Zonula Occludens-1 Is a Scaffolding Protein for Signaling Molecules

**Gα_{12} DIRECTLY BINDS TO THE Src HOMOLOGY 3 DOMAIN AND REGULATES PARACELLULAR PERMEABILITY IN EPITHELIAL CELLS**

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Zonula occludens proteins are multidomain proteins usually localized at sites of intercellular junctions, yet little is known about their role in regulating junctional properties. Multiple signaling proteins regulate the junctional complex, and several (including G proteins) have been co-localized with zonula occludens-1 (ZO-1) in the tight junction of epithelial cells. However, evidence for direct interactions between signaling proteins and tight junction proteins has been lacking. In these studies, we constructed Go-glutathione S-transferase (GST) fusion proteins and tested for interactions with [35S]methionine-labeled in vitro translated ZO-1 and ZO-2. Only Gα_{12} di- rectly interacted with in vitro translated ZO-1 and ZO-2. Using a series of ZO-1 domains expressed as GST fusion proteins and in vitro translated [35S]methionine-labeled Gα_{12}, we found that Gα_{12} and constitutively active (Q229L) α_{12} (QLα_{12}) bind to the Src homology 3 (SH3) domain of ZO-1. This binding was not detected with SH3 domains from other proteins. Inducible expression of wild-type α_{12} and QLα_{12} in Madin-Darby canine kidney (MDCK) cells was established using the Tet-Off system. In Gα_{12}-expressing cells, we found that ZO-1 and Gα_{12} co-localize by confocal microscopy and co-immunoprecipitate. Gα_{12} from MDCK cell lysates bound to the GST-ZO-1-SH3 domain, and expression of QLα_{12} in MDCK cells reversibly increased paracellular permeability. These studies indicated that ZO-1 directly interacts with Gα_{12} and that Gα_{12} regulates barrier function of MDCK cells.

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To provide a barrier function, epithelial cells have evolved a highly organized junctional complex that includes tight junctions (TJs), adherens junctions, gap junctions, and desmosomes. A variety of proteins have been identified within the junctional complex; they include integral membrane proteins (such as claudins, occludin, and E-cadherin), signaling molecules (protein kinase C, Src tyrosine kinases, small G proteins, and heterotrimeric Gα subunits), and potential scaffolding proteins (ZO-1, ZO-2, and ZO-3; for reviews, see Refs. 1 and 2). The ZO proteins are multidomain MAGUK (membrane-associated guanylate kinase) family members that contain a potential guanylate kinase domain, multiple PDZ domains, and a SH3 domain (see Fig. 1). Although ZO-1 is expressed in multiple cell types, in epithelial cells it is localized only in the tight junction, the most apical component of the junctional complex. The two related family members, ZO-2 and ZO-3, form a complex with ZO-1 in the TJ and link to the actin cytoskeleton (3). The function of ZO proteins in regulating the junction is unknown, but based upon their domain structure, they have been proposed to be scaffolding proteins (4).

Several Gα subunits, including Gα_{12}, partially co-localize within the epithelial cell junction (1). Regulation of the junctional complex by heterotrimeric G proteins was suggested in early experiments (5). Subsequent studies have demonstrated that pertussis toxin-sensitive family members, Gα_{12} and Gα_{16}, localize in the tight junction of Madin-Darby canine kidney (MDCK) cells and affect both tight junction assembly and baseline properties (6, 7). Recently we showed that Gα_{16} stimulates tight junction assembly and co-localizes with a ZO-1 complex (8). However, to date, there has been no direct demonstration of an interaction between G protein signaling molecules and TJ proteins. Utilizing in vitro binding studies and inducible expression in MDCK cells, we demonstrate that Gα_{12} directly binds to ZO-1 through the SH3 domain and that activated Gα_{12} regulates paracellular permeability.

EXPERIMENTAL PROCEDURES

Reagents—cDNAs for mouse wild-type α_{12} (wtα_{12}) and constitutively active (Q229L) QLα_{12}, were provided by Henry Bourne (University of California, San Francisco), Gα_{12} was provided by D. Barber (University of California, San Francisco), and ZO-1, ZO-2, and anti-ZO-1 rat monoclonal antibody (R40.76) were from Dr. Goodenough (Harvard Medical School, Boston, MA). ZO-1-GST fusion protein constructs were the generous gift of M. Balda (University College, London) and are described in Ref. 9. The SH3 domains of n-Src, e-Src, Crk, Abl, and Grb2 were provided by Bruce Mayer (Children's Hospital, Boston, MA) (10). Gα_{12} antibodies were from Santa Cruz Biotechnology (S-20, Santa Cruz, CA). Tet-Off MDCK type II epithelial cells (MDCK-T23), Tet-Off cloning vectors, and Tet-free fetal calf serum were obtained from CLONTECH (Palo Alto, CA).

In Vitro Protein Binding Studies—Gα_{12}-, Gα_{16}-, Gα_{16}-, and Gα_{12}- GST fusion protein constructs were cloned as N-terminal GST-Gα fusion proteins using standard techniques into pGEX4T (Amersham Biosciences). GST fusion proteins were expressed in Escherichia coli and purified from bacterial lysates as described previously (11). In vitro translation of the Gα subunits, ZO-1, and ZO-2 was performed using [35S]methionine in a coupled rabbit reticulocyte lysate system (Promega, Madison, WI) as described previously (11). Similar amounts of [35S]-labeled proteins were incubated with 1 μg of GST fusion protein

1 The abbreviations used are: TJ, tight junction; ZO, zonula occludens; SH3, Src homology 3; GST, glutathione S-transferase; QL, Q229L; wt, wild type; MDCK, Madin-Darby canine kidney; dox, doxycycline; FITC, fluorescein isothiocyanate; TER, transepithelial resistance; GTPγS, guanosine 5′-O-(thio)triphosphate; Tet, tetracycline.
prebound to glutathione-agarose 4B overnight at 4 °C in 50 mM Tris-HCl, pH 7.4, 75 mM sucrose, 6 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Triton X-100 (buffer A). Samples were washed in buffer A, eluted, and analyzed by SDS-PAGE and autoradiography.

Tet-Off Inducible MDCK Cell Lines—wt12 and QL12 were subcloned into the pTRE expression vector (CLONTECH) by standard techniques. Tet-Off MDCK cells were cultured as described by Jou et al. (12). Cells were transfected using linearized pTK-Hyg with FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Resistant colonies were maintained in medium containing 100 μg/ml hygromycin, 100 μg/ml G418, and 40 ng/ml doxycycline. Subsequent experiments were carried out in medium without hygromycin and G418 and with or without doxycycline.

Immunohistochemistry—Ga12 and QL12-transfected Tet-Off MDCK cells were grown to confluency with or without doxycycline for specific times. Cells were fixed with ice cold 100% methanol for 20 min at −20 °C and blocked for 45 min at room temperature in 5% (w/v) nonfat milk containing 0.05% Triton X-100. Ga12 antibody was diluted 1:100 into ZO-1 hybridoma and incubated at 4 °C for 1.5 h and rinsed three times with 0.05% Triton X-100 followed by incubation with goat anti-rabbit Texas Red and goat anti-rat FITC-conjugated secondary antibodies (Pierce) at a 1:100 dilution. Slides were viewed on a Bio-Rad MRC-1024/2p confocal microscope, and images were processed using Photoshop software (Adobe, San Jose, CA).

Immunoprecipitation and Western Immunoblot Analysis—wt12 or QL12 cells were cultured with or without doxycycline for 3 days. Whole cell lysates were obtained by scraping monolayers in buffer B (100 mM NaCl, 2 mM EDTA, 10 mM HEPES, pH 7.5, 1 mM NaVO4, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) and brief sonication on ice. Antibodies against ZO-1 (rat) or Ga12 (rabbit) were added to the lysate overnight at 4 °C. Beads were washed with buffer B, and proteins were eluted in SDS-PAGE sample buffer. Western blot analysis using chemiluminescence detection (Pierce) was performed as described previously (13).

Transsepithelial Resistance (TER) and Paracellular Flux Measurements—MDCK cells were plated on polycarbonate filters (Transwell, Costar) at confluent density (2 × 104 cells/cm2), and TER was measured at different time points using a Millipore ERS electrical resistance system. Measurements are expressed in ohm × cm2 as a mean of the original readings after subtraction of background values. Paracellular flux was measured as described previously (14) using cells cultured on Transwell filters for 3 days with or without doxycycline. FITC-labeled 70-kDa dextran (concentration, 3.5 μM; Molecular Probes, Eugene, OR) was added into the apical chamber, and aliquots were taken from the lower chamber after 20, 40, and 60 min. FITC-dextran concentrations were determined using a fluorescent plate reader and standard curves (excitation, 485 nm; emission, 530 nm; CytoFluor 2300, Millipore Corp., Waters Chromatography, Bedford, MA) as described previously (14).

RESULTS AND DISCUSSION

The function(s) for multiple ZO proteins within the TJ is unknown, but other PDZ family members have been shown to provide a scaffold for signaling complexes. In Drosophila, a G protein signaling complex has been identified in association with unknown, but other PDZ family members have been shown to provide a scaffold for signaling complexes. In Drosophila, a G protein signaling complex has been identified in association with unknown, but other PDZ family members have been shown to provide a scaffold for signaling complexes. In Drosophila, a G protein signaling complex has been identified in association with unknown, but other PDZ family members have been shown to provide a scaffold for signaling complexes. In Drosophila, a G protein signaling complex has been identified in association with unknown, but other PDZ family members have been shown to provide a scaffold for signaling complexes.

Fig. 1. Interaction of Ga subunits with ZO-1 and ZO-2. A, GST alone, Ga12, and Ga12-GST fusion proteins (1 μg) were incubated overnight with in vitro translated [35S]methionine-labeled ZO-1 and ZO-2 as described under “Experimental Procedures.” 1 μl of in vitro translated ZO-1 and ZO-2 were used in overnight incubations, and the autoradiogram was exposed for 72 h. Ga12, structure of ZO-1 with PDZ domains, guanylate kinase (GUK), SH3, acidic, α, and proline-rich domains. Ga12 constructs A–F with specific amino acids and relationship to overall structure are shown underneath. Ga12, Ga12A, and Ga12-B-GST fusion proteins (1 μg) were incubated with 10 μl of [35S]methionine-labeled wt12, wt12, wt12, and QL12 as described under “Experimental Procedures.” 1 μl of each in vitro translated protein is shown on the right. A discrete separation of these Ga subunits was consistently seen under these electrophoresis conditions. Exposure time was 72 h. D, Ga12 and fusion proteins C, D, E, and F (1 μg) were incubated with [35S]methionine-labeled wt12 and QL12 (10 μl) as described above. 1 μl of wt12 was shown in the first lane. Exposure time was 72 h. E, [35S]methionine-labeled wt12 was incubated with equivalent amounts of GST-ZO-1-A and GST-SH3 from Abl, Csk, n-Src, c-Src, and Grb2 and analyzed as described above. Exposure time was 72 h. IVT, in vitro translated.

Ga12 is difficult to study in cells due to its low levels of expression. Therefore, we established a model system in which...
40 ng/ml doxycycline (dox) and then switched to doxycycline-free dox medium. We also measured paracellular flux of 70-kDa FITC-dextran in MDCK cells. Similar to other reports (12, 20), we utilized Western blot and confocal microscopy to analyze Gα12 protein expression (Fig. 2A), expression, localization, and interactions with ZO-1 in MDCK cells. Cells were cultured for 72 h ± dox, and the rate of movement for a 70-kDa FITC-dextran molecule was determined in triplicate. * indicates significant difference (p < 0.005).

MDCK cells. Similar to other reports (12, 20), we utilized Tet-Off inducible MDCK cell lines to express wtα12 and QLα12. wtα12 and QLα12 proteins were expressed within 24–48 h after the removal of doxycycline (Fig. 2A). Protein levels remained constant for up to 10 days in the absence of doxycycline, and expression of Gα12 proteins could be reexpressed within 48 h with the readdition of doxycycline (not shown). Gα12 had previously been demonstrated to co-localize with ZO-1 in MDCK cells by confocal microscopy (16). Endogenous Gα12 was barely detectable by Western blot and confocal microscopy in the presence of doxycycline (Fig. 2, A and panels a and e in B), but after 48 h of Gα12 expression (dox) there was co-localization with ZO-1 in the TJ (Fig. 2B, panels b and d). Expression of QLα12 in MDCK cells resulted in an altered cell phenotype and a complex staining pattern for QLα12 and ZO-1 (Fig. 2B, panels f and h). Some QLα12 was localized intracellularly, and this could occur from overexpression and/or reduced affinity for the plasma membrane. Although the staining patterns of QLα12 and ZO-1 were different, there appeared to be partial overlap in the lateral margins of the cell. Consistent with this observation, immunoprecipitation of Gα12 from both wild-type and QL-expressing cells co-precipitated ZO-1 (Fig. 2C). In the absence of Gα12 expression (+ dox) or with no added antibody (Fig. 2C, last lane) there was no detectable ZO-1. The expression levels of ZO-1 were not affected by wtα12 or QLα12 expression (determined by Western blot, not shown), and the increased immunoreactivity in QL lysates was unique to this experiment. We next utilized the ZO-1-GST fusion proteins (Fig. 1B) and Western blots to determine whether wtα12 or QLα12 expressed in MDCK cells could bind to specific ZO-1 domains. The ZO-1 SH3 domain (E fusion protein) was capable of binding to both wtα12 and QLα12 from cell lysates prepared from −dox cells (Fig. 2D). Identical to the in vitro results (Fig. 1), the B and E fusion proteins did not interact (not shown), and GST alone was negative (Fig. 2D). These results indicate that the SH3 domain is sufficient for interactions with Gα12 but do not exclude contributions from other ZO-1 domains.

The disrupted appearance of ZO-1 in the lateral membrane of QLα12-expressing MDCK cells (Fig. 2B) suggested that barrier function might be altered in these cells. To address this question, we measured TER and paracellular flux. Base-line TER was comparable in MDCK Tet-Off cells, uninduced wtα12, and QLα12 MDCK cell lines (Fig. 3A, + dox at T = 0). There was some variability in base-line TER as seen with the two sets of QLα12 cells at T = 0, but this was comparable to other reports (20). Expression of wtα12 protein resulted in a small decrease in TER after 24 h but remained nearly identical to Tet-Off MDCK cells throughout the remainder of the experiment (Fig. 3A, open and closed squares). Although we cannot exclude a subtle role for overexpressing wtα12 on the junction, these effects did not correlate with wtα12 protein expression (± dox). However, inducing QLα12 expression consistently caused a significant fall in TER to ~25 Ω cm² within 48 h (Fig. 3A, n = 7). This fall in TER was reversible as switching to + dox medium resulted in TER returning to normal over the next 24–48 h. There was a brief “overshoot” of base-line TER during TJ formation in QLα12 MDCK cells similar to what is typically observed during TJ assembly (21). The decrease in TER was sustained in QLα12 MDCK cells if doxycycline remained absent but could be reversed at any time by the readdition of doxycycline (not shown). We also measured paracellular flux of 70-kDa FITC-dextran in both Gα12, MDCK cell lines cultured ± doxycycline for 3 days (Fig. 3B). Induction of QLα12 expression led to a greater than 3-fold increase in flux rate (12.8 ± 1.5–40.3 ± 10.4 pmol/min × cm², n = 7), while there was no significant change in wtα12 MDCK cells ± doxycycline (6.5 ± 1.4 versus 9.4 ± 2.5 pmol/
min × cm², n = 7, Fig. 2B, left panel). As an additional control, we counted trypan blue-positive cells in QL12 monolayers ± doxycycline and found no significant difference. Taken together, these results indicate that expression of QL12, but not wt12, reversibly increases paracellular permeability, and these observations are consistent with the changes in ZO-1 staining pattern seen in QL12-expressing cells.

In recent years, the identification of integral membrane proteins in the TJ has resulted in significant progress toward understanding the structural features of the barrier. Claudins are essential for barrier function (2) and for paracellular regulation of specific ions (22, 23). Claudin family members interact with the scaffolding proteins ZO-1, ZO-2, ZO-3, and MUPP-1 (4, 24), and there are multiple interactions of these scaffolding proteins with other tight junction proteins (occludin and junctional adhesion molecule, Ref. 25) and signaling molecules (26, 27). Several different signaling pathways regulate the barrier of intact epithelia. Activation and overexpression of protein kinase C isoforms or monomeric G proteins results in disruption of junctions and increased leakiness in kidney epithelial cells (12, 21, 29). Furthermore, we have previously shown that activated Gα12 and Gαq increase base-line TER in MDCK cells (7, 8). The finding that activation of Gα12 lowers TER and increases paracellular permeability raises the possibility that multiple G proteins function in concert to regulate base-line TJ properties.

For the first time, we have demonstrated that ZO-1 directly interacts with a G protein α subunit and propose that ZO-1 functions to scaffold Gα12. ZO proteins may organize signaling complexes within a discrete membrane microdomain, the TJ. Considering the complex signaling pathways that regulate the TJ, we would suggest that there are likely to be other direct interactions between signaling proteins and ZO proteins. We show that Gα12 can also interact with ZO-2 (Fig. 1A), and other lower affinity interactions may not have been detected. The finding that wto12 and QL12 interact similarly with ZO-1 suggests that the interaction is independent of Gα12 conformation and supports the notion that ZO-1 scaffolds Gα12. Although we cannot exclude the possibility that ZO-1 directly regulates Gα12, we found that the GST-ZO-1-A fusion protein (Fig. 1B) has no effect on GTPγS binding to Gα12. The mechanism(s) for Gα12 regulation of paracellular properties within the TJ and the role of the interaction with ZO-1 remain to be determined. One possibility is that through its interaction with the scaffolding proteins ZO-1 and ZO-2, but not ZO-3, within the C-terminal domain of E-cadherin has been shown to be required for Gα12 binding (amino acids 854–864, Ref. 31). Comparison of these amino acids with the SH3 domain of ZO-1 identifies a highly conserved group of charged residues at amino acids 514–518 (EXID/E/KD/E). The SH3 domain of ZO-2 contains an identical sequence, and ZO-2 also binds to Gα12 (Fig. 1A). However, the SH3 domain of ZO-3 is lacking the last two charged amino acids, and we were unable to detect an interaction of in vitro translated ZO-3 with Gα12 (results not shown). Finally, a recent report identified an interaction between Gα12 and Hsp90, and Hsp90 also contains this identical cluster of charged residues (32). Other motifs have also been identified for Gα12 binding (33). Taken together, these findings support a scaffolding function for ZO-1 and suggest a similar role for ZO-2 and ZO-3 within the TJ. Unraveling the specific connections between ZO proteins and other signaling molecules will help provide the keys to understanding TJ regulation.

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