EphA2 Phosphorylates the Cytoplasmic Tail of Claudin-4 and Mediates Paracellular Permeability*

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Eph receptors and ephrin ligands are widely expressed in epithelial cells and mediate cell-cell interaction. EphA2 is expressed in various cancer tissues and cell lines. Although the mechanism of action of EphA2 is unknown, its expression correlates with progression of the malignant phenotype of cancerous tissues. Here, we have shown that EphA2 modulates the localization and function of claudin-4, a constituent of tight junctions. EphA2 associates with claudin-4 via their extracellular domains. This association, in turn, leads to phosphorylation of the cytoplasmic carboxyl terminus of claudin-4 at Tyr-208. The tyrosine phosphorylation of claudin-4 attenuates association of claudin-4 with ZO-1, decreasing integration of claudin-4 into sites of cell-cell contact and enhancing paracellular permeability. These results indicate that EphA2 moderates the function of tight junctions via phosphorylation of claudin-4.

The members of the Eph receptor family can be classified into two groups based on their sequence similarity and their preferential binding to ligands tethered to the cell surface by a glycosylphosphatidylinositol anchor (ephrin-A) or by a transmembrane domain (ephrin-B) (1–4). Although the studies of Eph receptors and ephrins have focused on their neuronal targeting and neural plasticity (5–9), they are also widely expressed in epithelial cells, and overexpression of Eph receptors and ephrins has also been reported in various tumors. In particular, the overexpression of EphA2, an Eph receptor, was shown to correlate with poor prognosis and high vascularity in these cancer tissues (10–15).

In epithelial cells, cell-cell adhesion appears to be closely connected with the function of Eph receptors. For example, E-cadherin-mediated cell-cell adhesion is required for EphA2 localization at cell contacts, and the loss of E-cadherin decreases the phosphotyrosine content of EphA2 (16, 17). In addition, disrupting signaling through some of the Eph receptors and ephrins leads to impaired cell-cell adhesion in early stage Xenopus embryos (18, 19).

Tight junctions locate at the most apical part of lateral membranes and serve as a paracellular barrier to restrict the movement of molecules, including ions and proteins, across cell boundaries. Claudins, a family of tetraspan transmembrane proteins containing more than 20 members, are a major constituent of tight junctions (20, 21). Carboxyl-terminal YV sequences conserved among claudin families are involved in interaction with PDZ domain-containing molecules such as ZO-1, ZO-2, ZO-3, MUPP1, and PATJ (22–24). ZO family proteins maintain plaque structures underlying tight junctions. Claudins are one of the most frequently overexpressed genes in various malignant cells (25–27).

During the screening of cross-talk between Eph-ephrin family molecules and intercellular adhesion molecules, we found that EphA2 makes a complex with claudin-4, which led us to investigate whether claudin-4 is a biochemical target of EphA2.

In this study, we have described the biological interaction of EphA2 with claudin-4. Upon cell-cell contact, a tyrosine residue located in the carboxyl-terminal region of claudin-4 was phosphorylated by activated EphA2. This phosphorylation event led to reduced association of claudin-4 with ZO-1 and decreased integration of claudin-4 into sites of cell-cell contact. Analysis of paracellular flux indicated that activation of EphA2 delayed assembly of tight junctions in Madin-Darby canine kidney (MDCK) cells, and this depended on EphA2 kinase activity. These results show that, by regulating the localization and function of claudin-4, EphA2 moderates tight junction permeability.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—Plasmids encoding full-length human EphA2 and a mutant EphA2I94N were previously described (28). The Fc fusion protein expression constructs EphA2, ephrin-A1, EphB2, and ephrin-B1 were constructed using PCR-generated amplicons of the entire extracellular domain of each protein as previously described (28, 29). Mutants of EphA2, lacking either the cytoplasmic domain (EphA2-(1–563)) or the extracellular domain (EphA2-(541–977)) were constructed by tagging PCR-generated amplicons with GFP at the carboxyl or amino terminus, respectively. The plasmids encoding claudin-4 and N-ZO-1 (amino acids 1–862) were donated by S. Tsukita. Mutants of claudin-4 (Y193F, Y197F, Y208F, Y193/197F, and Y193/197/208F) and EphA2 (I94N) were generated using the Altered Sites mutagenesis system (Promega). The truncated mutants of claudin-4 shown in Fig. 2a were constructed by cloning PCR-generated amplicons into pEBB with the addition of the FLAG epitope tag at the carboxyl terminus. The antibodies for the FLAG (M2) and hemagglutinin (Y-11) tags were obtained from Sigma and Santa Cruz, respectively. The monoclonal antibodies for EphA2 and phosphotyrosine (4G10) were purchased from Upstate Biotechnology. The antibodies for claudin-4 and ZO-1 were purchased from ZyMed. Anti-GFP was from Nacalai Tesque. Alexa Fluor-labeled secondary antibodies of anti-goat IgG, anti-rabbit IgG, and anti-mouse IgG were purchased from Molecular Probes. Fusion proteins of Eph and ephrin with the Fc region of immunoglobulin were purified by passing the culture medium of COS1 cells transfected with plasmids encoding the Fc fusion proteins through a protein A-Sepharose column as previously described (29).

Cell Culture and Transfection—HT29 colon carcinoma cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. MDCK cells and COS1 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. For transient expression

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2 The abbreviations used are: MDCK, Madin-Darby canine kidney; GST, glutathione S-transferase; ECD, extracellular domain; GFP, green fluorescent protein; FITC, fluo- rescein isothiocyanate.
FIGURE 1. EphA2 associates with claudin-4. a, COS1 cells were transiently transfected with a plasmid encoding EphA2 together with a plasmid encoding claudin-4. Cells were lysed and immunoprecipitated (IP) with anti-claudin-4, anti-EphA2, or mouse IgG1 as indicated. The precipitates were subjected to immunoblotting (IB) with the indicated antibodies. Arrowheads indicate coprecipitated EphA2 (left) and claudin-4 (right). Asterisk and sharp sign indicate IgG heavy chain and light chain, respectively. b, EphA2 physiologically associates with claudin-4 in HT29 cells. HT29 cells were lysed and subjected to immunoprecipitation (IP) and immunoblotting (IB) with the indicated antibodies. Arrowheads indicate coprecipitated EphA2 (left and claudin-4 (right). c, stimulation of the EphA2 receptor induces the redistribution of claudin-4. HT29 cells were untreated (left column) or incubated with clustered ephrin-A1-Fc for 5 min or 1 h as indicated above the middle and right column. The cells were stained with antibodies against either EphA2 or Fc together with anti-claudin-4 as indicated at the bottom.

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Assays, COS1 cells were transfected with plasmid DNA using FuGENE 6 reagent (Roche Applied Science).

Generation of Adenoviruses and Adenoviral Infection—To generate recombinant adenoviruses, cDNAs encoding wild type, the K646M mutant of EphA2, or EphA2-(1–563)-GFP were subcloned into the vector pShuttle-CMV (Stratagene). They were transformed into an Escherichia coli strain containing the Ad5-based adenovirus vector pADEasy-1 (Stratagene). Transposition of the EphA2 cDNAs from the pShuttle-CMV into pADEasy-1 created the adenoviral vectors pADE-EphA2 (wild type, K646M, and EphA2-(1–563)-GFP) where the transgenes were under the control of the cytomegalovirus promoter. Recombinant adenoviral DNA was transfected into 293 human embryonic kidney cells to allow production of adenoviral particles. The titer of recombinant adenoviruses, cDNAs encoding wild type, the K646M mutant of EphA2, or EphA2-(1–563)-GFP were subcloned into the vector pShuttle-CMV (Stratagene) according to the manufacturer’s instructions. Confluent MDCK cells grown on Transwell filters or glass coverslips were infected with adenoviruses at a multiplicity of infection of 5 in medium containing 10% fetal bovine serum. After incubation for 12 h the virus-containing medium was removed and fresh medium containing 10% fetal bovine serum was added. The infected cells were used for permeability assays or immunostaining 48 h after the infection.

Immunoprecipitation and Immunoblotting—Transfected cells were harvested 48 h after transfection, and cell lysates were prepared with protease inhibitors in PLC buffer (50 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 100 mM NaF, 1 mM Na3VO4, and 1% Triton X-100). The lysates were precleared by incubation with protein G-agarose (Roche Applied Science) for 1 h at 4 °C. To purify target proteins, 1 μg of monoclonal or affinity-purified polyclonal antibody was incubated with 500 μg of precleared cell lysate for 2 h at 4 °C and then precipitated with protein G-agarose for 1 h at 4 °C. Immunoprecipitates were extensively washed with PLC buffer, separated by SDS-PAGE, and subjected to immunoblotting. After blocking, blots were incubated with appropriate primary antibodies. Blots were then washed four times with TBST (150 mM NaCl, 10 mM Tris (pH 8.0), and 0.05% Tween20), incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit whole IgG antibodies (Amersham Biosciences) for 30 min, washed, and visualized by autoradiography using chemiluminescence reagent (Western Lightning; PerkinElmer).

Cell Staining—Cells were fixed for 5 min at room temperature with 4% paraformaldehyde in phosphate-buffered saline and permeabilized for 10 min with 0.2% Triton X-100. The cells were preincubated in 2% bovine serum albumin with 5% normal serum for 0.5 h and incubated with specific primary antibodies for 1 h at room temperature. After washing, cells were incubated with Alexa-conjugated secondary antibodies (Molecular Probes) for 0.5 h at room temperature. In some experiments, membrane-bound Fc fusion proteins were stained with Alexa488-conjugated anti-mouse IgG Fab for 0.5 h. Photos were taken with a Radiance 2100 confocal microscope (Bio-Rad).

In Vitro Binding Assay—The recombinant GST-tagged claudin-4 containing the second cytoplasmic domain was prepared in TTK11-competent cells (Stratagene) by transformation with pGEX4T claudin-4. Tyrosine-phosphorylated GST-claudin-4 was prepared by induction of a nonspecific tyrosine kinase in TTK11 cells according to the manufacturer’s instructions. GST-tagged proteins (2.5 μg) were purified using glutathione-agarose. Each GST-tagged protein (2.5 μg) was incubated with lysate prepared from COS1 cells transfected with N-ZO-1 in PLC buffer for 4 h at 4 °C. The beads were washed four times with the same buffer, and bound proteins were separated by SDS-PAGE. Precipitated N-ZO-1 was detected by immunoblotting with anti-ZO-1 antibody.

Paracellular Permeability Assay—MDCK cells were seeded into the upper well of transwell chambers (0.4-μm pore size; Becton Dickinson Labware). After the cells reached confluence and established tight junctions, the medium of both the upper and lower wells was replaced with Hanks’ balanced salt solution containing 2 mM EGTA. The extent of cell-cell contact after treatment with EGTA was confirmed by microscopic observation. The medium was replaced from calcium-free to normal growth medium with 0.5% fetal bovine serum (calcium switch), and monolayers were allowed to recover for the indicated period. FITC-dextran (M, 3,000 or 2,000) was added to the upper well at a concentration of 10 μg/ml and incubated for 30 min. Samples were then taken from the lower compartment of the transwell chamber. The amount of
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FITC-dextran in the lower wells was determined using Beacon 2000 (Takara) with an excitation wavelength of 490 nm and detection of emissions at 530 nm.

RESULTS

Claudin Interacts with the Extracellular Domain of EphA2—Physical association between EphA2 and claudin-4 was first detected in COS1 cells transiently expressing both EphA2 and claudin-4. In these cells, EphA2 coprecipitated with claudin-4 immunoprecipitated with claudin-4-specific antibodies (Fig. 1a, left panel). Formation of an EphA2-claudin-4 complex was confirmed by immunoprecipitation using EphA2-specific antibodies (Fig. 1a, right panel). Endogenous EphA2 also coprecipitated with endogenous claudin-4 in extracts of the HT29 colon cancer cell line (Fig. 1b). Thus, endogenous EphA2 and claudin-4 physically interact in HT29 colon cancer cells.

Stimulation of EphA2 with ephrin-A1-Fc induces formation of membrane patches through the clustering of receptors (30–32). We examined changes in the localization of claudin-4 after stimulation of EphA2 with ephrin-A1-Fc. For efficient binding of the Fc fusion protein to the receptors, cells were plated at low density. In unstimulated HT29 cells, claudin-4 is diffusely expressed both on the cell membrane and in the cytoplasm (Fig. 1c, left row). Several minutes after exposure to ephrin-A1-Fc, claudin-4 redistributed as fine patches in the membrane that overlapped EphA2/ephrin-A1-Fc complexes (Fig. 1c, middle row). At 1 h after stimulation, partial colocalization of claudin-4 with EphA2/ephrin-A1-Fc was clearly observed as patchy complexes in the cytoplasm (Fig. 1c, right row). The dynamic colocalization of EphA2 and claudin-4 further indicates physiological association of these two molecules.

Next, a series of mutants of claudin-4 were generated to determine the region required for interaction with EphA2 (Fig. 2a). Among the mutants of claudin-4, the one containing the amino-terminal region including the first extracellular domain (ECD) tightly bound to EphA2 (Fig. 2a). Next, several mutants of EphA2 were utilized to determine the region involved in the interaction with claudin-4 (Fig. 2b). The extracellular domain, but not the cytoplasmic domain of EphA2, associates with claudin-4 (Fig. 2b, left panel). The amino-terminal region of EphA2 (amino acids 28–206) binds to its ligand, ephrin-A1 (33). Claudin-4 binds to EphA2 (amino acids 207–540), which contains the core region necessary for the interaction with its cognate ligand, ephrin-A1 (Fig. 2b, right panel). On the other hand, specific EphA2 mutation (I94N), which was incidentally found to impair binding with ephrin-A1 (Fig. 2c, left panel), did not significantly affect EphA2 binding to claudin-4 (Fig. 2c, right panel).
**FIGURE 3. Claudin-4 is tyrosine phosphorylated by the interaction with EphA2.**

- **a.** COS1 cells were transiently transfected with claudin-4 and wild type (wt) or a kinase-inactive mutant (K646M) of EphA2. Upper panel, claudin-4 was immunoprecipitated (IP) from cell lysates and subjected to immunoblotting with anti-phosphotyrosine (4G10). Arrowheads indicate tyrosine-phosphorylated EphA2 (open) and tyrosine-phosphorylated claudin-4 (filled). Asterisk and sharp sign indicate IgG heavy and light chains, respectively, which are not detected in the lysate of untransfected COS1 cells. Middle panel, EphA2 was immunoprecipitated (IP) from the cell lysates, and tyrosine phosphorylation of EphA2 was detected by immunoblotting (IB) with 4G10. Bottom two panels, EphA2 and claudin-4 detected in the lysate prior to immunoprecipitation. b, the kinase-inactive mutant of EphA2 competitively blocked the phosphorylation of claudin-4 by wild type EphA2. COS1 cells were transiently transfected with claudin-4 and hemagglutinin-tagged wild type EphA2 together with increasing amounts of kinase-inactive EphA2K646M as indicated above the lanes. Assays were performed as in panel a. The expression level of wild type EphA2 (shown as immunoblotting with anti-hemagglutinin, bottom panel) was the same in each lysate. Arrowheads indicate tyrosine-phosphorylated EphA2 (open) and claudin-4 (filled). Asterisk and sharp sign indicate IgG heavy and light chains, respectively. c, claudin-4 lacking the binding region to EphA2 was not phosphorylated. COS1 cells were transiently transfected with wild type EphA2 and FLAG-tagged wild type claudin-4 or a mutant lacking the first extracellular domain of claudin-4 (ΔECD1) as indicated above the lanes. Upper panel, claudin-4 was immunoprecipitated (IP) from cell lysates, and tyrosine-phosphorylated claudin-4 was detected by immunoblotting (IB) with anti-phosphotyrosine (4G10). Arrowheads indicate wild type (filled) and ΔECD1 claudin-4 (open). Sharp sign indicates IgG light chain. Lower panels, EphA2 and FLAG-tagged claudin-4 detected in the lysate prior to immunoprecipitation. d, EphA2 phosphorylates claudin-4 at Tyr-208. The amino acid sequence of the carboxyl tail of human claudin-4 is shown at the top with the tyrosine residues numbered. COS1 cells were transiently transfected with EphA2 together with FLAG-tagged wild type (wt) claudin-4 or claudin-4 with mutations at the indicated tyrosine residues. Assays were performed as in panel c. Upper panel, arrowheads indicate phosphorylated EphA2 (open) and claudin-4 (filled). Asterisk and sharp sign indicate IgG heavy and light chains, respectively. Lower panel, FLAG-tagged claudin-4 detected in the lysate prior to immunoprecipitation.

Claudin-4 has three tyrosine residues in the second (carboxy-terminal) cytoplasmic domain, but there is no tyrosine in the first (amino-terminal) cytoplasmic domain. Among the three tyrosine residues in the second cytoplasmic domain, the one located at the carboxyl terminus is well conserved in most claudin families (22). EphA2-induced phosphorylation of claudin-4 was completely abolished when the tyrosine at the carboxyl terminus was replaced by phenylalanine (claudin-4Y208F), whereas substitution of other tyrosine residues did not affect the phosphorylation level of claudin-4 (Fig. 3d).

It was next determined whether cell-cell contact stimulates tyrosine phosphorylation of claudin-4 by physiological activation of EphA2. HT29 cells were used because they express the ephrin-A1 ligand, which can activate EphA2 upon cell-cell adhesion (34). In HT29 cells, both EphA2 and claudin-4 were highly phosphorylated on tyrosine residues when the cells were plated at high density, whereas their phosphoryla-
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FIGURE 4. Tyrosine phosphorylation of claudin-4 is induced by cell-cell contact. a, HT29 cells were grown until confluent. The cells were either left untreated (confluent) or incubated in 2 mM EGTA in Hanks' balanced salt solution until cell-cell contacts were abolished but the cells remained attached to the plate (EGTA). EGTA was removed, and the cells were incubated in normal cell culture medium for 4 h (restoration). The cells were lysed at each time point and subjected to immunoprecipitation (IP) with anti-claudin-4 or anti-EphA2. Tyrosine-phosphorylated claudin-4 (left column, top panel) and tyrosine-phosphorylated EphA2 (left column, second panel) were detected by immunoblotting (IB) with anti-phosphotyrosine (4G10). EphA2 that coprecipitated with claudin-4 was detected by immunoblotting with anti-EphA2 (left column, third panel). Tyrosine-phosphorylated claudin-4, tyrosine-phosphorylated EphA2, and coprecipitated EphA2 are indicated by arrowheads. Sharp sign and asterisk indicate IgG light and heavy chains, respectively. EphA2 and claudin-4 detected in the lysate prior to immunoprecipitation are shown in the lower panels. The appearance of the cells at each time point is shown in the right panel. b, HT29 cells plated at low cell density were treated with ephrin-A1-Fc for 10 min or left untreated (–). Claudin-4 was immunoprecipitated from each cell lysate, and tyrosine-phosphorylated claudin-4 was detected by immunoblotting (IB) with anti-phosphotyrosine (4G10). Sharp sign indicates IgG light chain. Claudin-4 detected in the lysate prior to immunoprecipitation is shown in the lower panel. c, confluent HT29 cells were infected with Ad-GFP or Ad-EphA2-(1–563)-GFP as indicated above the lanes. The infected cells were lysed, and the tyrosine phosphorylation of claudin-4 was examined as described in panel b. Sharp sign indicates IgG light chain. Claudin-4 and GFP and GFP-tagged EphA2 detected in the lysate prior to immunoprecipitation are shown in the lower panels.

tion level was very low when cell-cell interaction was disrupted by depletion of calcium in the medium (Fig. 4a). Treatment with nifedipine, a blocker of calcium channels, had no effect on the status of tyrosine phosphorylation of claudin-4 and EphA2 (data not shown), excluding the possibility that a decrease of intracellular calcium by EGTA affected the phosphorylation level of claudin. Treatment of HT29 cells with ephrin-A1-Fc enhanced phosphorylation of endogenous claudin-4 (Fig. 4b). Tyrosine phosphorylation of claudin-4 in HT29 cells was blocked by expression of EphA2-(1–563)-GFP, which does not contain the cytoplasmic region of EphA2 (Fig. 4c). These results suggest that claudin-4 is phosphorylated in HT29 cells when EphA2 is activated upon cell-cell interaction.

EphA2, however, remained associated with claudin-4 after disruption of cell-cell adhesion (Fig. 4a), even after the cells were dispersed as a single cell suspension through prolonged incubation with the calcium-chelating agent (data not shown). Therefore, the majority of association between EphA2 and claudin-4 occurred on the cell membrane of the same cells in cis, rather than on two different cells in trans.

Tyrosine Phosphorylation of Claudin-4 Attenuates Association with ZO-1—The carboxy-terminal YV sequence of claudin associates with the PDZ domain of ZO-1 (22). We examined whether tyrosine phosphorylation of claudin-4 at the cytoplasmic tail affects interaction between claudin-4 and ZO-1. Non-phosphorylated and tyrosine-phosphorylated forms of recombinant GST-tagged claudin-4-(188–209), which contains the cytoplasmic tail, were purified using the TKX1 E. coli expression system (see “Experimental Procedures”). Non-phosphorylated claudin-4-(188–209) could effectively pull down the amino-terminal region of ZO-1 (N-ZO-1) expressed in COS1 cells. On the other hand, phosphorylated claudin-4-(188–209) precipitated significantly lower amounts of N-ZO-1 (Fig. 5a, lanes 1 and 2). Because claudin-4-(188–209) contains three tyrosine residues (Fig. 3d), we also generated recombinant GST-claudin-4-(188–209)Y193/197F, in which two residual tyrosines were mutated, to confirm that phosphorylation of Tyr-208 of claudin-4 is responsible for attenuation of its binding ability with ZO-1. Non-phosphorylated wild type and the Y193/197F mutant of claudin-4-(188–209) equally coprecipitated ZO-1 (Fig. 5a, lanes 1 and 3), whereas phosphorylated claudin-4-(188–209)Y193/197F, in which only Tyr-208 was phosphorylated, did not effectively bind to ZO-1 (Fig. 5a, lane 4). Furthermore, neither claudin-4 lacking the carboxy-terminal YV sequence nor GST alone bound to ZO-1 (Fig. 5a, lanes 5 and 6).

Next, experiments were conducted to determine whether EphA2 activation leads to reduction of association between claudin-4 and ZO-1. The amount of claudin-4–comunmunoprecipitated with ZO-1 was reduced in EphA2-overexpressing COS1 cells (Fig. 5b). Association between claudin-4 and ZO-1 was also attenuated in MDCK cells expressing EphA2, and this association was further decreased by stimulation with ephrin-A1-Fc (Fig. 5c). From these results we conclude that EphA2 activation can reduce association of claudin-4 with ZO-1 by inducing phosphorylation of Tyr-208 of claudin-4 in vivo.

To examine the biological significance of EphA2-mediated attenuation of the binding affinity between claudin-4 and ZO-1, the change in localization of claudin-4 was monitored during the dynamic process of reestablishment of cell-cell adhesion. Wild type or the kinase-inactive mutant of EphA2 was expressed at high levels by adenovirus-mediated gene transfer in MDCK cells, which express endogenous claudin-4. When the cells were initially treated with calcium-chelating agent, ZO-1 and claudin-4 localization to cell-cell contact sites was completely abolished (data not shown). 30 min after replacing the calcium-free medium with normal growth medium (calcium switch), ZO-1 was found to relocalize to the cell-cell contact sites regardless of the presence of kinase-active or kinase-inactive EphA2 (Fig. 5d). On the other hand, in cells expressing kinase-dead EphA2, claudin-4 also relocalizes...
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FIGURE 5. Phosphorylation of claudin-4 at Tyr-208 inhibits its interaction with ZO-1. a, the second cytoplasmic domain of wild type (WT), the Y193/197F mutant, or the ΔYW mutant of claudin-4 was tagged with GST. The recombinant proteins were purified as either non-phosphorylated (−) or tyrosine phosphorylated (+) from TKX1-competent cells and incubated with lysate from N-ZO-1-transfected COS1 cells. The cell lysates were then incubated with protein G-Sepharose to precipitate claudin-4. Upper panel, coprecipitated N-ZO-1 was detected by immunoblotting (IB). Middle panel, purified GST fusion proteins added to the lysate are shown in a Coomassie Blue-stained gel. Lower panel, the tyrosine phosphorylation of recombinant GST-tagged claudin-4 was detected by immunoblotting with anti-phosphotyrosine (4G10). b, COS1 cells were transiently transfected with Myc-tagged ZO-1 and EphA2 as indicated above the lanes. ZO-1 was immunoprecipitated (IP) with anti-Myc antibody from cell lysates, and coprecipitated claudin-4 was detected by immunoblotting (IB). Claudin-4, ZO-1, and EphA2 detected in the lysates prior to immunoprecipitation are shown in the lower panels. c, MDCK cells infected with Ad-wild type EphA2 or control Ad-GFP were treated with EGTA. After removal of EGTA, the cells were incubated for 30 min in normal growth medium with (+) or without (−) ephrin-A1-Fc (4 μg/ml), ZO-1 was immunoprecipitated (IP) from the cell lysate, and coprecipitated claudin-4 was detected by immunoblotting (IB). ZO-1, claudin-4, and EphA2 detected in the lysates prior to immunoprecipitation are shown in the lower panels. d, MDCK cells infected with Ad-wild type EphA2 (WT) or Ad-EphA2K646M were incubated with EGTA until cell-cell contacts were abolished. After removal of EGTA, the cells were incubated for 30 min or 3 h in normal growth medium with (+) or without (−) ephrin-A1-Fc (4 μg/ml) as indicated. The cells were then fixed and stained with anti-cadinl-4 antibody or anti-ZO-1 antibody. Representative fields are shown.

to cell-cell contact sites 30 min after calcium switch, but in cells expressing kinase-active EphA2, claudin-4 did not relocalize to these sites (Fig. 5d). However, 3h after calcium switch claudin-4 had relocalized to cell-cell contact sites in cells expressing kinase-active EphA2 (Fig. 5d). These results suggest that EphA2 causes a delay in reassembly of claudin-4 to tight junctions but has no effect on ZO-1.

EphA2 Affects Paracellular Permeability of Normal Epithelial Cells Depending on Its Kinase Activity—To examine the biological effect of the interaction of EphA2 with claudin-4 on tight junctions, paracellular permeability was measured using low molecular size dextran in MDCK epithelial cells. The amount of the dextran flux through a monolayer of confluent MDCK cells was almost undetectable even when EphA2 was overexpressed (data not shown). Therefore, we decided to examine the effect of EphA2 on reestablishment of the paracellular barrier after calcium switch. Overexpression of EphA2 effectively inhibited the prompt decrease of paracellular permeability through an MDCK monolayer after calcium switch, and this delay was further enhanced when cells were stimulated by ephrin-A1-Fc (Fig. 6a). On the other hand, kinase-inactive EphA2 did not affect paracellular permeability (Fig. 6a), indicating that the kinase activity of EphA2 affects permeability. In this experiment, the phosphorylation level of claudin-4 3 h after calcium switch had decreased to approximately the same level as in the confluent untreated cells (Fig. 6b, compare lanes 2 and 4). The paracellular flux of large molecular sized dextran (M, 2,000) was below detectable levels at all time points shown in Fig. 6a (data not shown), indicating no massive destruction of junctional complexes had occurred in our experiment.

DISCUSSION

This is the first report showing tyrosine phosphorylation of claudin in relationship with its biological function. EphA2 induced the specific phosphorylation of Tyr-208 in the cytoplasmic tail of claudin-4 in COS1 cells, and endogenous claudin-4 was phosphorylated in response to EphA2 activation in HT29 cells. Both the kinase activity of EphA2 and association between EphA2 and claudin-4 are required for phosphorylation of claudin-4. Considering that EphA2 and claudin-4 associate on the same cell surface in cis, claudin-4 is most probably a direct substrate of EphA2. Our results indicate that activation of EphA2 by its ligand decreases integration of claudin-4 into tight junctions and enhances paracellular permeability.

Tyrosine phosphorylation of claudin-4 in its carboxyl-terminal tail inhibits binding to ZO-1. It is also reported that phosphorylation of the carboxyl-terminal tail of occludin by c-Src also attenuated its association with ZO-1, ZO-2, and ZO-3 in vitro (36). Thus, tyrosine phosphorylation of claudin-4 at Tyr-208 may be involved in regulation of paracellular permeability through the inhibition of its association with ZO-1.
The claudin family is still uncertain. At least one other Eph family member, EphA4, is likely to be involved in this cross-talk, as claudin-4 is also tyrosine phosphorylated by coexpression with EphA4 in COS1 cells (data not shown). Also, the tyrosine residue located at the carboxyl terminus is conserved among at least 8 claudin family members, claudin-1–8. Therefore, other claudins also have the potential to be phosphorylated by Eph receptors. We are currently testing this hypothesis.

In tumors overexpressing EphA2, assembly of tight junctions may be negatively regulated via phosphorylation of claudin. This has the potential to affect the malignant phenotype of the carcinoma, such as loss of cell polarity. Moreover, in tumors overexpressing both EphA2 and claudin-4, like HT29 colon cancer cells, phosphorylation of claudin-4 might transduce signals distinct from tight junction function. We are currently attempting to identify other downstream targets of signaling through tyrosine phosphorylation of claudin-4 and determine their roles in the regulation of epithelial cells and cancer cells.

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FIGURE 6. EphA2 affects paracellular permeability and reassembly of claudin-4. a, MDCK cells were grown until confluent in Transwell chambers. The cells were then infected with Ad-wild type EphA2 (WT), Ad-EphA2K646M (K646M), or Ad-GFP. Two days later, the cells were treated with EGTA to disrupt cell-cell contacts, and then the EGTA was removed and replaced with normal growth medium (calcium switch). After the calcium switch, the cells were incubated in normal growth medium with (+) or without (−) ephrin-A1-Fc (4 μg/ml) for the indicated times. FITC-dextran was then added to the upper wells and the cells incubated for a further 30 min. After 30 min, the fluorescence of FITC-dextran in the lower wells was measured. The results are presented in terms of the % fluorescence in the lower wells when FITC-dextran was added to cell monolayers just after EGTA treatment. Each bar represents the mean value ± S.D. of three independent experiments. The expression of wild type EphA2 and EphA2K646M is shown in the middle panel. The phosphorylation levels of claudin-4 in wild type EphA2-expressing cells with or without ephrin-A1-Fc 30 min after the calcium switch is shown in the bottom panel. b, confluent MDCK cells infected with Ad-wild type EphA2 (lanes 2–4) or not infected (lane 1) were either left untreated (lanes 1 and 2) or subjected to calcium switch as in panel a (lanes 3 and 4). After the calcium switch, the cells were incubated in normal growth medium containing ephrin-A1-Fc (4 μg/ml) for 30 min (lanes 3) or 3 h (lane 4). The cells were then lysed, and claudin-4 was immunoprecipitated (IP) from the lysate. Upper panel, tyrosine-phosphorylated EphA2 and claudin-4 were detected by immunoblotting (IB) with anti-phosphotyrosine (4G10). Arrowheads indicate immunoprecipitated EphA2 phosphorylated on tyrosine (open) and immunoprecipitated claudin-4 phosphorylated on tyrosine (filled). Asterisk and sharp sign indicate IgG heavy chain and light chain, respectively. Lower panel, claudin-4 detected in the lysate prior to immunoprecipitation.
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