Epidermal Growth Factor Receptor Activation Differentially Regulates Claudin Expression and Enhances Transepithelial Resistance in Madin-Darby Canine Kidney Cells*

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Tight junctions (TJs) are the most apical cell-cell junctions, and claudins, the recently identified TJ proteins, are critical for maintaining cell-cell adhesion in epithelial cell sheets. Based on their in vivo distribution and the results of overexpression studies, certain claudins, including claudin-1 and -4, are postulated to increase, whereas other claudins, especially claudin-2, are postulated to decrease the overall transepidermal resistance. The overall ratio among claudins expressed in a cell/tissue has been hypothesized to define the complexity of TJs. Disruption of the TJs contributes to various human diseases, and a correlation between reduction of TJ function and tumor dedifferentiation has been postulated. The epidermal growth factor (EGF) receptor (EGFR) is overexpressed in a wide spectrum of epithelial cancers, and its expression correlates with a more metastatic cancer phenotype. However, normal functioning of EGFR is essential for normal epithelial cell proliferation and differentiation. The role of EGFR-dependent signaling in the development and maintenance of epithelial TJ integrity has not been studied in detail. This study demonstrates that, in polarized Madin-Darby canine kidney II cells, EGF-induced EGFR activation significantly inhibited claudin-2 expression while simultaneously inducing cellular redistribution and increased expression of claudin-1, -3, and -4. Accompanying these EGF-induced changes in claudin expression was a 3-fold increase in transepithelial resistance, a functional measure of TJs. In contrast, there were no alterations in protein expression and/or intracellular localization of other TJ-related proteins (ZO-1 and occludin) or adherens junction-associated proteins (E-cadherin and β-catenin), suggesting that EGF regulates TJ function through selective and differential regulation of claudins.

The maintenance of an epithelial barrier with regulated paracellular permeability is vital to the functioning of many multicellular organs, including the kidney (1). Tight junctions (TJs) create this barrier, regulating paracellular movement of water and solutes across vertebrate epithelia, and are composed of a belt of anastomosing strands of proteins and lipids that surround the lateral membrane of epithelial cells (2–5). Transepithelial resistance (TER) is a functional measure of the integrity of TJs (6, 7). Dynamic regulation of TJ function is fundamental to many physiological processes, and disruption of TJs drastically alters paracellular permeability and is a hallmark of many pathological states (8, 9). Many insults to epithelia such as ischemia/reperfusion, inflammation, and toxic injury lead to the loss of this barrier function (10–12). Previous studies demonstrated a correlation between reduction of TJs and tumor differentiation, and evidence for altered permeability of TJs in transformed epithelia has long been recognized (13, 14). More recent studies have demonstrated a decrease in the number of TJ strands in transitional carcinoma of the urinary bladder (15, 16), decreased transepithelial impedance across the colons of mice treated with chemical carcinogens (17), and increased TJ permeability in inflammatory bowel disease (18).

Claudins, the recently identified integral transmembrane protein of TJs, are members of a superfamily of transmembrane proteins (3, 4). To date, 24 members of this family have been identified, and they encode proteins with molecular masses that range from ~20 to 27 kDa (19). They are predicted to have four transmembrane-spanning helices with cytoplasmic N and C termini (3). Claudins in different organs and even within the same organ differ qualitatively in their overall ability to regulate paracellular solute and ion movement, and this difference is defined by the quality and complexity of the TJs. Claudins demonstrate homo- and heterophilic interactions with other claudins (3, 20) as well as with the TJ-related proteins occludin and ZO-1 through their PDZ binding domains (21, 22), and the overall ratio among claudins expressed in a particular cell type helps define the characteristics of its TJs (23).

The epidermal growth factor (EGF) is a 53-amino acid polypeptide that regulates the proliferation and differentiation of a variety of cell types, including polarized epithelial cells (24, 25). The receptor for EGF and related ligands (EGFR, HER1, ErbB1), the prototypical member of a superfamily of receptors with intrinsic tyrosine kinase activity, is widely expressed in many cell types, including cells of epithelial lineage (26, 27). Upon activation, the intrinsic kinase is activated, and EGFR tyrosyl-phosphorylates itself and numerous intermediary effec-

* This work was supported by National Institutes of Health Grant DK51265 and the Department of Veterans Affairs (to R. C. H.) and by American Heart Association Southeast Affiliate Grant 403756112-2003 (to A. B. S.). Work performed at the Vanderbilt University Medical Center Imaging Core Resource was supported by National Institutes of Health Grants CA68485 and DK20593. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: TJs, tight junctions; TER, transepithelial resistance; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MDCK, Madin-Darby canine kidney; BrdUrd, bromodeoxyuridine; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; ERR, extracellular signal-regulated kinase; HGF, hepatic growth factor.
EGF Regulates TER in MDCK Cells

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—MDCK II cells were grown in Dulbecco’s modified Eagle’s medium containing Earle’s balanced salt solution supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B in 5% CO₂ and 95% air at 37°C. For polarization studies, cells were electrophoretically denatured on Transwell filters (12- or 24-mm, 0.4-μm pore size; Corning Costar, Cambridge, MA). EGF was purchased from Upstate Biotechnology, Inc. Anti-claudin-1, -2, -3, and -4; anti-occludin; and anti-ZO-1 antibodies were purchased from Zymed Laboratories Inc. (South San Francisco, CA), and anti-E-cadherin and anti-β-catenin antibodies were purchased from Transduction Laboratories (Lexington, KY). The bromodeoxyuridine (BrdUrd) staining kit was obtained from Roche Applied Science. Rho- damine X-conjugated anti-rabbit and fluorescein isothiocyanate-conju- gated anti-mouse secondary antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA).

Measurement of TER—MDCK cells were plated on Transwell filters at confluence (∼2 × 10⁶ cells) and allowed to attach for 12–20 h to form a tight monolayer in normal Ca²⁺-containing medium. Thereafter, cells were washed with phosphate-buffered saline (PBS) and placed in ser- um-free culture medium for 24 h to render them quiescent. EGF (100 ng/ml) was then added to the quiescent cells and left in the medium. One Transwell chamber was left blank intentionally to determine the intrinsic membrane resistance. TER was measured at various time points after treatment using a Millipore electrical resistance sys- tem. Final values were obtained by subtracting the blank value, and the results are expressed as ohms/cm².

Preparation of Triton X-100-soluble and -insoluble fractions—The method for preparation of Triton X-100-soluble and -insoluble fractions was adapted from Stuart et al. (42) with modifications. In brief, cells were scraped in cell lysis buffer (10 mM HEPES, pH 7.2, 1% Triton X-100, 100 mM NaCl, 2 mM EDTA, 1 mM benzamidine, 1 mM phenyl- methylsulfonyl fluoride, 10 μg/ml leupeptin, and soybean and lima bean trypsin inhibitors) and incubated at 4°C for 20 min, followed by cen- trifugation at 10,000 × g for 20 min. The resulting supernatant was considered the Triton X-100/detergent-soluble fraction. For the insoluble fraction, the pellet from the soluble fraction was dissolved by pipet- ting in pellet solubilization buffer (10 mM HEPES, pH 7.2, 1% SDS, 100 mM NaCl, 2 mM EDTA, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and soybean and lima bean trypsin inhibitors) and the suspensions were sonicated for three sonication pulses (10 s each) at level 2. The resulting preparation was incubated at 4°C for 20 min, followed by centrifugation at 10,000 × g for 20 min; the superna- tant was considered the Triton X-100/detergent-insoluble fraction. Pro- tein concentration was determined separately for both fractions using the respective lysis buffer as a blank, and equal amounts of protein were subjected to analysis. To ensure equal loading, the same immuno- blots were stripped and probed for different antigens with different patterns of expression.

Immunoblotting—Equal amounts of proteins were subjected to SDS- PAGE and immunoblotted with the specific antibodies for different antigens. Signals were detected using an ECL detection kit (Amersham Biosciences). Following densitometry, the value obtained for protein extracted from the Triton X-100-soluble fraction in the control cells was taken as 100%, and the ratio between detergent-soluble and -insoluble fractions and changes in protein expression were determined relative to this value. When total cellular protein was used, the protein loading was normalized to actin values obtained from immunoblotting with anti-actin antibody after stripping the same membrane, and the per- centage decrease/increase was determined relative to control values.

Immunofluorescence Microscopy—Cells plated on Transwell filters or glass coverslips were rinsed with PBS with 0.2% bovine serum albumin and fixed with 3.7% formaldehyde in PBS for 30 min on ice. For permeabilization, fixed cells were treated with 0.2% Triton X-100 in PBS for 10 min and thereafter washed three times with PBS. After permeabilization in PBS containing 5% normal goat serum and 1% bovine serum albumin, the samples were incubated with primary antibodies for 1 h in a moist chamber at 37°C. The samples were then washed three times with PBS, followed by incubation for 30 min with the respective conjug- gated secondary antibody. The samples were again washed three times with PBS, embedded in glycerol/PBS-based mounting medium, and examined using a Zeiss 410 confocal microscope at the Vanderbilt University Medical Center Cell Imaging Core Resource. Image analysis was performed using the Metamorph cell imaging program (Universal Imaging Corp., Downingtown, PA).

Northern Blot Analysis—Northern blot analysis was performed as described previously (43). In brief, 25 μg of total RNA isolated from control and EGF-treated cells was electrophoresed under denaturing conditions on a 1.2% agarose gel and blotted onto Immobilon N nylon membrane (Millipore Corp, Bedford, MA). The membrane was baked under vacuum for 1 h and hybridized with 32P-labeled full-length clau- din-2 cDNA amplified from MDCK II cells under standard conditions. After washing, the membrane was subjected to autoradiography at 70°C to detect mRNA signals. The same membrane was stripped and probed with glyceraldehyde-3-phosphate dehydrogenase probe to en- sure equal loading.

BrdUrd Incorporation and Claudin Staining in Subconfluent Cells—MDCK II cells were plated at low density on glass coverslips and were left to develop into individual colonies. Thereafter, cells were serum- starved for 24 h, and EGF (100 ng/ml) was added. After 20 h of EGF treatment, BrdUrd (following the manufacturer’s recommendation) was added to the cells for 2 h. Preliminary experiments indicated peak thymidine incorporation 20–24 h post-EGF treatment.

Statistics—The graphic data are presented as means ± S.E. Statis- tical analysis was performed, when appropriate, using Student’s t test. A difference of p < 0.05 was considered statistically significant.

RESULTS

Exogenous EGF Increases TER—Exogenous EGF (100 ng/ml) was administered to confluent quiescent MDCK II cells plated on Transwell filters, and TER was measured at 0, 1, 4, 8, 18, and 24 h as described under “Experimental Procedures.” EGF administration resulted in a significant increase in TER begin- ning at 4 h after the administration of EGF, with TER reaching a maximum value (3-fold increase) 24 h following EGF treat- ment (Fig. 1A). The peak in TER was followed by a gradual decline starting from 30 h post-treatment; however, the values remained higher compared with those in control cells even at 48 h post-EGF treatment. To ensure that this response was dependent on EGFR activation, one group of cells was pre- treated for 30 min with a specific and stable pharmacological inhibitor of EGFR tyrrosine phosphorylation, PD153035 (44), before administration of EGF. Pretreatment of cells with PD153035 completely blocked the EGF-induced increase in TER (Fig. 1A), whereas PD153035 alone had no effect on basal TER in non-EGF-treated cells (data not shown). In addition, we tested SB2494002, wortmannin (specific phosphatidylinosi- tol 3-kinase inhibitors), SB203580 (a specific p38 MAPK inhibit- or), PD98059 (a specific ERK1/2 MAPK inhibitor), and stauro- sporine (a broad-range inhibitor of protein kinase C signaling pathways) to determine the possible signaling pathway(s) re- sponsible for the EGF-induced increase in TER downstream of EGFR. Although LY294002, wortmannin, and SB203580 were

a A. B. Singh and R. C. Harris, unpublished data.
without effect, PD98059 and staurosporine both blocked the EGF-induced increase in TER (Fig. 1B).

**EGF Down-regulates Claudin-2 Expression—MDCK II cells express claudin-1, -2, -3, and -4 (23).** Overexpression of claudin-2 decreased TER in MDCK I cells, the high resistance clone of MDCK cells (23), suggesting an inhibitory effect of claudin-2 on MDCK cell TJ function. Therefore, we first determined the effect of EGF treatment on claudin-2 protein expression at 15 min and 1, 4, 8, and 18 h post-EGF treatment. EGF treatment significantly down-regulated expression of total cellular claudin-2 protein, beginning as early as 15 min after EGF treatment, and claudin-2 expression reached its nadir by 18 h post-EGF treatment (Fig. 2A). Pretreatment of cells with PD153035 prevented the EGF-induced decrease in claudin-2 expression (Fig. 2B).

To investigate whether decreased claudin-2 protein expression is the result of a decrease in mRNA transcription, we performed Northern blot analysis. Quiescent cells were treated as described above, and samples were collected at 15 min and 1, 3, 6, 9, 12, and 18 h post-EGF treatment. One group of cells was pretreated with PD153035, and samples were collected at 8 h post-EGF treatment. The results indicate that EGF treatment induced a decrease in claudin-2 mRNA expression starting at 15 min post-EGF treatment, and mRNA expression reached its nadir after 3–6 h of EGF treatment (Fig. 2C). Pretreatment of cells with PD153035 also prevented this decrease in mRNA expression (Fig. 2D).

To determine whether the EGF-induced decrease in claudin-2 expression is specific to the protein fraction associated with the TJs, we further analyzed the changes in expression of claudin-2 in detergent-soluble (cytosolic or loosely associated with membrane) and detergent-insoluble (representing protein incorporated in the TJs) fractions in response to EGF treatment. Under basal conditions, the majority of the claudin-2 protein was extracted in the detergent-insoluble fraction; EGF treatment resulted in significantly decreased protein expression in both detergent-soluble and -insoluble fractions beginning at 15 min post-EGF treatment without altering the relative percentages of the protein in the detergent-soluble and -insoluble fractions (Fig. 3A). This decrease in claudin-2 protein expression was persistent at 24 and 48 h post-EGF treatment (data not shown). Pretreatment of cells with PD153035 markedly prevented the EGF-induced decrease in claudin-2 protein expression in both detergent-soluble and -insoluble fractions (Fig. 3B).

Immunofluorescent staining with anti-claudin-2 antibody confirmed the results from biochemical analysis and indicated predominant lateral membrane staining in control cells (Fig. 3C). The lateral membrane staining was significantly decreased after 8 h and was almost undetectable after 18 h of EGF treatment, supporting the findings from immunoblot analysis. At the same time, a punctuate pattern of cytoplasmic/perinuclear staining was observed after 18 h of EGF treatment. Because little full-length immunoreactive claudin-2 protein was present in the detergent-soluble fraction at the same time points, this cytoplasmic/perinuclear immunoreactivity may represent claudin-2 protein degradation products trafficked to subcellular organelles.

**Lactacysteine Pretreatment Prevents EGF-induced Claudin-2 Protein Degradation**—Although Northern blot analysis showed that EGF decreased steady-state levels of claudin-2 mRNA, there was a relatively greater decrease in claudin-2 protein content in the detergent-insoluble fraction at early time points. We therefore determined whether pretreatment of the cells with an inhibitor of ubiquitination, lactacysteine (45), before EGF treatment would affect the EGF-induced decrease in claudin-2 protein expression. Experiments were performed as described above, except that one group of cells received lactacysteine (5 μM) for 30 min before EGF treatment, and samples were collected at 4 and 8 h post-EGF treatment. Lactacysteine pretreatment almost completely prevented the EGF-induced decrease in claudin-2 content in the detergent-insoluble fractions at 4 h post-EGF treatment and partially prevented the decrease after 8 h of EGF treatment. In contrast, lactacysteine

**EGF Regulates TER in MDCK Cells**

A

![Graph](http://example.com/graph1.png)

**Fig. 1. Effect of EGF on TER in MDCK II cells.** A, EGF (100 ng/ml) was added to the media of polarized and quiescent MDCK II cells plated on 12-mm filters. TER was measured as described under "Experimental Procedures." The values presented are means ± S.E. and are representative of at least three independent experiments. Pretreatment of cells with a specific EGFR tyrosine kinase inhibitor, PD153035 (2.5 μM), prevented the EGF-induced increase in TER. B, pretreatment of cells with PD98059 (50 μM), a specific inhibitor of MAPK (ERK1/2), and staurosporine (50 nM), a broad-range inhibitor of protein kinase C, prevented the EGF-induced increase in TER. LY294002 (1 μM) and wortmannin (100 nM), pharmacological inhibitors of phosphatidylinositol 3-kinase activation, and SB203580 (20 μM), a specific p38 MAPK inhibitor, did not inhibit the EGF-induced increase in TER. CON, control.
had no effect on the EGF-induced decrease in claudin-2 protein content in the detergent-soluble fraction at the same time points (Fig. 3D). EGF Induces Intracellular Redistribution and Increases Protein Synthesis of Claudin-1—Overexpression of claudin-1 has been shown to increase TER and the number of TJ strands in MDCK II cells (46, 47), and bryostatin-1-induced TJ tightness in T84 epithelial cells correlates with enhanced membrane association of claudin-1 (48). In this study, in contrast to claudin-2, EGF treatment induced significant increases in claudin-1 expression selectively in the detergent-insoluble fractions within 1 h after EGF treatment, and claudin-1 expression was 213 and 233% of the control values after 8 and 18 h of EGF treatment, respectively (Fig. 4A). Interestingly, EGF treatment also induced a simultaneous and progressive decrease in claudin-1 protein content in the detergent-soluble fraction as early as 1 h post-EGF treatment, with protein expression in this fraction reaching a nadir (65% compared with the control value) after 8 h of EGF treatment (Fig. 4A). After 18 h of EGF treatment, claudin-1 was slightly higher in the detergent-soluble fraction compared with 8-h post-EGF treatment values. This suggests that the EGF-induced increase in the membrane-specific claudin-1 protein expression is the result of both translocation of the protein from cytoplasmic fractions and increased protein synthesis. The timing of the EGF-induced increases in claudin-1 expression paralleled the EGF-induced increases in TER. Interestingly, the shift in subcellular localization and the increase in claudin-1 protein expression specific to detergent-insoluble fractions observed at 18 h post-EGF treatment remained unchanged at 48 h post-treatment (data not shown). Pretreatment of cells with PD153035 completely blunted the EGF-induced changes in claudin-1 expression (Fig. 4B). Confocal microscopic analysis indicated weak lateral membrane and intense perinuclear/nuclear staining for claudin-1 in control cells. Increased lateral membrane staining was observed after 8 h of EGF treatment, with predominant immunolocalization at the lateral membrane by 18 h (Fig. 4C).

EGF Induces Increased Protein Synthesis of Claudin-3 and -4—Overexpression of claudin-4 in MDCK II cells increases TER through selective regulation of ion flux (49). Although the functional role of claudin-3 in the regulation of TER in MDCK cells has not yet been determined, recent studies have shown that claudin-3 is expressed exclusively in the tight epithelia (50). Similar to claudin-1, EGF treatment also induced significant increases in claudin-3 and -4 expression selectively in the detergent-insoluble fractions within 1 h after EGF treatment, with expression increased by 184 and 263% at 8 h and by 202 and 295% at 18 h, respectively (Figs. 5A and 6A), which remained unchanged at 24 and 48 h post-EGF treatment. In contrast to claudin-1, claudin-3 and -4 protein content in the detergent-soluble fractions was decreased by only 16 and 14%, respectively, after 8 h of EGF treatment (Figs. 5 and 6A). Pretreatment of cells with PD153035 also prevented EGF-induced changes in claudin-3 and -4 expression (Figs. 5 and 6B).
These two Claudins demonstrated patterns of expression distinct from those of Claudin-1. In control cells, Claudin-3 exhibited weak but predominant lateral membrane staining, confirming the extraction of relatively higher amounts of Claudin-3 from the detergent-insoluble fractions compared with the detergent-soluble fractions (Fig. 5C), whereas Claudin-4 exhibited apical/submembranous immunolocalization (Fig. 6C). After EGF administration, lateral membrane staining for Claudin-3 progressively increased, with intense lateral membrane staining detectable after 18 h (Fig. 5C). For Claudin-4, EGF induced distinct lateral membrane staining between the apical/submembranous rings by 8 h; and after 18 h of treatment, the lateral membrane and submembranous staining merged together to provide predominant lateral membrane staining (Fig. 6C), consistent with the marked increase in Claudin-4 extracted from the detergent-insoluble fraction at that point.

Effect of Cycloheximide on EGF-induced Changes in Claudin Intracellular Localization—Because EGF treatment induced a significant decrease in Claudin-1 protein content in the detergent-soluble fractions and a simultaneous increase in protein content in the detergent-insoluble fractions, we investigated whether these alterations would occur if protein synthesis was inhibited. Experiments were performed as described above, except that one group of cells was pretreated with Cycloheximide (100 ng/ml) for 30 min before adding EGF. Cycloheximide pretreatment partially inhibited the EGF-induced increases in Claudin-1 expression observed in the detergent-insoluble fractions; however, it decreased the Claudin-1 protein content in the detergent-soluble fractions to 34 and 12% of control values at 8 and 18 h, respectively (Fig. 7A), compared with 65 and 84% in the cells without cycloheximide treatment (Fig. 4A). At the same time, the percent increases in Claudin-1 protein content in the detergent-insoluble fractions were 134 and 154% at 8 and 18 h post-EGF treatment in cells pretreated with cycloheximide, respectively (Fig. 7A), compared with 213 and 233% in cells not treated with cycloheximide (Fig. 4A). Cycloheximide pretreatment did not prevent the EGF-induced redistribution of Claudin-1 from the detergent-soluble to -insoluble fractions.

Fig. 3. Effect of EGF on Claudin-2 localization. A, equal amounts of protein from Triton X-100-soluble (S, Tri-Sol) and -insoluble (I, Tri-Insol) fractions were prepared as described under “Experimental Procedures,” separated by 12% SDS-PAGE, and immunoblotted with anti-Claudin-2 antibody. The amount of Claudin-2 protein extracted from control samples in the detergent-soluble fraction was taken as 100% and was used as a reference to determine the ratio between soluble and insoluble fractions and the changes in protein expression. The values presented are means ± S.E. B, shown is the effect of PD153035 (2.5 μM) on the EGF-induced decrease in Claudin-2 protein expression after 8 h of EGF treatment, as determined by Western blot analysis. C, immunofluorescence microscopy was performed using rabbit anti-Claudin-2 polyclonal antibody with MDCK cells left untreated (left panel) or fixed at 8 h (middle panel) or 18 h (right panel) post-EGF treatment. Note the decrease in lateral membrane staining following EGF treatment (arrow). Punctuate granular staining was observed after 18 h of EGF treatment (arrowheads). The lower panels show the XZ sectioning of the corresponding panels (original magnification ×400). D, shown is the effect of lactacysteine (Lac; 5 μM) on the EGF-induced decrease in Claudin-2 protein expression after 4 and 8 h of EGF treatment, as determined by Western blot analysis. m, minutes.
Cycloheximide pretreatment induced a greater decline in claudin-3 protein content in the detergent-soluble fraction and almost completely prevented EGF-induced increases in the detergent-insoluble fraction-specific expression of claudin-3 (Fig. 7B). Although cycloheximide pretreatment had little effect on claudin-4 protein content in the detergent-soluble fractions, the detergent-insoluble fraction-specific claudin-4 protein expression was markedly decreased at 8 and 18 h to 119 and 123% in cycloheximide-treated cells, respectively, compared with 263 and 295% in cells that did not receive cycloheximide (Fig. 6A). Cycloheximide pretreatment had no effect on the EGF-induced decrease in claudin-2 expression in either the detergent-soluble or -insoluble fractions (data not shown).

To investigate whether EGF treatment has any effect on the adherens junction, the protein expression and detergent solubility of E-cadherin and β-catenin were determined. Similar to ZO-1 and occludin, the protein expression and/or intracellular localization of E-cadherin and β-catenin was not significantly affected by EGF treatment at any time point (Fig. 8B). Cycloheximide pretreatment had no significant effect on the protein expression or intracellular localization of ZO-1, occludin, E-cadherin, or β-catenin (data not shown).

EGF Administration Alters Claudin Expression in Subconfluent and Non-polarized MDCK II Cells—To determine whether the EGF-dependent changes in claudin expression are specific to cells grown under polarized conditions, we investigated the effect of EGF treatment on quiescent MDCK II cells grown as small subconfluent colonies on glass coverslips. In control cells, no BrdUrd staining was observed, and predominant lateral membrane staining was observed for claudin-1 and -2 (Fig. 9). Claudin-2 lateral membrane localization was accompanied by nuclear staining in some cells. EGF treatment induced significant cell proliferation compared with the control cells, as determined by intense BrdUrd nuclear staining; BrdUrd incorporation was mostly confined to the cells at the periphery of the colonies. In contrast, the lateral membrane and nuclear staining for claudin-2 were completely lost in EGF-treated cells, whereas in...
creased lateral membrane staining for claudin-1 was detected (Fig. 9).

Pattern of Hepatic Growth Factor (HGF)-induced Alterations in Claudin Expression Is Distinct from That of EGF—To determine whether these growth factor-induced changes in claudin expression are specific to EGFR activation, responses to HGF were examined. HGF is known to decrease TER in many cell lines (51, 52); and unlike EGF, HGF induces epithelial-mesenchymal transitions in MDCK II cells grown on plastic (53). Quiescent and polarized MDCK cells were treated with 20 ng/ml HGF, a concentration known to induce cell scattering in MDCK cells (54); and changes in claudin expression were determined. In contrast to EGF, HGF treatment induced a marked increase in claudin-1 and -4 protein content in the detergent-soluble fractions within 15 min after treatment, which reached a maximum value after 18 h. Interestingly, no major differences were observed in claudin-3 expression, except for a small and delayed decrease in protein content in the detergent-soluble fraction. For claudin-2 protein expression, a small decrease was observed in total claudin-2 expression in response to HGF treatment (data not shown), but this decrease was mainly in the detergent-soluble fraction (Fig. 10). It is noteworthy that claudin-1, -2, -3, and -4 expression in the detergent-insoluble fraction was largely unaffected by HGF treatment.

DISCUSSION

In this study, using polarized and quiescent MDCK II cells as a model, we have shown that EGFR activation regulates epithelial TJs through selective and differential modulation of both expression and intracellular localization of the integral TJ proteins, claudins. This modulation of claudin expression and localization was accompanied by a 3-fold increase in TER in response to EGFR activation. These EGF-induced changes in TJ composition appear to be specific to claudins because no significant changes in the protein expression and/or intracellular localization of either adherens junction proteins (E-cadherin or β-catenin) or TJ-associated proteins (ZO-1 and occludin) were observed in response to EGF. Another growth factor, HGF, which is known to decrease TER in many epithelial cell lines (51, 52) and to induce epithelial-mesenchymal transitions in MDCK II cells (53), did not reproduce these EGF-induced alterations in claudin expression and localization.

Of the claudins identified to date, MDCK II cells express claudin-1, -2, -3, and -4, whereas MDCK I cells, a clonal variant of MDCK II cells, are deficient in claudin-2 expression (23, 55). MDCK I cells exhibit 40–60-fold higher TER compared with MDCK II cells (23, 56), and overexpression of claudin-2 in MDCK I cells decreases TER to the level in MDCK II cells (23). Recently, overexpression of claudin-2 in another high resistance clone of MDCK cells (MDCK7) was shown to decrease
TER through induction of cation-selective channels in the TJ (57). These observations suggest a functional role for claudin-2 in the regulation of TER in MDCK cells such that expression of the claudin-2 protein is inversely proportional to TER. This hypothesis is further supported by the fact that, in the kidney, claudin-2 is predominantly expressed in the proximal tubule, which is considered to be a “leaky” epithelium (50, 57, 58, 60).

The observation in this study that EGF administration to MDCK II cells led to increased TER accompanied by marked decreases in total claudin-2 expression and lateral membrane localization supports the hypothesis that claudin-2 protein expression could be inversely proportional to TER.

The EGF-induced increases in TJ expression of claudin-1, -3, and -4 observed in this study are also consistent with previous observations concerning the role of these claudins in TJ function. Overexpression of claudin-1 induces formation of TJs in fibroblasts (61) and increases TER in MDCK II cells (46, 47). Claudin-1-deficient mice manifest a severely compromised epidermal barrier and die within 24 h of birth due to dehydration (62). Furthermore, bryostatin-1-induced tightness of the TJs correlates with enhanced membrane association of claudin-1 in T84 epithelial cells (48). Overexpression of claudin-4 has been shown to increase TER in MDCK II cells (49), and a recent study describing segmental distribution of various claudins in the mouse nephron showed that claudin-3 and -4 are expressed mainly in the “tight” epithelia of the kidney and that only claudin-1, -3, -4, and -8 are expressed in the collecting segment of the kidney nephron (60), the segment with the highest TER.

This study suggests that the intracellular mechanisms for regulation of individual claudins by EGF may also be different. Decreased claudin-2 expression appears to be the result of decreased transcription as well as increased intracellular degradation. Increases in claudin-1, -3, and -4 were due in part to increased protein synthesis, but the cycloheximide studies suggested that translocation of existing claudin-1 protein from the fractions containing cytosolic and loosely membrane-bound proteins to the cytoskeletal fractions may partially underlie early increases in TJ specific expression. In contrast, increases in TJ expression of claudin-3 and -4 proteins appear to be predominantly due to synthesis of new protein and suggest that newly synthesized claudin-3 is targeted to both detergent-soluble and -insoluble fractions, whereas newly synthesized claudin-4 is targeted predominantly to the detergent-insoluble fraction. However, further studies will be required to examine intracellular trafficking of these proteins. Furthermore, the fact that EGF induced cell proliferation as well as changes in claudin expression in subconfluent cells under non-polarized culture conditions suggests that these effects, although regu-
lated by the same receptor, are distinct from each other.

The EGF-induced increase in TER was completely prevented by inhibiting EGFR tyrosine phosphorylation. Furthermore, the increase in TER was inhibited by pretreatment of cells with PD98059, a specific inhibitor of MAPK (ERK1/2), as well as by staurosporine, a broad-range inhibitor of protein kinase C signaling pathways. EGFR activation is known to induce MAPK activation through the Ras-Raf-MAPK pathway (63), and protein kinase C has been shown to be downstream of EGFR activation (64). However, further studies will be required to determine the specific targets of these kinases.

Based on localization studies in the kidney, it has been postulated that junctional complexity and the designation of tight versus leaky epithelia are dependent upon both the expression of specific claudins and the ratio of claudin family

![FIG. 7. Effect of cycloheximide (100 ng/ml) on EGF-induced changes in claudin expression. A, claudin-1; B, claudin-2; C, claudin-3. S and Tri-Sol, Triton X-100-soluble; I and Tri-Insol, Triton X-100-insoluble; m, minutes.](image)

![FIG. 8. Effect of EGF treatment on protein expression and/or intracellular redistribution of TJ-related proteins ZO-1 and occludin (A) and adherens junction proteins E-cadherin and β-catenin (B). m, minutes.](image)

![FIG. 9. EGF-induced BrdUrd incorporation and changes in claudin-1 and -2 expression in subconfluent MDCK II cells plated under non-polarized culture conditions. Upper panels, untreated control cells; lower panels, cells after 22 h of EGF treatment. a, anti-BrdUrd antibody; b, anti-claudin-1 antibody; c, anti-claudin-2 antibody. Original magnification was ×400.](image)
members expressed (20, 23, 65). This study provides direct evidence supporting this concept and indicates that differential regulation of members of the Claudin family of proteins in MDCK cells and suggest one potential mechanism by which EGFR may promote differentiation and integrity of polarized epithelia. Furthermore, this study provides evidence in support of the hypothesis that changes in TER are the outcome of the ratio of the different Claudins expressed in the TJ. To our knowledge, this is the first report showing that EGFR regulates Claudin expression and that activation of the same receptor can differentially regulate different Claudins.

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FIG. 10. Effect of HGF treatment (20 ng/ml) on Claudin expression and intracellular distribution in polarized and quiescent MDCK II cells. Experiments were performed and the detergent-soluble and -insoluble fractions were prepared exactly as described for experiments using EGFR, soluble, I; insoluble, m, minutes.
Epidermal Growth Factor Receptor Activation Differentially Regulates Claudin Expression and Enhances Transepithelial Resistance in Madin-Darby Canine Kidney Cells

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J. Biol. Chem. 2004, 279:3543-3552.
doi: 10.1074/jbc.M308682200 originally published online October 30, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308682200

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