erbB1 Functions as a Sensor of Airway Epithelial Integrity by Regulation of Protein Phosphatase 2A Activity*

Paola D. Vermeer‡, Lacey Panko§, Michael J. Welsh*,†, and Joseph Zabner‡†

From the ‡Department of Internal Medicine, §Howard Hughes Medical Institute, University of Iowa Roy J. and Lucille A. Carver College of Medicine, Iowa City, Iowa 52242 and †Program in Forensic Science, University of Nebraska, Lincoln, Nebraska 68588

Two enzymes, protein phosphatase 2A and atypical protein kinase C, are associated with the tight junction and regulate its function. For example, phosphorylation of the tight junction protein occludin is required for its incorporation into the junction. The association of a kinase and phosphatase with the tight junction suggests that a balance between their activities exists and is required for normal tight junction function. This hypothesis predicts that loss of epithelial integrity may disrupt this balance in such a way as to facilitate restoration of epithelial integrity. Our previous data have shown that apically localized growth factors segregate from their basolaterally localized erbB receptors. Loss of epithelial integrity allows ligand access to the basolateral membrane where it immediately binds to and activates erbB receptors. We found that activation of erbB1 leads to phosphorylation of protein phosphatase 2A, inhibiting its activity. Importantly, this phosphorylation event was dependent on factors in the overlying airway surface liquid; washing away this liquid prevented phosphorylation. erbB1-mediated inhibition of phosphatase activity would shift the balance in favor of the kinase such that tight junction proteins would regain their phosphorylation, allowing for their incorporation into the junction complex. This mechanism provides a rapid means of sensing the loss of epithelial integrity and subsequently restoring barrier function.

Polarized epithelia line the cavities of the body, forming a physical barrier between the external environment and the body compartments. Essential to the function of an epithelium is its polarity. Cellular polarity is established and maintained by cell-cell adhesion junctions, including the tight junction and the adherens junction. We have previously demonstrated that maintenance of barrier function is required for silencing signaling cascades in differentiated primary cultures of human airway epithelia (1). Airway epithelial secretory growth factors enter the airway surface liquid (ASL). Among these is heregulin-α, a ligand for the family of erbB tyrosine kinase receptors that localize to the basolateral membrane in airway epithelia. This arrangement allows for receptor activation the instant epithelial integrity is compromised. In the presence of intact tight junctions, erbB receptors are silent (not phosphorylated). Disruption of cell-cell junctions allows ASL factors immediate access to the basolateral membrane, resulting in erbB receptor activation. Receptor activation triggers signaling cascades involved in injury repair. Following restoration of epithelial integrity, heregulin-α regains its restriction to the ASL, and erbB receptors are once again silenced. These data emphasize the importance of epithelial polarity and the cell-cell adhesion junctions that maintain it. In the absence of tight junctions, polarity ceases to exist and erbB signaling cascades become activated. Such aberrant signaling can lead to many downstream effects, including cellular proliferation, migration, and even apoptosis. Given the essential role of the tight junction in polarity and signaling, regulation of the tight junction itself becomes important to understand.

The tight junction is composed of a complex of proteins, including transmembrane proteins (e.g. occludin and the claudins), scaffolding proteins (e.g. ZO-1), and enzymes (e.g. atypical protein kinase C, aPKC, and protein phosphatase 2A, PP2A) (2, 3). The presence of a kinase and phosphatase, two enzymes with opposing activities, suggests they may regulate the tight junction complex. Moreover, their localization at the tight junction leaves them poised to immediately sense and signal the loss of epithelial integrity. In fact, Suzuki et al. (4) demonstrated that a dominant negative mutant of aPKC blocked reformation of the tight junction following Ca²⁺ chelation, suggesting that aPKC activity is required for resealing the junction. Atypical PKC phosphorylates tight junction proteins, enabling their insertion into the junction complex (5, 6). In fact, enteropathogenic Escherichia coli, the major cause of diarrhea worldwide, dephosphorylates occludin causing its dissociation from intestinal epithelial tight junctions and increasing transepithelial conductance (7). This molecular mechanism may explain the resultant clinical outcome (diarrhea). Importantly, this effect was inhibited by calycin A, a serine/threonine phosphatase inhibitor, suggesting that as a consequence of infection a serine/threonine phosphatase had been activated. Similarly, Nunbhakdi-Craig et al. (2) demonstrated that inhibition of PP2A promotes tight junction protein phosphorylation and assembly. Conversely, increasing PP2A activity decreases phosphorylation of tight junction proteins while increasing transepithelial conductance and paracellular permeability (2).

Taken together, these data suggest that both aPKC and PP2A activities regulate the state and function of the tight junction. Therefore, we hypothesized that a balance exists between aPKC and PP2A activity such that at equilibrium the tight junction complex is intact and functional. This hypothesis predicts that any shifts in this balance would alter epithelial integrity. Given the essential roles of cell-cell adhesion junctions in the establishment and maintenance of polarity as well as their function as barriers against infection, we wondered whether the junction itself is able to sense the loss of integrity and respond in such a way as to restore it.

EXPERIMENTAL PROCEDURES

Primary Human Airway Epithelial Cell Culture Model—Airway epithelial cells were isolated from trachea and bronchi of donor lungs. Cells were seeded onto collagen-coated, semipermeable membranes (0.6 cm² Millicell-HA; Millipore, Bedford, MA) and grown at an air-liquid inter-
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face as previously described (8–11). Epithelial cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium that was supplemented with a serum substitute, 2% Ultroser G, (BioSepa; Villeneuve, La Garenne, France) and 100 milliliters/ml of penicillin, 100 mg/ml of streptomycin, and 10 mg/ml of gentamicin. Basolateral culture medium was changed every 2–4 days. Samples were collected with approval from the University of Iowa Institutional Review Board.

Immunoprecipitation and Western Blot—Lysate was made by incubation in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM NaVO₄, 10 mM NaPi, 100 mM NaF, phenylmethylsulfonyl fluoride, leupeptin, pepstatin, aprotonin) with 1% Triton X-100 for 20 min at 4 °C. Prior to lysis, some cultures were first treated with 10 mM EGTA to decrease transepithelial resistance. Lysate and cells were collected, homogenized for 30 s, and following a 1500–rpm spin, the soluble fraction was incubated with antibody for immunoprecipitation for 2 h or overnight at 4 °C. Following immunoprecipitation, protein G-Sepharose (Pierce) was added and incubated for an additional 30 min at 4 °C. Complexed proteins were washed several times, eluted with sample buffer (4% SDS, 100 mM dithiothreitol, 20% glycerol, 0.005% bromphenol blue, 0.065 M Tris, pH 6.8), and separated by SDS-PAGE. Protein was then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). Polyvinylidene difluoride membranes were blocked in 5% bovine serum albumin in PBS for 1 h followed by three washes in 1 × TTBS (137 mM NaCl, 2.7 mM KCl, 2.5 mM Tris, 0.1% Tween 20). Membranes were incubated with antibody for 2 h at room temperature, followed by washes in 1 × TTBS. Secondary antibodies were conjugated to horseradish peroxidase (Amersham Biosciences) and used at 1:10,000 for 1 h. Bound antibody was detected with SuperSignal solution (Pierce) and exposed to film (Kodak Scientific Imaging Film, X-OMAT AR; Eastman Kodak Co., Rochester, NY).

Antibodies—For immunoprecipitation, mouse anti-PP2A catalytic α (BD Transduction Laboratories, San Diego, CA) and rabbit anti-occludin-1 (Zymed Laboratories, San Francisco, CA) were each used at 1:500. For Western blotting the following antibodies were used: rabbit anti-occludin (1:500; Zymed Laboratories), mouse anti-PCα (1:500; BD Transduction Laboratories), mouse anti-PP2A catalytic α (1:500; BD Transduction Laboratories), rabbit anti-occludin-1 (1:100; Zymed Laboratories), and mouse anti-phosphotyrosine (1:500; Upstate Biotechnology, Lake Placid, NY). For immunocytochemistry, the following antibodies were used: rabbit antibody specific for the catalytic subunit of PP2A (mouse anti-PP2A catalytic α, 1:500; BD Transduction Laboratories) for 2 h or overnight at 4 °C. Protein G-Sepharose (Pierce) was added and the immunoprecipitation returned to 4 °C for 30 min. Pelleted beads were washed several times with ice-cold wash buffer (250 mM imidazole, pH 7.2, 1 mM EGTA, 0.1% 2-mercaptoethanol, 0.5 mg/ml of bovine serum albumin) and a final wash with ice-cold phosphate-free dH₂O. Beads were pelleted and the supernatant discarded. Fifty microliters of assay buffer (165 μM imidazole, pH 7.2, 660 μM EDTA, 0.066% 2-mercaptoethanol, 0.066% bovine serum albumin) containing 330 μM RRA(pT)VA synthetic phospho-peptide (Promega, Madison, WI) were added to the beads and incubated with rocking for 2 h at room temperature. Following incubation, beads were again pelleted and the supernatant transferred to a 96-well plate. An equal volume of tolysaldehyde/malachite green dye solution was added (Promega) to each well and incubated for 15–30 min at room temperature. Optical densities were measured at 600 nm. A standard curve using free phosphate was generated for every run. Controls included the addition of 1 nM okadaic acid (negative control), a phosphatase inhibitor, to all conditions and addition of increasing concentrations of purified PP2A (positive control) to 200 μM phospho-peptide.

Reverse Transcriptase PCR—RNA isolation was performed using the RNAqueous-4PCR kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Briefly, epithelia were lysed, precipitated with ethanol, RNA-bound to a filter, and washed several times following elution. RNA was reverse transcribed with Thermoscript RT (Invitrogen). Controls included the omission of RT. The generated cDNA was then used as the template in PCR reactions using erbB ligand-specific primers. The erbB ligand-specific forward and reverse primers for betacellulin, transforming growth factor-α (TGF-α), heparin-binding epidermal growth factor, epiregulin, heregulin-α, and amphiregulin were used. Following heat denaturation, DNA was amplified in an Eppendorf MMastercycler thermocycler (Westbury, NY) as follows: 94 °C for 30 s, 56 °C for 30 s, 72 °C for 40 s for 40 cycles followed by a 72 °C postdwell for 2 min. Amplified DNA was separated by agarose gel electrophoresis and imaged (Polaroid, Cambridge, MA) under UV light.

RESULTS

Unphosphorylated PP2A Is Associated with Tight Junction Proteins in Differentiated Human Airway Epithelia—To study the cellular mechanisms regulating the integrity of tight junctions in epithelia, we characterized the tight junction in our model system of primary cultures of differentiated human airway epithelia as well as in Calu-3 cells, a pulmonary adenocarcinoma cell line that forms epithelia. In both these systems, cells are grown at the air-liquid interface.

 Though the tight junction is composed of a large complex of proteins (12), our studies focused on occludin, claudin-1, PP2A, and aPKC. Following immunoprecipitation of PP2A, Western blot analysis showed that occludin, claudin-1, and aPKC co-immunoprecipitated with PP2A, suggesting all four proteins form a complex in airway epithelia (Fig. 1A). Similarly, occludin and claudin-1 co-immunoprecipitate from intact epithelia (Fig. 1B).
Studies by Guo et al. (13), Chen et al. (14), and Chen et al. (15) suggested that the catalytic subunit of PP2A can be tyrosine phosphorylated. Based on these data, we asked what is the phosphorylation status of PP2A in intact epithelia. Fig. 2A shows that when tight junctions are undisturbed, the catalytic subunit of PP2A is not tyrosine phosphorylated.

Compromised Epithelial Integrity Dissociates the Tight Junction Complex—Diseases of the airway are generally associated with loss of epithelial barrier function, suggesting that the maintenance of barrier function is critical for normal respiratory physiology. Maintenance of epithelial barrier function is directly related to the state of the tight junction. Therefore, we asked what is the state of the tight junction complex when epithelial integrity is compromised. Calcium chelation with EGTA leads to a decrease in transepithelial resistance (16), a hallmark of compromised barrier function. Fig. 1 shows that following EGTA treatment, PP2A no longer associates with occludin, claudin-1, or PKC, suggesting that disrupting epithelial integrity is associated with dissociation of the tight junction complex. The co-immunoprecipitation of occludin and claudin-1 is similarly lost following EGTA (Fig. 1B).

Compromised Epithelial Integrity Leads to Phosphorylation of PPSA—As mentioned above, PP2A activity can be regulated by tyrosine phosphorylation. In fact, EGF stimulation of cells overexpressing erbB1 leads to tyrosine phosphorylation of PP2A and inhibition of its phosphatase activity (14, 15). erbB receptors localize to the basolateral membrane in intact airway epithelia where they are inactive. However, loss of epithelial integrity leads to erbB receptor activation (1). As shown in Fig. 1, loss of epithelial integrity correlates with disruption of the tight junction complex. Because this also activates erbB1, we asked whether an additional consequence is the tyrosine phosphorylation of PP2A. To specifically activate erbB1, epithelia were first treated with EGTA and then stimulated with EGF. Fig. 2A shows that under this condition, PP2A was tyrosine phosphorylated. Interestingly, basolateral EGF treatment alone was not sufficient to result in tyrosine phosphorylation of PP2A (data not shown), suggesting that PP2A remains complexed at the tight junction and inaccessible to phosphorylation by activated erbB1. These data also suggest a geographical relationship exists between erbB1 and PP2A. To study this more closely, intact or EGTA-treated epithelia were immunostained and analyzed by confocal microscopy. Fig. 2D shows that in intact epithelia erbB1 predominantly localizes basal to the tight junction as previously demonstrated (1). Though PKC immunolocalizes to areas within the cytoplasm, as well as at the apical surface, some also localizes to cell–cell junctions. This is most clearly visualized en face (Fig. 2E). Likewise, though PP2A immunolocalizes to several cellular sites, some also localizes to the tight junction (Fig. 2C). Interestingly, following EGTA treatment, though PKC remains at cell–cell junctions, the level of immunostaining of PP2A in this region seems to significantly diminish. Similarly, EGTA treatment leads to a change in erbB1 localization. These immunocalization data suggest that a breakdown of the junction mediated by EGTA changes the geographical relationship between these proteins. EGF, airway epithelial cultures were treated basolaterally with EGTA. Control cultures were not treated. Following lysis, PP2A was immunoprecipitated and analyzed by phosphatase assay. PP2A phosphatase activity is shown relative to control (100%) activity.
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Both sets of epithelia were then basolaterally treated with EGTA. Basolateral treatment allowed for disruption of tight junctions without dilution of the endogenous apical ASL. Under these conditions we found that PP2A was tyrosine phosphorylated in those epithelia where the ASL was present (Fig. 2B). Washing away of the ASL resulted in a weakened signal. These data suggest that disruptions of epithelial integrity activate a system (or systems) that result in tyrosine phosphorylation of PP2A. Based on our data and that of others (15), this system likely includes erbB receptors.

Tyrosine Phosphorylation of PP2A Decreases Its Activity—Phosphorylation of PP2A has been associated with inhibition of its activity (13, 15). To determine the functional consequences of tyrosine phosphorylation of PP2A in airway epithelia, phosphatase assays were performed. In this assay, a synthetic peptide containing serine- and threonine-phosphorylated residues is incubated with immunoprecipitated PP2A. PP2A activity leads to phosphate release that, when combined with a molybdate-malachite green solution, results in a color change easily quantified by optical density. Fig. 2F shows that when epithelia were basolaterally treated with EGTA, PP2A activity decreased to ∼50% of control untreated epithelia. It is important to note that immunoprecipitation of PP2A would pull down total cellular PP2A, only a small fraction of which associates with the tight junction. Therefore, complete loss of PP2A activity was not expected (or generated). We hypothesize that the PP2A activity remaining in treated cultures comes from non-tight junction-associated PP2A. However, the assay does not allow for experimental distinction of these different PP2A populations. These data suggest that loss of epithelial integrity results in inhibition of PP2A activity.

Inflammation Activates erbB Receptors—Diseases of the airway characterized by compromised barrier function are generally associated with inflammation. In fact, Coyne et al. (17) demonstrated that treatment of airway epithelia with the inflammatory cytokines TNFα/IFNγ leads to decrease in transepithelial resistance in vitro. Similarly, we found that 48 h of TNFα/IFNγ treatment significantly reduced transepithelial resistance (Fig. 3A). In addition, our data demonstrating disruption of the tight junction complex following EGTA treatment (Fig. 1) predict that the localization of tight junction proteins would also be altered under conditions that compromise integrity, including TNFα/IFNγ treatment. Fig. 3B shows that the localization of both occludin and claudin-1 is altered following TNFα/IFNγ treatment.

An additional consequence of compromised epithelial integrity is the activation of erbB receptors. We have previously demonstrated that EGTA treatment, as well as mechanical injury, leads to erbB receptor activation (1). As expected, treatment with TNFα/IFNγ also leads to erbB receptor activation. Fig. 3C shows the presence of phosphorylated (activated) erbB1 following TNFα/IFNγ, EGTA, and mechanical injury. In the presence of intact junctions, erbB1 was not phosphorylated. These data suggest that any interventions that compromise epithelial integrity (and thus the tight junction), including inflammation, lead to activation of erbB1.

Activation of PKC-α with 20 nM phorbol 12-myristate 13-acetate for up to 5 days resulted in a time-dependent increase in Rt from 858 ± 41 Ω·cm² at baseline to 1771 ± 64 Ω·cm² by day 5 (p < 0.01). After 5 days, untreated epithelia showed a resistance of 1276 ± 46 Ω·cm² (p < 0.01 compared with phorbol 12-myristate 13-acetate). However, phorbol 12-myristate 13-acetate has been shown to decrease amiloride-sensitive sodium currents, and thus we cannot conclude that the increase in Rt is solely due to an effect on the paracellular pathway (18).

We wondered whether, like EGTA, TNFα/IFNγ treatment would lead to inhibition of PP2A activity. Fig. 3D shows that these inflammatory cytokines decrease PP2A activity to the same level as EGTA treatment. Taken together, these data suggest that inflammatory-mediated loss of epithelial integrity also functionally compromises PP2A activity. As a further test, we asked whether inhibition of PP2A with okadaic acid could reverse the effect of TNFα/IFNγ on transepithelial resistance. Fig. 3E shows that inclusion of 1 nM okadaic acid to TNFα/IFNγ reverses the effect of TNFα/IFNγ alone on transepithelial resistance, suggesting a role of phosphatase activity in tight junction function.

FIGURE 3. A, epithelia were treated basolaterally with TNFα/IFNγ for up to 48 h. Transepithelial resistance was measured to determine cytokine effect on epithelial integrity. Control cultures were not treated with cytokine. B, en face confocal images of control and cytokine-treated epithelia immunostained for occludin and claudin-1. C, three mechanisms of compromising epithelial integrity (TNFα/IFNγ, mechanical wound, and EGTA) were tested for their effect on the activation (phosphorylation) of erbB1 by immunoprecipitation/Western blot analysis. All three methods of compromising transepithelial resistance led to phosphorylation of erbB1. In intact, untreated epithelia erbB1 was not phosphorylated. D, phosphatase assay demonstrates that epithelial treatment with TNFα/IFNγ also leads to a decrease in PP2A activity that is equivalent to inhibition of total cellular PP2A by inclusion of okadaic acid. E, inclusion of okadaic acid to TNFα/IFNγ reverses the effect of TNFα/IFNγ alone on transepithelial resistance, suggesting a role of phosphatase activity in tight junction function.

erbB1-activating Ligands Are Expressed Apically in Airway Epithelia—The intimate association between tight junction integrity and silencing/activation of the erbB receptors (1) prompted us to hypothesize that signaling between these two systems is essential for sensing and regulating epithelial integrity. Based on the demonstrated ability of erbB1 to tyrosine phosphorylate PP2A (15), we focused on erbB1. It should be stressed, however, that in airway epithelia all four erbB receptors are expressed and redundant functions are likely, including the regulation of PP2A activity. In fact, phosphatase assays were performed following EGTA treatment in the presence of PD153035, an inhibitor of erbB1 tyrosine kinase activity. However, despite this inhibition, PP2A activity was still decreased to the same degree as in untreated controls (data not shown). These data suggest that other erbB receptors or other systems are able to inhibit PP2A activity following the loss of epithelial integrity.

Our previous work demonstrated that airway epithelia lack EGF, a ligand for erbB1. Therefore, we wondered what, if any, erbB1-activating ligands were expressed by airway epithelia. RT-PCR demonstrated that transcripts for TGF-α, heparin-binding epidermal growth factor, epiregulin, and amphiregulin are present (Fig. 4A). Transcript for betacellulin was absent. Heregulin-α, the ligand for erbB3 and -4, was included...
as a positive control (1). Given that erbB1 is not phosphorylated (activated) in intact epithelia (Fig. 3C), erbB1-activating ligands must be apically localized. Immunolocalization of amphiregulin (Fig. 4B) confirmed this prediction. These data suggest that several erbB1-activating ligands are expressed by the airway and sorted apically.

erbB1 Signaling Maintained until Epithelial Integrity Restored—Taken together, the above data suggest a mechanism that instantly signals the loss of epithelial integrity, possibly via erbB receptor activation. This system relies on the availability of ligands in the ASL. Therefore, we wondered whether chronic inflammatory conditions (such as are present in cystic fibrosis and asthma) might, in time, deplete ASL ligands and short-circuit this regulatory mechanism. Given the importance of sensing loss of integrity, we hypothesized that erbB1 activation triggers either ligand synthesis, release of intracellular ligand stores, or cleavage of transmembrane ligand precursors to ensure the continued presence of ligand. To test this hypothesis, we first determined the levels of one erbB1 ligand at steady state. Fig. 5 shows enzyme-linked immunosorbent assay data from human airway epithelial cell lysate demonstrating low levels of TGF-α (~10 pg/ml). Analysis of several other donors suggested variability in TGF-α levels ranging from low to undetectable (data not shown). Interestingly, when the erbB1 was activated by basolaterally administered ligand for 24 h prior to assay, TGF-α levels increased significantly (to ~25–30 pg/ml). These data suggest that activation of the erbB1 initiates a signaling cascade(s) that increases the availability of at least one of its own ligands, TGF-α. Such a cyclic system could serve to ensure continued erbB1 activation until restoration of tight junction integrity is completed. Restoration of integrity silences erbB signaling by restricting ligand access to receptor.

DISCUSSION

Here we have described a mechanism utilized by polarized airway epithelia to immediately detect the loss of epithelial integrity while simultaneously activating signaling cascades to quickly restore tight junction competency. Such a system serves a protective function for epithelia when even a transient loss of tight junction function leaves the epithelium vulnerable to environmental insults (e.g. bacterial, viral, allergen). Therefore, the ability to sense and restore barrier function is essential for normal respiratory function.

The presence of aPKC and PP2A at the tight junction positions these signaling molecules at a pivotal location where they not only function to maintain tight junction proteins at the membrane and as components of the complex but also work in reassembly of the tight junction following an insult. We have shown that in primary cultures of differentiated human airway epithelia, occludin, claudin-1, PP2A, and aPKC form a complex localized to cell-cell junctions. When epithelia are intact, tight junction proteins co-immunoprecipitate, demonstrating the presence of a complex (Fig. 1, A and B). Immunolocalization studies demonstrated that some expressed aPKC and PP2A localize to cell-cell junctions (Fig. 2C). The loss of epithelial integrity correlates with the loss of co-immunoprecipitation of these junctional proteins, demonstrating a breakdown of the complex (Fig. 1, A and B). Interestingly, though EGTA treatment decreased the amount of PP2A localized at cell-cell junctions, aPKC junctional localization remained unchanged (Fig. 2C). We speculate that its retention may be required to direct newly phosphorylated junctional proteins to the appropriate contact site. EGTA treatment, though effective, remains an in vitro tool with little physiologic relevance. Therefore, we tested whether two inflammatory cytokines, TNFα and IFNγ, were able to alter transepithelial resistance as previously demonstrated by Coyne et al. (17). After 48 h, cytokine treatment resulted in a significant decrease in Rt (Fig. 3A). In addition when immunolocalized, occludin and claudin-1 staining was absent in the majority of TNFα/IFNγ-treated cells. This was in stark contrast to control cells where occludin and claudin-1 expression was evident at all cell-cell contacts in a characteristic chicken wire pattern (Fig. 3B). These changes could account for the decrease in Rt. Importantly, inclusion of okadaic acid to the cytokine mixture reversed their effect on Rt, again (Fig. 3E) supporting a role of phosphatase activity in the regulation of epithelial integrity.

Loss of epithelial integrity allows ASL growth factors access to their basolaterally localized receptors. Here, we have shown phosphorylation of erbB1 following mechanical injury and EGTA and TNFα/IFNγ treatment, all of which decrease transepithelial resistance (Fig. 2 and Ref. 1). Importantly, in controls where the tight junctions are intact, erbB1 is silenced (not phosphorylated). Based on the report of Chen et al. (15) demonstrating tyrosine phosphorylation of PP2A by erbB1, we wondered whether a consequence of erbB1 activation in epithelia was the phosphorylation of PP2A. We have shown that PP2A was tyrosine phosphorylated only following the loss of epithelial integrity (Fig. 2A) and was dependent on the presence of ASL factors (Fig. 2B). Moreover, phosphorylation of PP2A also correlated with a decrease in its activity (Fig. 2F). Taken together, these data suggest that ASL-dependent activation of the erbB receptors leads to phosphorylation and subsequent inhibition of PP2A.

Our initial hypothesis was that a balance exists between PP2A and aPKC activities when epithelial integrity is intact. Thus, the inhibition of PP2A activity would disrupt this balance and increase the rate of tight junction reassembly by shifting the balance in favor of aPKC activity. In
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this way, restoration of epithelial integrity is ensured. Our data support this hypothesis.

The literature suggests that erbB1 activation is required for the regulation of PP2A activity. However, when erbB1 tyrosine kinase activity was inhibited with PD153035, basolateral EGTA treatment still resulted in decreased PP2A activity (data not shown), suggesting that other erbB receptors also regulate PP2A activity. Given the importance of maintaining airway epithelial barrier function intact, it may not be surprising that redundancy exists in this regulatory mechanism.

Though ASL factors are present at equilibrium and able to activate erbB receptors following loss of integrity, we wondered whether in the context of chronic injury or infection/inflammation, ASL components become depleted and thus ineffective in stimulating receptor activation. Interestingly, we found that though basal TGF-α levels are low in intact epithelia, chronic activation of erbB1 resulted in an increase of TGF-α protein (Fig. 5). This mechanism would ensure the continued supply of ligand, and thus erbB1 activation, until tight junctions are resealed. Once resealed, ASL factors become apically restricted and unable to activate erbB receptors. The receptor is thus silenced. This auto-shutoff mechanism elegantly maintains epithelial polarity regulation. Meyer et al. (19) highlight the roles of tyrosine kinases and protein kinase C in the

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reassembly of tight junctions. In their reversible model of H2O2-induced disassembly of tight junctions in Madin-Darby canine kidney cells, they found that only inhibitors of tyrosine kinases (PP-2 and genistein) and protein kinase C (calphostin C, bisindolylmaleimide I, and chelerythrine) were able to inhibit tight junction reassembly. These data support our findings that aPKC and erbB1 activities play a role in tight junction reassembly.

The regulatory mechanism described here is similar to that described for the adherens junction. The adherens junction is another specialized cell-cell complex of proteins that lies just below the tight junction. Cadherin proteins form homotypic associations between adjacent cells and are intracellularly linked to the cytoskeleton via association with a complex of proteins, including β-catenin. Xu et al. (20) demonstrated that PTP1B, a non-receptor tyrosine phosphatase, associates with N-cadherin and regulates the function of the adherens junction via its dephosphorylation of β-catenin. Dephosphorylation of β-catenin stabilizes and maintains the integrity of the adherens junction. In addition to interacting with a phosphatase, the adherens junction complex also associates with a kinase, Fic (21, 22). In fact, phosphorylation of β-catenin by Fic has the opposite effect on the adherens junction as its dephosphorylation by PTP1B, that is dissociation of the adherens junction (23, 24). Thus, a balance between kinase and phosphatase activity at the adherens junction also appears to regulate the integrity of this cell-cell adhesion complex. Taken together with the data presented here, the above studies suggest that the regulation of cell-cell adhesion complexes by kinases and phosphatases may be a general mechanism of regulating epithelial integrity.

Given the importance of maintaining polarity and barrier function of epithelia, there are likely redundant systems that could compensate for the loss of any one of the components in the mechanism we describe (e.g. erbB1 activity may be compensated for by erbB2–4, also basolaterally localized in the airway epithelium). This study serves to highlight the importance of preserving polarity and the tight junction. Understanding the mechanisms regulating epithelial integrity will identify new therapeutic targets for diseases that compromise epithelial integrity.

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