The tight junction (TJ) of polarized epithelial cells is critical for maintaining an impermeant barrier and epithelial cell polarity. The signaling events important for TJ assembly require regulated calcium stores and protein kinase C (PKC), but the earliest signaling events in the cascade have not been well defined. We now show that Goα2 in Madin Darby canine kidney (MDCK) cells localizes to a region overlapping with the TJ. To further analyze the localization of Go subunits in epithelial cells, rat Goα2, Q205LGoα2 (Goα2 “activated” by point mutation) and plasmid without insert (PC) were transfected into MDCK cells and localized by immunofluorescence and confocal microscopy. Similar to endogenous Goα2, Goα2-MDCK cells localize Goα2 (84% similar to Goα2) in the subapical region overlapping with ZO-1 (zona occludens-1), a key component of the TJ. PC-MDCK cells have no detectable Goα. In Goα2-MDCK cells, a physical association of Goα with components of the TJ was detectable by immunoprecipitation of ZO-1. Immunoprecipitates of ZO-1 from Goα2-MDCK cells consistently coprecipitated Goα. Constitutively active Q205LGoα localized to the subapical lateral membrane similar to wild-type Goα. To determine if constitutively activated Go subunits can affect TJ biogenesis, the formation of tight junctions in PC, Goα2, and Q205LGoα2-MDCK cells was followed by measurement of transepithelial resistance (TER) during the Ca2+ switch. Cells were transfected with a plasmid containing a Ca2+ switch, which allows the process to be followed by immunocytochemical or biochemical analyses. The results indicate that internal calcium stores, which are known to substantially overlap with the IP3-sensitive stores in the endoplasmic reticulum, are critical for TJ formation during polarized epithelial biogenesis. These results also suggest that TJ molecules are sorted in a calcium-dependent manner to the lateral surface of the plasma membrane where they associate with the actin cytoskeleton.

Because regulated calcium stores are critical for TJ formation and because these stores are released in parallel with activation of protein kinase C (PKC), the role of this kinase in TJ formation has been studied. When PKC was inactivated by independent mechanisms (calphostin C or low concentrations of H7), both the sorting and assembly of TJ proteins was perturbed, as determined by immunocytochemistry and TER (10, 11). Consistent with this, diacylglycerol analogs accelerate TJ assembly (12). Moreover, PKC was translocated from the junctional complex, associate with at least one TJ protein, and that activated Goα accelerates TJ biogenesis without significantly affecting the maintenance of the TJ. Together, these results suggest an important role for heterotrimeric G proteins in TJ assembly.

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The tight junction (TJ) serves not only as a barrier function in epithelial tissue, but may also play an essential role in establishing and maintaining lipid and protein polarity (reviewed in Refs. 1 and 2). In developing tissues as well as cultured cell models, mechanisms involved in regulating the bioassembly of tight junctions appear significantly different from those necessary for their maintenance. MDCK cells in culture provide an excellent and widely utilized model for the assembly of tight junctions. MDCK cells maintained in low calcium media (LC media) lack cell-cell contact, intercellular junctions, and apical-basolateral polarization of lipids and protein (3, 4–7). Upon “switching” to normal calcium media (NC media), the cells rapidly develop characteristics of a polarized, tight, transporting epithelium. In many respects, the MDCK cell calcium switch model recapitulates in vitro key events in epithelial morphogenesis (8, 9). The presence of a single cell type undergoing intercellular junction formation in a synchronous manner allows the process to be followed by immunocytochemical and biochemical, as well as physiological means.

We have previously shown that, as MDCK cells form junctions and polarize, there are impressive changes in intracellular calcium concentration (3, 5). Most interestingly, at sites of cell-cell contact, where tight junctional assembly occurs, we observed localized increases in intracellular calcium (5). When these increases were buffered with cell-permeant calcium chelators or when endoplasmic reticulum calcium stores were depleted with thapsigargin (3, 7), the assembly of the TJ was perturbed, as determined by immunocytochemistry and trans-epithelial electrical resistance (TER, a functional measure of TJ integrity). Furthermore, the association of TJ proteins (e.g. ZO-1) with the cytoskeletal fraction was disrupted, as determined by their solubility profile in detergent (Triton X-100) extracts. These results indicate that internal calcium stores, which are known to substantially overlap with the IP3-sensitive stores in the endoplasmic reticulum, are critical for TJ formation during polarized epithelial biogenesis. The results also suggest that TJ molecules are sorted in a calcium-dependent fashion to the lateral surface of the plasma membrane where they associate with the actin cytoskeleton.

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cytosol to the membrane fraction, and at least one PKC isoform was translocated directly to the vicinity of the TJ (11). During TJ assembly, several key TJ proteins, including ZO-1 and ZO-2, appear to be phosphorylated in a PKC-dependent fashion (11).

A key question remains to be answered regarding the early signaling events that are critical for TJ biogenesis. If regulated calcium stores and PKC are necessary for TJ assembly, it is likely that a heterotrimeric G protein(s) plays an essential role early in the process. Reagents that modulate G protein function such as cholera toxin, pertussis toxin, and AlF3 have been shown to affect TJ biogenesis during the Ca2+ switch in MDCK cells (4) and Gαi2 has been localized in the vicinity of the tight junction overlapping with ZO-1 in LLC-PK1 cells (13). In the course of studying the localization of Ga subunits in MDCK cells, we have expressed Ga, and activated Ga, (Q205L) in MDCK cells. The highly conserved Gln-205 residue in Ga is important during the transactivation of GTP hydrolysis. The Gln → Leu mutation significantly slows GTP hydrolysis and results in a constitutively “activated” Ga subunit (14, 15). We now show that transfection of Ga and Q205L into MDCK cells leads to their localization near the junctional complex, association with at least one TJ protein (ZO-1), and that expression of activated Ga (Q205L) accelerates TJ biogenesis.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Cell Culture—Rat Gaα cDNA was cloned into Bluescript (Stratagene) as described previously (16) and recloned into the EcoRI/ApaI sites of pcDNA3 (Invitrogen). pMN-Q205Lα,α,α,α was provided by Dr. R. Iyengar, and the cDNA was amplified by PCR using primers to the 5’-end and to the opposite strand 3’-end. The resulting cDNA was cloned into pcDNA3 and sequenced by T7-double-stranded DNA sequencing (U. S. Biochemical Corp). MDCK cells were maintained in Dulbecco’s modified Eagle’s Medium, supplemented with antibiotics plus 5% fetal calf serum. Transfected cell lines were maintained in G418 (500 μg/ml; Life Technologies, Inc.).

Transfections—Subconfluent MDCK cells (ATCC, Rockville MD) were transfected with 10 μg of linearized pU1, Gaα, Q205Lα,α,α,α or pcDNA3 by the calcium phosphate precipitation method. G418-resistant colonies were analyzed for expression by Western blot analysis using R4 polyclonal antibody provided by Dr. E. Neer as described previously (17). Two clones expressing Q205Lα,α,α,α and four Gaα clones were further analyzed. There were no significant differences between clones.

Immunolocalization—Untransfected, Pc-, Ga-, and Q205Lα,α,α,α MDCK cells were plated at confluence on coverslips or Transwell filters (12 mm) (Costar), washed with PBS, and fixed with methanol (100%, −70 °C) for 10 min and stained as previously described (11). Samples were incubated with one or two of several antibodies; rabbit polyclonal Gaα (RA) at 1:100 dilution, rat monoclonal to ZO-1 (undiluted; hybridoma courtesy of D. Goodenough), mouse monoclonal to E-cadherin (courtesy of B. Gumbiner) (undiluted), mouse monoclonal to α-catenin (apical marker; courtesy of G. Ajakian). The Gaα antibody (A57; DuPont NEN) was used at 1:50 dilution. One or two of the following secondary antibodies were used: goat anti-rabbit Texas Red or fluorescein, goat anti-rat Texas Red or fluorescein, or goat anti-mouse Texas Red or fluorescein (all from Jackson ImmunoResearch, West Grove, Pa.). Coverslips or filters were visualized on a Nikon Labphot-2 microscope equipped with epifluorescence or a Bio-Rad MRC 600 confocal microscope equipped with 2 filter blocks for dual label image acquisition.

Immunoprecipitation—Confluent Gaα- and Pc-MDCK cells were scraped in cold PBS, resuspended in 1 ml of buffer A (50 mM Tris, pH 7.6, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 100 mM NaCl, 2 mM EDTA, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and soy and lima bean trypsin inhibitors), frozen and thawed three times, and triturated through a 27-gauge needle. Samples were centrifuged at 10,000 rpm for 10 min, and the supernatant was precleared with 25 μl of goat anti-rat Sepharose beads (Cappel). The supernatant was incubated with ZO-1 hybridoma supernatant (200 μl) or an equivalent amount of rat serum or control rat hybridoma and 50 μl of goat anti-rat Sepharose beads overnight at 4 °C with rocking. These conditions quantitatively immunoprecipitate ZO-1 and several other proteins of the tight junction (11). Beads were washed and samples were analyzed by SDS-PAGE and Western with the R4 Gaα antibody as described previously (17). The nitrocellulose was de-

**Fig. 1. Immunolocalization of Gaα in MDCK cells.** A, wild-type MDCK cells were grown to confluence on coverslips and double-stained with rabbit Gaα antibody and ZO-1 hybridoma as described under “Experimental Procedures.” The coverslips were visualized on a Nikon Labphot-2 microscope, and the identical field was photographed in the same focal plane with Tnax 3200 film (Kodak) for 3–6 s using the FITC filter for ZO-1 and Texas Red filter for Gaα. Magnification = ×1030. B, coverslips of wild-type MDCK cells were prepared as described in A, and confocal images of two cells (marked 1 and 2) were obtained on Bio-Rad MRC 600 confocal microscope with dual filters that permits simultaneous detection of fluorescein and Texas Red. The same cells (1 and 2) labeled with Gaα and ZO-1 antibodies are displayed in the XZ orientation with the apical (Ap) membrane marked with a white line. Digital images were labeled and merged with images from A using Adobe Photoshop Mountain View, CA. Magnification = ×1050.

RESULTS AND DISCUSSION

Epithelial cells are known to constitutively express a variety of Ga subunits, including Gaα2, Gaα3, Gaα5, and Gaα1. In rat enterocytes, Gaα2 and Gaα3 are localized in the basolateral membrane while Gaα1 appears evenly distributed in both apical and basolateral membranes (18). Along the renal tubule, Ga subunits can be found in either the apical, basolateral, or both domains depending upon the cell type (19, 20). In cultured renal epithelial cells such as LLC-PK1 cells (porcine renal cell line with proximal tubule characteristics), Gaα2 overlaps with the tight junction (13), and Gaα3 is found predominantly in the Golgi (21, 22). Fig. 1 shows that Gaα2 in wild-type MDCK cells has a distribution similar to that reported for LLC-PK1 cells. Gaα2 partially overlaps with the tight junction protein, ZO-1 when identical fields are visualized by standard immunofluorescent microscopy. Fig. 1A shows linear staining of both Gaα2 and ZO-1 that is consistent with basolateral localization. In addition, there is detectable intracellular staining of Gaα2 that...
is more prominent than is seen for ZO-1. To refine the localization of Gαo and ZO-1 in MDCK cells, confocal images in the XZ orientation were obtained. Fig. 1B shows two cells double-labeled with Gαo and ZO-1 antibodies. The distribution of Gαo and ZO-1 along the lateral membrane is similar but not identical. There is overlap of the two proteins although it appears that Gαo has a slightly broader distribution along the lateral membrane and some intracellular staining. In our studies aimed at identifying elements involved in the targeting of G proteins to specific locales in epithelial cells, we began with a G protein not normally expressed in epithelial cells; Gαo, the major G protein in neuronal tissues. We used MDCK cells, which have been widely employed to study epithelial junctions and polarity.

When Gαo was transfected into MDCK cells, the protein was found to be highly expressed and localized predominantly laterally by standard immunofluorescence (not shown). More detailed examination of the cellular localization using confocal microscopy revealed that the Gαo subunit had been efficiently targeted to the lateral-subapical region of the transfected cells in the vicinity of the apical junctional complex (Fig. 2A). Subsequent double-labeling studies and confocal microscopy with antibodies against the adherens junctional protein, E-cadherin, and the TJ protein, ZO-1, revealed that the transfected Gαo subunit localized to a region between the TJ and adherens junctions, at least partially overlapping both (Fig. 2A). Thus, the distribution of Gαo in Gαo-MDCK cells is remarkably similar to the distribution of Gαo in wild-type MDCK cells (Fig. 1). Fig. 2B, left panel, shows that there is no detectable staining of PC-MDCK cells with Gαo antibody.

Given the close proximity of Gαo to the tight junction, we sought to determine whether the transfected Gαo subunit associated with proteins of the TJ. A number of laboratories have previously shown that antisera against ZO-1 immunoprecipitates at least three TJ proteins: ZO-1, ZO-2, and p130 (11, 12). When transfected MDCK cells were immunoprecipitated with antibodies against ZO-1, Gαo could be consistently detected in Gαo-MDCK cells but not in PC-MDCK cells (Fig. 3). The conditions for immunoprecipitation have previously been determined to quantitatively immunoprecipitate almost all of the ZO-1 (11). This result indicates that a fraction of Gαo in transfected cells associates into a complex containing at least one TJ protein and raises the possibility of a functional interaction between Gαo and the TJ. In order to further examine this possibility, we transfected a constitutively activated Gαo mutant (Q205L) into MDCK cells. This point mutation is constitutively “activated” and maintained in a receptor-activated conformation.

Standard and confocal microscopy revealed that, like wild-type Gαo, Q205Lαo is also targeted to the lateral membrane in the vicinity of tight junction. Fig. 2B, right panel, demonstrates linear lateral surface staining of Q205Lαo when imaged by confocal microscopy. To address the role of constitutively activated Gαo in the TJ, transepithelial electrical resistance (TER), a reliable measure of TJ integrity, was monitored both in confluent monolayers and also during the MDCK cell “Ca2+ switch.” The MDCK cell Ca2+ switch is a model widely employed to study the bioassembly of the TJ and has been particularly useful in elucidating signaling events (3, 4, 5, 10–12). The time course for TER development at various times is shown in Fig. 4A, and the rates of TER development for the early phase (0–4 h) and later phase (4 h-peak) are shown in Fig. 4B. In confluent monolayers, there was no significant difference between the cell lines in baseline TER values (Fig. 4A; p > 0.05) indicating that expression of Gαo and Q205Lαo had little if any effect on the maintenance of TJ integrity. The baseline TER values of nontransfected MDCK cells was similar to PC cells (not shown). In the early phase of the Ca2+ switch (from 0–4 h), Q205Lαo cells had significantly faster (p < 0.006) TER development than Gαo or PC cells (54 ± 4 versus 23 ± 3 (PC); 12 ± 1 (Gαo) Ωcm²/h). In the later stages of the Ca2+ switch (4 h-peak), Q205Lαo-MDCK continued to demonstrate accelerated TER development in comparison to Gαo- and PC-MDCK cells (117 ± 10 versus 45 ± 5 (PC); 66 ± 7 (Gαo) Ωcm²/h). The significantly faster rate of TER development in Q205Lαo-MDCK cells results in much higher TER values at all time points and peak TER values that are significantly (p < 0.02) higher (1168 ± 107 Ωcm²) when compared with Gαo-.
(548 ± 54 Ωcm²) and PC-MDCK (437 ± 37 cells) (Fig. 4A). These data suggest an important role for signaling initiated by a heterotrimeric G protein in TJ assembly and distinct from a role in the maintenance of the formed TJ. Consistent with this notion, TER measurements 28 h after cell contact was established were similar to those in confluent monolayers with no significant difference between transfected cells and mock-transfected controls (Fig. 4A). Thus, the localization of an activated Ga subunit into the TJ accelerates TJ biogenesis. Together, these results suggest a critical role for a heterotrimeric G protein in TJ bioassembly, presumably through activation of calcium signaling and PKC, although a direct link remains to be established. Of course, a limitation of these experiments is their heterologous nature since the key G protein in TJ assembly in MDCK cells must be something other than Ga. This could be Ga, which is found in the vicinity of the TJ or another unidentified G protein associated with the TJ.

The association of at least a fraction of Ga with Zo-1 is interesting not just because it suggests an intimate physical association of a G protein with a critical component of the TJ, but also because of similarities with neuronal cells. Ga is concentrated in the distal tip of neuronal processes and is regulated by GAP-43, a protein essential for accurate pathway development of growing neurites (23, 24). Expression of constitutively activated Ga increases the number of neurites per cell possibly by affecting protein kinase C and intracellular calcium release (25, 26). Zo-1 is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins, and, apart from Zo-2, its greatest reported homology is with an essential protein of the postsynaptic density, PSD-95 (27). Ga is also enriched in the post-synaptic density (28), although it is unknown if Ga associates with PSD-95. Ga also regulates N-type calcium channels in the heart (29) as well as several other signaling pathways (reviewed in Ref. 30). Thus, the effect of Ga in the Ca²⁺ switch of MDCK cells could conceivably result from modulation of intracellular and/or extracellular calcium. This intriguing parallel raises the possibility that these proteins (ZO-1, Zo-2, and PSD-95), and perhaps other MAGUK family members, may possess structural motifs that confer an ability to associate with G proteins. Zo-1 and PSD-95 share 55% amino acid sequence similarity, and each of these proteins is about 40% similar to GAP-43. However, there are no obvious sequence motifs in the primary sequences of these proteins that suggest a site for interaction with Ga subunits. Important questions in TJ biogenesis remain regarding events upstream and downstream to the activation of G proteins and precisely which G protein(s) are involved. Nevertheless, the results of this study provide evidence for an important role of Ga subunits in the early events of tight junction biogenesis.

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