Phosphorylation of Tyr-398 and Tyr-402 in Occludin Prevents Its Interaction with ZO-1 and Destabilizes Its Assembly at the Tight Junctions

Bertha C. Elias1, Takuya Suzuki1, Ankur Seth1, Francesco Giorgianni1, Gautam Kale2, Le Shen2, Jerrold R. Turner2, Anjaparavanda Naren1, Dominic M. Desiderio3, and Radhakrishna Rao1,2

From the 1Department of Physiology, 3Charles B. Stout Neuroscience Mass Spectrometry Laboratory, University of Tennessee Health Science Center, Memphis, Tennessee 38163 and the 2Department of Pathology, University of Chicago, Chicago Illinois 60637

Occludin is phosphorylated on tyrosine residues during the oxidative stress-induced disruption of tight junction, and in vitro phosphorylation of occludin by c-Src attenuates its binding to ZO-1. To determine the phosphorylation sites, mass spectrometric analyses of C-terminal domain of occludin identified Tyr-379 and Tyr-383 in chicken occludin as the phosphorylation sites, which are located in a highly conserved sequence of occludin, YETDYTT; Tyr-398 and Tyr-402 are the corresponding residues in human occludin. Deletion of YETDYTT motif abolished the c-Src-mediated phosphorylation of occludin and the regulation of ZO-1 binding. Y398A and Y402A mutations in human occludin also abolished the c-Src-mediated phosphorylation and regulation of ZO-1 binding. Y398D/Y402D mutation resulted in a dramatic reduction in ZO-1 binding even in the absence of c-Src. Similar to wild type occludin, its Y398A/Y402A mutant was localized at the plasma membrane and cell-cell contact sites in Rat-1 cells. However, Y398D/Y402D mutants of occludin failed to localize to the cell-cell contacts. Calcium-induced reassembly of Y398D/Y402D mutant occludin in Madin-Darby canine kidney cells was significantly delayed compared with that of wild type occludin or its T398A/T402A mutant. Furthermore, expression of Y398D/Y402D mutant of occludin sensitized MDCK cells for hydrogen peroxide-induced barrier disruption. This study reveals a unique motif in the occludin sequence that is involved in the regulation of ZO-1 binding by reversible phosphorylation of specific Tyr residues.

Epithelial tight junctions (TJs) form a selective barrier to the diffusion of toxins, allergens, and pathogens from the external environment into the tissues in the gastrointestinal tract, lung, liver, and kidney (1). Disruption of TJs is associated with gastrointestinal diseases such as inflammatory bowel disease, celiac disease, infectious enterocolitis, and colon cancer (2–4) as well as in diseases of liver and kidney (5, 6). Numerous inflammatory mediators such as tumor necrosis factor α, interferon γ, and oxidative stress (7–12) are known to disrupt the epithelial TJs and the barrier function. Several studies have indicated that hydrogen peroxide disrupts the TJs in intestinal epithelial by a tyrosine kinase-dependent mechanism (11, 12).

Four types of integral proteins, occludin, claudins, junctional adhesion molecules, and tricellulin are associated with TJs. Occludin, claudins, and tricellulin are tetraspan proteins, and their extracellular domains interact with homotypic domains of the adjacent cells (1, 2, 13). The intracellular domains of these proteins interact with a variety of soluble proteins such as ZO-1, ZO-2, ZO-3, 7H6, cingulin, and symplekin (14–23); this protein complex interacts with the perijunctional actomyosin ring. The interactions among TJ proteins are essential for the assembly and the maintenance of TJs. Therefore, regulation of the interactions among TJ proteins may regulate the TJ integrity. A significant body of evidence indicates that numerous signaling molecules are associated with the TJs. Protein kinases and protein phosphatases such as protein kinase Cζ (PKCζ), PKCα (24), c-Src (25), c-Yes (26, 27), mitogen-activated protein kinase (28), PP2A, and PP1 (29) interact with TJs, indicating that TJs are dynamically regulated by intracellular signal transduction involving protein phosphorylation. Additionally, other signaling molecules such as calcium (30), phosphatidylinositol 3-kinase (31), Rho (32), and Rac (33) are involved in the regulation of TJs.

Occludin, a ~65-kDa protein, has been well characterized to be assembled into the TJs. Although occludin knock-out mice showed the formation of intact TJs in different epithelia (34), numerous studies have emphasized that it plays an important role in the regulation of TJ integrity. Occludin spans the membrane four times to form two extracellular loops and one intracellular loop, and the N-terminal and C-terminal domains hang into the intracellular compartment (35–37). In epithelium with intact TJs, occludin is highly phosphorylated on Ser and Thr residues (38), whereas Tyr phosphorylation is undetectable. However, the disruption of TJs in Caco-2 cell monolayers by oxidative stress and acetaldehyde leads to Tyr phosphorylation of occludin; the tyrosine kinase inhibitors attenuate the disruption of TJs (39, 40). Furthermore, a previous in vitro study dem-
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onstrated that Tyr phosphorylation of the C-terminal domain of occludin leads to the loss of its interaction with ZO-1 and ZO-3 (25).

In the present study we identified the Tyr residues in occludin that are phosphorylated by c-Src and determined their role in regulated interaction between occludin and ZO-1 and its assembly into the TJs. Results show that 1) Tyr-379 and Tyr-383 in chicken occludin and Tyr-398 and Tyr-402 in human occludin are the exclusive sites of phosphorylation by c-Src, and these Tyr residues are located in a highly conserved sequence of occludin, YET-DYT, 2) deletion of YEDTYTT or point mutation of Tyr-398 and Tyr-402 in human occludin attenuates the phosphorylation-dependent regulation of ZO-1 binding, 3) Y398D/Y402D mutation of human occludin leads to loss of ZO-1 binding and prevents its translocation to the plasma membrane and cell-cell contact sites in Rat-1 cells, 4) Y398D/Y402D mutation of occludin delays its assembly into the intercellular junctions during the calcium-induced assembly of TJs, and 5) expression of Y398D/Y402D mutant occludin sensitizes cell monolayers for hydrogen peroxide-induced disruption of barrier function.

EXPERIMENTAL PROCEDURES

Chemicals

Cell culture reagents and supplies, G418, Lipofectamine-R, and Plus reagent were purchased from Invitrogen. FuGENE was purchased from Roche Diagnostics, and glutathione (GSH), leupeptin, aprotonin, pepstatin A, phenylmethylsulfonyl fluoride, protease inhibitor mixture, GSH-agarose, Triton X-100, and vanadate were purchased from Sigma. The QuikChange XL site-directed mutagenesis kit was from Stratagene, La Jolla, CA. Active c-Src (recombinant protein) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). All other chemicals were of analytical grade and were purchased either from Sigma or Fisher.

Plasmids and Recombinant Proteins

cDNA for the C-terminal tail of chicken occludin (amino acids 358–504) was a kind gift from Dr. James Anderson (University of North Carolina, Chapel Hill, NC); this was used to amplify and insert into pGEX2T vector. The C-terminal tail of human occludin 378–522 was amplified from a full-length cDNA for the C-terminal tail of chicken occludin (amino acids 358–504) was a kind gift from Dr. James Anderson (University of North Carolina, Chapel Hill, NC). The amplified product was inserted into the pGEX2T vector to create GST-cOcl-CWT. The C-terminal domain of occludin was cloned into the pGEX2T vector using the manufacturer’s protocol. Peptides extracted from the ZipTip were subjected to phosphopeptide extraction.

Mass Spectrometric Analysis

Tryptic Digestion—GST-cOcl-CWT, tyrosine-phosphorylated by c-Src, was suspended in 10% acetonitrile in 10 mM ammonium bicarbonate, pH 8.5, and incubated at 37 °C overnight with TPCK-treated trypsin (enzyme to substrate molar ratio of 1:10). The digest was passed through 0.45-μm filters. Clear supernatant was lyophilized to dryness. Air-dried samples were equilibrated in an aqueous solution containing 0.1% trifluoroacetic acid and desalted by passing through C-18 ZipTip (Millipore, Bedford, MA) using the manufacturer’s protocol. Peptides extracted from the ZipTip were subjected to phosphopeptide extraction.

3.5) and eluted in 50 mM ammonium bicarbonate at pH 10. Phosphopeptide extracts were then subjected to MALDI and LC/MS/MS analysis.

MALDI-TOF—Phosphopeptide extracts were dried under vacuum and reconstituted in 2 μl of matrix (α-cyano-4-hydroxycinnamic acid) and spotted for crystallization. Crystals were analyzed by MALDI-TOF using Voyager Biospectrometry work station DE (delayed extraction technology) (Perseptive Biosystems Inc., Framingham, MA) and Data Explorer (Perseptive Biosystems). A Prescriptive Biosystems MALDI time-of-flight instrument incorporating a nitrogen laser (Laser Science, Newton, MA) was used to obtain MALDI mass spectra. Samples solubilized in 85% acetic acid and mixed (1:3 v/v) with α-cyano-4-hydroxycinnamic acid matrix were spotted in 1-μl aliquots and air-dried. Typically, 100–250 laser shots were used to obtain one mass spectrum. Mass scale was calibrated with peptide internal standards.

LC/MS/MS Analysis—Sequence analysis of tryptic peptides was performed by injecting 3 μl of the ZipTip-purified sample onto a capillary C-18 LC column on-line with a Finnigan LCQ DECRA (Thermoquest, San Jose, CA) ion-trap mass analyzer that is equipped with a nanoelectrospray ionization source. The capillary C-18 column was prepared in-house using New Objective Pico Frit (360-μm outer diameter, 75-μm inner diameter, 15-μm tip, 10.4-cm length) and Magic C18AQ packing material (5-μm beads, 200 Å pores). The peptides were fractionated using 0.1% formic acid in water as solvent A and 90% acetonitrile as solvent B. The acquired spectra were visualized using Qual-browser in the X-Calibur software suite. Raw

| Chicken occludin  | Predicted mutant | Forward primer sequence 5′–3′ |
|-------------------|------------------|-------------------------------|
| Δ378–385          | Y398A            | GMAACAGACAGACTCACCTGCAGACACTACATGCG |
| Y379A             | Y379/383         | GGATGAGTCCCAGGTAGACCGAGGACCGAC |
| Y379/383          | Y383A            | GTAGACAGACACAGAGGACACACG |
| Y379/383          | Y383/383         | GTAGACAGACACAGAGGACACACG |
| Chicken occludin  | Y398A            | GMAACAGACAGACTCACCTGCAGACACTACATGCG |
| Y379A             | Y379/383         | GGATGAGTCCCAGGTAGACCGAGGACCGAC |
| Y379/383          | Y383A            | GTAGACAGACACAGAGGACACACG |
| Y379/383          | Y383/383         | GTAGACAGACACAGAGGACACACG |
| Human occludin    | Y398A            | GMAACAGACAGACTCACCTGCAGACACTACATGCG |
| Y379A             | Y379/383         | GGATGAGTCCCAGGTAGACCGAGGACCGAC |
| Y379/383          | Y383A            | GTAGACAGACACAGAGGACACACG |
| Y379/383          | Y383/383         | GTAGACAGACACAGAGGACACACG |
| Human occludin    | Y398A            | GMAACAGACAGACTCACCTGCAGACACTACATGCG |
| Y379A             | Y379/383         | GGATGAGTCCCAGGTAGACCGAGGACCGAC |
| Y379/383          | Y383A            | GTAGACAGACACAGAGGACACACG |
| Y379/383          | Y383/383         | GTAGACAGACACAGAGGACACACG |

TABLE 1

Sequences of primers used to generate various mutations

Sequences in bold substitute tyrosine residues in the wild type occludin.

- GST-cOcl-CWT, tyrosine-phosphorylated by c-Src, was suspended in 10% acetonitrile in 10 mM ammonium bicarbonate, pH 8.5, and incubated at 37 °C overnight with TPCK-treated trypsin (enzyme to substrate molar ratio of 1:10). The...
data thus obtained was analyzed against a protein data base generated from Swissprot using the Sequest software suite (Sequest Technologies Inc., Lisle, IL).

Antibodies—Mouse monoclonal anti-ZO-1, rabbit polyclonal anti-ZO-1, and rabbit polyclonal anti-ZO-3 were purchased from Zymed Laboratories Inc., and AlexaFluor 488-conjugated antimouse IgG antibody was from Molecular Probes (Eugene, OR). Anti-phosphotyrosine antibody conjugated with horseradish peroxidase (HRP) was from BD Transduction Laboratories. HRP-conjugated mouse monoclonal anti-GST antibody was from Upstate Biotechnology. Cy3-conjugated anti-rabbit IgG, HRP-conjugated anti-mouse IgG, and HRP-conjugated anti-rabbit IgG antibodies were purchased from Sigma.

Cell Culture and Transfection

Caco-2, Rat-1, and MDCK cells were cultured in Dulbecco’s modified Eagle’s medium from Invitrogen and supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 2 mM glutamine as per the ATCC guidelines. MDCK cells were seeded on 6-well plates a day before transfection to achieve 50–60% confluency. The cells were transfected using 1 μl of antibiotic-free Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1 μg of DNA plasmid (empty vector pEGFP or vector carrying hOclWT or its mutants), 1 μl of Plus reagent, and 3 μl of Lipofectamine-R for each well. After 20 h, the cell monolayers were trypsinized and seeded onto 100-mm plates. The cells were subjected to G418 selection (0.7 mg/ml) for 2 weeks. Resistant cells were sorted to obtain only GFP-expressing cells by fluorescence-activated cell sorter. Cells were maintained in the medium that was supplemented with 0.3 mg/ml G418. Rat-1 cells were transfected using FuGENE reagent as per the manufacturer’s protocol, and the GFP-positive cells were sorted by fluorescence-activated cell sorter. Stably transfected cells were selected using G418 as described above.

Immunofluorescence Microscopy

Cell monolayers (12-mm transwells) were washed with phosphate-buffered saline 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 8.0, containing 150 mM NaCl and 0.5% Tween 20) and incubated for 1 h with primary antibodies (rabbit polyclonal anti-ZO-1 and mouse monoclonal anti-GFP) followed by incubation for 1 h with secondary antibodies (Cy3-conjugated anti-rabbit IgG and AlexaFluor 488-conjugated anti-mouse IgG). The fluorescence was visualized using a Zeiss LSM 5 laser scanning confocal microscope, and images from Z-series sections (1 μm) were collected by using Zeiss LSM 5 Pascal 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).

GST Pulldown Assay

To determine the interaction of occludin with ZO-1 and ZO-3, GST-hOcl-C GST-cOcl-C (2.5–10 μg) was incubated with Caco-2 whole cell extract made in phosphate-buffered saline containing 0.2% Triton X-100, 1 mM sodium vanadate, and 10 mM sodium fluoride for 16 h at 4 °C on an inverter. GST-occludin-C (GST-conjugated C-terminal tail of occludin) was pulled down with 20 μl of 50% GSH-agarose slurry at 4 °C for 1 h. The amounts of ZO-1 and ZO-3 bound to GSH-agarose were determined by immunoblot analysis. Nonspecific binding was determined by carrying out the binding with GST.

Immunoblot and Densitometric Analysis

Proteins were separated by 7% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blotted for ZO-1, ZO-3, and p-Tyr 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 ECL chemiluminescence method (Amersham Biosciences). Quantitation was performed by densitometric analysis of specific bands on immunoblots by using the software, Image J.

Hydrogen Peroxide Treatment and Paracellular Permeability

MDCK cell monolayers that stably express GFP-hOclWT, GFP-hOclY398A/Y402A, or GFP-hOclY398D/Y402D were exposed to varying concentrations (20–2500 μM) of hydrogen peroxide for 2 h, and paracellular permeability was evaluated by measuring the unidirectional flux of inulin as described before (11).

TJ Assembly by Calcium Switch

MDCK cell monolayers that stably express GFP-hOclWT, GFP-hOclY398A/Y402A, or GFP-hOclY398D/Y402D, were incubated overnight with low calcium medium followed by calcium replacement as described before (29). TJ assembly was evaluated by measuring transepithelial electrical resistance, inulin permeability, and confocal microscopy.

Immunoprecipitation

GFP was immunoprecipitated from cells under native or denatured conditions as described before (29). Anti-GFP immunocomplexes at native conditions were immunoblotted for ZO-1, whereas complexes under denatured conditions were immunoblotted for p-Tyr.

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

Tyr-379 and Tyr-383 in Chicken Occludin Are the Sites of Phosphorylation by c-Src—A previous study showed that Tyr phosphorylation of occludin C-terminal domain by c-Src resulted in the loss of its interaction with ZO-1 (25). In the present study we identified the phosphorylation sites in occludin.
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A C-terminal domain by mass spectrometric analysis. GST-fused C-terminal region (150 amino acids) of chicken occludin (GST-cOcl) was prepared and phosphorylated by incubation with c-Src and ATP. Tyr-phosphorylated GST-cOcl-C was digested with trypsin. Generation of five different tryptic peptides containing Tyr residues was predicted (Fig. 1A); the mass of these peptides was expected to increase by 80 Da with phosphorylation of each Tyr residue. MALDI mass spectrometric analysis of the phosphopeptide extracts from the tryptic digest detected several phosphopeptides with masses slightly deviated from the predicted mass analysis (Fig. 1B). Fig. 1C shows the ion fragmentation spectrum from LC/MS/MS analysis of the phosphopeptide with a mass of 2918 Da. The sequences of peptides as determined by LC/MS/MS analyses of the phosphopeptide extract are summarized in Fig. 1D. Three different Tyr-phosphorylated peptides were identified. All three peptides were identified as the derivatives of tryptic peptide, P1 with single or double phosphorylation of Tyr residues. These results determine that two Tyr residues in occludin C-terminal region corresponding to the sequence of P1 were singly or doubly phosphorylated. These two Tyr residues correspond to Tyr-379 and Tyr-383 in chicken occludin.

Sequence alignment of occludin from different species (Fig. 2A) demonstrated that Tyr-379 and Tyr-383 are located in a highly conserved sequence of occludin (YETDYTT) and that Tyr-398 and Tyr-402 are the corresponding Tyr residues in human occludin. Therefore, we induced mutations in chicken and human occludin C-terminal region (cOcl-C and hOcl-C). The YETDYTT was deleted or the tyrosine residues in this region were subjected to point mutation in cOcl-C and hOcl-C (Fig. 2B) and inserted into pGEX2T vector to generate GST-fused mutant proteins. Tyr-398 and Tyr-402 in full-length human occludin in pEGFP vector were mutated to phenylalanine or aspartic acid and expressed in Rat-1 or MDCK cells as GFP fusion proteins.
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Deletion of YEDYTT in Chicken Occludin C-terminal Region Prevents Phosphorylation and Attenuates Regulation of ZO-1 Binding—The ability of GST-cOcl-C or GST-cOcl-C(Y378–385) to bind ZO-1 was evaluated by pulldown assay. Both GST-cOcl-C and GST-cOcl-C(Y378–385) showed dose-dependent binding to ZO-1 and ZO-3 (Fig. 3A). However, ZO-1 binding to GST-cOcl-C(Y378–385) was low at higher doses compared with the ZO-1 binding to GST-cOcl-C under similar conditions (Fig. 3B). Incubation with c-Src in the presence of ATP induced Tyr phosphorylation of GST-cOcl-C, whereas no phosphorylation was detected in GST-cOcl-C(Y378–385) (Fig. 3C). Incubation of GST-cOcl-C with c-Src in the presence of ATP significantly reduced ZO-1 binding (Fig. 3C, D), whereas ZO-1 binding was similar for GST-cOcl-C(Y378–385) that was incubated with c-Src in the presence or absence of ATP.

Y379F and Y383F Mutation of Chicken Occludin Attenuates Its Phosphorylation and Regulation of ZO-1 Binding—GST pulldown assay for ZO-1 binding showed that GST-cOcl-CWT, GST-cOcl-CY379F, and GST-cOcl-CY383F bind ZO-1 and ZO-3 in a dose-dependent manner (Fig. 4A). Incubation with c-Src in the presence of ATP showed a partial phosphorylation of single mutants, GST-cOcl-CY379F and GST-cOcl-CY383F, whereas phosphorylation was undetectable in the double mutant, GST-cOcl-CY379F/Y383F (Fig. 4B). ZO-1 binding was not significantly different among unphosphorylated occludin (Fig. 4, A and B), except that ZO-1 binding of GST-cOcl-CY379F was slightly greater than that of GST-cOcl-CWT. Incubation in the presence of ATP and c-Src resulted in a reduced ZO-1 binding by GST-cOcl-CWT and GST-cOcl-CY379F (Fig. 4, B and C). However, incubation with c-Src in the presence of ATP did not alter the ZO-1 binding by GST-cOcl-CY383F and GST-cOcl-CY379F/Y383F (Fig. 4, B and C). GST-cOcl-CY379D and GST-cOcl-CY383D mutation in Chicken Occludin Attenuates Its Binding to ZO-1—GST pulldown assay showed a dose-dependent binding of GST-cOcl-CWT, GST-cOcl-CY379D, GST-cOcl-CY383D, and GST-cOcl-CY379D/Y383D to ZO-1 and ZO-3 (Fig. 5A). However, the ZO-1 binding to GST-cOcl-CY379D and GST-cOcl-CY383D was significantly low as compared with ZO-1 binding to GST-cOcl-CWT (Fig. 5A). ZO-3 binding to GST-cOcl-CY383D and GST-cOcl-CY379D/Y383D were significantly low compared with ZO-3 binding to GST-cOcl-CWT, whereas ZO-3 binding to GST-cOcl-CY379D was significantly greater. c-Src-induced Tyr-phosphorylation of GST-cOcl-CY379D and GST-cOcl-CY383D was low compared with Tyr phosphorylation of GST-cOcl-CWT, and the Tyr-phosphorylation was absent in GST-cOcl-CY379D/Y383D double mutant (Fig. 5B). Incubation with c-Src in the presence of ATP resulted in reduced ZO-1 binding to GST-cOcl-CWT and GST-cOcl-CY379D (Fig. 5, B and C). However, incubation of GST-cOcl-CY383D and GST-cOcl-CY379D/Y383D with c-Src in the presence of ATP did not alter ZO-1 binding (Fig. 5, B and C).

Mutation of Tyr-398 and Tyr-402 in Human Occludin Prevents Phosphorylation and Alters ZO-1 Binding—The sequence analysis indicated that Tyr-398 and Tyr-402 are the residues in human occludin that correspond to Tyr-379 and Tyr-383 in chicken occludin. Therefore, we mutated Tyr-398 and Tyr-402 in hOcl-C. Similar to GST-cOcl-CWT, incubation with c-Src in the presence of ATP induced Tyr phosphorylation of GST-hOcl-CWT, whereas phosphorylation was undetectable in GST-hOcl-CY389F/Y402D and GST-hOcl-CY398D/Y402D mutants (Fig. 6A). GST pulldown assay showed that GST-hOcl-CWT binds to ZO-1, and this binding was reduced by incubation with c-Src in the presence of ATP. GST-hOcl-CY398D/Y402D showed only a trace amount.

Figure 1. Identification of phosphorylation sites in occludin. GST-cOcl-CWT was phosphorylated by incubation with c-Src in the presence of ATP and subjected to digestion by trypsin. A, predicted peptide fragments (P1–P5) that contained one or two Tyr residues generated by tryptic digestion of GST-cOcl-CWT. The position of peptides in the sequence of occludin and their predicted mass are listed. B, phosphopeptides extracted from tryptic digests were subjected to mass analyses by MALDI mass spectrometry. P1(p–p) represents P1 peptide with two phospho-Tyr residues and one additional Arg residue as a result of misdigestion by trypsin, and P1(2p) represents the P1 peptide with two phospho-Tyr residues. C, the ion fragmentation spectrum for P1(2p) as obtained by LC/MS/MS analyses. D, the summary of peptides sequenced by LC/MS/MS analysis. Peptides corresponding to these are listed in panel A, and their phosphorylation states are provided. Asterisks represent the phosphorylated Tyr residues.

Figure 2. Mutation of occludin. A, the alignment of amino acid sequence of occludin from different mammalian species indicate that the Tyr residues phosphorylated by c-Src correspond to Tyr-379 and Tyr-383 in chicken occludin and Tyr-398 and Tyr-402 in human occludin. B, list of the mutations induced in the C-terminal region of chicken and human occludin.

N. musculus

R. norvegicus

R. aequinocutis

C. familiaris

G. gallus

$$\begin{align*}
\text{A} & \quad \text{N. musculus} \\
\text{B} & \quad \text{R. norvegicus} \\
\text{C} & \quad \text{R. aequinocutis} \\
\text{D} & \quad \text{C. familiaris} \\
\text{E} & \quad \text{G. gallus}
\end{align*}$$

**FIGURE 2.** Mutation of occludin. A, the alignment of amino acid sequence of occludin from different mammalian species indicate that the Tyr residues phosphorylated by c-Src correspond to Tyr-379 and Tyr-383 in chicken occludin and Tyr-398 and Tyr-402 in human occludin. B, list of the mutations induced in the C-terminal region of chicken and human occludin.
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Binding to GST (3 μg) was performed as a control. The labels p44, p42, and p22 correspond to the molecular weight of GST-cOcl-CWT, GST-cOcl-CΔ378–385, and GST, respectively. B, densitometric analysis of ZO-1 and ZO-3 bands from three different experiments described in panel B. In each experiment, ZO-1 band density for GST-cOcl-CWT incubated without ATP was designated to 100, and corresponding bands in other groups were normalized as a percent of that number. Values are the mean ± S.E. (n = 3). The asterisk indicates the value that is significantly (p < 0.05) different from corresponding value for −ATP group.

of ZO-1 binding. ZO-1 binding to GST-hOclCΔ378–385 was lower than GST-hOcl-CWT; however, the ZO-1 binding was not further reduced by incubation with c-Src in the presence of ATP (Fig. 6, A and C). Unlike reduced binding to GST-hOcl-CΔ378–385, the ZO-1 binding to GST-hOclCΔ378–385 was similar to that of GST-hOcl-CWT (Fig. 6B). Once again, GST-hOclCΔ378–385 showed no Tyr phosphorylation or regulation of ZO-1 binding when incubated with c-Src in the presence of ATP (Fig. 6B). Densitometric analysis (Fig. 6C) confirmed that ZO-1 binding to GST-hOcl-CΔ378–385 and GST-hOclCΔ378–385 was significantly lower than that of GST-hOcl-CWT, whereas the binding to GST-hOclCΔ378–385 was similar to that of GST-hOcl-CWT. Furthermore, incubation with c-Src in the presence of ATP significantly reduced ZO-1 binding to GST-hOcl-CWT but not to GST-hOcl-CΔ378–385, GST-hOclCΔ378–385, or GST-hOclCΔ378–385 when compared with corresponding ZO-1 binding in the absence of ATP.

Y398D and Y402D Mutation of Human Occludin Prevents Its Localization at Plasma Membrane and Cell-Cell Contact Sites in Rat-1 Cells—The regulation of ZO-1 binding by Tyr-398 and Tyr-402 raised the question of whether phosphorylation of Tyr-398 and/or Tyr-402 of occludin affects its localization at the TJs. To determine the effect of mutation of

Y398D and Y402D Mutation of Occludin Delays Its Assembly at the TJs and Sensitizes MDCK Cells for TJ Disruption by Hydrogen Peroxide—Unlike Rat-1 cells, in MDCK cell monolayers, GFP-hOclCΔ378–385 was expressed at the cell-cell contact sites, which was associated with the redistribution of ZO-1 at the cell-cell contact and the plasma membrane. In contrast, GFP-hOclCΔ378–385 was localized exclusively at the intracellular compartment with no trace of distribution at the plasma membrane or cell-cell contact sites (Fig. 7).

Tyr-398 and Tyr-402 on the distribution of occludin at the plasma membrane and the intercellular junctions, we transfected Rat-1 cells (occludin null) with GFP-hOcl, GFP-hOclCΔ378–385, and visualized the cells by confocal microscopy. GFP-hOclCWT and GFP-hOclCΔ378–385 were localized to both the plasma membrane and the intracellular compartment (Fig. 7). A greater level of occludin was found at the cell-cell contact sites, which was associated with the redistribution of ZO-1 at the cell-cell contact and the plasma membrane. In contrast, GFP-hOclCΔ378–385 was localized exclusively at the intracellular compartment with no trace of distribution at the plasma membrane or cell-cell contact sites (Fig. 7).
Fig. 9. ZO-1 distribution in hydrogen peroxide-treated cell monolayers paralleled the distribution of GFP. Tyr-398- and Tyr-402-phosphorylated Occludin Results in Loss of Interaction with ZO-1 in MDCK Cell Monolayers—To confirm the H$_2$O$_2$-induced phosphorylation of occludin in the cell, we evaluated H$_2$O$_2$-induced Tyr-phosphorylation of GFP-hOcl$_{WT}$, GFP-hOcl$_{Y398A/Y402A}$, and GFP-hOcl$_{Y398D/Y402D}$ in MDCK cell monolayers. Immunoprecipitation of GFP followed by immunoblot analysis in H$_2$O$_2$-treated cells expressing GFP-hOcl$_{WT}$ indicated that occludin was phosphorylated on Tyr residues (Fig. 9C). However, GFP-hOcl$_{Y398A/Y402A}$ and GFP-hOcl$_{Y398D/Y402D}$ were not Tyr-phosphorylated in H$_2$O$_2$-treated cells.

Coimmunoprecipitation studies (Fig. 9D) indicated that ZO-1 coimmunoprecipitated with GFP-Ocl$_{WT}$ and GFP-hOcl$_{Y398A/Y402A}$ whereas ZO-1 failed to co-immunoprecipitate with GFP-hOcl$_{Y398D/Y402D}$, indicating that phosphorylation of Tyr-398 and Tyr-402 results in loss of interaction with ZO-1 in MDCK cells.

DISCUSSION

A significant body of evidence suggests that Tyr phosphorylation of TJ proteins may play an important role in the regulation of epithelial TJs. Occludin is highly phosphorylated on Ser and Thr residues in the intact epithelium, and Tyr phosphorylation is undetectable (38). Previous studies, however, demonstrated that occludin undergoes Tyr phosphorylation during the disruption of TJs by hydrogen peroxide (11, 39). Furthermore, a recent in vitro study demonstrated that Tyr phosphorylation of the C-terminal region of occludin reduces its ability to interact with ZO-1, a TJ plaque protein (25). In the present study we identified the Tyr phosphorylation sites in the C-terminal region of occludin and demonstrated their role in regulation of ZO-1 binding.

The Tyr phosphorylation sites in the C-terminal region of chicken occludin were determined by mass spectrometric analysis of phospho-occludin. Mass analysis of phosphopeptide extracts from tryptic digests detected the presence of one phosphopeptide corresponding to the predicted peptide fragment of chicken occludin (amino acids 371–393) with phospho-Tyr residues. There are two Tyr residues within this sequence (Tyr-379 and 383), which were found to be phosphorylated as the mass of this peptide was 160 daltons greater than the predicted mass value. Another phosphopeptide detected by MALDI showed a molecular mass of 2915.7, which is 156 daltons greater than the predicted mass of the monophosphate peptide fragment. LC/MS/MS analysis determined that this peptide corresponds to the sequence 370–383 of chicken occludin.
of chicken occludin with an extra Arg (Arg-370) at the N-terminus compared with the predicted 371–393 fragment. This is possibly caused by a misdigestion by trypsin due to the presence of sequential Arg residues in this region of the occludin sequence. LC/MS/MS analysis also detected two types of 2915.2-dalton peptides; one in which Tyr-379 was phosphorylated and another in which Tyr-383 was phosphorylated. Therefore, the sequences of all three phosphopeptides identified in the study demonstrate that Tyr-379 and Tyr-383 are the phosphorylation sites in chicken occludin; Tyr-398 and Tyr-402 are the corresponding tyrosines in human occludin. These two tyrosines are located in a highly conserved sequence of occludin HYETDYTT. BLAST analysis of this sequence demonstrated that this is a unique motif that is not present in other proteins, including claudins.

Deletion of HYETDYTT (378–385) from chicken occludin abrogated c-Src-induced Tyr-phosphorylation of the occludin C-terminal region, confirming the mass spectrometric data that Tyr-379 and Tyr-383 are the phosphorylation sites in chicken occludin. Point mutation of Tyr-379 or Tyr-383 to phenylalanine resulted in a partial decrease in c-Src-induced Tyr-phosphorylation. Decrease in Tyr phosphorylation was greater in Y383F mutants compared with that in Y379F mutants, sug-
gesting that Tyr-383 is a preferred phosphorylation site. Double mutation of Tyr-379 and Tyr-383 abolished c-Src-induced Tyr phosphorylation. Similarly, mutation of Tyr-398 and Tyr-402 in the C-terminal region of human occludin also abrogated the c-Src-induced Tyr phosphorylation. Previous studies showed that c-Src plays an important role in hydrogen peroxide-induced disruption of TJs and barrier dysfunction in Caco-2 cell monolayers (11). Expression of inactive c-Src significantly reduced hydrogen peroxide-induced Tyr-phosphorylation of occludin and TJ disruption, suggesting that c-Src-induced Tyr phosphorylation may be involved in this process. The present study determined the role of phosphorylation of specific Tyr residues in the C-terminal region of occludin in the regulation of its interaction with ZO-1. GST pulldown assays demonstrated that C-terminal regions of both chicken and human occludin bind to ZO-1. Deletion of the sequence HYET-DYTT in chicken occludin resulted in a significant reduction in binding to ZO-1 at higher concentrations of occludin; however, at low concentrations the deletion mutant bound to ZO-1 at a similar level to that of the wild type occludin. When wild type occludin C-terminal domain was incubated with c-Src and ATP, there was a significant reduction in ZO-1 binding; however, this was not observed with the deletion mutant, indicating that the phosphorylation of Tyr-379 and Tyr-383 is important in the regulation of interaction between occludin and ZO-1. This was confirmed by point mutations of Tyr-379 and -383. Y379F mutation partially reduced c-Src-induced regulation of ZO-1 binding, whereas Y383F or Y379F/Y383F mutation completely attenuated c-Src-induced regulation of ZO-1 binding. Similarly, Y398F/Y402F mutation in human occludin attenuated c-Src-induced regulation of ZO-1 binding. However, Y398F/Y402F mutation by itself resulted in significant reduction in ZO-1 binding. On the other hand, Y398A/Y402A mutation did not affect ZO-1 binding in the absence or presence of c-Src. Therefore, the results of this study demonstrate that Tyr-398 and Tyr-402 are important in regulation of ZO-1 binding by human occludin.

The crystal structure of the occludin C-terminal region (383–522) has been determined recently (15). This coiled-coil region C-terminal to the Tyr phosphorylation sites does bind to ZO-1 quite well. This indicates that the main function of the phosphorylation is not ZO-1 binding; rather, it plays a role in the regulation of ZO-1 binding. Similar to ZO-1 binding, ZO-3 binding to GST-hOcl-C was also altered by mutation of Tyr-379 and -402. Interestingly, ZO-3 binding to Y398F mutant was greater than its binding to GST-hOcl-CWT. However, at present, the reason for this enhanced binding is not clear and needs further studies.
Tight Junction Regulation by Tyr Phosphorylation of Occludin

To determine the effect of Tyr-398 or Tyr-402 phosphorylation on the assembly of occludin into intercellular junctions, we induced point mutations to GFP-tagged full-length human occludin. The GFP-hOclWT and its mutants, GFP-hOclY398A/Y402A, GFP-hOclY398D/Y402D, and the corresponding single mutants were transfected into Rat-1 fibroblasts or MDCK cells. Confocal immunofluorescence microscopy demonstrated that the GFP-hOclWT and its Y398A/Y402A mutant were localized at the plasma membrane of Rat-1 cells, forming intercellular contact sites. Rat-1 cells express high levels of ZO-1, but it is predominantly localized in the intracellular compartment. However, transfection of GFP-hOclWT or GFP-hOclY398A/Y402A induced a recruitment of ZO-1 at the plasma membrane and the intercellular contact sites. Y398A/Y402A mutant occludin did not alter the distribution of occludin at the plasma membranes. However, Y398D, Y402D, and Y398/Y402D mutants of occludin failed to localizes to the plasma membrane or cell-cell contact sites; rather, they were distributed in the intracellular compartment. This indicates that mimicking the phosphorylation of Tyr-398 and Tyr-402 by mutation to aspartic acid results in the loss of its ability to assemble at the plasma membranes and cell-cell contact sites. This may be due to the loss of its ability to bind to ZO-1.

A significant portion of GFP-occludin in vesicular structure did appear near the plasma membrane, suggesting that the mutation did not result in a defect in its ability to integrate into the plasma membrane; rather, these occludin mutants are internalized into the cells due to the lack of their interaction with ZO-1 and inability to integrate into TJ structure. Both single mutants, Y398D and Y402D, similarly failed to localize at the intercellular junctions, indicating that phosphorylation of either Tyr residues is enough to alter its ability to bind ZO-1 and integrate into TJs. In vitro phosphorylation and ZO-1 binding studies indicated that Tyr-383 is more important than Tyr-379 in chicken occludin. However, this is in contrast to cell data, which shows that both Tyr-398 and Tyr-402 in human occludin are important for its localization at the cell-cell contact sites. This may be explained by the lower preference of c-Src for Tyr-379 compared with Tyr-383. In the cell, Tyr-379 may be phosphorylated by some other tyrosine kinase.

In MDCK cells, wild type and Y398A/Y402A mutant occludin were localized at the intercellular junctions. However, during the early stages of TJ assembly by calcium replacement, we saw a delay in the organization of GFP-hOclY398D/Y402D at the intercellular junctions. One hour after calcium replacement, Y398D/Y402D mutant occludin was distributed predominantly in the intracellular compartment, whereas WT and Y398A/Y402A mutant occludin were organized at the intercellular junctions. This confirmed that phosphorylation of Tyr-398 and Tyr-402 of occludin does prevent its ability to integrate into the TJs. The organization of Y398D/Y402D mutant at the intercellular junctions may be mediated by dimerization of mutant with the endogenous occludin. Expression of Y398D/Y402D mutant of occludin did disrupt the junctional distribution of ZO-1. This dominant negative effect is evident only at higher levels of mutant expression. The expression of mutant in relation to endogenous occludin is difficult to assess. However, ZO-1 redistribution was seen only in cells with higher level of mutant expression. Therefore, it is not clear whether such an effect can be seen at endogenous levels of phospho-occludin.

Furthermore, MDCK cell monolayers that express Y398D/Y402D mutant of occludin were dramatically more sensitive to hydrogen peroxide-induced disruption of barrier function, whereas cell monolayers expressing Y398A/Y402A mutant occludin showed significant resistance to hydrogen peroxide compared with cell monolayers expressing wild type occludin. The present study also shows that hydrogen peroxide failed to induce Tyr phosphorylation in MDCK cell monolayers, demonstrating that Tyr-398 and Tyr-402 are the phosphorylation sites in hydrogen peroxide-treated cells. The loss of co-immunoprecipitation of ZO-1 with GFP-hOclY398D/Y402D in MDCK cells confirms our observation made in in vitro studies that...
phosphorylation of Tyr-398 and Tyr-402 does prevent its interaction with ZO-1.

As shown in Fig. 9, the GFP-hOclY398D/Y402D eventually tends to organize at the intercellular junctions, although not as discretely as GFP-hOclWT or GFP-hOclY398A/Y402A. This is possibly due to oligomerization of GFP-hOclY398D/Y402D with the endogenous occludin. The inulin flux in hydrogen peroxide-treated cell monolayers that express GFP-hOclY398D/Y402D is significantly higher than that in cells that express GFP-hOclWT or GFP-hOclY398A/Y402A. The mechanism for greater sensitivity to hydrogen peroxide or delayed assembly is not clear at this point. However, we speculate that mixed oligomerization of GFP-OclY398D/Y402D and endogenous occludin facilitates the dissociation of ZO-1 by hydrogen peroxide due to weak interaction between GFP-OclY398D/Y402D with ZO-1; this may work synergistically with the hydrogen peroxide-induced Tyr phosphorylation of endogenous occludin and loss of its interaction with ZO-1. In summary, this study identifies Tyr-398 and Tyr-402 as the phosphorylation sites in human occludin and demonstrates that phosphorylation of these Tyr residues results in the loss of interaction between occludin and ZO-1 and attenuation of its integration into the epithelial TJ.

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