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Early molecular events in murine gastric epithelial cells mediated by *Helicobacter pylori* CagA

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Abstract

Background—Murine models of *Helicobacter pylori* infection are used to study host-pathogen interactions, but lack of severe gastritis in this model has limited its usefulness in studying pathogenesis. We compared the murine gastric epithelial cell line GSM06 to the human gastric epithelial AGS cell line to determine if similar events occur when cultured with *H. pylori*.

Materials and Methods—The lysates of cells infected with *H. pylori* isolates or an isogenic *cagA*-deficient mutant were assessed for translocation and phosphorylation of CagA, and for activation of stress pathway kinases by immunoblot.

Results—Phosphorylated CagA was detected in both cell lines within 60 minutes. Phospho ERK 1/2 was present within several minutes and distinctly present in GSM06 cells at 60 minutes. Similar results were obtained for phospho JNK, although the 54 kDa phosphoprotein signal was dominant in AGS whereas the lower molecular weight band was dominant in GSM06 cells.

Conclusion—These results demonstrate that early events in *H. pylori* pathogenesis occur within mouse epithelial cells similar to human cells and therefore support the use of the mouse model for the study of acute CagA-associated host cell responses. These results also indicate that reduced disease in *H. pylori* infected mice may be due to lack of the Cag PAI, or by differences in the mouse response downstream of the initial activation events.

Introduction

Helicobacter pylori (*H. pylori*) colonizes the human gastric mucosa and induces a variety of disease states including asymptomatic and symptomatic gastritis(1), peptic ulcer disease(2), and increased risk of developing gastric cancer(3, 4). The bacteria reside at the apical surface of the epithelium where they persist for the life of the host. The means by which *H. pylori* induce diseases that vary from one host to another remain undefined, although host genetics (5-9), diet (10, 11), environmental factors(12), and bacterial factors (13-15) likely

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all contribute to pathogenesis. One of the most intensely studied *H. pylori* virulence factors is the CagA oncoprotein and the *cagA* pathogenicity island (Cag PAI). The Cag PAI is approximately 40kb of DNA including the *cagA* gene and genes for the structural proteins of a type IV secretion system (T4SS) required for translocation of the CagA protein into host epithelial cell cytoplasm(16-22). Culture of *H. pylori* with human gastric epithelial cell lines has been useful for the study of virulence factors including the translocation and phosphorylation of the CagA protein, and in characterizing the role of the T4SS in the induction of interleukin 8 (IL-8).

Several mouse models of *H. pylori* colonization have now been utilized extensively to study *H. pylori* infection and immunity. These models have been useful for identification of subunit vaccine candidates and characterization of host immunity (reviewed in (23), as well as evaluating the contribution of specific proteins for colonization of the gastric mucosa(24-27). Although chronic gastritis and gastric atrophy from infection of mice with *H. pylori* SS1 strain (HpSS1) has been reported(28), *H. pylori* infection of wild type mice commonly results in only mild gastritis, and therefore it has been less informative regarding *H. pylori* pathogenesis(29-31). The induction of severe gastritis has often required the use of transgenic knockout mice deficient in regulatory cytokines or Treg cell activity, which can complicate the interpretation of results (32-35). Several laboratories have utilized a derivative of HpSS1 taken prior to its adaptation to mice (36-38). Whereas HpSS1 possesses a nonfunctional T4SS, the original isolate termed pre-mouse SS1 (PMSS1) has a fully functional T4SS and more reliably induces gastritis in mice (36, 39).

Since most strains of *H. pylori* fail to induce severe gastritis in the mouse, a comparison of the molecular events associated with *H. pylori* infection of human and mouse gastric epithelial cells may provide a means of identifying differences in the host response that may be critical for pathogenesis. The GSM06 mouse gastric epithelial cell line developed from transgenic mice harboring the temperature sensitive SV40 large T-antigen gene has been a useful model for the *in vitro* study of the gastric mucosa for physiological and pharmacological investigations (40). In the present study, we compare GSMO6 cells to the human AGS gastric cell line to study early events in *H. pylori* infection. We demonstrate that, similar to the association of *H. pylori* with human gastric epithelial cells, CagA is translocated into the cell cytoplasm of GSM06 cells where it becomes phosphorylated. Infection of GSM06 cells with *H. pylori* also activates stress pathway intermediates but with subtle distinctions in kinetics and phopshorylation patterns. These data indicate that early molecular events occur in mouse epithelium during *H. pylori* infection, and that the lack of inflammation observed in the mouse model relative to human disease may be due to downstream events in *H. pylori* pathogenesis, or to variations in the Cag PAI of mouse adapted strains.

Materials and methods

H. pylori strains

H. pylori strain 26695 and a Cag PAI deletion mutant developed by Yoshio Yamaoka (41) were provided with permission by Ellen Beswick (University of New Mexico, Albuquerque). *H. pylori* strain HpM5 was a clinical isolate from an adult gastric biopsy

collected at University Hospitals in Cleveland, OH and mouse-adapted by repeated *in vivo* passage in mice as previously described(27). *H. pylori* isolates were grown on Columbia blood agar supplemented with 7% horse blood and antibiotics including trimethoprim (20 μg/ml), vancomycin (6 μg/ml), amphotericin B (2.5 μg/ml) and cefsulodin (16 μg/ml) as we have previously reported(42). Cultures were grown for 96 hours in a microaerophilic environment. For infection experiments, bacteria were first transferred to liquid cultures of 10 ml Brucella broth supplemented with 10% fetal bovine serum (FBS) and the selective antibiotics described above in a 37°C incubator with 5% CO_2.

Construction of isogenic CagA deficient *H. pylori*.

The *cagA* gene was amplified by PCR to generate two overlapping fragments. The 5' fragment was amplified using forward primer cagA/Pst1-F (5'-GCTACTGCAGACAATGACTAACTAAACCATTGAC-3') to incorporate a Pst1 site upstream of the start codon, and reverse primer cagA/BamH1-R (5'-CCATGAAATCTATTTTGTTTTGGATCCCTTCTTGACTTAATGCTC-3') to incorporate a BamH1 site. The overlapping 3' fragment was amplified using forward primer cagA/BamH1-F (5'-GAGCATTAAGTCAAGAAGGGATCCAAAACAAAATAGATTTCATGG-3') containing the BamH1 site, and reverse primer cagA/Pst1-R (5'-CGATCTGCAGCCTTTAAGATTTTTGGAAACCACC-3') which incorporated a Pst1 site immediately downstream of the stop codon. The two amplicons were combined and amplified by standard overlap extension PCR using primers cagA/Pst1-F and cagA/Pst1-R. The resulting amplicon was digested with Pst1 and ligated into the Pst1 site of pGEM-5zf (Promega Corporation, Madison, WI). A 1.8 kb kanamycin resistance cassette (*aph*) was inserted at the BamH1 site within the *cagA* gene. Kan^r colonies were selected, and the plasmid construct was sequenced. The *H. pylori* strain HpM5, which transforms with high efficiency(27), was transformed using a modification of the method described by Wang *et al* (43). Briefly, 1 μg of the plasmid construct was combined with 100 μl of liquid HpM5 culture, spotted onto a Brucella agar plate containing the standard antibiotic mix and grown overnight as described above. Bacteria were then resuspended in 100 μl PBS, and plated on selective media containing 20 μg/ml kanamycin. Bacteria were grown for 96 hours and the kanamycin-resistant colonies were assayed by PCR for the presence of the disrupted *cagA* gene.

Gastric cell lines

The human gastric cell line, AGS (ATCC, Manassas, VA) was maintained in Ham's F12 supplemented with 10% FBS and glutamine under standard cell culture conditions. The mouse gastric cell line, GSM06, was obtained from the RIKEN Cell Bank (Tsukuba Science City, Japan), and maintained in DMEM/F12 media supplemented with 2% FBS, ITES (2 μg/ml insulin, 2 μg/ml transferrin, 0.122 μg/ml ethanolamine, and 0.009 μg/ml sodium selenite), and 10 ng/ml EGF, at 33°C as previously described(40). The GSM06 cell line, derived from the fundic mucosa of C57BL/6 mice expressing a temperature sensitive SV40 large T-antigen, were maintained at 33°C. Cells were switched to 37°C at least 24 hours prior to use in all experiments. All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA)

Infection of epithelial cells

Epithelial cell lines were seeded at either 1×10^6 cells/well in 24 well tissue culture dishes, or 5×10^5 cells/well in 48 well dishes and grown overnight in 5% CO_2 at 37°C. Cells were then infected with *H. pylori* at a bacteria to epithelial cell ratio of 25:1 for the indicated lengths of time. The density of *H. pylori* culture was determined by comparing the OD_{450nm} to a previously established growth curve. Cell supernatants were removed at previously determined time points and stored at −70°C until analyzed for cytokine levels as described below. Epithelial cells were lysed and prepared for immunoblot analysis as described below.

Western Blot

Epithelial cells from infection experiments were washed three times in PBS and lysed in 150 μl lysis buffer on ice. Lysis buffer consisted of 50 mM Tris, pH 8.0, 0.2 mM EDTA, 200 mM NaCl, 1% NP-40, and 10% glycerol, and was supplemented with 200 mM sodium orthovanadate, 100 mM DTT and protease inhibitors. Bacterial lysates were prepared by resuspending bacterial cell pellets in SDS-PAGE sample buffer. The protein content of cell lysates was determined by the Bradford assay (BioRad, Hercules, CA), normalized and resolved on 10% SDS-PAGE gels. Proteins were transferred to nitrocellulose, blocked with 4% nonfat dry milk, and incubated with primary antibody (1:1000) overnight at 4°C. Blots were subsequently incubated with secondary antibody conjugated to horse radish peroxidase (1:2500) and developed using the ECL plus western blotting detection system (GE Healthcare Life Sciences, Pittsburgh,PA). Rabbit polyclonal primary antibodies specific for JNK, phopho-JNK, p38, phopho-p38, ERK 1/2, and phosphor-ERK 1/2 were purchased from Cell Signaling Technology (Danvers, MA). Polyclonal rabbit anti-CagA antibody was purchased from Austral Biologicals (San Ramon, CA). Monoclonal anti-phosphotyrosine monoclonal antibody 4G10 was purchased from EMD Millipore (Billerica, MA) and polyclonal rabbit anti-beta-actin was purchased from Thermo Scientific (Waltham, MA).

Immunofluorescence

AGS or GSM06 cells were grown to 70% confluency on glass cover slips in the wells of 24 well cluster plates. Following infection with *H. pylori* the cells were washed in PBS, fixed in methanol at –20°C, and blocked in 1% normal goat serum. Cells were stained using sera from mice immunized with HpSS1 lysate antigen as the primary antibody, followed by goat anti-mouse FITC conjugate. The cells were counter-stained with AlexaFluor 633 phalloidin (Molecular Probes, Inc., Eugene, OR), mounted in Vectasheild mounting medium with DAPI (Vector Laboratories, Inc., Burlingame,CA), and examined for fluorescence by confocal microscopy using Leica Confocal Software.

Statistics

Differences between control and experimental groups in the amount of cytokine produced by stimulated cell lines was evaluated by student's t test. Differences were considered statistically significant if *P* values were less than 0.05.

Results

Association of *H. pylori* with epithelial cell membranes

H. pylori has been shown to closely associate with human gastric epithelial cells in vivo and in vitro. To determine if mouse adapted *H. pylori* also associates with gastric epithelial cells *in vitro* we incubated our mouse-adapted HpM5 strain(27) with human AGS cells and mouse GSM06 cells for microscopic analysis following fluorescent antibody staining of the bacteria (Figure 1 A). HpM5 closely associated with AGS cells after 30 minutes of infection. Bacteria uniformly associated along the periphery of the cells. Similarly, HpM5 cultured with the mouse gastric epithelial cell line GSM06 associated with the cell membrane. No bacteria were observed within the cytoplasm of either AGS cells or GSM06 cells. Since GSM06 cells often display spindle and angular shapes when less than confluent, no analysis could be made on the ability of HpM5 to induce the hummingbird phenotype as has been demonstrated for infected AGS cells (21).

CagA expression by *H. pylori* strains

The *cagA* gene of HpM5 was interrupted by insertion of a kan^r cassette to create an isogenic CagA-deficient strain of *H. pylori*. The creation of HpM5 *aph::cagA* was confirmed by PCR (data not shown). Immunoblot analysis of bacterial lysates from HpM5 and HpM5 *aph::cagA*, as well as 26695 and its corresponding Cag PAI deletion mutant, was performed to assess CagA expression (Figure 1B). Both 26695 and HpM5 were positive for large molecular weight proteins of approximately 130 kDa and 90 kDa. No protein was detected in the HpM5 *aph::cagA* lysate or the 26695 Cag PAI mutant by the CagA-specific antibody.

Translocation of CagA into epithelial cells

Translocation of CagA into the epithelial cell cytoplasm is an important step in *H. pylori* pathogenesis. To validate the mouse model for the study of *H. pylori* virulence, it is important to establish that CagA translocation occurs with murine epithelial cells. Human AGS and mouse GSM06 gastric epithelial cells were infected with HpM5 and then washed prior to examination of cell lysates for translocated CagA by immunoblot with a CagA-specific antibody. Translocation of CagA (T-CagA) was noted in both cell types as indicated by the 90/130 kDa doublet (Figure 1C). T-CagA was evident in both cell types by 60 minutes post infection. Stripped membranes were also probed for phosphorylated CagA and both cell types had detectable phosporylated CagA as early as 30 minutes post-infection. The detection of phosphorylated cagA earlier than translocated CagA is most like due to the sensitivity of the respective antibodies. Similar results were reported in a phagocyte model of CagA translocation where a CagA polyclonal antisera and commercially available phosphotyrosine specific antibody were used to detect CagA and phosphorylated CagA respectively (44).

Variation in JNK isoform phosphorylation between host species

Strains of *H. pylori* that carry *cagA* vary in CagA production, phosphorylation potential, and virulence(45) and we observed similar variations between strains when used in coculture with GSM06 cells (supplementary Figure 1). Whereas the translocation of CagA is largely

associated with molecular events that influence cellular morphology and cell cycle dynamics, interaction of the T4SS with the host cell membrane are more directly related to the induction of inflammation(46). Therefore, we compared the stress response of mouse and human epithelial cells when cocultured with wild type, TFSS deficient, and CagA deficient *H. pylori*. Culture of cells with *H. pylori* strains induced the phosphorylation of extracellularly regulated kinase 1/2 (ERK 1/2) within minutes (Figure 2). The presence of phospho-ERK however was significantly elevated only in GSM06 cells at 60 minutes when co-cultured with all strains except the PAI deficient 26695In most cases this significant phosphorylation was still evident for GSM06 cells at 120 minutes.

Infection of the epithelial cell lines with *H. pylori* also induced the phosphorylation of C-Jun N-terminus kinase 1/2 (JNK1/2) but the intensity and kinetics of expression were more varied than for phospho-ERK (Figure 3). Although the expression of total JNK was evident, significant increases in phosphorylated JNK in AGS cells was only noted at 120 minutes and only for HpSS1. This significant increase was restricted to the p54 isoform. Although not statistically significant, the p54 isoform was also visable above control levels for HpM5 and HpM5 *aph::cagA*. When GSM06 cells were infected with the bacterial strains, phospho-JNK was elevated for 26695, HSS1, and HpM5 *aph::cagA* by 30 minutes but significance was lost in all three groups by 60 minutes, although phosphorylation was noted again for HpM5 at 120 minutes. In all cases, significant increases in phosphorylation were restricted to the p46 isoform. Signaling of the p38 pathway was also evaluated (Figure 4). Significant increases in phosphorylated p38 were evident by 5 minutes in both cell lines and remained throughout the 120 minute incubation period. No major differences were evident however, between signaling in the AGS and GSM06 cell lines nor could any events be attributed to either the pathogenicity island or the CagA protein.

Discussion

The present study demonstrates that Cag PAI-positive strains of *H. pylori* interact with mouse gastric epithelial cells similar to previous reports using human epithelial cells (16, 18-22). The bacteria were shown to associate with the epithelial cell surface and to effectively translocate CagA protein into the host epithelial cell cytoplasm where it becomes phosphorylated at tyrosine residues. *H. pylori* also activated mouse gastric epithelial cells similar to human cells in that phosphorylation of ERK-1/2, JNK-1/2 and p38 was noted. The pattern of phosphorylated JNK was distinct between AGS and GSM06 cells however as the limited phosphorylation of AGS was observed for the p54 subunit whereas the p46 subunit was primarily phosphorylated in GSM06 cells. These results provide evidence that subtle differences may occur in early molecular signaling that may contribute to limiting the host response to *H. pylori* in mice.

Mouse models of *H. pylori* infection have been useful in predicting candidate molecules for vaccine development and determining the minimal requirements for the development of vaccine-induced protective immunity (reviewed in (23)), and in determining factors that are essential for colonization and chronic infection(24-27). The use of mouse models for the study of *H. pylori* virulence and pathogenesis has been limited as laboratories have found this model to induce limited gastritis, or disease that progresses slowly(29-31). To

compensate, novel methods of grading inflammation associated with infection have been employed, such as limiting the evaluation to the microscopic field displaying the most severe inflammation, or focusing on the antral-fundic junction(30, 32). More recently, some investigators have utilized the clinical isolate of *H. pylori* that was used to develop HpSS1 designated Pre mouse SS1. Unlike *H. pylori* SS1 which has been shown to have a nonfunctional PAI(47, 48), the pSS1 strain has a functional PAI and induces more gastric inflammation in the mouse(36, 39).

The *H. pylori* Cag PAI induces disease through at least two distinct mechanisms. The interaction of the T4SS with the host cell membrane, most likely through CagL binding to α5β1 integrin, induces proinflammatory factors including IL-8(46, 49-51). These events are believed to promote a local inflammatory response *in vivo*. Additionally, the T4SS translocates CagA into the epithelial cell cytoplasm where it becomes phosphorylated, and alters cell signaling events in the host that impact cell structure and cell cycle events(18-22). It is currently unknown to what extent these events occur in the infected mouse stomach. The use of transgenic mice expressing CagA in gastric epithelial cells however, indicates that CagA maintains oncogenic activity in mice(52).

Variations in disease among mouse models of *H. pylori* infection may be partially explained by differences in the host genetics among mouse strains(31). Limited pathogenesis also appears to be due to deficiencies in *Cag* PAI function in many cases. Philpott *et al.* showed that *H. pylori* strains that are deficient in *Cag* PAI genes are more successful at colonizing mice(48). Additionally, continued passage in mice tends to ameliorate the ability of the bacteria to induce pro-inflammatory responses. The most commonly used strain for infecting mice, HpSS1, produces the CagA protein but has a nonfunctional T4SS(47). Recently, the use of the clinical isolate that is the parental strain of SS1 has been used in mice and found to induce increased pathogenesis(36-38). This strain has a fully functional T4SS. The increased gastritis and host response induced with the PMSS1 strain suggest that *H. pylori* has the potential to employ the same virulence mechanisms in play when interacting with the human host, and are consistent with the present results demonstrating T4SS positive *H. pylori* induce proinflammatory cytokines in mouse gastric epithelial cells and successfully translocate CagA into host cells where it becomes phosphorylated.

Although infection with *H. pylori* strains induced similar events in human and mouse epithelial cell lines, slight variations were observed in the duration of stress signaling pathway intermediates. Additionally, whereas the phophorylated 54 kd JNK protein was predominant in human cells, in mouse cells, it was the 46 kd JNK protein that was phosphorylated. These results suggest that the mild inflammatory response observed in *H. pylori*-infected mice may be due in part to subtle differences between humans and mice in the cell signaling events associated with *H. pylori* interaction with host epithelial cells. It should be noted that *H. pylori* can activate epithelial cells through the EGF receptor (53-55). It is possible therefore that the addition of EGF for maintenance of GSM06 cells may result in signaling events that influence the response of these cells to T4SS contact. However, we did not observe any signaling differences in control AGS cells and GSM06 cells without *H. pylori* infection.

These results differ from those reported using primary mouse gastric epithelial cells in which translocation of CagA could not be detected in mouse epithelial cells (56). However, several differences in experimental design may explain this discrepancy. First, whereas we relied on GSM06, a T antigen-driven cell line derived from transgenic C57BL/6 mice, others used primary cell cultures from the stomachs of CD1 mice. Our reliance upon the GSM06 cell line was necessitated by the inability to maintain viability of freshly isolated murine gastric epithelial cells. Second, the *H. pylori* strains differed between the two studies, as did the source of anti-CagA antibody.

The present study using a murine gastric epithelial cell line demonstrate that events similar to those observed in human cell lines occur in mice as illustrated by the ability of *H. pylori* to translocate CagA into GSM06 cells. Ultimately, an *in vivo* demonstration of the ability of *H. pylori* to translocate CagA into epithelial cells of the mouse may be necessary to determine the role this mechanism plays in pathogenesis. However, the present study provides evidence that CagA translocation and phosphorylation may occur, and that there may be subtle differences between the signaling pathways employed between humans and mice. Further characterization of these differences and how the murine response digresses from that of humans may help elucidate how *H. pylori* induces disease in its natural host.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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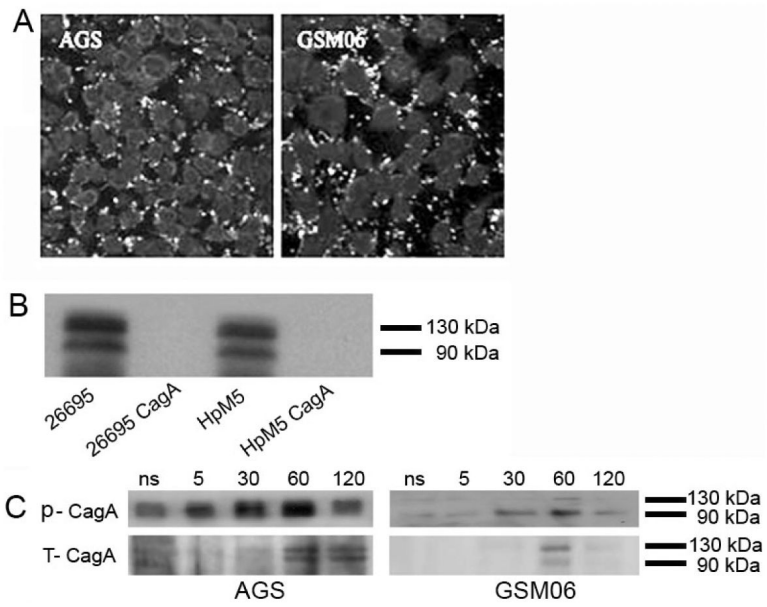

Figure 1.

H. pylori associates with mouse gastric GSM06 epithelial cells and translocates CagA. (A) Epithelial cells grown on glass coverslips were infected with HpM5, washed, fixed, and stained with *H. pylori*-specific mouse antisera and anti-mouse IgG-FITC conjugate. Cellular morphology was detected using phalloidin and DAPI counter-stains. (B) Western blot analysis of bacterial lysate from strain 26695, 26695 with a PAI deletion (26695 CagA), mouse-adapted HpM5 and the isogenic mutant HpM5 *aph::cagA* (HpM5 CagA) using anti-CagA polyclonal antibody. (C) CagA-specific immunoblot analysis of AGS and GSM06 cell lysates following infection with HpM5 demonstrating phospho-CagA (P-CagA) and total CagA (T-CagA).

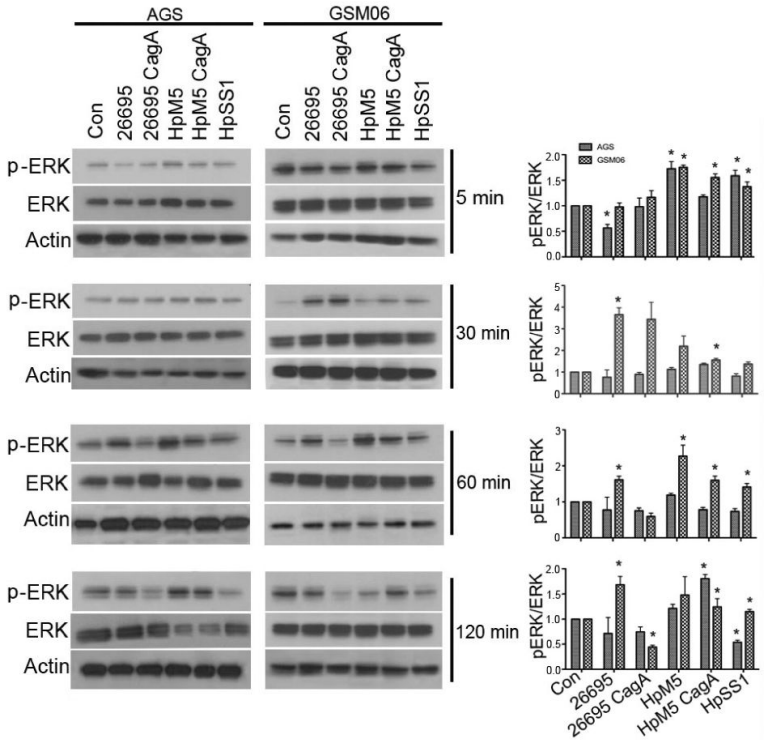

Figure 2.

Infection of mouse epithelial cells with *H. pylori* activates the ERK 1/2 stress pathway. Lysates from AGS and GSM06 cells infected with 26695, 26695 CagA, HpM5 or HpM5 CagA for either 5, 30, 60 or 120 minutes were resolved by SDS-PAGE and analyzed by immunoblot using total and phospho-ERK specific monoclonal antibodies. Data were evaluated as the ratio of phospho-ERK to total ERK. Significant changes in phosphorylation were determined as compared to control, unstimulated cells. $*P < 0.05$.

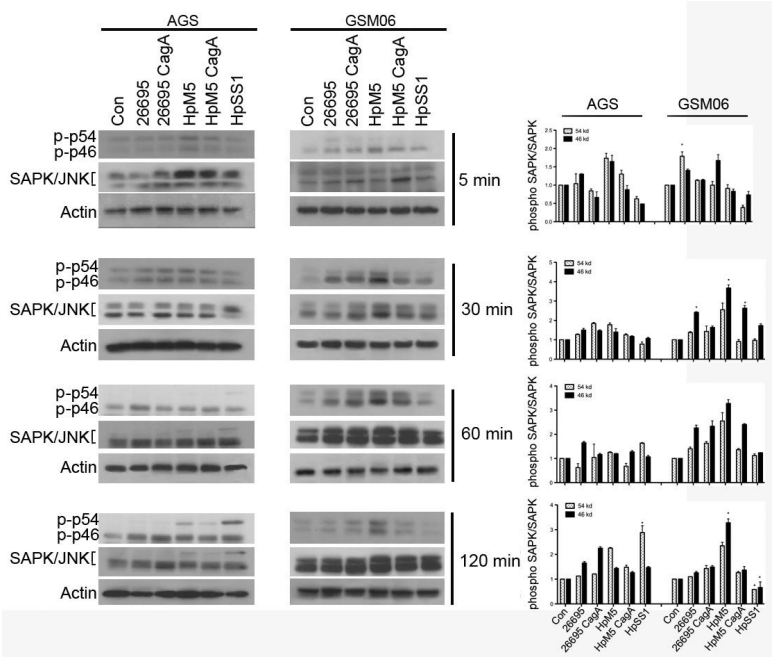

Figure 3.

Infection of mouse epithelial cells with *H. pylori* activates the JNK stress pathway by phosphorylation distinct from human cells. Lysates from AGS and GSM06 cells infected with 26695, 26695 CagA, HpM5 or HpM5 CagA for either 5, 30, 60 or 120 minutes were resolved by SDS-PAGE and analyzed by immunoblot using total and phospho-JNK 1/2 specific monoclonal antibodies. Data were evaluated as the ratio of phospho-JNK P-54 of P-46 to total JNK 54 or 46 respectively. Significant changes in phosphorylation were determined as compared to control, unstimulated cells. $*P < 0.05$.

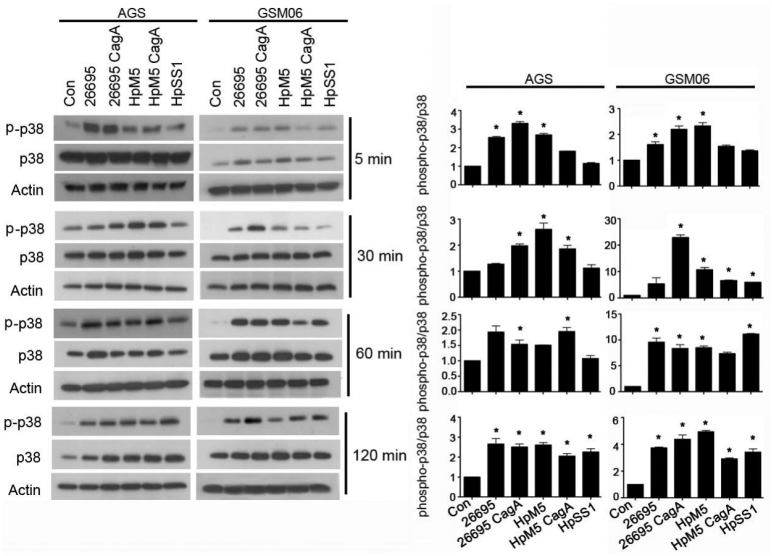

Figure 4.

Infection of mouse epithelial cells with *H. pylori* activates the p38 stress pathway. Lysates from AGS and GSM06 cells infected with 26695, 26695 CagA, HpM5 or HpM5 CagA for either 5, 30, 60 or 120 minutes were resolved by SDS-PAGE and analyzed by immunoblot using total and phospho-p38 specific monoclonal antibodies. Data were evaluated as the ratio of phospho-p38 to total p38. Significant changes in phosphorylation were determined as compared to control, unstimulated cells. $*P < 0.05$.