Gα12 Regulates Epithelial Cell Junctions Through Src Tyrosine Kinases

Tobias N. Meyer¹, Jennifer Hunt² Catherine Schwesinger¹, Bradley M. Denker²

Renal Division, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School¹, Boston, MA and Division of Surgery, Children’s Hospital², Boston, MA.

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¹Current Address:
Universitätsklinik Hamburg-Eppendorf,, Department of Internal Medicine
Division of Nephrology
Martinistrasse 52
20246 Hamburg, Germany

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To whom correspondence should be addressed:
Bradley M. Denker, Division of Nephrology, Brigham and Women’s Hospital and Harvard Medical School, Harvard Institutes of Medicine, 77 Avenue Louis Pasteur, Boston, MA, 02115. Phone: (617) 525-5809, Fax: (617) 525-5830, Email: bdenker@rics.bwh.harvard.edu

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ABSTRACT

Regulation and assembly of the epithelial cell junctional complex involves multiple signaling mechanisms including heterotrimeric G-Proteins. Recently, we demonstrated that Gα12 binds to the tight junction scaffolding protein ZO-1 through the SH3 domain, and activated Gα12 increases paracellular permeability in MDCK cells (Meyer et al., J. Biol. Chem 277; 24855 (2002)). In the present studies, we explore the effects of Gα12 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 Gα12 (QL) expressing MDCK cells. The normal distribution of ZO-1 and Na/K-ATPase was altered in QLα12-MDCK cells consistent with loss of polarity. We found that the tyrosine kinase inhibitor genistein and the Src specific inhibitor PP-2 reversibly abrogated the QLα12 phenotype on the junctional complex. Junctional protein localization was preserved in PP-2 or genistein treated, QLα12 expressing cells, and the increase in paracellular permeability as measured by TER and [H]3-mannitol flux was prevented by the inhibitors. Src activity was increased in QLα12-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 Gα12 regulates MDCK cell junctions, in part, through Src tyrosine kinase pathways.
INTRODUCTION

Heterotrimeric G proteins, comprised of Gα and Gβγ subunits, are expressed in all eukaryotic cells and provide a major mechanism for signal transduction. Binding of an agonist to a G-protein-coupled-receptor activates the Gα subunit by promoting GDP release and GTP binding. GTP-liganded Gα dissociates from Gβγ, and both subunits interact with downstream effector molecules (reviewed in (42)). There are at least 16 Gα subunits grouped into four major families based upon sequence similarities of the α subunit (Gs, Gi, Gq, and G12), and multiple family members are typically expressed within the same cell. One mechanism to allow specific signal transduction is to localize signaling molecules into discrete membrane domains. Several Gα subunits, including Gα12, partially colocalize within the epithelial cell junction (8, 10, 11, 21). Regulation of the junctional complex by heterotrimeric G proteins was suggested in early experiments (1), and subsequent studies have demonstrated that pertussis toxin sensitive family members Gαi2 and Gαo localize in the tight junction of MDCK cells and affect both tight junction assembly and baseline properties (8, 10, 21, 50). Recently, the role of G proteins modulating the tight junction has been expanded to include Gαs, which also stimulates tight junction assembly and indirectly associates with a ZO-1 complex (49).

The tight junction is the most apical component of the junctional complex, which includes gap junctions, adherens junctions and tight junctions. The tight junction (TJ) separates apical and basolateral membrane domains, an essential feature necessary for cellular polarity, and also regulates paracellular flux through the interaction of
transmembrane proteins (claudin and occludin families). Understanding mechanisms regulating maintenance and assembly of junctions is essential for insights into developmental processes and pathophysiologic disorders including malignant transformation and ischemic/toxic injuries. Multiple signaling pathways including; regulated calcium stores, protein kinase C, Src tyrosine kinases, small G proteins and heterotrimeric Gα subunits have been shown to regulate this complex process (reviewed in (9, 39)).

The G12 protein family consists of the ubiquitously expressed members Gα12 and Gα13 (54), and they regulate a variety of cellular responses including transformation of fibroblasts (23, 63, 66), activation of JNK and serum response element (7, 15, 32, 46), stress fiber formation (3), and neurite retraction in PC12 cells (27). In addition, Gα12 and Gα13 can stimulate phospholipase D and transcription of cyclooxygenase-2 and Egr-1 (44, 53, 64). Utilizing inducible Gα12 (wtα12) and constitutively active Q229Lα12 (QLα12) expressing MDCK cell lines, we demonstrate that QLα12 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/Cell Lines: cDNAs for wt\(\alpha\)12 and QL\(\alpha\)12 were kindly provided by Henry Bourne (Univ. of California, San Francisco). Establishment and characterization of Tet-off MDCK cell lines with inducible G\(\alpha\)12 expression is described in (37). Polyclonal anti-G\(\alpha\)12 antibodies were purchased from Santa Cruz Biotechnology (S-20, Santa Cruz, CA). Anti-\(\alpha\)- or \(\beta\)-catenin, anti-occludin, anti-claudin-1 and anti-E-cadherin were from Zymed Laboratories (San Francisco, CA). Monoclonal anti-phosphotyrosine (4G10), monoclonal anti-Na/K-ATPase and the Rho activation assay were purchased from Upstate (Lake Placid, NY). Anti-c-Src, anti-pTyr\(^{419}\)-Src and anti-pTyr\(^{530}\)-Src were from Biosource International Inc. (Camarillo, CA). Anti-ZO-1 rat monoclonal antibody (R40.76) was kindly supplied by D. Goodenough (Harvard University). Phalloidin coupled to was purchased from Molecular Probes (Eugene, OR). Tet-Off Madin Darby canine kidney Type II epithelial cells (T-23 MDCK), Tet-Off cloning vectors and tetracycline-free fetal calf serum were obtained from Clontech (Palo Alto, CA). Genistein was purchased from Sigma, PP-2 was purchased from Calbiochem (San Diego, CA). Plasticware was from Falcon (Lincoln Park, NJ). Transwells (polycarbonate permeable filters (0.4 \(\mu\)m pore, 6 and 12 mm diameter) were obtained from Costar (Cambridge, MA), the ohm meter (Millicell ERS) from Millipore (Bedford, MA).

Cell culture: Tet-Off MDCK cells were incubated at 37°C in 5% CO\(_2\) and maintained in Dulbecco`s modified Eagle`s medium (DMEM) from Cellgro (Herndon, VA) containing 5% Tet-system approved FBS (Clontech), 100 \(\mu\)g/ml G418, 50 IU/ml penicillin and 50 \(\mu\)g/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 doxycycline.

Subsequent experiments with conditional expression of the Gα subunits were carried out in the same medium without hygromycin and G418 and with or without doxycycline.

**Dome formation and inhibitor studies:** Gα12 and QLα12 transfected Tet-Off MDCK cells were grown in to confluence in 12 well plates with doxycycline (40 ng/ml) before switching 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 (Nikon eclipse, TE300) at 100X magnification in 10 consecutive visual fields.

**Immunohistochemistry:** Gα12 and QLα12 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% paraformaldehyde (EM Sciences, Fort Washington, PA) in PBS for 20 min at RT 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 RT in 5% w/v non-fat 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 RT preceded the 1 h at 4°C incubation 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 RT and mounted with Fluoromount G (Southern Biotechnology Associates, Inc. Birmingham, AL) and viewed through a 100X 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:** Wtα12 or QLα12-cells were cultured with or without doxycycline for 3 days. Whole cell lysates were obtained by scraping monolayers in lysate HEPES buffer (NaCl 100 mM, EDTA 2 mM, HEPES 10 mM, pH 7.5, NaVO₄ 1 mM, NaF 25 mM, PMSF 1 mM, Triton-X 100 1%, Na-deoxycholate 0.5%, SDS 0.1%) and 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 over night at 4°C on a shaker. Beads were washed 4 times with lysate buffer and proteins eluted in SDS-Page sample buffer. SDS-Page electrophoresed proteins were transferred over night (MSI nitrocellulose, Westboro, MA). Nonspecific binding was blocked with 2% nonfat milk or 2% BSA in blocking buffer (Tris/HCl 50 mM, EDTA 10 mM, Triton X-100 1%) prior to exposure of the membrane to the primary antibody for 1 h at room temperature. After washing (1 h in TBS + Tween 20, 0.05 %, 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 detected with SuperSignal West Pico-horseradish peroxidase substrate system (Pierce, Rockford, IL) and autoradiography (Kodak Biomax MR, Rochester, NY).
**TER Measurements:** MDCK cells were plated on polycarbonate filters (Transwell, Costar) at approximately 3x10^5 cells/cm² and maintained in culture media plus doxycycline for 48-72h to establish tight monolayers. Similar effects on TER were obtained when cells were plated at 1.2x10^6 cells/cm². TER was measured at different time points after changing to a doxycycline free medium with a Millipore ERS electrical resistance system, as described previously (55). Measurements are expressed in ohm × cm², as a mean of the original readings after subtraction of background values.

**Mannitol Flux Rates:** Wtα12 and QLα12-MDCK cells were cultured for 3 days on 6mm Transwells in the presence of doxycycline before switching half of the filters to dox medium +/- tyrosine kinase inhibitors for an additional 3 days. Filters were washed three times with Hanks Balanced Salt Solution (HBSS) and incubated in HBSS with 1mM mannitol in both chambers. At t=0, 4µCi of [H]³-mannitol (17Ci/mmol, PerkinElmer Life Sci. Inc., Boston, MA) was added to the apical chamber and plates stirred using an orbital shaker at 37°C. 100µl aliquots were removed from the basal chamber at 30, 60, 120 and 180min and replaced with an identical volume of HBSS+1mM mannitol. Counts were determined in scintillation fluid using a Packard Tri-Carb A2200 liquid scintillation counter. Background was subtracted, counts per minute converted to pmol of mannitol, and rates determined from linear regression analysis of the 4 time points using GraphPad Prism (GraphPad Inc.).
**Rho activation assay:** Wtα12 or QLα12-cells were cultured with or without doxycycline and tyrosine kinase inhibitors for 3 days. Lysis was performed with ice cold lysis buffer (Tris/HCl 50 mM, pH 7.2, Triton X-100 1%, sodium deoxycholate 0.5%, SDS 0.1%, NaCl 500 mM, MgCl₂ 10 mM, protease inhibitors) as supplied by the manufacturer (Upstate Biotechnology, Lake Placid, NY). Samples were sonicated on ice and centrifuged for 10 min at 13,000 rpm. 400 µl of supernatant were added to 30 ul of glutathione-agarose slurry linked to Rhotekin Rho binding domain that specifically binds activated (GTP-bound) Rho (buffer: Tris/HCl 50 mM, pH 7.5, Triton X-100 0.5%, NaCl 150 mM, MgCl₂ 5 mM, dithiothreitol 1 mM and protease inhibitors) and incubated for 45 min at 4°C. Agarose beads were centrifuged and washed times. Activated Rho was eluted with SDS-PAGE sample buffer, denatured for 10 min at 72°C and analyzed Western blot with purified polyclonal anti-Rho A,B,C antibody (provided by Upstate). Positive and negative controls were prepared with uninduced wtα12- or QLα12-cell lysates. Prior to incubation with glutathione-agarose slurry, GTPγS (final 100 µM) or GDP (final 1 mM) were added to 400 µl of supernatant at 30°C for 15 min. Binding was terminated on ice and addition of MgCl₂ (final 60 mM) as recommended by the manufacturer before addition to slurry as described.

**Statistics:** The data are given as mean values ± standard error of the mean (SEM) (n), where n refers to the number of measurements performed. The 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. A $P$-value of $<0.05$ was accepted to indicate statistical significance.
RESULTS

Expression of Activated Gα12 Affects Dome Formation in MDCK Cells.

We previously showed that activated Gα12 (QL) expressed in MDCK cells reversibly increased paracellular flux and lowered transepithelial resistance (37). Figure 1 demonstrates another consequence of leaky epithelia - 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 (such as ouabain) or interventions that disrupt the junctional complex (low calcium). Figure 1A shows QLα12 protein levels as a function of doxycycline concentration, and figure 1B and C show the inverse correlation between QLα12 protein levels and dome formation. In the absence of doxycycline, domes were completely absent (fig. 1B, panel d) and there was an inverse correlation between QLα12 protein levels and the number of domes visualized (fig. 1A and C). There were no differences among wtα12 and QLα12 MDCK cell lines in the presence of doxycycline (no Gα12 expression), and the number of domes was unchanged by inducing wtα12 protein for 72h (fig. 1B panel a and c; fig. 1C).

Expression of QLα12 in MDCK Cells Disrupts both Tight and Adherens Junction Proteins.
We next examined the staining pattern of junctional proteins in the presence and absence of wt\(\alpha_{12}\) and QL\(\alpha_{12}\) expression (fig. 2). We have previously shown that the pattern of ZO-1 staining was significantly altered with QL\(\alpha_{12}\) expression, and 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 wt\(\alpha_{12}\) expression (results not shown) nor where there any differences when cells were grown on coverslips or Transwell filters. In QL\(\alpha_{12}\) expressing MDCK cells, the tight junction proteins claudin-1 and occludin and the adherens junction proteins E-cadherin and \(\beta\)-catenin show altered localization. The patterns were similar for ZO-1, occludin, \(\beta\)-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 the development of punctate intracellular staining (fig. 2 panels c,d). Activation of \(\beta\)-catenin often results in translocation into the cytoplasm and to the nucleus (as an example, see (36)). In QL\(\alpha_{12}\) expressing MDCK cells, there was increased \(\beta\)-catenin tyrosine phosphorylation (see fig. 5), but we did not detect significant cytosolic localization of \(\beta\)-catenin nor any co-localization with the nuclear stain DAPI (not shown).

*Epithelial Cell Polarity is Disrupted and Cortical Actin StainingIncreased with QL\(\alpha_{12}\) Expression.*

Since expression of QL\(\alpha_{12}\), but not wt\(\alpha_{12}\), resulted in significant changes in junctional protein staining and increased paracellular permeability, polarity of the
epithelial cell monolayer was likely to be disrupted. The TJ scaffolding protein ZO-1 is normally restricted to a discrete plane in the TJ at the interface between the apical and basolateral membranes. 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-MDCK cells (fig. 3A, panels a,e and c,g). However, inducing QLα12 protein (-dox), caused loss of this separation with ZO-1 appearing diffusely along the lateral membrane and in the apical membrane (fig. 3A, panels f; fig. 3B, panel b, arrow) and the Na/K-ATPase partially localizing in the apical membrane (fig. 3A, panel d and fig.3B, panel d, arrow). Wtα12 expression caused no significant changes in the actin cytoskeleton (see fig. 6A) while inducing QLα12 protein resulted in altered cell shape and increased actin stress fibers (fig. 3B, panels e, f, and fig. 6B). Actin staining was similar in both QLα12- and wtα12-MDCK cells in the absence of Gα12 expression (+ dox, see fig. 6). These findings indicate 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 Gα12 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 Gα12 (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 MDCK cells expressing QLα12 (fig. 4A, n=7). Baseline TER in Tet-off MDCK cells was approximately 75 ohms·cm² which is a little lower than values reported by some groups (26), but consistent with others (34). Neither PP-2 nor genistein affected baseline TER under control conditions (+dox), 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 switching back to doxycycline free medium (at about 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 transcellular ion flow, we measured paracellular flux of [H]³-mannitol in both wildtype and QLα12 MDCK cells in the presence and absence of Gα12 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 both PP2 and genistein. Both inhibitors resulted in a small increase in flux of +dox monolayers perhaps due to non-specific effects from exposure to the inhibitors for 72h. In overexpressing wtα12 MDCK cells, there was a small increase in mannitol flux that was blocked by the inhibitors (fig. 4B).
Src is Activated and β-catenin Phosphorylation Increased Without Detectable Rho Activation in QLα12-MDCK Cells

Based on the observation that PP-2 blocked the QLα12 protein effects on the MDCK cell junction, and others have reported Src activation by Gα12 (28, 41), we investigated c-Src activity in QLα12- and wtα12 expressing cells. Western blots of wtα12- and QLα12-MDCK cell lysates prepared from cells grown +/- doxycycline showed no significant difference in total c-Src immunoreactivity (fig. 5A). Src is activated by phosphorylation of pTyr\textsuperscript{419} and normally maintained in an inactive conformation through intramolecular interactions between the SH2 domain and the phosphorylated C-terminal tail (pTyr\textsuperscript{530}) (reviewed in (58)). Therefore, increases in phosphorylation of Tyr\textsuperscript{419} or decreases in Tyr\textsuperscript{530} can be used as evidence of Src activation. Without Gα12 expression (+ dox), there was minimal phosphorylation of pTyr\textsuperscript{419} (as detected with pTyr\textsuperscript{419} specific antibody) in both wtα12 and QLα12 MDCK cells reflecting Src inactivation (fig. 5A, middle panel). Inducing QLα12 expression (-dox) caused a significant increase of pTyr\textsuperscript{419} phosphorylation (4.4 fold ± 0.4, n=5) with no detectable change in wtα12-MDCK cells (fig. 5A, middle panel). Also consistent with c-Src activation, there was a concomitant decrease of pTyr\textsuperscript{530} phosphorylation (3.9 fold ± 0.3, n=4) with QLα12 expression while there was no significant difference in pTyr\textsuperscript{530} Src phosphorylation relative to occludin in wtα12 cells (fig. 5A, right panel).

Our confocal studies showed that both E-cadherin and β-catenin localization were disrupted in QLα12-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 during adherens junction disassembly [72]. To determine if QLα12 expression caused increased phosphorylation of β-catenin, we immunoprecipitated α- and β-catenin from QLα12 MDCK cells +/- dox. In our gel system, α- and β-catenin migrate with nearly identical molecular weights of 98kDa. Nevertheless, fig. 5B (top panel) shows that α-catenin precipitates a significant amount of β-catenin while the β-catenin coprecipitates a much smaller amount of α-catenin. Both α- and β-catenin were immunoprecipitated from + and - dox cells and probed by Western blot with anti-phosphotyrosine antibodies (bottom panel). For both α- and β-catenin precipitates, there is a significant increase in tyrosine phosphorylation of - dox cells. This finding, combined with the results of the top panel, 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.

Since Gα12 can activate Rho through Src dependent and 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 the junction (25, 26, 47, 65). However, in Rhotekin binding assays (fig. 5C), the amount of activated Rho was not increased in either wt or QLα12 expressing MDCK cells 72 hours after doxycycline removal. There was no detectable difference in the quantity of Rho in cell lysates nor did treatment with PP2 or genistein significantly affect Rho expression or activation. In support of this observation, treatment of Gα12-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 MDCK Cells

Confocal analysis of Gα12-MDCK cells reveals that expressing 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 [24] wtα12 is found along the cell perimeter (fig. 6A panel 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 panels c,f), and not significantly affected by the inhibitors although a few more stress fibers were evident (panels i, l). In QLα12-MDCK cells the inhibitors had no major effect on QLα12 localization, but in QLα12-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 compare panels 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 panel k compared with panel 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, panel h). This effect of genistein on cell morphology was not seen in wtα12 expressing MDCK cells (fig. 6A panel h). Unlike wtα12-MDCK cells, actin staining of QLα12-MDCK cells revealed increased stress fibers (fig. 6B panel
f), and the nature of these stress fibers was different in the presence of the inhibitors. In genistein treated cells, the transcellular actin filaments appeared significantly shorter than in untreated cells (fig. 6B, panels f, i) while PP2 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. PP2 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 +dox there are very few stress fibers 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 (-dox), ZO-1 staining becomes fragmented and more diffuse along the lateral membrane (as seen in 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 inhibitor treated cells, ZO-1 staining is preserved, although in genistein treated cells, stress fibers are more visible and the increased intensity of cortical actin staining are still evident. PP2 treated cells appear more like the control condition with fewer stress fibers and less intense actin staining. In the presence of inhibitors and doxycycline the pattern is similar to the +dox only condition.
DISCUSSION

The regulation of the epithelial cell barrier is now 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 all contribute in interconnecting pathways to maintain an intact junction, and regulate its biogenesis (partially reviewed in (2, 9, 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 Gα12 and the tight junction scaffolding protein, ZO-1 (37). In addition, we showed that expression of activated Gα12 (QLα12) in these cells reversibly disrupted the localization of the TJ protein, ZO-1 and caused increased paracellular permeability as measured by TER and paracellular flux (37). The findings reported here indicate that proteins of the tight and adherens junction are disrupted, and there is significant reorganization of the actin cytoskeleton. These Gα12 mediated changes appear to be predominantly regulated through Src tyrosine kinases.

Expressing 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 QLα12 expression did not irreparably injure or alter the cell. The functional changes in paracellular permeability correlated with morphologic changes of the junctional complex. With QLα12 expression,
but not wtα12, 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 wildtype or
constitutively active mutants of Gαo, Gαi2, 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. Since claudins interact with Zona Occludens proteins
(22), and ZO-1 binds to Gα12, 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 since TJ
assembly starts with interactions of E-cadherin and E-cadherin also interacts with Gα12,
[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 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 PKC isoforms or monomeric G proteins in addition to lowering of extracellular calcium, results in disruption of junctions and increased leakiness in kidney epithelial cells (20, 25, 26, 40, 48). Furthermore, we have previously shown that activated Gαi2 and Gαs increase baseline TER in MDCK cells (49, 50). Tyrosine kinases are well described in regulating epithelial cell junctions and are important downstream effectors of Gα12. The tyrosine kinase inhibitor genistein and the Src-specific inhibitor PP-2 both prevented development of the typical phenotype seen 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 that resulted in a less permeable paracellular space. The effects of genistein in comparison with PP2 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 Gα12 and indirect activation of Src via another mechanism. Gα12 directly regulates other tyrosine kinases (Bruton’s tyrosine kinase and pp72syk (24, 28)), and several receptors (thrombin, endothelin, vasopressin) signal through Gα12 to indirectly activate Src (6). Furthermore, direct Src kinase activation by Gα12/13 was shown in thrombin-stimulated platelets, which coincided with platelet shape changes (28).
Although Gα12 signaling is linked to Rho mediated changes in the actin cytoskeleton (via Rho exchange factors (3, 16-18, 28, 29, 31)), there was no significant Rho activation 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 upon persistent QLα12 expression. It is also possible that other small G proteins (not detected with Rhotekin binding) such as Rac1 are downstream of Gα12 in this system. In an analogous experimental design, Postma et al., showed that Gα12 regulation of gap junctions via Src tyrosine kinases was also independent of Rho activation (45). The current experiments do not distinguish direct Src phosphorylation of β-catenin from other mechanisms causing increased phosphorylation (such as changes in E-cadherin and catenin localization induced by QLα12 expression). Future experiments will be needed to determine whether the increased β-catenin phosphorylation is important to the Gα12/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 viral 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, 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 QLα12-MDCK cells. We have previously shown 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 the 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 TJ. In addition, there are a surprising large number of interacting scaffolding proteins of the MAGUK family (membrane associated guanylate kinase) now identified within the epithelial cell TJ. These proteins share a common overall structure and typically include multiple protein-protein interaction domains including multiple PDZ domains (see (12, 13)). Examples of these proteins known to be located in the epithelial cell tight junction include Zona Occludens-1, 2, 3, and the related MAGI-1 (43) proteins. In addition, other interactions between Junctional Adhesion Molecule (JAM) 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 Gα12 and ZO-1 in vitro and in cells, and now show that Src tyrosine kinases are important for the Gα12 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 Gα12 have so far
been unsuccessful. This suggests to us that Src is in proximity to ZO-1 and Gα12, but may be in direct association with one of the other scaffolding proteins. Our previous results with activated Gαi2 and Gαs in MDCK cells have shown increased rates of TJ assembly in the calcium switch model in addition to increased baseline TER. Gα12 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 TJ 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 identify these interactions and begin to define these regulatory pathways in-vivo.

Acknowledgments

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References


FIGURE LEGENDS

Figure 1

A

![Western Blot](image)

B

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![Bar Graph](image)
Figure 1: QLα12 Expression and Dome Formation in MDCK Cells.  

A. Western blot of Gα12 after 48 h of induction of QLα12 cells with varying doxycycline concentrations (0-40 ng/ml). Expression was re-suppressed within 48 h when doxycycline was added back after 48 h in the absence of doxycycline (right lane). 

B. Phase contrast microscopy of wtα12- and QLα12-MDCK monolayers at baseline (+ dox) and 72 h after induction of Gα12 expression. Wtα12 expressing monolayers showed typical dome formation without (a, +dox) or with induction (c, -dox) of wtα12 expression. In QLα12-MDCK cells, dome formation was observed without induction of QLα12 expression (b). After 72 hours of QLα12 expression (d, -dox), dome formation was no longer observed. Bar 50 μm. 

C. Quantitation of dome formation as a function of doxycycline concentration at 72h of QLα12 or wtα12 expression. * indicates significant difference with uninduced monolayers (40 ng/ml).
Figure 2: Immunofluorescent Localization of Tight Junction and Adherens Junction Proteins in QLα12-MDCK Cells. Images were obtained before (+dox) and after (-dox) induction of QLα12 expression for 48h. Staining of ZO-1 (a, b), claudin-1 (c, d), occludin (e, f), β-catenin (g, h) and E-cadherin (i, j) was performed as described in Materials and Methods. Bar 25 μm.
**Figure 3: Immunofluorescent Localization of ZO-1, Na/K-ATPase and Actin in QLα12-MDCK Cells.**  

**A.** Confocal localization for ZO-1 and Na/K-ATPase in uninduced (+dox) and induced (-dox) QLα12-MDCK cell monolayers at an apical (a-d) and basal (e-h) plane.  

**B.** XZ-axis images of uninduced (+dox) and induced (-dox) QLα12 MDCK cells stained for ZO-1 (a, b), Na/K-ATPase (c, d) and actin (e, f). Note the altered distribution of these proteins in QLα12 (-dox) expressing cells (b, d, f) including some apical localization of the Na/K-ATPase (arrow, d). Arrows in b and d indicate apical staining. Bar 15 μm.
**Figure 4: Barrier Function in QLα12-MDCK Cells.**

**A.** TER was measured in confluent, uninduced monolayers at the beginning (0 h) and throughout Gα12QL induction (-dox) and re-suppression (+dox) as described in Materials and Methods. The identical protocol was followed in the presence of the tyrosine kinase inhibitors genistein (100 µM, open upward triangles) or PP-2 (10 µM, open downward triangles). The tyrosine inhibitors were added at the beginning of QLα12 induction (0 h) and were present throughout the experiment. At 124 h the tyrosine kinase treated monolayers were induced again, but without the tyrosine kinase inhibitors present (-dox). A simultaneous control of uninduced QLα12-MDCK cells (doxycycline present at all times, closed squares) is shown for comparison (n=7 for each time point).

**B.** Mannitol flux in wt and QLα12 MDCK +/- inhibitors. Cells were cultured on 6mm Transwell filters for 72h in the presence or absence of doxycycline and inhibitor as described in Material and Methods. Fluxes of [H³]-mannitol were measured at 0, 30, 60, 120 and 180min and rates determined as described in Material and Methods. A representative experiment is shown with n=3 for each condition and results expressed as the mean +/- S.D. The experiment was repeated 3-4 times with similar results.
Meyer et al., fig 5

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IB: occludin

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Catenin IP: α β α β

Western: α-Catenin β-Catenin

Dox: + - + -

IP: β-Catenin α-Catenin

Western: pTyr pTyr

C

Activated rho lysates

dox: + + + - - - - -

wtα12 GTPyS GDP gen gen PP-2 PP-2 PP-2
Figure 5: Analysis of Src Activation, β-catenin Tyrosine Phosphorylation and Rho Activation in Gα12-MDCK Cells

A. Western blot of Src in MDCK cells following QLα12 or wtα12 expression. Whole cell lysates were resolved by SDS-PAGE and Src was detected by Western blot using anti-c-Src antibodies (left panel), anti-pTyr^{419}-Src antibodies (middle panel) or anti-pTyr^{530}-Src antibodies (right panel). Uninduced lysates (+dox) were compared with lysates of QLα12 or wt expressing MDCK cells (-dox for 48 h). Equal loading of the lanes was shown with occludin antibody after stripping and reblotting of the nitrocellulose. B. Tyrosine phosphorylation of immunoprecipitated α-catenin and β-catenin. Top panel; α- and β-catenin were immunoprecipitated from -dox QLα12 MDCK after 72h and analyzed by Western with either α- or β-catenin antibodies. Bottom panel; α- or β-catenin were immunoprecipitated from cell lysates with (-dox) or without (+dox) QLα12 expression, analyzed by Western using anti-phosphotyrosine antibody as described in Materials and Methods. C. Rhotekin Binding Assay. Western blot of Rho in cellular lysates and after GST-Rhotekin pull down experiments from induced and uninduced wtα12 and QLα12 MDCK cells. Uninduced wtα12 and QLα12 cell lysates were treated with GTPγS (10 μM) to irreversibly activate Rho (left lane), or GDP (100 μM). In addition, GST-rhotekin pull downs from lysates prepared from uninduced (+dox) or induced (-dox) Gα12 MDCK cells treated with genistein (gen, 100 μM) or PP-2 (10 μM) are shown. Right panel: Western blot of the same lysates used for the pull down experiments (2% of total lysate). This experiment was performed 3 times with similar results.
Meyer et al. Fig. 6A

WTα12 cells

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Figure 6: Confocal Immunofluorescent Localization of Gα12, ZO-1 and Actin in wtα12 and QLα12 Expressing Cells in the Presence of Tyrosine Kinase Inhibitors.

A. Wtα12 expression was induced for 72h (d-l) in the absence (d-f) or presence of genistein (100µM) (g-i) or PP-2 (10µM) (j-l) Cells were stained for Gα12 (a-c), ZO-1 (d-f) and actin (g-l) as described in Materials and Methods. B. QLα12 expression was induced for 72 h (d-l) in the absence (d-f) or presence of either (100µM) (g-i) or PP-2 (10µM) (j-l) Bar 20 µm.
Fig 7 meyer et al.,

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Figure 7: Actin Staining at the level of ZO-1 in QLα12-MDCK Cells.

QLα12 MDCK cells cultured on Transwell filters for 72h in +dox and then switched to -dox in the presence and absence of genistein (100µM) or PP-2 (10µM) for an additional 72h. Cells were fixed in 4% paraformaldehyde and costained with ZO-1 and phalloidin as described in the Materials and Methods. Images were obtained at the level of ZO-1 using a Nikon Labophot-2 microscope and Spot Digital camera and software (www.diaginc.com/SpotSoftware/spotrt.htm version 3.5.7). Images were then processed in Adobe Photoshop as described in Material and Methods. Bar = 10µM.)