordance with the properties of known Na⁺-coupled glucose transporters (Table 1 and Ref. 50). In most cases, application of phlorizin (1 mM), an inhibitor of the Na⁺-coupled glucose transporter, SGLT1 (18), reversed the glucose-induced ΔI_SC. These data suggest that a Na⁺-coupled glucose transporter of the SGLT-SLCA5 cotransporter family is localized at the apical membrane of epithelial cells in the mouse trachea.

DISCUSSION

The goal of this study was to gain an understanding of the physiological roles of ATB⁰⁺ by investigating the tissue distribution, subcellular localization, and function in mouse and human tissues. Antibodies created against both human and mouse ATB⁰⁺ were specific and effective for recognizing the recombinant proteins on Western blots (Fig. 1A) and in transfected cells by immunostaining (Fig. 1, B and C). After initial characterization of the antibodies, the tissue distribution of the ATB⁰⁺ proteins was explored. The mATB⁰⁺ protein was detected by α-mATB⁰⁺ on a Western blot in mouse stomach, colon, testis, and blastocysts and throughout the lung (Fig. 2). The tissue distribution of the ATB⁰⁺ protein correlates with functional measurements in various cells and tissues (Figs. 2 and 3). Na⁺-dependent neutral and cationic amino acid transport, designated system B⁰⁺, was described in mouse blastocysts (45), Xenopus oocytes (31), a human colon cell line (5), rabbit conjunctiva (20, 21), rabbit small intestine (34, 35), rat anterior pituitary cultures (46), bullfrog lung (30), and human bronchial (10) and rat alveolar (22) epithelial cells in culture. Studies of ATB⁰⁺ mRNA expression in human (39) and mouse (17, 43) are also in agreement with the distribution of the protein. hATB⁰⁺ mRNA analysis showed high expression in the trachea, lung, fetal lung, mammary gland, and salivary gland and lower levels in the pituitary, stomach, colon, uterus, prostate, and testis (39). mATB⁰⁺ mRNA was detected by Northern blot in mouse lung, distal small intestine, cecum, and colon (17, 43). In contrast, mATB⁰⁺ mRNA levels were present at low levels in the small intestine (17); however, the protein was not detected in our preparation (Fig. 2). Further investigation is required to determine if the ATB⁰⁺ protein is expressed in all cells and tissues in which the mRNA was detected.

Similar to the mouse lung (Fig. 2), human cartilaginous airways and distal lung, comprised mostly of alveoli, showed expression of the ATB⁰⁺ protein (Fig. 3). Interestingly, we observed that the ATB⁰⁺ protein obtained from human airway and human distal lung

Fig. 5. ATB⁰⁺ localization in mouse trachea. Mouse trachea 10-μm frozen sections were costained with rabbit α-mATB⁰⁺ and mouse α-β-tubulin IV. Secondary antibodies, goat anti-rabbit IgG Alexa Fluor 488 and goat anti-mouse IgG Alexa Fluor 594, were used to visualize β-tubulin IV (red) and ATB⁰⁺ (green). To stain nuclei, sections were incubated with TO-PRO3 (blue). Images were captured with a Leica TCS-NT confocal microscope using a ×40 objective. A: β-tubulin IV was localized to the cilia at the apical membrane of tracheal epithelial cells. B: mATB⁰⁺ was localized to the apical membrane of tracheal epithelial cells. C: mATB⁰⁺ and β-tubulin IV were colocalized at the apical membrane of tracheal epithelial cells. Scale bar = 15 μm.
showed an apparent difference in molecular mass (Fig. 3). Similarly, ATB0⁺/H11001 from mouse blastocysts and testis also showed different migration on SDS-PAGE compared with ATB0⁺/H11001 from mouse lung (Fig. 2). The variation in the apparent molecular mass might be a result of alteration in the protein primary structure or differential posttranslational modification. Because of the single size of ATB0⁺ mRNA (39, 43), it is likely that the difference in mobility of the transporter protein is because of alteration in posttranslational modification such as the glycosylation state. It will be important to directly determine the biological significance of the different ATB0⁺ isoforms.

To investigate the subcellular localization of the mATB0⁺ protein, immunohistochemistry was performed on mouse trachea and lung sections. This study revealed that mATB0⁺ is expressed throughout the mouse lung in the epithelial cells of the trachea, bronchioles and alveoli (Fig. 4–7), and bronchi (data not shown). mATB0⁺ was predominantly expressed on ciliated epithelial cells of the bronchiole (Figs. 5 and 6). Colocalization with β-tubulin IV, a cilia-enriched protein, indicated apical localization (Figs. 5 and 6). We were unable to determine if ATB0⁺ was localized to the cilia, microvilli, or...
have demonstrated Na⁺/H¹⁺-induced physiologically. In mined directly by uptake of radiolabeled amino acids or fi transporter. Together these (Table 1 and Fig. 8). We applied a concentration sev-ments of cells in culture (22), ATB₀⁺ and functional measurements in alveolar epithelial is also likely to express in surfactant-secreting alveolar type I pneumocytes but may also be expressed in surfactant-secreting alveolar type II cells (Fig. 7). Based on our immunolocalization data and functional measurements in alveolar epithelial cells in culture (22), ATB₀⁺ is also likely to express in the apical membrane of alveolar epithelial cells in vivo.

Functional measurements of hATB₀⁺ and mATB₀⁺ in Xenopus oocytes (39, 43) and mammalian cells (17) have demonstrated Na⁺- and Cl⁻-dependent neutral and cationic amino acid transport as the hallmark of ATB₀⁺ activity. Transporter function can be determined directly by uptake of radiolabeled amino acids or by measuring the transport-associated current electrophysiologically. In Xenopus oocytes expressing human or mouse ATB₀⁺, amino acids generated an inward current resulting from the electrogenic nature of the transport process (36, 39). To demonstrate that ATB₀⁺ is functional in native tissue, we conducted measurements of Iₛₐₖ in freshly excised mouse tracheas mounted in Ussing chambers. We showed that, upon initial application of the amino acids (arginine and leucine), an amiloride-insensitive Iₛₐₖ was generated (Table 1 and Fig. 8). We applied a concentration several-fold higher than the EC₅₀ values of arginine and leucine so that each amino acid could generate maximal transport current and, thus, consecutive application of the other amino acid would not generate additional current (Fig. 8). As predicted, the arginine- and leucine-induced current was not additive, suggesting that they both act via a common mechanism. Transport of arginine and leucine by ATB₀⁺ is Na⁺- and Cl⁻-dependent in heterologous systems. Similarly, the arginine-induced Iₛₐₖ depends on the presence of both ions (Table 1). Importantly, ATB₀⁺ is the only known Na⁺-dependent cationic (e.g., arginine) amino acid transporter. Together these findings strongly suggest that the current is mediated by ATB₀⁺. Therefore, our Iₛₐₖ measurements in excised mouse tracheas are the first studies to definitively demonstrate the activity of ATB₀⁺ in native tissue. Furthermore, these data confirm the immunolocalization results and suggest that the transporter is functional at the apical membrane of the airway epithelium. Although the physiological function of ATB₀⁺ is not known, the expression of ATB₀⁺ throughout the airways and alveoli suggests an important role in lung function.

Physiological significance of ATB₀⁺ in the lung. Expression of an ion-coupled, high-affinity, broad-specificity amino acid transporter throughout the lumen of the airways and alveoli raises two interesting questions. First, what is the source of amino acids? Second, what is the functional significance of their removal under normal and pathophysiological conditions? Little is known about the amino acid composition of the fluid that lines the alveolar lumen and airway surface (15, 41), and the source of the amino acids in the airway lumen has not been studied. Amino acids may be secreted by the epithelium, filtrated from the pulmonary vasculature, or result from degradation of proteins in the lung lumen. From alveoli to trachea, there is a significant decrease in the surface area requiring a reduction in ASL volume and its contents of more than 4,000-fold. To account for the relatively constant height of the ASL and similar longitudinal transport rate (27, 48), the total volume of ASL and its protein contents must decrease from lower to upper airway. Consequently, the airway epithelium must remove proteins and/or their degradation products (2, 27).

There are several possible mechanisms of transepithelial protein transport as follows: 1) paracellular

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<th>Table 1. Changes in short-circuit current</th>
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<td>Arginine (500 μM)</td>
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<td>NaCl 2.06 ± 0.37</td>
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<td>Na⁺ free 0</td>
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<td>Cl⁻ free 0.25 ± 0.15</td>
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Data are means ± SE; n, no. of experiments. NT, not tested.
diffusion, 2) endocytosis/transcytosis, and 3) absorption of protein degradation products, small peptides, and amino acids via ion-coupled transporters (Fig. 9). In airway epithelial cells in culture, albumin was transported in an apical-to-basolateral direction by nonspecific protein transcytosis (7, 24). In alveolar preparations, several studies that utilized exogenously applied proteins showed that large proteins, such as albumin, transferrin, and IgG, are cleared by specific processes, for example, receptor-mediated endocytosis (6, 16, 23, 29, 33), and smaller proteins, such as granulocyte macrophage colony-stimulating factor and cytochrome C, are removed from the alveolar space by paracellular diffusion (6, 8, 9, 29, 33). Although transcytosis and paracellular diffusion were determined to be important for removal of exogenously applied protein in epithelial cultures, the fate of endogenous protein in the lung has not been studied. There is some evidence of protein degradation in airway epithelial cells (24), and several proteases and peptidases are expressed in the lung (4, 26, 28, 40, 44, 51). Therefore, we propose that protein degradation followed by transporter-mediated removal of the degradation products is a major pathway for protein removal from the ASL and alveolar space. In this study, we determined that the broad-specificity neutral and cationic amino acid transporter ATB$^{0+}$ is predominantly localized to the apical membrane of ciliated epithelial cells lining the airways and in alveolar type I cells (Fig. 4-7). We also showed that the transporter is functional in native mouse trachea (Fig. 8 and Table 1). Therefore, ATB$^{0+}$ may be involved in the removal and recycling of amino acids from the ASL and alveolar space. In addition, the H$^+$-coupled peptide transporter, PEPT2, is localized at the apical membrane of airway epithelial cells (11, 12), and it is also likely to contribute to the removal of protein degradation products. The model in Fig. 9 demonstrates the possible protein removal pathways and specifically shows that ion-coupled transporters, such as ATB$^{0+}$ and PEPT2, might play central roles in this process. In addition to amino acids and peptides, transporters for mono- and oligosaccharides might mediate the removal of sugar moieties of degraded glycoproteins such as mucin. In the present study, we showed that glucose also generated an amiloride-insensitive, Na$^+$-dependent change in $I_{SC}$ (Fig. 8 and Table 1). Similar currents were observed in horse trachea (25).

Ion-coupled transporters may also play an essential role during bacterial infection. Nutrients such as sugars and amino acids are required for bacterial colonization and growth. Thus the removal of these nutrients from the ASL and alveolar space may be critical for maintenance of a low nutrient environment and consequently prevention of bacterial growth. Interestingly, in cystic fibrosis patients, an increase in ASL amino acid concentration correlated with severity of respiratory disease (1, 41). Although the cause of this increase in amino acid concentration is unknown, it may contribute to sustained bacterial infection. We therefore can speculate that ATB$^{0+}$ and other nutrient transporters are critical for lung defense.

Amino acid removal might be particularly crucial when proteins accumulate in the airway or alveolar space. Acute respiratory distress syndrome (ARDS) is characterized by damage of the alveolar-capillary barrier resulting in pulmonary edema where immune cells, inflammatory molecules, and protein-rich fluid enter the alveolar space and can result in alveolar cell death, inhibited respiratory function, and respiratory failure (47, 49). Pulmonary alveolar phospholipoproteinosis (PAP) is a rare pulmonary disease in which surfactant homeostasis is dysregulated, resulting in
surfactant protein accumulation in the alveolar space and inhibited gas exchange (3, 37). For the resolution of these diseases, removal of protein from the alveolar space is necessary. Under these inflammatory conditions where immune cells infiltrate the alveoli and proteases accumulate (4, 19, 38), protein degradation and absorption might be even more critical for lung protein clearance. In pathological conditions such as ARDS and PAP, transporters such as ATB\(^{0+}\) and PEPT2 may be important for disease resolution. Interestingly, mutations in the amino acid transporter y\(^+\)LAT1 result in lysinuric protein intolerance, a human disease characterized by hyperammonemia, hepatosplenomegaly, osteoporosis, and alveolar proteinosis (36a). The distribution of y\(^+\)LAT1 in the lung has not been studied, but this phenotype suggests that amino acid transporters play important roles in protein removal from the alveolar space. Future studies may determine the role ATB\(^{0+}\) and transporters for other organic molecules in normal lung function and under pathophysiological conditions.

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