Distinct Domains of Paracingulin Are Involved in Its Targeting to the Actin Cytoskeleton and Regulation of Apical Junction Assembly*†§

Serge Paschoud‡, Laurent Guillemot‡, and Sandra Citi‡§†

From the ‡Department of Molecular Biology and §Department of Cell Biology, University of Geneva, 4 Boulevard d’Yvoy, 1205 Geneva, Switzerland

**Background:** Paracingulin is a junctional protein that regulates Rho GTPase activities in epithelial cells.

**Results:** Overexpressed mutated paracingulin constructs show different subcellular localizations and effects on junction assembly.

**Conclusion:** The domains of paracingulin involved in targeting to the actin cytoskeleton and in regulation of junction assembly were identified.

**Significance:** The structural basis for the functional differences between cingulin and paracingulin was clarified.

Paracingulin is an Mr 150–160 kDa cytoplasmic protein of vertebrate epithelial tight and adherens junctions and comprises globular head, coiled-coil rod, and globular tail domains. Unlike its homologous tight junction protein cingulin, paracingulin has been implicated in the control of junction assembly and has been localized at extrajunctional sites in association with actin filaments. Here we analyze the role of paracingulin domains, and specific regions within the head and rod domains, in the junction and localization of paracingulin by inducible overexpression of exogenous proteins in epithelial Madin Darby canine kidney (MDCK) cells and by expression of mutated and chimeric constructs in Rat1 fibroblasts and MDCK cells. The overexpression of the rod + tail domains of paracingulin perturbs the development of the tight junction barrier and Rac1 activation during junction assembly by the calcium switch, indicating that regulation of junction assembly by paracingulin is mediated by these domains. Conversely, only constructs containing the head domain target to junctions in MDCK cells and Rat1 fibroblasts. Furthermore, expression of chimeric cingulin and paracingulin constructs in Rat1 fibroblasts and MDCK cells identifies specific sequences within the head and rod domains of paracingulin as critical for targeting to actin filaments and regulation of junction assembly, respectively. In summary, we characterize the functionally important domains of paracingulin that distinguish it from cingulin.

The apical junctional complex (AJC) is characteristic of polarized epithelial cells and comprises the tight junction (TJ) and the adherens junction (AJ). AJC proteins not only orchestrate epithelial cell-cell adhesion and tissue barrier function (1–4) but are also key players in transducing extracellular and intracellular signals to regulate cytoskeletal architecture, cell motility, gene expression, and differentiation (5–9). Both TJ and AJ are connected to the actin cytoskeleton and contain cytoplasmic proteins that regulate actin polymerization and contractility through their physical and functional interactions with actin, actin-binding proteins, and small GTPases of the Rho family. In turn, the establishment and maintenance of the AJC depend critically on the assembly and contractility of the cortical actin cytoskeleton (10–18, 19, 20). Thus, the interplay between AJC proteins and the actin cytoskeleton translates signaling cues into changes in cell shape, motility, barrier function, and gene expression.

Paracingulin (also known as JACOP or cingulin-like protein (CGNL1)) is unique among proteins of the AJC because it has been detected either both at TJ and AJ, or only at TJ, depending on tissue (21, 22). The structure and domain organization of paracingulin are similar to that of the TJ protein cingulin, with globular head, coiled-coil rod, and globular tail domains, but these two proteins are recruited to junctions independently (21–24). Recently, we identified sequences in the head domain of paracingulin that are responsible for targeting paracingulin to TJ and to AJ through its interaction with the TJ protein ZO-1 and the AJ protein PLEKHA7, respectively (25). Interestingly, unlike cingulin, in some tissues paracingulin has been detected in the basal region of epithelial cells, associated with non-junctional actin filaments (21). The molecular basis for the interaction of paracingulin with the actin cytoskeleton is not known. Although in transfected NIH3T3 fibroblast only exogenous constructs containing the globular head domain of paracingulin associate with actin filaments (21), it is not clear whether distinct regions of the head domain target paracingulin to junctions and to the actin cytoskeleton.

The issue of the mechanisms regulating the subcellular distribution and actin association of paracingulin is important, because paracingulin has been implicated in the regulation of RhoA and Rac1 activities at junctions through its interaction with the guanidine nucleotide exchange factors GEF-H1 and
Functional Domains of Paracingulin

Tiam1, respectively (26). MDCK monolayers depleted of paracingulin show increased RhoA activity at confluence, similar to what was observed in MDCK cells depleted of cingulin (26, 27). However, paracingulin-depleted MDCK cells also show altered development of the TJ barrier and impaired Rac1 activation during junction assembly by the calcium switch protocol (26), whereas cingulin depletion has no detectable effect on TJ assembly (27). In vitro binding experiments with recombinant proteins indicate that regions both in the globular head and coiled-coil rod domains of paracingulin can interact directly with the RhoA and Rac1 guanidine exchange factors GEF-H1 and Tiam1, providing a molecular mechanisms for the control of Rho GTPases and TJ assembly by paracingulin (26). However, it is not known whether the head, the rod, or both domains regulate Rac1-dependent TJ assembly within cells.

Here, to identify structural domains of paracingulin that are involved in its subcellular targeting and its function as a modulator of junction assembly, we generated stable lines of MDCK cells that overexpress either full-length paracingulin, or its head, or its rod + tail domains, or chimeric molecules containing switched sequences of cingulin and paracingulin. By examining the subcellular localization of these constructs in MDCK cells and Rat1 fibroblasts and junction assembly in MDCK cells, we define the roles of specific head and coiled-coil rod sequences of paracingulin in modulating junction assembly and targeting to the actin cytoskeleton.

EXPERIMENTAL PROCEDURES

Materials—Antibodies against cingulin (36-4401), claudin-2 (32-5600), claudin-3 (34-1700), occludin (71-1500), GFP (A11122), and ZO-1 (33-9100) were from Invitrogen. Anti-RhoA (sc-179) and anti-ZO-3 antibodies (H-130) were from Santa Cruz Biotechnology. Anti-Rac1 (610650) was from BD Transduction Laboratories, and TRITC-phalloidin was from Sigma (P1951). Secondary antibodies for immunofluorescence were from Jackson Laboratories.

Generation of Full-length, Head, and Rod + Tail Paracingulin Constructs—Constructs coding for full-length (residues 1–1296), head (residues 1–579), and rod + tail (residues 580–1296) domains of canine paracingulin were obtained by reverse transcription (Superscript II reverse transcriptase, Invitrogen) of MDCK RNA (RNAeasy mini kit, Qiagen), followed by subcloning into the NotI-SalI sites of pBluescript. The head region was amplified with primers 5’-AGACAGGGGCAGTGAGCTGTATTTGCCG-3’ (forward) and 5’-ATCCAGTTGTCGACGATTTTCTCAAGACCAGGT-3’ (reverse) and cloned into NotI-SalI sites of pBluescript. The rod + tail region was amplified with primers 5’-AGACAGGGGCAGTGAGCTGTATTTGCCG-3’ (forward) and 5’-ATCCAGTTGTCGACGATTTTCTCAAGACCAGGT-3’ (reverse) and cloned into NotI-SalI sites of pBluescript. All PCR steps were performed with the Expand high fidelity PCR kit (Roche), and all constructs were verified by sequencing. Constructs for Tet-regulated expression of YFP fused to either the full-length paracingulin, paracingulin head, or rod + tail domains were generated in pTRE2-hyg (Clontech). First, we substituted the GFP sequence in the pBS-GFP-CGN-myc plasmid described previously (28) with YFP by subcloning the amplified sequence (from pEYFP-N1, Clontech) into BamHI-NotI sites. Second, we replaced the cingulin sequence, excised by digestion of the pBS-YFP-CGN-myc plasmid with NotI-Clal, with the canine paracingulin cDNAs (either full-length, head, or rod + tail), excised from the pBluescript plasmids, by digestion with NotI-AccI. The tagged paracingulin sequences were then subcloned into the BamHI-Sall sites of pTRE2-hyg (Clontech).

Generation of Chimeric Constructs—Four cingulin/paracingulin chimeras were generated by swapping the “B” and “D” regions of cingulin and paracingulin. In paracingulin, the B region is a 169-residue fragment located in the C-terminal half of the head (residues 252–421), and the D region is a 292-residue fragment in the N-terminal half of the rod (residues 585–877) (26), respectively (Fig. 6B). In the CGN/CGNL1 chimeras, named CGN-CGNL1/B and CGN-CGNL1/D, respectively, the cingulin backbone contained either the head B domain or the rod D domain of paracingulin replacing the corresponding B (residues 157–222) and D (residues 375–804) sequences of cingulin. CGNL1/CGN chimeras (CGNL1-CGN/B and CGNL1-CGN/D) were generated conversely using the backbone of paracingulin and replacing the B and D sequences of paracingulin with the topologically corresponding regions of cingulin. The cloning strategy for the CGNL1-CGN/B chimera consisted of ligating together three PCR-amplified fragments (residues 1–251 CGNL1 flanked by NotI-HindIII sites, residues 157–222 CGN flanked by HindIII and EcoRV, and 422–579 CGNL1 flanked by Smal-NotI) and inserting the resulting sequence in frame into the NotI site of the rod + tail CGNL1 construct downstream of YFP and upstream of the rod + tail sequence. The cloning strategy for the CGN-CGNL1/B chimera was the same, with the following fragments/cloning sites to insert downstream of GFP into the CGN rod + tail construct (28): residues 1–156 CGN (NotI-EcoRI), 252–421 CGNL1 (EcoRI-EcoRV), and 223–374 CGN (Smal-NotI). The cloning strategy for the CGNL1-CGN/D and the CGN-CGNL1/D chimeras also included multiple steps of PCR amplification and subcloning (details available upon request). All constructs were completely sequenced to check that all coding regions (YFP/GFP, head, rod, and tail) were in frame, that no residue mutation was generated, and that the 28-residue repeat of the coiled-coil was not disrupted. The predicted apparent molecular size of each chimera (CGN-CGNL1/B = 176 kDa, CGN-CGNL1/D = 148 kDa, CGNL1-CGN/B = 166 kDa, and CGNL1-CGN/D = 192 kDa) was consistent with the observed migration by SDS-polyacrylamide gel electrophoresis.

Cell Culture and Transfection—Culture, transfection, and selection of MDCKII Tet-Off cells to generate stable clones that inducibly express either the full-length, head, or rod + tail domains of paracingulin were as described previously (28). Transgene expression was induced by replacing doxycycline (Dox)-containing medium (40 ng/ml) with medium without doxycycline for 3 days. Clones were isolated by cloning rings and selected for strong induction and low background by fluorescence-activated cell sorting (FACSCalibur, BD Biosciences). The calcium switch assay was performed as described previously (26). Rat1 fibroblasts were cultured in DMEM (Sigma) containing 10% FBS and 1× minimal essential medium non-essential amino acids (Invitrogen). Cell populations expressing
fluorescently tagged proteins were selected in antibiotic medium and by FACS sorting.

**RhoA Activity and Wound Healing Assay**—RhoA activity was measured by a GST-pull-down assay using GST-rhotekin (26, 27). For the wound healing assay, two-day confluent monolayers were scratched with a sterile 200-ml pipette tip, and phase contrast images of the wound were taken at time 0 and 7 h after scratching, using a Zeiss S100 Axiovert microscope. The distances between the edges of the wound were measured, and the decrease in the distance observed in control cells (expressing YFP alone) was taken as 100%. Data for clones expressing paracingulin constructs were expressed as a ratio of decreased distance in individual clones versus decreased distance in the control clone. Data from three to five independent experimental sets were averaged, and standard errors and statistical significance were calculated using Student’s unpaired t test.

**Measurement of Transepithelial Resistance and Rac1 Activity**—Measurement of transepithelial resistance (TER) and Rac1 activation during the calcium switch-induced junction assembly was as described previously (26, 27). TER was measured in duplicate 6.5-mm diameter Transwell filters (Costar). For inducible lines, parallel cultures of cells were incubated either in the absence or in the presence of Dox for 3 days before plating. Data from at least three independent experiments were averaged and expressed as ohm·cm².

**Immunoblotting and Immunofluorescence**—Lysates were prepared for total cell extract analysis or GST pull-down assays, normalized for protein content, and processed for immunoblotting as described previously (26–28). For immunofluorescence, cells were either permeabilized and fixed with a Triton/paraformaldehyde protocol or only fixed with paraformaldehyde and processed as described previously (26–28). Confocal images were acquired using a Zeiss 510Meta confocal microscope in multitracking mode to prevent bleed-through between channels.

**RESULTS**

**Exogenous Expression of Either Full-length Paracingulin, Its Globular Head, or Its Rod + Tail Domains in MDCK Cells Does Not Result in Altered TJ Protein Expression**—To dissect the functions of paracingulin domains we prepared constructs of
either full-length paracingulin (FL), the paracingulin globular head domain (H), or the paracingulin coiled-coil rod plus globular tail domains (R+T) in the vector pTRe2Hyg (Fig. 1A). The constructs were tagged N-terminally with the fluorescent protein YFP and C-terminally with the myc sequence. A control construct with myc-tagged YFP was also prepared (Fig. 1A). MDCK cells expressing the Tet repressor under the control of a doxycycline-inducible promoter (MDCK Tet-Off) were transfected with the constructs, and stable clones that inducibly expressed the recombinant proteins upon Dox removal were isolated.

To assess transgene expression, cells were cultured either in the presence or in the absence of Dox for 3 days, and cell lysates were prepared, normalized for total protein content, and analyzed by immunoblotting with antibodies against GFP, which recognize the YFP tag (Fig. 1B). In the presence of Dox, little or no leaked protein expression was detected, whereas upon the removal of Dox, proteins of the expected molecular size were detected by the anti-GFP antibodies: 28 kDa for YFP, 177 kDa for the YFP-FL paracingulin construct, 92 kDa for the YFP-H construct, and 113 kDa for the YFP-(R+T) construct (Fig. 1B). Clones expressing the rod+ tail domain construct showed the highest levels of transgene expression upon Dox removal, resulting in a strong immunoblotting signal (Fig. 1B).

Paracingulin depletion in MDCK cells up-regulates the expression of the TJ protein ZO-3 (26). To test whether inducible overexpression of either paracingulin or its structural domains results in a perturbation of TJ protein expression, we analyzed the expression of the TJ proteins ZO-3, claudin-2, and occludin, as a control, in our MDCK cell clones. As shown in Fig. 1B, the expression levels of endogenous claudin-2 were low in two of the three MDCK cell lines expressing the rod+ tail domains of paracingulin, suggesting that this variability was due to clonal variations. Importantly, no reproducible increase or decrease in the expression of these endogenous proteins was detectable when comparing the induced versus uninduced samples of the different constructs. Thus, no reproducible effect on TJ protein expression was detected following overexpression of the transgenics.

In MDCK Cells, the Full-length and Head Constructs of Paracingulin Show Functional and Cytoplasmic Localizations, the Rod+ Tail Construct Accumulates in Cytoplasmic Aggregates, and None of the Constructs Perturb Junction Organization—To examine the subcellular localization of the different paracingulin constructs, stable MDCK lines were analyzed by confocal immunofluorescence under either induced or non-induced conditions. Cells were counterstained with antibodies against either ZO-1 (Fig. 2) or occludin (not shown) to assess the integrity of the AJC. Under uninduced conditions, no fluorescence was detected in cells expressing either YFP, YFP-tagged paracingulin full-length (FL), head (H), or rod+ tail constructs (R+T) (Fig. 2, A, C, E, and G), indicating a tight repression of expression. Upon removal of Dox, MDCK cells expressing YFP alone showed diffuse cytoplasmic fluorescence, confirming that YFP does not associate with junctions (arrows in Fig. 2, B–B'). In contrast, cells expressing full-length paracingulin showed clear labeling at cell-cell junctions that was partially colocalized with ZO-1 (arrowheads in Fig. 2, D–D') as well as cytoplasmic submembrane staining (arrow in D). In cells expressing the paracingulin head construct, cells showed diffuse cytoplasmic and nuclear labeling (arrows in Fig. 2, F–F') and also weaker but detectable junctional labeling (arrowheads), consistent with the notion that the head domain of paracingulin contains the key sequences required for its junctional recruitment (25).

In summary, overexpression of paracingulin and its domains in MDCK cells does not perturb the molecular organization of the TJ. The full-length and head constructs are targeted to junctions, but the head construct without the rod+ tail domains is mostly accumulated in the cytoplasm.
Inducible Overexpression of the Rod + Tail but Not the Head Domain of Paracingulin Perturbs TJ Barrier Development and Rac1 Activation during Junction Assembly by the Calcium Switch—In MDCK cells, paracingulin depletion results in delayed junction assembly, and abnormal development of the TJ barrier, through impaired Rac1 activation (26). To examine whether the inducible overexpression of either full-length paracingulin or its head or rod + tail domains has an effect on junction assembly, we measured the TER of different clonal lines during the calcium switch (Fig. 3A–G). In all cell lines under uninduced conditions, the peak in TER, which represents a characteristic spike in TER value during TJ assembly by the calcium switch, occurred between 4 and 8 h after the calcium switch, and its value was between 350–450 ohm cm$^{-2}$ (Fig. 3, A–G), similarly to WT cells (not shown). On the other hand, under induced conditions, the value of the TER peak in the different clones was variable, depending on the construct that was expressed. Upon Dox removal and transgene expression, cells overexpressing the full-length paracingulin showed a slightly decreased value of the TER peak by about 40% (from 406 ohm cm$^{-2}$ to 246 ohm cm$^{-2}$) in one clone (clone A, Fig. 3A), and by about 44% (from 336 ohm cm$^{-2}$ to 189 ohm cm$^{-2}$) in the second clone (clone B, Fig. 3B). In cells expressing YFP fused to the head domain of paracingulin (Fig. 3, C and D), the TER temporal profile and peak values were similar to those observed in the presence of Dox, similarly to cells expressing the YFP protein alone (G). In contrast, in cells overexpressing the rod + tail construct, induction by removal of Dox resulted in a 75% drop in the peak value of TER, from 440 ohm cm$^{-2}$ to 112 ohm cm$^{-2}$ in one clone (clone A, Fig. 3E) and a 73% drop, from 339 ohm cm$^{-2}$ to 91 ohm cm$^{-2}$ in the second clone (clone B, F). Immunofluorescence analysis showed that the decrease in the peak of TER correlated with a delayed assembly of actin and ZO-1 at the apical junctional complex, which was stronger in the cells

**FIGURE 3.** Overexpression of the paracingulin rod + tail domains and, to a lesser extent, of full-length paracingulin affects the development of the TJ barrier and Rac1 activation during the calcium switch assay. A–G, analysis of the TER (expressed in ohm cm$^{-2}$) of MDCK monolayers (A and B = full-length clones, C and D = head clones, E and F = rod + tail clones, and G = YFP) during the calcium-switch assay, either in the absence (-Dox, continuous line) or presence (+Dox, dotted line) of doxycycline. Values represent the mean ± S.D. (error bar) of three to five independent experiments. H, analysis or Rac1 activity in lysates of MDCK cell lines expressing YFP, full-length, head, and rod + tail domains (one clone each). Lysates were prepared at time 0 min and 15 min of the calcium switch procedure, normalized for total Rac1 activity, and active Rac1 was isolated by GST pull-down assays and detected by immunoblotting. Numbers below the lanes of active Rac1 indicate densitometric values, normalized to total value, from one representative experiment of three.
expressing the rod+tail construct compared with the cells expressing the full-length protein (supplemental Figs. 1 and 2). Because the peak in TER is linked to Rac1 activation (26), we next examined the effect of overexpression of the different constructs on Rac1 activation under induced conditions. GST-pull-down of active Rac1 showed that before the calcium switch, all clones had similarly low Rac1 activity (Fig. 3H). At 15 min after the beginning of the switch, when a first wave of Rac1 activation occurs (26, 29, 30), cells overexpressing either the full-length or the head constructs had a fraction of active Rac1, similar to that of cells expressing YFP alone (Fig. 3H). However, cells overexpressing the rod+tail domain had a roughly 50% lower fraction of active Rac1 when compared with control cells (Fig. 3H). In addition, cells overexpressing the full-length constructs had a 10% decrease in the fraction of activated Rac1 (Fig. 3H). Thus, the effect of overexpression of the rod+tail domain on barrier recovery correlated with decreased Rac1 activation, mimicking the effect of paracingulin depletion (26). The smaller effect of overexpression of the full-length molecule correlated with a lesser effect on Rac1 activation. These results demonstrate that the rod+tail region of paracingulin, but not the head domain, is critically involved in the Rac1-dependent regulation of TJ assembly. Furthermore, they suggest that within the full-length protein, the head domain may somehow prevent the rod+tail domain from suppressing Rac1 activation.

Because depletion of paracingulin in MDCK cells results in up-regulation of Rac1 activity at confluence (26), we also measured RhoA activation in confluent monolayers of cells expressing either YFP or each of the paracingulin constructs. No reproducible change in RhoA activity was observed when comparing uninduced and induced conditions (Fig. 4A), indicating that overexpression of either full-length paracingulin or of its domains does not affect RhoA activity in confluent MDCK monolayers. In addition, a wound-healing assay failed to reveal any significant effect of overexpression of the different constructs on the rate of wound closure (Fig. 4B), suggesting that overexpression of either full-length paracingulin or its head or rod+tail domain does not significantly affect cell motility.

The Rod+Tail Domain of Paracingulin Modulates the Association of the Head Domain with the Actin Cytoskeleton—To extend the analysis of structure-function relationships in paracingulin, we investigated the role of its head and rod+tail domains in targeting to the extrajunctional actin cytoskeleton. Because none of the constructs could be detected associated with extrajunctional actin filaments in MDCK cells (Fig. 2), we expressed the constructs in Rat1 fibroblasts, which have no TJ but display spot-like AJ that contain ZO-1. In agreement with previous observations (25), the full-length construct was targeted both to AJ, where it colocalized with ZO-1 (arrowheads in Fig. 5, A–Ar) and to filamentous structures anchored to the AJ (arrows). Importantly, full-length paracingulin labeling was mostly colocalized with phalloidin staining in sharply defined filamentous structures, demonstrating that the cytoplasmic filaments with which paracingulin associates are made of actin (arrows in Fig. 5, C–Cr). The localization of full-length paracingulin along sharply defined filamentous structures was also visualized in non-permeabilized cells (fixed only with 3% paraformaldehyde) examined directly for YFP fluorescence (arrow in Fig. 5B). Furthermore, the filamentous localization of paracingulin did not require ZO-1 interaction, because it was also observed in cells expressing a construct (Δ1–209) lacking the ZO-1 interacting region (arrow in Fig. 5D) (see also Ref. 25).

The head domain of paracingulin was detected at ZO-1-containing spot-like AJ (arrowheads in Fig. 5, E–E′, and inset), in the nucleus, and along cytoplasmic filaments (arrows). In fixed cells, the exogenous head domain colocalized with actin filaments (arrows in Fig. 5, G–G′), although the filamentous distribution was less sharply defined than the full-length construct. Confirming this observation, in non-permeabilized cells the distribution of the exogenous head domain was along thin filaments, more diffuse than that of the full-length molecule (Fig. 5, F–F′). This suggests that the rod+tail domain helps to stabilize the association of the paracingulin head domain with the actin cytoskeleton. In contrast, the rod+tail construct showed no colocalization with ZO-1 (Fig. 5, H–H′) and was detected throughout the cytoplasm, in meshwork that was morphologically distinct from that observed with full-length cingulin and that only randomly colocalized with actin (J–J′). Similarly, in non-permeabilized cells, the distribution was diffuse throughout the cytoplasm (Fig. 5, I–I′). This confirmed that only sequences in the head domain can target paracingulin to AJ (25) and showed that the rod+tail domains do not efficiently associate with actin filaments in Rat1 fibroblasts, although they may stabilize the interaction of the head domain with the actin cytoskeleton.
Functional Domains of Paracingulin

The B Region in the Head Domain of Paracingulin Is Required to Target Paracingulin to the Actin Cytoskeleton—Cingulin and paracingulin show distinct localizations when exogenously expressed in Rat1 fibroblasts (Fig. 6A). Although cingulin accumulates exclusively at ZO-1-containing AJ (arrowheads in Fig. 6A, panels A–A’), paracingulin associates both with ZO-1-containing spot-like AJ (arrowheads, panels B–B’) and with cytoplasmic actin filaments (arrows, panels B–B’) (25). Thus, we sought to identify the paracingulin sequences that are responsible for this distinct behavior. We focused on two regions of the paracingulin sequence, called the B and D regions (see “Experimental Procedures”), which may have important roles, because they interact in vitro with GEF-H1 and Tiam1 (26). Importantly, the B and D regions of paracingulin show only 33 and 29% sequence identity with the topologically corresponding regions (central part of the globular head domain and N-terminal part of the rod domain) of cingulin. Therefore, we tested whether one or both of these regions would direct the distinct subcellular localizations of these proteins when exogenously expressed in Rat1 fibroblasts. Because the paracingulin head domain alone showed a different association with actin filaments than the full-length construct (Fig. 5), we decided to examine the role of these sequences within the context of a complete molecule comprising both head and rod+tail domains. Therefore, we constructed chimeric molecules where either the cingulin or paracingulin backbones were mutated by replacing either the B or D region with the corresponding regions of paracingulin or cingulin, respectively (Fig. 6B). These constructs were transfected into Rat1 fibroblasts, and their subcellular distribution was analyzed by confocal immunofluorescence.

The substitution of the B region of cingulin by the larger B region of paracingulin (CGN-CGNL1/B chimera) resulted in a protein that was targeted mainly to ZO-1-containing AJ, similar to the cingulin molecule (Fig. 6C, panel A–A’). In cells expressing larger amounts of exogenous protein or in cells visualized without Triton permeabilization, it was possible to detect some cytoplasmic filamentous staining (arrow in Fig. 6C, panel B). This suggested that although the head domain of paracingulin associates with actin filaments, the presence of its B region within cingulin is not sufficient to override the high affinity of cingulin for the ZO-1-containing AJ unless expression levels are high. Conversely, the CGN-CGNL1/D construct was exclusively localized at ZO-1-containing AJ, regardless of fixation protocol (arrowheads in Fig. 6C, panels C–C” and D), indicating that the presence of the D region of the paracingulin rod within the cingulin backbone does not redirect the subcellular distribution of the chimera. Importantly, when the B region of cingulin replaced the B region within the paracingulin backbone (CGNL1-CGN/B chimera), the ability of paracingulin to localize to cytoplasmic actin stress fibers was lost (compare Fig. 6C, panels E and F, with A, panel B), and virtually all the exogenous protein was detected either at ZO-1-containing spot-like AJ (arrowheads and insets in panels E–E’) or in a diffuse cytoplasmic localization in non-permeabilized cells (panel F). Conversely, when the B region of paracingulin was left intact, and instead the D region of cingulin was inserted in the rod domain to replace the endogenous D region (CGNL1-CGN/B chimera), the construct was detected both at ZO-1-containing AJ (insets in Fig. 6C, panels G–G’) and along cytoplasmic filaments (arrows in panels G–G’ and H). Double-labeling with phalloidin confirmed that these cytoplasmic filaments colocalize with actin (supplemental Fig. 3). Taken together, these data show that the B region within the head domain of paracingulin is required for targeting of paracingulin to actin filaments.

The D Region in the Rod Domain of Paracingulin Is Critically Involved in the Modulation of Barrier Recovery by Paracingulin—To examine the role of the B and D regions of paracingulin in modulation of TJ barrier recovery, we overexpressed the different chimeras in MDCK cells and analyzed the profile of TER development during the calcium switch. All chimeras were targeted to junctions in MDCK cells, and were expressed at similar levels (supplemental Fig. 4). Overexpression of the CGN/CGNL1 chimeras with swapped B regions did not result in...
major changes in the profile of TER (Fig. 7, A and C), indicating
that this region is not critically involved in the modulation of
barrier recovery by paracingulin. In contrast, cell overexpress-
ing the cingulin backbone containing the paracingulin D region
(CGN-CGNL1/D) showed a 61% higher peak in TER (from 320
ohm-cm² to 515 ohm-cm²) when compared with control cells
(Fig. 7B). Moreover, cells overexpressing paracingulin contain-
ing the D region of cingulin (CGNL1-CGN/D chimera) showed
a delay in reaching the peak in TER with respect to control cells,
although the height of the peak was similar (Fig. 7D). These
observations indicate that the D region within the paracingulin rod domain is critically involved in modulation of junction assembly and that, when overexpressed in the context of a full-length molecule, this region does not inhibit, but rather promotes, an efficient TJ barrier recovery.

DISCUSSION

Cingulin and paracingulin are structurally similar adaptor proteins that dynamically associate with the cytoplasmic plaque of the AJC (24). Evidence from knockdown and knock-out studies shows that although neither protein is required to maintain the structural organization of the AJC at steady state, both are required to down-regulate RhoA activity in confluent MDCK cells, whereas paracingulin is also required to promote Rac1 activation during junction assembly (26, 27, 31). Here we show that the inducible overexpression of either full-length paracingulin or its domains does not result in the redistribution of TJ proteins or changes in TJ structure and barrier function at steady state. However, overexpression of either the rod/H11001 tail domains or, to a lesser extent, of the full-length molecule perturbs TJ assembly, junctional assembly of actin and ZO-1, and Rac1 activation during the calcium switch. Thus, we identify the rod/H11001 tail moiety as the key functional region of paracingulin involved in Rac1-dependent regulation of TJ assembly.

The precise mechanisms controlling the height of the TER peak during TJ barrier recovery in the calcium switch-mediated junction assembly are not known. However, Rac1 activation is critical (20, 26, 32–35), and we believe that it may influence the TER value by modulating the dynamics of the cytoskeletal associations of claudins, the transmembrane TJ proteins that directly control the passage of ions. Our studies indicate that the D region within the paracingulin rod domain is critically involved in modulation of junction assembly and that, when overexpressed in the context of a full-length molecule, this region does not inhibit, but rather promotes, an efficient TJ barrier recovery.

The lack of effect of the overexpression of full-length paracingulin or its domains on RhoA activation at confluence, or in downstream behaviors such as cell motility, is similar to what was observed previously in cells overexpressing cingulin (28). We interpret these results as indicating that, under these conditions, RhoA activity is already maximally down-regulated at junctions by the presence of endogenous cingulin and paracingulin and that exogenous proteins cannot further sequester GEF-H1 and inhibit RhoA. Thus, only the depletion of these proteins can result in a phenotype of increased RhoA activity, through the release of the inhibitory effect on GEF-H1.

Besides the unique role of paracingulin in Rac1 activation, one major difference between cingulin and paracingulin is that paracingulin has been detected not only at TJ but also at AJ and at extrajunctional sites, associated with actin filaments (21). Recently, we clarified the molecular basis for the selective localization of paracingulin at AJ through its interaction with PLEKHA7 and AJ protein complexes (23, 25). Here we show that, when transfected into Rat1 fibroblasts, only paracingulin and not cingulin colocalizes with actin filaments. Furthermore, although the isolated head domain of paracingulin can be detected in filamentous structures when expressed in Rat1...
fibroblasts, this localization is not as sharply defined as that of the full-length protein, suggesting that the rod+tail domain promotes the association of the head with actin filaments, possibly by stabilizing its conformation. Experiments using chimeric molecules clearly indicate that the B region within the head domain of paracingulin is required for paracingulin targeting to actin filaments. Future studies should investigate whether the B region interacts with actin directly or indirectly through actin-binding protein(s). Previous experiments with recombinantly expressed proteins showed that cingulin interacts directly with actin and that sequences located in the C-terminal part of the head domain of cingulin are implicated (36). However, the lack of colocalization of cingulin with actin filaments in fibroblasts, shown here, suggests that the affinity of interaction of cingulin with the actin cytoskeleton must be significantly lower than that of paracingulin.

In summary, the results presented here, together with previous studies (25), allow us to clarify the structure-function relationships of paracingulin (Fig. 8). The N-terminal region of the head domain contains distinct sequences that are essential for paracingulin targeting to TJ through the interaction with ZO-1 and to A through the interaction with PLEKHA7 (25). The central region of the head domain contains sequences that are required for the association with actin filaments. The coiled-coil rod, through its N-terminal, Tiam1-interacting D region, is essential for regulating the Rac1-dependent establishment of the TJ barrier. Thus, the head domain of paracingulin can be viewed as a docking module that targets the molecule to different subcellular compartments, whereas the rod domain is responsible for modulation of Rho GTPase activity. In conclusion, the results presented here define the structural basis for the functional differences between cingulin and paracingulin, and further contribute to understanding the molecular mechanisms of regulation of Rho GTPases, protein switches of fundamental importance in the morphogenesis and physiology of epithelia.

Acknowledgments—We thank Dr. Christoph Bauer and the Bioimaging Platform of the National Centre of Competence in Research “Frontiers in Genetics” for providing a confocal microscopy facility, Lionel Fond for technical support, Carolina Shore for participation during her extramuros internship, and members of the Citi laboratory for comments on the manuscript.

REFERENCES
25. Pulimeno, P., Paschoud, S., and Citi, S. (2011) A role for ZO-1 and...
PLEKHA7 in recruiting paracingulin to tight and adherens junctions of epithelial cells. *J. Biol. Chem.* **286**, 16743–16750


SUPPLEMENTARY FIGURE 1
SUPPLEMENTARY FIGURE 3
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. The effect of overexpression of the full-length and rod+tail constructs of paracingulin on the cortical junctional assembly of actin during the calcium switch assay. Double immunofluorescence analysis of MDCK clones stably expressing either the full-length paracingulin (A-C), the rod+tail (D-F) or the GFP control (G-I) constructs, at different times after the calcium switch, using phalloidin to visualize actin, and YFP/GFP fluorescence to visualize the overexpressed constructs. Arrows indicate the accumulation of circumferential labeling for actin, which is delayed in cells expressing the rod+tail construct. Bar= 10 µm.

Supplementary Figure 2. The effect of overexpression of the full-length and rod+tail constructs of paracingulin on the cortical junctional assembly of ZO-1 during the calcium switch assay. Double immunofluorescence analysis of MDCK clones stably expressing either the full-length paracingulin (A-C), the rod+tail (D-F) or the GFP control (G-I) constructs, at different times after the calcium switch, using anti-ZO-1 antibodies to visualize ZO-1, and YFP/GFP fluorescence to visualize the overexpressed constructs. Arrows indicate the accumulation of circumferential labeling for ZO-1, which is delayed in cells expressing the rod+tail construct and, to a lesser extent, the full-length construct. Bar= 10 µm.

Supplementary Figure 3. Localization of paracingulin/cingulin chimaeras in Rat1 fibroblasts. Immunofluorescence analysis of Rat1 fibroblasts stably expressing the different chimaeras (see Fig. 6 for schemes of constructs), after fixation with paraformaldehyde/Triton and immunolabeling with TRITC-phalloidin to visualize actin. Either GFP or YFP fluorescence was used to visualize the overexpressed constructs. Arrows indicate the paracingulin constructs containing the “B” region, which accumulate at filamentous, actin-containing structures. Nuclei are counterstained in blue with DAPI. Bar= 10 µm.

Supplementary Figure 4. Expression of paracingulin/cingulin chimaeras in MDCK cells. Immunoblot (A) and immunofluorescence (B) analysis of MDCK cells stably expressing the different chimaeras (see Fig. 6 for schemes of constructs). Lysates were normalized for total protein content for beta-tubulin, and then analyzed with antibodies against GFP. For immunofluorescence, monolayers were fixed with paraformaldehyde/Triton and directly visualized by confocal microscopy. Nuclei are counterstained in blue with DAPI. Bar= 10 µm.