Occludin is hyperphosphorylated on Ser and Thr residues in intact epithelial tight junction (TJ); however, the role of this phosphorylation in the assembly of TJ is unclear. The influence of protein phosphatases PP2A and PP1 on the assembly of TJ and phosphorylation of occludin was evaluated in Caco-2 cells. Protein phosphatase inhibitors and reduced expression of PP2A-Cα and PP1α accelerated the calcium-induced increase in transepithelial electrical resistance and barrier to inulin permeability and also enhanced the junctional organization of occludin and ZO-1 during TJ assembly. Phosphorylation of occludin on Thr residues, but not on Ser residues, was dramatically reduced during the disassembly of TJ and was gradually increased during the reassembly. PP2A and PP1 co-immunoprecipitate with occludin, and this association was reduced during the assembly of TJ. Glutathione S-transferase (GST) pull-down assay using recombinant GST-occludin demonstrated that cellular PP2A and PP1 bind to the C-terminal tail of occludin, and these interactions were also reduced during the assembly of TJ. A pairwise binding assay using GST-occludin and purified PP2A and PP1 demonstrates that PP2A and PP1 directly interact with the C-terminal tail of occludin. In vitro incubation of phospho-occludin with PP2A or PP1 indicated that PP2A dephosphorylates occludin on phospho-Thr residues, whereas PP1 dephosphorylates it on phospho-Ser. This study shows that PP2A and PP1 directly interact with occludin and negatively regulate the assembly of TJ by modulating the phosphorylation status of occludin.

An important function of the epithelial tight junction (TJ) is to form a barrier to the diffusion of pathogens, toxins, and allergens from the external environment into the tissues. The disruption of TJ plays a crucial role in the pathogenesis of a number of diseases related to the gastrointestinal tract, lung, and kidney (1–3). The TJ is organized by specific interactions between a wide spectrum of proteins. Three types of transmembrane proteins, occludin (4), claudins (5), and junctional adhesion molecule (6) interact with other intracellular plaque proteins such as ZO-1, ZO-2, ZO-3, cingulin, and 7H6, which in turn anchor the transmembrane proteins to the actin cytoskeleton (7–9).

A significant body of evidence indicates that the activities of various intracellular signaling molecules regulate the integrity of TJ. The signaling pathways involving protein kinases and GTPase switch proteins regulate the TJ permeability in different epithelial monolayer (10–22). Tyrosine kinases such as c-Yes, c-Src, and focal adhesion kinase are localized in the vicinity of TJ (4). A number of previous studies have shown that oxidative stress induces disruption of TJ and an increase in paracellular permeability by a tyrosine kinase-dependent mechanism (15–17, 19, 20). Oxidative stress induces Tyr phosphorylation of a wide spectrum of proteins, including occludin, ZO-1, E-cadherin, and β-catenin (19). Phosphorylation of occludin on Tyr residues results in the loss of its interaction with ZO-1, ZO-2, and ZO-3 (23). Furthermore, recent studies have demonstrated that activation of c-Src (20) and phosphatidylinositol 3-kinase (22) is involved in the oxidative stress-induced disruption of TJ in Caco-2 cell monolayer.

Occludin is hyperphosphorylated on Ser and Thr residues (24–27) in an intact epithelium. Although the significance of Ser/Thr phosphorylation of occludin in TJ assembly is unclear, occludin is shown to undergo dephosphorylation on Ser/Thr residues during the disruption of TJ by calcium depletion, phorbol esters, or bacterial infection (28–30). Phosphorylation of occludin on Ser/Thr residues may be mediated by atypical protein kinase C (PKC), such as PKCζ and PKCα, which are localized in the vicinity of TJ (10). A previous study indicated that PP2A, a Ser/Thr-phosphatase, interacts with TJ protein complex and suggested that it may influence the integrity of TJ in MDCK cells that overexpress PP2A (31). Therefore, the balance between atypical PKC and PP2A may determine the Ser/Thr phosphorylation status of occludin.

In the present study we examined the influence of endogenous PP2A and PP1 on the calcium switch–induced assembly of TJ in Caco-2 cells. This study demonstrates that 1) inhibition of PPase activity by selective inhibitors and reduced expression of PP2A-Cα or PP1α by siRNA and/or antisense oligonucleotides accelerates the assembly of TJ, 2) the assembly...
of TJ is associated with the phosphorylation of occludin on Thr residues, which is enhanced by reduced expression of PP2A, 3) PP2A and PP1 directly interact with occludin, and their interaction with occludin is reduced during the assembly of TJ, and 4) PP2A dephosphorylates occludin on p-Thr residues, whereas PP1 dephosphorylates it on p-Ser.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Cell culture reagents and supplies and Oligo-fectamine® were purchased from Invitrogen. Fluorescein isothiocyanate-inulin, GSH, leupeptin, aprotinin, bestatin, pepstatin A, phenylmethylsulfonyl fluoride, GSH-agarose, Triton X 100, vanadate, Malachite green, protein-A-Sepharose, and protein-G-Sepharose were purchased from Sigma. Okadaic acid, foscarnecin, and calcyulin-A were purchased from Calbiochem. Purified PP2A, PP1, and phosphopeptide, RRApSVA (pS is phosphoserine), were purchased from Upstate (Charlottesville, VA). The Pase substrate phosphopeptide (KRpTIRR) and p-Tyr peptide DADEpYLIPQQG were custom synthesized by Sigma Genosys (St. Louis, MO). All other chemicals were of analytical grade purchased either from Sigma or Fisher.

**Antibodies**—Mouse monoclonal anti-PP2A-Cα, anti-PP1α, HRP-conjugated anti-GST, and anti-PKCζ antibodies were purchased from BD Transduction. Mouse monoclonal anti-occludin, rabbit polyclonal anti-ZO-1, HRP-conjugated anti-occludin, rabbit polyclonal anti-p-Thr, and anti-p-Ser antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA). AlexaFluor 488-conjugated anti-mouse IgG antibody was obtained from Molecular Probes (Eugene, OR). Cy3-conjugated anti-rabbit IgG, HRP-conjugated anti-mouse IgG, antiantin, and HRP-conjugated anti-rabbit IgG antibodies were purchased from Sigma. The specificity of anti-p-Thr and anti-p-Ser antibodies was confirmed by immunoblotting control cell extracts for p-Thr and p-Ser in the presence of varying concentrations of different peptides; RRApSVA, KRpTIRR, or DADEpYLIPQQG. Results are presented in Fig. 7, C and D.

**Antisense Oligonucleotide and siRNA**—Antisense oligonucleotides were designed against the nucleotide sequence of the genes for catalytic subunits of human PP2A and human PP1. The nucleotide sequences of PP2A and PP1 were compared with each other using the ClustalW program, and unique sequences were selected for each protein. To determine the specificity of these oligos, the sequences were further verified by BLAST search of the known human genome databases, and no matches were found other than the respective proteins, confirming the uniqueness of these nucleotide sequences. The sequences of antisense oligos for PP2A and PP1 and the missense olo are as follows: AS-PP2A-1, ttcggctagcggcagcgt; AS-PP2A-2, cccttacatcagcgcgct; AS-PP2A-3, cagctcagctgcgttacaggg; AS-PP1-1, gattcgctggctcagaggt; AS-PP1-2, ctcagctgccgcttcaggg; AS-PP1-3, 3tgcgctattgccctggagg; missense oligo, tatagctgctagcttga.

The sequence of the missense oligo did not match any sequence for human proteins. These antisense oligonucleotides were custom synthesized in the phosphorothioate and Cy3-conjugated form by Sigma Genosys and were purified by high performance liquid chromatography. siRNA to human PP2A-Cα and scrambled RNA control were purchased from Dharmacon (Lafayette, CO).

**Cell Culture**—Caco-2 cells, purchased from American Type Culture Collection (Manassas, VA), were grown under standard cell culture conditions as described before (19). Cells were grown on polycarbonate membranes in Transwells (6.5, 12, or 24 mm; Costar, Cambridge, MA), and experiments were conducted 11–13 days (6.5 or 12 mm Transwells) or 17–19 days (24 mm Transwells) post-seeding.

**TJ Assembly by Calcium Switch**—Caco-2 cell monolayers were treated with 4 mM EGTA in both the apical and basal compartments until the transepithelial electrical resistance (TER) was reduced to about 15–17% that of basal values (usually reduced from 300 to 50 ohms-cm²); this was monitored by measuring TER every 5 min, and the average time recorded for EGTA treatment was 30 min. The cells were then quickly washed three times with DMEM to remove all traces of EGTA and incubated in regular DMEM containing calcium for varying times. The integrity of TJ was analyzed by measuring TER and unidirectional flux of fluorescein isothiocyanate-conjugated inulin.

**Treatment with PPase Inhibitors**—Caco-2 cells were treated with PPase inhibitors as previously described (32). Cell monolayers were incubated with 100 nM foscarnecin, 4 nM okadaic acid, and 0.5 mM calcyulin-A for 24 h before the experiment. The cells were then washed with DMEM, and the inhibitors were added again during the calcium switch experiments to maintain the inhibitory effect.

**Transfection of Antisense Oligos and siRNA**—Caco-2 cells (125,000 cells/well) were seeded in six-well plates. The cells were then allowed to grow and attach for 24 h. After the incubation, the cells were treated with serum-free, antibiotic-free DMEM, and the incubation was continued for an additional 24 h. The cells were then transfected using 1 ml of antibiotic-and serum-free DMEM with or without 80 pmol of the antisense oligonucleotides or siRNA and 3.15 µl of Oligo-fectamine® reagent in each well and incubated for 6 h at 37 °C. Serum was then added to the medium to make a final concentration of 10% serum and incubated at 37 °C. After 24 h the cell monolayers were trypsinized and seeded on to Transwell inserts, and the TER was monitored every day. Calcium switch experiments were performed on day 4. For controls, cells were transfected with missense oligos or control RNA with scrambled nucleotide sequence at similar dose and transfection conditions.

**Measurement of TER**—TER was measured as described previously (15) using a Millicell-ERS Electrical Resistance System (Millipore, Bedford, MA). TER was calculated as ohms-cm² by multiplying it with the surface area of the monolayer. The resistance of the polycarbonate membrane in Transwells (~30 ohms-cm²) was subtracted from all readings.

**Unidirectional Flux of Inulin**—Transwells with the cell monolayers were incubated under different experimental conditions in the presence of fluorescein isothiocyanate-inulin (0.5 mg/ml) in the basal well. At varying times during the assembly of TJ, 100 µl each of apical and basal media were withdrawn, and fluorescence was measured using a fluorescence plate reader (BioTEK Instruments, Winooski, VT). The flux into the...
apical well was calculated as the percentage of total fluorescence administered into the basal well/μm² of surface area.

**Immunofluorescence Microscopy**—At varying times during the calcium switch, cell monolayers (12 mm) were washed with PBS and fixed in acetone:methanol (1:1) at 0 °C for 5 min. Cell monolayers were blocked in 3% nonfat milk in TBST (20 mM Tris, pH 7.2, and 150 mM NaCl) and incubated for 1 h with primary antibodies; rabbit polyclonal anti-ZO-1, anti-PP2A-Cα, or mouse monoclonal anti-occludin antibodies followed by incubation for 1 h with secondary antibodies; goat AlexaFluor 488-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG antibodies. The fluorescence was visualized using a Zeiss LSM 5 laser scanning confocal microscope, and the images from Z-series sections (1 μm) were collected by using Zeiss LSM 5 Pascal, the confocal microscopy software (Release 3.2). Images were stacked using the software, Image J (NIH), and processed by Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

**Preparation of Detergent-insoluble Fractions**—Triton-insoluble fractions were prepared as previously described (33). Cell monolayers in Transwells (24 mm) were washed twice with ice-cold PBS and incubated for 5 min with lysis buffer-CS (50 mM Tris buffer, pH 7.4, containing 1.0% Triton X-100, 2 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml bestatin, 10 μg/ml pepstatin-A, 1 mM vanadate, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at low speed (15,600 × g) for 4 min at 4 °C to sediment the high density actin-rich fraction. The pellet was suspended in 200 μl of lysis buffer-CS. Protein concentration in different fractions was measured by the BCA method (Pierce). Triton-insoluble and Triton-soluble fractions were mixed with an equal volume of Laemmli sample buffer (2 × concentrated) and heated at 100 °C for 10 min.

**Immunoprecipitation**—At varying times during the assembly of TJ, Caco-2 cell monolayers (24 mm Transwells) were washed with ice-cold 20 mM Tris, pH 7.4, and actin-rich Triton-insoluble and Triton-soluble fractions were prepared. The suspension of Triton-insoluble fraction was sonicated for 10 s in lysis buffer-N (20 mM Tris, pH 7.4, containing 0.2% Nonidet P-40, 0.1% sodium deoxycholate, and a mixture of protease inhibitors). Sonication induced fragmentation of the F-actin filaments and released actin-bound protein complexes. Under such conditions greater than 80% of occludin and ZO-1 were recovered in the supernatant fraction; however, co-immunoprecipitation studies indicated that the interaction between occludin and ZO-1 was unaffected. The actin lysate and Triton-soluble fraction (1.0 mg protein/ml) were incubated with 2 μg of anti-occludin antibodies at 4 °C for 16 h. The immune complexes were isolated by precipitation using protein-A/G-Sepharose (for 1 h at 4 °C). For immunoprecipitation of p-Thr and p-Ser, Triton-insoluble and -soluble fractions were heated in the presence of 0.3% SDS for 10 min at 100 °C followed by centrifugation. The clear supernatant was then used for immunoprecipitation.

**Immunoblot Analysis**—Proteins were separated by SDS-polyacrylamide gel (7 or 4–12% gradient) electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked for occludin, ZO-1, p-Thr, p-Ser, PP2A-Cα, PKCζ, or PP1α by using specific antibodies in combination with HRP-conjugated anti-mouse IgG or HRP-conjugated anti-rabbit IgG antibodies. HRP-conjugated anti-GST antibody was used for immunoblot analysis of GST or GST-occludin. The blot was developed using the ECL chemiluminescence method (Amersham Biosciences). Quantitation was performed by densitometric analysis of specific bands on the immunoblots by using the software Image J.

**PP2A Assay**—Detergent-soluble or insoluble fractions or an immunocomplex was diluted in PPase buffer (50 mM HEPES, pH 7.2, 60 mM NaCl, 60 mM KCl, and protease inhibitors) to a final volume of 20 μl and incubated with 5 μl of phosphopeptide substrate, KRPTIRR (5 μg). After incubation at 30 °C for 10 min, free phosphate was assayed by adding 100 μl of Malachite green reagent to each sample in a 96-well microtiter plate. After 10 min of incubation at 30 °C, absorbance was measured at a 650-nm wavelength in a microplate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA). Zero-minute incubation was used for the control assay. Assay was also performed in the presence of 100 nM fostriecin. To determine PP2A-specific activity, the phosphatase activity measured in the presence of 100 nM fostriecin was subtracted from the corresponding total activity. The units of PP2A activity represent pmol of free phosphate generated in 1 h under assay conditions.

**Preparation of GST-occludin-C**—C-terminal tail of chicken occludin was prepared as a GST fusion protein (GST-occludin-C) in Escherichia coli BL21DE (3) cells and purified using GSH-agarose as described before (23). cDNA for the C-terminal tail of occludin (amino acids 354–503) in pGEX vector was received as a gift from Dr. J. M. Anderson, University of North Carolina (Chapel Hill, NC).

**GST Pulldown Assay**—To prepare protein extracts, Caco-2 cell monolayers at different stages of calcium switch were lysed in 0.2% Triton X-100 in PBS containing 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate (3 ml/100 mm plate). Cell lysates were centrifuged at 15,000 × g for 15 min, and the supernatant was used for the pulldown assay. Cell lysate (0.6 ml) was incubated with 10 μg of GST-occludin-C and 20 μl of GFP-agarose at 4 °C for 15 h on an inverter. Agarose beads were washed 3 times with PBS, and proteins were extracted by heating at 100 °C for 10 min in 20 ml of Laemmli sample buffer. The amounts of PP2A, PP1, and PKCζ present in GFP-agarose pulldown were determined by immunoblot analysis. At the end of the experiment the blots were stained with Ponceau S and immunoblotted for GST to confirm the use of equal amounts of GST-occludin-C in different samples.

**Pairwise Binding Assay**—To determine the direct interaction between occludin and PP2A-Cα or PP1α, GST-occludin-C (5–20 μg) was incubated with purified PP2A-Cα (0.1 or 0.25 μg) or purified PP1α (0.1 or 0.25 μg) in PBS containing 0.2% Triton X-100, 1 mM vanadate, and 10 mM sodium fluoride for 3 h at 30 °C on an inverter. GST-occludin-C was pulled down by binding to 20 μl of 50% GFP-agarose slurry at 30 °C for 1 h. The amounts of PP2A-Cα and PP1α bound to GFP-agarose pulldown were determined by immunoblot analysis. Nonspecific binding was determined by carrying out the binding with GST instead of GST-occludin-C.
Protein Phosphatases and Tight Junction Regulation

FIGURE 1. Inhibition of PPase activity accelerates the assembly of TJ. Caco-2 cell monolayers were preincubated with or without okadaic acid, fostriecin, or calyculin A. TJ assembly in cell monolayers was evaluated by the calcium switch method. After pretreatment with PPase inhibitors, cell monolayers were exposed to EGTA for 30 min. EGTA was washed off, and regular medium with calcium was added. A, after incubation with okadaic acid, PPase activity was measured in extracts from Triton-insoluble (▪) and Triton-soluble (●) fractions. Values are the mean ± S.E. (n = 4). B, after incubation with varying doses of okadaic acid, PP2A activity was immunoprecipitated from cell extracts. PPase activity was measured in the anti-PP2A immunocomplexes. Values are the mean ± S.E. (n = 3). C, basal TER was measured after treatment of cell monolayers with different PPase inhibitors. Values are the mean ± S.E. (n = 8). D, TER was measured in cell monolayers during EGTA treatment and calcium replacement in untreated cells (●) or cells treated with okadaic acid (▴), fostriecin (●), or calyculin-A (▲). TER was also measured in the control cell monolayer without calcium switch (■). Values are the mean ± S.E. (n = 8). Asterisks indicate the values that are significantly (p < 0.05) different from values for inhibitor-treated cells at the corresponding time period. E, inulin permeability was measured before and after EGTA treatment and at 3 h after calcium replacement. Values are the mean ± S.E. (n = 8). Asterisks indicate the values that are significantly (p < 0.05) different from control (None) values.

Dephosphorylation of Occludin by PP2A and PP1—Occludin was immunoprecipitated from the Triton-soluble fraction of Caco-2 cells. Occludin immunocomplexes were then incubated with 0.5 μg PP2A or PP1 in PPase buffer for 60 min. For control assay, PPase formulation buffer was used in place of PP2A and PP1. After incubation for 10–60 min at 30 °C, the reaction mixture was heated with Laemmli sample buffer and immunoblotted for p-Thr, p-Ser, and occludin.

Statistics—Comparison between two groups was made by Student’s t tests for grouped data. Significance in all tests was set at 95% or greater confidence level.

RESULTS

Inhibition of Protein Phosphatase Activity Accelerates the Calcium Switch-induced Assembly of TJ—The effect of endogenous PP2A and PP1 activities on the assembly of TJ was evaluated in Caco-2 cells. Assembly of TJ was monitored by the calcium switch method. PP2A activity in Caco-2 cells was selectively inhibited by pretreatment of cells with low concentrations of okadaic acid, fostriecin, or calyculin-A as reported before (32). Incubation with okadaic acid dose-dependently reduced PP2A activity in both detergent-insoluble and detergent-soluble fractions of Caco-2 cells (Fig. 1A). PP2A activity measured by immunocomplex PP2A assay also demonstrated a reduction in the PP2A activity in okadaic acid-treated cells (Fig. 1B). Similarly, incubation with fostriecin and calyculin-A also reduced PP2A activity (data not shown).

Basal TER of cell monolayers treated with PPase inhibitors was not significantly different from the TER values for control (Fig. 1C). Calcium depletion by EGTA rapidly reduced the TER, and replenishment of calcium in the EGTA-treated cell monolayers gradually restored the TER to the basal level (Fig. 1D). Increased TER by calcium replenishment was accompanied by reduced permeability to inulin. Pretreatment of cells with okadaic acid, fostriecin, and calyculin-A significantly accelerated the restoration of TER during the reassembly of TJ by calcium replacement (Fig. 1D). The inulin permeability measured during the restoration of TER was significantly lower in inhibitor-treated cells compared with that in untreated cells (Fig. 1E).

Confocal immunofluorescence microscopy showed that EGTA treatment resulted in the disruption of junctional organization of occludin and ZO-1 and redistribution of these proteins in the intracellular compartments (Fig. 2A). Three hours after calcium replacement a partial reorganization of occludin and ZO-1 at the intercellular junctions was achieved. However, in cells pretreated with fostriecin, both occludin and ZO-1 realocalization to the intercellular junctions was almost complete by 3 h. Densitometric analysis indicated that intracellular fluorescence of occludin (Fig. 2B) and ZO-1 (Fig. 2C) increased during EGTA treatment, whereas the junctional fluorescence was significantly reduced. During the reassembly, this process appeared to be reversed. However, the decrease in intracellular and inercrease in junctional fluorescence of both occludin and ZO-1 during the reassembly was significantly greater in fostriecin-treated cells compared with that in untreated cells. The z-sections of these images (Fig. 2D) confirm the accelerated reassembly of occludin and ZO-1 in cells pretreated with fostriecin. Confocal microscopy also indicates that occludin and ZO-1 are localized predominantly in vesicular structures in EGTA-treated cell monolayers.
Reduced Expression of PP2A by Antisense Oligonucleotides or siRNA Accelerates the Assembly of TJ

To confirm the role of endogenous PP2A in the regulation of the assembly of TJ, we designed three different antisense oligonucleotides against the nucleotide sequence of human PP2A-Cα. Antisense oligonucleotides, AS-PP2A-2, AS-PP2A-3, or a combination of AS-PP2A-2 and AS-PP2A-3 were transfected into Caco-2 cells. Cy3-conjugated AS-PP2A-3 showed that Oligofectamine® effectively introduced the antisense oligonucleotides into the cell (Fig. 3A). Transfection with AS-PP2A-2, AS-PP2A-3, or AS-PP2A-2 + AS-PP2A-3 resulted in a marked decrease in the levels of PP2A-Cα compared with cells transfected with the missense oligonucleotide (Fig. 3B and C). However, these oligonucleotides did not alter the level of PP1α in these cells. Similarly, transfection of cells with siRNA (siPP2A-1, siPP2A-2, and siPP2A-3) designed against the nucleotide sequence of human PP2A-Cα gene also reduced the levels of PP2A-Cα without affecting the levels of PP1α (Fig. 3B and C). siPP2A-3 was the more effective in reducing the level of PP2A-Cα compared with siPP2A-1 and siPP2A-2. Transfection with antisense oligos did not significantly alter the basal TER (Fig. 3D). However, calcium switch experiments in these cells showed that transfection with antisense oligonucleotides significantly accelerated the restoration of TER during calcium-induced reassembly of TJ (Fig. 3D). Double transfection of AS-PP2A-2 and AS-PP2A-3 was more effective than transfection of individual antisense oligonucleotides. The inulin permeability measured 3 h after calcium restoration was significantly low in cells transfected with antisense oligonucleotides compared with that in cells treated with missense oligonucleotides (Fig. 3E). Transfection with each siRNA resulted in only a slight increase in basal TER (Fig. 3F), but it significantly accelerated the restoration of TER during TJ reassembly by calcium switch compared with restoration of TER in cells transfected with scrambled RNA (Fig. 3F). Transfection with different siRNAs reduced the basal inulin permeability compared with inulin permeability in cell monolayers transfected with scrambled RNA (Fig. 3G). Inulin permeability measured 3 h after calcium restoration was significantly low in cells transfected with siRNA. The accelerated recovery of both TER and the barrier to inulin was more pronounced in cell monolayers transfected with siPP2A-3 compared with those in cell monolayers transfected with siPP2A-1 or siPP2A-2 (Fig. 3G).

Confocal immunofluorescence microscopy showed that transfection with siPP2A-3 (Fig. 4A) enhanced the assembly of occludin and ZO-1 at the intercellular junctions during the TJ reassembly by calcium restoration. Densitometric analysis of intracellular and junctional fluorescence for occludin and ZO-1 showed that the calcium switch-induced decrease in intracellular and increase in junctional occludin (Fig. 4B) and ZO-1 (Fig. 4C) was significantly greater in siPP2A-3-transfected cells compared with that in control RNA-transfected cells. Similar
antisense oligonucleotides were designed against the sequence of human PP1α. Transfection of Cy3-conjugated AS-PP1-1 showed successful incorporation of oligonucleotides into the cells (Fig. 5A). Transfection with AS-PP1-1, AS-PP1-2, or AS-PP1-3 markedly reduced the levels of PP1α without affecting the levels of PP2A-Cα (Fig. 5B). The basal TER of cell monolayers transfected with antisense oligonucleotides was not significantly different from that of cell monolayers transfected with missense oligonucleotides (Fig. 5C). Transfection with AS-PP1-1, AS-PP1-2, or AS-PP1-3 significantly accelerated the recovery of TER (Fig. 5D) and the barrier to inulin (Fig. 5E) during the calcium-induced reassembly of TJ. Confocal microscopy showed that occludin and ZO-1 were reorganized at the intercellular junctions 3 h after calcium restoration in cells transfected with AS-PP1-1 (Fig. 6A), whereas very little junctional organization of occludin and ZO-1 was achieved in cells transfected with missense oligonucleotide. Calcium-induced decrease in intracellular and increase in junctional fluorescence of occludin (Fig. 6B) and ZO-1 (Fig. 6C) was significantly greater in AS-PP1-1-transfected cells compared with the missense oligo-transfected cells.

### Phosphorylation of Occludin on Thr Residues Was Altered during Disassembly and Reassembly of TJ—Occludin is known to be hyperphosphorylated on Ser and Thr residues in a normal epithelial monolayer, and hyperphosphorylated occludin was found to be localized predominantly in the detergent-insoluble fractions of epithelial cells (24-27). Therefore, Ser/Thr phosphorylation of occludin is likely to be involved in the assembly and/or stabilization of TJ. We examined the Ser/Thr phosphorylation of occludin during the calcium switch-induced disassembly and reassembly of TJ. Immunoprecipitation of p-Thr followed by immunoblot analysis for occludin in detergent-insoluble fractions showed that disruption of TJ by EGTA treatment is accompanied by a dramatic reduction in the level of Thr-phosphorylated occludin (Fig. 7, A and B). Thr phosphorylation of occludin was gradually restored during the TJ reassembly by calcium restoration. Occludin in

### Reduced Expression of PP1 by Antisense Oligonucleotides Accelerates the Assembly of TJ—Okadaic acid and fostriecin are more selective inhibitors of PP2A, whereas calyculin A is known to be equally efficient in inhibiting both PP2A and PP1. Therefore, we evaluated the role of endogenous PP1 in regulating the assembly of TJ during calcium switch. Three different

results were obtained in cells transfected with AS-PP2A-3 (data not shown).

Reduced Expression of PP1 by Antisense Oligonucleotides Accelerates the Assembly of TJ—Okadaic acid and fostriecin are more selective inhibitors of PP2A, whereas calyculin A is known to be equally efficient in inhibiting both PP2A and PP1. Therefore, we evaluated the role of endogenous PP1 in regulating the assembly of TJ during calcium switch. Three different
control cell monolayers was also phosphorylated on Ser residues (Fig. 7, A and B). Ser phosphorylation of occludin was slightly and gradually reduced during EGTA-induced disassembly and calcium-mediated reassembly of TJ. The total amount of occludin and ZO-1 present in the detergent-insoluble fraction remained unaffected during the EGTA-induced disassembly and calcium-mediated reassembly of TJ (Fig. 7, A and B). To determine the specificity of anti-p-Thr and anti-p-Ser antibodies used in this study, we immunoblotted control cell extracts for p-Thr or p-Ser in the absence or presence of different peptides that contained either a p-Thr or p-Ser or p-Tyr residue. The results show that immunoblotting for p-Thr by anti-p-Thr antibody was reduced by p-Thr peptide but not by p-Ser peptide or p-Tyr peptide (Fig. 7D). Therefore, our results confirm the specificity of these antibodies.

**Association of PP2A and PP1 with the TJ Protein Complex Is Reduced during the Assembly of TJ**—The role of PP2A and PP1 in the regulation of TJ assembly and the changes in the Thr phosphorylation of occludin during the disassembly and reassembly suggested that PP2A and PP1 may interact with the TJ protein complex and regulate Thr phosphorylation of occludin. A previous study showed that PP2A-Cα is associated with the anti-occludin immunoprecipitates in MDCK cell monolayers (31). To determine the regulated association of PP2A and PP1 with the TJ, we studied the co-immunoprecipitation of PP2A-Cα and PP1 with occludin during the disassembly and reassembly of TJ. PP2A-Cα was co-immunoprecipitated with occludin in detergent-insoluble fractions of control cell monolayers (Fig. 8, A and B). EGTA treatment slightly increased the level of PP2A-Cα. On the contrary, co-immunoprecipitation of PP2A-Cα with occludin was gradually reduced during the calcium-mediated reassembly of TJ. The PP2A activity associated with anti-occludin immunocomplexes was also significantly increased by EGTA treatment, whereas it was decreased below the basal levels during the reassembly of TJ (Fig. 8C). Co-immunoprecipitation of PP1 with occludin was also reduced during the reassembly of TJ in a time-dependent manner (Fig. 8, D and E).

Co-immunoprecipitation with occludin indicates an association of PP2A-Cα and PP1α with the TJ protein complex. However, it does not demonstrate a direct interaction of PP2A-Cα or PP1α with occludin. To determine the direct interaction between occludin and PP2A or PP1, pairwise binding of GST-occludin-C (C-terminal 150 amino acids) with purified PP2A (AC dimer) and PP1α was conducted. PP2A binds directly to GST-occludin-C in a dose-dependent manner (Fig. 8F). Similarly, PP1α also binds to GST-occludin-C directly in a dose-dependent manner (Fig. 8G). Neither PP2A nor PP1α interacts with GST.

The regulated binding of PP2A to occludin during the assembly of TJ was further studied by GST pulldown assay for interaction of GST-occludin-C with PP2A-Cα and PP1α in Triton-
soluble protein extracts prepared from cells at different stages of calcium switch-mediated disassembly and reassembly of TJ. PP2A-Cα binds to GST-occludin-C in control cells, which was dramatically increased by EGTA treatment. However, the binding of PP2A-Cα to GST-occludin-C was gradually reduced during the reassembly of TJ (Fig. 8H). Binding of PP1α to GST-occludin-C was also dramatically increased by EGTA treatment and reduced during the reassembly of TJ; the binding of PKCζ, however, was unaffected. The total amount of PP2A, PP1, and PKCζ remained unaffected during the disassembly and assembly of TJ (Fig. 8I).

PP2A and PP1 Dephosphorylate Occludin on Ser/Thr Residues—It is not clear if PP2A and PP1 are directly responsible for dephosphorylation of occludin on Ser/Thr residues. A previous study suggested that PP2A might regulate the activity of PKCζ, which in turn may regulate the phosphorylation of occludin (31). To determine the direct role of PP2A in dephosphorylation, occludin was immunoprecipitated from the detergent-insoluble fraction of Caco-2 cells. Anti-occludin immunocomplexes were incubated with purified PP2A or PP1 for 10 min. Dephosphorylation of occludin on Ser and Thr residues was measured by immunoblot analysis for p-Thr and p-Ser. Incubation with PP2A almost completely reduced the p-Thr levels in occludin (Fig. 9, A and B), whereas the level of p-Ser was unaffected. Incubation with PP1 on the contrary rapidly dephosphorylated occludin on Ser residues, whereas it had no effect on Thr phosphorylation of occludin (Fig. 9, A and B). Continued incubation for 60 min showed gradual dephosphorylation of both p-Thr and p-Ser by both phosphatases, but at 60 min the control was found to be unstable.

To determine the role of endogenous PP2A on dephosphorylation of occludin, phosphorylation of occludin on Ser and Thr residues was analyzed during the calcium switch-induced disassembly and assembly of TJ in missense, AS-PP2A-3 or AS-PP1-3 oligo-transfected cells. The basal phosphorylation of occludin on Thr residues was slightly greater in both AS-PP2A-3 and AS-PP1-3-transfected cells compared with that in missense oligo-transfected cells (Fig. 9, C and D). Thr phosphorylation of occludin during the calcium-induced reassembly of TJ was also significantly greater in AS-PP2A-3-transfected cells compared with that in missense-transfected and AS-PP1-3-transfected cells (Fig. 9, C and D). Basal level of Ser phosphorylation of occludin were unaffected by transfection with AS-PP2A or AS-PP1 (Fig. 9E). Ser phosphorylation of occludin was slightly reduced during calcium-induced reassembly of TJ in missense oligo or AS-PP2A-transfected cells, whereas that in AS-PP1-transfected cells was increased (Fig. 9, E and F).

DISCUSSION

Hyperphosphorylation of occludin on Ser and Thr residues in intact epithelium (24–27) and its dephosphorylation during the disruption of TJ by several factors (28, 29) indicates that phosphorylation of occludin on Ser/Thr residues plays a crucial role in maintaining the integrity of epithelial TJ. The present study shows that protein phosphatases PP2A and PP1 directly interact with the C-terminal tail of occludin and negatively regulate the assembly of TJ in Caco-2 cells. Association of PP2A and PP1 with occludin is reduced during the calcium switch-mediated assembly of TJ. The inhibition of PP2A activity or reduced expression of PP2A-Cα accelerates the assembly of TJ by promoting Thr phosphorylation of occludin.

Acceleration of the recovery of TER and the barrier to inulin permeability during calcium switch by PP2A inhibitors such as okadaic acid, fostriecin, and calyculin-A indicates that the endogenous PP2A activity negatively regulates the assembly of TJ. Confocal immunofluorescence microscopy showed that EGTA treatment disrupts the intercellular organization of occludin and ZO-1. Occludin and ZO-1 appear to be redistrib-
uted in vesicular forms in the intracellular compartments. Replacement of calcium gradually reorganized occludin and ZO-1 at the intercellular junctions. Three hours after calcium replacement, the organization of occludin and ZO-1 at the intercellular junctions was partially restored. However, in cells pretreated with fostriecin, the reorganization of occludin and ZO-1 was almost complete. These results indicate that reduced PP2A activity accelerates the assembly of occludin and ZO-1 and, therefore, promotes the assembly of TJ and the development of barrier function. The role of endogenous PP2A in the regulation of the assembly of TJ was further confirmed by selectively reducing the expression of PP2A-Cα by antisense oligonucleotides and siRNA. The recovery of the barrier function and the reassembly of occludin and ZO-1 were much faster in cells transfected with siRNA to PP2A-Cα compared with that in cells transfected with control RNA. These results confirm that endogenous PP2A negatively influences the assembly of TJ in Caco-2 cells.

Although okadaic acid and fostriecin are selective inhibitors of PP2A, calyculin-A is known to equally inhibit PP2A and PP1 activities. Our study showed that calyculin-A also accelerated the assembly of TJ. Therefore, we examined the role of PP1 in the TJ regulation. Antisense oligonucleotides designed against the nucleotide sequence of human PP1α reduced the levels of PP1α without affecting the levels of PP2A-Cα, indicating a selective reduction in the levels of PP1α. The recovery of TER and the barrier to inulin during calcium switch was much faster in cells transfected with antisense oligonucleotides to PP1, suggesting that PP1α also negatively regulates the assembly of TJ. Confocal microscopy showed that the reduced expression of PP1α accelerated the reassembly of occludin and ZO-1 at the intercellular junctions compared with cells transfected with missense oligonucleotides. Therefore, these studies demonstrate that both PP2A and PP1 play a role in the regulation of TJ assembly in Caco-2 cells.

The changes in the level of phosphorylation of occludin on Ser and Thr residues were examined during the EGTA-induced disruption of TJ and calcium-mediated reassembly. The results demonstrate that occludin in the detergent-insoluble fraction undergoes dephosphorylation on Thr residues during the disassembly of TJ; however, phosphorylation of occludin on Ser residues was unaffected. Occludin is rapidly rephosphorylated on Thr residues during reassembly by calcium restoration, whereas phosphorylation of occludin on Ser residues was slightly reduced. The amount of occludin present in the detergent-insoluble fraction remained unchanged during the disassembly or reassembly of TJ, indicating that although occludin and ZO-1 are disrupted into vesicular structures, these structures continue to associate with the actin cytoskeleton.

Previous studies indicated that occludin is phosphorylated predominantly on Ser residues rather than Thr residues in MDCK I cells, and the occludin isolated from detergent-insoluble fraction was found to be phosphorylated exclusively on Ser residues (24). Similarly, another study showed that 62–65-kDa

![Figure 6](image-url)

**Figure 6.** Reduced expression of PP1α accelerates the assembly of occludin and ZO-1. A, Caco-2 cells were transfected with antisense (AS) oligonucleotides AS-PP1-1 or with missense oligonucleotide. Four days after transfection cell monolayers were subjected to calcium switch-mediated TJ assembly. Cell monolayers were fixed before and after EGTA treatment and 3 h after calcium restoration. Fixed cells were stained for occludin and ZO-1 by immunofluorescence methods, and images were collected by confocal microscopy. B and C, densitometric analysis of intracellular and junctional fluorescence for occludin (B) and ZO-1 (C). Values are the mean ± S.E. (n = 6). Asterisks indicate the values that are significantly different from the corresponding values for reassembly (Ca²⁺) in cells transfected with missense (MS) oligonucleotides.
occludin was phosphorylated on both Ser and Thr residues, whereas 71-kDa occludin was phosphorylated exclusively on Ser residues (28). The overall phosphorylation (studied by $^{32}$P incorporation) of both 62–65-kDa and 71-kDa occludins was reduced during disassembly of TJ by prolonged exposure to low calcium medium or treatment with phorbol esters (28). On the contrary, our present study demonstrates that Thr phosphorylation is more important than Ser phosphorylation during the assembly of TJ in Caco-2 cells. One possible explanation for this discrepancy may be the cell type-dependent differences. Similar to our present observation, a recent study demonstrated that phorbol ester-induced disruption of TJ was associated with a rapid decrease in Thr phosphorylation of occludin in LLCK1 cells (30). Alternatively, the difference in the model system may have contributed to the discrepancy in occludin phosphorylation on Ser or Thr residues during reassembly. In the previous studies, MDCK cells were exposed to low calcium medium for 18–24 h to disrupt the TJ. In our present study, to study the role of immediate signaling mechanism, TJ was quickly disrupted by exposure to EGTA for 30 min. Under the present conditions, occludin and ZO-1 were localized in vesicular form, but they continued to associate with the detergent-insoluble fractions. In contrast, disruption of TJ by long-term exposure to low calcium results in dissociation of occludin and ZO-1 from the detergent-insoluble fractions (24, 28).

A previous study showed that PP2A can be co-immunoprecipitated with occludin and ZO-1 in MDCK cells that overexpress PP2A (31). The present study shows that PP2A can be co-immunoprecipitated with occludin in detergent-insoluble fractions of Caco-2 cells, and this co-immunoprecipitation is slightly increased by EGTA treatment but gradually reduced during the calcium-induced reassembly of TJ. The PP2A activity associated with occludin immunocomplexes was also significantly increased by EGTA treatment and decreased during the calcium-induced assembly of TJ. The increased association of PP2A with the TJ protein complex during EGTA treatment and decrease in the association of PP2A during the reassembly may contribute to the rapid changes in Thr phosphorylation of occludin during the disassembly and reassembly of TJ. PP1$\alpha$ was also co-immunoprecipitated with occludin, suggesting that PP1$\alpha$ interacts with the TJ protein complex. Co-immunoprecipitation of PP1$\alpha$ with occludin was also reduced during the reassembly of TJ. Therefore, both PP2A and PP1 are associated with the TJ protein complex, and loss of their interactions with occludin may contribute to rapid phosphorylation of occludin during the calcium-induced reassembly of TJ.

Co-immunoprecipitation of PP2A and PP1 with occludin does not clearly demonstrate if the interaction is direct or if it is mediated by their interaction with other TJ proteins. However, our pairwise binding studies using GST-occludin-C and purified PP2A or PP1 demonstrate that both PP2A and PP1 directly interact with the C-terminal tail of occludin. Occludin is a transmembrane protein, spanning the membrane four times to form two extracellular loops, and the C-terminal and N-terminal regions hang into the intracellular compartment (4). The C-terminal region of occludin interacts with other TJ proteins such as ZO-1, ZO-2, and ZO-3 (7, 23) and with signaling proteins such as PKC$\zeta$ (10), c-Yes (21), c-Src (20), phosphatidylinositol 3-kinase (22), etc.

The regulated interaction of PP2A-Ca and PP1$\alpha$ during the reassembly of TJ was studied by GST pulldown assay using GST-occludin-C and protein extracts prepared from the cells at different stages of EGTA-induced disassembly and calcium-mediated reassembly of TJ. GST-occludin-C pulled down both PP2A and PP1 in extracts from EGTA-treated cells, and this binding was dramatically higher as compared with the binding in control cell extracts. Binding of both PP2A-Ca and PP1$\alpha$ was gradually reduced during the reassembly of TJ. These results are complimentary to the above-described changes in co-immunoprecipitation of PP2A-Ca and PP1$\alpha$ with occludin during the reassembly of TJ, suggesting that the interaction between PP2A-Ca and PP1$\alpha$ with the C-terminal region of occludin may be regulated during the disassembly and reassembly of TJ. Because the total amount of PP2A and PP1 in the Triton-soluble fraction used for the binding assay did not change under different conditions, one can speculate that the change in PP2A and PP1 binding during calcium-induced TJ assembly was caused by changes in their affinity for occludin. The mechanism involved in the change in affinity is not clear. It could be due to modification of PP2A and PP1 subunits by phosphorylation. The binding of PKC$\zeta$ was unaffected during the disassembly.
Protein Phosphatases and Tight Junction Regulation

The mechanism involved in PP2A- and PP1-mediated prevention of TJ assembly is unclear. It is likely that PP2A and PP1 induce dephosphorylation of occludin on Ser/Thr residues. The previous study in MDCK cells suggested that PP2A may inhibit the activity of PKCε, which in turn results in reduced phosphorylation of occludin. To determine a direct role of PP2A and PP1 in dephosphorylation of occludin, we evaluated the effect of PP2A and PP1 on dephosphorylation of p-Thr and p-Ser in occludin isolated from the detergent-insoluble fraction of Caco-2 cells. Rapid dephosphorylation of p-Thr on occludin indicates that PP2A can directly dephosphorylate occludin on Thr residues. Interestingly, PP2A showed no significant dephosphorylation of p-Ser on occludin. On the contrary, PP1-dephosphorylated occludin on p-Ser residues, but showed no significant effect on p-Thr residues at 10 min. These results demonstrate that PP2A and PP1 may have distinct roles in the regulation of Ser/Thr phosphorylation of occludin. The role of PP2A in occludin dephosphorylation was further confirmed by evaluating the Thr phosphorylation of occludin during calcium switch induced TJ assembly in Caco-2 cells that were transfected with missense oligo or antisense oligonucleotide, AS-PP2A-3 or AS-PP1-3. Transfection of antisense oligonucleotides, AS-PP2A-3 and AS-PP1-3 increased the basal level of occludin phosphorylation on Thr residues to some extent and partially prevented EGTA-induced dephosphorylation. AS-PP2A enhanced the Thr phosphorylation of occludin during calcium-induced reassembly more rapidly compared with that in missense oligo-transfected and AS-PP1-3-transfected cells. Reduced expression of PP1 by AS-PP1-3 did not significantly influence the Thr phosphorylation of occludin during reassembly, suggesting that PP2A may be more important in regulation of Thr phosphorylation of occludin.

Although PP1-dephosphorylated occludin on Ser residues in in vitro experiments, the Ser phosphorylation of occludin was only
In summary, this study demonstrates that both PP2A and PP1 negatively regulate the assembly of TJ by interacting with the TJ protein complex and modulating the phosphorylation status of occludin on Thr and/or Ser residues.

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FIGURE 9. PP2A and PP1 dephosphorylate occludin on Ser/Thr residues. A, anti-occludin immunocomplexes were prepared from Triton-insoluble fractions of Caco-2 cells and incubated with PP2A, PP1, or the formulation buffer (Carrier) for 10 min. Samples were then immunoblotted for p-Thr, p-Ser, and occludin. Untreated sample represents the immunocomplex that was not incubated. B, densitometric analysis of p-Thr and p-Ser bands corresponding to occludin in experiments shown in panel A. Values were normalized to the density of corresponding band for untreated group and are presented as the mean ± S.E. (n = 3). Asterisks indicate the values that are significantly different from the corresponding values for samples incubated in the absence of PP2 or PP1 (Untreated). C, calcium switch experiment was performed in cells transfected with missense oligo or antisense oligos, AS-PP2A-3, and AS-PP1-3. D, detergent-insoluble fractions were prepared at various stages of TJ assembly, and p-Thr was immunoprecipitated (IP) followed by immunoblot analysis for occludin. D, densitometric analysis of occludin bands in experiments is shown in panel C. Values were normalized to the density of corresponding band for the untreated group (Control) and are presented as the mean ± S.E. (n = 3). Asterisks indicate the values that are significantly different from the corresponding values for missense oligo or AS-PP1-transfected cells. E, calcium switch experiment was performed in cells transfected with missense oligo or antisense oligo, AS-PP2A-2 + AS-PP2A-3 or AS-PP1-1. D, detergent-insoluble fractions were prepared at various stages of TJ assembly, and occludin was immunoprecipitated followed by immunoblot analysis for occludin. F, densitometric analysis of occludin in experiments is shown in panel E. Values were normalized to the density of the corresponding band for untreated group (Control) and are presented as the mean ± S.E. (n = 3). Asterisks indicate the values that are significantly different from corresponding values for missense oligo and AS-PP2A-transfected cells.