p21-activated Kinase 1 Activates the Nuclear Factor κB (NF-κB)-inducing Kinase-IκB Kinases NF-κB Pathway and Proinflammatory Cytokines in Helicobacter pylori Infection*

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Helicobacter pylori, the causative agent of several human gastric diseases, induces activation of the immediate early response transcription factor nuclear factor κB (NF-κB), which subsequently triggers release of proinflammatory cytokines in colonized epithelial cells. Here we report that in H. pylori infection p21-activated kinase 1 (PAK1) activates NF-κB. Activated PAK1 associates with NF-κB-inducing kinase, which upon activation directs the activity of IκB kinases to IκBo. Our results indicate that in epithelial cells PAK1 participates in a unique pathway that links H. pylori-dependent effector molecules to the activation of NF-κB and the induction of the innate immune response.

Exposure of cells to various stimuli results in phosphorylation, ubiquitination, and subsequent degradation of IκB molecules. The liberated nuclear factor κB (NF-κB) dimers are translocated to the nucleus, where they activate transcription of target genes (1). Key components of the intracellular signal transduction pathways regulating NF-κB activation are represented by NIK (2) and the IκB kinases (IKKα) (3). IKKα and IKKβ are the catalytic subunits of a protein kinase complex that phosphorylates IκB molecules (4). IKKγ (or NF-κB essential modulator (NEMO)) represents the regulatory subunit (5, 6).

We have studied the mechanism of NF-κB activation in response to human pathogenic Helicobacter pylori in epithelial cells where NF-κB, one of the main activators of the inflammatory response, triggers the induction of immune function including cytokine/chemokine production, growth control, and apoptosis (1). The epithelial cytokine/chemokine response is particularly important in the early stages of H. pylori-induced inflammation and is often followed by diseases like gastritis, peptic ulcer (7), gastric cancer (rarely), and low grade B-cell mucosa-associated lymphoid tissue gastric lymphoma (8).

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1 The abbreviations used are: NF-κB, nuclear factor κB; NIK, NF-κB-inducing kinase; IKK, IκB kinase; NEMO, NF-κB essential modulator; PAI, pathogenicity island; MOI, multiplicities of infection; TNF, tumor necrosis factor; PAK, p21-activated kinase; SDSL-PAGE, SDS-polyacrylamide gel electrophoresis; MEEKK1, mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (ERK) kinase kinase 1; JNK, c-Jun NH₂-terminal kinase.

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reaction was carried out by polymerase chain reaction with the primers as described previously (19). A sub saturating number of cycles allowed a semi-quantitative analysis within the infection kinetics. For inhibition of IkBa degradation by the 26 S proteasome, the cells were preincubated for 60 min using 10 μM lactacystin (Affiniti) before the bacteria were added. Polymerase chain reaction products were visualized by ethidium bromide staining after agarose gel electrophoresis.

**Transient Transfections and Reporter Assays—**Transactivating activity of NF-κB was analyzed in 293 or HeLa cells by cotransfection of a luciferase expression plasmid (400 ng) containing three repeats of the NF-κB human immunodeficiency virus–binding site and expression constructs using cationic liposomes (DAC-30, Eurogentec). 16 h after transfection the cells were infected with *H. pylori*, treated with 10 ng ml−1 tumor necrosis factor α (TNFα) (Promega), or left untreated. Luciferase assays were performed 3–4 h after treatment as recommended by the manufacturer’s instructions (Promega). The data presented are representative of more than three independent experiments. A PSE-β-galactosidase vector (Promega) was used for normalization of transfection efficiency. The results were recorded on a Wallac 405 B-counter (Berthold-Wallac) and given as fold induction or as percent induction compared with the control.

**Immunoprecipitation and Protein Kinase Assays—**From the radiom immune precipitation buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 2.5% glycerol, 1 mM EGTA, 50 mM NaF, 1 mM Na3VO4, 10 μM Na2OPO4, 100 μM phenylmethylsulfonyl fluoride, 10 μM pepstatin A, and 4 μM aprotonin) lysed cell immunoprecipitations were performed with anti-α-Pak (sc-881, Santa Cruz Biotechnology), anti-IKKα (Pharmingen), anti-IKKβ (BIO SOURCE), anti-Ita (sc-805, Santa Cruz Biotechnology), anti-Vsv (Roche Molecular Biochemicals), anti-Myc (Pharmingen), or anti-Flag (Sigma) antibodies. The immunoprecipitated and co-immunoprecipitated proteins were separated via SDS-PAGE and blotted as described previously (18). Immunodetection was achieved with antibodies as indicated (anti-α-Pak1, sc-882, Santa Cruz Biotechnology; anti-IκBα, sc-847, Santa Cruz Biotechnology; anti-NEMO (BIO SOURCE), etc.). For *in vitro* kinase reactions, immunocomplexes were recovered, washed, and incubated with the kinase buffer (100 mM KCl, 0.1 mM CaCl2, 6 mM MgCl2, 30 mM Tris, pH 7.5, 0.1 mM Na3VO4, 1 μM dithiotreitol, 10 μCi of [γ-32P]ATP) using 2.5 μg of myelin basic protein (Upstate Biotechnology) and 0.5 μg of IκBα (18) as substrates for PAK1 and IKKs, respectively. The samples were separated via SDS-PAGE and dried, and phosphorylation was visualized by autoradiography. Equal amounts of the total lysates were analyzed in an immunoblot to indicate equivalent protein amounts in all lanes. *In vitro* translation was performed using a TNT kit (Promega), and coimmunoprecipitation was performed with translation reactions diluted in phosphate-buffered saline.

**RESULTS**

**NF-κB Activation in *H. pylori* Infection Involves IKK Activity—**AGS cells (neuroendocrine differentiated gastric carcinoma cells) infected with *H. pylori* or treated with TNFα for various periods of times, fractionated into cytoplasmic and nuclear components, and subjected to electrophoretic mobility shift assay showed an equivalent increase of NF-κB DNA binding activity within 90 and 10 min, respectively (Fig. 1a, lanes 1–5). The *H. pylori*-induced binding activity was strongly reduced and partially super-shifted when anti-p50 or anti-p65 antibodies were used, whereas an anti-c-Rel antiserum or preimmune serum did not show a reduction (Fig. 1a, lanes 10–15). The specificity of the DNA binding activity was examined by adding increasing amounts of the non-labeled double-stranded IκB oligonucleotide for competition (Fig. 1a, lanes 14–16). In contrast to the wild type *H. pylori* strain, an isogenic mutant strain (PAI, no expression of the type IV secretion machinery) did not show NF-κB activation (Fig. 1a, lanes 17–19). This experiment excludes a role for lipopolysaccharide in *H. pylori*-induced NF-κB activation in epithelial cells. To determine whether NF-κB activation is sustained at later stages of the infection, we studied *H. pylori* infection up to 6 h after infection. Maximal DNA binding of NF-κB was observed between 90 and 180 min after infection and was not detectable later than 240 min after infection (Fig. 1a, lanes 20–22). *De novo* protein synthesis was not required for *H. pylori*-induced NF-κB activation (data not shown). Furthermore, *H. pylori* infection or TNFα stimulation led to a rapid loss of IκBα as analyzed in an immunoblot (Fig. 1b). To assess changes in cytokine gene expression, AGS cells were challenged to infection with *H. pylori*. Cytokine mRNA expression was determined at different time points after infection by reverse transcriptase-polymerase chain reaction of total RNA prepared from infected cells. The cytokine mRNA levels were compared with the constitutive β-actin mRNA in the same polymerase chain reactions. Infection with *H. pylori* led to an increased synthesis of granulocyte-macrophage colony-stimulating factor (upper panel), interleukin-8 (middle panel), and TNFα (lower panel) in response to *H. pylori* in the absence (lanes 1–4) or presence (lanes 5–8) of 10 μM lactacystin. H3, *H. pylori*.

![Fig. 1. Induction of cytokine genes and activation of NF-κB in *H. pylori*-colonized AGS cells. a, NF-κB activation. Electrophoretic mobility shift assay of AGS cells colonized by *H. pylori* wild type or PAI strains (MOI 50) (lanes 1–8 and 17–22) or of TNFα-treated cells (10 ng ml−1) (lanes 7–9) at the indicated periods of time; antibody supershifting of the NF-κB DNA complex (lanes 10–13) or cold oligonucleotide competition (5, 10, or 50 ng) (lanes 14–16). pS, preimmune serum; ns, nonspecific complex. b, IκBα degradation. Immunoblot of *H. pylori*-infected AGS cells using anti-IκBα antibody (sc-847, Santa Cruz Biotechnology). c, induction of cytokine mRNAs. Duplex reverse transcriptase-polymerase chain reaction of the cytokine genes granulocyte-macrophage colony-stimulating factor (upper panel), interleukin-8 (middle panel), and TNFα (lower panel) in response to *H. pylori* in the absence (lanes 1–4) or presence (lanes 5–8) of 10 μM lactacystin. H3, *H. pylori*.](image-url)
FIG. 2. H. pylori-induced NF-κB activation involves the IKK complex. a and b, H. pylori induces IKKα and IKKβ. AGS cells were infected with H. pylori (MOI 50) or treated with TNFα for the indicated time periods. Endogenous IKKα (a) or IKKβ (b) was immunoprecipitated from lysed cells, and their kinase activity was determined using IκBα as substrate. Autoradiographs of SDS-PAGE are shown. IP, immunoprecipitation; IB, immunoblot. c and d, kinase-inactive IKKβ blocks the H. pylori-induced transcriptional activity of NF-κB. Relative luciferase activity in 293 cells infected with H. pylori (MOI 50) or TNFα (10 ng ml
−1) (c) or co-transfected with Iκκ reporter plasmid and kinase-inactive IKKβ(K44A) or MKK6(K116R) (d) and expression vectors, as indicated. The results are representative of at least three independent experiments. Data are expressed as fold-activation compared with non-treated cells or as percent induction. Hp, H. pylori.

Pacifically in an in vitro kinase assay using IκBα as a substrate. H. pylori infection as well as TNFα treatment of cells resulted in increased IKKα (Fig. 2a, lanes 1–8, upper panel) and IKKβ (Fig. 2b, lanes 1–8, upper panel) kinase activity on the substrate IκBα. Compared with H. pylori-infected cells, TNFα-treated cells induced IKKα and IKKβ faster in the analyzed time course as expected. Immunoblot analysis confirmed the presence of similar quantities of IKK proteins in each of the extracts used for immunoprecipitation (Fig. 2, a and b, lower panels).

To analyze the ability of H. pylori to induce NF-κB transactivation activity, we tested the effects of activation of H. pylori on the expression of an NF-κB-dependent reporter gene in transiently transfected 293 cells. H. pylori infection (MOI 50) or TNFα treatment of cells increased transcription of the reporter gene (Fig. 2c). When we tested the effect of a kinase-inactive IKKβ(K44A) construct on the expression of an NF-κB-dependent reporter gene in transiently transfected and H. pylori-infected or TNFα-treated 293 cells, we observed suppression of NF-κB activation. This indicates that H. pylori-induced NF-κB activation involves IKKβ (Fig. 2d). For a control, we analyzed the effect of kinase-inactive MAP kinase kinase kinase (MKK4(416)) on the H. pylori-induced NF-κB activation, which was not affected.

PAK1 Activates NIK and the IKK Complex—Several kinases (e.g., NIK, mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (ERK) kinase 1 (MEKK1), IKK-1, TANK-binding kinase 1 (TNF-α-activating kinase, mixed-lineage kinase 3) have been shown to be signaling intermediates that act as direct activators of the IKK complex (2, 20–24). The cellular selection of the kinases might be dependent on cell type specificity and distinct extracellular stimuli. To study the H. pylori-induced upstream kinase involved in activation of the IKK complex, we analyzed the role of NIK and the MEKK1 in H. pylori-induced expression of an NF-κB-dependent reporter gene in transiently transfected 293 cells. H. pylori- or TNFα-stimulated NF-κB-dependent reporter gene activity was suppressed when kinase-inactive mutants of NIK (NIK(624–947); NIK(K429A,K430A)) were expressed (Fig. 3a).

In contrast to kinase-inactive NIK, the kinase-inactive MEKK1(K432M) did not significantly block H. pylori-induced NF-κB activation (Fig. 3a), whereas phorbol 12-myristate 13-acetate-induced NF-κB activation was affected by MEKK1(K432M) (data not shown). These results suggest that H. pylori-induced NF-κB activation involves NIK but not MEKK1 in this kinase cascade and exclude a functional cooperation between those kinases in the activation of IKK activity. Compelling evidence that NIK is involved in the activation of NF-κB has been shown in response to CD40 and CD3/CD28 induction (25, 26).

Our experimental evidence that the MAPK kinase kinase NIK represents a crucial factor in H. pylori-directed activation of the IKK complex led us to search for putative upstream regulators of NIK. Colinear amino acid sequence alignment of the kinase domain in NIK revealed homology to MEKK1 and Ste11 apart from other kinases, and Ste11 represents a downstream kinase of Ste20, the yeast homologue of mammalian PAK1 (27). Therefore, we examined whether PAK1, which is a stress response kinase of the MAPK kinase kinase kinase level, represents an upstream kinase for NIK. In AGS cells the activity of PAK1 was induced severalfold in response to pathophysiological stress raised by H. pylori infection. The kinase activity of immunoprecipitated PAK1 was measured with myelin basic protein as a substrate (Fig. 3b, lanes 1–4, upper panel). TNFα weakly induced PAK1 (Fig. 3b, lanes 5–7, upper panel). Immunoblot analysis confirmed the presence of similar quantities of PAK1 in each of the extracts used for immunoprecipitation (Fig. 3b, lower panel).

To study the mechanism of NF-κB activation, we analyzed whether PAK1 affects the H. pylori-induced NF-κB-dependent transcriptional activity. Kinase-inactive PAK1(K299R) blocked the expression of an NF-κB-dependent reporter gene in transiently transfected and H. pylori-infected 293 cells in a dose-dependent manner (Fig. 3c). In contrast to H. pylori infection, kinase-inactive PAK1(K299R) only slightly affected the NF-κB transactivation activity in TNFα-treated cells (Fig. 3c). To exclude possible effects on the putative upstream components of PAK1 due to titration of Rho-GTPases, we performed experiments with a PAK1(K299R) construct mutated in histidine 83 and 86, which prevents the binding of Rho-GTPases. This construct clearly blocked H. pylori-induced NF-κB activation. Furthermore, we used a PAK1 construct containing the residues 83–149. PAK1(83–149) has been demonstrated to inhibit the autophosphorylation of PAK1 by blocking a critical phosphoacceptor site that is required for its kinase activity and does not bind to Rac1 (28). Overexpression of this construct blocked H. pylori-induced activation of NF-κB in a dose-dependent manner (Fig. 3c). For a control, we transiently transfected a PAK1(83–149) construct with the leucine 107 to phenylalanine mutation (PAK1(83–149.L107F)), which inactivates the autoinhibitory domain of PAK1. This construct did not block H. pylori-induced NF-κB activation. To determine the signaling pathways that couple PAK1 to NF-κB activation, we tested
whether kinase-inactive NIK(K429A,K430A) could block signaling from constitutive active PAK1(L107F) in transiently transfected HeLa cells. The inactive NIK mutant NIK(K429A,K430A) blocked the activation of NF-κB-dependent gene expression by PAK1(L107F), whereas overexpression of inactive PAK1 (PAK1(K299R) and PAK1(H83L,H86L,K299R)) had little effect on NIK-induced activation of NF-κB (Fig. 3d). Our results raise the possibility that PAK1 may function as a coactivator of NIK to mediate NF-κB activation in PAK1 expression.

To identify NIK as a potential downstream component of PAK1 in H. pylori infection, we studied the interaction of these kinases. AGS or HeLa cells were transfected with Ha-tagged PAK1 constructs or Flag-tagged NIK constructs. The kinases were then immunoprecipitated. The immunoprecipitates were subjected to SDS-PAGE and analyzed in an immunoblot with the indicated antibodies. We observed interaction of Ha-PAK1(K299R) with Flag-NIK (Fig. 4a, lane 1), high affinity interaction between Ha-PAK1(K299R) and Flag-NIK(K429A,K430A) (lane 2), and interaction of Ha-PAK1 with Flag-NIK(K429A,K430A) (lane 3). We also performed these experiments with constitutive active Ha-PAK1(L107F), which resulted in a detectable interaction with Flag-NIK(K429A,K430A) (lane 4). These results indicate that kinase-active PAK1 could target the MAPK kinase NIK. In contrast to the PAK1/NIK interaction, we observed no direct interaction between PAK1 and the IKK complex components IKKα, IKKβ, or NEMO/IKKγ (Fig. 4a, b and c). Appropriate controls show that NIK interacted with IKKα (Fig. 4b) and that IKKγ interacted with NEMO/IKKγ (Fig. 4c). Similar to the kinase-inactive PAK1(K299R), the constitutively active PAK1(L107F) did not interact with an IKK subunit (data not shown).

To analyze endogenous PAK1/NIK interaction, we tried to derive HeLa cells stably expressing an epitope-tagged version of NIK. Unfortunately, cells which had integrated the NIK wild type cDNA did not survive. To circumvent this problem we transiently transfected small amounts of kinase-inactive Flag-NIK(K429A,K430A) in a titration experiment and determined an amount that still allowed interaction with activated PAK1 induced in H. pylori infection. Interestingly, H. pylori infection caused an inducible interaction of the endogenous PAK1 and the kinase-inactive Flag-NIK(K429A,K430A) (Fig. 4d, lanes 2–4, upper panel), whereas the non-stimulated or TNFα-treated cells exhibited no PAK1/NIK interaction (lanes 1, 5, and 6, upper panel). Immunoblot analysis confirmed the presence of similar quantities of Flag-NIK and endogenous PAK1 in each of the extracts used for immunoprecipitation (Fig. 4d, middle and lower panels). To study whether this interaction is direct, we carried out an in vitro analysis using [35S]labeled proteins translated in wheat germ extracts. After cotranslation of Flag-NIK and HaPAK1 (Fig. 4e, lane 1) followed by anti-Flag immunoprecipitation (lane 4), we detected PAK1 in the immunoprecipitate. The converse experiment of immunoprecipitation with an anti-Ha antibody allowed the detection of Flag-NIK in the immunoprecipitate (data not shown).

Our results raised the possibility that PAK1 could function as the kinase that activates NIK in H. pylori infection. The analysis of H. pylori-infected cells, which were transfected with kinase-inactive NIK(K429A,K430A), resulted in the occurrence of an additional NIK form with a slightly decreased mobility in SDS-PAGE as detected in an immunoblot (Fig. 5a, lane 2). When NIK(K429A,K430A) was expressed without H. pylori infection, this slower migrating form of NIK was weakly visible (Fig. 5a, lane 1). Cells cotransfected with constitutive active PAK1(L107F) caused an accumulation of the more slowly mi-
PAK1 Directs NF-κB Activation

The text describes experiments and findings related to the role of PAK1 in the activation of NF-κB. PAK1 binds NIK, and this interaction is critical for NF-κB activation. The text also discusses the importance of PAK1 in response to infections, particularly by H. pylori, and its role in the innate immune response. The text outlines experimental procedures, including immunoprecipitation and kinase assays, to support these findings. The discussion section highlights the significance of these results in the context of NF-κB signaling and infection by H. pylori.
PAK1 Directs NF-κB Activation

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FIG. 5. PAK1 phosphorylates NIK. a, effect of PAK1 on phosphorylation of NIK. HeLa cells were transfected with Flag-NIK(K429A, K430A) (lane 3) or co-transfected with constitutive active PAK1(L107F) (lane 4). The identity of the phosphoprotein was determined by Western blotting. b, effect of PAK1 on phosphorylation of NIK. HeLa cells were transfected with NIK and stimulated with TNF, co-transfected with PAK1(K299R) (lane 3); cells were also infected with H. pylori (lane 4) or additionally co-transfected with PAK1(K299R) (lane 5) or NIK(K429A, K430A) (lane 6). The immunoprecipitations were performed using an anti-Vsv antibody, and the immunocomplex kinase reactions were analyzed by SDS-PAGE and autoradiography. The phosphorylated IKKα (IKKα-P) is indicated (top panel). The other panels show the immunoblot analysis, which presents the presence of similar quantities of Vsv-IKKα, Flag-NIK, and HA-PAK1 in each of the extracts used for immunoprecipitation. IP, immunoprecipitation; IB, immunoblot.

FIG. 6. Model for the role of PAK1 in the activation of the NIK-IKK NF-κB pathway and the induction of the innate immune response in H. pylori infection. See "Results".

A simultaneous interaction of negative regulatory factor with the SH3 domain of Vav, which induces its guanine nucleotide exchange factor activity for Cdc42 and Rac1. The activation of Rac1 and Cdc42 leads to their subsequent dissociation from Vav and strongly increases their affinity for PAK1 (40).

Based on these data, the PAK kinases in mammals can be divided into two subfamilies: the PAK subfamily, containing an NH2-terminal catalytic p21-binding domain (also known as cdc42/Rac1-interactive binding domain) and a COOH-terminal kinase domain, and the germinal center kinase-like subfamily, which contains an NH2-terminal catalytic domain and lacks the p21-binding domain. In the first description of PAK it was shown that the serine/threonine protein kinase activity of PAK could be stimulated by the binding of activated GTP-bound Rac1 and Cdc42. Recently, a variety of studies have suggested that PAKs can participate in a broad range of cellular events that include cytokines and other inflammatory factors during innate immune response. The identity of the effector molecule(s) of H. pylori that induce(s) NF-κB is not known so far. We speculate that PAK1 becomes activated directly or indirectly by H. pylori factor(s). The identification of the H. pylori factor(s) in this process of NF-κB activation will unravel the mechanism of the H. pylori-induced signaling in the future. Interestingly, the viral protein negative regulatory factor from human simian immunodeficiency virus targets PAK1 and induces nuclear responses. The negative regulatory factor-induced mechanism of PAK1 activation involves the simultaneous interaction of negative regulatory factor with the SH3 domain of Vav, which induces its guanine nucleotide exchange factor activity for Cdc42 and Rac1. The activation of Rac1 and Cdc42 leads to their subsequent dissociation from Vav and strongly increases their affinity for PAK1 (40).
