Developmental regulation of claudin localization by fetal alveolar epithelial cells

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Submitted 2 December 2003; accepted in final form 30 August 2004

FLUID PRODUCED BY THE FETAL LUNG creates mechanical disturbances that are critical for air space development (22, 23). Fetal lung fluid balance is maintained by the coordinated regulation of epithelial ion channel activity and barrier function (3, 5, 21, 31, 32). Epithelial barrier function is primarily mediated by tight junction proteins known as claudins. There are roughly two dozen different claudins that regulate paracellular ion permeability and epithelial barrier function (8, 35–37). Each tissue shows a distinct claudin expression pattern, which enables tissue-specific differences in paracellular permeability.

Previous work has shown that rodent and human lung epithelia express claudin-1, claudin-3, claudin-4, claudin-5, claudin-7, claudin-8, and claudin-18 (9, 24, 28, 38). The pattern of claudin expression is sensitive to epithelial cell phenotype. For instance, rat type II cells express more claudin-3 than type I cells, whereas type I cells express more claudin-7 than type II cells (28, 38). However, expression of claudin-4 and claudin-5 does not appear to be tightly linked to rat alveolar epithelial cell phenotype (38). Changes in claudin expression by lung epithelial cells have also been linked to changes in paracellular permeability (9, 38).

A recent screen of gene expression by human fetal lung (HFL) alveolar epithelial cells indicates that claudin-5 is one of the major genes upregulated during differentiation in culture (14). In addition to claudin-5, claudin-18 expression has been demonstrated in fetal mouse lung (24). Although there is some information about claudins expressed in the lung, little is known about regulation of claudin expression during human fetal lung development. Thus we examined tight junction protein expression by HFL cells cultured under conditions where cells either remain undifferentiated or differentiate to a type II cell-like phenotype (2, 14). We found that HFL cells modulate epithelial barrier function, in part, by regulating claudin expression and localization to the plasma membrane.

MATERIALS AND METHODS

Cell culture. Enriched populations of epithelial cells were isolated from second-trimester (13–20 wk gestation) human fetal lung tissue under Institutional Review Board-approved protocols. After overnight culture as explants without hormones, explants of fetal lung tissue were digested with a combination of trypsin, collagenase, and DNase. Fibroblasts were removed by preferential adherence to 60-mm plastic culture dishes, and each culture dish was washed and cultured for an additional 3–5 days in 1 ml of serum-free Waymouth control medium or Waymouth medium containing 10% fetal calf serum. After overnight culture (day 1), the cells were washed and cultured for an additional 3–5 days in 1 ml of serum-free Waymouth control medium or Waymouth medium containing 10 mM dexamethasone plus 0.1 mM 8-bromo-cAMP and 0.1 mM IBMX (DCI). These concentrations maximally induce surfactant components in human lung explant cultures, as previously assessed by mRNA expression analysis (14). Induction of the type II cell phenotype by DCI was routinely assessed using Nile red to label lamellar bodies (Fig. 1).

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A–D Fig. 1. Differentiated human fetal lung (HFL) cells form tight epithelial barriers. A–D: isolated HFL cells were cultured on permeable supports for 4 days in either medium containing 10 nM dexamethasone + 0.1 μM 8-bromo-cAMP and 0.1 mM IBMX (DCI, A, C) or control medium (B, D). DCI-treated cells had refractile vesicles consistent with lamellar bodies as determined by phase contrast microscopy (A; arrowheads), which were not present in control cells (B). HFL cell cultures were treated with a vital dye, Nile red, to label lamellar bodies and then imaged by fluorescence microscopy (C, D). Lamellar bodies present in DCI-treated cells were labeled by Nile red to varying extents (C). By comparison, untreated control cells were not labeled by Nile red (D). Bars = 10 μm. E: monolayer barrier function was determined by measuring transepithelial resistance (TER). TER was significantly higher for DCI-treated HFL cells compared with control cells (*P < 0.001). Bars represent means ± SE for n = 39 (DCI) or n = 45 (control) individual data points collected from 6 independent experiments.

Transepithelial resistance (TER) of HFL cells cultured on permeable supports was measured using an Ohmmeter (World Precision Instruments, Sarasota, FL) (38). HFL cells showed similar responses to DCI, regardless of whether they were plated on extracellular matrix-coated or uncoated substrata (13). However, medium volume was critical for a maximal response to DCI (13). Thus cells in 35-mm dishes were cultured in 1 ml of medium. For cells cultured on permeable supports, 0.5 ml of medium was added to the bottom part of the well, and 0.1 ml of medium was added to the top part of the well.

Immunofluorescence. Antibodies to claudins, zonula occludens (ZO)-1, occludin, junctional adhesion molecule (JAM)-1, and CD63 were from Zymed (San Francisco, CA); anti-ZO-2 and -ZO-3 were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-early endosome antigen (EEA)-1 was from Affinity Bioreagents (Golden, CO); and antiammoniosidase II was from Covance (Berkeley, CA). Anticlaudin antibodies are routinely tested for cross-reactivity using cells transfected with claudin cDNAs (38). Isolated cells plated onto glass coverslips or Transwell permeable supports were washed with PBS, fixed, and permeabilized with MeOH/acetonitrile 1:1 for 2 min and then washed with PBS + 0.5% Triton X-100 (PBS/TX) and PBS + 0.5% Triton X-100 + 2% rat serum (PBS/TX/GS) (38). The cells were then incubated with primary antiserum diluted into PBS/GS for 1 h at room temperature, washed, and then incubated with Cy2-conjugated goat anti-rabbit IgG and Cy3-conjugated goat anti-mouse IgG (Jackson Immunoresearch, Malvern, PA) for 1 h at room temperature. The cells were then imaged using an Olympus IX-70 inverted fluorescence microscope outfitted with a Hamamatsu Orca charge-coupled device, and images were captured using Image Pro software. For each claudin, intracellular fluorescence was measured from 60–75 cell images obtained from duplicate experiments of matched, simultaneously labeled samples captured with the same exposure times. Cell interiors were manually traced using the Image Pro feature analysis function, and the average pixel intensity was calculated and corrected for background fluorescence. Relative fluorescence was calculated as the average ± SE so that DCI-treated HFL cells gave an average intracellular fluorescence intensity of 100 for each claudin.

For in situ Triton X-100 extraction (11), cells were incubated in PBS containing 0.675 mM CaCl2, 0.2 mM MgCl2, and 0.5% Triton X-100 for 30 min at 15°C, washed with PBS, fixed with methanol-acetone, and analyzed by immunofluorescence microscopy as described above.

Endocytosis inhibitors. For potassium depletion experiments, cells were preincubated for 15 min in potassium-free HEPES-buffered saline (0.14 M NaCl, 2 mM CaCl2, 1 mM MgCl2, 1 mg/ml glucose, and 20 mM HEPES, pH 7.4) diluted 1:1 with water to make it hypotonic (15). After treatment, the cells were further incubated for 3 h at 37°C in either potassium-free HEPES-buffered saline or complete HEPES-buffered saline (0.14 M NaCl, 10 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 1 mg/ml glucose, and 20 mM HEPES, pH 7.4), then fixed, and immunolabeled. Monodansylcadaverine (MDC) was prepared as a 5 mM stock in HEPES-buffered saline slightly acidified with 0.1 M HCl. MDC was diluted into HEPES-buffered saline to a final concentration of 100 or 200 μM and adjusted to pH 7.4 (30).

Immunoblot. Cultured cells were scraped and microfuged at 10,000 rpm, and the resulting pellets were frozen at −80°C. The cell pellets were then thawed and vigorously resuspended in PBS containing 1.0% Triton X-100, 0.2% SDS, 1 mM sodium vanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonil fluoride, 2 μg/ml leupeptin, and 1 μg/ml pepstatin. Protein concentration was measured with the DC protein reagent (Bio-Rad, Hercules, CA). Samples normalized for total amount of protein (10 μg/lane) were resolved using SDS-PAGE, transferred to polyvinylidene difluoride membrane, and then blocked overnight with 4% powdered milk, and 0.1% Tween 20 (Blotto). The blots were incubated with Blotto containing specific antiserum, washed, and then further incubated with goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson Immunoresearch). Specific signals corresponding to a given protein were detected with the enhanced chemiluminescence reagent (Amersham) and quantified with a Kodak EDAS system. Data were from triplicate determinations ± SE, and significance was determined with Student’s t-test. For biochemical analysis of Triton X-100 extractability (18), cells were harvested, homogenized using a ball bearing homogenizer, and centrifuged at 500 g for 5 min, and the resulting postnuclear supernatant was brought to 0.5% Triton X-100. The samples were incubated for 30 min at 4°C, centrifuged at 100,000 g for 30 min, and separated into Triton-soluble (supernatant) and -insoluble (pellet) fractions, which were analyzed by SDS-PAGE and immunoblot as described above.
mRNA analysis. Microarray analysis was from a previously described data set (14). Real-time PCR on a single-plex format was done using an ABI Prism 7000 (Applied Biosystems, Foster City, CA). Total RNA was prepared with the RNeasy kit (Qiagen, Valencia, CA) from day 4 control and DCI-treated cells isolated from four separate type II cell preparations. Total RNA (10–15 μg) was used to prepare cDNA. The reactions contained 1× Assay-on-Demand Gene Expression Assay Mix, 1× TaqMan Universal PCR Master Mix, and cDNA diluted in RNase-free water in 25 μl of total reaction volume. Assay-on-Demand Gene Expression Assay Mix contains both the forward and reverse primers plus MGB Eclipse probe. The primer and probe sets used were found in the Applied Biosystems website (http://www.allgenes.com) and consisted of GAPDH: GGGCGCTTTTGTACACCGAGGTCACCAGGGCTGTCT (exon 3 with midpoint at base 130 of GenBank sequence NM_002046), claudin-5: GTTACCTTGACCGTGCTCAGTGCACC (exon 1 with midpoint at base 452 of GenBank sequence NM_003277), claudin-18: ACTGTTCAGACCAGGGCGCTCAGTGCACC (exon 3 with midpoint at base 511 of GenBank sequence NM_016369). The two-step PCR protocol involved 2 min at 50°C and 10 min at 95°C followed by 15 s and 60°C for 1 min and 40 cycles. The 2-min/50°C step is required for optimal AmpErase UNG activity when using the TaqMan Universal PCR Master Mix. Fluorescence intensity was recorded during the annealing step of each cycle of the reaction. The assays were determined to be in the linear amplification range by cDNA standards derived from RNA from type II cells treated for 5 days with hormones to allow for comparisons both within and between experiments.

RESULTS

Undifferentiated lung epithelial cells isolated from second-trimester HFL develop type II cell characteristics when cultured in the presence of dexamethasone, cAMP, and IBMX (DCI) (14). Cells cultured without DCI remain undifferentiated. Markers of type II cell differentiation include development of microvilli, reduction in glycogen deposits, appearance of lamellar bodies, induction of surfactant protein and phospholipid enzyme expression, and secretagogue-responsive release of lamellar body contents. DCI-treated HFL cells cultured on permeable supports differentiated toward a type II cell phenotype, as confirmed by the development of refractile, perinuclear lamellar bodies that are labeled by Nile red (Fig. 1). The majority of DCI-treated cells showed varying levels of Nile red labeling characteristic of type II cells cultured under these conditions (14).

To determine whether there were phenotype-specific differences in tight junction barrier formation, TER was measured for HFL cells cultured on permeable supports. We found that DCI-treated HFL cells formed high resistance monolayers, where the average TER was 1.758 ± 220 (n = 39) ohm/cm² (Fig. 1E). In contrast, undifferentiated HFL cells showed significantly lower barrier function, where the average TER was 248 ± 45 (n = 45) ohm/cm². DCI treatment seems critical for tight junction formation, since a previous study showed that isolated HFL cells cultured in Ham’s F-12 medium containing insulin, epithelial growth factor, transferrin, and endothelial cell growth supplement showed some type II cell characteristics but had a TER of ~360 ohm/cm² (3).

Given the difference between DCI-treated and undifferentiated control HFL cells in barrier function, we examined the expression of claudins and five other tight junction proteins by HFL cells (Table 1). Claudin-1, claudin-3, claudin-4, claudin-5, claudin-7, claudin-9, claudin-10, and claudin-18 mRNAs were expressed by HFL cells. mRNAs for occludin, JAM-1, ZO-1, ZO-2, and ZO-3 were also expressed by HFL cells. In comparing the relative expression of these mRNAs, we found claudin-5 and claudin-18 to have the most dramatic increases in relative expression by DCI-treated HFL cells compared with undifferentiated cells. These data were confirmed by real-time RT-PCR, where DCI-treated HFL cells showed 19.5 ± 7.8 (n = 3)-fold more claudin-5 expression and 13.3 ± 3.5 (n = 4) (Fig. 2) fold more claudin-18 expression than undifferentiated HFL cells. On the other hand, claudin-1 showed a decrease in relative mRNA expression by DCI-treated HFL cells. These differences suggest that transcriptional control of claudin mRNA expression accompanies HFL cell differentiation. This was not restricted to claudins, since DCI-treated HFL cells also showed decreased ZO-2 mRNA expression and increased ZO-3 mRNA expression compared with undifferentiated control cells.

Tight junction protein expression was then examined by immunoblot (Fig. 2). Occludin and ZO-1 resolved as a set of multiple bands, consistent with previous reports (7, 16, 25, 33, 34). Also, claudin-18 isolated from HFL cells migrated as both monomer and higher molecular mass forms, perhaps due to incomplete dissociation of claudin-18 complexes during SDS-PAGE. One possibility is that these represent claudin-18 dimers or heterodimers with another claudin. Analysis of claudin-18 is also complicated by the potential presence of an alternatively spliced 22-kDa form, which is not recognized by the anticlaudin-18 antibody used in this study (24). Thus, for the purposes of quantitation, densitometric measurements from all of the protein species observed by immunoblot were combined to give a single value for total protein expression. Of the
proteins examined, DCI-treated HFL cells had roughly half the expression of claudin-1 and two- to threefold more claudin-3, claudin-18, occludin, and JAM-1 expression than undifferentiated HFL cells. These changes in tight junction protein expression are expected to contribute to the increased barrier function of DCI-treated HFL cells. Also, expression of claudin-1, claudin-5, and claudin-18 protein followed the trend of mRNA expression levels in response to DCI. However, claudin-3, occludin, ZO-2, ZO-3, and JAM-1 protein content in response to DCI treatment was not accompanied by comparable changes in mRNA, suggesting that these proteins are not strictly regulated at the transcriptional level.

A clue to another possible basis for increased barrier function by DCI-treated HFL cells came when we examined the intracellular distribution of claudins by immunofluorescence microscopy (Fig. 3). Consistent with a functional tight junction barrier, DCI-treated HFL cells showed claudin-1, claudin-3, claudin-4, claudin-5, and claudin-7 predominantly localized to the plasma membrane. In contrast, control cells showed increased intracellular claudin fluorescence (Fig. 3). This was confirmed by a semiquantitative analysis of intracellular claudin fluorescence. Average intracellular pixel intensity for control cells was between approximately three to six times higher than the average intracellular pixel intensity for DCI-treated cells, depending on the claudin examined (see Fig. 7). Also, plasma membrane claudin localization was more discontinuous for control cells compared with DCI-treated cells. Thus it appeared that differential claudin localization to the plasma membrane might play a role in regulating barrier function of DCI-treated HFL cells.

In contrast to claudins, occludin, ZO-1, and ZO-2 were predominantly plasma membrane localized, whether or not the HFL cells were treated with DCI (Fig. 4). Thus the other tight junction proteins examined did not show intracellular accumulation, although we could detect plasma membrane discontinuities in the labeling for each of these proteins. The lack of
intracellular accumulation for occludin, ZO-1, and ZO-2 suggests that HFL cells may use different mechanisms to regulate localization of claudins compared with these other tight junction proteins.

Previous studies have shown that incorporation of proteins into tight junctions frequently correlates with enhanced binding to the cytoskeleton, which, in turn, increases resistance to Triton X-100 extraction (12, 25, 33, 39). Consistent with this, the Triton X-100-resistant pool of occludin increased when HFL cells were treated with DCI, particularly compared with the more slowly migrating forms of occludin (Fig. 5A). However, resistance to detergent extraction was not a universal feature of tight junction proteins, since JAM-1 was readily extracted by Triton X-100 under conditions where occludin was resistant to solubilization. We then determined whether changes in claudin localization by HFL cells correlated with a change in Triton X-100 solubility (Fig. 5B). Claudin solubility was comparable for both DCI-treated and undifferentiated HFL cells, although claudin-4 and claudin-5 appeared to be more readily extracted than claudin-3 and claudin-7. The Triton X-100-resistant fraction of claudin-7 present in control HFL cells was not restricted to the plasma membrane, as determined by in situ Triton X-100 extraction. By comparison, the Triton X-100-resistant pool of ZO-1 was mainly localized to the plasma membrane. Together, these observations suggest that a strong interaction with the cytoskeleton was not likely to be required for the shift in claudin localization in response to DCI treatment. However, we cannot rule out the possibility that DCI treatment might alter association of claudins with tight junction scaffold proteins that are not resistant to Triton X-100 extraction.

We used immunofluorescence microscopy to determine whether different claudins localized to the same intracellular compartment of undifferentiated HFL cells. As shown in Fig. 6, there was considerable immunofluorescence colocalization for each of the claudin pairs tested. Given this and the morphological similarities for the intracellular claudin-containing compartments (e.g., Fig. 3), it seems most likely that all of the claudins expressed by control HFL cells localized to comparable intracellular compartments. The intracellular claudin-5-containing pool was further characterized with markers for different membrane compartments. By double-label immunofluorescence, intracellular claudin-5 showed the most colocalization with an early endosomal marker, EEA-1. There was considerably less claudin-5 colocalization with markers for late endosomes (CD63) or the Golgi apparatus (mannosidase II).

Localization of claudin-5 to early endosomes in undifferentiated HFL cells suggested that the intracellular claudin pool formed after internalization from the plasma membrane. To further test this, we examined the effect of inhibiting HFL cell endocytosis on claudin localization and TER. Inhibition of clathrin-mediated endocytosis by either potassium depletion or MDC treatment enhanced the relative localization of claudins to the plasma membrane of undifferentiated HFL cells (Fig. 7).
MDC-treated cells showed increased barrier function in a dose-dependent manner, as measured by increases in TER. In response to DCI treatment, HFL cells altered expression of a number of tight junction components at both the mRNA and protein levels (Table 1). Although these changes were fairly modest at the protein level (on the order of approximately two- to threefold), changes in expression of tight junction proteins are consistent with altered barrier function. Because epithelial barrier function critically depends

DISCUSSION

Using an in vitro model, we found that HFL type II cell differentiation induced by DCI treatment is associated with increased barrier function. In response to DCI treatment, HFL cells altered expression of a number of tight junction components at both the mRNA and protein levels (Table 1). Although these changes were fairly modest at the protein level (on the order of approximately two- to threefold), changes in expression of tight junction proteins are consistent with altered barrier function. Because epithelial barrier function critically depends

Fig. 7. Effect of endocytosis inhibitors on claudin localization and HFL barrier function.

A–C: isolated undifferentiated HFL cells were cultured for 4 days and then further cultured in control medium (A), potassium-depleted medium (B), or treated with 200 μM monodansylcadaverine (MDC, C) for 3 h. The cells were then fixed, permeabilized, and immunostained for claudin-7. Potassium depletion (B) and MDC treatment (C) enhanced claudin-7 localization to the plasma membrane. Bar, 10 μm.

D: the amount of intracellular claudin immunofluorescence was quantified by image analysis (see MATERIALS AND METHODS) for either control cells that were untreated (black bars), potassium depleted (white bars), or treated with 200 μM MDC (hatched bars) or cells cultured in DCI-containing medium (gray bars) (means ± SE, *significantly greater than treated cells, P < 0.05). Inhibiting endocytosis decreased the amount of intracellular claudin fluorescence for each claudin examined.

E, F: isolated HFL cells were cultured for 4 days on permeable supports in either control medium (E) or DCI-containing medium (F). The cells were then treated with vehicle alone, 100 μM MDC, or 200 μM MDC for 3 h, and transepithelial resistance (TER) was then measured (means ± SE, n = 6 from 2 independent experiments). Cells treated with 200 μM MDC showed significantly higher TER than untreated controls (*P < 0.05).
on the claudin composition of tight junction strands (37), altering the expression of a few specific claudins might be expected to have a significant effect on barrier function. DCI-treated HFL cells, which formed a high resistance monolayer, also had claudins primarily localized to the plasma membrane. In contrast, undifferentiated cells with poor barrier function showed plasma membrane discontinuities in tight junction protein labeling and had a significant intracellular claudin pool. Thus HFL barrier function is not only due to transcriptional and translational regulation of claudins expressed by the cells but might be influenced by claudin subcellular localization upon type II cell differentiation. Interestingly, other tight junction proteins, such as occludin, ZO-1, and ZO-2, were predominantly plasma membrane localized, regardless of whether the cells were DCI treated or not. Thus HFL cells have the distinctive capacity to differentially regulate the localization of claudins and other classes of tight junction proteins as a function of cell differentiation.

We found that intracellular claudin-5 in undifferentiated cells localized to EEA-1-positive endosomes. Tight junction components internalized in response to calcium depletion also transit through early endosomes en route to a unique storage compartment (17). However, Matsuda et al. (20) found that internalized claudins bypassed early endosomes and were preferentially transported to rab-7-positive late endosomes. Whether there are multiple routes for internalization of tight junction proteins in general and claudins in particular is not known at present. It is also likely that different types of epithelial cells might use different pathways to regulate claudin turnover. Interestingly, Matsuda et al. (20) also found that internalized claudins were not accompanied by other tight junction proteins, such as occludin or ZO-1. This is consistent with our observation that HFL cells show phenotype-specific differences in claudin localization, as opposed to occludin, ZO-1, and ZO-2. A model for independent regulation of tight junction proteins is also suggested by Coyne et al. (10), who showed that treatment of airway epithelial cells with medium chain fatty acid decreases barrier function by inducing redistribution of claudin-1 and claudin-4, but not ZO-1 and occludin.

We found that treatments that inhibited clathrin-mediated endocytosis enhanced claudin localization to the plasma membrane for HFL alveolar epithelial cells. Consistent with this, Ivanov et al. (17) showed that clathrin-mediated endocytosis is required for calcium depletion-induced internalization of tight junction components by T84 intestinal epithelial cells. Inhibiting endocytosis also enhanced monolayer barrier function by HFL cells. This effect was extremely modest for control cells, suggesting that undifferentiated HFL cells require additional cofactors to establish a tight monolayer in addition to claudin localization to the plasma membrane. However, inhibiting endocytosis nearly doubled the barrier function of DCI-treated HFL cells. Together, these observations underscore the notion that tight junctions are dynamic structures that rapidly turn over through endocytosis (29) and that claudin internalization may serve as a control point for regulating HFL epithelial barrier function.

Differential claudin localization by HFL cells might also be due to a direct effect of cAMP and/or dexamethasone. For instance, activation of protein kinase A by cAMP has been implicated in increasing barrier function of intestinal epithelial cells (19). Similarly, dexamethasone treatment has been found to enhance barrier function and tight junction formation by endothelial cells and mammary epithelial cells (6, 27). However, this effect was predominantly attributed to increases in ZO-1 expression, which did not occur in response to DCI treatment in our model.

As alveolar epithelial cells differentiate to a type II cell phenotype, their membrane protein transport pathways are remodeled. The most prominent example of this is the appearance of lamellar bodies. As another example of this, we have previously shown that a gap junction protein, connexin46, was differentially localized as a function of alveolar epithelial cell phenotype (1). Here, we found that localization of claudins, another class of junction proteins, also changes in concert with alveolar epithelial cell phenotype. We speculate that increases in type II cell barrier function might contribute to a positive feedback loop that induces fetal type II cell differentiation by restricting fluid flux out of developing air spaces and promoting mechanical distension (22, 23). Enhancing barrier function between fetal type II cells might also be an initial step in preparing the lung epithelium for air breathing. During late gestation, the epithelium changes from a secretory surface to an absorptive surface (4, 26). The development of improved lateral barriers to solute and fluid flux may be a key component of this process.

In summary, we found that HFL cells regulate epithelial barrier function through coordinated regulation of tight junction protein expression levels and subcellular targeting. Although these processes were observed for cells from the developing lung, similar mechanisms may also control barrier function in the mature lung or in response to lung injury. Therapeutic manipulation of alveolar barrier function will likely require a multipronged approach. Our data suggest that regulation of claudin localization and/or alveolar epithelial cell endocytosis may be an important component of regimens designed to alter lung barrier function in vivo.

ACKNOWLEDGMENTS

We thank Lisa Miller, Ping Wang, and Sreedevi Angampalli for technical assistance. P. L. Ballard is the Gisela and Dennis Alter Endowed Chair in Pediatric Neonatology.

GRANTS

This work was supported by grants from National Institutes of Health R01-GM-61012 and P01-HL-019737, Program 3 (M. Koval), and Program 4 (P. L. Ballard). B. L. Daugherty is supported by a National Research Service Award Postdoctoral Training Grant from National Heart, Lung, and Blood Institute.

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