NF-κB- and C/EBPβ-driven Interleukin-1β Gene Expression and PAK1-mediated Caspase-1 Activation Play Essential Roles in Interleukin-1β Release from *Helicobacter pylori* Lipopolysaccharide-stimulated Macrophages*

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_Helicobacter pylori_ is a Gram-negative microaerophilic bacterium that causes chronic gastritis, peptic ulcer, and gastric carcinoma. Interleukin-1β (IL-1β) is one of the potent proinflammatory cytokines elicited by _H. pylori_ infection. We have evaluated the role of _H. pylori_ lipopolysaccharide (LPS) as one of the mediators of IL-1β release and dissected the signaling pathways leading to LPS-induced IL-1β secretion. We demonstrate that both the NF-κB and the C/EBPβ-binding elements of the IL-1β promoter drive LPS-induced IL-1β gene expression. NF-κB activation requires the classical TLR4-initiated signaling cascade leading to IκB phosphorylation as well as PI-3K/Rac1/p21-activated kinase (PAK) 1 signaling, whereas C/EBPβ activation requires PI-3K/Akt/p38 mitogen-activated protein (MAP) kinase signaling. We observed a direct interaction between activated p38 MAP kinase and C/EBPβ, suggesting that p38 MAPK is the immediate upstream kinase responsible for activating C/EBPβ. Most important, we observed a role of Rac1/PAK1 signaling in activation of caspase-1, which is necessary for maturation of pro-IL-1β. _H. pylori _LPS induced direct interaction between PAK1 and caspase-1, which was inhibited in cells transfected with dominant-negative Rac1. PAK1 immunoprecipitated from lysates of _H. pylori _LPS-challenged cells was able to phosphorylate recombinant caspase-1, but not its S376A mutant. LPS-induced caspase-1 activation was abrogated in cells transfected with caspase-1(S376A). Taken together, these results suggested a role of PAK1-induced phosphorylation of caspase-1 at Ser376 in activation of caspase-1. To the best of our knowledge our studies show for the first time that LPS-induced Rac1/PAK1 signaling leading to caspase-1 phosphorylation is crucial for caspase-1 activation. These studies also provide detailed insight into the regulation of IL-1β gene expression by _H. pylori _LPS and are particularly important in the light of the observations that IL-1β gene polymorphisms are associated with increased risk of _H. pylori_-associated gastric cancer.

1 The abbreviations used are: IL, interleukin; LPS, lipopolysaccharide; TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; PI-3K, phosphatidylinositol 3-kinase; IRAK, IL-1 receptor-associated kinase; TRAF, tumor necrosis factor receptor-associated factor; IKK, IκB kinase; PAK, p21-activated kinase; MAPK, mitogen-activated protein kinase; PMA, phorbol 12-myristate 13-acetate; z-YVAD-FMK, z-Tyr-Val-Ala-Asp(OMe)-CH₂F; z, benzyloxycarbonyl; DTT, dithiothreitol; NTA, nitrilotriacetic acid; C/EBP, CCAAT enhancer-binding protein; ELISA, enzyme-linked immunosorbent assay; PIPES, |-(3-cholamidopropyl)dimethylammonio|-1-propanesulfonic acid; GST, glutathione S-transferase; RT, reverse transcription; GSK, glycogen synthase kinase; ERK, extracellular signal-regulated kinase; dn, dominant-negative.

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Helicobacter LPS-stimulated IL-1β Release from Macrophages

17-kDa IL-1β (10). The active IL-1β is released and exhibits its diverse biological functions.

Soluble mediators of *H. pylori* are known to induce IL-1β. Of particular significance is the finding that IL-1β gene cluster polymorphisms suspected of enhancing production of IL-1β are associated with an increased risk of gastric cancer (11). This makes it worthwhile to explore the mechanism of induction of IL-1β by *H. pylori*, and in particular, the role of LPS. The expression of IL-1β is regulated at the level of transcription (12–14), mRNA stabilization, and post-translational proteolytic processing (15). C/EBPβ (13)- and NF-κB (14)-binding sites have been characterized in the human IL-1β promoter.

Toll-like receptors (TLRs) play central roles in innate immunity by recognition and discrimination of specific conserved patterns of molecules derived from bacteria, fungi, or viruses (16–18). Activation of TLRs results in stimulation of signaling pathways widely involving recruitment of the adaptor molecule myeloid differentiation factor 88 (MD88) (19–21). The serine/threonine kinase IL-1 receptor-associated kinase 1 (IRAK1) is subsequently recruited, becomes phosphorylated, dissociates from the complex and associates with tumor necrosis factor receptor-associated factor 6 (TRAF6) (22–24) leading to the activation of mitogen-activated protein kinases (MAPKs), transcription factors such as NF-κB and concomitant production of cytokines (25, 26). MAPKs comprise an important group of serine/threonine signaling kinases that transduce a variety of extracellular stimuli through a cascade of protein phosphorylations, which lead to the activation of transcription factors (27–29). There are three principal MAPK family members: (i) p46 and p54 c-Jun N-terminal kinase or stress-activated protein kinase (JNK or SAPK, respectively) with multiple subisoforms, (ii) p38 MAPK with α, β, γ, and δ isoforms, and (iii) p42 and p44 extracellular signal-regulated kinase (ERK). MAPKs are activated by specific upstream MAPK kinases (MKKs) (30).

Since TLR- and MAPK signaling lead to the control of gene transcription, we explored the role of these signaling pathways in *H. pylori*-LPS-mediated IL-1β release, and its regulation at the level of transcription. We also explored the pathways regulating proteolytic processing of pro-IL-1β. The study described here provides evidence that *H. pylori* LPS signals through TRIF to regulate IL-1β transcription driven by NF-κB and C/EBPβ elements in the IL-1β promoter. Whereas regulation at the level of NF-κB involves the canonical TRIF-directed phosphorylation of JNK-β leading to NF-κB activation, regulation of C/EBPβ involves a TLR4/phosphatidylinositol 3-kinase (PI3K)/Akt/p38 MAPK pathway. We observed a direct interaction between phospho-p38MAPK and C/EBPβ, suggesting that p38MAPK is the immediate upstream kinase responsible for activating C/EBPβ. In addition, PI3K/Rac1/p21-activated kinase 1 (PAK1) signaling also regulates NF-κB, as well as the caspase-1-mediated processing of pro-IL-1β to IL-1β. We present the novel finding that PAK1 interacts with caspase-1, and that this interaction is blocked in dominant-negative (dn)-Rac1-transfected cells. PAK1 immunoprecipitated from *H. pylori* LPS-challenged cells, phosphorylated caspase-1 at Ser222 and activation of caspase-1 was abrogated in caspase-1 (S376A)-transfected cells. We hypothesize that PAK1 is a key upstream kinase regulating the activation of caspase-1 at least in some cell types.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Akt inhibitor (1-(6-hydroxymethyl-chiro-inositol 2-((R)-2-O-methyl-3-O-octadecylcarbonate), SB203580, U0126, SP 600125, z-Tyr-Val-Ala-Asp(OMe)-CH₂F, (z-YVAD-FMK) N-acetyl-Tyr-Val-Ala-Asp(7-amino 4-trifluoromethylcoumarin) (Ac-YVAD-afc), phorbol 12-myristate 13-acetate (PMA), and wortmannin, were products of EMD Biosciences, San Diego, CA. Protease inhibitors were from Roche Applied Science. All other reagents were of analytical grade. The human interleukin-1β ELISA kit was from Amersham Biosciences. *Escherichia coli* LPS was purchased from Sigma. γ-32P/ATP was from Janski, BRT, Hyderabad, India.

**Antibodies**—Anti-p88 MAPK, -PAK1, -phospho-p83MAPK, -phospho-PAK1, -interleukin-1β, and -p-Tyr were from Cell Signaling Technology, Beverly, MA. Anti-p85 and supershift antibodies against p50, p65, and C/EBPβ were from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Anti-Rac1 and anti-caspase-1 were from BD Biosciences, San Jose, CA. Neutralizing TLR-4 antibody (HTA 125) was purchased from Imgenex Biotech, Bhubaneswar, India.

**ASSays**—*H. pylori* 26695 was cultured for 3 days on horse blood agar plates in a microaerophilic milieu (10% CO₂, 5% O₂, and 85% N₂) at 37 °C. The bacterial cells were harvested; LPS was prepared by the hot phenol-water method described by Westphal and Jann (31), dialyzed, and freeze-dried. LPS was estimated using the E-TOXATE amoeocyte lysate assay kit from Sigma.

**Cell Culture**—THP1 cells derived from a patient with acute monocytic leukemia are mature cells from the monocyte/macrophage lineage. These were obtained from the National Center for Cell Science, (Pune, India). The cell line was maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 ml glutamine, and 20 mM sodium bicarbonate. The cells were incubated at 5% CO₂ and 95% humidity in a 37 °C chamber. THP1 cells were treated with PMA to induce maturation of the monocytes to a macrophage-like adherent phenotype (32). Cells were washed thrice with culture medium and cell viability was determined to be >95% by the trypan blue dye exclusion method. The inhibitors U0126, SB203580, SP600125, Akt inhibitor, and wortmannin, were dissolved in Me₂SO. Appropriate vehicle controls were maintained in all experiments in which these inhibitors were used.

**RNA Isolation**—Cells were harvested and RNA was isolated from THP1 cells using TRIzol reagent (Invitrogen RNeasy Mini kit) and used as template to amplify the cDNA of the gene cluster. RNA isolated from THP1 cells using the sense and antisense primers 5′-ATGAGTCCGATGGAGCTGAATTTCTC′-3′ and 5′-ATAAAGCTTTCTAAGATTGTCCTGGCT′-3′, respectively and cDNA was amplified from RNA isolated from THP1 cells using RT-PCR using the sense and antisense primers 5′-TAGATTTGCTGAGCTGAGCTGCTGA′-3′ and 5′-TTGGATTACATGTCCTGGAGGAAGTTGA′-3′, respectively. LPS was purchased from Sigma. [32P]ATP was from Jonaki, India. [β-32P]-labeled Oligonucleotide probes were made from the above primers using the random primer labeling kit (United States Biochemicals). The primers were incubated with [β-32P]-ATP. After annealing to the template, the probe was purified using a nucleotide spin column and used for hybridization with the Southern Blot membrane.

**Plasmids and Transient Transfections**—TLR4 was amplified from DNA isolated from THP1 cells using the sense and antisense primers 5′-ATGAGTCCGATGGAGCTGAATTTCTC′-3′ and 5′-ATAAAGCTTTCTAAGATTGTCCTGGCT′-3′, respectively. LPS was generated using the same method as that used for DNA isolation from the bacteria. LPS was estimated using the E-TOXATE amoeocyte lysate assay kit from Sigma.

** reporter plasmid was used to normalize transfection efficiencies.**

**Treatment with LPS and Preparation of Cell Lysates**—THP1 cells were treated with PMA and cultured in 24-well tissue culture plates at 10⁵ cells per well followed by treatment with H. pylori LPS (or *E. coli* LPS where indicated). The cells were washed with ice-cold phosphate-buffered saline and lysed with lysis buffer (20 mM Tris-
**Phosphorylation of Caspase-1 in Vitro—**PAK1 was immunoprecipitated from lysates of cells challenged with *H. pylori* LPS by incubating with PAK1 antibody overnight followed by incubation with protein A/G-agarose for another 3 h. The immunoprecipitate was washed twice in kinase buffer (300 mM Tris, 1 mM KCl, 1 mM MgCl₂, 10 mM Na₃VO₄, 10 mM EDTA, pH 7.5) and incubated with 5 µg of purified His-tagged caspase-1 as substrate in 20 µl of kinase buffer containing 1 µCi of [γ³²P]ATP and 7.5 µM cold ATP for 15 min. The reaction was terminated by the addition of 5× Laemmli sample buffer and phosphorylated caspase-1 was analyzed by SDS-PAGE and Western blotting using phosphoserine-specific antibody.

**RNA Isolation and Reverse Transcription (RT)-PCR—**Total RNA was prepared from cells using the RNeasy™ Mini Kit (Qiagen) according to the manufacturer’s protocol. 100 ng of RNA was reverse-transcribed using the First-Strand cDNA Synthesis kit (Roche Applied Sciences). The sense primer 5’-AAACAATGAGCTTATACAGTGT-3’, and the antisense primer 5’-CCGGAGAACAACATCTCC-3’ were used to amplify 391 bp of IL-1β mRNA. Glyceraldehyde-3-phosphate dehydrogenase was amplified using the following primers: sense, 5’-CTTCAATGCCCTTGAACGATCC-3’ and antisense, 5’-GAGGCGCAGTGGAGCTCCTCC-3’ to generate a 604-bp product. The PCR conditions for IL-1β mRNA were: denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and extension at 72°C for 1 min for 35 cycles. The PCR products were separated on a 1% agarose gel, blotted onto a nitrocellulose membrane, and probed with anti-C/EBPβ antibody.

**Affinity Precipitation Assay for Rac—**This was done as described by Benard et al. (33). Cell extracts after treatment were incubated with GST-PBD (the p21-binding domain (PBD) of PAK1 fused to GST) in a maximal final volume of 500 µl for 1 h at 4°C. The bead pellet was then centrifuged for 2 min at 2,000 rpm at 4°C and resuspended in 25 mM Tris-HCl pH 7.6, 1 mM EDTA, 30 mM MgCl₂, 40 mM NaCl, 1% Nonidet P-40 and twice with the same buffer without Nonidet P-40. The bead pellet was then incubated for 2 h at 4°C with 1 µg of PAK1 antibody (PAK1 antibody (5’-CCGGAGAACAACATCTCC-3’) or the mutated oligonucleotide with mutated sites underlined (5’-CCGGGGAAACATCTCC-3’)) and the antibody complex was washed twice with ice-cold buffer (40 mM Tris-HCl, pH 7.5, 0.1 mM KCl, 1 mM EDTA), pelleted by centrifugation at 600 × g for 1 min, resuspended in 400 µl of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTG, 0.5 mM pefabloc, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 0.5 µg/ml benzamidine) and lysed by sonication (three bursts of 15 s each) in a cold sonicator. The reaction was terminated by the addition of 5× Laemmli sample buffer and phosphorylated caspase-1 was analyzed by SDS-PAGE and Western blotting using phosphoserine-specific antibody.

**Assays of C/EBPβ-p38 MAP Kinase Interaction—**In order to study the *in vivo* interaction between C/EBPβ and p38 MAP kinase, C/EBPβ was immunoprecipitated from lysates of cells treated without or with *H. pylori* LPS. The immunoprecipitate was boiled in Laemmli buffer, separated on SDS-PAGE, transferred onto polyvinylidene difluoride membrane and blotted for p38 MAPK. In separate experiments, lysates from cells treated without or with *H. pylori* LPS were incubated with immobilized phospho-p38 MAP kinase (Cell Signaling Technology) overnight at 4°C. Bead-bound proteins were separated by SDS-PAGE, electrotransferred, and probed with anti-C/EBPβ antibody.

**Affinity Precipitation Assay to Study the Interaction of Caspase-1 and PAK1—**His-tagged caspase-1 was expressed in *E. coli* DH5α by inducing with isopropyl-β-D-thiogalactopyranoside (for 2 h) and immobilized on Ni²⁺-NTA-agarose. The agarose beads were incubated for 2 h at 4°C with lysates from cells treated without or with *H. pylori* LPS. The bead pellets were washed, suspended in Laemmli buffer, separated on SDS-polyacrylamide (7.5%) gels, transferred onto nitrocellulose membranes and blotted for PAK1. In order to study the *in vivo* interaction between PAK1 and caspase-1, lysates from cells treated without or with *H. pylori* LPS were incubated overnight with PAK1 antibody at 4°C, followed by incubation with protein A/G-agarose for 3 h. Agarose-bound proteins were separated by SDS-PAGE, electrotransferred, and probed with anti-caspase-1 antibody.

**Caspase-1 Activity Assays—**Transfected or non-transfected THP-1 cells were either left untreated or were treated with LPS (20 ng/ml) and then lysed by incubating with 25 µl of lysis buffer (25 mM HEPES, pH 7.5, 2 mM EDTA, 0.1% (v/v) CHAPS, 5 mM DTT, 10 µg/ml pepstatin, 10 µg/ml aprotinin, 20 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) on ice for 15 min. The supernatant was kept frozen at –70°C until used. Lysates (20 µg of protein) were taken in 100 µl of assay buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM DTT, 10% (v/v) glycerol, 0.1% CHAPS, and 200 µM caspase-1 substrate Ac-YVAD-APC. Fluorescence of the released AFC was measured using excitation and emission wavelengths of 400 nm and 505 nm, respectively.

**Phosphorylation of Caspase-1 in Vitro—**PAK1 was immunoprecipitated from lysates of cells challenged with *H. pylori* LPS by incubating with PAK1 antibody overnight followed by incubation with protein A/G-agarose for another 3 h. The immunoprecipitate was washed twice in kinase buffer (300 mM Tris, 1 mM KCl, 1 mM MgCl₂, 10 mM Na₃VO₄, 10 mM EDTA, pH 7.5) and incubated with 5 µg of purified His-tagged caspase-1 as substrate in 20 µl of kinase buffer containing 1 µCi of [γ³²P]ATP and 7.5 µM cold ATP for 15 min. The reaction was terminated by the addition of 5× Laemmli buffer and phosphorylated caspase-1 was analyzed by SDS-PAGE and Western blotting using phosphoserine-specific antibody.

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1% (v/v) Triton X-100, 10% glycerol, 2 mM DTT, 10 μg/ml leupeptin). After centrifugation, the supernatant was stored at –70 °C. For promoter activation analysis, luciferase activity assays were performed in a luminometer, and the results were normalized for transfection efficiencies by assay of β-galactosidase activity.

RESULTS

IL-1β Induction by H. pylori LPS—The ability of H. pylori LPS to stimulate the release of IL-1β was studied in THP-1 cells. ELISA showed that LPS stimulated the release of IL-1β in a dose-dependent manner (Fig. 1A). IL-1β release peaked at 18 h (Fig. 1B).

Role of the Mitogen-activated Protein Kinases (MAPKs) in IL-1β Induction by H. pylori LPS—MAPKs are known to regulate the upstream signaling events that control cytokine induction. We therefore evaluated the role of MAPKs in LPS-induced IL-1β production by THP-1 cells using pharmacological inhibitors of MAPKs. E. coli LPS-triggered IL-1β release has been reported to be inhibited by inhibitors of ERK (35). This finding was reproduced in our present study (Fig. 1C). On the other hand, we observed that H. pylori LPS-mediated secretion of IL-1β was unaffected by the MEK inhibitor U0126 (10 μM) (Fig. 1C) ruling out the involvement of the MEK/ERK pathway in IL-1β production in this case. IL-1β production was inhibited by the p38 MAPK inhibitor SB203580 (5 μM) (Fig. 1C) suggesting a role of the p38 MAPK pathway in IL-1β production. H. pylori LPS-mediated secretion of IL-1β was not inhibited by the JNK inhibitor, SP 600125 (25 μM). The lack of involvement of JNK was again unlike what has been reported in the case of E. coli LPS (35) and also observed by us in this study (Fig. 1C).

FIG. 1. H. pylori LPS stimulates release of IL-1β from THP-1 cells. Cells were cultured in 96-well plates and either left untreated or treated with different concentrations of LPS for 20 h (A), or with LPS (20 ng/ml) for different time periods (B). Where indicated (C and D) cells were pretreated with different inhibitors for 30 min or with neutralizing anti-TLR4 antibody prior to treatment with LPS (20 ng/ml) for 20 h. For E and F, cells were transfected with different constructs as indicated, prior to LPS treatment. The supernatant was assayed for IL-1β release using an IL-1β-specific ELISA. Data represent the means ± S.E. of three different experiments.
The TLR4 Signaling Pathway in LPS-stimulated IL-1β Production in THP-1 Cells—The TLR signaling pathway is known to play a role in regulation of cytokine production through a signaling cascade leading to the activation of NF-κB (19, 20, 38). LPS is known to signal through TLR4. We therefore investigated the involvement of the TLR4 signaling pathway in LPS-stimulated IL-1β secretion by THP-1 cells. Pretreatment of cells with neutralizing antibodies against TLR4 prior to challenge with H. pylori LPS, blocked IL-1β secretion (Fig. 1D) supporting a role of TLR4 signaling in the present instance. Transfection with dominant-negative TLR2 had no effect on LPS-mediated IL-1β release (data not shown).

In the canonical pathway of NF-κB activation, TLR4 signals along the MyD88/IRAK/TRAF6 axis, leading to the phosphorylation of IκB-α and its subsequent degradation by the proteasome and releasing NF-κB facilitating its movement into the nucleus. The likely involvement of this classical pathway in H. pylori LPS-induced IL-1β production was tested by transfecting cells with dominant-negative constructs of MyD88, or IRAK1 or TRAF6 or mutated IκB-α (S32A, S36A) in which the two critical serine residues, which are phosphorylated by the IKKs were mutated. An abrogation of IL-1β release (Fig. 1F) in each case, confirmed the likely role of the classical pathway of NF-κB activation in this case.

Role of the PI-3K/Akt Signaling Pathway in IL-1β Induction by H. pylori LPS—PI-3K is known to signal downstream of TLR4 (39). We examined the role of the PI-3K/Akt signaling pathway using the PI-3K inhibitor wortmannin. Both E. coli and H. pylori LPS-stimulated IL-1β production was inhibited by wortmannin (200 nM) (Fig. 1C) or by using the Akt inhibitor (15 μM) prior to stimulation with LPS. In both cases, LPS-stimulated IL-1β release was abrogated by these inhibitors indicating the involvement of PI-3K and Akt in LPS-stimulated IL-1β release.

Activation of heterodimeric (p85-p110) PI-3K is most often promoted by tyrosine phosphorylation of p85 and recruitment to the plasma membrane (40). Therefore PI-3K activation was assessed by preparing cell lysates after treatment with LPS, immunoprecipitating with anti-p85 and analyzing the immunoprecipitates by Western blotting with anti-phosphotyrosine antibody. Prior treatment with neutralizing antibody against TLR4, abrogated the activation of PI-3K (Fig. 2A), suggesting the likely role of TLR4-dependent signaling in LPS-stimulated IL-1β production. A putative PI-3K-binding site (amino acids 257–260, YKAM) has been identified in the C terminus of MyD88 and LPS stimulation has been shown to result in the formation of the PI-3K-MyD88 complex (41). We therefore tested whether activation of PI-3K is dependent on MyD88. Dominant-negative MyD88 could indeed abrogate the activation of PI-3K (Fig. 2A).

p38 MAPK Activation Is Dependent on Akt Kinase—Since both Akt kinase and p38 MAPK were required for LPS-stimulated IL-1β release, we sought to examine whether the activation of either of these kinases was dependent on the other. The activation of p38 MAPK as assessed by Western analysis of phospho-p38 MAPK was abrogated in cells pretreated with neutralizing antibodies against TLR4 or with wortmannin or Akt inhibitor (Fig. 2, B and D). On the other hand LPS-stimulated Akt kinase activity as assessed on Akt immunoprecipitates of treated cells using GSK3 as substrate, was not affected by transfecting cells with dn-p38 MAPK (data not shown), but abrogated in cells pretreated with wortmannin (Fig. 2E). Finally, activation of p38 MAPK was independent of PAK1, since transfection of cells with PAK1 (KD) did not abrogate LPS-stimulated p38 MAPK activation (Fig. 2C). Taken together, these data suggested that LPS triggers TLR4/MyD88/PI-3K/Akt/p38 MAPK signaling.

Role of Rac1/PAK1 Signaling in LPS-Stimulated IL-1β Production—Exposure of macrophages to LPS activates the transcription of many cytokine genes in an NF-κB-dependent manner (42). In macrophages, Rac1/PAK1 signaling mediates NF-κB activation in response to E. coli LPS, leading to cytokine gene transcription (43). PAK family kinases were discovered by their ability to selectively bind the Rho GTPase family members such as Rac (44–47). Thus we felt it necessary to elucidate the role of Rac1 and PAK1 in H. pylori LPS-induced IL-1β release. Transfection of cells with dn-Rac1 or kinase-dead PAK1 prior to challenge with LPS, led to the abrogation of IL-1β release (Fig. 1F), suggesting a role of both Rac1 and PAK1 in LPS-mediated IL-1β production. Pretreatment with wortmannin or transfection of cells with dn-Rac1 abrogated PAK1 activation assessed using phospho-PAK antibody (Fig. 2, F and G). PAK1 activation was therefore dependent on PI-3K and Rac1 in the present instance.

LPS-mediated Activation of Rac1 Is Dependent on PI-3K—In order to assess the activation of Rac1 and formation of Rac1-GTP in response to stimulation with LPS, we performed affinity precipitation experiments with a GST fusion protein corresponding to the p21-binding domain (PBD) of PAK1 (GST-PBD) that specifically binds to and precipitates Rac-GTP from cell lysates (33). The presence of Rac1 in the precipitate was assessed using Rac1 antibody. As shown in Fig. 2H, GST-PBD effectively interacted with the active GTP-bound form of Rac1 in cell lysates obtained from LPS-treated cells. However, no interaction was observed between GST-PBD and GTP-Rac1 in cells treated with wortmannin, indicating that Rac1 activation is dependent on PI-3K in the present instance. Immunoblotting analysis of total lysates revealed no changes in the expression levels of Rac1. PI-3K has been demonstrated to be upstream of Rac activation in several instances such as Rac-dependent platelet-derived growth factor and insulin-stimulated membrane ruffling (48, 49).

LPS Stimulates Caspase-1 Activity in THP-1 Cells—Post-transcriptional regulation and processing of pro-IL-1β protein into mature IL-1β involves caspase-1 (10, 51). Preincubation of cells with caspase-1 inhibitor, z-YVAD-FMK followed by treatment with LPS, diminished mature IL-1β production to control levels (Figs. 1D and 3A), suggesting that caspase-1 is involved in LPS-induced IL-1β maturation. We examined whether LPS could stimulate caspase-1 activity in THP-1 cells. Caspase-1 activity of LPS-treated cells was maximum after 8 h (data not shown). LPS-stimulated caspase activity was blocked by transfection of cells with kinase-dead PAK1 or with dn-Rac1 (Fig. 3B) suggesting the involvement of Rac1/PAK1 signaling in the activation of caspase-1.

We next attempted to evaluate whether PAK1 interacts with caspase-1. PAK1 was immunoprecipitated from lysates of H. pylori LPS-challenged cells. The immunoprecipitates when subjected to Western analysis, showed the presence of caspase-1 (Fig. 4A), suggesting a direct interaction between PAK1 and caspase-1 following H. pylori LPS challenge of THP-1 cells. Control experiments where immunoprecipitation was carried out with isotype IgG did not show any band indicating the specificity of the interaction between caspase-1 and PAK1. In an alternative approach, caspase-1 was expressed as a recombinant His-tagged fusion protein immobilized on Ni2+-NTA agarose. When lysates of THP-1 cells challenged with H. pylori LPS were incubated with immobilized caspase-1-agarose, Western blotting showed that PAK1 could be pulled down from the lysate. Control experiments using Ni2+-NTA agarose alone, failed to pull down PAK1 (Fig. 4B, lane a). These results definitively demonstrated that PAK1 interacts with caspase-1. The interaction was abrogated when lysates from...
cells transfected with dn-Rac1 were used (Fig. 4C). We next attempted to evaluate whether interaction of PAK1 with caspase-1 was associated with the phosphorylation of caspase-1. We observed that immunoprecipitated PAK1 from lysates of cells treated with \textit{H. pylori} LPS, was able to phosphorylate recombinant caspase-1 (Fig. 4D). Some studies have examined the substrate specificity of the PAK family of kinases (52, 53). Based on these studies, the optimal minimal sequence for PAK1 appeared to be (R/K)(R/K)R\textsuperscript{X}S. Scanning the caspase-1 sequence, it appeared possible that RKVRFS\textsuperscript{376} could represent a PAK1-recognizable motif, with Ser\textsuperscript{376} representing the site of phosphorylation. This was supported by our observations that PAK1-mediated phosphorylation performed as described above, was abrogated when the S376A mutant of caspase-1 was used as a substrate, suggesting that PAK1 phosphorylates caspase-1 at serine 376. The relevance of this phosphorylation in the activation of caspase-1 was evident from the fact that caspase-1 activation was abrogated in \textit{H. pylori} LPS-challenged THP-1 cells transfected with caspase-1 (S376A) (Fig. 3B).

\textbf{LPS Regulates IL-1\textbeta Gene Expression in THP-1 Cells—}Semi-quantitative RT-PCR analysis for IL-1\textbeta mRNA expression was performed on total cellular RNA from THP-1 cells treated with LPS. LPS induced IL-1\textbeta mRNA expression (Fig. 5A). In order to determine whether PI-3K and p38 MAPK signaling influence IL-1\textbeta gene expression, cells were pretreated with wortmannin (200 nM) or SB203580 (5 \textmu M) before stimulation with LPS. IL-1\textbeta mRNA levels were assessed by RT-PCR. We observed that SB203580 and wortmannin both suppressed IL-1\textbeta mRNA levels (Fig. 5A), suggesting a role of PI-3K and p38 MAPK signaling in maintaining steady state levels of IL-1\textbeta mRNA.

\textbf{NF-\kappaB- and C/EBP\beta-Binding to the IL-1\textbeta Promoter in THP-1 Cells Treated with LPS—}The IL-1\textbeta gene is regulated by an inducible promoter element. These cis-acting DNA sequences are the ultimate targets of numerous transcription factors activated by distinct signal transduction pathways. Monocyte/macrophage-specific expression of the IL-1\textbeta gene depends upon its promoter located between positions −131 and +12 (36, 37).
The IL-1β promoter contains three important transcription factor-binding motifs, namely NF-IL6 (or C/EBPβ) which is the β-form of the C/EBP of the basic leucine zipper family, NF-κB and Spi-1 (PU.1), a myeloid and B-cell-specific winged helix-turn helix transcription factor that belongs to the ETS family of proteins. In order to determine whether H. pylori LPS- inducible expression of the IL-1β gene depends on transcription factor binding to these motifs regulated by upstream signal transduction pathways, we performed electrophoretic mobility shift assays (EMSAs) using oligonucleotides containing the NF-κB, the C/EBPβ and Spi-1 DNA-binding elements of the IL-1β promoter. Electrophoretic mobility shifts were observed using the IL-1β gene-specific NF-κB- and C/EBPβ-binding oligonucleotides (Fig. 5, B and D) following stimulation with LPS while no shift was observed in the case of Spi-1-specific oligonucleotide (data not shown). Lack of binding with the mutated NF-κB- and C/EBPβ-specific DNA probes showed that the complexes were specific. Supershift of the band obtained with the NF-κB-specific oligonucleotide with anti-p65 or anti-p50 suggested that p65 and p50 are the components of the protein complex that binds to the NF-κB element of the IL-1β promoter. Anti-C/EBPβ supershifted the band obtained with the C/EBPβ-specific DNA probe. Electrophoretic mobility shift of the NF-κB-specific oligonucleotide was inhibited in cells pretreated with wortmannin but not with SB203580 (Fig. 5C), suggesting the involvement of PI-3K but not p38 MAPK in NF-κB activation. On the other hand, electrophoretic mobility shift of the C/EBPβ-specific oligonucleotide was inhibited by both wortmannin and SB203580 (Fig. 5E), suggesting that both PI-3K and p38 MAPK are involved in C/EBPβ binding to specific elements of the IL-1β promoter.

p38 MAP Kinase and C/EBPβ Interact in H. pylori-challenged THP-1 Cells—To study whether p38 MAP kinase interacts with C/EBPβ in vivo, we immunoprecipitated lysates from H. pylori-LPS-challenged cells with C/EBPβ-specific antibodies from THP-1 cells treated with H. pylori LPS. Western analysis showed that p38 MAP kinase communoprecipitated in the complex (Fig. 6A). Isotype IgG failed to precipitate detectable p38 MAP kinase confirming the specificity of the communoprecipitation procedure. In order to study the interaction between C/EBPβ and p38MAPK, C/EBPβ was expressed as a recombinant GST fusion protein and immobilized on glutathione-Sepharose. When lysates of THP1 cells challenged with H. pylori LPS were incubated with immobilized C/EBPβ-GST Sepharose, Western blotting showed that p38 MAPK could be pulled down from the cell lysate (Fig. 6B). Control experiments with GST alone (lane a) did not show any band when Western blotting was carried out with p38 MAPK-specific antibody. Prior treatment with Akt inhibitor could abrogate the interaction between C/EBPβ and p38MAPK. The converse experiments were also performed. Immunoprecipitation was carried out using anti-phospho-p38 MAPK antibody immobilized on agarose. Immunoprecipitates showed the presence of C/EBPβ on Western analysis, whereas appropriate controls did not (Fig. 6C). These results supported the view that phospho-p38MAPK interacts with C/EBPβ in an Akt kinase-dependent manner.

Luciferase Reporter Gene Assays—The three luciferase reporter constructs C1 (encompassing both the NF-κB- and the C/EBPβ-binding sites, C2 (harboring only the NF-κB-binding site) and C3 (harboring only the C/EBPβ-binding site) were used to test luciferase expression following challenge with LPS for 3 h. When cells were transfected with the luciferase reporter construct C1, pretreatment of cells with Akt inhibitor prior to LPS challenge led to a 50% reduction in the fold-stimulation of luciferase activity (Fig. 7A). Akt inhibitor had no effect on LPS-stimulated luciferase activity when construct C2 was used as the luciferase reporter construct (Fig. 7B), whereas it completely inhibited LPS-stimulated luciferase activity when the reporter construct C3 was used (Fig. 7C). These results suggested that Akt kinase signaling regulates IL-1β promoter activity at the level of the transcription factor C/EBPβ but not at the level of NF-κB-driven promoter activation. On the other hand, transfection of cells with the kinase-inactive mutant of Ikkβ (Ikkβ-dn), or kinase-dead PAK1 (PAR-KP), led to a partial inhibition of luciferase activity driven by the NF-κB- and C/EBPβ-binding element-containing C1 (Fig. 7A), a complete inhibition of luciferase activity driven by the NF-κB-binding element-containing C2 (Fig. 7B), and to no inhibition of luciferase activity driven by the C/EBPβ-binding element-containing C3 (Fig. 7C). These results suggested that PAK1 and Ikkβ signaling leads to NF-κB activation and stimulation of IL-1β gene expression. TLR4 was central to signaling leading to IL-1β gene expression, since pretreatment of cells with neutralizing antibodies against TLR4 abrogated LPS-induced luciferase activity driven by the presence of both NF-κB- and C/EBPβ-binding elements in the luciferase reporter construct.

DISCUSSION

The modulation of host macrophage cell signaling by H. pylori has not been studied in detail. The host response to bacteria is initiated when the microorganism interacts with cell surface receptors including TLRs. Recognition through TLRs largely occurs through pathogen-associated molecular patterns of which the best known is LPS, a potent activator of macrophages. This leads to the release of several cytokines including IL-1β. Although H. pylori infection is associated with increased levels of proinflammatory cytokines especially IL-1β (8, 9), the exact mechanism of the release of IL-1β by cells of monocytic lineage is not clear as yet. Understanding this mechanism is of particular relevance in the light of the observations that IL-1β gene polymorphisms are associated with increased risk of gas-
tric cancer. Since it has been reported that aqueous extracts of \textit{H. pylori} could induce IL-1\(\beta\) release, we explored whether LPS, one of the components of the aqueous extract, could be responsible for IL-1\(\beta\) release. We observed that \textit{H. pylori} LPS mediates the secretion of IL-1\(\beta\) by THP1 cells. We report in-depth studies on \textit{H. pylori} LPS-mediated IL-1\(\beta\) release. We observed that LPS stimulation leads to electrophoretic mobility shifts of IL-1\(\beta\) gene-specific NF-\(\kappa\)B- and C/EBP\(\beta\)-binding sequences.

![FIG. 4](http://www.jbc.org/)

**H. pylori** LPS-driven interaction between PAK1 and caspase-1. A. THP1 cells were left untreated or treated with \textit{H. pylori} LPS followed by immunoprecipitation with anti-PAK1 antibody. The immunoprecipitate was blotted with anti-caspase-1 antibody. Negative control for the immunoprecipitation was performed in lysates from treated cells using anti-isotype IgG. As a positive control (last lane), caspase-1 was immunoprecipitated from the cell lysate, followed by blotting with anti-caspase-1 antibody. Reprobing was performed with anti-PAK1 antibody as a control for equal loading on the gels. B, cell lysates obtained before (lane b) and after (lanes a and c) treatment with LPS were incubated with Ni-NTA-agarose (lane a, negative control) or caspase-1 bound to Ni\(^{2+}\)-NTA-agarose (lanes b and c). The bound proteins were then subjected to Western analysis with anti-PAK1 antibody. Reprobing was carried out with anti-His antibody. C, cells were transfected either with empty vector or with dominant-negative Rac1 (dn-Rac1). Cells were then either left untreated or treated with \textit{H. pylori} LPS. Cell lysates were then treated with caspase-1-bound Ni\(^{2+}\)-NTA-agarose. The bound proteins were then subjected to Western analysis with anti-PAK1 antibody. D, THP1 cells were left untreated or treated with \textit{H. pylori} LPS followed by immunoprecipitation with anti-PAK1 antibody. The immunoprecipitate was used to phosphorylate recombinant wild-type caspase-1 in the presence of [\(\gamma\)^32P]ATP. The autoradiogram is representative of three independent experiments. E, PAK1 was immunoprecipitated from untreated and \textit{H. pylori} LPS-treated cell lysates and used to phosphorylate either wild type or mutant (S376A) recombinant His-tagged caspase-1 separately. The phosphorylated caspase-1 was detected by Western blotting using anti-phosphoserine antibody. The blot was reprobed with anti-His antibody.

![FIG. 5](http://www.jbc.org/)

**H. pylori** LPS stimulates IL-1\(\beta\) gene expression and NF-\(\kappa\)B and C/EBP\(\beta\) binding to the IL-1\(\beta\) promoter in THP-1 cells. Cells were left untreated or pretreated with the inhibitors SB203580 (5 \(\mu\)M) or wortmannin (200 nM) for 30 min, prior to challenge with LPS (20 ng/ml) for 3 h. A, total RNA was isolated and RT-PCR was performed using primers specific for the IL-1\(\beta\) or the glyceraldehyde-3-phosphate dehydrogenase (\textit{gapdh}) genes. The products were visualized on agarose gel by ethidium bromide staining. The data shown are representative of results obtained from three independent experiments. For electrophoretic mobility shift assays (EMSA), the nuclear extracts were subjected to EMSA using labeled IL-1\(\beta\)-specific NF-\(\kappa\)B (B and C) or C/EBP\(\beta\) (D and E) DNA probes. Where required, the binding reaction was performed in the presence of mutated oligonucleotides or supershift antibodies as indicated. The arrowheads indicate the position of supershifted bands. Gels are representative of results obtained from three independent experiments.
cytokine gene induction in macrophages, we evaluated the role of TLR signal transducers in the regulation of IL-1β gene expression and IL-1β release by H. pylori LPS. Pretreatment of cells with neutralizing antibodies against TLR4/MyD88 abrogated LPS-stimulated release of IL-1β. Transfection of cells along with empty vectors or PAK1 (Kd) or IKKα/β (dn) as indicated. In each case cells were cotransfected with β-galactosidase reporter plasmid and luciferase activities were normalized to β-galactosidase activity from contransfection. Where indicated cells cotransfected with luciferase construct and β-galactosidase reporter plasmid were treated with Akt inhibitor for 1 h or with neutralizing anti-TLR4 antibody. Transfected cells were then treated with LPS for 3 h, lysed, and luciferase as well as β-galactosidase activities were measured. Fold activation refers to the increase in luciferase activity over that found in cells transfected with the reporter and a control plasmid without any treatment. A, IL-1β promoter region (−356/−12)-luciferase (C1); B, IL-1β promoter region (−356/−131)-luciferase (C2); C, IL-1β promoter region (−131/−12)-luciferase (C3). The data represent the means ± S.D. of three separate experiments. Ac-YVAD-AFC, N-acetyl-Tyr-Val-Ala-Asp-(7-amino 4-trifluoromethylcoumarin); PBD, p21-binding domain.

We demonstrated that C/EBPβ and p38 MAPK interact in vivo and that GST-C/EBPβ can immunoprecipitate p38MAPK in its phosphorylated form from H. pylori LPS-challenged THP1 cell lysates. p38 MAPK has indeed been found to phosphorylate C/EBPβ in H. pylori LPS-challenged THP-1 cells.2

Whether PAK1 signaling is dependent or independent of IKKβ-mediated IκBα phosphorylation is presently under investigation. Expression of active PAK1 in macrophages stimulates the nuclear translocation of the p65 subunit of NF-κB without activation of IKKs (43). Whether stimulation of macro-

FIG. 6. H. pylori LPS-driven interaction between p38 MAPK and C/EBPβ. A, cells were left untreated (lane a) or treated with H. pylori LPS (lanes b and c) followed by immunoprecipitation with anti-C/EBPβ antibody. The immunoprecipitate was blotted with anti-p38 MAPK antibody. Negative control for the immunoprecipitation was performed in lysates from treated cells using anti-isotype IgG. As a positive control, p38 MAPK was immunoprecipitated from the cell lysate, followed by blotting with anti-p38 MAPK antibody (lane d). Reprobing was performed with anti-C/EBPβ antibody as a control for equal loading on the gels. B, cell lysates obtained after treatment without (lane b) or with H. pylori LPS (lanes a–d) were incubated with glutathione-Sepharose (lane a, negative control) or C/EBPβ bound to glutathione-Sepharose (C/EBPβ-GST) (lanes b–d). Resin-bound proteins were then subjected to Western analysis using anti-p38 MAPK antibody. Reprobing of the blot was carried out with anti-GST antibody. The immunoprecipitate was blotted with anti-p38 MAPK antibody (lane a) or treated with H. pylori LPS (lanes b–d) followed by immunoprecipitation with anti-phospho-p38 MAPK coupled to agarose (lanes b and c) or with an irrelevant protein coupled to agarose (lane a). The agarose-bound proteins were then subjected to Western analysis with anti-C/EBPβ antibody. Lane d is a positive control where C/EBPβ was detected by immunoblotting of the cell lysate using anti-C/EBPβ antibody.

FIG. 7. H. pylori LPS drives luciferase expression from luciferase constructs containing the IL-1β gene-specific promoter. The IL-1β promoter luciferase reporter constructs were transfected into THP1 cells along with empty vectors or PAK1 (Kd) or IKKα/β (dn) as indicated. In each case cells were cotransfected with β-galactosidase reporter plasmid and luciferase activities were normalized to β-galactosidase activity from contransfection. Where indicated cells cotransfected with luciferase construct and β-galactosidase reporter plasmid were treated with Akt inhibitor for 1 h or with neutralizing anti-TLR4 antibody. Transfected cells were then treated with LPS for 3 h, lysed, and luciferase as well as β-galactosidase activities were measured. Fold activation refers to the increase in luciferase activity over that found in cells transfected with the reporter and a control plasmid without any treatment. A, IL-1β promoter region (−356/−12)-luciferase (C1); B, IL-1β promoter region (−356/−131)-luciferase (C2); C, IL-1β promoter region (−131/−12)-luciferase (C3). The data represent the means ± S.D. of three separate experiments. Ac-YVAD-AFC, N-acetyl-Tyr-Val-Ala-Asp-(7-amino 4-trifluoromethylcoumarin); PBD, p21-binding domain.
phages with H. pylori is associated with PAK1-dependent, IKK-independent nuclear translocation of p65, is also presently under investigation.

Our study does not suggest that the signaling pathways described here provide a complete scheme of regulation of IL-1β gene expression. Luciferase reporter plasmids harboring the IL-1β gene-specific NF-κB- and C/EBPβ-binding elements showed that inhibiting either the NF-κB or the C/EBPβ activation pathways described here, led to a partial inhibition of luciferase expression. On the other hand, IL-1β release was brought down almost to control levels when either of these pathways was inhibited. Thus, the endogenous IL-1β promoter locus is likely to be controlled by additional mechanisms such as nucleosome remodeling. Nevertheless, we demonstrate that TLR4/MyD88 signaling leads to activation of the classical IRAK/TRAF6/IKK pathway as well as to activation of PI-3K both of which are necessary for IL-1β release. LPS-mediated signaling drives IL-1β gene expression by activation of both NF-κB and C/EBPβ. The TLR4/MyD88/PI-3K signaling axis is critical in LPS-mediated IL-1β secretion It controls Rac1/PAK1-associated NF-κB activation and Akt/p38 MAPK-associated C/EBPβ activation.

Most interestingly, we observed that PAK1 regulates mature IL-1β production by activating caspase-1. We demonstrated that following H. pylori LPS challenge, PAK1 associates with caspase-1 in vivo. In addition, recombinant caspase-1 was able to immunoprecipitate PAK1 from H. pylori LPS-treated cell lysates. This association was inhibited in cells transfected with dn-Rac1. Cells transfected with dn-PAK1 failed to show H. pylori LPS-induced activation of caspase-1. To the best of our knowledge, we demonstrate for the first time that in addition to its role in NF-κB activation, Rac1/PAK1 signaling is upstream of caspase-1 activation. Our studies provide evidence of a novel role of PAK1-mediated phosphorylation of S376 of caspase-1 in its activation. Caspase-1 activation has been reported to be mediated by formation of a molecular platform known as the inflammasome comprising caspase-1, PyCARD/Asc and NALP1 (50). However, the molecular events triggering assembly of the inflammasome remain unclear. Our observations suggest that Rac1/PAK1 signaling is likely to be involved upstream of inflammasome assembly. We have also observed that recombinant, constitutively active PAK1 can phosphorylate recombinant caspase-1 in vitro. The significance of PAK1-mediated caspase-1 phosphorylation is under active investigation.

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NF-κB- and C/EBPβ-driven Interleukin-1β Gene Expression and PAK1-mediated Caspase-1 Activation Play Essential Roles in Interleukin-1β Release from Helicobacter pylori Lipopolysaccharide-stimulated Macrophages
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