Involvement of Go12 in the Maintenance and Biogenesis of Epithelial Cell Tight Junctions*

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Polarized epithelial cells have highly developed tight junctions (TJ) to maintain an impermeant barrier and segregate plasma membrane functions, but the mechanisms that promote TJ formation and maintain its integrity are only partially defined. Treatment of confluent monolayers of Madin-Darby canine kidney (MDCK) cells with AlF4⁻ (activator of heterotrimeric G protein α sub-units) results in a 3-4 fold increase in transepithelial resistances (TER), a reliable indicator of TJ integrity. MOCK cells transfected with activated Goα (Q205L) have accelerated TJ formation (Denker, B. M., Saha, C., Khawaja, S., and Nigam, S. J. (1996) J. Biol. Chem. 271, 25750-25753). Goα has been localized within the tight junction, and a role for Goα in the formation and/or maintenance of the tight junction was studied by transfection of MDCK cells with vector without insert (PC), wild type Goα, or a GTPase-deficient mutant (constitutively activated), Q205L. Tryptic conformational analysis confirmed expression of a constitutively active Goα in Q205LGoα-MDCK cells, and confocal microscopy showed a similar pattern of Goα localization in the three cell lines. Q205LGoα-MDCK cells had significantly higher base-line TER values than wild type Goα or PC-MDCK cells (1187 ± 150 versus 576 ± 89 (Goα); 377 ± 52 mΩ·cm² (PC)), and both Goα- and Q205LGoα-transfected cell lines more rapidly develop TER in the Ca²⁺ switch, a model widely used to study the mechanisms of junctional assembly. Treatment of cells with AlF4⁻ during the Ca²⁺ switch had little effect on the kinetics of TER development in Goα- or Q205LGoα-MDCK cells, but PC cells reached half-maximal TER significantly sooner in the presence of AlF4⁻ (similar times to Goα-transfected cells). Base-line TER values obtained after the switch were significantly higher for all three cell lines in the presence of AlF4⁻. These findings indicate that Goα is important for both the maintenance and development of the TJ, although additional Goα subunits are likely to play a role.

Polarized epithelia have developed highly specialized membrane functions enabling vectorial transport across the cellular layer. The junctional complex of epithelial cells includes gap junctions, adherens junctions, and tight junctions. The tight junction (TJ) is the most apical component of the junctional complex and provides two essential functions: (i) the permeability barrier to paracellular fluxes and (ii) the “fence” separating the apical and basolateral membrane domains. In developing tissues as well as cell culture models, the critical signaling events important to junction formation appear to be quite different from mechanisms that maintain junctional integrity. The TJ is composed of a complex of proteins that includes occludin, the only transmembrane protein identified so far (1). There are several peripherally attached membrane proteins found in the TJ including the zona occludens family (ZO-1, -2, and -3) (2-4). ZO proteins are members of the MAGUK (membrane associated guanylate kinase) superfamily that are often found at sites of cell-cell contact and may function to couple extracellular signaling pathways with the cytoskeleton. Other proteins found in or near the TJ include cingulin, αH6, symplekin, unidentified phosphoproteins, and a series of signal transduction molecules (reviewed in Ref. 5).

MDCK cells are a cultured epithelial cell line that has been extensively utilized for studies of epithelial polarity, targeting of proteins, and the study of intercellular junctions (6). The Ca²⁺ switch model of TJ formation in MDCK cells has been widely utilized to gain insights into the function of polarized epithelial cells (7-11) and recapitulates many of the critical molecular events of epithelial morphogenesis. MDCK cells cultured in low calcium (µM) lack cell-cell contact, polarity, and junctions. “Switching” to normal calcium medium (NC) triggers a series of molecular events that leads to establishment of the polarized phenotype with characteristics of a tight transporting epithelium. Tight junction development can be followed by measuring the transepithelial resistance (TER), a rapid and reproducible assessment of tight junction integrity. Because MDCK cells are clonal and TJ development can be synchronized in the Ca²⁺ switch, the role of specific proteins on TJ biogenesis can be studied in this system by cDNA transfections.

The critical role of calcium in the formation of intercellular junctions is well established. Extracellular calcium is required for homotypic interactions of E-cadherin and is likely to be the initial event of junctional complex formation (12). Regulated intracellular calcium stores are also important for tight junction biogenesis. There are local increases in intracellular calcium concentration at the points of cell-cell contact (9), and chelation of intracellular calcium perturbs TER development (13). Thapsigargin depletes intracellular endoplasmic reticulum stores of calcium, and thapsigargin treatment of MDCK cells prior to initiation of cell-cell contact prevents TER development and the sorting of ZO-1 to the TJ (7). The signaling

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Role of G Proteins in Tight Junction Biogenesis

In recent years, the importance of G proteins in tight junction (TJ) formation and maintenance has been emphasized. G proteins are a family of heterotrimeric proteins involved in signal transduction and are localized to the membrane. They consist of three subunits: Gα, Gβ, and Gγ. Gα can be activated by various receptors and effectors, leading to changes in cellular functions such as calcium levels, ion permeability, and paracellular resistance.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Cell Culture—Rat Gαo cDNA was cloned into Bluescript (Stratagene) as described previously (20) and recloned into the EcoRI and ApaI sites of pcDNA3 (Invitrogen). Q205Lαo was provided by Dr. Gary Johnson and cloned into Bluescript using HindIII sites and then into pcDNA3 using XhoI and Xbal sites. MDCK cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with antibodies plus 5% fetal calf serum. Transfected cell lines were maintained in G418 (500 μg/ml; Life Technologies, Inc.)

Transfection—Subconfluent MDCK cells (ATCC, Manassas, VA) were transfected with 10 mg of linearized plasmid by calcium phosphate precipitation method as described previously (16). G418-resistant colonies were analyzed for increased Gαo expression by Western blot using a rabbit polyclonal antibody directed toward the C terminus of Gαo (AS7, NEN Life Science Products). Control cells were obtained by transfecting pcDNA3 without insert, and all cell lines were established in parallel.

Western Analysis of Transfected Clones—Confluent PC-12 cells, or Q205Lαo-MDCK cells were washed twice with PBS and then scraped into buffer A (50 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 75 mM sucrose, 1 mM dithiothreitol, 1 mM EDTA). Cells were frozen and thawed three times and triturated ten times through a 27 gauge needle. All samples were incubated at 30 °C with no added nucleotide or 100 μM GTPγS. Samples were immediately placed on ice, and trypsin was added (20 pmol of 1:1-tosylamide)-2-phenylthethyl chloromethyl ketone-treated trypsin (Sigma). All samples were incubated at 30 °C for 20 min, and digestion was terminated by the addition of SDS-polyacrylamide gel electrophoresis sample buffer following by boiling for 5 min. Samples were then analyzed by SDS-polyacrylamide gel electrophoresis and Western blot using AS7 anti-Gαo rabbit polyclonal antibody (1:1,000) and ECL (Pierce) with goat anti-rabbit horseradish peroxidase (1:10,000).

Immunohistochemistry—PC-12 cells, or Q205Lαo-MDCK cells were grown on coverslips or Transwell filters (12 mm) (Costar), rinsed with PBS, and fixed with methanol (100%, −70 °C) for 10 min. Cells were then washed with PBS and blocked as described previously (16). Samples were incubated with rabbit polyclonal Gαo (AS7, from NEN Life Science Products) at several dilutions and rat monoclonal to ZO-1 (undiluted supernatant; courtesy of D. Goodenough) for 1 h. Cells were washed with PBS three times at 5-min intervals and incubated with secondary antibodies (fluorescein- or Texas Red-conjugated goat anti-rabbit or anti-rat IgG; Jackson ImmunoResearch, West Grove, PA) at 1:100 with for 1 h. Coverslips were visualized on a Nikon Labophot-2 confocal microscope or a Bio-Rad 1024 confocal microscope using the 63× oil immersion objective. Images were processed in Adobe Photoshop (Adobe, CA) and figure compiled in Adobe Illustrator (Adobe, CA).

RESULTS AND DISCUSSION

Several lines of evidence have suggested the involvement of heterotrimeric G proteins in TJ formation. Early studies with G protein modulators such as pertussis toxin, cholera toxin, AlF4−, and a variety of other agents showed variable effects on TJ formation (10). Several confocal studies have localized Gαi2, Gαi3, and Gα12 in the vicinity of the tight junction (16, 17, 19, 21). Recently, we demonstrated that Gαo, a member of the Gα family inhibited by pertussis toxin (−80% similar to Gαi3), its receptors and effectors are distinct, and furthermore, Gαo is not detected in renal epithelia or MDCK cells (16, 18, 19). Several Gα family members are expressed in epithelial cells, and Gαo has been shown to overlap with the tight junction in epithelial cell lines (16, 17). Taken together, these observations raise the possibility that Gαo may be an important regulator of tight junctions. To test this hypothesis, we initially looked for effects of AlF4− (activator of Gα subunits) on tight junctions in control cell lines and then established MDCK cells overexpressing wild type Gαo and a constitutively activated Gαo (GTPase-deficient, Q205Lαo). We find that AlF4− significantly increases TER in control cells and accelerates TER development during the Ca2+ switch. The effects of AlF4− can be reproduced in MDCK cells expressing activated Gαo, indicating that this Gα subunit is critical to the development and maintenance of tight junctions.

Because AlF4− activates all Gα subunits in MDCK cells and several studies have placed Gαo in close proximity to the TJ, we tested the hypothesis that Gαo was important to this process by stably expressing Gαo and a constitutively activated Gαo (Q205Lαo) in MDCK cells. The amount of transfected Gαo was determined relative to the levels of endogenous Gαo in PC-MDCK cells. Western blots of the three cell lines using identical amounts of total protein were analyzed (not shown) using NIH image (Wayne Rasband, NIH). Relative to PC-MDCK cells, the level of Gαo in Gαo-MDCK cells was 3.9 ± 0.4-fold (n = 7) increased, and for Q205Lαo-MDCK the level of Gαo was 8.5 ± 0.7-fold (n = 7) increased.

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was of Goα2 was 1.8 ± 0.2-fold (n = 7) above PC-MDCK cells. To confirm that constitutively activated Goα2 was expressed in these transfected MDCK cells, we utilized a tryptic cleavage analysis of Goα2 (Fig. 2). This technique has been widely utilized as an indicator of Go subunit conformation (23, 24) and is based on the observation that Go subunits have a different cleavage pattern depending on whether they are folded into an active or inactive conformation. In the active conformation (GTP-ligated), there is only a single tryptic site accessible near the N terminus (approximately Arg21) resulting in a constitutively activated Goα resulting in a peptide of approximately 20 kDa. In the inactive (GDP-liganded) conformation, an additional site becomes accessible in the a2 helix or switch region (near Arg209) resulting in peptides of approximately 25 and 17 kDa. In the absence of added nucleotide, samples were preincubated at 30 °C for 10 min with no added nucleotide (−) or 100 μM GTPγS (+) followed by digestion with 20 pmol of trypsin for 20 min at 30 °C. Reactions were terminated with SDS-polyacrylamide gel electrophoresis sample buffer and boiling. Samples were analyzed by Western with AS7 antibody at 1:1000 and ECL as described under "Experimental Procedures." The times of exposure for each cell line differ and were deliberately overexposed to determine whether the expected 39-kDa peptide could be detected. Only the Q205Lα2-MDCK cells demonstrated a protected fragment at 39 kDa in the absence of added nucleotide.

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![Graph showing TER values in control cells](image)

**Graph 1.** The effects of aluminum fluoride on base-line TER values in control cells. Untransfected (WT-MDCK) and vector transfected (PC-MDCK) cells were plated at confluence on transwells filters and allowed to stabilize over 48–72 h. Medium was then changed to NC medium (− AlF4, black bars) or NC medium + (AlF4)3 (3 mm NaF + 50 μM AlCl3) for 24 h. TER measurements were obtained after 24 h. Results are the means ± S.E. for five experiments each with four to six individual TER values. The differences were significant (p < 0.05) for an effect of AlF4 on both cell lines.

**Graph 2.** Tryptic proteolysis of PC-, Goα2-, and Q205Lα2-MDCK cells. Confluent monolayers of each cell type were washed with PBS, scraped, and fractionated as described under "Experimental Procedures." Approximately 50–75 μg of total protein was used; samples were preincubated at 30 °C for 10 min with no added nucleotide (−) or 100 μM GTPγS (+) followed by digestion with 20 pmol of trypsin for 20 min at 30 °C. Reactions were terminated with SDS-polyacrylamide gel electrophoresis sample buffer and boiling. Samples were analyzed by Western with AS7 antibody at 1:1000 and ECL as described under "Experimental Procedures." The times of exposure for each cell line differ and were deliberately overexposed to determine whether the expected 39-kDa peptide could be detected. Only the Q205Lα2-MDCK cells demonstrated a protected fragment at 39 kDa in the absence of added nucleotide.

**Graph 3.** Confocal localization of Goα2 in transfected cells. A, PC-MDCK cells were grown on Transwell filters and double stained with antibody to Goα2 (AS7) at 1:25 dilution and undiluted ZO-1 monoclonal antibody as described under "Experimental Procedures." A single field was simultaneously visualized for ZO-1 and Goα2 localization. Magnification, 950×. B, transfected cells were simultaneously stained under identical conditions using AS7 antibody at 1:100 dilution and undiluted ZO-1 as described under "Experimental Procedures": PC-MDCK cells (panel a), Goα2-MDCK (panel b), and Q205Lα2-MDCK (panel c). Identical fields were simultaneously visualized by confocal microscopy as described. Magnification, 950×.
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**Fig. 4.** Base-line TER values and time course of TER development after Ca\(^{2+}\) switch. PC-, G\(\alpha\)\(i2\), and Q205L\(\alpha\)\(i2\)-MDCK cells were plated at confluent density on Transwell filters, and base-line TER values were obtained 36–40 h later. Cells were then placed in low calcium medium and TJ biogenesis followed over time using the Ca\(^{2+}\) switch as described under “Experimental Procedures.” Blanks were subtracted for each experiment. A, base line (BL) and time course of TER development. TER values were significantly higher for Q205L\(\alpha\)\(i2\)-MDCK cells (1187 ± 50 \(\Omega\) cm\(^2\); \(p < 0.001\)) than for G\(\alpha\)\(i2\)-MDCK (576 ± 89 \(\Omega\) cm\(^2\)) or PC-MDCK (377 ± 52 \(\Omega\) cm\(^2\)). The difference between G\(\alpha\)\(i2\)- and PC-MDCK was not significantly different. TER values were obtained every 2 h after switching from low calcium to NC medium at time 0. Post-Ca\(^{2+}\) switch base-line values are shown at 26 h. There was a decrease in base-line TER values for each of the three cell lines, but Q205L\(\alpha\)\(i2\) remained significantly higher than the other two cell types. Results are expressed as the means ± S.E. of 12 independent experiments with \(n = 4–6\) for each cell line in each experiment. Graphs were generated and statistical analyses were performed on data using Graphpad Prism 2.0 (Graphpad Software, Inc.) B, post-Ca\(^{2+}\) switch base-line TER values obtained at 26 h for the six independent experiments ± AlF\(_4^-\). The differences ± AlF\(_4^-\) were significant for each of the cell lines (\(p = 0.008\) for PC and \(p = 0.002\) for both G\(\alpha\)\(i2\) and Q205L\(\alpha\)\(i2\)-MDCK cells).

Transfected G\(\alpha\)\(i2\) subunits were localized in a similar manner to the endogenous G\(\alpha\)\(i2\), the G\(\alpha\)\(i2\) antibody (AS7) was diluted to a point where the endogenous G\(\alpha\)\(i2\) was barely detectable. Fig. 3B shows a confocal analysis of PC-, G\(\alpha\)\(i2\)-, and Q205L\(\alpha\)\(i2\)-MDCK stained and analyzed under identical conditions using a 1:100 dilution of the G\(\alpha\)\(i2\) antibody. In panel a, PC-MDCK cells only demonstrate faint intracellular staining, but in panels b and c, transfected G\(\alpha\)\(i2\) and Q205L\(\alpha\)\(i2\) can be visualized in the subapical lateral membrane overlapping with the TJ marker, ZO-1. Again, there is intracellular staining that is similar to the endogenous G\(\alpha\)\(i2\) (Fig. 3A). Overall the pattern of transfected G\(\alpha\)\(i2\) and Q205L\(\alpha\)\(i2\) is very similar to that seen with the endogenous G\(\alpha\)\(i2\) subunits. These results confirm that transfected G\(\alpha\)\(i2\) and Q205L\(\alpha\)\(i2\) partition between the lateral membrane overlapping with the TJ and intracellular compartments. This finding is similar to our prior findings with G\(\alpha\)\(i2\)-transfected MDCK cells (16).

Because transfected G\(\alpha\)\(i2\) and Q205L\(\alpha\)\(i2\) were localized in a manner similar to that of the endogenous G\(\alpha\)\(i2\), we next determined whether G\(\alpha\)\(i2\) localization in the TJ had any functional consequences for the tight junction. PC-, G\(\alpha\)\(i2\)-, and Q205L\(\alpha\)\(i2\)-MDCK cells were simultaneously analyzed under steady state conditions and also by using the Ca\(^{2+}\) switch. TJ integrity was followed by measurement of transepithelial resistance. Fig. 4A demonstrates the base-line resistances in these cells and the pattern of TER development after the Ca\(^{2+}\) switch. Overexpressing G\(\alpha\)\(i2\) had a small but insignificant (\(p = 0.07\)) effect on base-line resistances in comparison with PC-MDCK cells (576 ± 89 versus 377 ± 52; \(n = 12\), but Q205L\(\alpha\)\(i2\)-MDCK cells had significantly higher base-line TER values (1187 ± 150 \(\Omega\) cm\(^2\); \(p < 0.001\)). The base-line TER values for G\(\alpha\)\(i2\)- and PC-MDCK cells were similar to reported values of G\(\alpha\)\(i2\) (16), and several clones were analyzed with no significant differences seen among the clones. To gain insight into the mechanism of higher TER values observed in Q205L\(\alpha\)\(i2\)-MDCK cells, all three cell lines were simultaneously analyzed in the Ca\(^{2+}\) switch. The elevated TER in Q205L\(\alpha\)\(i2\)-MDCK cells could be achieved by differences in the kinetics of TER development. Nonlinear regression analysis of the TER data between 0–12 h for all of the cell lines (Fig. 4A) indicates an asymptotic approach to peak TER. Although the data do not precisely fit standard kinetic models, the kinetics of TER development in these cells is similar to what has been reported in other studies (15, 26).

The time to half-maximal TER is a useful value for discussing the effects of G\(\alpha\)\(i2\) expression on TER biogenesis, and these values were calculated for each cell line in the presence and absence of AlF\(_4^-\) (Table 1). Table 1 shows that the time to half-maximal TER (\(T_{50}\)) was significantly more rapid in G\(\alpha\)\(i2\)- and Q205L\(\alpha\)\(i2\)-MDCK cells (0.8 ± 0.3 and 1.2 ± 0.3 h, respectively) in comparison with PC cells (3.0 ± 0.5 h). AlF\(_4^-\) had no significant effect on G\(\alpha\)\(i2\)-transfected cells but significantly shortened \(T_{50}\) for PC cells (1.1 ± 0.7 h, a value similar to that of G\(\alpha\)\(i2\) cells). The observation that PC-MDCK cells treated with AlF\(_4^-\) develop TER nearly as rapidly as Q205L\(\alpha\)\(i2\)-MDCK cells suggests that G\(\alpha\)\(i2\) may be the predominant G\(\alpha\) subunit critical in TER development. Fig. 4B shows that the base-line TER values for the three cell lines on the day following the Ca\(^{2+}\) switch (26 h) are significantly higher in the presence of AlF\(_4^-\). In contrast to AlF\(_4^-\) effects on the rate of TER development, all three cell lines had significantly increased base-line TER at 26 h in the presence

### Table 1

|          | TER Max | \(T_{50}\) |
|----------|---------|-----------|
| G\(\alpha\)\(i2\) | 525     | 0.8 ± 0.3 |
| G\(\alpha\)\(i2\) + AlF\(_4^-\) | 600     | 0.5 ± 0.4 |
| Q205L\(\alpha\)\(i2\) | 975     | 1.2 ± 0.3 |
| Q205L\(\alpha\)\(i2\) + AlF\(_4^-\) | 975     | 1.1 ± 0.5 |
| PC       | 450     | 3.0 ± 0.5 |
| PC + AlF\(_4^-\) | 700     | 1.1 ± 0.7 |
of AlF$_4^-$, similar effects of AlF$_4^-$ were seen with the three cell lines cultured in the steady state (not shown). This raises the possibility that AlF$_4^-$ activates additional G subunits in the steady state that enhances transepithelial resistance.

Taken together, these studies offer direct evidence that Ga$_2$ is a critical regulator of tight junction biogenesis and affects baseline characteristics of the tight junction. The protein composition of the TJ is complex with one integral membrane protein identified so far (occludin), several peripherally attached proteins with partially defined functions (including ZO-1, -2, and -3) and a variety of signal transduction molecules including PKC isoforms, G subunits, and tyrosine kinases (see Ref. 5 for review). How these diverse proteins function to maintain and regulate the development of tight junctions is not well understood. G proteins could be activated within the TJ through a classical seven-transmembrane receptor (although none yet identified in the TJ) or alternatively through a modulatory protein that promotes GDP release or slows GTP hydrolysis. Additional transmembrane proteins must exist within the TJ (27), and there are multiple examples of modulatory proteins that affect G protein function. GTPase activating proteins (RGS proteins; regulators of G protein signaling; reviewed in Ref. 28) interact with G subunits, and nucleotide exchange factors that promote GDP release have been described for many small G proteins such as Ras (29). Although analogous proteins for G subunits have not yet been identified, such proteins may exist and could provide mechanisms for activation of G subunits in the TJ or within intracellular compartments (30). Our findings that Ga$_2$ is important for both the maintenance and development of the TJ does not exclude roles for other Ga subunits, and in fact the effects of AlF$_4^-$ on the steady state TER suggests that other Ga subunits are likely to enhance this barrier.

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