Ga12 regulates epithelial cell junctions through Src tyrosine kinases

Tobias N. Meyer, Jennifer Hunt, Catherine Schwesinger, and Bradley M. Denker

Regulatory and assembly of the epithelial cell junctional complex involve multiple signaling mechanisms, including heterotrimeric G proteins. Recently, we demonstrated that Ga12 binds to the tight junction scaffolding protein ZO-1 through the SH3 domain and that activated Ga12 increases paracellular permeability in Madin-Darby canine kidney (MDCK) cells (Meyer et al. J Biol Chem 277: 24855–24858, 2002). In the present study, we explore the effects of Ga12 expression on tight and adherens junction proteins and examine downstream signaling pathways. By confocal microscopy, we detect disrupted tight and adherens junction proteins with increased actin stress fibers in constitutively active Ga12 (QLa12)-expressing MDCK cells. The normal distribution of ZO-1 and Na-K-ATPase was altered in QLa12-expressing MDCK cells, consistent with loss of polarity. We found that the tyrosine kinase inhibitor genistein and the Src-specific inhibitor PP2 reversibly abrogated the QLa12 phenotype on the junctional complex. Junctional protein localization was preserved in PP-2- or genistein-treated QLa12-expressing cells, and the increase in paracellular permeability as measured by transepithelial resistance and [3H]mannitol flux was prevented by the inhibitors. Src activity was increased in QLa12-expressing MDCK cells as assessed by Src autophosphorylation, and β-catenin tyrosine phosphorylation was also increased, although there was no detectable increase in Rho activity. Taken together, these results indicate that Ga12 regulates MDCK cell junctions, in part through Src tyrosine kinase pathways.

Address for reprint requests and other correspondence: B. M. Denker, Renal Division, Brigham and Women’s Hospital and Harvard Medical School, Harvard Institutes of Medicine, 77 Ave. Louis Pasteur, Boston, MA 02115. (E-mail: bdenker@rics.bwh.harvard.edu).

http://www.ajpcell.org 0363-6143/03 $5.00 Copyright © 2003 the American Physiological Society C1281
expressing MDCK cell lines, we demonstrate that QL12 reversibly disrupts tight and adherens junction proteins, alters polarized localization of membrane proteins, and increases paracellular permeability through activation of Src tyrosine kinase pathways.

MATERIALS AND METHODS

Materials and cell lines. cDNAs for wt12 and QL12 were kindly provided by Henry Bourne (University of California, San Francisco). Establishment and characterization of Tet-Off MDCK cell lines with inducible QL12 expression are described elsewhere (37). Polyclonal anti-Gα12 antibodies (S-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-α-, β-catenin, anti-occludin, anti-claudin-1, and anti-E-cadherin from Zymed Laboratories (San Francisco, CA), monoclonal anti-phosphotyrosine (pTyr; 4G10), monoclonal anti-Na-K-ATPase, and the Rho activation assay from Upstate Biotechnology (Lake Placid, NY), and anti-c-Src, anti-pTyr1152-Src, and anti-pTyr253-Src from Biosource International (Camarillo, CA). Anti-ZO-1 rat monoclonal antibody (R40.76) was kindly supplied by D. Goodenough (Harvard University). Phalloidin coupled to Alexa Fluor 568 (excitation at 578 nm and excitation at 600 nm) was purchased from Molecular Probes (Eugene, OR). Tet-Off MDCK type II epithelial (T-23 MDCK) cells, Tet-Off MDCK II epithelial (T-23 MDCK) cells, Tert-Off cloning vectors, and genistein from Sigma, PP-2 from Calbiochem (San Diego, CA), and the ohm well, CA), plasticware from Falcon (Lincoln Park, NJ), Trans-well filters [polycarbonate, permeable (0.4 μm pore, 6 and 12 mm diameter)] from Costar (Cambridge, MA), and the ohm meter (Millicell ERS) from Millipore (Bedford, MA).

Cell culture. Tet-Off MDCK cells were incubated at 37°C in 5% CO2 and maintained in Dulbecco’s modified Eagle’s medium (Cellgro, Herndon, VA) containing 5% Tet system-approved PBS (Clonetics) and 100 μg/ml G418, 50 IU/ml penicillin, and 50 μg/ml streptomycin (Sigma, St. Louis, MO). MDCK clones were maintained in medium containing 100 μg/ml hygromycin, 100 μg/ml G418, and 40 ng/ml doxycy-cline. Subsequent experiments with conditional expression of the Ga subunits were carried out in the same medium without hygromycin and G418 and with or without doxycycline.

Dome formation and inhibitor studies. Ga12- and QL12-transfected Tet-Off MDCK cells were grown to confluence in 12-well plates with doxycycline (40 ng/ml) before they were switched to medium with or without doxycycline and with or without the tyrosine kinase inhibitors genistein (100 μM) or PP-2 (10 μM). Dome formation was evaluated by counting typical dome structures with an inverted microscope (Eclipse TE300, Nikon) at ×100 magnification in 10 consecutive visual fields.

Immunohistochemistry. Ga12- and QL12-transfected Tet-Off MDCK cells were plated on glass coverslips or Transwell filters and grown to confluence with or without doxycycline for specific times. The cells were fixed with 4% paraformal-dehyde (EM Sciences, Fort Washington, PA) in PBS for 20 min at room temperature or 100% methanol at −20°C and rinsed with PBS three times for 5 min. Immunohistochemistry was performed in various double-staining combinations. The cells were blocked for 45 min at room temperature in 5% (wt/vol) nonfat milk. Primary antibody combinations were diluted 1:100 in PBS or ZO-1 hybridoma medium and incubated at 4°C for 1.5 h with periodic gentle shaking. Three rinses with 0.05% Triton X-100 in PBS for 5 min each at room temperature preceded the 1-h incubation at 4°C with the corresponding secondary antibody mix. Texas red or fluorescein isothiocyanate-conjugated secondary antibodies (Pierce, Rockford, IL) were diluted 1:100 in 0.05% Triton X-100 in PBS. Coverslips were washed three times with 0.05% Triton X-100 in PBS for 5 min at room temperature, mounted with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL), and viewed through a ×100 oil immersion objective with a laser scanning confocal system (model MRC-1024/2p, Bio-Rad) coupled to a Zeiss Axiovert microscope. Localization of proteins was determined after separate excitation/emission of both labeled proteins. Images were processed using Photoshop software (Adobe).

Immunoprecipitation and Western immunoblot analysis. The wt12- or QL12-expressing cells were cultured with or without doxycycline for 3 days. To obtain whole cell lysates, monolayers were scraped in lysis HEPES buffer (100 mM NaCl, 2 mM EDTA, 10 mM HEPES, pH 7.5, 1 mM NaVO4, 25 mM NaF, 1 mM PMSF, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) and subjected to brief sonication on ice. Protein A-Sepharose beads and antibodies against α-catenin, β-catenin, ZO-1, occludin, and claudin-1 were added to the lysate overnight at 4°C on a shaker. Beads were washed four times with lysis buffer, and proteins were tracted. 1-h or QL12-expressing cells were cultured with or without doxycycline before exposure of the membrane to the primary antibody for 1 h at room temperature. After the lysates were washed (1 h in Tris-buffered saline + 0.05% Tween 20; Sigma), secondary horseradish peroxidase-conjugated antibodies (Jackson Laboratories, West Grove, PA) were used at 1:10,000 for 1 h at room temperature, and signal was detected with SuperSignal West Pico horseradish peroxidase substrate system (Pierce) and autoradiography (Biomax MR, Eastman Kodak, Rochester, NY).

Transepithelial resistance measurements. MDCK cells were plated on polycarbonate filters (Transwell, Costar) at 3 × 105 cells/cm2 and maintained in culture medium + doxycycline for 48–72 h to establish tight monolayers. Similar effects on transepithelial resistance (TER) were obtained when cells were plated at 1.2 × 106 cells/cm2. TER was measured at different times after change to a doxycycline-free medium with a Millipore electrical resistance system, as described previously (55). Measurements (in ohm·cm2) are expressed as a mean of the original readings after substraction of background values.

Mannitol flux rates. MDCK cells expressing wt12 and QL12 were cultured for 3 days on 6.5-mm Transwell filters in the presence of doxycycline before half of the filters were switched to doxycycline-free medium with or without tyrosine kinase inhibitors for an additional 3 days. Filters were washed three times with Hanks’ balanced salt solution (HBSS) and incubated in HBSS with 1 mM mannitol in both chambers. At time 0, 1.4 μCi of [3H]mannitol (17 Ci/mmol; Perkin-Elmer Life Sciences, Boston, MA) were added to the apical chamber, and plates were stirred using an orbital shaker at 37°C. Aliquots (100 μl) were removed from the basolateral chamber at 30, 60, 120, and 180 min and replaced with an identical volume of HBSS + 1 mM mannitol. Counts were determined in scintillation fluid using a liquid scintillation counter (Packard Tri-Carb A2200). Background was subtracted, and counts per minute were converted to picomoles of mannitol, and rates were determined from linear regression analysis of the four time points using GraphPad Prism.

Rho activation assay. After 3 days of culture with or without doxycycline and tyrosine kinase inhibitors, wt12- or QL12-expressing cells were lysed with ice-cold lysis buffer
Go12 regulation of cell junctions

[50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl2, and protease inhibitors as supplied by the manufacturer (Upstate Biotechnology)]. Samples were sonicated on ice and centrifuged for 10 min at 13,000 rpm. Four hundred microliters of supernatant were added to 30 μl of glutathione-agarose slurry linked to rhodotin Rho-binding domain, which specifically binds activated (GTP-bound) Rho (buffer: 50 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, 150 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, and protease inhibitors), and incubated for 45 min at 4°C. Agarose beads were centrifuged and washed three times. Activated Rho was eluted with SDS-PAGE sample buffer, denatured for 5 min at 95°C, and analyzed by Western blot with purified polyclonal anti-RhoA, anti-RhB, or anti-RhC antibody (Upstate Biotechnology). Positive and negative controls were prepared with uninduced wtα12- or QLα12-expressing cell lysates. Before incubation with glutathione-agarose slurry, guanosine 5′-O-(3-thiotriphosphate) (100 μM final concentration) or GDP (1 mM final concentration) was added to 400 μl of supernatant at 30°C for 15 min.

Statistics. Values are means ± SE; n refers to the number of measurements. Paired Student’s t-test was used to compare mean values within one experimental series. Data from two groups were compared by unpaired t-test. P < 0.05 was accepted to indicate statistical significance.

RESULTS

Expression of activated (QL) Go12 affects dome formation in MDCK cells. We previously showed that activated Go12 (QL) expressed in MDCK cells reversibly increased paracellular flux and lowered TER (37). Figure 1 demonstrates another consequence of leaky epithelia, i.e., the loss of domes. Domes are focal areas of fluid accumulation that appear in confluent monolayers of MDCK cells and arise from vectorial transport of fluid across the monolayer. Domes arise in areas where pressure from accumulated fluid exceeds the binding to the culture plate (4, 30), and dome formation can be inhibited by drugs that block transporters or ion pumps (e.g., ouabain) or interventions that disrupt the junctional complex (e.g., low calcium). Figure 1A shows QLα12 protein levels as a function of doxycycline concentration, and Fig. 1, B and C, shows the inverse correlation between QLα12 protein levels and dome formation. In the absence of doxycycline, domes were completely absent (Fig. 1B, d), and there was an inverse correlation between QLα12 protein levels and the number of domes visualized (Fig. 1, A and C). There were no differences among wtα12- and QLα12-expressing MDCK cell lines in the presence of doxycycline (no Go12 expression), and the number of domes was unchanged by inducing wtα12 protein for 72 h (Fig. 1, B, a and c, and Fig. 1C).

Expression of QLα12 in MDCK cells disrupts tight and adherens junction proteins. We next examined the staining pattern of junctional proteins in the presence and absence of wtα12 and QLα12 expression (Fig. 2). We previously showed that the pattern of ZO-1 staining was significantly altered with QLα12 expression, and we now extend these observations to other tight and adherens junction proteins. There were no observable differences in staining patterns for tight and adherens junction proteins with induced wtα12 expression (results not shown), and there were no differences between cells grown on coverslips and those grown on Transwell filters. In QLα12-expressing MDCK cells, the tight junction proteins claudin-1 and occludin and the adherens junction proteins E-cadherin and β-catenin show altered localization. The patterns were similar for ZO-1, occludin, β-catenin, and E-cadherin, with thickened and disrupted linear membrane staining (Fig. 2). The localization of claudin-1 was most perturbed, with nearly complete loss of membrane staining and development of punctate intracellular staining (Fig. 2, C and D). Activation of β-catenin often results in translocation into the cytoplasm and to the nucleus (36). In QLα12-expressing MDCK cells, β-catenin tyrosine phosphorylation was increased (see Fig. 5), but we did not detect significant cytosolic localization of β-catenin or any colocalization with the nuclear stain 4′,6-diamidino-2-phenylindole (not shown).

Epithelial cell polarity is disrupted and cortical actin staining increased with QLα12 expression. Because expression of QLα12, but not wtα12, resulted in significant changes in junctional protein staining and increased paracellular permeability, polarity of the epithelial cell monolayer was likely to be disrupted. The tight junction scaffolding protein ZO-1 is normally restricted to a discrete plane in the tight junction at the interface between the apical and the basolateral membrane domains. Na-K-ATPase is more broadly expressed along the basolateral membrane but restricted from the apical membrane domain. Normally, there is little overlap of these two proteins in MDCK cells, and in the presence of doxycycline, this separation is apparent in QLα12-expressing MDCK cells (Fig. 3A, a and e, and c and g). However, induction of QLα12 protein (doxycycline-free medium) caused loss of this separation, with ZO-1 appearing diffusely along the lateral membrane and in the apical membrane (Fig. 3A, f, and Fig. 3B, b, arrow) and the Na-K-ATPase partially localizing in the apical membrane (Fig. 3A, d, and Fig. 3B, d, arrow). Expression of wtα12 caused no significant changes in the actin cytoskeleton (see Fig. 6A), whereas induction of QLα12 protein resulted in altered cell shape and increased actin stress fibers (Fig. 3B, e and f; see Fig. 6B). Actin staining was similar in QLα12- and wtα12-expressing MDCK cells in the absence of Go12 expression (doxycycline-containing medium; see Fig. 6). These findings indicate that QLα12 expression causes cell rounding, increased stress fibers, and loss of separation between proteins that are normally restricted to specific membrane domains.

Paracellular regulation of MDCK cell junctions by Go12 involves Src tyrosine kinase(s). Tyrosine kinases have been shown to regulate epithelial cell junctions (56, 61), and several have been shown to be immediate downstream effectors of Go12 (24). We found that genistein (a well-described broad inhibitor of tyrosine kinases) and PP-2 [a highly specific Src kinase family inhibitor that has been crystallized with a Src family member (51)] prevented the decrease in TER seen in QLα12-expressing MDCK cells (Fig. 4A). Baseline TER in Tet-Off MDCK
cells was ~75 Ω·cm², which is a little lower than values reported by some groups (26) but consistent with values reported by others (34). Neither PP-2 nor genistein affected baseline TER under control conditions (doxycycline-containing medium), and neither compound affected Gα12 protein expression or levels of ZO-1 (by Western blot; not shown). PP-2 completely blocked the fall in TER typically seen in QLα12-expressing MDCK cells. Interestingly, genistein caused a significant increase in TER throughout the experiment. The effects of PP-2 and genistein on blocking the QLα12-induced fall in TER were completely reversible. With removal of the inhibitors and return to doxycycline-free medium (at ~120 h; Fig. 4A), there was a rapid fall in TER to the level seen in untreated monolayers. To confirm that the effects of the inhibitors were on paracellular flux and not on transcel-

---

Fig. 1. QLα12 expression and dome formation in Madin-Darby canine kidney (MDCK) cells. A: Western blot of Gα12 after 48 h of induction of QLα12-expressing cells with doxycycline (0–40 ng/ml). Expression was repressed within 48 h upon readdition of doxycycline after 48 h in the absence of doxycycline (right lane). B: phase-contrast microscopy of wtα12- and QLα12-expressing MDCK monolayers at baseline (in doxycycline-containing medium (+dox)) and 72 h after induction of Gα12 expression. Monolayers expressing wtα12 showed typical dome formation without (a, +dox) or with (c, −dox) induction of wtα12 expression. In QLα12-expressing MDCK cells, dome formation was observed without induction of QLα12 expression (b). After 72 h of QLα12 expression (d, −dox), dome formation was no longer observed. Scale bar, 50 μm. C: quantitation of dome formation as a function of doxycycline concentration at 72 h of QLα12 or wtα12 expression. There is a significant difference in domes of QLα2 MDCK cells starting at 1 ng/ml doxycycline from uninduced monolayers (40 ng/ml).
lular ion flow, we measured paracellular flux of [³H]mannitol in wild-type and QLα₁₂-expressing MDCK cells in the presence and absence of Go₁₂ expression and inhibitors. Figure 4B demonstrates a large (~7-fold) increase in paracellular flux with induction of QLα₁₂ expression. The increase in flux from QLα₁₂ expression was significantly but not completely inhibited by PP-2 and genistein. Both inhibitors resulted in a small increase in flux of monolay-

Fig. 2. Immunofluorescent localization of tight and adherens junction proteins in QLα₁₂-expressing MDCK cells. Images were obtained before (+dox) and after (−dox) induction of QLα₁₂ expression for 48 h. Cells were stained for ZO-1 (A and B), claudin-1 (C and D), occludin (E and F), β-catenin (G and H), and E-cadherin (I and J). Scale bar, 25 μm.
ers in doxycycline-containing medium, perhaps because of nonspecific effects from exposure to the inhibitors for 72 h. Although much less dramatic, similar findings were observed in wt/H925112-overexpressing MDCK cells. There was a small increase in mannitol flux that was also blocked by the inhibitors (Fig. 4B).

Src is activated and β-catenin phosphorylation increased without detectable Rho activation in QLα12-expressing MDCK cells. On the basis of the observation that PP-2 blocked the QLα12 protein effects on the MDCK cell junction and the report that Src is activated by Gα12 (28, 41), we investigated c-Src activity in QLα12- and wtα12-expressing cells. Western blots of wtα12- and QLα12-expressing MDCK cell lysates prepared from cells grown in medium with and without doxycycline showed no significant difference in total c-Src immunoreactivity (Fig. 5A). Src is activated by phosphorylation of pTyr419 but is normally maintained in an inactive conformation through intramolecular interactions between the SH2 domain and the phosphorylated COOH-terminal tail (pTyr530) (for review see Ref. 58). Therefore, increases in phosphorylation of Tyr419 or decreases in Tyr530 can be used as evidence of Src activation. Without Gα12 expression (doxycycline-containing medium), phosphorylation of pTyr419 (as detected with pTyr419-specific antibody) was minimal in wtα12- and QLα12-expressing MDCK cells, reflecting Src inactivation (Fig. 5A, middle). Inducing QLα12 expression (doxycycline-free medium) caused a significant increase of pTyr419 phosphorylation (4.4 ± 0.4 fold, n = 5) with no detectable change in wtα12-expressing MDCK cells (Fig. 5A, middle). Also, consistent with c-Src activation, there was a concomitant decrease of pTyr530 phosphorylation (3.9 ± 0.3 fold, n = 4) with QLα12 expression, whereas there was no significant difference in pTyr530 Src phosphorylation relative to occludin in wtα12-expressing cells (Fig. 5A, right).

Our confocal studies showed that E-cadherin and β-catenin localization were disrupted in QLα12-expressing MDCK cells (Fig. 2). Gα12 recently was demonstrated to interact with E-cadherin and regulate β-catenin release (35), and other studies have shown that β-catenin tyrosine phosphorylation is increased

---

**Fig. 3.** Immunofluorescent localization of ZO-1, Na-K-ATPase, and actin in QLα12-expressing MDCK cells. A: confocal localization for ZO-1 and Na-K-ATPase in uninduced (+ dox) and induced (− dox) QLα12-expressing MDCK cell monolayers at an apical (a−d) and basal (e−h) plane. B: zx-axis images of uninduced (+ dox) and induced (− dox) QLα12-expressing MDCK cells stained for ZO-1 (a and b), Na-K-ATPase (c and d), and actin (e and f). Note altered distribution of these proteins in QLα12 (− dox)-expressing cells (b, d, and f), including some apical localization of Na-K-ATPase (arrow, d). Arrows in b and d indicate apical staining. Scale bars, 15 μm.
During adherens junction disassembly (72). To determine whether QLα12 expression caused increased phosphorylation of β-catenin, we immunoprecipitated α- and β-catenin from QLα12-expressing MDCK cells in medium with and without doxycycline. In our gel system, α- and β-catenin migrate with nearly identical molecular mass of 98 kDa. Nevertheless, Fig. 5B (top) shows that α-catenin precipitates a significant amount of β-catenin, whereas the β-catenin coprecipitates a much smaller amount of α-catenin. Both α- and β-catenin were immunoprecipitated from cells in medium with and without doxycycline and probed by Western blot with anti-pTyr antibodies (Fig. 5B, bottom). For α- and β-catenin precipitates, there is a significant increase in tyrosine phosphorylation of cells in medium without doxycycline. This finding, combined with the results of Fig. 5B, top, suggests that most tyrosine phosphorylation is occurring on β-catenin, although we cannot rule out changes in α-catenin as well. Similar results were obtained when filters were stripped and reprobed with catenin or pTyr antibodies.

Because Gα12 can activate Rho through Src-dependent and Src-independent mechanisms, we determined whether Rho was activated with QLα12 expression. Gα12 regulation of the actin cytoskeleton is directly linked to Rho signaling via a novel family of Rho exchange proteins [p115 RhoGEF (29)], and Rho has been shown to regulate tight junctions (25, 26, 47, 65). However, in rhotekin-binding assays (Fig. 5C), the amount of activated Rho was not increased in wtα12- or QLα12-expressing MDCK cells 72 h after removal of doxycycline. There was no detectable difference in the quantity of Rho in cell lysates, nor did treatment with PP-2 or genistein significantly affect Rho expression or activation. In support of this observation, treatment of QLα12-expressing MDCK cells with the cell-permeant Rho kinase inhibitor Y-27632 had no effect on QLα12-induced changes in the junction (results not shown).

Tyrosine kinase inhibitors prevent disruption of junctional proteins in QLα12-expressing MDCK cells. Confocal analysis of QLα12-expressing MDCK cells reveals that expression of wtα12 has little effect on the pattern of ZO-1 localization and actin staining at the level of the tight junction (Fig. 6A). Similar to our previous report (37), wtα12 was found along the cell perimeter (Fig. 6A, d), and treatment with genistein or PP-2 did not appear to significantly change the Gα12 staining pattern. Actin staining was unchanged by wtα12 expression (Fig. 6A, c and f) and was not significantly affected by the inhibitors, although a few more stress fibers were evident (Fig. 6A, i and l). In QLα12-expressing MDCK cells, the inhibitors had no major effect on QLα12 localization, but in QLα12-expressing MDCK cells treated with PP-2 or genistein, the inhibitors prevented the disrupted ZO-1 staining pattern normally observed in MDCK cells expressing QLα12 (Fig. 6B, b, e, h, and k). In PP-2 treated cells, the ZO-1 staining pattern was indistinguishable from controls, with a fine linear continuous staining pattern along the lateral membrane (Fig. 6B, k and b). Interestingly, genistein had a novel effect on cell morphology. The ZO-1 staining remained narrow and continuous, but the membrane was no longer linear and contained “microspikes” (Fig. 6B, h). This effect of genistein on cell morphology was not seen in wtα12-expressing MDCK cells (Fig. 6A, h). Unlike wtα12-expressing MDCK cells, actin staining of QLα12-expressing MDCK cells revealed increased stress fibers (Fig. 6B, f), and the nature of these stress fibers was different in the presence of the inhibitors. Transcellular actin filaments appeared significantly shorter in genistein-treated cells with and without inhibitors. Cells were cultured on 6-mm Transwell filters for 72 h in the presence or absence of doxycycline and inhibitor. Fluxes of [3H]mannitol were measured at 0, 30, 60, and 180 min, and rates were determined. A representative experiment is shown with n = 3 for each condition, and results are expressed as means ± SD. Experiment was repeated 3–4 times with similar results.

Fig. 4. Barrier function in QLα12-expressing MDCK cells. A: trans-epithelial resistance (TER) was measured in confluent, uninduced monolayers at the beginning (0 h) and throughout QLα12 induction (−dox) and resuspension (+dox). An identical protocol was followed in the presence of tyrosine kinase inhibitors genistein (Gen, 100 μM) or PP-2 (10 μM). Tyrosine inhibitors were added at the beginning of QLα12 induction (0 h) and were present throughout the experiment. At 124 h, tyrosine kinase-treated monolayers were induced again, but without tyrosine kinase inhibitors (−dox). A simultaneous control of uninduced QLα12-expressing MDCK cells [doxycycline present at all times (+dox only)] is shown for comparison (n = 7 for each time point). B: mannitol flux in wild-type and QLα12-expressing MDCK cells with and without inhibitors. Cells were cultured on 6-mm Transwell filters for 72 h in the presence of doxycycline and inhibitor. Fluxes of [3H]mannitol were measured at 0, 30, 60, 120, and 180 min, and rates were determined. A representative experiment is shown with n = 3 for each condition, and results are expressed as means ± SD. Experiment was repeated 3–4 times with similar results.
than in untreated cells (Fig. 6B, f and i), whereas PP-2 appeared to increase the number and length of stress fibers. Although the inhibitors caused some changes in morphology, the effect on the barrier was less pronounced. PP-2 had no significant effect on the time course of TER development, and genistein caused a significant increase in TER (Fig. 4A). Furthermore, the effect of the inhibitors on the actin cytoskeleton at the level of the tight junction was less pronounced. Figure 7 shows the actin cytoskeleton at the level of the tight junction protein ZO-1. In doxycycline-containing medium, very few stress fibers are visible at the level of ZO-1, and actin staining is predominantly along the lateral margins of the cell, with only focal areas of overlap (yellow in the merged images). With induction of QLα12 (doxycycline-free medium), ZO-1 staining becomes fragmented and more diffuse along the lateral membrane (Figs. 2 and 6), and most of the ZO-1 is colocalizing with actin. Stress fibers are more abundant, and there is increased intensity of cortical actin staining. In the uninduced cells, ZO-1 staining is preserved, although in genistein-treated cells, stress fibers are more visible and the increased intensity of cortical actin staining is still evident. PP-2-treated cells appear more similar to the control condition, with fewer stress fibers and less intense actin staining. In the presence of inhibitors and doxycycline, the pattern is similar to that in medium containing doxycycline only.

**DISCUSSION**

The regulation of the epithelial cell barrier is appreciated to be a complex and highly regulated process. Multiple signaling mechanisms, including heterotrimeric G proteins, monomeric G proteins, serine-threonine protein kinases, tyrosine kinases, and regulated calcium stores, contribute in interconnecting pathways to maintain an intact junction and regulate its biogenesis (for review see Refs. 2, 9, and 14). Ultimately establishing the direct protein interactions and signaling pathways that regulate these processes will require a combination of protein biochemistry, cell culture models, and transgenic animals. Utilizing in vitro binding techniques and immunoprecipitations from cultured MDCK cells, we recently demonstrated a direct interaction between Gaα12 and the tight junction scaffolding protein ZO-1 (37). In addition, we showed that expression of activated Gaα12 (QLα12) in these cells reversibly disrupted the localization of the tight junction protein ZO-1 and increased paracellular permeability as measured by TER and paracellular flux (37). The findings reported here indicate that proteins of the

---

**Fig. 5. Analysis of Src activation, β-catenin tyrosine phosphorylation, and Rho activation in QLα12-expressing MDCK cells.** A: Western blot of Src in MDCK cells after QLα12 (QL) or wtα12 (wt) expression. Whole cell lysates were resolved by SDS-PAGE, and Src was detected by Western blot using anti-c-Src antibodies (left), antiphospho-Tyr419 (pY419) (Src) antibodies (pY419, middle), or anti-β-catenin antibodies. B: Western blot of the same lysates used for pulldown experiments (2% of total lysate). Expression protein kinases, tyrosine kinases, and regulated calcium stores, contribute in interconnecting pathways to maintain an intact junction and regulate its biogenesis (for review see Refs. 2, 9, and 14). Ultimately establishing the direct protein interactions and signal-
tight and adherens junctions are disrupted, and there is significant reorganization of the actin cytoskeleton. These Gα12-mediated changes appear to be predominantly regulated through Src tyrosine kinases. Expression of QLα12 results in a leaky epithelium (37) and is reflected in these studies by the loss of monolayer domes. Dome formation reflects the tightness of the monolayer and is a sensitive indicator of
Alterations in junctional integrity (4, 30). The effect of QLα12 on dome formation was reversible, indicating that QLα12 expression did not irreparably injure or alter the cell. The functional changes in paracellular permeability correlated with morphological changes of the junctional complex. With QLα12, but not wtα12, expression, a striking change was observed in the staining pattern of the tight junction proteins ZO-1, occludin, and claudin-1 and the adherens junction proteins E-cadherin and β-catenin. This effect of QLα12 on junctional proteins appears to be unique within the G protein family. Previous studies expressing wild-type or constitutively active mutants of Gαo, Gαq, or Gαs had no effect on the staining patterns of ZO-1, occludin, or E-cadherin (10, 49, 50). In the QLα12-expressing MDCK cells, claudin-1 localization was most severely affected. Claudins are essential for barrier function (60) and for paracellular regulation of specific ions (52, 62). We hypothesize that the loss of dome formation and barrier function was predominantly mediated by changes in claudin distribution. Because claudins interact with zona occludens proteins (22) and ZO-1 binds to Go12, we speculate that the QLα12-induced changes in ZO-1 localization resulted in the disrupted claudin distribution. However, we cannot exclude the possibility that QLα12 simultaneously disrupts the adherens junction, because tight junction assembly starts with interactions of E-cadherin and E-cadherin also interacts with Go12 (39). Similarly, we found the normal separation of ZO-1 and Na-K-ATPase disrupted in QLα12-expressing MDCK cells. The most likely explanation is the loss of junctional integrity, resulting in diffusion of proteins within the plasma membrane. Although we did not look at other membrane marker proteins and did not explicitly examine protein diffusion, this is most likely a generalized effect of QLα12 on the junction and is not specific to the Na-K-ATPase. This loss of polarity would be expected in epithelial cells with a disrupted junctional complex and changes in protein-protein interactions among junctional proteins.

Several different signaling pathways regulate the barrier of intact epithelia. Activation and overexpression of protein kinase C isozymes or monomeric G proteins, in addition to lowering extracellular calcium, result in disruption of junctions and increased leakiness in kidney epithelial cells (20, 25, 26, 40, 48). Furthermore, we previously showed that activated Go12 and Goα increase baseline TER in MDCK cells (49, 50). Tyrosine kinases are well described in regulation of epithelial cell junctions and are important downstream effectors of Go12. The tyrosine kinase inhibitor genistein and the Src-specific inhibitor PP-2 prevented development of the typical phenotype in QLα12-expressing MDCK cells. Although the effects of these two inhibitors were different, both prevented the increase in paracellular permeability as measured by the fall in TER and increase in mannitol flux. Both inhibitors prevented the disruption of ZO-1 staining, and genistein treatment resulted in a distinct cell shape and altered actin cytoskeleton, which resulted in a less permeable paracellular space. The effects of genistein compared with PP-2 indicate that, in addition to c-Src, other tyrosine kinase pathways are likely to regulate these processes. We further demonstrated a change in c-Src tyrosine phosphorylation pattern that is consistent with Src activation in QLα12-expressing cells. These studies were not designed to distinguish between direct Src activation by Go12 and indirect activation of Src via another mechanism. Go12 directly regulates other tyrosine kinases [Bruton’s tyrosine kinase and pp72y (24, 28)], and several receptors (thrombin, endothelin, and vasopressin) signal...
through Go12 to indirectly activate Src (6). Furthermore, direct Src kinase activation by Go12/13 was shown in thrombin-stimulated platelets and coincided with platelet shape changes (28). Although Go12 signaling is linked to Rho-mediated changes in the actin cytoskeleton [via Rho exchange factors (3, 16–18, 28, 29, 31)], no significant Rho activation was detected in these cell lines. We cannot entirely exclude the possibility that transient Rho activation occurred at earlier time points of junction disassembly, but we would suggest that this is unlikely, because the junctional phenotype was dependent on persistent QL12 expression. It is also possible that other small G proteins (not detected with rhotekin binding), such as Rac1, are downstream of Go12 in this system. In an analogous experimental design, Postma et al. (45) showed that Go12 regulation of gap junctions via Src tyrosine kinases was also independent of Rho activation. The present experiments do not distinguish direct Src phosphorylation of β-catenin from other mechanisms causing increased phosphorylation (e.g., changes in E-cadherin and catenin localization induced by QL12 expression). Future experiments are needed to determine whether the increased β-catenin phosphorylation is important to the Go12/Src-mediated effects on the junction.

Direct effects of Src on the junctional complex are well established. Tyrosine kinase signaling is important in junctional regulation, and Src, in particular, affects junctional assembly and baseline properties. Activation of Src reduces intercellular adhesion but differentially affects tight and adherens junctions. Oncogenic v-Src induced the redistribution of E-cadherin into the cytosol and caused the disassembly of adherens junctions with only marginally altered tight junction structure and function (19, 56). Avian c-Src expression, which does not cause cell transformation in fibroblasts (57), increased tyrosine phosphorylation of the tight junction proteins ZO-1 and ZO-2 and the adherens junction protein β-catenin but not α-catenin (56). This raises the possibility that Src could be directly phosphorylating β-catenin in QL12-expressing MDCK cells. We previously showed that tyrosine phosphorylation of proteins in the region of the tight junction (possibly by Src) is important for tight junction reassembly after exposure to oxidative stress (38). Similarly, in the ATP depletion-repletion model, tyrosine kinase inhibition prevented recovery of the tight junction after ATP depletion, and tyrosine phosphorylation of occludin was found to be critical for the tight junction reassembly (5, 59).

In recent years, there has been a dramatic increase in the number of proteins found in or near the epithelial cell tight junction. In addition, there are a surprisingly large number of interacting scaffolding proteins of the membrane-associated guanylate kinase (MAGUK) family, now identified within the epithelial cell tight junction. These proteins share a common overall structure and typically include multiple protein–protein interaction domains, including multiple PDZ domains (12, 13). Examples of these proteins, known to be located in the epithelial cell tight junction, include ZO-1, ZO-2, and ZO-3 and the related MAGI-1 (43) proteins. In addition, other interactions between junctional adhesion molecule and another MAGUK protein (CASK/LIN-2) have been identified (33). One question arising from these observations is why the tight junction requires so many interacting scaffolding molecules. We previously demonstrated a direct interaction between Go12 and ZO-1 in vitro and in cells and now show that Src tyrosine kinases are important for the Go12-mediated effects on the junction. This raises the possibility that Src is also contained within this multiprotein complex. However, our initial efforts to identify Src in complex with ZO-1 and Go12 have been unsuccessful. This suggests to us that Src is in proximity to ZO-1 and Go12 but may be in direct association with one of the other scaffolding proteins. Our previous results with activated Go12 and Goa in MDCK cells have shown increased rates of tight junction assembly in the calcium switch model in addition to increased baseline TER. Go12 appears to have the opposite effect on the junction, leading us to speculate that multiple G proteins regulate the properties of the junction in opposing directions. Therefore, one of the reasons for concentrating scaffolding molecules in the tight junction may be to coordinate the spatial and functional interactions of these (and other) signaling proteins. If this hypothesis is correct, then we expect to find more direct interactions between these MAGUK proteins and signaling molecules. The future challenge is to identify these interactions and begin to define these regulatory pathways in vivo.

Present address of T. N. Meyer and C. Schwesinger: Universitätsklinik Hamburg-Eppendorf, Dept. of Internal Medicine, Div. of Nephrology, Martinistrasse 52, 20246 Hamburg, Germany.

DISCLOSURES

This work was supported by National Institute of General Medical Sciences Grant GM-55223 (to B. M. Denker), National Institute of Diabetes and Digestive and Kidney Diseases National Research Service Award DK-62678 (to J. L. Hunt), a Clinical Scientist Award from the National Kidney Foundation (to B. M. Denker), and Deutsche-Forschungsgemeinschaft (Bonn, Germany) Grant Me 1760/1-1 (to T. N. Meyer).

REFERENCES


