Gap junction-microtubule associations in rat alveolar epithelial cells

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Efficient function of the lung in gas exchange depends on close interactions between type I and type II alveolar epithelial cells (AEC) (6), as well as between the alveolar epithelium and proteins of the extracellular matrix (ECM) (13) or basement membrane (1, 10, 34, 42, 43). These cell-cell and cell-matrix interactions play an essential role in pulmonary biology. It is now well known that in lung tissue, ECM components regulate expression of connexins, a group of well-defined transmembrane proteins that mediate intercellular communication via gated gap junction channels (18, 20, 21, 26). The latter structures establish direct communication between the cytoplasmic compartments of adjacent cells and thereby facilitate cell-to-cell interactions. Nevertheless, the pathways that establish and regulate these interactions in the alveolar region of the lung remain poorly understood.

Gap junction intercellular communication (GJIC) supports diverse functions in the alveolar region of the lung, including propagation of calcium oscillations (14), surfactant secretion (4, 36), and both cellular maturation and differentiation (17). Gap junction complexes, or plaques (46), are aggregates of intercellular channels formed by the connexin family of transmembrane proteins that range in molecular mass from 20 to 50 kDa (47). Connexins are the products of a family of closely related but distinct genes (48), divided into α- and β-subclasses according to sequence homology (49).

The present studies are based on connexin 43 (Cx43), which is highly expressed in primary AEC cultures (2, 5, 18, 34). Typical of integral membrane proteins, Cx43 is modified following translation. The protein exhibits multiple phosphorylation sites in the cytoplasmic COOH-terminal domain that assume a tissue-specific distribution (27). The state of Cx43 phosphorylation appears to play a role in regulation of GJIC, via gating of gap junction channels (37), as well as through regulation of connexin subcellular localization (38) and turnover (7, 24, 29).

The “life cycle” of connexins, like other integral membrane proteins, consists of de novo synthesis, post-translational modification and degradation, or turnover (29, 31). Turnover of Cx43 is rapid (7), with a reported half-life of 1–3 h. The above processes occur in diverse subcellular compartments and may differ according to tissue environment (24, 29). Movement of Cx43 among these compartments thus requires interactions with cytoskeletal elements (45), including actin filaments and microtubules that associate with the plasma membrane. The role of the latter associations in biological regulation of connexin expression and function is poorly understood in lung tissue.

Previous studies from this laboratory demonstrated that changes in alveolar cell-ECM interactions substantially alter connexin expression (18–21). Blocking cell-ECM interactions with antibodies against either fibronectin or relevant integrins redistributes Cx43 from the plasma membrane to the cytoplasm, where the connexin no longer supports intercellular communication. To address mechanisms that underlie ECM-mediated regulation of cell-cell communication, we...
cused on the role of microtubules in Cx43 expression, turnover, and intercellular interactions. Results from those experiments suggest that the cytoskeleton provides linkage in regulation of cell-cell communication through integrin-mediated signal transduction pathways.

MATERIALS AND METHODS

Isolation and culture of AEC. Type II pulmonary AEC were isolated from the lungs of male Sprague-Dawley rats (150–175 g body wt) obtained from Charles River Laboratories. Procedures for isolation of purified cell populations have been detailed elsewhere (44). Briefly, animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg). After washing of blood from the pulmonary circulation and lavage of the airways, we dispersed alveolar cells by intratracheal instillation of Joklik’s minimal essential medium containing elastase (25 U/ml) and 0.05% BaSO4. Purified populations of type II cells were subsequently isolated by density-gradient centrifugation. The day of cell isolation is designated as day 0. Freshly isolated day 0 type II AEC were plated (2.1 × 10⁴ cells/cm²) on glass coverslips or in six-well culture plates, as indicated. By day 2 of primary culture, the cells assemble and deposit a fibronectin-rich ECM on the culture surface.

Immunocytochemistry. Immunocytochemical methods were used to examine type II cell morphology. Day 3 cells plated on glass coverslips were fixed with 100% methanol for 60 min then incubated with a rabbit polyclonal antibody against Cx43 (Zymed) or with mouse monoclonal antibodies against tubulin (Zymed) or against the trans-Golgi network (TGN): L1/P5ASKPNFTSSENNPP17C (Affinity Bioreagents). Western blot analysis. Western blots were used to evaluate expression of Cx43 and tubulin, as detailed previously (19). Membrane-enriched cellular fractions of proteins from type II cell cultures were collected from suspended cells lysed in cold hypotonic buffer (10 mM Tris, pH 7.8) containing a standard protease inhibitor cocktail (in mg/ml): 0.1 aprotinin, 0.1 leupeptin, 0.1 antipain, and 0.01 pepstatin, in buffer containing 1 mM EDTA-Na and 0.2 mM PMSF. Crude membrane fractions were recovered from the pellet after centrifugation of the above extracts at 15,000 g (10 min). The resulting pellets were dissolved in 1% SDS containing the above-mentioned protease inhibitors and subsequently subjected to Western blot analysis, as above.

Pulse-chase experiments. Confluent day 3 AEC monolayers were incubated in methionine-deficient medium containing high-specific-radioactivity [³⁵S]methionine (100 µCi/well, NEN). After 2 h, the culture medium was replaced with medium containing 1 mM methionine. Cells were harvested at intervals of 1–24 h thereafter, as indicated.

Immunoprecipitation of Cx43. Cells radiolabeled with [³⁵S]methionine were rinsed twice with PBS containing 1 mM EDTA, harvested in lysis buffer, and then sonicated. After overnight incubation with antibody against Cx43 (1:50, 4°C), samples were incubated for 1 h with magnetic beads conjugated to IgG (Polysciences). The beads were washed with low-, then high-salt buffer, collected using a magnetic separation stand (MagneSphere Technology, Promega), and resuspended in gel sample buffer (0.625 M Tris (pH 6.8), 2% SDS, 20% glycerol, 0.0025% bromphenol blue, and 4.9% 2-mercaptopethanol). Immunoprecipitated proteins were separated using SDS-PAGE and quantified with a densitometer. Coimmunoprecipitations were carried out to determine whether internalized Cx43 plaques were associated with lysosomes. Cells were harvested on day 3, after having been treated with colchicine (100 µM) for 4 h. Immunoprecipitated Cx43 was transferred to nitrocellulose membranes for immunodetection of the lysosomal protein lamp 2 using ECL kits.

Antibodies and PD-98059. Freshly isolated type II cells were plated on a plastic culture surface and exposed either to antibodies against the α5-integrin subunit or to PD-98059 on day 0. Subsequently, cultures were analyzed on day 1, 2, or 3.

Evaluation of intercellular coupling. Inter cellular transfer of the fluorescent dye Lucifer yellow (Molecular Probes) was used to evaluate functional coupling of AEC via gap junction channels. Single cells were loaded (100 hPa for 30 s) with a solution of 6.7% Lucifer yellow dissolved in 1 M LiClO₄ using a glass micropipette and an Eppendorf micromanipulator. Spread of the dye from the injected cell to adjacent cells was visualized under epifluorescence illumination using an inverted Olympus IX70 microscope. The number of cells containing Lucifer yellow was determined 3 min after the micropipette was removed from the target cell.

Data analysis. Mean and SE were calculated based on data from three independent experiments and analyzed by a two-tailed Student’s t-test. Values of P < 0.05 are considered to be of statistical significance.

RESULTS

In primary cultures of AEC, both expression and subcellular localization of Cx43 are closely regulated by integrin-mediated cell-ECM interactions (21). For example, expression of both Cx43 mRNA and protein is elevated rapidly and substantially in primary AEC cultured on a fibronectin-rich ECM (20). Conversely, antibodies against fibronectin, against the fibronectin-binding α5-integrin subunit, or the MAP kinase kinase inhibitor PD-90859 downregulate this response (3). The latter effects initiate redistribution of Cx43 from the plasma membrane to the cell interior and consequently inhibit GJIC. Thus it is evident that intracellular connexin trafficking plays an essential role in regulation of cell-to-cell communication in the alveolar microenvironment. The present experiments extend the above observations through further examination of the potential role of microtubules in regulation of Cx43 expression and function in the pulmonary alveolar epithelium.

Altered microtubule morphology in cells exposed to antibodies against the α5-integrin subunit or to PD-98059. Recent observations demonstrated a significant reduction in Cx43 expression in AEC exposed either to antibodies against the α5-integrin subunit or to the MAP kinase kinase inhibitor PD-98059. The present experiments address the hypothesis that inhibition of Cx43 expression involves the cytoskeleton. AEC were cultured 3 days under control conditions (Fig. 1A). In parallel, additional cells were exposed to antibodies directed against either the α5-integrin subunit (Fig. 1B) or to the MAP kinase kinase inhibitor PD-98059 (Fig. 1C) for 1 h on day 3 of primary cell culture. Immunocytochemical staining was used to visualize the structure of microtubules. Either anti-α5-integrin antibodies or PD-98059 altered the typical radial structure of microtubules (compare control). PD-98059 also appeared to decrease the intensity of tubulin-positive immunofluorescence.
Gap junction plaques are associated with microtubules during internalization into AEC. In previous studies we observed significant changes in the distribution of alveolar Cx43-positive gap junction plaques between the plasma membrane and the cytosolic compartments (21). On the basis of those data, we applied immunocytochemical double-labeling methods to visualize both Cx43 (Fig. 2A) and tubulin (Fig. 2B) in parallel day 3 cell cultures.

Intracellular Cx43-positive gap junction plaques are not associated with the TGN. Day 3 cells were immunodouble labeled using antibodies against both Cx43 and proteins of the TGN, in which newly translated Cx43 is stored and modified (Fig. 3). Figure 3A shows control cells double labeled with antibodies against both Cx43 (green) and the TGN (red). Internalized Cx43 in colchicine-treated cells did not appear to colocalize with the Golgi (Fig. 3B).

Effect of anti-α5-integrin antibody and PD-98059 on tubulin expression. The data in Fig. 4 quantitate the time course of change in tubulin expression by AEC during 4 days of primary culture. Under control conditions, the pattern of tubulin expression was similar to that in previous reports (cited above), with low values on day 0 and day 1 increasing threefold ($P < 0.01$) to plateau on days 3 and 4. Patterns of expression similar to the control were observed in cells treated with antibodies against the α5-integrin subunit. In contrast, PD-98059 (50 μM) substantially inhibited tubulin expression over the same culture interval (Fig. 4).

Effect of colchicine on tubulin expression. The effect of colchicine on tubulin expression was examined by Western blot analysis (Fig. 5). Over an interval of up to 24 h, colchicine (100 μM) had no significant effect on tubulin expression, compared with the day 2 control. Over the same interval, tubulin expression increased threefold ($P < 0.01$) in the absence of colchicine.

Effect of colchicine on Cx43 expression. The effect of colchicine on Cx43 expression was also quantitated by Western blot analysis (Fig. 6), as outlined above. Effects of the drug were nearly identical to those on tubulin expression. During 24 h, colchicine had no
significant effect on Cx43 expression (compare day 2 controls). Similar to tubulin, Cx43 expression increased threefold ($P < 0.01$) in the absence of colchicine.

Turnover of Cx43 by type II cells in primary culture. Confluent monolayers of day 3 AEC (Fig. 7) were radiolabeled for 2 h with high-specific-radioactivity $[^{35}S]$methionine (“pulse,” 100 μCi/well), then rinsed (arrows), and cultured in the presence of 1 mM methionine (“chase”) for intervals of 1 to 24 h, as indicated. During the chase, Cx43 protein was immunoprecipitated at selected intervals using magnetic beads then subjected to SDS-PAGE to resolve the phosphorylated and nonphosphorylated forms of the connexin.

Data combined from three independent experiments showed no significant effect of colchicine on the half-life of the nonphosphorylated form of Cx43 (Cx43-NP, Fig. 7A). On average, the half-life of Cx43-NP was 1 h in
both control and colchicine-treated cultures. In contrast, the half-life of phosphorylated Cx43-P1 (Fig. 7B) in colchicine-treated cells is 3–4 h, significantly below that in control cells (>24 h).

**Modulation of gap junction formation by colchicine.** On the basis of the above observations, we explored the effects of colchicine on microtubule assembly and on formation of Cx43-positive gap junction channels. After 2 days’ culture on glass coverslips, alveolar type II cells were treated with colchicine (100 μM) for intervals of 0.5–24 h (Fig. 8). Control cells were incubated in parallel over the same interval. Cells were fixed for 60 min in 100% methanol and then immunostained at the intervals indicated using antibodies against tubulin (Fig. 8, A–D) or against Cx43 (Fig. 8, E–H).

Within 30 min in the presence of colchicine, microtubules began to depolymerize (not shown). Tubulin immunopositive staining was no longer detected as early as 1 h after colchicine treatment nor at 4 or 24 h thereafter (Fig. 8, B and C, respectively). In contrast, day 3 control cells (Fig. 8D) showed normal tubulin staining (compare Fig. 8, A–D). A second group of day 2 control cultures (Fig. 8, E–G) showed typical punctate immunopositive staining of Cx43 associated with the plasma membrane. Increasing internalization of Cx43-positive gap junction plaques was evident as early as 30 min following colchicine exposure (not shown). After 4 or 24 h (Fig. 8, F and G), cells continued to exhibit Cx43 staining in the cytosol, with diminished staining at the plasma membrane (compare with control, Fig. 8H). Depolymerization of tubulin occurred rapidly in the presence of colchicine, whereas endocytosis of Cx43 was less rapid, continuing over the entire interval of the experiment and showing strong cytosolic staining at 24 h.

**Colchicine inhibits GJIC in cultured AEC.** On day 3, Lucifer yellow was introduced into a single AEC by an Eppendorf microinjector (100 hPa, 30 s). After 3 min, the number of cells containing dye was quantified. Under control conditions, Lucifer yellow loaded into a single cell on day 3 spread rapidly to 64 ± 5 adjacent cells via gap junction channels (Fig. 9A). In contrast, intercellular transfer of Lucifer yellow was nearly abol-

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**Fig. 6.** Effect of colchicine on Cx43 expression. The effect of colchicine on Cx43 expression was also evaluated by Western blot analysis. With short exposures (0.5–4 h, gray bars) colchicine did not inhibit Cx43 expression compared with the day 2 control (D2). In contrast, after 24-h incubation, colchicine inhibited Cx43 expression compared with the day 3 control (D3). Values are the means and SE of 3 observations.

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**Fig. 7.** Pulse-chase labeling of type II cell Cx43. Confluent day 3 cells were pulse-labeled 2 h with high-specific-activity [35S]methionine (100 Ci/well, NEN), then rinsed (arrows), and cultured in the presence of 1 mM methionine for up to 24 h, as indicated (chase). Cx43 protein was then immunoprecipitated and subjected to SDS-PAGE. Data combined from 3 independent experiments show no significant effect of colchicine on nonphosphorylated Cx43, compared with control cells (A). On average, the half-life of Cx43 in both groups was 1 h. Turnover of phosphorylated Cx43 was more rapid in colchicine-treated cells, with a t1/2 of 3–4 h compared with >24 h in control cultures (B). Values represent the means and SE of 3 observations. □, Colchicine; ●, control.
ished in the presence of colchicine (100 \( \mu \text{M} \)), where in parallel cultures the dye moved into 3 ± 1 adjacent cells (Fig. 9B). These data offer direct functional evidence that colchicine inhibits intercellular communication via gap junction channels.

**DISCUSSION**

The mammalian lung is structured to optimize bidirectional exchange of oxygen and carbon dioxide between the alveolar compartment and pulmonary capillary blood. The efficiency of gas exchange, driven by gradients of partial pressure across the alveolar wall, reflects evolution of a pulmonary diffusion barrier of large surface area and minimal thickness, thereby optimizing diffusion of gas across permeable barriers.

Within the past few years, reports from several laboratories (2, 6, 9, 10, 20, 26, 34) established and confirmed the presence of functional gap junction channels linking adjacent AEC. These basic observations opened inquiry focused on the physiology and cell biology of alveolar tissue. Only limited information is available, however, concerning the physiological significance of gap

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**Fig. 8.** Effect of colchicine on microtubule assembly and gap junction formation. Alveolar cells cultured 2 days on glass coverslips were treated with colchicine (100 \( \mu \text{M} \), 0.5–24 h) then fixed in 100% methanol (60 min). Control cells were incubated 2 or 3 days in parallel, as indicated (A, E, D, and H). Cells were labeled immunocytochemically on day 1 using antibodies against tubulin (A–D) or against Cx43 (E–H). After 30 min in the presence of colchicine, microtubules began to depolymerize (not shown). One hour (not shown), 4 h, and 24 h (B and C, respectively) after colchicine exposure, tubulin-positive labeling was not detected. Day 3 control cells showed a normal pattern of tubulin staining (D). Control day 2 cultures (E) showed typical punctate immunopositive staining of Cx43 at the cell membrane. Increasing internalization of Cx43-positive gap junction plaques was evident within 30 min after colchicine exposure (not shown). At 24 h, the cells continued to exhibit Cx43 staining in the cytosol, with diminished staining in the plane of the plasma membrane (G). Day 3 control cells are shown in H.

**Fig. 9.** Colchicine inhibits gap junction intercellular communication in type II cell cultures. Lucifer yellow was introduced into a single control day 3 alveolar epithelial cell (arrow) using an Eppendorf microinjector (100 hPa, 30 s). Within 3 min, the dye spread to 64 ± 5 adjacent cells via gap junction channels (A). In contrast, the dye moved into 3 ± 1 adjacent cells in cultures exposed to colchicine (B, \( P < 0.001 \) vs. control). Values are the means ± SE of 6 independent observations.
junction-mediated intercellular communication (GJIC) between alveolar cells (2, 9, 25, 26, 34). The present studies address the role of microtubules in regulation of GJIC at the alveolar surface.

Antibodies against the α5-integrin subunit cause disruption of the AEC monolayer, resulting in subconfluent cellular patches with low transepithelial resistance (35). The response to antibodies against the α5-subunit was similar. The antibodies also changed the distribution of the microtubules, providing further evidence of the association of the cytoskeleton with the integrin-mediated signal transduction pathway. The MAP kinase inhibitor PD-98059 induced similar effects on the distribution and expression of microtubules. In addition, antibodies against the β1-integrin subunit inhibit both migration of human fetal tracheal epithelial cells following wounding (22). These data suggest that the α5-subunit is involved in cellular differentiation and migration, which require constant remodeling of cytoskeletal structure.

Gap junction hemichannels, connexons, comprise six polypeptide connexin monomers. Each connexon is assembled in the endoplasmic reticulum (15) and in the TGN (28, 39). The resulting hemichannel is then transported to the plasma membrane in 100- to 150-nm vesicles, where, depending on the connexin involved, it may be phosphorylated, as is the case with Cx43. Cx43 is a dynamic protein with a half-life on the order of hours (30, 40). This relatively rapid rate of turnover appears to be essential to regulation of Cx43-mediated GJIC. Either hyper- or hypophosphorylation of connexins may lead to internalization and consequent inactivation of the gap junction channel (19, 32).

Depolymerization or perturbation of actin filaments by cytochalasin B (45), cytochalasin D (23), or phalloidin (41) influences the assembly of gap junction channels. The present study confirms evidence that microtubules interact directly with Cx43, thus playing a role in the Cx43 life cycle. The latter premise is supported by a biochemical approach using the COOH-terminal tail of Cx43 to “pull down” the interacting protein, tubulin (16). The nature of the direct association of tubulin with Cx43 remains to be detailed. On the basis of the observations cited above, Cx43 may act as an anchor site to stabilize the distal ends of the microtubulin molecule (16). In the present study, microtubules were observed in close proximity to the plasma membrane when cells were treated either with antibodies against the α5-integrin subunit or with PD-98059. Thus changes in microtubules may be due to internalization of gap junction plaques induced by these agents, as it is evident that Cx43 and tubulin can establish physical interactions.

Cx43 is degraded by both proteasomal and lysosomal pathways (8). In general, the proteosomal pathway appears to be more effective on cytosolic and nuclear proteins, whereas lysosomes play a role in the degradation of membrane proteins and materials derived from endocytosis. The present observations suggest that cytosolic Cx43 staining is derived from the plasma membrane, as indicated by parallel internalization of Cx43-positive gap junction plaques (21), which appear to be targeted for degradation via a lysosomal pathway. Immunostaining of AEC for Cx43 in parallel either with the TGN or with the lysosomal marker lamp 2 confirmed that cytosolic Cx43-positive gap junction plaques are not associated with the Golgi, but colocalize with lysosomes. More lysosomal membrane protein was associated with Cx43 following short-term (4 h), compared with long-term (24 h), colchicine exposures, in which membrane Cx43 staining was reduced and internalized Cx43-positive gap junction plaques were aggregated within the cytosol.

Parallel pulse-chase experiments based on [35S]methionine labeling suggested a short half-life, ~50 min, for Cx43-NP (Fig. 7A), in contrast to the extended half-life (>24 h) of the corresponding phosphorylated species (Fig. 7B). Colchicine reduced the half-life of phosphorylated Cx43 to several hours (Fig. 7B, open symbols) but had little effect on the nonphosphorylated form of the connexin (Fig. 7A, open symbols). Rapid turnover of Cx43-NP has been observed in rat leptomeningeal cell cultures (24), suggesting that the cytoplasmic Cx43 pool may be subject to acute regulation.

Multiple factors influence the half-life of Cx43-NP. These include synthesis and turnover of the Cx43 protein, as well as transformation of Cx43-NP to its phosphorylated derivatives, Cx43-P1 and Cx43-P2. The half-life of Cx43-P1 is determined by two factors: phosphorylation of Cx43-NP to Cx43-P1 and -P2 and turnover of Cx43-P1 and -P2. The present experiments are focused on Cx43-NP and Cx43-P1 and reflect only the overall effects of these factors. Colchicine did not block Cx43-NP synthesis and phosphorylation but induced phosphorylated Cx43 turnover. Colchicine also blocked functional gap junction coupling in cultured AEC microinjected with Lucifer yellow. This phenomenon was not observed when Rat-1 cell microtubules were disrupted with nocodazole (33).

In summary, the present study offers initial insight relevant to the role of microtubules in regulation of GJIC between AEC. Further observations address the role of actin filaments in the Cx43 life cycle. The latter experiments reveal that disruption of actin microfilaments by cytochalasin B increased cytoplasmic Cx43 staining and decreased membrane-associated Cx43 protein, suggesting a consequent decrement in Cx43-mediated intercellular communication. The pattern of cytoplasmic Cx43 distribution differed, however, from that following disruption of microtubules, wherein immunopositive Cx43 was more evenly distributed within the cell.

It is essential to recognize that regulation of GJIC in cells of the alveolar epithelium is complex, involving multiple, simultaneous, interacting pathways, often regulated independently. Although microtubules appear to play a key role in Cx43 protein trafficking and conductance, definition of differences in the functional...
interactions of these cytoskeletal components in lung tissue requires further study.

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DISCLOSURES

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