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The Secreted Peptidyl Prolyl cis,trans-Isomerase HP0175 of Helicobacter pylori Induces Apoptosis of Gastric Epithelial Cells in a TLR4- and Apoptosis Signal-Regulating Kinase 1-Dependent Manner

Chaitali Basak, Sushil Kumar Pathak, Asima Bhattacharyya, Shresh Pathak, Joyoti Basu, and Manikuntala Kundu

Apoptosis contributes to the pathology of gastric epithelial cell damage that characterizes Helicobacter pylori infection. The secreted peptidyl prolyl cis, trans-isomerase of H. pylori, HP0175 executed apoptosis of the gastric epithelial cell line AGS in a dose- and time-dependent manner. The effect of HP0175 was confirmed by generating an isogenic mutant of H. pylori disrupted in the HP0175 gene. The apoptosis-inducing ability of this mutant was impaired compared with that of the wild type. The effect of HP0175 was mediated through TLR4. Preincubation of the gastric epithelial cell line AGS with anti-TLR4 mAb inhibited apoptosis induced by HP0175. Downstream of TLR4, apoptosis signal-regulating kinase 1 activated MAPK p38, leading to the caspase 8-dependent cleavage of Bid, its translocation to the mitochondria, mitochondrial pore formation, cytochrome c release, and activation of caspases 9 and 3. We show for the first time that a secreted bacterial Ag with peptidyl prolyl cis,trans-isomerase activity signals through TLR4, and that Ag executes gastric epithelial cell apoptosis through a signaling pathway in which TLR4 and apoptosis signal-regulating kinase 1 are central players. The Journal of Immunology, 2005, 174: 5672–5680.

Helicobacter pylori is a spiral, microaerophilic Gram-negative bacterium that colonizes gastric epithelial cells. Depending on the strain of H. pylori and the individual it infects, the bacterium can cause peptic ulcer disease and gastric mucosa-associated lymphoid tissue lymphoma (1). Apoptosis is involved in H. pylori-induced gastric epithelial cell damage (2–6). Apoptosis-related genes and proteins such as the Bcl-2 family, Fas-Fas ligand, and p53 have been implicated in epithelial cell apoptosis (7–9). Recent data have shown that caspases 8, 3, and 9 are activated in gastric epithelial cells during infection with H. pylori (10). This underscores the need to understand in detail the nature of the factors responsible for H. pylori-infected gastric epithelial cell apoptosis.

Apoptosis is an orchestrated suicide program in which the key morphological alterations are mediated by a family of cysteine proteases known as the caspases, which are activated by proteolytic cleavage (11–13). In death receptor-mediated (or extrinsic) cell death, procaspase 8 is recruited to the death-inducing signaling complex through its N-terminal death effector domain (14, 15). Activated caspase 8 can subsequently cleave downstream effector caspases 3 and 7 (16–18). Death via the intrinsic pathway is characterized by a decrease in mitochondrial transmembrane potential and release of cytochrome c (cyt c) (19), apoptosis-inducing factor (20), and Smac (DIABLO) (21) from the mitochondria. Cytosolic cyt c together with ATP/dATP induces a conformational change in apoptotic protease-activating factor-1, leading to peptidyl prolyl cis,trans-isomerase oligomerization, binding of procaspase 9 in a so-called apoptosome complex (22, 23), and proteolytic activation of procaspase 9 (24).

The MAPK cascade is one of the evolutionarily conserved phosphorylation-regulated protein kinase cascades that influences cell survival or cell death. JNK and p38 mediate various types of stress-induced apoptosis (25). Apoptosis signal-regulating kinase 1 (ASK1) is a member of the MAPK kinase kinase family that plays a role in stress-induced apoptosis, principally through activation of the JNK or p38 MAPK signaling cascades (26–29).

Secreted proteins of H. pylori, such as VacA (30), urease (31), and γ-glutamyl transpeptidase (32), are apoptosis-inducing proteins that contribute to gastric inflammation and epithelial cell damage. However, apoptosis is likely to be regulated by a number of proteins. With this in view, we focused on the H. pylori repertoire of secreted proteins identified by several groups in recent years (33, 34). We narrowed down to the protein HP0175, which is one of five Ags of H. pylori preferentially recognized by the Abs of patients with gastroduodenal ulcers rather than dyspepsia patients (35, 36). HP0175 is characterized by a C-terminal peptidyl prolyl cis,trans-isomerase (PPlase) core identified on the basis of sequence similarity. PPlases have been characterized as virulence factors of Legionella pneumophila (37) and Trypanosoma cruzi (38).

1 Abbreviations used in this paper: cyt c, cytochrome c; ASK1, apoptosis signal-regulating kinase 1; ΔΨm, mitochondrial membrane potential; DiOC6, 3′,3′-dihexyloxocarbocyanine; FMK, fluoromethyl ketone; KO, knockout; MBP, myelin basic protein; CCCP, carbonyl cyanide m-chlorophenylhydrazone; MPT, mitochondrial permeability transition; PARP, poly(ADP-ribose) polymerase; PPlase, peptidyl prolyl cis,trans-isomerase; t-Bid, truncated Bid; WT, wild type.

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2 Address correspondence and reprint requests to Dr. Manikuntala Kundu, Department of Chemistry, Bose Institute, 93/1 Acharya Prafulla Chandra, Road, Kolkata 700009, India. E-mail address: manikuntala@vsnl.net

Department of Chemistry, Bose Institute, Kolkata, India

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In the present study we demonstrate that HP0175 is a proapoptotic factor by two independent experimental approaches, namely, histone ELISA and annexin V-fluores binding to phosphatidylyserine exposed on the cell surface. The contribution of HP0175 to *H. pylori*-induced cell death in AGS cells was confirmed by the observation that an isogenic mutant of *H. pylori* 26695 disrupted in the HP0175 gene was impaired in its apoptosis-inducing ability. HP0175 appeared to signal through TLR4, because preincubation with a blocking anti-TLR4 Ab inhibited HP0175-mediated apoptosis in AGS cells. Also, HP0175 interacted directly with TLR4. ASK1 was activated downstream of TLR4, leading to the sequential activation of p38 MAPK and caspase 8 linked to the cleavage of Bid. The translocation of Bid to the mitochondria led to the disruption of mitochondrial membrane potential, cyt c release, and sequential activation of caspases 9 and 3, culminating in cell death. HP0175 emerges as a novel pathogen-associated molecular pattern, which signals through TLR4 to execute cell death. To the best of our knowledge, this is the first report of a secreted Ag of *H. pylori* capable of inducing apoptosis in a TLR4- and ASK1-dependent manner.

Materials and Methods

Reagents

Abs against total and phospho-specific MAPks, poly(ADP-ribose) polymerase (PARP), Bid, and cleaved caspases 3, 8, and 9 were purchased from Cell Signaling Technology. Abs against ASK1, cyt c, β-actin, and protein A/G Plus agarose were purchased from Santa Cruz Biotechnology. SB203580, U0126, z-Asp(OCH3)-Glu(OH2)-Val-Asp(OCH3)-FMK (z-DEVD-FMK), z-Leu-Glu(Ome)-His-Asp(Ome)-CH2F (z-LEHD-FMK), z-Ile-Glu(Ome)-Thr-Asp(Ome)-FMK (z-IETD-FMK), and protease inhibitors were purchased from EMD Biosciences. 3′,3′-Dihexyloxacarbocyanine (DiOC5) was obtained from Molecular Probes. Escherichia coli LPS and polymyxin B resin were purchased from Sigma-Aldrich. Annexin-fluor was purchased from Roche.

Cloning of the gene encoding HP0175

The gene encoding the protein HP0175 (accession no. P56112) was amplified by PCR using the genomic DNA of *H. pylori* strain 26695 as template and the primers 5′-TTG AGA TCC TGG ATC GTC AGC ATG AAA AAA AAT ATC ATC TTA ATT-3′ (sense) and 5′-TAT AGG TAG TCT TCT TAG ATT ACA ATT TTA-3′ (antisense) with BanHI and EcoRI sites (underlined) and cloned in pUC19. The cloned HP0175 gene was excised from pUC19 and cloned between NheI (indicated in bold) and EcoRI sites in the vector pET28a (Novagen) for expression. The resulting plasmid is designated pCMK101.

Expression and purification of rHP0175

Induction of HP0175 from *E. coli* BL21 (DE3)/pCMK101 was conducted at 37°C for 2 h with isopropl thio-β-galactoside (50 μM). Hexa-His-tagged HP0175 was purified from the cell lysates by chromatography on Ni2+NTA (Qiagen). Anti-HP0175 Abs were raised by Imugen Biotech with immunizing rabbits with purified HP0175.

Production of the HP0175 gene-disrupted mutant strain

The entire HP0175 gene region was amplified by PCR using genomic DNA of *H. pylori* strain 26695 as template and the primers 5′-CCG GGT ACC AAT TTT GTA ATC TTC TTT-3′ (sense); a) and 5′-TTT GGA TCC GGG TAG CGT TCG CAC ATG G-3′ (antisense); b) harboring KpnI and BamHI sites (underlined), respectively, and cloned in pBluescript (pBlue-NS). The PstI site was created 450 bp downstream from the N terminus of the HP0175 gene by overlap extension PCR (pBlue-NPS). The kanamycin resistance cassette (aphA3) from pUC4K (Amersham Biosciences) was cloned at the PstI site of pBlue-NS. The resulting plasmid, pBlue-NPS-aphA3, was electroporated into *H. pylori* 26695. The transformants were plated on BHI agar plates containing 5 μg/ml kanamycin, and kanamycin-resistant clones (knockout (KO) strain, disrupted in HP0175) were selected. The insertion of the aphA3 gene within the HP0175 gene was confirmed by PCR using genomic DNA from the KO strain as a template, followed by restriction digestion of the PCR product with PstI to excise out the kanamycin cassette.

Purification of VacA

The vacA gene of *H. pylori* 26695 was amplified by PCR (30 cycles and cloned in pET20b (Novagen). The VacA construct was transformed into *E. coli* strain BL21(DE3), and VacA was purified from the cell-free supernatant by chromatography on Ni2+-NTA-agarose.

Assay for LPS

LPS contamination of the purified protein was determined by Limulus amebocyte lysate assay using the E-TOXATE kit (Sigma-Aldrich) with a sensitivity limit of 0.1 U/Eml (39).

PPase assay

PPase activity was measured in a coupled assay with chymotrypsin, in which the oligopeptide, N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma-Aldrich; 20 μM) in 100 mM sodium phosphate buffer (pH 7.8) was used as the substrate (40). After preincubation at 15°C for 2 min, the reaction was started by adding 10 μM chymotrypsin and was monitored spectrophotometrically at 15°C for 5 min by recording the increase in absorbance corresponding to the release of p-nitroanilide. The arbitrary unit of PPase activity, Up, was calculated from Up = (kp – kr)k/kw where kp and kw are the first-order rate constants for p-nitroanilide release in the presence and absence of the protein, respectively.

Culture of the human gastric epithelial cell line AGS

The human gastric epithelial cell line AGS was obtained from the National Center of Cell Science and maintained in Ham’s F-12 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO2. Dishes were washed to remove nonadherent cells. Adherent cells were ≥95% viable as determined by trypan blue dye exclusion.

Bacteria and infection

For AGS cell infections, *H. pylori* strains were grown under microaerophilic conditions for 48 h on brain heart infusion agar plates containing 10% FBS and incubated with AGS cells monolayers at a multiplicity of infection of 100 on culture plates in Ham’s F-12 medium supplemented with 10% FBS.

Plasmid constructs and transient transfections

TLR4 was amplified from RNA isolated from THP-1 cells using the sense and antisense primers 5′-ATGGATCCGCATGGAGCTGAATTTCTA-3′ and 5′-ATAAAAGCTTCTAAGTTGCTCTTCG-3′, respectively, and cloned between the BamHI and HindIII sites of pcDNA3.1. TLR4 (1-643) (TLR4(dn)) encoding the first 643 aa of TLR4, was generated using the same sense primer and the antisense primer 5′-ATAAGGCTTTCACTACTGCTGTAATGAT-3′. Hemagglutinin-tagged wild-type ASK1 (ASK1(wt)) and a catalytically inactive mutant (K709M) of ASK1 (ASK1(KM)) were obtained from Dr. H. Ichijo (Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan). Flag-tagged p38 and its dominant negative mutant (p38(dag)) were obtained from Dr. R. Davis (University of Massachusetts Medical School, Worcester, MA). pCMVdwt caspase 9S was obtained from Dr. D. W. Seol (University of Pittsburgh School of Medicine, Pittsburgh, PA). Cells were transfected with 2 μg of each (empty vectors or recombinants) using FuGene 6 (Roche) according to the manufacturer’s instructions. β-Galactosidase reporter plasmid was used to normalize transfection efficiencies.

Cell death

For the detection of histones by ELISA, AGS cells were plated (6 × 104 cells/plate) on 96-well plates. After treatments, cell death was detected with the cell death detection ELISA Plus kit (Roche) according to the manufacturer’s protocol.

For measurements of annexin-fluor binding, 100 μl of cells in annexin-fluor binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl2) were mixed with 2 μl of annexin-fluor. The mixture was incubated for 15 min at room temperature, washed with binding buffer, and analyzed by flow cytometry. To assess necrosis, propidium iodide (1 μg/ml) was added to the cell suspension just before analysis.

Treatment of AGS with HP0175 and preparation of cell lysates

AGS cells were cultured in 24-well tissue culture plates at 4 × 104 cells/well, treated with HP0175, and lysed with lysis buffer (20 mM Tris-HCl (pH 7.4); 1% (v/v) Nonidet P-40; 10% (v/v) glycerol; 137 mM NaCl; 20 mM sodium fluoride; 1 mM EDTA; 40 mM sodium β-glycerophosphate; 4 μg/ml each of leupeptin, pepstatin, and aprotinin; 1 mM Na2VO3; 1 mM

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Pefabloc; and 1 mM benzamidine) on ice for 15 min. Cell lysates were boiled for 5 min after the addition of 5× Laemmli sample buffer and subjected to Western blotting. When performing Western blotting for detection of caspases, cells were pelleted and freeze-thawed three times in 20 μl of cell extraction buffer (50 mM PIPES/NaOH (pH 6.5), 2 mM EDTA, 0.1% (v/v) CHAPS, 5 mM DTT, 20 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 1 mM Pefabloc). The lysates were centrifuged at 10,000 × g for 5 min at 4°C, and the supernatants were collected for the detection of caspases.

Western blotting

Proteins were separated on SDS-polyacrylamide gels, then transferred electrophoretically to polyvinylidene difluoride membranes. The blots were blocked with 5% nonfat dry milk and subsequently incubated overnight at 4°C with primary Abs in TBST (1%, v/v) with 5% (w/v) BSA. After washing, the blots were incubated with HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technology; or appropriate secondary Ab) in blocking buffer for 1 h at room temperature, followed by development with BM chemiluminescence reagent (Roche) and exposed to x-ray film (Kodak XAR5; Eastman Kodak).

ASK1 kinase assay

AGS cells were cultured in 24-well tissue culture plates at 6 × 10^5 cells/ well. After treatment, cells were lysed with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 1% sodium deoxycholate, 0.5 mM sodium pyrophosphate, 1 mM sodium β-glycerophosphate, 1 mM Na3VO4, and 1 μg/ml leupeptin. The supernatant (equivalent to 200 μg of protein) was incubated overnight at 4°C with rabbit polyclonal ASK1 Ab. Protein A/G Plus agarose was added and incubated at 4°C for an additional 3 h. The beads were washed twice with lysis buffer and twice with kinase buffer (25 mM Tris-HCl (pH 7.5), 5 mM sodium-β-glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4, and 10 mM MgCl2). The pellet was washed once with kinase buffer without protease inhibitors. The beads were then incubated in 20 μl of kinase buffer in the presence of 0.5 μCi of [γ-32P]ATP (sp. act. 6000 Ci/mmol) with 1 μg of myelin basic protein (MBP) as substrate at 30°C for 15 min. The reaction was stopped by adding protein gel denaturing buffer. After SDS-PAGE, gels were dried and subjected to autoradiography.

Study of the interaction of HP0175 with TLR4

AGS cell (10^7 assay) lysates were incubated with Ni2+ -NTA or HP0175-bound Ni2+ -NTA agarose at 4°C for 2 h with shaking. The beads were washed and boiled in 2× Laemmli buffer for 5 min, proteins were separated by 7.5% SDS-PAGE, transferred onto polyvinylidene difluoride, and immunoblotted with anti-TLR4 Ab to detect His-tagged protein-bound TLR4. In another set of experiments, cells were transfected with empty vector or TLR4 construct, followed by lysis and immunoprecipitation with anti-TLR4 Ab as described above. The pellet was washed, boiled with 2× denaturing buffer, electrophoresed, and immunoblotted with anti-His Ab to detect TLR4-bound His-tagged protein.

Isolation of cytosol and mitochondrial fractions for detection of cyt c and Bid

After treatments, 18 × 10^6 cells were suspended in 400 μl of suspension buffer (20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM Pefabloc, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 150 mM sucrose) and broken with 20 passages through a 26-gauge needle. The homogenate was centrifuged at 750 × g for 10 min to remove nuclei and unbroken cells. The mitochondrial pellet was obtained by centrifugation at 10,000 × g for 15 min and resuspended in 40 μl of resuspension buffer (20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM Pefabloc, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). The supernatant obtained by centrifugation at 100,000 × g for 60 min at 4°C was the cytosolic fraction. Before Western blotting, protein concentrations were assayed, and all samples were normalized to equal protein concentrations. Blots were stripped and reprobed for β-actin expression.

Measurement of mitochondrial membrane potential

The induction of the mitochondrial permeability transition was determined in intact cells as the reduction in the accumulation of DIOC6. Briefly, 5 × 10^3 AGS cells were loaded with DIOC6 (100 nM) during the last 30 min of treatment with HP0175, then lysed in deionized water, and fluorescence of DIOC6 was read in a Hitachi F-4500 fluorescence spectrophotometer at excitation and emission wavelengths of 488 and 500 nm, respectively. Values for de-energized mitochondria were determined by simultaneous treatment of cells with 10 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) and DIOC6.

Statistical analysis

Data are represented as the mean ± SD of separate experiments. ANOVA and Student’s t test were performed to test statistical significance. A value of p < 0.05 was considered statistically significant.

Results

Purification of rHP0175

HP0175 was expressed as an N-terminally hexa-His-tagged protein in E. coli BL21(DE3)/pCMK101 and was purified by affinity chromatography on Ni2+-NTA agarose. It migrated as a single band of Mw 30 on SDS-PAGE (Fig. 1A). The catalytic activity confirmed that the rHis-tagged protein was biologically active. A Limulus amebocyte lysate assay demonstrated that <0.05 ng of LPS was present per microgram of protein in the different preparations of HP0175 tested.

HP0175-induced cell death

Taking into account previous reports that secreted proteins of H. pylori induce apoptosis of gastric epithelial cells, we asked whether HP0175 could be one of the apoptosis-inducing factors of H. pylori. Protein in E. coli protein in H. pylori (WT or KO) at a multiplicity of infection of 50 for 24 h (C). D. Cells were incubated with LPS or with equal amounts of His-tagged irrelevant protein (irr Ab). Western blot analysis of HP0175. Purified HP0175 or cell extracts from wild-type H. pylori (WT) or an isogenic mutant inactivated in the HP0175 gene (KO) were run on SDS-PAGE, electrotransferred onto a polyvinylidene difluoride membrane, and probed with anti-HP0175 Ab or Ab raised against an irrelevant His-tagged protein (irr Ab). Western blot analysis of HP0175. Purified HP0175 or cell extracts from wild-type H. pylori (WT) or an isogenic mutant inactivated in the HP0175 gene (KO) were run on SDS-PAGE, electrotransferred onto a polyvinylidene difluoride membrane, and probed with anti-HP0175 Ab or Ab raised against an irrelevant His-tagged protein (irr Ab). F. AGS cells were cocultured with H. pylori (WT or KO) at a multiplicity of infection of 50 for 24 h, and cell death was measured as described above, *p < 0.05 vs WT (by t test).

FIGURE 1. Purification of HP0175 and HP0175-induced cell death of AGS cells. A, Coomassie Blue-stained gels of unduplicated lane a), induced (lane b) E. coli cells expressing Hs-tagged HP0175, and purified HP0175 (lane c). The arrow indicates the position of HP0175. AGS cells were incubated with HP0175 (100 ng/ml) for various periods of time (B) or with various concentrations of HP0175 or Vac A for 24 h (C). D. Cells were incubated with LPS or with equal amounts of His-tagged irrelevant protein or His-tagged HP0175. In separate experiments, HP0175 was subjected to boiling for 1 h or was treated with polymyxin B resin as indicated in the figure. For the latter, 100 μl of His-HP0175 was mixed with 100 μl of polymyxin B bead slurry and incubated at 4°C for 1 h. Beads were removed, and the supernatant was assayed for protein content and used for apoptosis induction. In each case, the inducer was removed at the indicated time point, cells were washed and lysed, and cell death was measured using the Cell Death ELISA kit (Roche) as described in Materials and Methods. Results are expressed as the fold increase in the release of histone compared with that in the control (untreated AGS); values are the mean ± SD of three different experiments. *, p < 0.05 vs HP0175 (by t test)
His-HP0175 induced cell death of AGS cells in a time- and dose-dependent manner (Fig. 1, B and C). HP0175 was a more potent apoptosis-inducing factor than Vac A (Fig. 1C), which was reported to be an inducer of apoptosis. Cell death was also assessed by measuring the binding of annexin-fluos to treated cells. This method also supported the view that HP0175 was an apoptosis-inducing protein (Table I). Using propidium iodide staining, no necrosis was observed in the treated cells.

**HP0175-induced apoptosis is not due to His tag or LPS**

To control for nonspecific effects of the His tag present in recombinant HP0175, an irrelevant His-tagged protein was used in parallel. The irrelevant His-tagged protein failed to induce apoptosis above the level observed in control cells (Fig. 1D and Table I), ruling out the likelihood of the His tag contributing to the apoptosis-inducing ability.

As shown in Fig. 1D and Table I, heat treatment inhibited the apoptosis-inducing ability of HP0175, whereas polymyxin B treatment was without effect, ruling out the possibility of contaminating LPS being the factor responsible for induction of apoptosis. In addition, AGS cells were exposed to doses of *E. coli* LPS ranging from 5 ng to 5 μg/ml up to 36 h, and apoptosis was assessed using histone ELISA. *E. coli* LPS was unable to induce apoptosis in AGS cells (Fig. 1D). These findings indicate that LPS contamination was not responsible for the apoptosis observed in cells exposed to HP0175. Moreover, the fact that heat treatment abrogated the apoptosis-inducing ability of HP0175 suggested that induction of apoptosis was a property specific to the protein.

**Apoptosis-inducing ability of an isogenic mutant deficient in HP 0175**

An isogenic mutant disrupted in the HP0175 gene (KO) was constructed. Successful disruption of the HP0175 gene in the KO strain was confirmed by the absence of any band in cell extracts upon Western blotting and probing with anti-HP0175 Ab (Fig. 1E). The strain grew normally in vitro. However, inactivation of the HP0175 gene led to a significant reduction in the induction of apoptosis compared with the wild-type parent strain (Fig. 1F and Table I). Apoptosis-inducing ability was not totally abolished in the KO. This was expected, because several other apoptosis-inducing factors, such as VacA, urease, and γ-glutamyl transpeptidase, also contribute to cell death. These results confirmed the involvement of HP0175 in the induction of apoptosis in the gastric epithelial cell line AGS.

**HP0175 signals through TLR4**

TLR4 is expressed constitutively in AGS cells (42). AGS cells lysates were incubated with HP0175 immobilized on Ni²⁺-NTA agarose. Western blots probed with anti-TLR4 Ab showed that TLR4 is pulled down with immobilized HP0175 in the absence or the presence of polymyxin B (Fig. 2A), suggesting a direct interaction between HP0175 and TLR4. TLR4 could not be pulled down in controls using Ni²⁺-NTA agarose alone or when an irrelevant His-tagged protein was immobilized on Ni²⁺-NTA agarose, confirming a specific interaction between HP0175 and TLR4.

![FIGURE 2. HP0175 signals through TLR4 to induce apoptosis in AGS cells.](http://www.jimmunol.org/)

**Table I. TLR4-dependent HP0175-induced apoptosis in AGS cells**

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<tr>
<th>Treatment</th>
<th>Annexin V-Fluos-Positive Cells (%)</th>
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<tr>
<td>Control</td>
<td>6 ± 1a</td>
</tr>
<tr>
<td>His-HP0175</td>
<td>60 ± 5</td>
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<tr>
<td>Irrelevant His-protein</td>
<td>7 ± 1</td>
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<tr>
<td>Heat-treated His-HP0175</td>
<td>10 ± 3</td>
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<tr>
<td>His-HP0175 + anti-TLR4b</td>
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</tr>
<tr>
<td>His-HP0175 + iC</td>
<td>58 ± 7</td>
</tr>
<tr>
<td>His-HP0175 + PBd</td>
<td>61 ± 6</td>
</tr>
<tr>
<td>His-HP0175 + SB203580 (5μM)</td>
<td>7 ± 1c</td>
</tr>
<tr>
<td>His-HP0175 + U0126 (25μM)</td>
<td>58 ± 6</td>
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<td>HP(WT)</td>
<td>75 ± 5</td>
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<tr>
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<td>IETD + His-HP0175</td>
<td>6 ± 1f</td>
</tr>
<tr>
<td>DEVD + His-HP0175</td>
<td>10 ± 2e</td>
</tr>
<tr>
<td>LEHD + His-HP0175</td>
<td>9 ± 3f</td>
</tr>
</tbody>
</table>

* Results represent the means ± SD.

* Cells were pretreated with anti-TLR4 mAb or isotype-matched Ab (iC) before challenge with His-HP0175.

p < 0.001 vs His-HP0175.

p < 0.005 vs His-HP0175.

His-HP0175 was incubated with polymyxin B resin as described in Fig. 1 before incubation with AGS cells.

p < 0.05 vs His-HP0175.

p < 0.05 vs control + His-HP0175.

p < 0.05 vs vector control + His-HP0175.

* * p < 0.005 vs HP0175-treated cells only (by ANOVA). C and D, Cell death was measured as described in Fig. 1. Values are the mean ± SD of three different experiments.
No band appeared in Western blots performed using anti-CD14 or anti-TLR2 Ab (data not shown), confirming that the band that was visible after pull-down could be attributed specifically to TLR4. The interaction between HP0175 and TLR4 was also confirmed by the converse experiment. Cells were transfected with empty vector or with TLR4 construct, cell lysates were incubated with HP0175, immunoprecipitated with anti-TLR4 Ab, followed by Western analysis using anti-His Ab. HP0175 was specifically pulled down with TLR4 (Fig. 2B). No band was visible when immunoprecipitation was performed using an isotype-matched Ab or when cells were transfected with empty vector or treated with an irrelevant His-tagged protein. These results argued in favor of a specific interaction between HP0175 and TLR4. TLRs recognize various pathogen-derived molecular motifs (43). A recent study has shown that _H. pylori_ infection leads to up-regulation of TLR4 and MD-2 expression in gastric mucosa (44). Transfection of AGS cells with TLR4(dn) could inhibit HP0175-induced cell death (Fig. 2C). At the same time, preincubation of AGS cells with blocking anti-TLR4 mAb could also block HP0175-mediated cell death (Fig. 2D and Table I), whereas control isotype-matched Ab was without effect. Taken together, these findings indicate that TLR4 signaling effects HP0175-induced cell death.

**HP0175-induced cell death involves MAPK**

The stress-activated MAPK p38 is known to effect cell death under a variety of conditions (25). The p38 MAPK inhibitor SB203580 could inhibit HP0175-induced cell death in a dose-dependent manner (Fig. 3A and Table I), whereas U0126, an inhibitor of ERK1/2 MAPK, was without effect. These results suggested that p38 MAPK was involved in mediating cell death effected by HP0175. This was also supported by our observations that transfection of cells with dominant negative p38 MAPK (p38(agf)) inhibited HP0175-induced cell death (Fig. 3B and Table I). ASK1 is a member of the MAPK kinase kinase family that plays a role in stress-induced apoptosis through activation of the stress-activated MAPK signaling cascades (26–29). Catalytically inactive ASK1 (ASK1(KM)) inhibited HP0175-induced cell death (Fig. 3B and Table I), supporting the view that cell death was dependent on ASK1.

**Activation of ASK1 and p38 MAPK by HP0175**

Incubation of AGS cells with HP0175 led to a time-dependent increase in ASK1 kinase activity, as evidenced by in vitro phosphorylation of MBP (Fig. 4A). The activation of ASK1 was dependent on TLR4, because transfection of cells with TLR4(dn) before HP0175 challenge led to an inhibition of ASK1 activity (Fig. 4B). Activation of p38 MAPK was assessed by Western analysis of the phospho form of the kinase. Although p38 MAPK was activated in cells transfected with wild-type ASK1 before challenge with HP0175, ASK1(KM) inhibited HP0175-induced activation of p38 MAPK (Fig. 4C), suggesting that ASK1 was upstream of p38 MAPK activation.

**Role of caspases in HP0175-mediated cell death**

Members of the caspase family are crucial mediators of apoptosis. The involvement of caspases 8, 9, and 3 was evident from the death inhibitory effects of z-IETD-FMK, z-LEHD-FMK, and z-DEVD-FMK, respectively (Fig. 5A and Table I). Caspase 9 is an upstream effector of the intrinsic apoptotic pathway. Caspase 8 is a member of the death receptor caspase family (30). The caspase inhibitors z-IETD-FMK, z-LEHD-FMK, and z-DEVD-FMK, respectively, could inhibit HP0175-induced apoptosis (Fig. 5A and Table I). These results suggest that caspase 8 is an important mediator of HP0175-induced cell death.

**FIGURE 3.** HP0175-induced cell death depends on ASK1, MAPKs, and caspases. AGS cells were left untreated or were treated with the cell-permeable MAPK inhibitors U0126 and SB203580 at the indicated concentrations (A) for 60 min, followed by removal of the inhibitors and incubation without or with HP0175 (100 ng/ml) for 24 h. *p < 0.001 vs control treated with HP0175 (by ANOVA). B, AGS cells were transfected with different constructs as indicated in the figure, followed by treatment without or with HP0175 (100 ng/ml) for 60 min. Cell death was measured as described in Fig. 1. *p < 0.001 vs WT (by ANOVA). Values are the mean ± SD of three different experiments.

**FIGURE 4.** HP0175 activates ASK1 and p38 MAPK. AGS cells were incubated with HP0175 (100 ng/ml) for various periods of time (A), or cells were transfected with empty vector or TLR4(dn), followed by treatment with HP0175 (100 ng/ml) for 60 min (B). Whole-cell lysates were prepared, ASK1 was immunoprecipitated, and in vitro ASK1 kinase activity was determined using MBP as substrate as described in Materials and Methods. The bottom panel is a representative Western blot with anti-ASK1 to show that the same amount of ASK1 was present in each sample. The bar diagrams represent densitometric scans of the phospho-MBP bands obtained in three different experiments (mean ± SD). C, AGS cells were transfected with ASK1(WT) or a catalytically inactive mutant of ASK1(KM), followed by incubation without or with HP0175 (100 ng/ml) for 2 h. Whole-cell lysates were prepared, followed by immunoblotting with anti-phospho-p38 MAPK Abs. The lower blots show that the same amount of p38 MAPK was present in each sample. The right panel represents the densitometric analysis of the phospho-p38 MAPK bands (error bars represent the SD from three independent experiments).
Western blotting using Abs against caspase 8 (fected with p38(WT) or p38(agf), followed by incubation without or with z-DEVD-FMK (50 μM; H9262) or z-LEHD-FMK (50 μM; H9252) for 60 min. Cell death was measured as described in Fig. 1. Results are expressed as the fold increase in the release of histone compared with that in the control (untreated, non-transfected AGS). Values are the mean ± SD of three different experiments.

The MPT refers to the regulated opening of a large, nonspecific pore in the inner mitochondrial membrane that causes loss of mitochondrial membrane potential (Δψm) (46). The MPT reduces the accumulation of the fluorescent dye DiOC6 in the mitochondria as a consequence of the loss of Δψm. Loss of Δψm is associated with the release of cyt c, formation of the apoptosome, and activation of caspase 9. Treatment of AGS cells with HP0175 produced a time-dependent decline in the retention of DiOC6 (Fig. 6A). Within 4 h, 25% of the dye was lost from the cells, and within 8 h, retention of DiOC6 was reduced by 76%. Treatment of AGS cells with CCCP, a proton ionophore that dissipates Δψm, resulted in a similar loss of DiOC6 fluorescence. Transfection of cells with ASK1(KM) or p38(Δn) prevented the loss of DiOC6 fluorescence (Fig. 6B), suggesting that ASK1 and p38 are involved in HP0175-induced loss of Δψm in AGS cells.

Generation of truncated Bid (t-Bid) and release of cyt c
Taking into consideration that caspase 8 was activated in HP0175-treated cells, it appeared likely that caspase 8 activation was linked to the mitochondrial death pathway. Linkage of caspase 8 to mitochondrial caspase 8 inhibitor-treated cells (Fig. 6C). Consistent with the effect of caspase inhibitors on HP0175-induced cell death, Western analysis using Abs specific for the cleaved form of caspase 3 showed that HP0175 challenge activated the executioner caspase 3 (Fig. 5C). The initiator caspase 9 (Fig. 5E) was also activated in a time-dependent manner, as evidenced by detection of the cleaved, activated form of the caspase by Western blotting. Caspase 3 activation was also supported by detection of the cleaved form of the caspase 3 substrate PARP in HP0175-challenged AGS cells (Fig. 5D). The generation of cleaved caspase 3 could be blocked by pretreatment with the caspase 3-specific inhibitor z-DEVD-FMK as well as the caspase 9-specific inhibitor z-LEHD-FMK (Fig. 5C). The latter result suggested that caspase 9 activation was necessary for the generation of cleaved caspase 3.

Generation of cleaved caspase 9 could be blocked by transfection of cells with p38(Δn) (Fig. 5F). Generation of cleaved caspase 9 could also be blocked by pretreating cells with the caspase 8-specific inhibitor z-IETD-FMK (Fig. 5E). These data supported the role of p38 MAPK as well as caspase 8 in the activation of caspase 9 in HP0175-challenged AGS cells. The role of caspase 8 in HP0175-induced death signaling was supported by Western analysis of active caspase 8. Cleaved caspase 8 was detected in HP0175-challenged AGS cells (Fig. 5G). This could be blocked in cells pretreated with the caspase 8-specific inhibitor z-IETD-FMK.

HP0175-induced caspase 8 activation was also blocked in cells transfected with p38(Δn) (Fig. 5H), suggesting a role for p38 in caspase 8 activation.

Induction of mitochondrial permeability transition (MPT)
Consistent with the effect of caspase inhibitors on HP0175-induced cell death, Western analysis using Abs specific for the cleaved form of caspase 3 showed that HP0175 challenge activated the executioner caspase 3 (Fig. 5C). The initiator caspase 9 (Fig. 5E) was also activated in a time-dependent manner, as evidenced by detection of the cleaved, activated form of the caspase by Western blotting. Caspase 3 activation was also supported by detection of the cleaved form of the caspase 3 substrate PARP in HP0175-challenged AGS cells (Fig. 5D). The generation of cleaved caspase 3 could be blocked by pretreatment with the caspase 3-specific inhibitor z-DEVD-FMK as well as the caspase 9-specific inhibitor z-LEHD-FMK (Fig. 5C). The latter result suggested that caspase 9 activation was necessary for the generation of cleaved caspase 3.

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FIGURE 6. HP0175-induced loss of mitochondrial membrane permeability transition, cleavage of Bid, and release of cyt c from the mitochondria. A: HP0175 was expressed in E. coli as a hexahistidine-tagged protein. It was found to possess PPlase activity, indicating that the recombinant protein was biologically active. The caspase 8 inhibitor z-IETD-FMK (50 nm) inhibited caspase activity in A549 cells transfected with p38WT, p38agf, ASK1WT, or ASK1KM before incubation without or with HP0175 (100 ng/ml) for 8 h (B). Cells were washed, and the fluorescence of DiOC6 was determined at excitation and emission wavelengths of 488 and 500 nm, respectively. Values for de-energized mitochondria were determined by simultaneous treatment of cells with 10 μM CCCP and DiOC6 (A). Without HP0175; □, with HP0175. Values are the mean ± SD of three different experiments. Cells were left untreated or were treated with the caspase 8 inhibitor z-IETD-FMK (50 μM; C and D) for 1 h before treatment with HP0175 (100 ng/ml) for 6 h (C) or for various periods of time (D). In a separate set of experiments, cells were transfected with p38WT, p38agf, ASK1WT, or ASK1KM before incubation without or with HP0175 (100 ng/ml) for 8 h (E). Cells were processed to obtain mitochondrial (C) or cytosolic (D and E) fractions as described in Materials and Methods. Truncated Bid (15 kDa; C) or cyt c (12 kDa) and actin (D and E) were detected by SDS-PAGE, followed by Western blotting. Blots are representative of results obtained from three separate experiments.

Discussion

Apoptosis is an orchestrated suicide program, and the key morphological alterations of apoptosis are mediated by caspases. H. pylori induces gastric epithelial cell apoptosis (49). Apoptosis contributes to the pathological outcome of the infection by disturbing the balance between the rate of new cell production and the rate of cell loss by apoptosis (50, 51). Atrophic gastritis and gastric dysplasia after H. pylori infection are associated with accelerated apoptosis of the gastric epithelium (52). It is therefore of considerable importance to understand the nature of the apoptosis-inducing factors of H. pylori. With this in view, we narrowed down to the secreted protein HP0175, a putative PPlase to evaluate its effects on gastric epithelial cells. HP0175 was expressed in E. coli as a hexahistidine-tagged protein. It was found to possess PPlase activity, indicating that the recombinant protein was biologically active. The gastric epithelial cell line AGS was challenged with HP0175 to evaluate its effects on gastric epithelium. The apoptosis-inducing ability was tested using two independent approaches, namely measurement of histone release by ELISA and measurement of annexin binding to cells using annexin-fluos. HP0175-challenged cells underwent apoptosis in a time- and dose-dependent manner, suggesting that HP0175 is one of a number of secreted apoptosis-inducing factors of H. pylori. The apoptosis-inducing property of HP0175 was not due to LPS contamination, because LPS (up to a concentration of 5 μg/ml) was not able to induce apoptosis in AGS cells, and treatment of HP0175 with polymyxin B resin did not affect its ability to induce apoptosis. The inability of irrelevant His-tagged recombinant protein to induce apoptosis clearly suggested that the apoptosis-inducing ability was a property specific to HP0175. Appreciable reduction in the induction of apoptosis by the isogenic strain lacking HP0175 also confirmed the apoptosis-inducing ability of HP0175. The signaling pathway downstream of HP0175 challenge was then dissected. HP0175 was observed to interact directly with TLR4, and HP0175-induced cell death was dependent on TLR4, because pre-incubation of cells with blocking anti-TLR4 Ab could block HP0175-mediated cell death. Not surprisingly, we observed that caspases 3, 8, and 9 are activated after challenge of macrophages with HP0175, and that these are all required for HP0175-induced death of AGS cells, because inhibition of any one of these caspases with caspase-specific peptide inhibitors was sufficient to block cell death. Previous reports have shown that H. pylori induces activation of caspase 8 and 3 and subsequent cleavage of PARP, leading to an increased level of apoptosis in gastric epithelial cells both in vivo and in vitro (53). Caspase 3 activation also occurs when NF-κB signaling is suppressed in H. pylori-challenged gastric epithelial cell lines as well as primary gastric epithelial cells (54).

H. pylori is known to activate MAPKs (55), which are mediators of stress-induced apoptosis. Choi et al. (56) have reported that the inhibition of p38 MAPK by SB203580 decreased H. pylori-mediated apoptosis of epithelial cells. Inhibition of HP0175-mediated cell death in cells transfected with a dominant negative mutant of p38 MAPK suggested that p38 MAPK is required to trigger HP0175-mediated cell death. ASK1 is a MAPK kinase kinase that affects stress-activated cell death by activation of p38 in many cell types. We observed that HP0175 challenge activated ASK1 in a TLR4-dependent manner.

The link between ASK1-p38 MAPK signaling and caspase activation was evident from the observation that a catalytically inactive mutant of ASK1 could prevent activation of caspases 8, 9, and 3, suggesting that ASK1 plays a central role in HP0175-induced cell death. The role of the mitochondrial death pathway appeared likely, because inactive caspase 9 blocked death. The loss of retention of the probe DiOC6 from cells challenged with HP0175, concomitant with the release of cyt c, confirmed the role of the mitochondrial pathway in HP0175-induced cell death. p38 MAPK regulated this pathway, because loss of DiOC6 retention could be inhibited in cells transfected with p38dn (or ASK1KM).

The release of cyt c from the mitochondria could be blocked by caspase 8 inhibitor, suggesting that the death signal triggered by caspase 8 activation is amplified by the mitochondrial release of cyt c. Cyt c release could also be blocked in p38dn-transfected cells, suggesting that ASK1/p38 MAPK signaling plays a crucial role not only in activation of caspase 8, but also in downstream events leading to the release of cyt c and activation of caspases 9 and 3 (Fig. 6). The use of inhibitors as well as the kinetics of caspase activation and cyt c release suggested sequential activation of caspases 8, 9, and 3, as depicted in the model proposed in Fig. 7. Caspase 3 inhibition did not affect the activation of caspase 8, ruling out caspase 3 as the mediator of caspase 8 activation.

Death signals are in several instances amplified through the mitochondrial pathway by the caspase 8-mediated cleavage of Bid and its translocation to the mitochondria. Bid-deficient mice are resistant to hepatocellular apoptosis (57). We analyzed the status of Bid in the mitochondrial fractions of HP0175-challenged macrophages. Western blotting showed an increase of t-Bid in the mitochondria as a function of time of challenge with HP0175, whereas caspase 8 inhibitor could block the generation of t-Bid in the mitochondrial fraction, suggesting that caspase 8-mediated Bid cleavage activates the mitochondrial death pathway in this case. This was supported by the concomitant detection of cyt c in the cytosolic fraction.
HP0175-TLR4 interaction are under investigation. ASK1 plays a central role in this pathway by activating p38 MAPK (Fig. 7). Signaling downstream of p38 leads to the sequential activation of caspase 8, truncation of Bid, its translocation to the mitochondria, and loss of mitochondrial membrane potential initiating the release of cyt c and the activation of caspases 9 and 3. It has been reported that H. pylori-mediated apoptosis of gastric epithelial cells involves translocation of Bax, followed by mitochondrial depolarization and subsequent activation of caspase 3 (58), and that reduced levels of Bcl-2 are associated with H. pylori-mediated apoptosis of gastric epithelial cells (59). Our studies strengthen the view that the mitochondrial pathway plays a critical role in H. pylori-mediated apoptosis in gastric epithelial cells. Our present investigation provides insight into the pathways through which a secreted protein of H. pylori with PPIase activity signals to induce apoptosis. HP0175 may be regarded an addition to the growing list of molecules that effect TLR-mediated apoptotic cell death (60).

HP0175 may therefore contribute to the pathology of gastric cancer by inducing hyperproliferation of the gastric epithelium. These studies provide new insights into H. pylori pathogenicity and reveal for the first time a novel characteristic of a secreted protein with PPIase activity.

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References


