Characterization of the Interaction between Protein 4.1R and ZO-2
A POSSIBLE LINK BETWEEN THE TIGHT JUNCTION AND THE ACTIN CYTOSKELETON* 

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Multiple isoforms of the red cell protein 4.1R are expressed in nonerythroid cells, including novel 135-kDa isoforms. Using a yeast two-hybrid system, immunocolocalization, immunoprecipitation, and in vitro binding studies, we found that two 4.1R isoforms of 135 and 150 kDa specifically interact with the protein ZO-2 (zonula occludens-2). 4.1R isoforms interact with ZO-2 and occludin at Madin-Darby canine kidney (MDCK) cell tight junctions. Both isoforms of 4.1R coprecipitated with proteins that organize tight junctions such as ZO-2, ZO-1, and occludin. Western blot analysis also revealed the presence of actin and α-spectrin in these immunoprecipitates. Association of 4.1R isoforms with these tight junction and cytoskeletal proteins was found to be specific for the tight junction and was not seen in nonconfluent MDCK cells. The amino acid residues that sustain the interaction between 4.1R and ZO-2 reside within the amino acids encoded by exons 19–21 of 4.1R and residues 1054–1118 of ZO-2. Exogenously expressed 4.1R containing the spectrin/actin- and ZO-2-binding domains was recruited to tight junctions in confluent MDCK cells. Taken together, our results suggest that 4.1R might play an important role in organization and function of the tight junction by establishing a link between the tight junction and the actin cytoskeleton.

Erythrocyte protein 4.1R is an 80-kDa cytoskeletal protein critical in circulating red cells for the dynamic organization and maintenance of the spectrin/actin cytoskeleton and for the attachment of the cytoskeleton to the cell membrane through interactions with integral membrane proteins such as glycoporphin C and band 3 (1). In nonerythroid cells, an additional and prevalent 135-kDa 4.1R isoform class has been detected. It contains a 209-amino acid extension at its N terminus end (2). Multiple isoforms of 4.1R exist. They arise through complex alternative pre-mRNA splicing pathways (2, 3), post-translational modifications (4), and use of at least two translation initiation sites (5, 6). In nucleated erythrocyte and nonerythroid cells, a spectrum of proteins ranging from 30 to 210 kDa has been detected that contains epitopes for 4.1R (7, 8). Unlike the strict peripheral distribution of 4.1R in mature erythrocytes, 4.1R isoforms in nonerythroid cells are localized in the nucleus and nuclear matrix (9–13), at points of cell-cell or cell-matrix contact (14, 15), and in centrosomal and Golgi structures (16, 17). In addition, 4.1R may also interact with microtubules and stress fibers (14, 18). However, the precise identity of 4.1R isoforms in these subcellular structures, their binding partners, and their biological significance in nonerythroid cells are not well understood.

The 80-kDa erythrocyte 4.1R protein is composed of four different domains as defined by chymotryptic digestion (19). The 30-kDa N-terminal domain (aa1 207–474; GenBank™/EBI Data Bank accession number J03796) interacts with glycoporphin C (20), calmodulin (21), p55 (20), and band 3 (22). The 30-kDa domain has also been shown to bind to CD44 (23) and pICln, a protein involved in cellular volume control (24). The 10-kDa domain (aa 579–624) forms a ternary complex with spectrin and actin (25–27). The function of the 16-kDa (aa 472–578) and 22–24-kDa (aa 625–774) domains in erythrocytes is not known. In nonerythroid cells, 4.1R interacts with calmodulin in a Ca2+-dependent manner through its N-terminal extension (28), with human CASK through its 30-kDa domain (29), with an α-subunit of the human nuclear shuttle complex importin (Rch1) through its 30- and 10-kDa domains (12), and with the immunophilin FKBP13 (30) and NuMA (13) through its 22–24-kDa C-terminal domain.

Like 4.1R, many members of the protein 4.1 superfamily, including ezrin, radixin, moesin, merlin, and myosin, are known to associate with the plasma membrane (reviewed in Ref. 31). A Drosophila homolog of protein 4.1, Coracle, is localized at septate junctions in ectodermally derived epithelial cells (32). Drosophila Neurexin and DLG (Discs Large), homologs of proteins that are known to bind to 4.1, glycoporphin C, and human DLG, respectively, are also localized at the septate junctions (33, 34). Collectively, these studies suggest the localization of 4.1 to the septate junction, an invertebrate specific junction with molecular components analogous to the vertebrate tight junction (TJ) (35). In addition to membrane binding activity, members of this family participate in important cell signaling events. For example, merlin is involved in growth regulation (36), and ezrin, radixin, and moesin are involved in Rho-dependent signaling pathways (37, 38).

To explore the function of 4.1R isoform(s) in nonerythroid cells, we searched for their binding partners. In this study, ...
using a combination of molecular/genetic, biochemical, and immunofluorescence studies, we establish that two nonerythroid 4.1R isoforms bind to the C-terminal proline-rich domain of X-104, a human homolog of the canine protein ZO-2 (henceforth referred to as hZO-2), through its C-terminal domain. ZO-2 belongs to the membrane-associated guanylate kinase (MAGUK) family of proteins and is known to organize the TJ in association with other TJ-associated proteins (39–41). We report here that these isoforms of 4.1R colocalize with ZO-2 and occludin and also form an intracellular complex with ZO-2, ZO-1, occludin, actin, and α-spectrin at MDCK cell TJs. Our results suggest that in nonerythroid cells, 4.1R may have a role in the organization and function of the TJ by establishing a direct link between the TJ and the actin cytoskeleton.

**Experimental Procedures**

**Construction of Expression Vectors—Construction of full-length 4.1R (135-kDa/pAS2-1); 80-kDa 4.1R (80-kDa/pAS2-1); its 30-kDa (30-kDa/pAS2-1), 16-kDa (16-kDa/pAS2-1), 10-kDa (10-kDa/pAS2-1), and 22–24-kDa domains (22–24-kDa/pAS2-1) or with pAS2-1 alone. The Gal4-AD plasmids recovered from positive clones were sequenced as described above.

**Antibodies—**Antibodies to synthetic peptides of segments of the 4.1R protein were generated by Dr. Robert J. Bloch (University of Maryland, Baltimore).

**Immunoprecipitation and Immunoblotting—**Immunoprecipitation of 4.1R, ZO-1, ZO-2, and occludin was performed using MDCK cell extracts. About 4 × 10⁶ cells were collected by scraping with a rubber policeman, washed three times with ice-cold PBS, resuspended in immunoprecipitation buffer (20 mM Tris-HCl, 150 mM NaCl, 2% CHAPS, 0.1% (w/v) SDS, 1 mg/ml bovine serum albumin, 0.2 mM EDTA, 5 mM iodoacetamide, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 4 μg/ml antipain, 12.5 μg/ml chymostatin, 12 μg/ml pepstatin, 130 μg/ml e-aminoacaproic acid, 200 μg/ml p-amino benzenesulphonic acid, and 1 μg phenylmethylsulfonyl fluoride (pH 7.5), and given 20 strokes in a tight-fitting glass homogenizer. The homogenate was centrifuged at 4°C for 15 min at 14,000 rpm. The supernatant was collected; and the protein content was determined using a protein determination kit (Bio-Rad).
supernatant was collected by centrifugation at 3000 × g for 5 min at 4 °C and split equally into seven tubes. Preimmune serum, affinity-purified anti-HA, anti-ZO-1 Ab, anti-ZO-2 Ab, anti-occludin Ab, rabbit IgG, or anti-p53 Ab (an irrelevant antibody as a control) containing 6 μg of IgG was added to different tubes and incubated for 2 h at 4 °C. Protein A-Sepharose CL-4B (100 μl of a 50% suspension) was then added and incubated overnight at 4 °C on a rocking platform. Following incubation with protein A-Sepharose, the samples were centrifuged at 3000 × g for 10 s at 4 °C and washed 10 times with 1 ml of immuno-precipitation buffer. The samples were resuspended in 80 μl of SDS sample buffer (62.5 mM Tris-HCl, 10% glycerol, 2% (w/v) SDS, 5% 2-mercaptoethanol, and 10 μg/ml bromphenol blue, pH 6.8) and boiled in a water bath for 5 min. The samples were centrifuged at 10,000 × g for 15 min at 4 °C, and the supernatants were fractionated on 6–12% SDS-polyacrylamide gels.

Transfer of proteins to nitrocellulose or polyvinylidene difluoride membranes and detection of 4.1R, ZO-1, ZO-2, occludin, actin, and spectrin were carried out by immobiloblotting using an ECL detection kit (Amersham Pharmacia Biotech) as described earlier (13). Quantitation of proteins from chromilumograms was done with NIH Image software for the Apple Macintosh computer. To visualize proteins, the gels were stained with GelCode™ SilverStain™ from Pierce.

In Vitro Binding Assay—Recombinant 4.1R/GST proteins were expressed and affinity-purified by coupling to glutathione-Sepharose beads as described (13). The recombinant proteins were quantitated by Coomasie Brilliant Blue staining using bovine serum albumin as a standard. Desired [35S]methionine-labeled segments of hZO-2, hZO-1, and human occludin were in vitro translated using a TNT™ SP6 Quick Coupled transcription/translation system (reticulocyte lysate system; Promega) according to the manufacturer’s recommendations. The translation products were quantitated based on percent of methionine incorporation and [35S]-specific activity. For in vitro binding, equal amounts of proteins (25 μg) were incubated with 20 μg of 4.1R/GST fusion proteins coupled to glutathione-Sepharose beads for 2 h at 4 °C in 300 μl of binding buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 10 mM sodium pyrophosphate, 1.5 mM sodium orthovanadate, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 4 μg/ml antipain, 12.5 μg/ml chymostatin, 12 μg/ml pepstatin, 130 μg/ml e-aminocaproic acid, 200 μg/ml p-aminoazobenzamidine, and 1 mM phenylmethylsulfonyl fluoride). After incubation, the beads were washed 10 times with 1 ml of binding buffer containing 0.5% Triton X-100 each time. The beads were then resuspended in 30 μl of SDS sample buffer, boiled for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis (12% gel; National Diagnostics, Inc., Atlanta). The gels were treated with Enlighting™ (NEN Life Sciences Products), and binding of [35S]-labeled proteins was detected by fluorography.

To determine the dissociation constants between 4.1R and ZO-2, ZO-1, or occludin, 5 μg of 24-kDa/GST or GST (as a control) coupled to glutathione-Sepharose beads was incubated with 0.0025–0.015 μg of [35S]-labeled translated products as described above. After extensive washing, the bound proteins were eluted with 50 mM Tris-HCl containing 20 mM glutathione, pH 8.0. The amount of bound and free labeled protein was determined from [35S]-specific activity and adjusted for binding to the GST-conjugated Sepharose beads. A Scatchard plot of the data was generated. Each experiment was repeated three times for estimation of Kd.

Fluorescence and Confocal Microscopy—MDCK cells were grown on poly-D-lysine-coated cover slips to confluence. The cover slips were washed three times with PBS and fixed in freshly prepared 2% paraformaldehyde in PBS for 25 min at room temperature. Cells were washed with PBS and permeabilized with a solution of 0.2% Triton X-100 and 10% normal goat serum in PBS for 15 min. The cells were blocked with 10% normal goat serum and 50 mM NH4Cl for 1.5 h at room temperature and washed three times with PBS containing 0.3% bovine serum albumin. Cells were incubated with primary antibodies (1:100 dilution of anti-occludin Ab, 1:100 dilution of anti-ZO-2 Ab, 1:200 dilution of anti-ZO-1 Ab, 1:50 dilution of anti-16-kDa Ab, and 1:25 dilution of anti-HA Ab) for 1 h at room temperature and washed three times as described above. They were then incubated with a 1:100 dilution of Texas Red-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature and washed three times again as described above. When double staining was desired, after the first set of staining, cells were blocked with un conjugated goat anti-rabbit Fab fragment (10 μg/ml in PBS; Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature and washed three times as described above. Incubation with the second set of antibodies was the same as with the first set of antibodies. Mounting of the coverslips and processing of images using a Noran confocal laser scanning image system and Intervision software (Noran Instruments Inc., Middleton, WI) were the same as described previously (13).

Immunoelectron Microscopy—Confluent MDCK cells were washed with PBS and fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in PBS overnight at 4 °C. The cells were then infiltrated with 15% polyvinylpyrrolidone and 1.95 μt sucrose in PBS (48), and ultrathin cryosections were cut at −100 °C with a Reichert FCS cryoultramicrotome (Leica Inc.). Cryosections were stretched using 2.3 μt sucrose and mounted on Formvar/carbon-coated grids. Sections were blocked with 10% normal goat serum and 20 μg/ml glycine in PBS for 30 min. Sections were sequentially incubated for 60 min with anti-ZO-2 (1:12 dilution) or anti-HP (1:15 dilution) antibody and AuroProbe EM goat anti-rabbit IgG (1.50 dilution; Amersham Pharmacia Biotech). The sections were post-fixed in 2% glutaraldehyde in PBS. The immunogold-labeled sections were examined in an electron microscope (EM 10, Carl Zeiss, Inc.).

RESULTS

Identification of hZO-2 as a 4.1R-binding Protein—We first attempted to identify 4.1R-binding partners by use of the yeast two-hybrid method. We screened ~107 transformants of a human brain cDNA library (constructed in Gal4-AD vector pACT2) using full-length 4.1R (135-kDa/pAS2-1) and its 22–24-kDa (22–24-kDa/pAS2-1) and 10-kDa (10-kDa/pAS2-1) domains (Fig. 1A) as bait constructs. 124 positive clones were obtained; five clones encoded a segment of the C-terminal domain of X-104 (referred to as hZO-2; aa 567–1185, hZO-2A/N/ pACT2; GenBank™ accession number L27476 as corrected in Ref. 43) (Fig. 1B). These clones of hZO-2 were obtained using 135-kDa/pAS2-1 and 22–24-kDa/pAS2-1 as bait constructs, but not 10-kDa/pAS2-1. Secondary screening and cotransformation of fresh yeast cells with the hZO-2AN/pACT2 and 135-kDa/pAS2-1, 22–24-kDa/pAS2-1, or pAS2-1 vector alone confirmed that hZO-2AN/pACT2 bound specifically to Gal4-BD fusion proteins of 135-kDa 4.1R or its 22–24-kDa domain.

The X-104 (hZO-2) gene was identified in relation to the
Friedreich ataxia locus protein (44) and by cDNA direct selection (49). It was subsequently shown to be identical to human ZO-2 (43). hZO-2 belongs to the MAGUK protein family along with the other TJ-associated proteins ZO-1 and ZO-3 (40). Although isoforms of hZO-2 have not been described, hZO-2 contains a region (amino acids 1000–1036) (50) homologous to the \( \beta \)-motif of canine ZO-2 (36 amino acids) and amino acids 991–1028 of chicken ZO-2, which are alternatively spliced (39, 50, 51). All five hZO-2 clones that interacted with 4.1R in yeast two-hybrid assays encoded amino acids 980–1168, which correspond to the C-terminal proline-rich domain and the putative \( \beta \)-motif of hZO-2.

**Protein 4.1R in Tight Junctions**

**Fig. 2.** Immunofluorescence and ultrastructural localization of 4.1R and tight junction proteins in confluent MDCK cells. A–F, subcellular colocalization of 4.1R, ZO-2, and occludin in confluent MDCK cells. Confluent MDCK cells were fixed and processed for immunofluorescence using antibodies for 4.1R, ZO-2, and occludin as described under “Experimental Procedures.” A scanning laser confocal microscope was used to collect sequential 0.35-\( \mu \)m-thick face-on (A, B, D, and E) or transverse (\( D' \) and \( E' \)) sections. ZO-2 (A) and 4.1R (B) or occludin (D) and 4.1R (E) localize at cell-cell contacts in a continuous fashion. The yellow color in C and F indicates the colocalization of ZO-2 and 4.1R or occludin and 4.1R, respectively. A–C are two-dimensional confocal images, and \( D'–F' \) are three-dimensional confocal images. Bar = 25 \( \mu \)m. Note that the transverse sections of occludin (\( D' \)) and 4.1R (\( E' \)) indicate that both 4.1R and occludin localize at the tight junction. G and H, immunoelectron micrographs of the tight junctional complex region in MDCK cells stained with anti-ZO-2 Ab or anti-HP Ab, respectively. The gold particles for ZO-2 (10 nm) and 4.1R (5 nm) are concentrated in the tight junctional complex of MDCK cells. Bar = 200 nm.

Colocalization of 4.1R and ZO-2 in MDCK Cell Tight Junctions—To verify the interaction of 4.1R with ZO-2 and to locate the site(s) of their intracellular interaction, we examined the subcellular distribution of 4.1R and ZO-2 in confluent MDCK cells by double-label immunofluorescence microscopy. As shown in Fig. 2 (A and B), ZO-2 (red) and 4.1R (green) localized at the cell-cell contacts and displayed honeycomb-like staining patterns. Both ZO-2 and 4.1R also showed some diffuse cyto-
plasmic staining. Cytoplasmic staining of ZO-2 has also been observed by others (51). By superimposing Fig. 2 (A and B), the yellow color (Fig. 2C) produced due to the combination of the green and red colors suggested the colocalization of 4.1R and ZO-2 at cell-cell junctions of confluent MDCK cells.

Ocludin has been shown to exclusively localize at TJs of epithelial and endothelial cells (52, 53) in association with ZO-2 andZO-1. To verify the localization of 4.1R at tight junctions, we performed double immunofluorescent staining of confluent MDCK cells with 4.1R and ocludin. As shown in Fig. 2, ocludin (red; panel D) and 4.1R (green; panel E) localized at apical cell borders and, to some extent, were diffuse in the cytoplasm. A transverse view of the cells taken at the position of the dotted line (in Fig. 2, D and E) showed that both ocludin (Fig. 2D) and 4.1R (Fig. 2E) localized at the TJ along the apical side of the cells. As shown in Fig. 2F, 4.1R precisely colocalized with ocludin at the MDCK cell TJs. Colocalization of 4.1R with ZO-2 and ocludin at MDCK cell TJs was also observed when cells were stained with anti-10-kDa Ab or anti-16-kDa Ab (data not shown). The colocalization of 4.1R with ocludin and ZO-2 indicated that 4.1R localizes specifically to TJs of confluent MDCK cells.

To study the subcellular localization of ZO-2 and 4.1R in more detail, we performed high resolution immunogold electron microscopy using fixed confluent MDCK cells. Anti-ZO-2 Ab was used as a control. As shown in Fig. 2, G and H, labeling for ZO-2 (10-nm gold particles) and 4.1R (5-nm gold particles) was found concentrated at the tight junctions as clusters (arrows). This further supports our contention that 4.1R localizes at tight junctions.

In Vivo Association of 4.1R and ZO-2—To examine the interaction of 4.1R with ZO-2 in vivo, we performed immunoprecipitation using confluent MDCK cell extracts. The extracts were subjected to immunoprecipitation with anti-HP Ab or anti-ZO-2 Ab. Preimmune serum (rabbit), purified rabbit IgG, and anti-p53 Ab (an irrelevant Ab) were used as controls. Analysis of immunoprecipitates by immunoblotting using anti-ZO-2 Ab showed that anti-ZO-2 Ab coprecipitated two polypeptides of 135 and 150 kDa; these comigrated with two polypeptides of similar molecular mass that were immunoprecipitated by anti-HP Ab (Fig. 3A). Similarly, analysis of the immunoprecipitates by immunoblotting using anti-ZO-2 Ab showed that ZO-2 coprecipitated with 4.1R (Fig. 3B). Neither 4.1R nor ZO-2 was coprecipitated with preimmune serum, rabbit IgG, or anti-p53 Ab (Fig. 3, A and B). These results suggest that 4.1R and ZO-2 are associated together in vivo. Analysis of the same immunoprecipitates by immunoblotting using anti-24-kDa Ab also revealed the presence of 135- and 150-kDa polypeptides (Fig. 3C), confirming that these polypeptides are 4.1R isoforms. However, these isoforms of 4.1R were not detected in anti-HP Ab or anti-ZO-2 Ab supernatants by anti-24-kDa Ab, but were detected by anti-HP Ab. Because anti-24-kDa Ab is raised against exon 19, these data suggest that most of the 135- and 150-kDa 4.1R isoforms that contain alternative exon 19 coprecipitate with ZO-2 and therefore are not detected in the supernatant, whereas those isoforms not containing exon 19 do not coprecipitate with ZO-2 and thus are detected by anti-HP Ab. This is consistent with the results of yeast two-hybrid mapping data that exons 19–21 of 4.1R are required for interaction with ZO-2 (Fig. 4B).

We determined the efficiencies of immunoprecipitation and coprecipitation to discern the fractions of 4.1R and ZO-2 that associate together. Quantitation from three different experiments (representative gels are shown in Fig. 3, A and B) showed that 80–90% of 4.1R was precipitated by anti-HP Ab, which in turn coprecipitated 35–48% of ZO-2. In addition, >95% of ZO-2 was precipitated by anti-ZO-2 Ab, which coprecipitated 45–55% of 4.1R. Analysis of the post-extraction pellet (insoluble fraction) revealed that only 5–10% of both 4.1R and ZO-2 were not extracted (data not shown). This is consistent with the notion that both 4.1R and ZO-2 move out of the nucleus at confluent density in MDCK cells (75, 76). Thus, ~35–45% of total cellular 4.1R and ZO-2 associate together in confluent MDCK cell TJs. Taken together, all of these results strongly suggest that about half of the cytoplasmic 135-kDa nonerythroid isoforms of 4.1R in MDCK cells interact with ZO-2.

The complex splicing pattern of 4.1R generates distinct isoforms with insignificant differences in molecular mass, but with a significant effect on their subcellular targeting. Although the isoform(s) of 4.1R that interact with hZO-2 are also of the same higher molecular class (~135 kDa) that interacts with NuMA in interphase nuclei and during mitosis (13), the same isoform(s) may not interact with NuMA because of their distinct subcellular localization. However, it has been shown that a given 4.1R isoform can adopt several localizations within the cell (11).
segments of 4.1R and hZO-2 or ZO-2 that interact, we examined the interactions between different domains or their segments of 4.1R and hZO-2 or ZO-2 in the yeast two-hybrid assays. We expressed different domains or segments of 4.1R as fusion products of the Gal4 DNA-binding domain in pAS2-1 and cotransformed them into Y190 with different segments of hZO-2 or ZO-2. The latter were expressed as Gal4-AD fusion products in pACT2, as shown in Fig. 4A. Full-length 4.1R (135 kDa), its 80-kDa isoform, and its 22–24-kDa domain interacted with 1) full-length hZO-2, 2) a segment of the C-terminal proline-rich domain of hZO-2 (aa 980–1168), and 3) amino acids 187–1174 of ZO-2. They did not interact with the N-terminal part of ZO-2 (aa 1–189) (Fig. 4A). The N-terminal extension (HP) and the 30-, 16-, and 10-kDa domains of 4.1R failed to interact with hZO-2 or ZO-2. None of the domains of 4.1R, when expressed as Gal4-BD fusion proteins in Y190, expressed the reporter gene(s) by themselves or in combination with Gal4-AD in pACT2 alone (data not shown). These data suggest that the C-terminal domains of 4.1R and hZO-2 or ZO-2 are necessary and sufficient for their interaction.

As illustrated in Fig. 4B, exons 17 and 18 were not required for interaction of hZO-2 with 4.1R. Fusion proteins consisting of exons 19–21 of 4.1R (exons 19–21/pGBT9) bound to hZO-2, but none of these exons alone (exon 19/pGBT9, exon 20/pGBT9, or exon 21/pGBT9) was sufficient for binding. Amino acids 980–1168 were encoded by the hZO-2 clones obtained from the two-hybrid screening. As shown in Fig. 4C, the C-terminal truncation of the fusion protein from amino acids 1119 to 1168 did not impede binding, but further truncation from the C-terminal end (aa 1074–1168) abolished the binding of hZO-2 to 4.1R. From the N-terminal end, truncation of the hZO-2 fusion protein from its N terminus up to amino acid 1053 did not affect...
its binding to 4.1R. The fusion protein encoding amino acids 1054–1118 of hZO-2 bound to 4.1R. The binding strength was comparable to the fusion protein encoding amino acids 980–1168 of hZO-2, as assessed by expression of the reporter gene lacZ (data not shown). Therefore, it appears that amino acids 1054–1118 of hZO-2 are sufficient for its interaction with 4.1R. These results suggest that the amino acids through which 4.1R and hZO-2 interact reside within amino acids encoded by exons 19–21 of 4.1R and amino acids 1054–1118 of hZO-2.

Protein 4.1R Associates with Tight Junction Proteins ZO-1, ZO-2, and Occludin in Confluent MDCK Cells—To examine the association of 4.1R with the TJ protein complex, we asked if it occurred in association with other TJ components such as ZO-1 and occludin (54–56). Therefore, we performed immunoprecipitation using confluent MDCK cell extracts and looked for 4.1R in the immunoprecipitates of ZO-1 and occludin and vice versa. Rabbit preimmune serum, purified rabbit IgG, and anti-p53 Ab (an irrelevant Ab) were used as negative controls to rule out nonspecific aggregation. ZO-2, which is known to coprecipitate with and bind directly to ZO-1 (57, 58) and occludin (41, 58), was used as a positive control.

As stated earlier and shown in Fig. 5A, two 4.1R isoforms (≈135 and ≈150 kDa) were immunoprecipitated by anti-HP Ab. These isoforms of 4.1R were also found to coprecipitate with ZO-2, ZO-1, and occludin when immunoprecipitation was carried out with anti-ZO-2 Ab, anti-ZO-1 Ab, and anti-occludin Ab, respectively, but not with preimmune serum, rabbit IgG, or anti-p53 Ab. Analysis of the same immunoprecipitates by immunoblotting using antibodies specific for the 16-kDa domain of 4.1R (anti-16-kDa Ab) also revealed the ≈135- and ≈150-kDa 4.1R isoforms in the immunoprecipitates of anti-HP Ab, anti-ZO-2 Ab, anti-ZO-1 Ab, and anti-occludin Ab. No other 4.1R isoforms were seen in the immunoprecipitates of ZO-2, ZO-1, and occludin, suggesting that the isoforms of 4.1R that associate with these TJ proteins are of the 135-kDa molecular mass class. This study also confirmed the ≈150-kDa protein as a 4.1R isoform.

Analysis of duplicates of the blot shown in Fig. 5A by immunoblotting using anti-ZO-2 Ab (Fig. 5C), anti-occludin Ab (Fig. 5D), and anti-ZO-1 Ab (Fig. 5E) showed that ZO-1, ZO-2, occludin, and two 4.1R isoforms (≈135 and ≈150 kDa) coprecipitated together. None of these proteins were detected in immunoprecipitates of any of the control antibodies (Fig. 5, A–E). These results suggest that two 4.1R isoforms, ZO-2, ZO-1, and occludin associate together in vivo.

To examine the involvement of other 4.1R-binding cytoskeletal proteins such as actin and spectrin in this complex, we analyzed the anti-HP Ab, anti-ZO-2 Ab, anti-ZO-1 Ab, and anti-occludin Ab immunoprecipitates by immunoblotting using the relevant antibodies. As shown in Fig. 5F, actin was found to coprecipitate with 4.1R, ZO-2, ZO-1, and occludin, but not with p53, preimmune serum, or rabbit IgG immunoprecipitates. Anti-spectrin Ab detected both the α- and β-isoforms of spectrin in MDCK cell lysates (Fig. 5G). Interestingly, only α-spectrin was detected in the anti-HP Ab, anti-ZO-2 Ab, anti-ZO-1 Ab, and anti-occludin Ab immunoprecipitates. The absence of actin and spectrin (despite their abundance in the cell) in immunoprecipitates of preimmune serum, rabbit IgG, and anti-p53 Ab indicated that their presence in the immunoprecipitates of anti-HP Ab, anti-ZO-2 Ab, anti-ZO-1 Ab, and anti-occludin Ab was due to a specific intracellular association with these proteins. The coprecipitation of actin and α-spectrin with the TJ proteins was not surprising because the C-terminal half of ZO-1 has been shown to cosediment with actin filaments (58, 59), and α-spectrin has been shown to coprecipitate with ZO-1 (60, 61).

To examine the presence of other 4.1R-binding and/or TJ-associated proteins in this protein complex, anti-HP Ab immunoprecipitates were examined by SDS-polyacrylamide gel electrophoresis and silver staining (Fig. 5H). About 17 protein bands were visualized in the anti-HP Ab immunoprecipitates, but not in the preimmune serum immunoprecipitates. The molecular mass of some of these proteins was identical to that of some of the TJ-associated proteins. A duplicate of the gel in Fig. 5H was transferred to polyvinylidene difluoride membrane...
Protein 4.1R Binds to ZO-2, ZO-1, and Occludin in Vitro—Because the results from immunoprecipitation experiments suggested that 4.1R associates with TJ proteins ZO-2, ZO-1, and occludin in vivo, we asked if there were additional binary interactions between 4.1R and ZO-1 or occludin. We constructed yeast expression vectors expressing full-length ZO-1, occludin, or their different segments in frame with Gal4-AD and performed yeast two-hybrid assays with 4.1R and its subdomains. The results of filter lift β-galactosidase activity assays were inconclusive. Therefore, chlorophenol red β-d-galactopyranoside-based liquid β-galactosidase activity assays were performed. As shown in Fig. 7A, the N-terminal cytoplasmic domain of occludin (aa 1–57) did not show substantial β-galactosidase activity over the control. However, full-length occludin (data not shown) as well as its C-terminal cytoplasmic domain (aa 250–504) showed weak interactions with the 135- and 80-kDa isoforms and C-terminal domain of 4.1R. A weak interaction was also observed between the N-terminal cytoplasmic domain of occludin (aa 1–57) and HP of 4.1R. In addition, weak interactions were detected between the 135- and 80-kDa isoforms and the 24-kDa domain of 4.1R and the C-terminal proline-rich domain (aa 1045–1737) (Fig. 7B), but not the N-terminal part (aa 1–1044) of ZO-1 (data not shown).

To confirm direct binding of 4.1R to ZO-2, ZO-1, or occludin, we performed in vitro binding assays. Different isoforms and domains of 4.1R were expressed as GST fusion proteins and purified by coupling to GST-Sepharose beads as described (13).

In vitro binding was performed between 4.1R/GST and in vitro translated 125I-methionine-labeled hZO-2 (aa 905–1054, hZO-2-1/TOPO; or aa 980–1168, hZO-2-2/TOPO), hZO-1 (aa 1042–1454, ZO-1-1/TOPO; or aa 1453–1747, ZO-1-2/TOPO), or occludin (aa 130–283, Ocl-1/TOPO; or aa 284–522, Ocl-2/TOPO). As shown in Fig. 8A, aa 980–1168 of hZO-2, aa 1042–1454 of ZO-1, and aa 284–522 of occludin bound to 135-kDa/GST, 80-kDa/GST, and 24-kDa/GST, but not to 30-kDa/GST, 16-kDa/GST, 10-kDa/GST, HP/GST, or GST alone. The binding constants of ZO-2, ZO-1, and occludin with the 24-kDa domain of 4.1R were determined. Each binding was saturable; and from Scatchard analyses, $K_d$ values of $-4.5 \times 10^{-8}$, $7.5 \times 10^{-7}$, and $7.1 \times 10^{-7} \mu M$ for hZO-2, ZO-1, and occludin (Fig. 8G) were
revealed, respectively. These results suggest direct binding of ZO-2, ZO-1, and occludin to 4.1R and are consistent with results from the yeast two-hybrid assays.

Recruitment of 4.1R/GFP Fusion Proteins to Tight Junctions in Confluent MDCK Cells—To verify that 4.1R interacts with ZO-2 and/or other tight junction-associated proteins in intact cells, we constructed expressions vectors with segments of 4.1R fused to GFP and transfected them into cultured MDCK cells. Because results from yeast two-hybrid and in vitro binding assays suggest that the 22–24-kDa domain of 4.1R is sufficient for interaction with ZO-2, ZO-1, and occludin, cells were transfected with 24-kDa/GFP. GFP alone and 10-kDa/GFP were used as controls. However, as shown in Fig. 9, GFP alone, 10-kDa/GFP, or 24-kDa/GFP was not recruited to cell-cell junctions (Fig. 9, A–C). The state of confluency and localization of ZO-1 at cell-cell junctions in the same cells were revealed by staining the cells with anti-ZO-1 Ab (Fig. 9, A–E). Confocal microscopy also revealed that the 10+24-kDa/GFP fusion proteins colocalized with ZO-2 and ZO-1 in cultured MDCK cell tight junctions (data not shown). All these fusion proteins were also concentrated in the nucleus.

**DISCUSSION**

Several studies suggest that the cortical cytoskeleton is involved in the structural and functional organization of TJs (reviewed in Ref. 56; Ref. 62), but little is known about the molecule(s) that link these two distinct structures. In this study, we demonstrate that two nonerythroid isoforms of 4.1R (~135 and ~150 kDa) interact with hZO-2. In addition to ZO-2, these isoforms of 4.1R also associate with other TJ proteins such as ZO-1 and occludin and the cytoskeletal proteins α-spectrin and actin in one protein complex. Endogenous components of TJs also efficiently recruit tagged 4.1R segments containing the spectrin/actin- and ZO-2-binding domains to TJs. We thus hypothesize that 4.1R isoforms in epithelial cells may participate in regulation of TJ by mediating a direct link between the TJ proteins with the underlying cytoskeleton.

ZO-2 belongs to the MAGUK protein family. MAGUK proteins such as p55, human DLG, and the human LIN2 homolog are known to associate with the cortical actin cytoskeleton. In erythrocytes, the MAGUK protein p55 links the transmembrane protein glycophorin C to the spectrin/actin cytoskeleton through the 30-kDa domain of protein 4.1R (20). Human DLG and the human LIN2 homolog are also known to bind to 4.1R in epithelial cells (15, 29). Similarly, ZO-1, a member of the MAGUK family of proteins, binds to ZAK, a serine-threonine kinase (63), and links the transmembrane protein occludin to cytoskeleton by binding to F-actin (59). However, the interaction between hZO-2 and 4.1R was unexpected because hZO-2 lacks the 4.1R-binding motif used by the MAGUK proteins p55, human DLG, and human LIN2, a conserved lysine-rich sequence motif located between the SH3 and guanylate kinase domains (15, 20, 29).

Although the C-terminal domain encoded by exons 19–21 of 4.1R interacts with multiple PDZ domains containing hZO-2, the expected PDZ domain-tail interaction (64) was not ob-
served. Our data suggest that the amino acids required for the interaction of 4.1R and hZO-2 reside within the amino acids encoded by exons 19–21 of the C-terminal domain of 4.1R and amino acids 1054–1118 of the proline-rich domain of hZO-2.

Because deletion of exon 19, 20, or 21 abolishes this interaction, it appears that the amino acids of 4.1R that interact with hZO-2 may be distributed in these three exons and that there may be multiple contact sites between these proteins. It is also

FIG. 8. Protein 4.1R binds to hZO-2, hZO-1, and occludin in vitro. GST alone or 4.1R/GST fusion proteins coupled to glutathione-Sepharose beads were incubated with in vitro translated[^S]methionine-labeled segments of hZO-2, ZO-1, or occludin. After washing, the proteins complexed to beads were analyzed by SDS-polyacrylamide gel electrophoresis and detected by fluorography. A, in vitro binding between 4.1R/GST and amino acids 905–1059 of hZO-2. This segment of hZO-2 did not bind to 4.1R isoforms or to any of the 4.1R domains. B, in vitro binding between 4.1R/GST and amino acids 980–1168 of hZO-2. This segment of hZO-2 bound to the 135- and 80-kDa 4.1R isoforms and to the 4.1R 24-kDa domain. C, in vitro binding between 4.1R/GST and amino acids 1453–1747 of hZO-1. This segment of hZO-1 did not bind to 4.1R isoforms or to any of the 4.1R domains. D, in vitro binding between 4.1R/GST and amino acids 1042–1454 of hZO-1. This segment of hZO-1 bound to the 135- and 80-kDa 4.1R isoforms and to the 4.1R 24-kDa domain. E, in vitro binding between 4.1R/GST and amino acids 1453–1747 of hZO-1. This segment of human occludin did not bind to 4.1R isoforms or to any of the 4.1R domains. F, in vitro binding between 4.1R/GST and amino acids 284–522 of human occludin. This segment of occludin bound to the 135- and 80-kDa 4.1R isoforms and to the 4.1R 24-kDa domain. G, quantitative analysis of binding between the 24-kDa domain of 4.1R and aa 980–1168 of hZO-2, aa 1042–1454 of ZO-1, and aa 284–522 of human occludin. A glutathione-Sepharose bead slurry containing 24-kDa/GST was incubated with 0.0025–0.015 μg of in vitro translated[^S]methionine-labeled hZO-2, hZO-1, or occludin (from the top). The amount of translated peptide that bound to the bead product in each case was quantitated as described under "Experimental Procedures." Each value represents the mean value of triplicate experiments. The binding was saturable, and Scatchard analysis (inset) indicated $K_D$ values of $4.5 \times 10^{-8}$, $7.5 \times 10^{-7}$, and $7.1 \times 10^{-7}$ M for hZO-2, ZO-1, and occludin, respectively.
possible that the presence of these three exons of 4.1R together gives rise to a particular folding of 4.1R required for its interaction with hZO-2 or ZO-2. A recent study shows that these amino acids of 4.1R are highly conserved among other 4.1R-like genes (30), raising the possibility that these gene products may also interact with hZO-2 and ZO-2.

ZO-2 is known to associate with the cytoplasmic surface of the TJ (51, 57). It shares a strong homology with ZO-1, especially within the conserved MAGUK domains (39). It is expressed in almost every tissue (44) and associates with ZO-1 through its second PDZ domain at both the adherens junctions and TJs (41, 51, 58, 65). ZO-2 binds directly to the C-terminal 147 amino acids of occludin (66) and coprecipitates with occludin and α-catenin (41). It is a component of the TJ along with ZO-1, ZO-3, cingulin, 7H6 antigen, symplekin, several unidentified proteins, and transmembrane proteins such as occludin (reviewed in Ref. 56) and members of the claudin family (67).

ZO-1, ZO-2, and occludin have been shown to localize at TJs of confluent MDCK cells (51–53, 61). Using anti-HP Ab, an antibody specific for nonerythroid isoforms of 4.1R, we found that 4.1R epitopes are also localized at TJs of MDCK cells by confocal microscopy and high resolution electron microscopy (Fig. 2, B and H, respectively). Confocal microscopic analysis of double-labeled confluent MDCK cells showed that these 4.1R epitopes colocalize at TJs with ZO-2 and occludin (Fig. 2, C and F). In immunoprecipitation studies using the same antibodies, ZO-2 and two nonerythroid 4.1R isoforms were found to coprecipitate together. About 40–50% of 4.1R and ZO-2 appear to remain associated together under our experimental conditions. Unlike ZO-2 and ZO-1, which also localize at adherens junction in addition to TJs, occludin is exclusively localized at the TJs (52, 53). We found that 4.1R, ZO-1, ZO-2, and occludin coprecipitated together. This association of 4.1R with ZO-2, ZO-1, or occludin was not seen in cells that were not confluent (and thus in which the tight junction was not organized) (Fig. 6), strongly suggesting that these proteins associate together at TJs.

Coprecipitation does not reveal whether there is a direct interaction between 4.1R and ZO-1 or occludin. We thus tested this hypothesis in yeast two-hybrid and in vitro binding assays. As shown in Fig. 7, only weak interactions between the C-terminal domain of 4.1R and the proline-rich domain of ZO-1 (Fig. 7B) or the C-terminal cytoplasmic domain of occludin (Fig. 7A) were observed in yeast two-hybrid assays. These interactions were only 25–30% of the interaction between 4.1R and hZO-2 or ZO-2 as detected in yeast two-hybrid assays. ZO-2, ZO-1, and occludin bound to 4.1R in in vitro binding assays (Fig. 8). Interestingly, a recent study suggested that a third protein might mediate the interaction between ZO-1 and ZO-2 (58). Similarly, a mutant occludin that bound to ZO-1 in vitro failed to localize at TJs (68). Both ZO-1 and ZO-2 also localize to TJs in occludin-deficient TJs (41, 69). These findings imply that association of other factors, in addition to ZO-1 and ZO-2, may be required for localization of occludin at TJs.

Both the 135- and 80-kDa isoforms interact with ZO-2 in yeast two-hybrid assays, whereas HP alone does not. Thus, our results do not define the precise isoform(s) of 4.1R that interact with ZO-2. Because of the complex splicing pathway that 4.1R undergoes, it is difficult to know exactly which exons are included in the isoform(s) that interact with ZO-2. However, epitopes for anti-HP Ab are observed at TJs; moreover, the two isoforms of 4.1R that coprecipitate with ZO-2 and other TJ proteins are exclusively of the higher molecular mass. Finally, no 80-kDa isoform containing exon 19 (which is required for its interaction with ZO-2) is expressed in MDCK cells (13). We thus believe that isoforms of 4.1R that interact with ZO-2 are of the 135-kDa class or of similar molecular mass. The 80-kDa isoform binds to ZO-2 in yeast two-hybrid and in vitro binding assays, but does not coprecipitate with ZO-2. These data are compatible because the increased concentration and proximity of molecules containing the binding sites in yeast two-hybrid and in vitro binding assays could allow the 80-kDa isoform to interact with ZO-2. In the intact cells, factors such as post-translational modifications and compartmentalization or presence of modifying cofactors will affect their interaction. In the same context, the amino acids encoded by exons 19–21 of 4.1R are required and sufficient for interaction of 4.1R with ZO-2 in vitro, but not in vivo. Indeed, the 22–24-kDa domain is not sufficient to target GFP to tight junctions, although per se it binds well to ZO-2. These amino acids are highly conserved among the 4.1-like gene family (30). It can thus be argued that other 4.1R-like gene products interact with ZO-2. However, as discussed above, our results strongly implicate 4.1R isoforms as opposed to 4.1-like gene products because the anti-HP Ab used in our assays does not react with other 4.1-like proteins.

A noteworthy finding is the association of 4.1R with ZO-1,
ZO-2, occludin, α-spectrin, and actin apparently in one protein complex, implying a connection of TJs to the cortical cytoskeleton, as previously suggested (Refs. 62 and 70; reviewed in Ref. 56). Several studies also suggest that actin plays a role in regulation of TJ permeability (71, 72). ZO-1 has been shown to interact with members of the MAGUK family, ZO-1, and ZO-2 and occludin. Several studies also suggest that actin plays a role in regulation of TJ permeability (71, 72). ZO-1 has been shown to interact with members of the MAGUK family, ZO-1, and ZO-2 and occludin. Several studies also suggest that actin plays a role in regulation of TJ permeability (71, 72). ZO-1 has been shown to interact with members of the MAGUK family, ZO-1, and ZO-2 and occludin. Several studies also suggest that actin plays a role in regulation of TJ permeability (71, 72). ZO-1 has been shown to interact with members of the MAGUK family, ZO-1, and ZO-2 and occludin. Several studies also suggest that actin plays a role in regulation of TJ permeability (71, 72). ZO-1 has been shown to interact with members of the MAGUK family, ZO-1, and ZO-2 and occludin. Several studies also suggest that actin plays a role in regulation of TJ permeability (71, 72). ZO-1 has been shown to interact with members of the MAGUK family, ZO-1, and ZO-2 and occludin. Several studies also suggest that actin plays a role in regulation of TJ permeability (71, 72). ZO-1 has been shown to interact with members of the MAGUK family, ZO-1, and ZO-2 and occludin. Several studies also suggest that actin plays a role in regulation of TJ permeability (71, 72). ZO-1 has been shown to interact with members of the MAGUK family, ZO-1, and ZO-2 and occludin. Several studies also suggest that actin plays a role in regulation of TJ permeability (71, 72). ZO-1 has been shown to interact with members of the MAGUK family, ZO-1, and ZO-2 and occludin. Several studies also suggest that actin plays a role in regulation of TJ permeability (71, 72). ZO-1 has been shown to interact with members of the MAGUK family, ZO-1, and ZO-2 and occludin.
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