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Molecular Characterization of *Helicobacter pylori* VacA Induction of IL-8 in U937 Cells Reveals a Prominent Role for p38MAPK in Activating Transcription Factor-2, cAMP Response Element Binding Protein, and NF- κ B Activation¹

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Helicobacter pylori VacA induces multiple effects on susceptible cells, including vacuolation, mitochondrial damage, inhibition of cell growth, and enhanced cyclooxygenase-2 expression. To assess the ability of *H. pylori* to modulate the production of inflammatory mediators, we examined the mechanisms by which VacA enhanced IL-8 production by promonocytic U937 cells, which demonstrated the greatest VacA-induced IL-8 release of the cells tested. Inhibitors of p38 MAPK (SB203580), ERK1/2 (PD98059), I κ B α ((E)-3-(4-methylphenylsulfonyl)-2-propenenitrile), Ca²⁺ entry (SKF96365), and intracellular Ca²⁺ channels (dantrolene) blocked VacA-induced IL-8 production. Furthermore, an intracellular Ca²⁺ chelator (BAPTA-AM), which inhibited VacA-activated p38 MAPK, caused a dose-dependent reduction in VacA-induced IL-8 secretion by U937 cells, implying a role for intracellular Ca²⁺ in mediating activation of MAPK and the canonical NF- κ B pathway. VacA stimulated translocation of NF- κ Bp65 to the nucleus, consistent with enhancement of IL-8 expression by activation of the NF- κ B pathway. In addition, small interfering RNA of activating transcription factor (ATF)-2 or CREB, which is a p38MAPK substrate and binds to the AP-1 site of the IL-8 promoter, inhibited VacA-induced IL-8 production. VacA activated an IL-8 promoter containing an NF-IL-6 site, but not a mutated AP-1 or NF- κ B site, suggesting direct involvement of the ATF-2/CREB binding region or NF- κ B-binding regions in VacA-induced IL-8 promoter activation. Thus, in U937 cells, VacA directly increases IL-8 production by activation of the p38 MAPK via intracellular Ca²⁺ release, leading to activation of the transcription factors, ATF-2, CREB, and NF- κ B. *The Journal of Immunology*, 2008, 180: 5017–5027.

Helicobacter *pylori* is a Gram-negative, strongly motile, spiral-shaped, microaerophilic bacterial pathogen found in the stomach mucosa of >50% of the world population. Its presence in the stomach is associated with an increased risk of peptic ulcer disease, gastric lymphoma, and gastric adenocarcinoma. Persistent infection by *H. pylori* causes prolonged inflammation, including

intraglandular infiltration of neutrophils, lymphocytes, and plasma cells in gastric mucosa (1–6). Inflammation mediated by cytokines, adhesion molecules, active oxygen species, NO, and PGs has been implicated in the pathogenesis of gastric mucosal injury induced by *H. pylori* (7).

The cytokines induced by *H. pylori* infection include TNF- α , IFN γ , IL-1, IL-6, and IL-8 (8–10). Induction of IL-8 secretion by *H. pylori* strains is associated with the presence of *cag* pathogenicity island (PAI),⁴ especially the *cagA* gene (11, 12). It has been shown, however, that a *cag* PAI-negative strain can stimulate IL-8 production to an extent similar to that of a *cag* PAI-positive strain (13, 14). In addition, long-term infection by *cag* PAI-deficient *H. pylori* results in gastric damage in mice (15–17). However, in support of a role for *cag* PAI, Viala et al. (18) demonstrated recently that the peptidoglycan, which is translocated by the type 4 secretion system encoded in the *cag* PAI, activated Nod1 and, subsequently, NF- κ B, leading to IL-8 release. In addition, Brandt et al. (19) provided several lines of evidence showing that CagA is able to induce IL-8 in a strain-dependent process, and that IL-8 release induced by CagA occurs via a Ras→Raf→Mek→ERK→NF- κ B signaling pathway in a Shp-2- and c-Met-independent manner. Although there are reports describing an association between *cag*

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⁴ Abbreviations used in this paper: PAI, pathogenicity island; 2-AG, 2-arachidonoyl glycerol; ATF, activating transcription factor; BAY11-7082, (E)-3-(4-methylphenylsulfonyl)-2-propenenitrile; BHA, butylated hydroxyanisole; DAPI, 4',6'-diamidino-2-phenylindole; iVacA, heat-inactivated VacA; NC-siRNA, negative control small interfering RNA; ROS, reactive oxygen species; siRNA, small interfering RNA.

PAI and progression of gastric disease (11, 20, 21), the pathogenic role of *cag* PAI is not completely understood.

One gene in *H. pylori* (*hopH*) was assigned the name outer inflammatory protein (OipA) on the basis of evidence that it played a role in stimulating gastric epithelial cells to produce IL-8 (22). The mechanism by which HopH stimulates IL-8 expression was reported to be different from that of *cag* PAI. However, Dossumbekova et al. (23) showed that, in their collection of *H. pylori* strains, HopH did not play a role in stimulating IL-8 production by gastric epithelial cells.

Kundu et al. (24) recently showed that the levels of secreted IL-1 β , TNF- α , and IL-6 were significantly increased in mouse gastric tissues infected with either *cag*⁺ or *cag*⁻ strains of *H. pylori*, suggesting that *cag* PAI is not the sole factor responsible for induction of proinflammatory cytokines. Cells responded to increased IL-1 β , TNF- α , and IL-6 secretion by enhancing matrix metalloproteinase-6 production. Furthermore, gastric epithelial cells infected in vitro with *H. pylori* expressed matrix metalloproteinase-9 in response to release of proinflammatory cytokines (8).

VacA, a protein toxin produced by *H. pylori*, has multiple effects on susceptible cells (e.g., epithelial and lymphatic cells), including vacuolation with alterations of endo-lysosomal function, mitochondrial damage, and inhibition of T cell proliferation (1–6). These different effects of VacA appear to result from activation of different signal transduction pathways. In AZ-521 cells, VacA induced activation of the p38/activating transcription factor (ATF)-2-mediated signal transduction pathway, independent of cellular vacuolation (25) or cytochrome *c* release secondary to mitochondrial damage (26, 27). Interestingly, in AZ-521 cells, we found that VacA enhanced PGE₂ production through induction of cyclooxygenase-2 expression via a p38 MAPK/ATF-2 cascade (28). VacA may modulate the activity of other *H. pylori* products; for example, VacA counteracted CagA-induced activation of NF-AT in AGS cells (29). With regard to a potential role for VacA in inflammation, the toxin was shown to induce bone marrow-derived mast cells to produce proinflammatory cytokines, TNF- α , MIP-1 α , IL-1 β , IL-6, IL-10, and IL-13 (30). To understand better mechanisms by which *H. pylori* induces IL-8, an important mediator in the immunopathogenesis of chronic gastritis, we examined whether IL-8 production by promonocytic U937 cells is enhanced by VacA, and observed that VacA directly increases IL-8 by activation of p38 MAPK, leading to activation of the transcription factors, ATF-2, CREB, and NF- κ B.

Materials and Methods

Cell lines

Human monocytic cell line U937, human gastric carcinoma cell line MKN1, human gastric cell line AGS, human colon cancer cell line DLD-1, Jurkat T cell line, and HL-60 cell line were grown in RPMI 1640 (Sigma-Aldrich) containing 10% FCS. Wilm's human kidney tumor cell line G401 was grown in DMEM (Sigma-Aldrich) containing 10% FCS. Human gastric adenocarcinoma cell line AZ-521 was grown in Eagle's MEM containing 10% FCS under 5% CO₂ at 37°C.

Purification of VacA

The toxin-producing *H. pylori* strain ATCC49503 was the source of VacA for purification by our published procedure (25). In brief, after growth of *H. pylori* in *Brucella* broth containing 0.1% β -cyclodextrin at 37°C for 3–4 days with vigorous shaking in a controlled microaerobic atmosphere of 10% O₂ and 10% CO₂, VacA was precipitated from culture supernatant with 50% saturated ammonium sulfate. Precipitated proteins were dialyzed against RX buffer (10 mM KCl, 0.3 mM NaCl, 0.35 mM MgCl₂, 0.125 mM EGTA, 1 mM HEPES (pH 7.3)) and applied to an anti-VacA-specific IgG Ab column equilibrated with RX buffer. After washing the column with RX buffer, VacA was eluted with 50 mM glycine-HCl buffer (pH 1.0), which was promptly neutralized with 1 M Tris-HCl (pH 10). After gel filtration on Superose 6HR 10/30 equilibrated with TBS buffer (60 mM

Tris-HCl buffer (pH 7.7), containing 0.1 M NaCl), purified VacA was stored at –20°C.

Infection of U937 cells by *H. pylori*

H. pylori standard strain ATCC43504 and its *vacA* mutant strain were used. VacA mutants were constructed, as previously described (31), with the exception that we used kanamycin resistance gene cassette (a gift from R. Haas, Max von Pettenkofer Institut, Munchen, Germany) in this study. Before challenging U937 cells, *H. pylori* strains were cultured in *Brucella* broth supplemented with 5% FBS under the microaerobic conditions for 12–24 h at 37°C with vigorous shaking, and then incubated with U937 cells (5 \times 10⁵) at a multiplicity of infection of 100 for 12 h. After incubation in a 5% CO₂ atmosphere for the time indicated in each figure, IL-8 in culture medium of infected cells was quantified by ELISA.

IL-8 ELISA

Immuno-reactive IL-8 was quantified in cell culture supernatants by a double-Ab ELISA kit using rIL-8 as a standard (BD Biosciences) following the manufacturer's protocol. This assay has a lower limit of detection of 200 pg/ml.

Detection of MAPK phosphorylation

U937 cells were incubated with 120 nM VacA for 0, 15, 30, 60, 120, or 240 min. Cells were then solubilized by incubation for 10 min on ice in 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM Na₃VO₄, 10 mM NaF, 1 mM PMSF, and leupeptin (10 μ g/ml). After centrifugation (15 min, 15,000 \times g), samples (20 μ g protein) of supernatants were subjected to SDS-PAGE and Western blotting using anti-phospho-MAPKs or anti-MAPK Abs.

Western blotting

To avoid the enzymatic effects on the samples of endogenous phosphatases and proteases, 1 mM Na₃VO₄ was added to block phosphatase activity, and 50 mM NaF, 1 mM PMSF, and leupeptin (10 μ g/ml) were added to inhibit proteases during cell lysis. After SDS-PAGE and transfer to Hybond ECL membranes (GE Healthcare), followed by blocking the membranes with 5% (w/v) defatted dried milk, immunodetection of phosphorylated MAPK was conducted by incubation of each membrane with the primary anti-phospho-specific p38 MAPK, ERK, or JNK Abs (Cell Signaling Technology). In all experiments, nonphosphorylated p38 MAPK, ERK, and JNK (Cell Signaling Technology) were detected simultaneously to confirm equal protein loading. All primary Abs were used at a dilution of 1/1000, and all secondary Abs at a dilution of 1/5000. To detect phosphorylated ATF-2 and CREB, similar experimental conditions were used.

Preparation of nuclear and cytosolic extracts

To prepare cytoplasmic and nuclear extracts, U937 cells (1 \times 10⁷) were washed twice at 4°C with PBS and once with PBS containing 1 mM Na₃VO₄ and 10 mM NaF. Subsequently, the cells were washed with 2 ml of 1 \times hypotonic buffer (20 mM HEPES (pH 7.9), 1 mM EDTA, and 1 mM EGTA), and lysed in 1 \times hypotonic buffer supplemented with 0.2% Nonidet P-40. Thereafter, the supernatants (cytosolic extracts) were transferred to a fresh tube, and the nuclear pellets were collected by centrifugation at 15,000 \times g for 10 min and resuspended in 100 μ l of 1 \times high-salt buffer (420 mM NaCl, 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, and 20% glycerol), after which they were incubated at 4°C for 30 min under constant rotation. Subsequently, the nuclear extracts were collected by centrifugation and stored at –80°C. Purity of the cell fractions was confirmed by Western analysis using Abs against NF- κ Bp65, lamin B2 (Abcam), and GAPDH (Santa Cruz Biotechnology), and then NF- κ Bp65 in cytosolic and nuclear extracts was normalized to GAPDH.

Immunostaining

U937 cells (1.0 \times 10⁵) seeded on a Lab-tek 8 chamber (Nunc) were incubated with 120 nM VacA or inactivated VacA at 37°C for 1 h. After incubation, the cells were fixed with 2% paraformaldehyde in PBS at room temperature for 15 min, and then washed three times with PBS. Cells permeabilized with PBS containing 0.1% Triton X-100 for 5 min were treated with blocking buffer (Block Ace solution; Snow Brand Milk Products) for 30 min, and stained with 1 μ g/ml 4',6'-diamidino-2-phenylindole (DAPI) for 5 min. They were then incubated with primary Abs for 1 h, followed by incubation at room temperature for 1 h with appropriate secondary Abs, such as anti-rabbit Abs or anti-mouse Abs, conjugated with Alexa Fluor 546. Anti-rabbit Abs were against phospho-ATF-2 and phospho-CREB, and anti-mouse Ab was against NF- κ Bp65. Stained cells were visualized using confocal microscopy (Leica Microsystems).

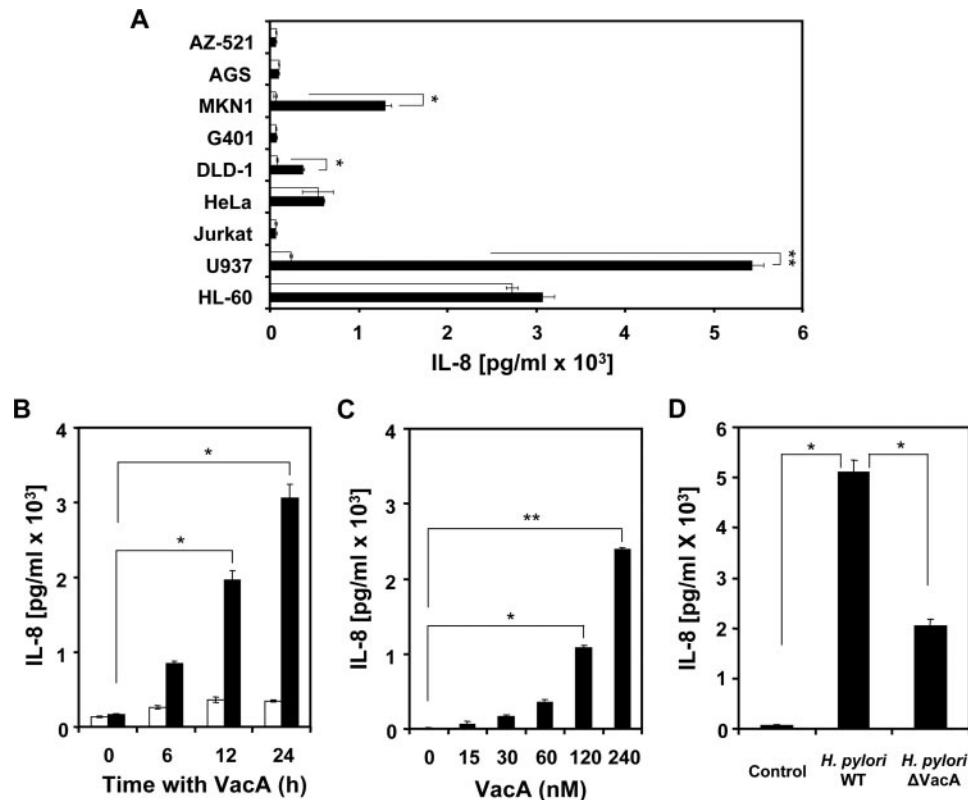


FIGURE 1. A, AZ-521, AGS, MKN1, G401, DLD-1, HeLa, Jurkat, U937, and HL-60 cells were incubated with VacA (■) or iVacA (iVacA, □) for 12 h in serum-free medium. IL-8 production in the culture medium was determined, as described in *Materials and Methods*. Representative results are shown as the mean \pm SE calculated from the results of three independent experiments. B, Confluent U937 cells were incubated with 120 nM VacA or iVacA for the indicated times in serum-free medium. IL-8 production in the medium was measured by ELISA. Results shown are the mean \pm SE of three independent experiments. C, U937 cells were incubated with the indicated amounts of VacA at 37°C for 12 h, and the medium was subjected to ELISA to determine IL-8 production. Representative results are shown as the mean \pm SE, with $n = 3$ per experiment, calculated from the results of three independent experiments after subtracting the value of cells incubated without toxin from that of toxin-treated cells. Statistical significance: *, $p < 0.05$; **, $p < 0.01$. D, U937 cells were infected with *H. pylori* ATCC43504 (wild-type strain) or its isogenic VacA-knockout mutant strain (Δ VacA strain) for 12 h. Cells incubated without infection (uninfected cells) were used as a negative control. After incubation, IL-8 in the culture medium was quantified by ELISA. Data are means \pm SD of values from three independent experiments with assays in duplicate. Statistical significance: *, $p < 0.01$.

Detection of changes in cytosolic free Ca^{2+} concentration

U937 cells were loaded with 5 μ M fura 2-AM (Dojindo Laboratories) by incubation in RPMI 1640 medium at 37°C for 30 min, and washed with the medium. After incubation with VacA or heat-inactivated VacA (iVacA), fluorescence images of the cells were analyzed with confocal microscopy, as reported by Ricken et al. (32). Fura 2 fluorescence at an emission wavelength of 510 nm was observed at room temperature by exciting fura 2 at 355 nm.

Detection of reactive oxygen species (ROS)

Serum-starved U937 (5×10^5 cells/well) plated in 96-well plates were loaded with 5 μ M redox-sensitive dye, 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes), for 30 min at 37°C, washed with RPMI 1640 serum-free medium, and incubated with VacA or 2-arachidonoyl glycerol (2-AG, positive control). ROS formation was measured for the indicated time in a multiwell fluorescence plate reader (PerkinElmer) using excitation and emission filters of 485 and 535 nm, as reported by Siegmund et al. (33). To quantify ROS generation, 2',7'-dichlorodihydrofluorescein diacetate fluorescence formed in VacA-treated cells was determined by subtracting the fluorescence of cells incubated without toxin from that of toxin-treated cells. The effect of 2-AG on ROS generation was quantified by the same method.

Transfection with ATF-2 or CREB small interfering RNA (siRNA)

U937 cells were seeded (2.0×10^5 cells in 4 ml of RPMI 1640/dish) in 60-mm culture dishes and grown overnight; ATF-2 (ATF-2-siRNA, 1 μ g), CREB (CREB-siRNA; 1 μ g), or negative control siRNA (NC-siRNA; 1 μ g) duplexes were introduced into cells using Lipofectamine RNAiMAX

transfection reagent (Invitrogen Life Technologies), according to the manufacturer's recommendations. A mock transfection without siRNA was also performed. ATF-2-siRNA, CREB-siRNA, and NC-siRNA were purchased from Santa Cruz Biotechnology. Silencing of the *ATF-2* gene, *CREB* gene, or *GAPDH* gene was determined by measuring ATF-2, CREB, or GAPDH protein expression at 24 h after transfection by Western blotting using anti-ATF-2, anti-CREB, or anti-GAPDH Abs (Cell Signaling Technology).

Reporter gene assay

The 5'-flanking region spanning from -133 to +44 bp of the IL-8 gene was subcloned into a firefly luciferase expression vector, and then site-directed mutagenesis of the AP-1, NF-IL-6, and NF- κ B sites was conducted by replacement of TGACTCA with TATCTCA for AP-1, CA GTTGCAAATCGT with AGCTTGCAAATCGT for NF-IL-6, and GGAATTTTCCT with TAACTTTTCCT for NF- κ B site using PCR, as noted in figure legends (34). U937 (5×10^5) cells transfected with a luciferase reporter plasmid containing an IL-8 promoter were cultured in 24-well plates in RPMI 1640 medium. A reporter construct (1 μ g) was mixed with 20 ng/ml control vector pRL-CMV (Toyo Ink) in 50 μ l of RPMI 1640 medium. The solution was mixed with 1 μ l of Lipofectamine 2000 reagent, diluted in 150 μ l of RPMI 1640 medium, and incubated at room temperature for 20 min; the two vectors in 200- μ l solutions were cotransfected into U937 cells after the cells were washed twice with RPMI 1640 medium. The cells were incubated at 37°C for 5 h in a 12% CO₂ atmosphere. After transfection with plasmid, the medium was replaced with 150 μ l of fresh RPMI 1640 without FCS. The next day, the cells were treated with 120 nM VacA. After incubation at 37°C for 6 h, cells were washed with 500 μ l of PBS and lysed by adding 100 μ l of lysis buffer (Toyo Ink). After incubation for 15 min at room temperature, the lysate was centrifuged

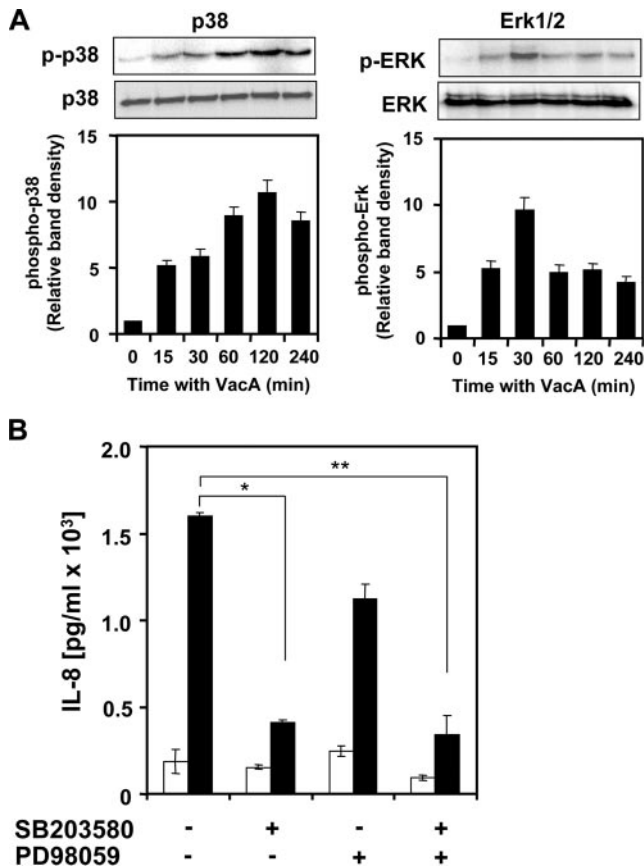


FIGURE 2. A, U937 cells were incubated with 120 nM VacA for the indicated times. Cell lysates were prepared at indicated incubation times and subjected to Western blot analyses using Abs recognizing MAPKs and phosphorylated MAPKs (*upper panel*). Data are representative of three independent experiments. Relative densities of phospho-p38 and phospho-ERK, as determined by densitometry scan analysis, were compared with densities obtained at 0 min (*bottom panel*). Data are mean \pm SE of values from triplicate experiments. B, Confluent U937 cells were pretreated with SB203580 (10 μ M), PD98059 (10 μ M), or both inhibitors (10 μ M) for 1 h before incubation with VacA or iVacA (120 nM) in serum-free medium. IL-8 production was measured by ELISA. The data are representative of at least three experiments. Statistical significance: *, $p < 0.05$; **, $p < 0.01$.

(15,000 \times g, 5 min, 4°C) and the supernatant was harvested and assayed with a PicaGene Dual-Luciferase Assay kit (PG-DUAL SP; Toyo Ink), according to the manufacturer's instructions.

Isolation of primary human CD14 blood monocytes

Human PBMC were isolated from peripheral blood of healthy donors using Ficoll-Hypaque gradients. PBMC were then further purified using the autoMACS sort system (Miltenyi Biotec) using positive selection with immunomagnetic beads specific for CD14 (Miltenyi Biotec), as described by the manufacturer. Freshly isolated cells were counted and 95–99% pure as assessed by staining using an FITC-labeled CD14 Ab and flow cytometric analysis (FACSCalibur; BD Biosciences).

Other reagents

Inhibitor of I κ B phosphorylation ((E)-3-(4-methylphenylsulfonyl)-2-propanenitrile (BAY11-7082)), an antioxidant (butylated hydroxyanisole (BHA) (35)), and a Ca²⁺ chelator, BAPTA-AM, were purchased from Sigma-Aldrich. Dantrolene and SKF-96365 were purchased from Calbiochem.

Statistical analysis

To establish the significance of the results, Student's *t* test was used for numerical data. Fisher's exact test or χ^2 test was used for categorical data as appropriate. A *p* value < 0.05 was considered statistically significant.

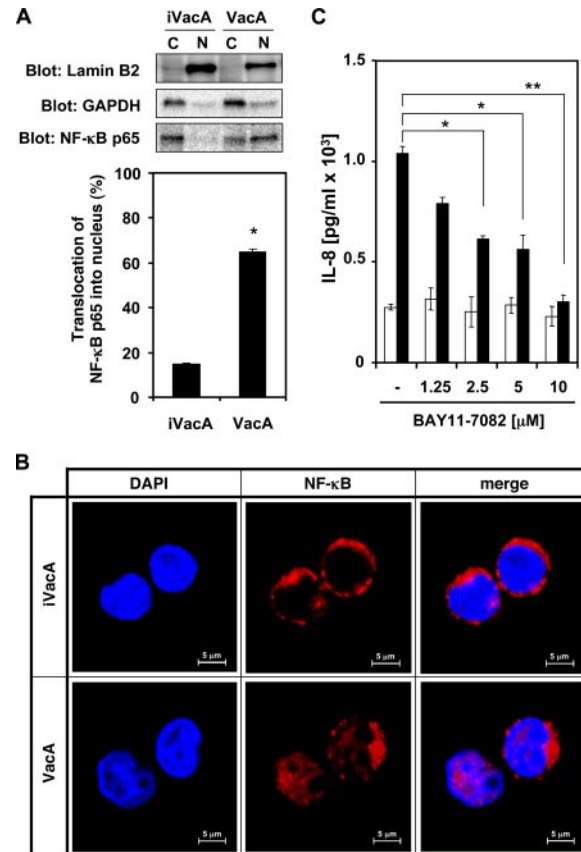


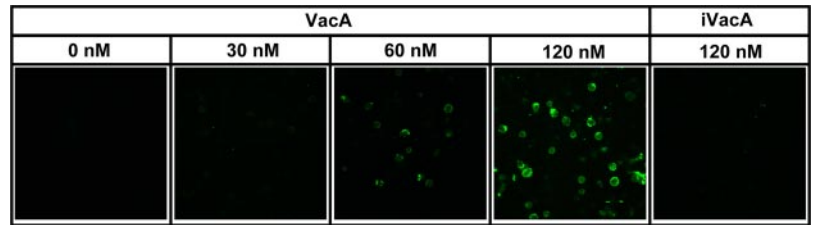
FIGURE 3. A, U937 cells were treated with 120 nM VacA or iVacA at 37°C for 60 min. The cells were collected by centrifugation and lysed in lysis buffer. Nuclear (N) and cytosolic (C) extracts were prepared, as described in *Materials and Methods*. Subsequently, NF- κ Bp65 in cytosolic and nuclear extracts was normalized to GAPDH after Western blotting. The percentage in the nucleus is based on densitometric quantification. The data are representative of at least three experiments. Statistical significance: *, $p < 0.05$. B, U937 cells were incubated with 120 nM VacA or iVacA at 37°C for 60 min. The cells were fixed using 2% paraformaldehyde, and treated for 5 min with 0.1% Triton X-100 for membrane permeabilization. The fixed cells were stained with 1 μ g/ml DAPI for 5 min, followed by incubation with anti-NF- κ B mAbs (1/100) in TBS containing 1% BSA. After treatment with the respective primary Abs, cells were incubated with secondary Ab in TBS containing 1% BSA and anti-mouse polyclonal Abs conjugated with Alexa fluor 546 (1/1000). Scale bar, 5 μ m. C, Confluent U937 cells were pretreated with BAY11-7082 (0, 1.25, 2.5, 5, and 10 μ M) for 1 h before incubation with 120 nM VacA (■) or iVacA (□) in serum-free medium. IL-8 production was measured by ELISA. The data are representative of at least three experiments. Statistical significance: *, $p < 0.05$; **, $p < 0.01$.

Results

VacA stimulates IL-8 secretion by various human cell lines

After a 12-h incubation with 120 nM VacA or iVacA (control), a 26.0-, 11.5-, and 6.7-fold increase was observed in IL-8 concentrations in the medium of human monocytic cell U937, gastrointestinal epithelial cell MKN1, and colon epithelial cell DLD-1 (Fig. 1A). VacA-induced IL-8 release was not observed with AZ-521, AGS, G401, HeLa, Jurkat, and HL-60 cells, implying that VacA-induced IL-8 production is limited to certain cell types. VacA-induced IL-8 production by U937 cells, which demonstrated the greatest release, was time and concentration dependent (Fig. 1, B and C). In addition, production of IL-8 in U937 cells was observed after challenging with *H. pylori* ATCC43504, whereas its

FIGURE 4. U937 cells were loaded with fura 2-AM (5 μ M, 37°C) in RPMI 1640 serum-free medium. Cells were washed with medium, and then treated with 0, 30, 60, or 120 nM VacA, or iVacA (120 nM) in the medium. Changes in cytoplasmic free Ca²⁺ concentration were determined by confocal microscopy, as described in *Materials and Methods*. The data are representative of at least three experiments.



isogenic VacA-knockout mutant strain did not induce IL-8 production in U937 cells, implying that VacA production by *H. pylori* is responsible for IL-8 production (Fig. 1D).

Effects of MAPKs on VacA-induced IL-8 production by U937 cells

To determine whether VacA activates MAPKs, U937 cells were incubated with 120 nM VacA (Fig. 2A). Phosphorylation of p38 and ERK1/2 was clearly evident after a 15-min incubation with VacA; phospho-p38 declined by 240 min. Phospho-ERK1/2 was maximal at 30 min and declined after 60 min. JNK was not activated by incubation of U937 cells with VacA (data not shown). Consistent with a role for p38 MAPK phosphorylation in U937 cells incubated with 120 nM VacA for 30 min in IL-8 release, SB203580, a p38 MAPK inhibitor, reduced VacA-induced IL-8 release from U937 cells (Fig. 2B). Inhibitors of ERK1/2 (PD98059) abolished the ability of VacA to induce IL-8 production by U937 cells. The inhibitors did not reduce cell number or induce morphological changes in cells (data not shown). These data suggest that activation of both p38 and ERK is involved in IL-8 production.

VacA-induced translocation of NF- κ B to nucleus in U937 cells and inhibition of IL-8 release by BAY11-7082

Activation of the IL-8 promoter is believed to require activation of the transcription factor NF- κ B by *H. pylori*. To examine the ability

of VacA to stimulate IL-8 induction through an NF- κ B-mediated pathway in U937 cells, we determined the translocation of NF- κ B to the nucleus in VacA-treated U937 cells and its association with IL-8 release. In agreement with the recent study of Kim et al. (36), VacA induced translocation of NF- κ Bp65 to the nucleus (Fig. 3A), as visualized by immunostaining with anti- NF- κ Bp65 Ab (Fig. 3B), whereas iVacA did not cause NF- κ B translocation. BAY11-7082 (37), which inhibits I κ B α , resulted in a dose-dependent reduction in VacA-induced IL-8 secretion by U937 cells (Fig. 3C). These data indicate that VacA induces IL-8 expression by activation of the canonical NF- κ B pathway.

VacA induces a rise in cytosolic free Ca²⁺ concentration

Intracellular Ca²⁺ concentration was monitored in U937 cells treated with VacA and iVacA using fura 2 as an indicator. Application of 120 nM VacA, but not iVacA, to U937 cells induced a rise in cytosolic free Ca²⁺ concentration within 2-h incubation (Fig. 4).

Effect of BAPTA-AM or BHA on VacA-induced IL-8 production and NF- κ B activation

BAPTA-AM (Fig. 5, A and C), a calcium chelator, but not BHA (Fig. 5, B and C), an antioxidant, caused a dose-dependent reduction in VacA-induced IL-8 production by U937 cells and blocked VacA-induced translocation of NF- κ Bp65 to the nucleus, suggesting that IL-8 production and NF- κ B activation in U937 cells

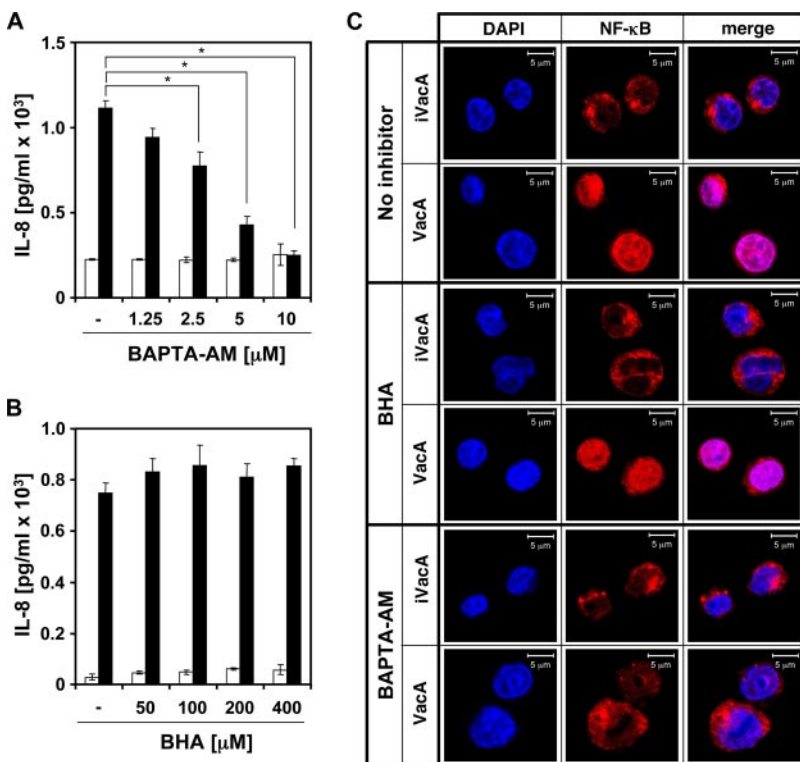


FIGURE 5. A, Confluent U937 cells were pretreated with BAPTA-AM (A) or BHA (B) for 1 h before incubation with VacA or iVacA (120 nM) in serum-free medium. IL-8 production was measured by ELISA. The data are representative of at least three experiments. C, U937 cells were preincubated with 50 μ M BHA or 10 μ M BAPTA-AM for 1 h before incubation with VacA or iVacA (120 nM) in serum-free medium. After incubation for 60 min, the cells were fixed using 2% paraformaldehyde, and treated for 5 min with 0.1% Triton X-100 for membrane permeabilization. The fixed cells were stained with 1 μ g/ml DAPI for 5 min, followed by incubation with anti-NF- κ B mAbs (1/100) in TBS containing 1% BSA. After treatment with the respective primary Abs, cells were incubated with secondary Ab in TBS containing 1% BSA and anti-mouse polyclonal Abs conjugated with Alexa fluor 546 (1/1000). Scale bar, 5 μ m. Statistical significance: *, $p < 0.01$.

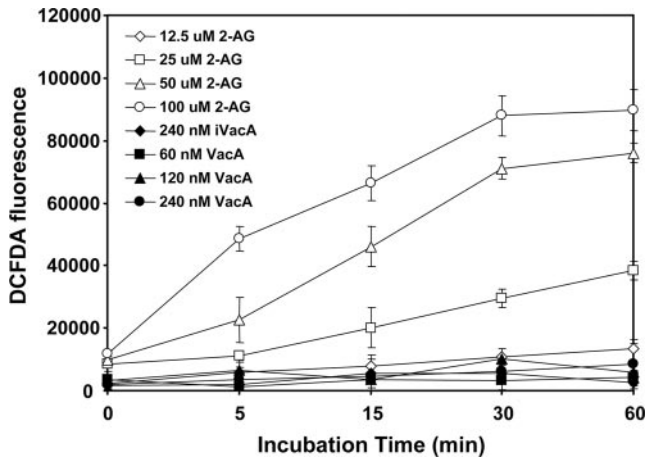


FIGURE 6. Confluent U937 cells were loaded with 5 μ M 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate for 30 min, washed with serum-free medium, and treated with 60, 120, or 240 nM VacA, 240 nM iVacA, or 2-AG (12.5, 25, 50, or 100 μ M, as positive control). ROS formation was measured in a multiwell plate reader in triplicate. Data are mean \pm SE of values from triplicate experiments, with $n = 3$ per experiment.

treated with VacA are independent of mitochondrial generation of ROS. In agreement, VacA did not affect ROS generation by U937 cells, whereas 2-AG induced ROS generation by U937 cells in a concentration- and time-dependent manner (Fig. 6).

Effects of BAPTA-AM and BHA on VacA-induced p38 MAPK activation in U937 cells

It is known that, in Vero cells, shiga toxin activates p38MAPK through an increase in intracellular Ca^{2+} (38), a signaling mechanism similar to the muscarinic agonist carbachol (39). To assess whether VacA-induced p38 MAPK activation is dependent on an increase in cellular Ca^{2+} , we examined the effects of the intracellular Ca^{2+} chelator BAPTA-AM on VacA-induced IL-8 production by U937 cells and found that pretreatment of cells with BAPTA-AM practically abolished VacA-induced IL-8 production, implying a role for intracellular Ca^{2+} in mediating p38 MAPK activation (Fig. 7A). In contrast, BHA did not affect VacA-induced p38 MAPK activation (Fig. 7B). These results support our hypothesis that an increase in intracellular Ca^{2+} , but not mitochondrial reactive oxygen intermediates generation, as is the case with eosinophils (36), is critical in U937 cells for p38 MAPK activation by VacA, leading to IL-8 production.

Effects of thapsigargin, SKF96365, and dantrolene on VacA-induced IL-8 production

To examine whether IL-8 production due to VacA-mediated increase in cytosolic free Ca^{2+} concentration is induced by emptying inositol 1,4,5-triphosphate-sensitive Ca^{2+} stores, we examined the effect of a specific inhibitor of sarcoplasmic and endoplasmic reticulum ATPases, thapsigargin (40). Thapsigargin led to a concentration-dependent increase in IL-8 release similar to what was measured with VacA, but not iVacA (Fig. 8A). This observation suggests that VacA-induced IL-8 production is mediated by an increase in cytosolic free Ca^{2+} via the Ca^{2+} store depletion by inositol 1,4,5-triphosphate-dependent Ca^{2+} release. In addition, as shown in Fig. 8, B and C, VacA-induced IL-8 production was inhibited by the imidazol derivative, SKF96365, a blocker of receptor-activated Ca^{2+} entry, which inhibits various types of ion channels, including receptor-activated channels (41) and weakly and significantly inhibited by dantrolene, which inhibits intracellular Ca^{2+} channels (ryanodine receptor channels) controlling Ca^{2+} release from in-

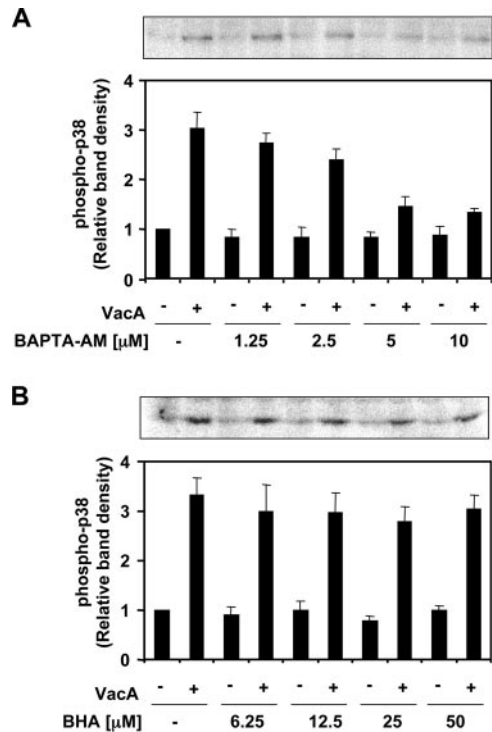


FIGURE 7. U937 cells were incubated with the indicated amounts of BAPTA-AM (A) or BHA (B) for 1 h before incubation with VacA or iVacA (120 nM) in serum-free medium. After incubation for 60 min, the cells were solubilized, followed by SDS-PAGE in 10% gels and Western blotting using anti-phospho-p38 Ab. Results are representative of three independent experiments. Relative densities of phospho-p38 as determined by densitometry scan analysis were compared with densities obtained without VacA and inhibitor. Data are mean \pm SE of values from triplicate experiments, with $n = 3$ per experiment. Statistical significance: *, $p < 0.05$.

tracellular stores (42). Thus, an increase in cytosolic free Ca^{2+} concentration, induced by emptying Ca^{2+} store as well as Ca^{2+} influx, may be responsible for VacA-induced IL-8 production.

Effect of VacA on phosphorylation of ATF-2 and CREB in U937 cells

These data suggest that VacA is responsible for translocation of NF- κ B into the nucleus and, hence, activation of IL-8 transcription. To obtain further evidence for the site on the IL-8 promoter responsible for cytokine release in response to VacA, we examined phosphorylation of ATF-2 and CREB in VacA-treated U937 cells. VacA enhanced phosphorylation of ATF-2 and CREB in a time-dependent manner (Fig. 9, A and B). Consistent with this result by confocal microscopy, the phosphorylation of ATF-2 and CREB in U937 cells treated with VacA for 60 min was markedly increased, compared with cells treated with iVacA (Fig. 9, C and D).

CREB and ATF-2 involvements in IL-8 expression

To determine further the roles of CREB and ATF-2 in regulating IL-8 expression in response to VacA treatment, U937 cells were transfected with CREB-siRNA or ATF-2-siRNA. Reduction of CREB and ATF-2 expression in U937 cells treated with CREB-siRNA or ATF-2-siRNA, respectively, resulted in suppression of IL-8 expression (Fig. 10).

VacA up-regulates IL-8 through both CRE and NF- κ B sites in the IL-8 promoter

To determine which sites in the IL-8 promoter are responsible for IL-8 expression, we next transfected U937 cells with IL-8 reporter

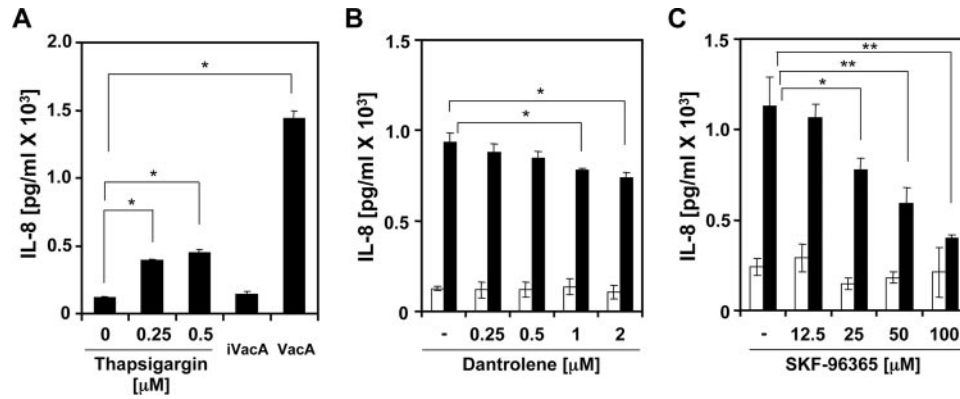


FIGURE 8. To examine whether thapsigargin (TG), an endoplasmic reticulum Ca^{2+} -ATPase inhibitor, induces IL-8 expression in U937 cells via increasing cytosolic free Ca^{2+} concentration induced by emptying Ca^{2+} stores, confluent U937 cells were treated with the indicated amounts of TG for 12 h in serum-free medium (A). In parallel with this experiment, U937 cells were incubated with 120 nM VacA or iVacA. IL-8 production was measured by ELISA. The data are representative of at least three experiments. To examine the effects of dantrolene, an inhibitor of intracellular Ca^{2+} channels, or SKF-96365, a blocker of Ca^{2+} influx, on VacA-induced IL-8 expression, confluent U937 cells were treated with the indicated amounts of Dantrolene (B) or SKF-96365 (C) for 1 h before incubation with 120 nM VacA or iVacA for 12 h in serum-free medium. IL-8 production was measured by ELISA. The data are representative of at least three experiments, with $n = 3$ per experiment. Statistical significance: *, $p < 0.05$; **, $p < 0.01$.

plasmids or with promoters containing deletion or its site-specific mutations of the AP-1 region, which is a binding site for ATF-2 and CREB, or NF-IL-6 or NF- κ B regions (see diagram in Fig. 11A). VacA increased luciferase activity of the wild-type promoter by >4-fold, and this effect was partially reduced by deletion of

AP-1 region, completely abolished by deletion of AP-1, NF-IL-6, and NF- κ B regions, and blocked by mutation of AP-1 or NF- κ B regions (Fig. 11B), suggesting that the VacA effect was mediated at least in part through the AP-1 and NF- κ B regions. In contrast, the effects of VacA were not altered in U937 cells transfected with

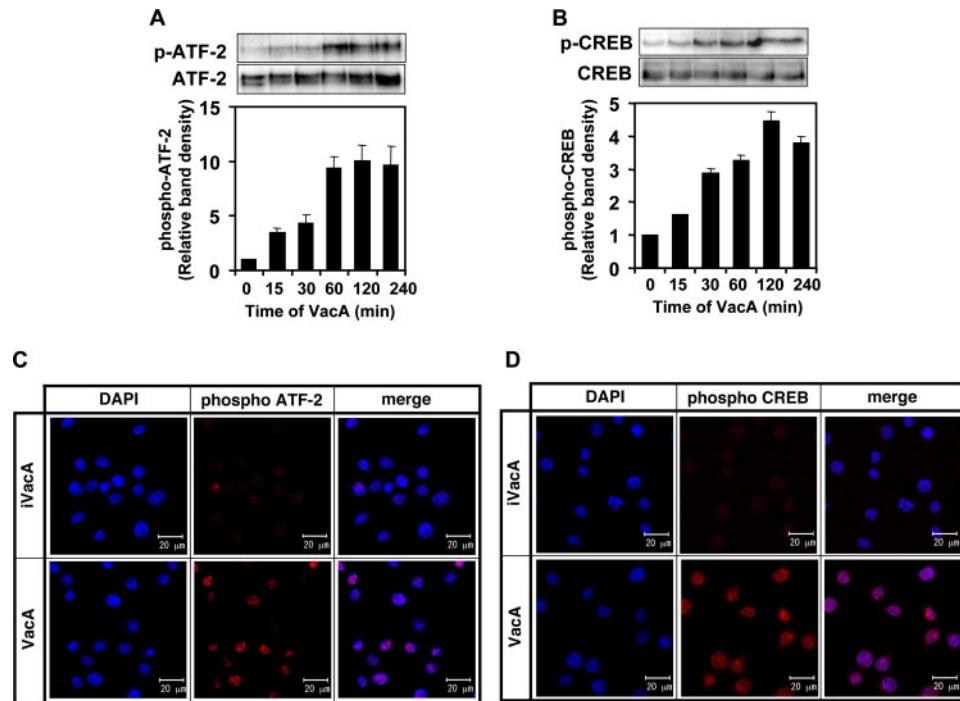


FIGURE 9. U937 cells were incubated with 120 nM VacA for the indicated times. Cell lysates were prepared and subjected to Western blot analyses using anti-phospho-ATF-2 (A) or anti-phospho-CREB (B) Abs. Relative amounts of phospho-ATF-2 and phospho-CREB, as determined by densitometry scan analysis, were compared with densities obtained at 0-min incubation. Data are mean \pm SE of values from triplicate experiments. C, U937 cells were incubated with 120 nM VacA or iVacA at 37°C for 60 min. The cells were fixed using 2% paraformaldehyde, and permeabilized by incubation with 0.1% Triton X-100 for 5 min. Fixed cells, stained with 1 μ g/ml DAPI for 5 min, were incubated with 1 μ g/ml DAPI for 5 min, then incubated with anti-phospho-ATF-2 polyclonal Abs (1/100) in TBS containing 1% BSA. After treatment with the respective primary Abs, cells were incubated with secondary Ab in TBS containing 1% BSA, either anti-rabbit polyclonal Abs conjugated with Alexa fluor 546(1/1000) or anti-mouse polyclonal Abs conjugated with Alexa fluor 546(1/1000). Data are representative of three experiments. D, U937 cells were incubated with 120 nM activated VacA or iVacA at 37°C for 60 min. The cells were fixed using 2% paraformaldehyde, and permeabilized for 5 min with 0.1% Triton X-100. The fixed cells were stained with 1 μ g/ml DAPI for 5 min, then incubated with anti-phospho-CREB polyclonal Abs (1/100) as primary Ab in TBS containing 1% BSA, followed by incubation with secondary Ab in TBS containing 1% BSA, either anti-rabbit polyclonal Abs conjugated with Alexa fluor 546(1/1000) or anti-mouse polyclonal Abs conjugated with Alexa fluor 546(1/1000).

FIGURE 10. U937 cells were grown overnight, and silencing of CREB or ATF-2 gene was performed with CREB-siRNA, ATF-2-siRNA, NC-siRNA, or without siRNA, as described in *Materials and Methods*. After a 24-h transfection, cells were suspended in serum-free medium and treated with VacA or iVacA for 2 h. **A**, Reduction of CREB, ATF-2, or GAPDH protein level was confirmed by Western blotting with anti-CREB, anti-ATF-2, or anti-GAPDH Abs (*left upper panel*), and relative amounts determined by densitometry scan analysis (*bottom panels and right upper panel*) were compared with densities obtained by mock transfection (without siRNA) or NC-siRNA transfection. The data are representative of at least two experiments, with $n = 3$ plates per experiment. **B**, U937 cells were grown overnight, and silencing of CREB or ATF-2 gene was performed with CREB-siRNA, ATF-2-siRNA, or NC-siRNA, as described in *Materials and Methods*. After 24-h transfection, cells were suspended in serum-free medium and treated with VacA or iVacA for 2 h. IL-8 production was measured by ELISA. The data are representative of at least three experiments, with $n = 3$ per experiment. Statistical significance: *, $p < 0.05$.

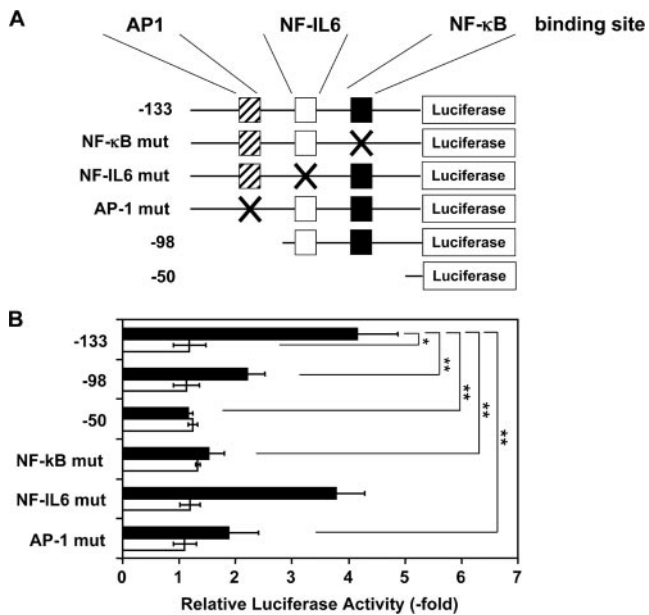
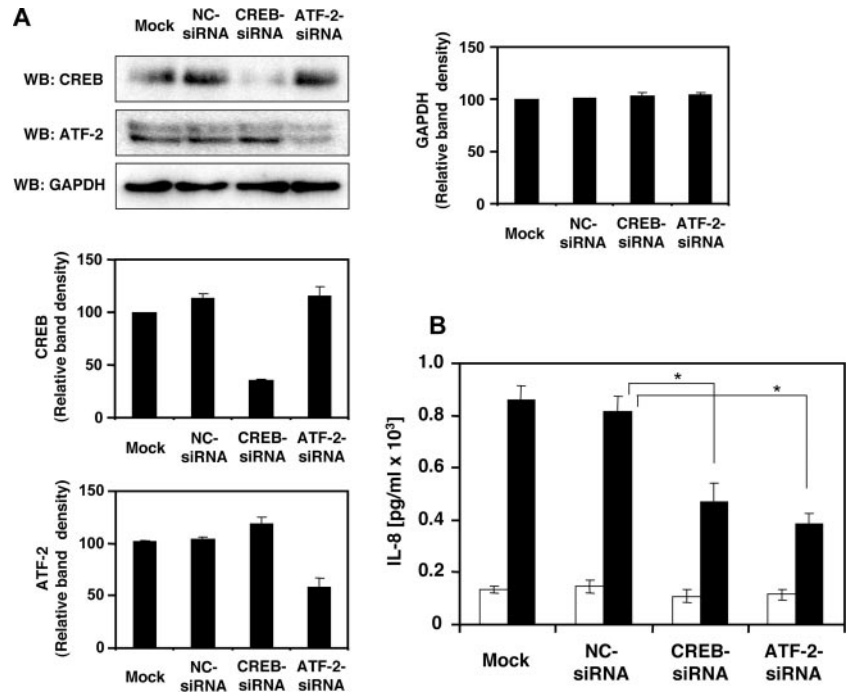


FIGURE 11. **A**, Schematic representation of wild-type and mutant IL-8 reporter constructs. The AP-1 site (-126 to -120; TGACTCA), NF-IL-6-like site (-94 to -81; CAGTTGCAAATCGT), or κ B-like site (-80 to -71; GGAATTCCT) in the IL-8 promoter (-133 to +44), linked to a luciferase reporter gene, was mutated to TatCTCA, agcT-TGCAAATCGT, and taAcTTTCCT, respectively. **B**, Effect of point mutations in the IL-8 promoter on the inducibility of luciferase activity. U937 cells were transiently transfected with IL-8 promoter-luciferase reporter plasmids with the -133/+44, -98, -50, NF- κ B mut, NF-IL-6 mut, or AP-1 mut promoters, as well as the reference plasmid pRL-CMV. Cells were either treated with 120 nM VacA or iVacA (0 or 6 h) at 37°C. Relative changes in luciferase expression were measured. □, Represent incubations with iVacA, and ■, with VacA. Luciferase activity was normalized for Renilla luciferase activity. Data are means \pm SD of values from three independent experiments, with $n = 3$ per experiment. Statistical significance: *, $p < 0.05$; **, $p < 0.01$.

an IL-8 promoter plasmid mutated at the NF-IL-6 site (Fig. 11B). These data demonstrate direct involvement of the ATF-2/CREB binding region in VacA-induced activation of the IL-8 promoter. In agreement, as noted earlier, reduction of ATF-2 and CREB expression in U937 cells by ATF-2 or CREB siRNA, respectively, resulted in suppression of IL-8 production.

Release of IL-8 in VacA-treated human PBMC

To characterize the proinflammatory effect of VacA on human PBMC, IL-8 release from PBMC in response to VacA was quantified by ELISA (Fig. 12). After incubation for 12 and 24 h, significant VacA-stimulated induction of IL-8 by CD14⁺ PBMC was observed, similar to the observations with U937 cells.

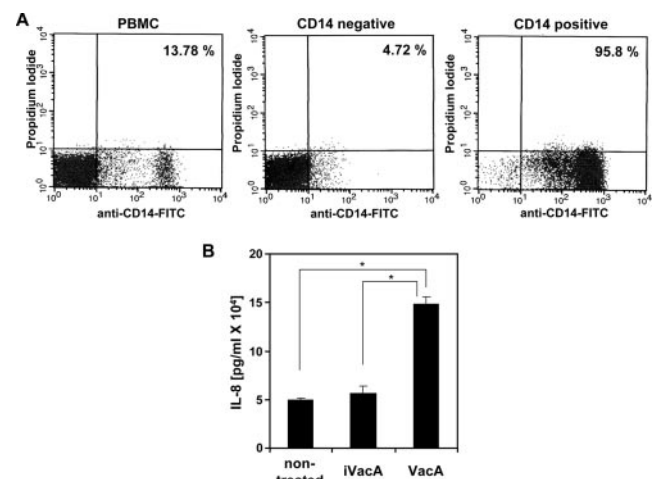


FIGURE 12. **A**, Flow cytometry of CD14⁺ monocytes. Monocytes were generated from PBMC by autoMACS; >90% of the isolated cells were CD14⁺. **B**, Isolated CD14⁺ cells were incubated with 120 nM VacA, iVacA, or as negative control in medium alone. After 24 h, the supernatants were analyzed in a human IL-8 ELISA. Data are means \pm SD of values from three independent experiments with assays in duplicate. Statistical significance: *, $p < 0.01$.

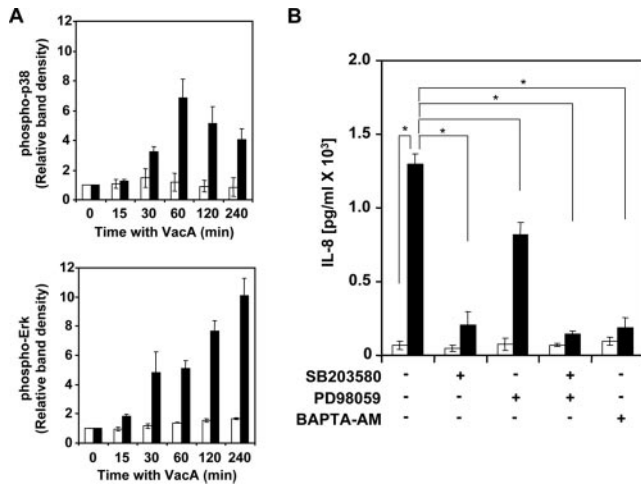


FIGURE 13. A, MKN1 cells were incubated with 120 nM iVacA or VacA for the indicated times. Cell lysates were prepared at indicated incubation times and subjected to Western blot analyses using anti-MAPKs and phosphorylated MAPK Abs. Data are representative of three experiments. Relative densities of phospho-p38 (*upper panel*) and phospho-ERK (*bottom panel*), as determined by densitometry scan analysis, were compared with densities obtained at 0 min. Data are mean \pm SE of values from triplicate experiments, with $n = 3$ per experiment. B, Confluent MKN1 cells were pretreated with SB203580 (10 μ M), PD98059 (10 μ M), or both inhibitors (10 μ M), or BAPTA-AM (10 μ M) for 1 h before incubation with VacA or iVacA (120 nM) in serum-free medium. IL-8 production was measured by ELISA. The data are representative of at least three experiments. Statistical significance: *, $p < 0.05$.

Activation of MAPKs and inhibition of IL-8 production in VacA-treated MKN1 cells

We also examined whether VacA induced phosphorylation of p38 and ERK1/2 and stimulated the IL-8 production by MKN1 cells. Phosphorylation of p38 and ERK1/2 (Fig. 13A) as well as IL-8 production (Fig. 13B) was induced in MKN cells treated with 120 nM VacA at indicated incubation periods. A p38 MAPK inhibitor, SB203580, blocked VacA-induced IL-8 production by MKN1 cells, whereas PD98059 partially suppressed the increase (Fig. 13B). Furthermore, VacA-induced IL-8 production was reduced in MKN1 cells treated with BAPTA-AM. These results suggest that VacA increased IL-8 production in U937 cells through a similar signaling pathway that included an increase in cellular Ca^{2+} and activation of p38 MAPK.

Discussion

Numerous studies report that gastric epithelial cells infected with *H. pylori* show enhanced IL-8 production (1–3, 11–14, 43–46). In analyzing the release of IL-8 by *H. pylori*, it is necessary to understand the effectors of *H. pylori* driving IL-8 induction in macrophages or gastric epithelial cells. Inflammation-associated factors of *H. pylori*, such as CagA, urease, and bacterial endotoxins, all may enhance IL-8 gene expression. No significant difference was observed between IL-8 production induced by a *cagA*-positive wild-type strain and a *cagA*-negative isogenic mutant strain of *H. pylori*. *H. pylori*-induced IL-8 production was reduced by PD98059, an ERK pathway inhibitor. Thus, ERK activation of NF- κ B, leading to enhanced IL-8 production by human gastric cell line, MKN 45, was CagA-independent (14). However, in AGS cells, CagA induced IL-8 in a strain-dependent manner through a Ras \rightarrow Raf \rightarrow Mek \rightarrow ERK \rightarrow NF- κ B signaling pathway (19). Thus, the role of CagA in induction of IL-8 is not defined.

He et al. (47) reported that mitochondrial generation of ROS/reactive oxygen intermediates induced by *Clostridium difficile* toxin A is involved in the nuclear translocation of NF- κ B. More recently, Kim et al. (36) indicated that, in eosinophils, VacA increased mitochondrial generation of ROS. Pretreatment with antioxidant BHA, before VacA exposure, significantly inhibited ROS formation, suggesting that mitochondrial generation of ROS is involved in IL-8 production by eosinophils treated with VacA. In addition, pretreatment with an intracellular Ca^{2+} chelator BAPTA-AM significantly decreased ROS production, NF- κ B activation, and chemokine secretion, suggesting that VacA induces intracellular Ca^{2+} influx, mitochondrial ROS generation, NF- κ B activation, and, finally, IL-8 expression in human eosinophils. In contrast, in U937 cells, we found that BHA did not block IL-8 production, suggesting that it is independent of ROS generation (Fig. 5B). In agreement with this result, VacA did not affect ROS generation by U937 cells (Fig. 6). The inhibitory effect of BAPTA-AM on p38 MAPK activation (Fig. 7), which was responsible for activation of the IL-8 promoter, is consistent with a role for intracellular calcium influx. VacA increased cytosolic free Ca^{2+} concentration (Fig. 4), which was inhibited by dantrolene, an intracellular Ca^{2+} channel inhibitor as well as SKF96365, a blocker of Ca^{2+} entry, suggesting that an increase in cytosolic free Ca^{2+} concentration, induced by emptying Ca^{2+} stores and Ca^{2+} influx may be responsible for VacA-induced IL-8 production (Fig. 8). This result was supported by the finding that thapsigargin, which increases cytosolic free Ca^{2+} via depletion of Ca^{2+} stores, also increased IL-8 release from U937 cells. Thus, the mechanisms for IL-8 release by U937 cells appear to be cell specific and different from those used by eosinophils.

H. pylori-induced IL-8 production in human gastric epithelial MKN45 cells was abolished by treatment with intracellular Ca^{2+} chelators as well as by calmodulin inhibitors, suggesting that Ca^{2+} /calmodulin signaling is involved in *H. pylori*-induced IL-8 production (14). It appears that MAPKs trigger NF- κ B-mediated IL-8 production, as suggested by the reports that a Korean *H. pylori* isolate activates MAPKs, AP-1, and NF- κ B and induces chemokine expression in AGS cells (48). Because we showed that VacA activated the p38 MAPK/ATF-2 cascade (25, 49), these findings led us to examine whether VacA triggers p38 MAPK-mediated IL-8 production via activation of ATF-2, which can bind to the AP-1 region in the IL-8 promoter, as well as its cell specificity. Our data reveal that, in macrophages, VacA is responsible for induction of IL-8 via an increase in cytosolic free Ca^{2+} concentration, resulting in p38 MAPK activation, leading to ATF-2, CREB, and NF- κ B activation. As shown in Fig. 13, in MKN1 cells, VacA increased IL-8 production through a similar signaling pathway that included an increase in cellular Ca^{2+} and activation of p38 MAPK, similar to what was observed in U937 cells.

It is well known that mast cells play important roles in innate immune responses against bacteria by releasing cytokines and by neutrophil recruitment through TNF- α (50). We found that in mouse bone marrow-derived mast cells, VacA induced the production of proinflammatory cytokines such as TNF- α , MIP-1 α , IL-1 β , IL-6, IL-10, and IL-13 (30). Furthermore, de Bernard et al. (51) reported that VacA and IgE stimulated RBL-2H3 mast cells to produce TNF- α after an increase in cytosolic Ca^{2+} . VacA depolarizes the T cell plasma membrane, resulting in the closing of a plasma membrane calcium channel, leading to inhibition of the rise of cytosolic Ca^{2+} , which mediates IL-2 induction under the control of transcription factors such as NF-AT (52). Because VacA-induced p38 MAPK was not inhibited by 5-nitro-2-(3-phenylpropylamino)-benzoic acid (49), it is likely that VacA-induced IL-8 production is independent of VacA anion channel formation, as

suggested by reports that its inhibitory effect was blocked by 5-nitro-2-(3-phenylpropylamino)-benzoic acid. In contrary to mast cell, VacA did not induce TNF- α production by U937 cells despite an increase in cytosolic Ca²⁺ (data not shown), suggesting that the mechanisms for TNF- α production also appear to be cell specific.

Consistent with our previous finding, which showed that p38 MAPK phosphorylation in AZ-521 cells treated with VacA was completely inhibited by addition of anti-VacA IgG (25), VacA-stimulated IL-8 induction was blocked by addition of anti-VacA IgG (data not shown), suggesting that the activation of p38 MAPK and subsequent effects are not due to the presence of contaminants in the purified VacA (e.g., endotoxin). Infection of U937 cells by a *vacA* mutant strain of *H. pylori* failed to induce IL-8 production, suggesting that VacA is responsible for IL-8 production (Fig. 1D). In addition, our observation that VacA induced IL-8 production by a premonocytic cell line, U937, was also found in PBMC (Fig. 12). Thus, not only mast cells (30, 52), neutrophils (53, 54), and eosinophils (36), but also macrophages, are involved in the inflammatory response against *H. pylori* through VacA-induced IL-8 production via intracellular Ca²⁺ movement.

Macrophages are important coordinators of an immune response to *H. pylori* and activate adaptive immunity by producing factors such as IL-12 that stimulate Th1 cells, resulting in production of cytokines such as IFN- γ (55, 56). Macrophages are also involved in the amplification of the inflammatory response by production of cytokines such as IL-1, TNF- α , and IL-6 (57, 58). It has been shown that a secreted peptidyl prolyl *cis*-, *trans*-isomerase (HP0175) elicits the release of IL-6 from human macrophages in a TLR4/MAPK-dependent manner by activating NF- κ B-driven *IL-6* gene transcription (59). Inflammatory cytokines, including IL-1 and TNF- α , activate NF- κ B and AP-1 and induce the expression of the MCP-1 gene in human endothelial cells (60). The promoter region of the human MCP-1 gene has been shown to contain putative consensus binding sites for NF- κ B and AP-1 (61, 62). It is likely that the gene products of the *cag* PAI are involved in the induction of MCP-1 gene expression, because a *cag* PAI-negative strain of *H. pylori* is incapable of inducing MCP-1 expression (63). We detected a significant increase in MCP-1, but not of IL-4, IL-6, IL-10, IL-12, or TNF- α expression by U937 cells treated with VacA (data not shown), which is not a product of *cag* PAI. The VacA-induced MCP-1 production might be mediated by activation of AP-1 and NF- κ B via MAPK activation. Thus, the host response to *H. pylori* infection might involve VacA, leading to release of inflammatory mediators.

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Disclosures

The authors have no financial conflict of interest.

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