Muc1 Cell Surface Mucin Attenuates Epithelial Inflammation in Response to a Common Mucosal Pathogen*

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Helicobacter pylori infection of the gastric mucosa causes an active-chronic inflammation that is strongly linked to the development of duodenal and gastric ulcers and stomach cancer. However, greater than 80% of individuals infected with H. pylori are asymptomatic beyond histologic inflammation, and it is unknown what factors influence the incidence and character of bacterial-associated gastritis and related disorders. Because previous studies demonstrated that the Muc1 epithelial glycoprotein inhibited inflammation during acute lung infection by Pseudomonas aeruginosa, we asked whether Muc1 might also counter-regulate gastric inflammation in response to H. pylori infection. Muc1−/− mice displayed increased bacterial colonization of the stomach and greater TNF-α and keratinocyte chemotractant transcript levels compared with Muc1+/+ mice after experimental H. pylori infection. Knockdown of Muc1 expression in AGS human gastric epithelial cells by RNA interference was associated with increased phosphorylation of IkBα, augmented activation and nuclear translocation of NF-κB, and enhanced production of interleukin-8 compared with Muc1-expressing cells. Conversely, Muc1 overexpression was correlated with decreased NF-κB activation, reduced interleukin-8 production, and diminished IkB kinase (IKK)/IKKγ coimmunoprecipitation compared with cells expressing Muc1 endogenously. Cotransfection of AGS cells with Muc1 plus IKKβ, but not a catalytically inactive IKKβ mutant, reversed the Muc1 inhibitory effect. Finally, Muc1 formed a coimmunoprecipitation complex with IKKγ but not with IKKβ. These results are consistent with the hypothesis that Muc1 binds to IKKγ, thereby inhibiting formation of the catalytically active IKK complex and blocking the ability of H. pylori to stimulate IkBα phosphorylation, NF-κB activation, and downstream inflammatory responses.

Epithelial cells lining mucosal surfaces provide a protective barrier against infectious pathogens (1). In addition, intact epithelia are major sources of inflammatory mediators (e.g. TNF-α2 and IL-8) that initiate and amplify host responses to microbial infection (2). A critical balance between pro- and anti-inflammatory pathways maintains a homeostatic environment at epithelia that effectively neutralizes harmful environmental insults without excessive bystander tissue damage. Although a large body of literature has characterized the microbial-stimulated pro-inflammatory pathways, relatively fewer research efforts have focused on elucidating the counterbalancing anti-inflammatory responses.

Our prior studies demonstrated that expression of mucin-1 (Muc1) on the epithelial cell surface down-regulated inflammation in response to Pseudomonas aeruginosa, an opportunistic lung pathogen (3). Using an experimental model of acute P. aeruginosa infection, we showed that Muc1−/− mice possessed higher levels of TNF-α and KC and enhanced NF-κB activation in airway epithelia compared with Muc1+/+ littermates. Subsequent studies by others confirmed that Muc1 also counter-regulated inflammatory responses against Staphylococcus, Streptococcus, and Corynebacterium (4). However, the molecular mechanism whereby Muc1 inhibits mucosal inflammation remains to be elucidated.

Helicobacter pylori is another human pathogen that induces a robust mucosal inflammatory response (5, 6). Currently greater than 50% of the world’s population is infected with H. pylori, making it the most globally common bacterial infectious agent. The high prevalence of H. pylori infection is due in large part to the inability of host immunity to clear bacterial colonization despite the vigorous gastric inflammatory response (7). H. pylori colonization of the stomach is initiated through the ability of the pathogen to bind to specific cell surface receptors expressing the Lewis b (Leb), sialyl-Lewis α (sLeα), and sialyl-Lewis x (sLeβ) antigens (5). However, the vast majority of infected individuals are asymptomatic apart from histologic gastritis.

Within the subgroup of H. pylori-infected individuals who do display clinical symptoms, epidemiologic and pathologic studies have confirmed a progressive increase in disease symptomatology and severity over several decades, starting with chronic inflammation followed by the development of noncancerous and precancerous lesions and, finally, gastric adenocarcinoma (8). The current paradigm of H. pylori-induced gastric cancer proposes that chronic gastritis during a lifetime of infection generates an environment awash with inflammatory

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2 The abbreviations used are: TNF, tumor necrosis factor; IL, interleukin; Muc1, mucin-1; KC, keratinocyte chemotractant; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; SSI, Sidney strain 1; CFU, colony forming unit; siRNA, small interfering RNA; pMuc1, plasmid encoding Muc1; IKK, IκB kinase; IκB, inhibitor of NF-κB; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PBS-T, PBS, 0.05% Tween 20; HEK, human embryonic kidney; BabA, blood group antigen binding adhesion; coIP, coimmunoprecipitation.
mediators and characterized by the activation of signaling pathways that cross-talk between inflammation and carcinogenesis (9). In support of this hypothesis, stomach infection by *H. pylori* is associated with epithelial cell gene mutations, inhibition of apoptosis, and stimulation of angiogenesis and cell proliferation, all of which contribute to development of a cancer phenotype (10). Therefore, a better understanding of host pro-inflammatory pathways as well as the counter-regulatory mechanisms that normally attenuate inflammation will provide important new information to treat the subset of *H. pylori*-infected patients who are at increased risk for developing life-threatening gastric diseases.

**EXPERIMENTAL PROCEDURES**

**Animals**—All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Maryland, Baltimore, MD. Muc1−/− mice on an FVB genetic background were generated by homologous recombination (11). Age (8–12 week)- and gender-matched FVB Muc1+/- littermates were from The Jackson Laboratories (Bar Harbor, ME). Muc1 genotypes and phenotypes were confirmed by polymerase chain reaction and Western blot analysis, respectively, using gastric epithelial tissues. All mice were caged in Static Micro-Isolator LPTM cages (Lab Products, Seaford, DE), bedded in combination size corncob bedding, fed solid chow, and housed under specific pathogen-free conditions.

**Bacteria and Bacterial Products**—*H. pylori* strains SS1 (12) and 26695 (13) were maintained on Columbia blood agar containing 7% defibrinated horse blood (Cleveland Scientific, Bath, OH), 20 μg/ml bacitracin, 20 μg/ml trimethoprim, 16 μg/ml ceftsulodin, 6 μg/ml vancomycin, and 2.5 μg/ml Fungizone (Sigma) under microaerophilic conditions as described (12). For challenge infections and treatment of AGS cells, bacteria were grown in static liquid cultures of Brucella broth (Difco) containing 10% heat-inactivated fetal bovine serum and antibiotics at 37 °C with 10% CO2. Bacterial lysate for immunization was prepared as described (12). Briefly, bacteria were concentrated by centrifugation and lysed using a probe sonicator. After centrifugation, the soluble fraction was filtered (0.45 μm), the protein concentration was determined using a bicinchoninic acid assay (Pierce), and the extract was stored at −80 °C.

**H. pylori Stomach Infection**—Mice were infected by gastric intubation on 2 consecutive days with 0.5 ml of log-phase cultures of *H. pylori* at 107 colony forming units (CFUs)/animal. Mice were sacrificed by intraperitoneal pentobarbital injection at 4 weeks post-infection. For immunization before challenge infection, mice were given 4 weekly intranasal inoculations containing 100 μg of *H. pylori* lysate and 5 μg of cholera toxin adjuvant in 20 μl applied directly to the nares (12). Unimmunized mice received adjuvant alone. All animals were challenged 2 weeks after the last immunization as above.

**CFU Determination**—The glandular stomach was divided longitudinally along the lesser curvature, the tissue fragments were dispensed into preweighed tubes, and weighed sections were homogenized in 200 μl of Brucella broth using 1.5-ml disposable polypropylene tissue grinders and pestles (Kontes, Vineland, NJ). The homogenate was serially diluted in sterile phosphate-buffered saline (PBS) from 1/10 to 1/1000, and 10 μl of each dilution was plated. Colonies were counted after 5–7 days of incubation. Representative colonies were tested for urease, oxidase, and catalase activities to confirm their identity as *H. pylori*.

**H. pylori Urease PCR**—DNA was isolated from stomachs using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). PCR amplification of the *H. pylori* urease C gene was performed with an Eppendorf Realplex instrument (Eppendorf, Hamburg, Germany) using 96-well microtiter plates and 0.4 μM primers as described (14) (Table 1). For each sample the PCR was performed in duplicate with SYBR Green supermix (Fermentas, Glen Burnie, MD). Samples were heated at 95 °C for 10 min and subjected to 40 cycles of 95 °C for 15 s and 72 °C for 30 s. Standard curves for the urease C gene were generated using serial 1/10 dilutions from 1/10 to 1/106 of a plasmid encoding the cloned gene.

**Quantitative Reverse Transcription-PCR**—Total RNA was isolated from stomachs using the RNeasy Mini Kit (Qiagen). RNA (0.2 μg) was reverse-transcribed into cDNA using the First Strand cDNA Synthesis kit (Qiagen) as described (13). PCR amplification conditions were as described above using 0.4 μM of primers for mouse TNF-α, KC, and glyceraldehyde-3-phosphate dehydrogenase (Table 1). Transcript levels were calculated from standard curves of the respective cloned genes, and the amounts of TNF-α and KC mRNAs were normalized to glyceraldehyde-3-phosphate dehydrogenase transcripts. Differences in transcript levels were calculated as 2−ΔΔCt, where Ct is the threshold cycle.

**Knockdown of Muc1 Expression**—AGS cells (CRL-1739, ATCC, Manassas, VA) were seeded in 24-well plates, incubated for 24 h in Dulbecco’s modified Eagle’s medium/F-12 medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen), and transfected with 20

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**TABLE 1**

| Target                     | Primer sequences                        | PCR product size | GenBank™ accession no. |
|----------------------------|----------------------------------------|------------------|------------------------|
| *H. pylori* urease C       | F: 5′-TTATCGTGAAAGACACAGAGAAA-3′       | 148              | M60398.1               |
|                            | R: 5′-ATCACACGCAGATGTC-3′              |                  |                        |
| Mouse GAPDH                | F: 5′-CACATGGGGGTTAGAAGACAC-3′         | 170              | NM_008084.2            |
|                            | R: 5′-ACCCAGGAAACTGTAGGG-3′            |                  |                        |
| Mouse TNF-                 | F: 5′-CACCACGGCTCTCCTGTCATAC-3′       | 149              | NM_013693.2            |
|                            | R: 5′-GGCTACAGGCTGTGTCATCT-3′         |                  |                        |
| Mouse KC                   | F: 5′-AATGAGCTGGGGTGCATG-3′           | 133              | NM_008176.3            |
|                            | R: 5′-CGCGACCATTCTTGAGTGTG-3′         |                  |                        |

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pm/well of a Muc1-targeting small interfering RNA (siRNA) (3, 15) or a nontargeting control siRNA (Dharmacon, Lafayette, CO) using 2.0 μl/well of Lipofectamine (Invitrogen) according to the manufacturer’s instructions.

**Muc1 Overexpression**—AGS cells in 24-well plates were transfected with a 0.8 μg/well of the pcDNA empty vector (Invitrogen) or a Muc1 expression plasmid (pMuc1) encoding a protein with 32 tandem repeats (16) using Lipofectamine as described (17). In some experiments cells were cotransfected with pMuc1 and 0.8 μg/well of plasmids encoding wild type IkB kinase β (IKKβ) or mutant IKKβ (K44M) (Addgene, Cambridge, MA).

**Luciferase Assay**—AGS cells in 24-well plates were cotransfected with the Muc1 or control siRNAs or with pcDNA vector or pMuc1 plus 0.8 μg/well of the pGL4.32 plasmid containing NF-κB response elements linked to a Photinus pyralis luciferase reporter gene plus 40 ng/well of the pRL-SV40 plasmid encoding Renilla luciferase (Promega, Madison, WI). The cells were incubated for 24 h either untreated or treated with *H. pylori* 26695 at a multiplicity of infection of 100:1, and luciferase activities were measured in cell lysates using the Dual luciferase assay system (Promega) according to the manufacturer’s instructions.

**IL-8 ELISA**—IL-8 was quantified by ELISA using commercially available capture and detection antibodies (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Briefly, microwells containing capture antibody were blocked with PBS containing 1% bovine serum albumin, and cell culture media were added and incubated for 2 h at room temperature followed by incubation with biotinylated detection antibody, peroxidase-labeled streptavidin, and tetramethylbenzidine substrate. Optical density values at 450 nm (A450) were measured and IL-8 levels were determined from standard curves constructed with serial dilutions of purified chemokine. All samples were analyzed in triplicate, and standard curves were performed on each plate.

**Western Blotting**—AGS cells were washed with PBS and lysed at 4 °C with PBS containing 0.1% SDS, 0.5% sodium deoxycholate, 1.0% Triton X-100, 5.0% glycerol, 1.0% protease inhibitor mixture, 10 mM NaF, and 1.0 mM NaVO₄ (Sigma) (17). Cell nuclei were isolated and lysed with the ProteoJET Cytoplasmic and Nuclear Protein Extraction kit (Fermentas). Equal protein aliquots (20 μg) were resolved on 4–20% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Bio-Rad), and the membrane was blocked for 1 h with PBS containing 0.05% Tween 20 (PBS-T) and 5% nonfat dry milk (Sigma) and reacted with 1.0 μg/ml antibodies to phospho-IκBα, total IκBα, IKKβ, or NF-κB p65 (Cell Signaling Technology, Danvers, MA). Membranes were washed with PBS-T, incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (KPL, Gaithersburg, MD), and developed with enhanced chemiluminescence substrate (GE Healthcare). To confirm equivalent protein loading and transfer, the blots were stripped with 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7, reblocked, and probed with 1.0 μg/ml antibodies to lamin B1 (for nuclear protein blots) or β-actin (for cytoplasmic protein blots) (Cell Signaling Technology) followed by secondary anti-body and chemiluminescence substrate. Immunoreactive bands were identified by co-migration of prestained protein size markers (Bio-Rad).

**ColP**—Equal protein aliquots (1.0 mg) of AGS cell lysates were immunoprecipitated with antibodies to IKKγ (Cell Signaling) or Muc1 (Neomarkers, Fremont, CA) plus protein G-agarose (Invitrogen) as described (17) and analyzed by Western blotting with antibodies to IKKβ or IKKγ as above.

**Statistical Analysis**—Replicates of 2–6 samples were used for each group. Mean ± S.D. values were calculated, and differences between groups were assessed using Student’s t test and considered significant at *p* < 0.05. All experiments were repeated at least two times.

**RESULTS**

**Muc1 Limits* H. pylori* Colonization of the Stomach**—Mouse models of *H. pylori* infection have shown that the bacterium induces a KC-dependent, neutrophil-dominated chronic gastritis that mimics human infection (12, 18). Initially, we determined whether loss of Muc1 expression affected *H. pylori* stomach colonization using Muc1+/- and Muc1/-/- mice. After experimental infection with the mouse-adapted *H. pylori* SS1 strain, greater bacterial CFUs and urease C gene copy numbers were detected in gastric tissues of Muc1-/- mice compared with Muc1+/+ mice (Figs. 1, A and B). Interestingly, the levels of anti-*H. pylori* serum IgG and IgA antibodies were equal in the two mouse strains (Figs. 1, C and D). Thus, Muc1 normally serves to limit *H. pylori* colonization of the murine gastric mucosa in an antibody independent manner. Because these experiments failed to demonstrate a role for humoral
immunity in H. pylori stomach infection of mice, we next focused on a potential role for a cellular immune response. H. pylori-infected Muc1−/− mice exhibit increased IL-8 production in response to infection (12, 18), TNF-α and KC mRNAs again were increased 4–6-fold in immunized and infected Muc1−/− mice compared with Muc1+/+ mice (Figs. 2A and C). In both experiments transcript levels were equivalent in uninfected Muc1+/+ and Muc1−/− mice, indicating that Muc1 expression did not influence basal cytokine gene expression. Furthermore, the increased IgG and IgA responses of H. pylori-immunized Muc1−/− mice (Figs. 1, C and D) indicated that type mice are capable of mounting a normal immune response to infection.

Down-regulation of Muc1 in AGS Cells Increases H. pylori-stimulated IL-8 Production—Given the mixed cell types (epithelial cells, inflammatory cells, fibroblasts) present in stomach biopsies potentially confounding the interpretation of the TNF-α/KC mRNA read-outs, AGS human gastric epithelial cells were used to confirm the in vivo results in an in vitro cell monoculture system. Initially, when AGS cells were treated with strain SS1, relatively little IL-8 was detected in spent culture media (Fig. 3A), which has previously been correlated with the presence of a compromised cytotoxin-associated gene A (cagA) pathogenicity island in this strain (19). By contrast, H. pylori 26695 induced robust IL-8 production in AGS cells (Fig. 3A). Next, to investigate the effect of Muc1 expression on H. pylori-induced chemokine production, the cells were transfected with a Muc1-targeting siRNA or nontargeting control siRNA, and IL-8 levels in culture media were measured after H. pylori treatment. AGS cells transfected with a Muc1-targeting siRNA exhibited >95% reduction in Muc1 protein levels at 72 h post-transfection compared with cells transfected with a control siRNA (Fig. 3B). Knockdown of Muc1 expression increased H. pylori 26695-stimulated IL-8 levels compared with Muc1-expressing cells (Fig. 3C). Although the increase in IL-8 production by Muc1 siRNA-transfected cells was modest compared with control siRNA-transfected cells (40–55% increase), the differences between the two groups were statistically significant between 3 and 24 h post-H. pylori treatment.

Overexpression of Muc1 Decreases H. pylori-stimulated IL-8 Production—As an independent measure of the effect of Muc1 expression on IL-8 production in response to H. pylori, AGS were transfected with the Muc1 expression plasmid or the empty pcDNA vector, the cells were treated with H. pylori, and IL-8 levels in culture media were measured. AGS cells transfected with the Muc1 plasmid displayed ~15-fold increased Muc1 protein levels compared with cells transfected with the empty pcDNA vector, the cells were treated with H. pylori, and IL-8 levels in culture media were measured. AGS cells transfected with the pMuc1 expression plasmid or the empty pcDNA vector, the cells were treated with H. pylori, and IL-8 levels in culture media were measured. AGS cells transfected with the pMuc1 expression plasmid or the empty pcDNA vector, the cells were treated with H. pylori, and IL-8 levels in culture media were measured.

**FIGURE 2.** Muc1−/− mice display increased TNF-α and KC transcript levels after H. pylori gastric infection. A, Muc1−/− and Muc1−/− mice were uninfected or infected with 107 CFUs/mouse of H. pylori SS1, and transcripts encoding TNF-α and KC were measured by quantitative reverse transcription-PCR in gastric tissue homogenates at 4 weeks post-infection. B and C, Muc1−/− and Muc1−/− mice were unimmunized and unchallenged (U/U), immunized and challenged (I/C), or immunized and challenged (I/C). Intranasal immunization at 1, 2, 3, and 4 weeks was with a bacterial lysate of H. pylori SS1 (100 μg/mouse). Unimmunized mice received cholera toxin adjuvant alone in the absence of H. pylori lysate. Challenge infection was by gastric intubation on 2 consecutive days with 107 CFUs/mouse of SS1 at 2 weeks after the last immunization. At 4 weeks post-infection, transcripts encoding TNF-α (A) and KC (C) were measured by quantitative reverse transcription-PCR in gastric tissue homogenates. Values are the means ± S.D. of 4–6 replicates per sample. The results are representative of two independent experiments.
empty vector (Figs. 4, A and B). Overexpression of Muc1 increased \textit{H. pylori}-induced IL-8 production compared with empty vector-transfected cells (Fig. 4C). In summary, Muc1 expression was inversely correlated with \textit{H. pylori}-stimulated KC and IL-8 production in mouse and human systems.

\textbf{Muc1 Counter-regulates \textit{H. pylori}-dependent NF-κB Activation}—Because NF-κB is a major transcription factor that regulates IL-8 gene expression, \textit{H. pylori}-driven NF-κB activation in Muc1-expressing or -nonexpressing AGS cells was compared using a plasmid containing the NF-κB response element linked to a firefly luciferase reporter gene. As shown in Fig. 5A, \textit{H. pylori}-treated cells transfected with the Muc1 siRNA had significantly greater luciferase activity compared with cells transfected with the control siRNA between 3 and 12 h post-treatment. Conversely, AGS cells overexpressing Muc1 displayed decreased luciferase activity at 6–12 h after \textit{H. pylori} treatment compared with cells transfected with empty vector (Fig. 5B). These results demonstrating an inverse relationship between Muc1 expression and NF-κB activation were not inconsistent with the IL-8 data presented above.

\textbf{Muc1 Blocks \textit{H. pylori}-stimulated NF-κB Activation and IL-8 Production through Inhibition of IκBα Phosphorylation}—The RelA (p65)/p50 NF-κB heterodimer normally resides in the cytosol as an inactive complex with IκBα (20). Phosphorylation of IκBα leads to its dissociation from NF-κB, allowing the activated transcription factor to translocate into the nucleus. Therefore, we examined the effect of Muc1 expression on \textit{H. pylori}-induced IκBα phosphorylation and NF-κB nuclear translocation. Transfection of AGS cells with the Muc1 siRNA was associated with increased IκBα phosphorylation after \textit{H. pylori} treatment compared with control siRNA-transfected cells.
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FIGURE 5. Muc1 expression correlates inversely with NF-κB activation. AGS cells were transfected with Muc1 or control siRNAs (A) or with pcDNA empty vector or pMuc1 (B). Cells were incubated for 48 h and cotransfected with the pGL4.32 plasmid containing NF-κB response elements linked to a P. pyralis (firefly) luciferase reporter gene plus the pRL-SV40 plasmid encoding Renilla luciferase. The cells were incubated for an additional 24 h either untreated or treated with H. pylori strain 26695 for the indicated times, and luciferase activities were measured in cell lysates. Each point represents the mean ± S.D. value of triplicate samples. *, p < 0.05; **, p < 0.01 comparing H. pylori-treated, Muc1 siRNA-transfected cells with H. pylori-treated, control siRNA-transfected cells (A) or comparing H. pylori-treated, pcDNA-transfected cells with H. pylori-treated, pMuc1-transfected cells (B). The results are representative of two independent experiments.

cells (Fig. 6A). Total IκBα levels were unaffected by Muc1 knockdown.

Next, Bay 11-7082, a pharmacological inhibitor that selectively and irreversibly blocks IκBα phosphorylation (21), was used to demonstrate that H. pylori-stimulated IκBα phosphorylation was upstream of IL-8 production. Initially, we confirmed that Bay 11-7082 inhibited IκBα phosphorylation and NF-κB activation in H. pylori-treated AGS cells. AGS cells pretreated with 10 μM Bay 11-7082 displayed reduced levels of cytoplasmic phospho-IκBα (Fig. 6B) and decreased levels of nuclear NF-κB predominantly at 1 and 3 h post-H. pylori treat-
subunit (IKKγ, or NEMO). Activation of NF-κB in response to infectious agents or proinflammatory cytokines is primarily dependent on IKKβ and IKKγ (22). By contrast, IKKα is dispensable for canonical NF-κB activation (20). Therefore, we focused on the roles of IKKβ and IKKγ in H. pylori-stimulated NF-κB activation and the effect of Muc1 expression on the interaction between these two IKK subunits. Transfection of AGS cells with an IKKβ expression plasmid increased H. pylori-stimulated IlkBα phosphorylation compared with cells transfected with a catalytically inactive mutant IKKβ (K44M) (Fig. 8A). By densitometry and normalization with the β-actin loading controls, the increase in phospho-IkBα levels in wild type IKKβ-transfected cells treated with H. pylori compared with cells not treated with H. pylori (3.8-fold) was significantly greater than the 2.0-fold increase seen in similarly treated cells transfected with the K44M mutant (Fig. 8B). Transfection efficiencies of wild type and mutant IKKβ were equal as assessed by Western blotting with an antibody recognizing both proteins. Thus, H. pylori-induced IlkBα phosphorylation was mediated by IKKβ.

Next, NF-κB activation and IL-8 production by Muc1-expressing or -nonexpressing AGS cells were compared in cells overexpressing IKKβ or the inactive mutant IKKβ. As before, in

FIGURE 7. A, Muc1 overexpression increases nuclear translocation of NF-κB in response to H. pylori treatment. AGS cells were transfected with pcDNA empty vector or pMuc1 for 72 h and treated for 6 h with H. pylori 26695, and NF-κB in nuclear and cytoplasmic fractions was detected by Western blotting (IB). B, shown is densitometric quantification of the NF-κB bands in A. C, Bay 11-7082 inhibits H. pylori-stimulated IL-8 production. AGS cells were pretreated with medium alone or with 10 or 20 μM Bay 11-7082 for 1 h. Cells were washed and either untreated or treated with H. pylori 26695 for the indicated times, and IL-8 levels in culture media were determined by ELISA. Each point represents the mean ± S.D. value of triplicate samples. The results are representative of two independent experiments.

FIGURE 8. A and B, IKKβ phosphorylates IkBα after H. pylori treatment. A, AGS cells were transfected with plasmids encoding wild type IKKβ (WT) or catalytically inactive IKKβ (K44M), the cells were untreated or treated with H. pylori 26695 for 6 h, and pIkBα and IKKβ levels were determined by Western blotting (IB). As a loading control, the blot was stripped and probed for β-actin. The results are representative of two independent experiments. B, shown is densitometric quantification of the IKKβ bands in A normalized to the loading controls.* p < 0.05. C and D, overexpression of IKKβ reverses Muc1-mediated reduction in H. pylori-stimulated NF-κB activation. C, AGS cells were transfected with the pcDNA empty vector, pMuc1, or plasmids encoding wild type IKKβ (pIKKβ) or catalytically inactive pIKKβ (K44M), and the cells were incubated for 24 h and cotransfected with the NF-κB-luciferase reporter plasmid plus pRL-SV40. The cells were incubated for an additional 24 h and treated with H. pylori 26695 for the indicated times, and luciferase activities were measured in cell lysates. D, AGS cells were transfected with empty vector, pMuc1, wild type pIKKβ, or pIKKβ (K44M), the cells were incubated for 24 h and treated with H. pylori 26695, and IL-8 levels in culture media were determined by ELISA. Each point represents the mean ± S.D. value of duplicate samples.* p < 0.05 comparing pcDNA- or pMuc1/pIKKβ-transfected cells with pMuc1- or pMUC1/pIKKβ (K44M)-transfected cells. The results are representative of two independent experiments.
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the absence of IKKβ transfection, Muc1 overexpression resulted in significantly decreased H. pylori-induced NF-κB activation (Fig. 8C) and IL-8 production (Fig. 8D) compared with cells transfected with empty vector. However, cotransfection of IKKβ with Muc1 reversed the inhibitory effect such that NF-κB activation and IL-8 production were restored to the original levels seen in the absence of Muc1 transfection. By contrast, cotransfection of the inactive mutant IKKβ with Muc1 failed to reverse the inhibitory effect of Muc1 expression. In conclusion, Muc1 inhibited H. pylori-induced NF-κB activation and IL-8 production in gastric epithelial cells at the level of the IKK complex.

Muc1 Inhibits IKKβ-IKKγ CoIP and Binds to IKKγ—Protein-protein interaction between IKKβ and IKKγ is required for activation of the IKK complex. May et al. (23) reported that a synthetic peptide corresponding to the region of IKKγ that is responsible for binding to IKKγ not only blocked IKKβ-IKKγ interaction but also inhibited downstream NF-κB activation. Therefore, we predicted that Muc1 binding to IKKβ and/or IKKγ would inhibit formation of the catalytically active IKKβ-IKKγ complex, thereby restraining H. pylori-stimulated NF-κB activation. To test this hypothesis, IKKβ-IKKγ coIPs were performed in AGS cells transfected with pcDNA empty vector or the pMuc1 plasmid. IKKβ-IKKγ coIP was reduced in cells overexpressing Muc1 compared with cells transfected with empty vector (Fig. 9A). Next, Muc1/IKKβ and Muc1/IKKγ reciprocal coIPs were performed to ascertain whether Muc1 interacted with either of the IKK subunits. Although Muc1 did not interact with IKKβ, a coIP complex was detected between Muc1 and IKKγ (Figs. 9, B and C). In summary, these results are consistent with the hypothesis that Muc1 binds to IKKγ, thereby inhibiting formation of the catalytically active IKKβ-IKKγ complex and blocking the ability of H. pylori to stimulate NK-κB activation and downstream inflammatory responses in gastric epithelial cells.

Muc1 Inhibits H. pylori-stimulated NF-κB Activation through Its Intracellular COOH Terminus—Muc1 is a membrane-tethered mucin expressed on the apical surface of polarized epithelial cells with a 72-amino acid cytoplasmic COOH terminus that contains 7 tyrosines as phosphorylation sites (24). Given the ability of membrane-bound Muc1 to colP with cytosolic IKKγ, we predicted that Muc1 inhibits H. pylori-induced NF-κB activation through its intracellular domain. To test this hypothesis, NF-κB activation was determined using the luciferase reporter plasmid in human embryonic kidney 293 (HEK293) cells transfected with a plasmid encoding only the COOH-terminal tyrosine residues. As documented in our prior publication, CD8/Muc1, CD8, and CD8/7YF proteins were all expressed on the surface of transfected HEK293 cells in equal amounts (25). Finally, a coIP complex between CD8/Muc1 and IKKγ, but not between the CD8 ectodomain and IKKγ, was seen in transfected HEK293 cells (Fig. 10B). By contrast, a coIP complex could not be demonstrated between CD8/Muc1 and IKKβ (Fig. 10C). In summary, these results suggest that the Muc1 intracellular COOH terminus is responsible for its ability to interact with IKKγ.

DISCUSSION

The results of this study are summarized as follows. (a) Muc1+/− mice displayed increased bacterial load and greater TNF-α and KC transcript levels in the stomach compared with Muc1+/+ mice after experimental H. pylori infection, (b) knockdown of Muc1 expression in AGS cells was correlated with increased iKBα phosphorylation, NF-κB activation,
mucin expression, and IL-8 production compared with Muc1-expressing cells, (c) overexpression of Muc1 was associated with decreased NF-κB activation and nuclear translocation, and IL-8 production compared with cells expressing Muc1 endogenously, (d) cotransfection of cells with Muc1 plus IKKβ reversed the Muc1 inhibitory effect, (e) transfection of cells with Muc1 inhibited IKKβ-IKKγ coIP, (f) Muc1 formed a protein complex with IKKγ, but not with IKKβ, and (g) Muc1 reduced H. pylori-stimulated NF-κB activation through its intracellular domain.

Two potential mechanisms can be envisioned to rationalize how binding of Muc1 to IKKγ blocks H. pylori-stimulated NF-κB activation. First, Muc1 may sequester the IKKγ monomer or IKKβ-IKKγ heterodimer complex on the inner leaflet of the plasma membrane, thus reducing the ability of the enzymatically active kinase complex to interact with and phosphorylate cytosolic IκBα. Alternatively, Muc1 and IKKβ may interact with the same or overlapping regions on IKKγ such that binding of Muc1 competitively inhibits binding of IKKβ to IKKγ. Given that these protein-protein interactions are noncovalent and reversible, the fact that overexpression of IKKβ repealed the inhibitory effect of Muc1 on NF-κB activation is more consistent with the latter possibility. However, both possible mechanisms are not mutually exclusive, and current studies in our laboratory are focused on distinguishing between these and other possibilities.

Although H. pylori colonization of the stomach induces a high incidence of chronic gastritis, it is also the case that most infected individuals never exhibit overt symptoms. Within the subset of H. pylori-infected individuals who do display gastric disease, clinical studies have revealed a gradual progression in disease severity over several decades (the Correa pathway) (8, 26). The initial gastritis consists of both active (neutrophilic) and chronic (monocytic and lymphocytic) inflammation, which may be followed by gastric atrophy (loss of differentiated epithelium), intestinal metaplasia (altered mucin structure and expression), and dysplasia (abnormal cell growth and development) before stomach cancer (27). Approximately 10–20% of infected subjects will develop gastric and duodenal ulcers, 1–2% will develop adenocarcinoma, and less than 1% will acquire gastric MALT lymphoma (28). However, it remains to be determined what factors (bacterial, host, and/or environmental) affect the development of gastric disorders associated with H. pylori infection.

Although the molecular and cellular mechanisms of H. pylori pathogenesis have yet to be fully elucidated, multiple hypotheses have been proposed that can be broadly classified as bacteria-initiated or host-initiated. In support of a direct effect of H. pylori on carcinogenesis, Ohnishi et al. (29) reported that uninfected transgenic mice expressing the H. pylori cagA protein under control of the mouse H+/K+-ATPase promoter, to predominantly restrict expression to the stomach, developed greater gastric epithelial hyperplasia and adenocarcinomas compared with non-transgenic mice. Alternatively, a chronic inflammatory microenvironment during a lifetime of H. pylori infection has been suggested to lead to progressive alterations in gastric epithelial cell phenotypes that ultimately culminate in neoplastic transformation. One possible mechanism involves the enhanced production of proinflammatory mediators in response to bacterial colonization that increase host cell gene mutations (10). Another mechanism, the perigenetic pathway, proposes the conversion to a transformed cell phenotype by altered expression of proteins normally involved in cell adhesion or proliferation (30). According to the perigenetic mechanism, H. pylori-induced up-regulation of inflammation-associated proteins, such as TNF-α, alters gastric epithelial cell adhesion and/or proliferation, thus leading to the dispersion and propagation of precancerous epithelial cells
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into mature tumors without the need for additional genetic mutations (e.g. in tumor suppressor genes) (31).

On the basis of the latter propositions and combined with the ability of Muc1 to down-regulate ongoing gastric inflammatory responses documented herein, we hypothesize that defective Muc1 protein structure and/or expression may be responsible, in part, for progression from local inflammation to more severe gastric disease that occurs in a subgroup of H. pylori-infected subjects. A review of the literature reveals anecdotal evidence that is not inconsistent with this hypothesis. For example, previous investigations have revealed an increased risk for intestinal metaplasia and gastric carcinoma in H. pylori-infected individuals expressing Muc1 alleles with fewer Muc1 exomedomain tandem repeats compared with individuals with longer alleles (32). Because Muc1 with altered glycans is internalized faster compared with molecules with normal glycosylation (33), it is possible that Muc1, with fewer tandem repeats, has a modified stability/turover and could, thus, account for altered disease progression. Our results, however, are more suggestive of a structural alteration in the Muc1 COOH terminus, which is known to interact with a variety of different cytosolic proteins (34). In particular, coIP of the Muc1 COOH and IKKγ in breast cancer cells was previously reported (35). Many of these protein-protein interactions are controlled by tyrosine phosphorylation of the Muc1 intracellular domain and, it is interesting to note, at least four Muc1 mRNA splice variants have been described that encode a gene product with a complete or partially deleted COOH terminus (36, 37). Future studies to characterize the expression of these and other Muc1 COOH-terminal splice variants in H. pylori-infected subjects with advanced gastric pathologies may be instructive in this regard.

Alternatively, decreased expression of full-length Muc1 may contribute to increased gastric inflammation and progression to stomach cancer, for example, through previously described Muc1 gene modifications, such as DNA methylation (38–40) and/or through the action of one or more negative regulatory elements located in the its promoter (41–43). Indeed, Muc1 expression in the stomach has been reported to be inversely correlated with the incidence of H. pylori-associated gastric diseases. Vinall et al. (44) demonstrated that whereas gastric epithelial Muc1 apical staining was seen in 34 of 41 biopsy specimens, whereas under neutral pH conditions BabA can bind to both Muc5AC and Muc1 (49). Lindén et al. (50) demonstrated that not only does H. pylori bind to Muc1 expressed on gastric epithelium but also that Muc1 shedding from the cell surface after bacterial binding limited cell adhesion by acting as a releasable decoy receptor (51). It is also clear, however, that host cells express surface receptors other than Muc1 that serve as adhesion sites for H. pylori. Some of these that have been reported in the literature include Toll-like receptors, integrins, and CD74 (52–54). On the basis of the data presented in this report, we hypothesize that, in the context of expression of the Muc1 intracellular COOH terminus, downstream signaling initiated by H. pylori is suppressed through the ability of the Muc1 cytoplasmic domain to interact with IKKγ.

In summary, the results of the present study demonstrate that Muc1 gastric expression both restricts H. pylori colonization and counter-regulates inflammatory responses, which we speculate to occur through interference with the canonical IKK → IκBα → NF-κB pathway. Although this conclusion does not preclude other potential mechanisms for the Muc1 anti-inflammatory effect, ongoing studies are directed at further defining the role of Muc1 on gastric epithelial cells in response to H. pylori infection with the goal of developing novel therapeutic strategies to lessen bacteria-associated disease burdens.

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