Helicobacter pylori Dysregulation of Gastric Epithelial Tight Junctions by Urease-Mediated Myosin II Activation

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Background & Aims: ***Helicobacter pylori*-induced gastritis predisposes to the development of gastric cancer. Increased epithelial tight junction permeability and alterations in apical-junctional complexes are also associated with an increased risk of carcinogenesis. Phosphorylation of myosin regulatory light chain (MLC) by MLC kinase (MLCK) regulates tight junction function. We determined whether MLCK was activated by *H pylori* and defined the mechanisms through which such activation dysregulates gastric epithelial barrier function. **Methods:** MKN28 gastric epithelial cells were cocultured with the *H pylori cag*$^+$ strain 60190 or *cagA*$^-$, *cagE*$^-$, *ureB*$^-$, or *vacA*$^-$ mutants. MLC phosphorylation and barrier integrity were determined by immunoblot analysis and transepithelial electrical resistance measurements, respectively. Localization of the tight junction protein occludin was determined by immunocytochemistry in MKN28 cells and INS-GAS mice. **Results:** *H pylori* induced a progressive loss of barrier function that was attenuated by inactivation of *ureB,* but not *cagA, cagE,* or *vacA*. Reductions in transepithelial electrical resistance were also dependent on functional urease activity. *H pylori* increased MLC phosphorylation in epithelial monolayers; this was significantly decreased by inhibition of MLCK or Rho kinase or by loss of UreB. *H pylori* infection of either cultured monolayers or hypergastrinemic INS-GAS mice induced occludin endocytosis, reflecting cytoskeletally mediated disruption of tight junctions. **Conclusions:** *H pylori* increases MLC phosphorylation, occludin internalization and barrier dysfunction in gastric epithelial cells. This process requires functional urease activity and is independent of the *cag* pathogenicity island or VacA. These data provide new insights into the mechanisms by which *H pylori* disrupts gastric barrier function.**

Helicobacter pylori colonizes the gastric mucosa of over half of the world's population. Although most infected individuals only develop chronic gastritis, a subset progress to gastric adenocarcinoma.[1–3] *H pylori* strains that possess a functional *cag* pathogenicity island (PAI) induce more severe disease than *cag*$^-$ strains.[4,5] The *cag* PAI contains several genes that encode components of a type IV secretion system which translocates bacterial products such as CagA into host cells following microbial contact. An independent *H pylori* locus linked with increased disease risk is *vacA*, which encodes the secreted toxin VacA.[3] VacA induces apoptosis in vitro and suppresses T cell responses to *H pylori*, which may contribute to the longevity of infection.[1,3] Another virulence factor required for colonization within the acidic gastric environment is urease.[6] *H pylori* urease is a complex consisting of multiple proteins encoded by 2 structural subunit genes, *ureA* and *ureB,* and 7 associated genes, and functions to hydrolyze urea to ammonia and CO_2.[7] In addition to modulating pH, urease, and ammonia produced by functional urease activity, can mediate tissue inflammation and injury.[8]

Gastric mucosal barrier function is essential for preventing potentially harmful elements present in the gastric lumen from gaining access to the gastric mucosa. Barrier function is compromised in *H pylori*-induced gastritis[9] and in intestinal epithelial cells infected with *H pylori.*[10] Tight junctions are dynamic structures located at the most apical region of cell-cell contact points and play critical roles in maintenance of barrier function, cell polarity, and intercellular adhesion. Disruption of tight junction complexes is associated with a variety of human diseases including cancers of the gastrointestinal tract.[11]

H pylori adhere to gastric epithelial cells in close proximity to tight junctions[12,13] and can alter localization of the component proteins that constitute junctional complexes.[10,13,14] In addition, *H pylori* has been detected in intraepithelial intercellular spaces directly beneath tight junctions leading investigators to hypothesize that the tight junction may be a site of entry for the bacterium.[15] Recently, *H pylori* has also been identified within the

Abbreviations in this paper: MLC, myosin II regulatory light chain; MLCK, myosin light chain kinase; MYPT1, myosin phosphatase target subunit 1; PIK, membrane-permeant inhibitor of MLCK; TER, transepithelial electrical resistance; ZO-1, zonula occludens-1.

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lamina propria, gastric lymph nodes and within the intracellular canaliculi of parietal cells.[16,17] Specific *H pylori* constituents have been reported to be involved in disruption of tight junctions. For example, ectopic expression of CagA in polarized MDCK cells increases tight junction permeability,[18] while addition of purified VacA to MDCK cells disrupts barrier function.[19]

Tight junction complexes are composed of integral membrane proteins, such as occludin, claudins, and junctional adhesion molecules (JAMs),[11] as well as membrane-associated proteins such as zonula occludens-1 (ZO-1). Occludin has been implicated in regulation of barrier function[20] and is linked directly to the actin cytoskeleton via its C-terminus[21] and indirectly through its interactions with ZO-1.[22] Expression of occludin at the level of the tight junction is disrupted by *H pylori* in cultured canine duodenal epithelial cell monolayers.[10]

Cytoskeletal contraction induced by phosphorylation of myosin II regulatory light chain (MLC) can regulate tight junction barrier function in response to physiological and pathophysiological stimuli and can result in disruption of tight junction structure.[23,24] Phosphorylation of MLC is regulated by 2 distinct signal transduction cascades. One pathway requires phosphorylation of MLC by MLCK, while the second pathway involves Rho-associated kinase (Rho kinase) activation. Rho kinase can either directly phosphorylate MLC or can increase phosphorylation of MLC by phosphorylating myosin phosphatase, thus inhibiting its phosphatase activity.[25]

H pylori selectively colonizes gastric epithelium in humans and in rodent models of infection; however, the mechanisms by which *H pylori* alters apical junctional complexes in gastric epithelial cells are not well defined. Therefore, the aim of this study was to define the bacterial and host epithelial constituents involved in gastric epithelial cell barrier dysfunction induced by *H pylori*.

Materials and Methods

Cell culture and reagents. MKN28 gastric epithelial cells were cultured at 37°C under 5% CO_2/95% O_2 in RPMI medium 1640 (GIBCO/BRL, Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Sigma, St Louis, MO) and gentamycin (20 μg/mL). To form monolayers, cells were grown on polyester Transwell (Sigma) permeable tissue culture inserts (0.4 μm pore size). The Rho-associated kinase inhibitor Y-27632 (Calbiochem, San Diego, CA) was used at a final concentration of 10 μM. Recombinant *H pylori* urease was obtained from Orovax (Cambridge, MA) and was added apically at a final concentration of 25 μg/mL. Ammonium chloride (Sigma) was used at a final concentration of 10 mM. Membrane permeant inhibitor of MLC kinase (PIK) was added apically and used at a final concentration of 250 μM.

***H pylori* strains and culture conditions.** The *H pylori cag*$^+$ strains 7.13 and 60190 were grown in *Brucella* broth with 10% FBS for 16 hours, harvested by centrifugation and cocultured with MKN28 cells at a multiplicity of infection (MOI) of 100:1. Isogenic *cagA*$^-$, *cagE*$^-$, *vacA*$^-$, and *ureB*$^-$ and null mutants were constructed in strain 60190 and were selected with kanamycin (25 μg/mL) as previously described.[26] Urease test media were used to confirm that the *ureB* mutant does not produce urease.[27]

Animals and *H pylori* challenge. All procedures were approved by the Institutional Animal Care Committee of Vanderbilt University. INS-GAS mice were orogastrically challenged with either sterile *Brucella* broth or *H pylori* strain 7.13 (5×10^9 cfu) as previously described.[28] Mice were euthanized 21 days after inoculation. One-half of the glandular stomach was embedded in OCT compound for immunohistochemical analysis. The remaining glandular stomach was homogenized in sterile PBS, plated on selective Trypticase (BBL; Becton Dickinson, Franklin Lakes, NJ) soy agar plates and incubated at 37°C for 5–6 days. All mice challenged with *H pylori* were successfully infected.

Measurement of transepithelial electrical resistance (TER). MKN28 cells were cultured on 0.33 cm^2 Transwell (Sigma) supports. TER was measured using a Millipore Millicell-ERS apparatus (Billerica, MA). TER was calculated as ohms × cm^2 by multiplying TER by the surface area of the monolayer. The resistance of the Transwell (Sigma) membrane was subtracted from all measurements, as previously described.[23]

Analysis of cell viability and apoptosis. Cell viability was determined using a trypan blue exclusion assay. A 1:1 suspension of MKN28 cells and 0.4% trypan blue solution was prepared and cells were counted using a hemocytometer. Apoptosis was assessed using the Caspase-Glo 3/7 assay (Promega, Madison, WI), according to the manufacturer's instructions.

Immunofluoresence. Gastric epithelial cells were cultured on 4-well chamber-slides (Nalge Nunc, Roskilde, Denmark), washed twice in 1X PBS, and formalin-fixed. Cells were permeabilized using 1X PBS containing 0.1% Triton X-100 (30 minutes, room temperature), washed 3 times in 1X PBS, and non-specific sites were blocked by incubation in 1% BSA. Formalin-fixed MKN28 cells were stained with rabbit anti-occludin antibody (1:400; Zymed Laboratories, San Francisco, CA) and Alexa Fluor 488-conjugated anti-rabbit IgG antibody (1:200; Molecular Probes, Eugene, OR). Images were captured using a Zeiss LSM 510 Confocal Microscope (Oberkochen, Germany). Image acquisition was performed using Zeiss LSM Image Examiner 3.2 software. OCT-embedded gastric sections were stained with mouse anti-occludin (Zymed) followed by Alexa fluor 594 conjugated anti-mouse IgG antibody Hoechst 33342. Images were captured using a Leica DM-LB microscope (Solms, Germany) under the control of Metamorph software (Molecular Dynamics, Amersham Biotech, Sunnyvale, CA).

Western blot analysis. For analysis of total cellular occludin, MKN28 cells were cultured for 72 hours and then cocultured with or without *H pylori* for 48 hours. Cells were lysed in RIPA buffer (50mM Tris, 150mM NaCl, 1% Triton X-100, 0.1% SDS) for 30 minutes at 4°C, centrifuged at 13,000 rpm for 30 minutes and the supernatant was collected. For analysis of subcellular occludin, detergent soluble and insoluble MKN28 cell fractions were separated by differential detergent extraction as previously described.[29] Cells were cultured for 72 hours to confluence, cocultured with *H pylori* for 48 hours, and then lysed in lysis buffer (0.5% Triton X-100, 150mM NaCl, 50mM Tris-HCL) for 30 minutes at 4°C with rotation. Cells were centrifuged (14,000 rpm, 10 minutes) and the supernatant was collected as the detergent soluble fraction. The pellet was then resuspended in lysis buffer containing 0.02% SDS, incubated for 30 minutes at 4°C, centrifuged (14,000 rpm, 10 minutes) and the supernatant was collected as the detergent insoluble fraction. Protein concentrations were quantified by the Bradford assay (Pierce, Rockford, IL). Proteins (20 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Occludin was detected using an anti-occludin antibody (1:400; Zymed Laboratories) in combination with goat anti-rabbit IgG (1:20,000; Sigma) HRP-conjugated secondary antibody. Samples were re-probed with a goat anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as an additional control for equal loading.

For analysis of MLC phosphorylation and MYPT phosphorylation, confluent monolayers of MKN28 cells were cocultured with *H pylori*, extracted in reducing sample buffer buffer (0.75M Tris-base, 30% glycerine, 6% SDS, 3% bromophenol-blue, 7.5% BME), and heated at 100°C for 5 minutes. Myosin light chain phosphorylation was detected using an anti phospho-myosin light chain 2 antibody (1:400; Cell Signaling, Danvers, MA) and samples were re-probed with an anti-myosin antibody (1:400; Sigma). Primary antibodies were detected using goat anti-mouse HRP-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology). Phosphorylated MYPT was detected using an anti phospho-MYPT1 antibody (1:200; Santa Cruz Biotechnology) and samples were re-probed with an MYPT1 antibody (1:200; Santa Cruz Biotechnology). Primary antibodies were detected using donkey anti-goat HRP-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology) and goat anti-rabbit HRP-conjugated secondary antibody (1:20,000; Sigma), respectively. Primary antibodies were visualized by Western Lightning Chemiluminescence Reagent Plus, and band intensities were quantified using the ChemiGenius Gel Bio Imaging System (Syngene, Frederick, MD).

Statistical analysis. Results are expressed as means ± s.e.m. Comparisons were made using the Student *t* test, and were considered significant at $P < .05$.

Results

H pylori *Alters Barrier Function in Gastric Epithelial Cells*

MKN28 cells are human gastric epithelial cells that form confluent monolayers. To determine whether this was an appropriate model to investigate barrier function, we first established that MKN28 cells form functi-

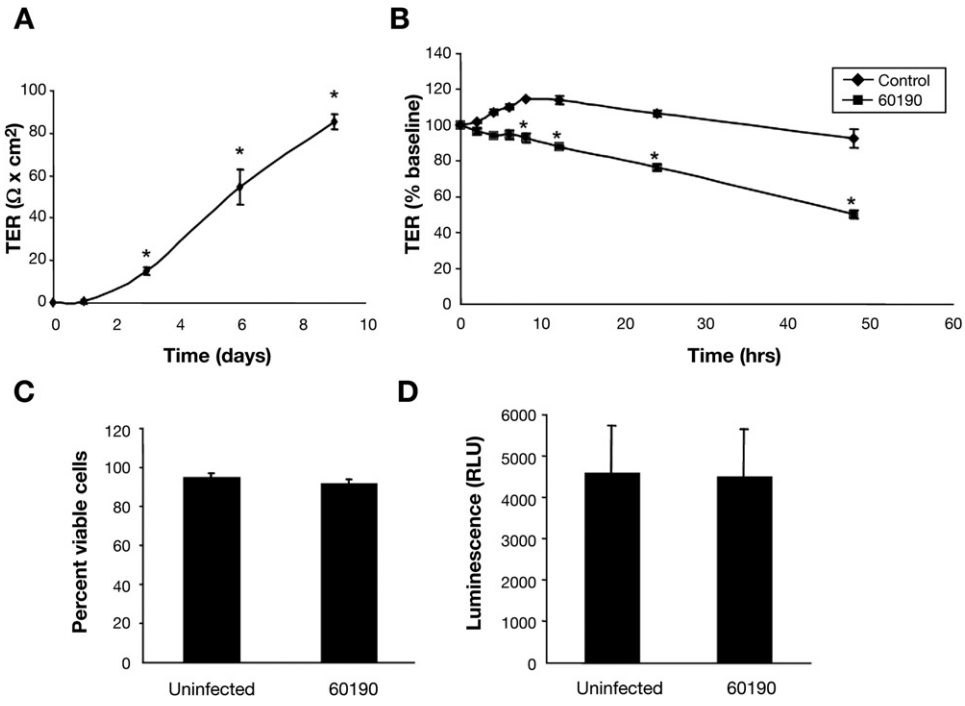

Figure 1. *H pylori* infection decreases TER in MKN28 cells. (A) TER of MKN28 cell monolayers was measured for 9 days. Cells reached confluence at day 3 (*$P < .05$ vs TER at day 0). (B) Coculture with *H pylori* strain 60190 (48 hours, MOI 100:1) significantly decreased TER compared with uninfected monolayers. (C) Coculture with *H pylori* strain 60190 (24 hours) did not alter MKN28 cell viability. (D) Caspase 3/7 activity was not changed in MKN28 cells following coculture with *H pylori* strain 60190 (8 hours). Data are expressed as means ± s.e.m., *$P < .05$, n=3.

onal tight junctions. MKN28 cells were grown to confluence and transepithelial electrical resistance (TER) was quantified. We found that as MKN28 cells reached confluence, there was a progressive increase in TER, indicating that these cells form functional tight junctions (Figure 1*A*).

To determine the effect of *H pylori* on tight junction barrier function, we next measured TER in MKN28 cells cocultured with a strain, 60190, that induces pathogenic gastric epithelial cell responses in vitro.[30] Coculture with *H pylori* caused a progressive decrease in TER in a time-dependent manner compared with uninfected control monolayers (Figure 1*B*). TER loss was apparent within 8 hours and fell by 50% following 48 hours of coculture with *H pylori*. Antibiotic treatment of infected MKN28 cells re-established TER to control levels (data not shown), indicating that loss of barrier function was clearly induced by *H pylori* and was reversible.

To determine that the decrease in TER was not due to *H pylori*-induced alterations in cell viability or apoptosis, we measured MKN28 cell viability and assessed caspase activity. Figure 1*C* and *D* demonstrate that coculture of MKN28 cells with *H pylori* did not significantly alter cell viability or caspase activity, indicating that, in this system, TER loss following coculture with *H pylori* is likely due to modulation of tight junction barrier function.

H pylori-*Induced Disruption of Tight Junction Barrier Function Is Mediated by Functional Urease*

Having determined that *H pylori* induced a time-dependent decrease in TER, we next examined the effects of *cagA*, *cagE*, *vacA*, and *ureB* on these events, since these genes encode products that induce pathogenic epithelial responses.[13,18,31] MKN28 cells were cocultured with wild-type strain 60190 or its *cagA*, *cagE*, *vacA*, or *ureB* mutants. As expected, coculture with wild-type strain 60190 resulted in loss of TER, and inactivation of *cagA*, *cagE,* or *vacA* had no effect on barrier dysfunction (Figure 2*A*). However, decreases in TER were significantly attenuated in cells cocultured with the 60190 *ureB*$^-$ mutant strain (Figure 2*A*). These findings indicate that *ureB,* but not *cagA*, *cagE*, or *vacA*, is required for disrupting tight junction barrier function in MKN28 cells and is consistent with previous findings examining the effects of ammonium on TER in colonic epithelial monolayers.[31]

Having determined that *ureB* is required for *H pylori*-induced loss of TER, we next tested the ability of recombinant urease and the product of urease activity, ammonium chloride, to modulate TER in this gastric cell system. TER was not decreased in MKN28 cells exposed to urease that was functionally inactive; however, ammonium chloride decreased TER (Figure 2*B*), suggesting that TER is modified by the functional, but not the structural, effects of urease.

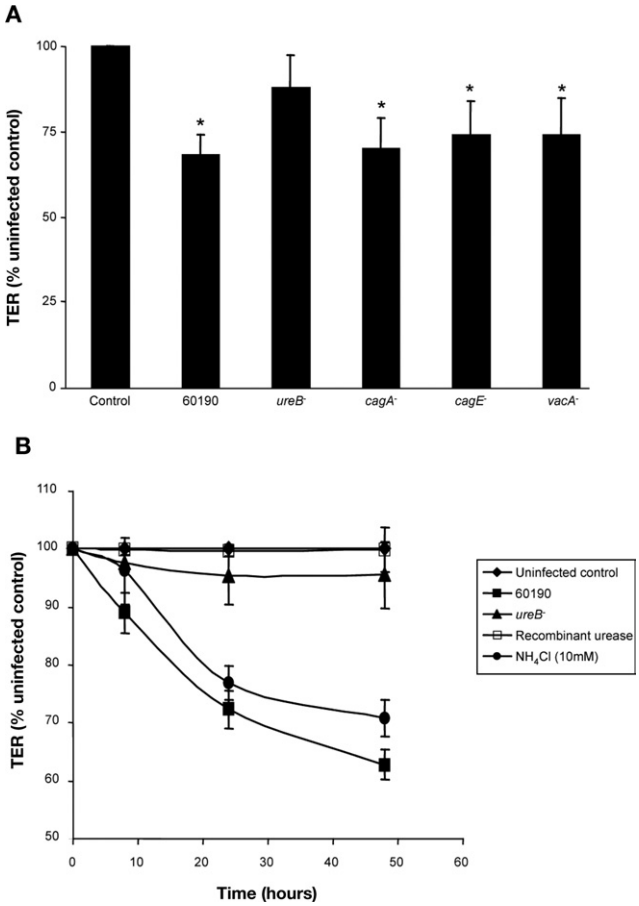

Figure 2. *H pylori*–induced decreases in TER are dependent on specific microbial genes. (*A*) *H pylori* wild-type strain 60190 (8 hours) decreased TER of MKN28 cells compared with uninfected controls and this was attenuated by inactivation of *ureB*. (*B*) *H pylori* wild-type strain 60190 decreased TER in MKN28 cells compared with uninfected controls and this was attenuated by inactivation of *ureB*. Addition of NH_4Cl (10mM) decreased TER similar to strain 60190 while addition of functionally inert recombinant urease (25μg/mL) had no effect. Data are expressed as means ± SEM, $^*P < .05$ vs uninfected cells, n=3.

Occludin Distribution Is Altered by H pylori

Occludin is a well-characterized tight junction component and has been reported to be disrupted in other nongastric cell systems by *H pylori*.[10] Therefore, we assessed localization of occludin in MKN28 cells by immunofluoresence. In uninfected monolayers, occludin was sharply localized to the cellular margins (Figure 3*A*). Similar to localization of occludin, the tight junction proteins ZO-1 and JAM-A were also localized to the cellular margins (data not shown). In contrast, by 48 hours of infection, the distribution of occludin was aberrant, with focal accumulation in the cytosol (Figure 3*B*). To extend these results, we cocultured MKN28 cells with another *H pylori* strain, 7.13, a rodent adapted isolate that induces gastric cancer in Mongolian gerbils and INS-GAS mice.[32] Mislocalization of occludin seen in response to strain 60190 was similarly induced by strain

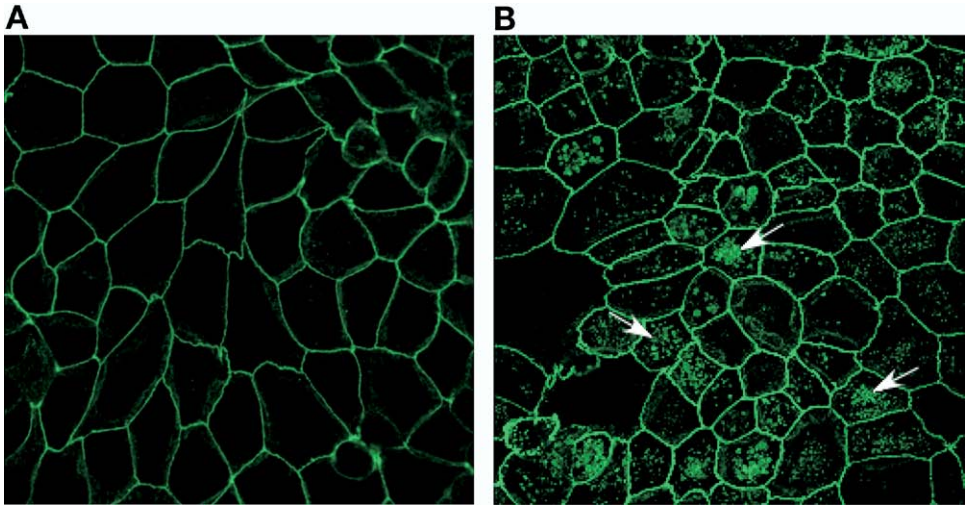

Figure 3. Coculture with *H pylori* induces occludin internalization in MKN28 cells. (*A*) Immunocytochemical localization of occludin (*green*) in uninfected MKN28 cells shows occludin localized to the cell membrane. (*B*) Occludin localization following coculture with *H pylori* strain 60190 (48 hours) showing redistribution of occludin from the cell membrane to focal areas within the cytosol. *Arrows* show occludin in the cytoplasm (bar, 50 μm).

7.13 (data not shown), indicating that these effects are not unique to a single *H pylori* strain.

Having determined that *H pylori* alters occludin distribution, we next examined the kinetics of this response. Exposure of MKN28 cells to *H pylori* strain 60190 for 8 hours induced occludin accumulation at contact points of three or more cells, but occludin was not visualized in the cytosol at this time point (Figure 4*B*). Following 24 hours of coculture, occludin staining at cellular margins was disrupted and nascent accumulation began to occur

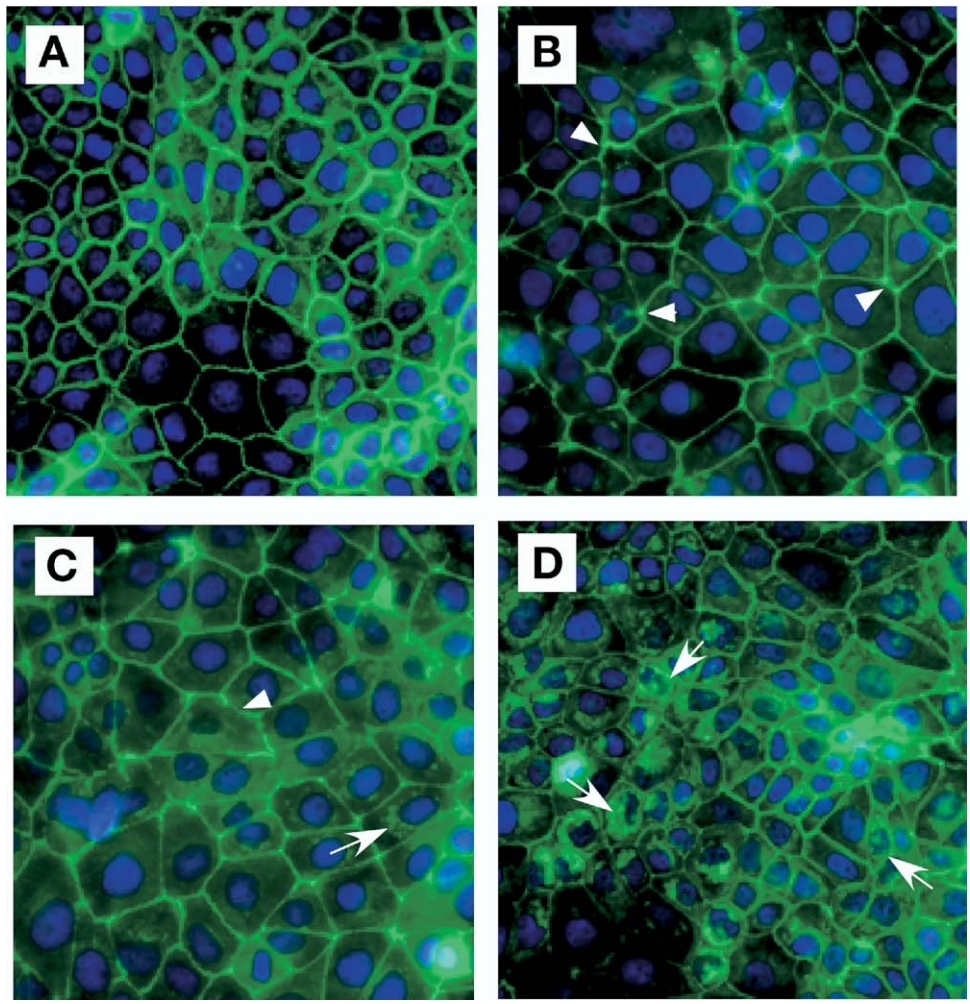

Figure 4. Time-dependent redistribution of occludin induced by *H pylori* strain 60190. Immunocytochemical localization of occludin in (*A*) uninfected MKN28 cells, (*B*) cells infected with *H pylori* for 8 hours showing accumulation of occludin at sites where 3 or more cells make contact, (*C*) cells infected for 24 hours demonstrating reduction of occludin at the cell membrane, and (*D*) 48 hours of infection revealing accumulation of occludin in the cytoplasm and discontinuous occludin staining at the cell membrane. *Arrowheads* indicate occludin at the cell membrane, *arrows* show occludin in the cytoplasm.

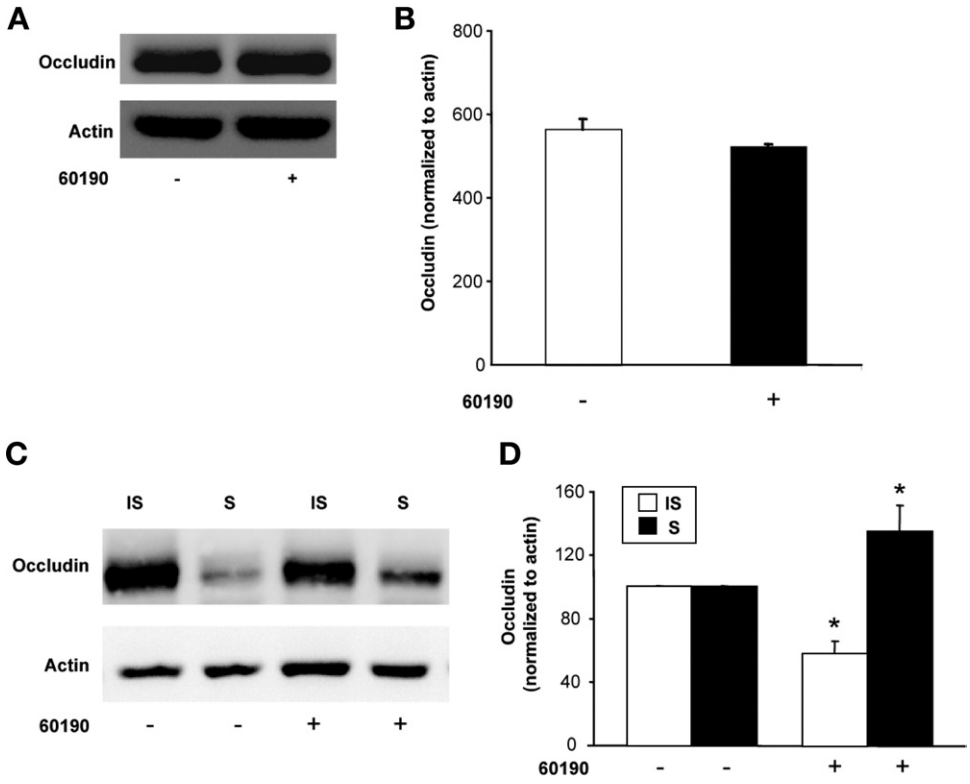

Figure 5. Levels of occludin following coculture with *H pylori* are decreased in the insoluble fraction and increased in the soluble fraction of MKN28 cells. MKN28 cells were cocultured for 48 hours with *H pylori* strain 60190 and occludin levels were determined by Western blotting. (*A*) Representative Western blot for occludin in total cell lysates and (*B*) densitometric analysis of occludin expression in 3 independent experiments showing no change in total occludin levels. (*C*) Representative Western blot for occludin in detergent-insoluble (IS) and detergent-soluble (S) MKN28 fractions in the presence or absence of strain 60190. (*D*) Densitometric analysis demonstrating loss of occludin from the insoluble fraction and increased occludin in the soluble fraction of *H pylori*-infected cells. Data are expressed as means ± SEM, $^*P < .05$, n=3.

in the cytosol of infected cells (Figure 4C). This phenotype was accentuated by 48 hours of *H pylori* infection (Figure 4*D*). To confirm these results, Western immunoblotting on cellular fractions was performed. Analysis of MKN28 whole cell lysates demonstrated that *H pylori* did not affect total cellular levels of occludin (Figure 5*A* and *B*). However, immunoblotting for occludin using soluble (cytosolic) and insoluble (membrane-associated) epithelial cell fractions confirmed that redistribution of occludin occurred via a reduction of occludin in the insoluble fraction in conjunction with an increase in occludin in the soluble fraction (Figure 5*C* and *D*).

H pylori *Increases MLCK Activity and Phosphorylation of MLC in Gastric Epithelial Cells*

To define the molecular mechanisms by which *H pylori* regulates occludin redistribution and alters tight junctions, we investigated whether this pathogen induced MLC phosphorylation, which regulates epithelial permeability, and is, in turn, regulated by MLCK. MKN28 cell monolayers were cocultured with *H pylori* strain 60190 and MLC phosphorylation was assessed by Western immunoblotting using a phospho-specific antibody. Strain 60190 significantly increased phosphorylation of MLC at 8 hours compared to uninfected cells (Figure 6). To determine whether phosphorylation of MLC by *H pylori* is regulated by MLCK, MKN28 cells were co-cultured with *H pylori* in the presence or absence of PIK (250 μM), a membrane-permeant inhibitor of MLCK.[33] Phosphorylation of MLC by *H pylori* was abolished by the addition of PIK (Figure 6*A* and *B*) indicating that MLCK is required for modification of MLC by *H pylori*.

Having established that inactivation of *ureB* attenuates *H pylori*-induced decreases in TER and that *H pylori* increases phosphorylation of MLC, we next determined whether phosphorylation of MLC is dependent on UreB. MKN28 cells were cocultured with wild-type strain 60190 or its isogenic *ureB* mutant and MLC phosphorylation was determined by Western blot analysis. As shown in Figure 6*C*, phosphorylation of MLC by *H pylori* was significantly attenuated by inactivation of *ureB* which mirrored results investigating the effects of UreB on TER (Figure 2).

To assess whether MLCK activation was similarly required for *H pylori*-induced reduction of TER, we cocultured MKN28 cells with *H pylori* in the presence or absence of PIK and quantified TER. Addition of PIK abolished the reduction in TER seen in response to *H pylori*, while PIK alone had no effect on TER in uninfected cells (Figure 6*D*). These results indicate that *H pylori* increases MLCK activity in MKN28 cells, which subsequently leads to a loss of TER, and that these events are mediated by UreB.

Rho Kinase Is Required for H pylori*–Induced Phosphorylation of MLC in Gastric Epithelial Cells*

In addition to MLCK, MLC phosphorylation can be regulated by Rho kinase and *H pylori* has previously been shown to activate Rho in vitro.[30,34] Rho kinase can either directly phosphorylate MLC or, alternatively, can inhibit

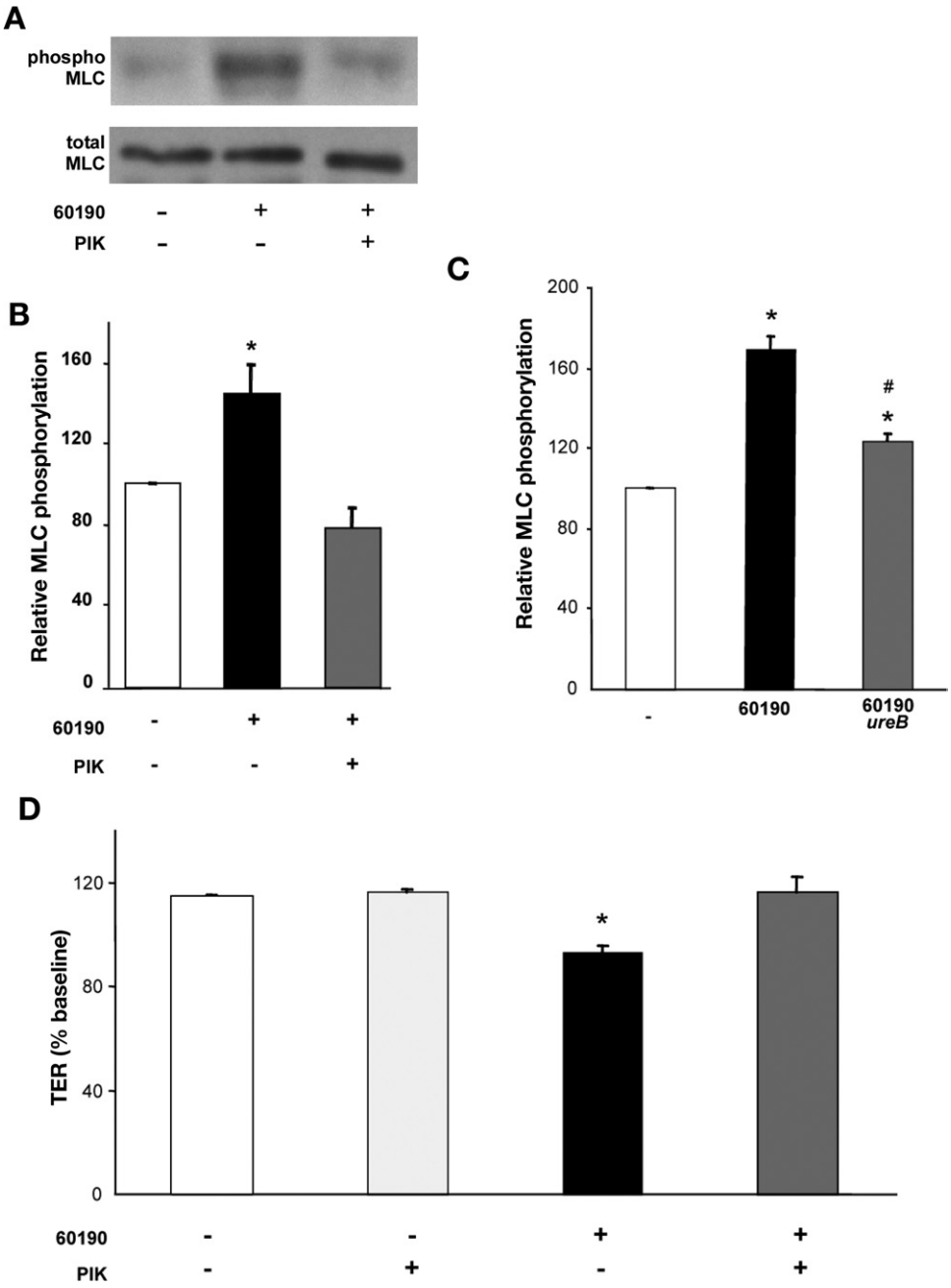

Figure 6. *H pylori* disrupts tight junction function via MLCK activation which involves UreB. Levels of total and phosphorylated (phospho) MLC were determined by Western blot analysis in MKN28 cells cocultured for 8 hours with *H pylori* strain 60190 in the presence or absence of PIK (250 μM). (*A*) Representative Western blot and (*B*) densitometric analysis of 3 independent experiments. Data were normalized to uninfected control and are expressed as means ± SEM, $^{*}P <$ .05 compared with uninfected cells. (*C*) Densitometric analysis of total and phosphorylated (phospho) MLC levels determined by Western blot analysis in MKN28 cells cocultured for 8 hours with *H pylori* wild-type strain 60190 or its isogenic *ureB* mutant. Data were normalized to uninfected control and are expressed as means ± SEM, $^{*}P <$ .05 compared with uninfected cells, $^{\#}P <$ .05 compared with cells infected with 60190, n=3. (*D*) Confluent MKN28 cells were cocultured with *H pylori* strain 60190 and after 4 hours, PIK was added to the apical media and TER was measured 4 hours later. Data were normalized to baseline TER and are expressed as means ± SEM, $^{*}P <$ .05 compared with uninfected cells, n=3.

MLC phosphatase activity by phosphorylation of myosin phosphatase target subunit 1 (MYPT1). To determine whether Rho kinase is involved in *H pylori*–induced phosphorylation of MLC, MKN28 cells were cocultured with *H pylori* in the presence or absence of the Rho kinase inhibitor Y27632. Western blot analysis demonstrated that MLC phosphorylation was attenuated by inhibition of Rho kinase (Figure 7*A*). Based on these results, we next investigated whether MYPT1-mediated signaling was responsible for increased MLC phosphorylation following coculture with *H pylori*. MKN28 cells were cocultured with wild-type strain 60190 and levels of total and phosphorylated (representing inactive) MYPT1 were determined by Western blot. No significant changes were observed in levels of phosphorylated MYPT1 in *H pylori*-infected versus uninfected cells (Figure 7*B* and *C*). Collectively, these data suggest that phosphorylation of MLC in response to *H pylori* is mediated via at least 2 distinct pathways that involve both MLCK and Rho kinase and that modification of MLC is not mediated by inhibition of myosin phosphatase.

H pylori *Infection Leads to a Focal and Marked Redistribution of Occludin in Gastric Epithelial Cells In Vivo*

To determine if occludin mislocalization is physiologically relevant within the context of *H pylori* coloni-

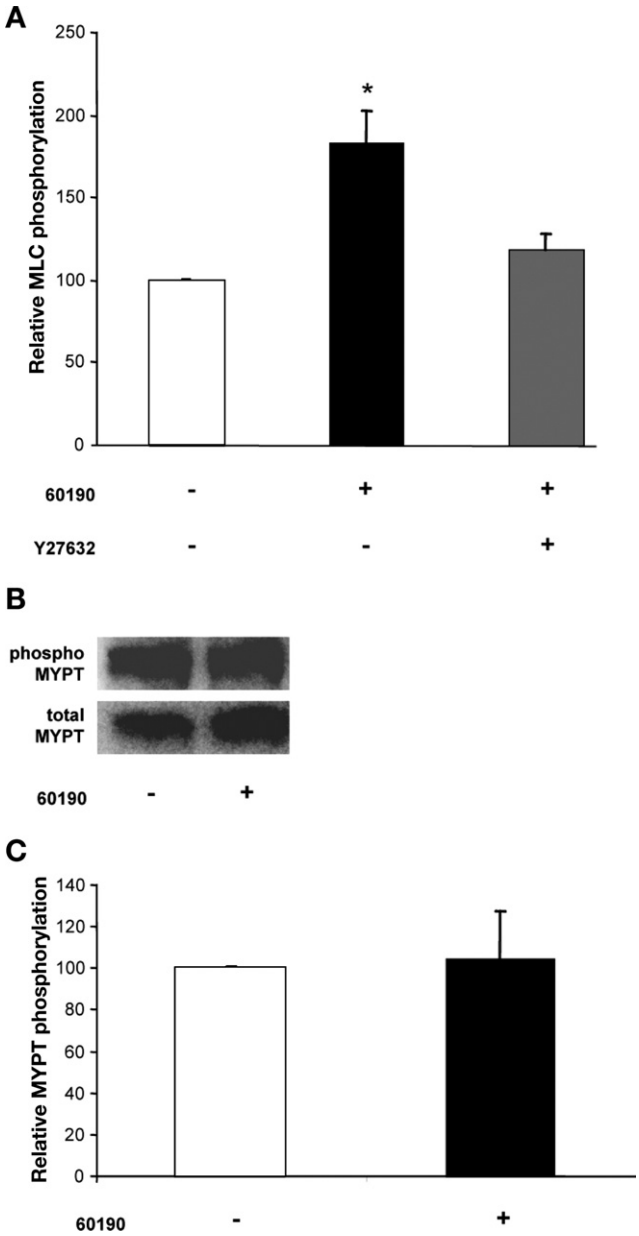

Figure 7. *H pylori*–induced phosphorylation of MLC requires Rho kinase activation. (*A*) Levels of total and phosphorylated MLC were determined by Western blot analysis in MKN28 cells cocultured with *H pylori* for 8 hours in the presence or absence of the Rho inhibitor Y27632 (10 μmol/L). Densitometric analysis of 3 independent experiments. (*B*) Levels of total and phosphorylated (phospho) MYPT were determined by Western blot analysis in MKN28 cells cocultured for 8 hours with strain 60190. Representative Western blot and (*C*) densitometric analysis of 3 independent experiments. Data were normalized to uninfected control and are expressed as means ± SEM, $^*P < .05$ compared with uninfected cells.

zation in vivo, we infected INS-GAS mice with the rodent adapted carcinogenic *H pylori* strain 7.13, which alters occludin localization in vitro. Concordant with our cell culture data, *H pylori* strain 7.13 decreased gastric epithelial cell membrane occludin and increased cytoplasmic occludin in a focal pattern, when compared to broth-treated control mice (Figure 8). The pattern of occludin mislocalization mirrored the altered topography of occludin observed in *H pylori*–infected cells in vitro (Figure 3), indicating that occludin mislocalization may also contribute to the ability of *H pylori* to induce injury within the gastric niche.

Discussion

Previous investigations into mechanisms by which *H pylori* disrupts the tight junction complex have been limited by the lack of testable gastric epithelial cell models that form functional barriers. The present study has utilized a biologically relevant in vitro model of *H pylori*–gastric epithelial cell interactions to demonstrate that infection induces a progressive loss of TER which is attenuated by disruption of *ureB* and which is followed by disruption of occludin at the level of the tight junction. *H pylori*-induced MLCK activation may link these events, as has been demonstrated in both in vitro and in vivo models of intestinal inflammatory disease.[23,24,35] Our results have also identified MLCK and Rho kinase as critical downstream targets of *H pylori* that mediate phosphorylation of MLC and tight junction barrier dysfunction, data that are consistent with other studies using a pharmacological inhibitor of MLCK in canine intestinal epithelial cells.[10]

Regulation of barrier function by occludin and MLC has precedence in other inflammatory conditions such as inflammatory bowel disease and immune-mediated diarrheal diseases.[24,29] Intestinal barrier dysregulation in an in vivo model of T cell–mediated acute diarrhea was previously demonstrated to be regulated by TNF-mediated MLCK-dependent MLC phosphorylation and was associated with MLCK-dependent redistribution of occludin.[24] Although we can not exclude the possibility that *H pylori*-induced cytokines alter barrier function in a similar manner, our data have shown that these events are due, at least in part, to a bacterial factor, since loss of *ureB* abrogated barrier disruption and MLC phosphorylation. This is consistent with previous studies demonstrating that urease contributes to *H pylori* virulence and that exposure to ammonium disrupts barrier function in colon carcinoma cells.[31]

Although a primary role for urease is generation of pH-altering ammonia, urease has been detected in the lamina propria of *H pylori*–infected individuals and exhibits chemotactic activity for monocytes and neutrophils.[36] *H pylori* urease and functionally inactive recombinant urease also stimulate macrophage inducible NO synthase expression,[8] which may result in the generation of substances that induce nitrosative damage. Taken together, these data suggest urease may exert effects that are not related to the metabolism of urea to ammonia. Our findings, however, indicate that *H pylori*–induced disruption of tight junction barrier function requires

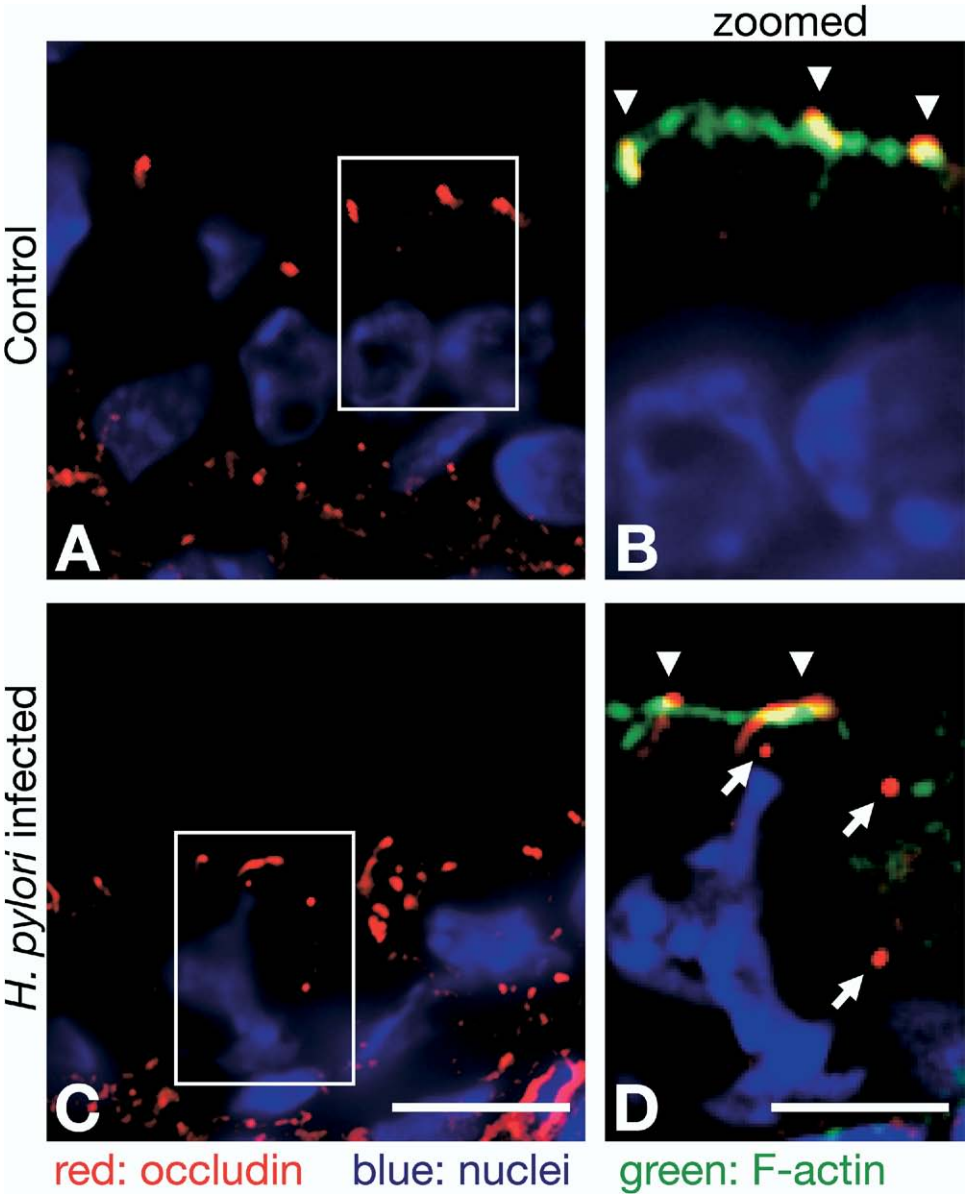

Figure 8. *H pylori* infection induces occludin mislocalization in mouse gastric epithelial cells in vivo. Immunohistochemical localization of occludin (*red*) in gastric epithelial cells from uninfected INS-GAS mice (*A* and *B*) and INS-GAS mice infected with *H pylori* strain 7.13 (*C* and *D*) for 21 days. Occludin was localized at tight junctions in uninfected INS-GAS mice; however, in *H pylori*–infected mice, occludin was found in the cytoplasm of epithelial cells. *B* and *D* show high magnification images in boxed areas of *A* and *C* with F-actin staining (*green*). *Arrowheads* indicate tight junction-associated occludin, colocalizing with apical actin. *Arrows* show occludin in the cytoplasm of gastric epithelial cells. Nuclei were stained with Hoechst 33342 (*blue*) (bar, 10 μm).

ammonium production, and not simply the presence of urease per se.

Although urease is a conserved *H pylori* constituent and is required for colonization of rodent models, the level of urease activity may allow strains to induce variable pathogenic responses. In addition, urease activity may facilitate other bacterial factors to induce epithelial damage by promoting disruption of the tight junction. For example, in conjunction with urease, *cag* PAI-encoded gene products may be able to encounter host cell constituents, such as $\alpha5\beta1$ integrins, which are normally secluded from elements in the gastric lumen. CagL has recently been shown to interact with $\alpha5\beta1$ integrin, and activate signal transduction pathways as well as facilitate translocation of CagA into the host cell.[37] Since $\alpha5\beta1$ integrins preferentially localize to the basolateral surface of polarized epithelial cells, our results raise the hypothesis that *H pylori* may gain access to intracellular spaces and the basolateral surface through urease-mediated disruption of the tight junction.

Our in vitro results indicate that the loss of TER induced by *H pylori* does not require VacA or components of the *cag* PAI. Consistent with these findings, studies using different systems have demonstrated that *H pylori* increases epithelial permeability in a manner independent of CagA and VacA,[10] and that the addition of ammonia decreases barrier function.[31] In contrast to our findings, other investigators have shown that transfection of CagA into MDCK cells or addition of purified VacA to MDCK cells disrupts tight junction barrier function.[18,19] The disparity in these results may be explained by differences in cell model systems and/or by differences in experimental design. Our current results were obtained using viable, pathogenic bacteria and gastric epi-

thelial monolayers while prior experiments utilized transfection techniques, purified bacterial proteins, or non-gastric cell types. Although MDCK cells form polarized monolayers, these cells are not of gastric origin and may not faithfully replicate *H pylori*–epithelial interactions in the gastric niche. Certain gastric cell models (eg, AGS cells) that are commonly used to study aspects of *H pylori* pathogenesis, such as cytokine secretion, are limited for studying the effects of *H pylori* on apical-junctional complexes since they express only low levels of the adherens junction protein E-cadherin,[38] and they do not form tight junctions.[13] In contrast, MKN28 cells express E-cadherin and occludin as well as other adherens junction and tight junction proteins, including ZO-1 and JAM-A, and these proteins localize to junctional components at sites of cell-cell contact.

In conclusion, the results of this study demonstrate that *H pylori* disrupts gastric epithelial barrier function. This requires *ureB* and functional urease activity but not the translocated effector protein CagA, the secreted toxin VacA, or a functional *cag* PAI. Disruption of barrier function also involves occludin mislocalization and MLCK activity and *H pylori* phosphorylates MLC in a Rho kinase- and MLCK-dependent manner. In vivo, *H pylori* similarly mislocalizes the tight junction protein occludin. Collectively, these data identify mechanisms through which *H pylori* disrupts gastric epithelial tight junction barrier function, and have identified a novel role for *H pylori*-induced activation of Rho kinase in the phosphorylation of MLC, events which may permit *H pylori* to lower the threshold for clinical disease.

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