Coordinate Activation of Activator Protein 1 and Inflammatory Cytokines in Response to *Neisseria gonorrhoeae* Epithelial Cell Contact Involves Stress Response Kinases

By Michael Naumann,* Thomas Rudel,* Björn Wieland,* Cornelia Bartsch,* and Thomas F. Meyer*‡

*From the *Max-Planck-Institut für Infektionsbiologie, Abteilung Molekulare Biologie, 10117 Berlin, Germany; and ‡Max-Planck-Institut für Biologie, Abteilung Infektionsbiologie, 72076 Tübingen, Germany*

Summary

Neisseria gonorrhoeae (*Ngo*), the etiologic agent of gonorrhea, induce a number of proinflammatory cytokines by contact to epithelial cells. Cytokine genes and a variety of other immune response genes are activated as a result of the regulatory function of immediate early response transcription factors including activator protein 1 (AP-1). Since it is established that phosphorylation of c-Jun, the central component of AP-1, by the stress-activated c-Jun NH_2-terminal kinase (JNK) increases the transcriptional activity of AP-1, we studied whether *Ngo* could induce stress response pathways involving JNK. We found that virulent *Ngo* strains induce phosphorylation and activation of JNK but not of p38 kinase. Analysis of a nonpathogenic *Ngo* strain revealed only weak JNK activation. In respect to the molecular components upstream of the JNK signaling cascade, we show that a dominant negative mutant of MAP kinase kinase 4 (MKK4) represses transcription of an AP-1–dependent reporter gene. Regarding upstream stress response factors involved in *Ngo*-induced MKK4/JNK/AP-1 activation, we identified p21-activated kinase (PAK) but not MAPK/ERK kinase kinase (MEKK1). Inhibition of small GTPases including Rac1 and Cdc42 by Toxin B prevented JNK and AP-1 activation. Our results indicate that *Ngo* induce the activation of proinflammatory cytokines via a cascade of cellular stress response kinases involving PAK, which directs the signal from the Rho family of small GTPases to JNK/AP-1 activation.

Key words: *Neisseria* • inflammation • activator protein 1 • c-Jun NH_2-terminal kinase • p21-activated kinase

The production of immunostimulatory cytokines by epithelial cells is an essential part of the biological response to the infection with human pathogenic microorganisms (1). Any inflammatory reaction requires the de novo synthesis of defined proteins. These include chemokines that attract macrophages and inflammatory cytokines that serve to amplify and spread the primary pathogenic signal. The mechanism by which these proteins are newly synthesized involves an inducible transcriptional initiation of their respective genes. This is governed by several transcription factors playing a role in regulating immune response genes including the early transcription factor activator protein 1 (AP-1; reference 2).[1]

Human pathogenic *Neisseria gonorrhoeae* (*Ngo*) induce a number of cytokine genes in epithelial cells involving the activity of the transcription factor AP-1 (3). The natural host benefit of cytokine release in response to pathogen infection is exploited by *Ngo* which cause a massive localized inflammation characterized by the infiltration of polymorphonuclear leukocytes and mononuclear phagocytes into the infected tissue of the urethra or the endocervix (4). As a consequence, the infected epithelial cell layer is destroyed and the bacteria may gain better access to deeper tissues (5). The colonization of the epithelia is a prerequisite of infection by *Ngo* and is largely dependent on the expression of a set of variable surface proteins. The hair-like type 4 pili

[1]*Abbreviations used in this paper:* AP-1, activator protein 1; ASM, acidic sphingomyelinase; CRIB, Cdc42/Rac interactive binding; DAG, diacylglycerol; DN, dominant negative; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; HSP, heparan-sulfate proteoglycan; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAP/ERK; MEKK, MEK kinase; MKK, MAPK kinase; MKKK, MKK kinase; MOI, multiplicities of infection; NF-κB, nuclear factor κB; *Ngo*, *Neisseria gonorrhoeae*; PAK, p21-activated kinase; PLC, phosphatidylcholine–dependent phospholipase C; SAPK, stress-activated protein kinase.

mediate the first contact between bacteria and host cells and are indispensable for infection in vivo (6, 7). Variant outer membrane proteins of the opacity-associated outer membrane protein family (Opa proteins) tightly interact with the host cell surface and trigger the engulfment of *Ngo* (8). There are at least three independent routes by which opa proteins induce the uptake of *Ngo* into host cells each mediated by interaction with a certain receptor. Heparan-sulfate proteoglycan (HSP) receptors (9) or the concerted action of HSPR and integrin receptors (10, 11) account for the major pathways for uptake into epithelial cells. Uptake of *Ngo* by the HSPR pathway involves the action of phosphatidylcholine-dependent phospholipase C (PLC) and the acidic sphingomyelinase (ASM; reference 12), the HSPR–integrin pathway possibly PKC (11). In phagocytic cells, Opa proteins occupy receptors of the CD66 family that results in a rapid, opsonin-independent phagocytosis of *Ngo* (13–15) involving Src kinases and Rac1 (16).

In contrast to the well-studied *Neisseria* adherence and invasion process, very little is known about the nature of the pathogen-induced proinflammatory signals. Our previous results showed that the infection of epithelial cells with gonococci induced the activation of the transcription factor AP-1 (3). In line with the known immunostimulatory function of AP-1 and nuclear factor κB (NF-κB; references 17, 18) epithelial cells infected with *Ngo* produced increased amounts of numerous proinflammatory cytokines (3). The secretion of cytokines in the cultured epithelial cell model was not initiated by LPS suggesting that the endotoxin-mediated mechanisms known to trigger inflammatory reactions of lymphoid cells are not relevant for epithelial cells (3). However, neither the signals nor the signaling pathways activating AP-1, which lead to production of cytokines in *Ngo*-infected epithelial cells, are defined.

Environmental stress or engagement of cytokine receptors leads to the activation of AP-1 and a family of mitogen-activated protein kinases (MAPK), which consecutively activate their members by phosphorylation (18, 19). In such a cascade, the AP-1 subunit c-Jun is activated by the c-Jun NH_2-terminal kinase (JNK); JNK itself is activated by MAPK kinase 4 and 7 (MKK4, MKK7); and MKK4 is activated by MAP/ERK (extracellular signal–regulated kinase) kinase 1 (MEKK1) at the level of MAPK, MKK, and MKK kinase (MKKK), respectively. Numerous MKKK beside MEKK1 have recently been identified, all able to activate the JNK pathway (20–25). The reason why mammalian cells express so many MKKK involved in signaling to stress-activated transcription factors is still unknown. It is still unclear in particular how the MKKK themselves are activated in mammalian cells in response to stress signals or cytokine receptor activation.

The small GTPases Rac1 and Cdc42 have been identified as far upstream regulators of the JNK-kinase cascade (20, 26, 27). The p21-activated kinases (PAKs) were the first kinases identified as direct effectors for the active GTPases Rac1 and Cdc42 (28). GTP-loaded Rac1 or Cdc42 bind to a Cdc42/Rac interactive binding (CRIB) domain (29) and thereby increase the activity of PAK significantly (28). Recently, MEKK1 and MEKK4 have also been shown to interact with Rac and Cdc42 (30). Blocking either PAK or MEKK1 activity prevents the activation of JNK either in response to activated GTPases (30, 31) or to apoptotic stimuli independent of GTPases (32, 33). However, blocking MEKK kinase does not prevent JNK activation by active PAK suggesting that both classes of kinases signal independently.

Here we show that *Ngo* induces the activation of the AP-1 transcription factor by a distinct kinase cascade involving GTPases, PAK, MKK4, and JNK1, but not MEK kinase 1. The identification of pathogen-specific signaling pathways to inflammatory cytokine production might be a basis for the therapeutic intervention of infections.

Materials and Methods

Human Cell Culture and Infection. Epithelial cells (HeLa and Chang) were grown in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS in a humidified 5% CO_2 atmosphere. Epithelial cells were seeded in tissue culture plates for 48 h before infection. 16 h before infection, the medium was replaced by fresh RPMI 1640 medium supplemented with 10% FCS. The epithelial cells were infected with *Ngo* given in multiplicities of infection (MOI) as described in the figure legends for different periods of time. For infection experiments, bacteria were centrifuged onto the epithelial cell monolayer for 5 min at 500 *g*. In the experiments using 10–50 ng/ml Toxin B (supplied by F. Hofmann and K. Aktories, University of Freiburg, Freiburg, Germany), the cells were preincubated for 15 min before the bacteria were added. Stimulation of the cells with 10 ng/ml TNF-α (Promega, Heidelberg, Germany) or 50 nM PMA (Sigma Chemical Co., St. Louis, MO) was performed for the indicated time points.

Bacteria. Three different *Ngo* strains were used for infection of human epithelial cell lines: the nonpiliated, but invasive Opa^+ strain N242, (VP1, P.IA, P^-, Opa_{27}, $Opa_{27.5}$, Opa_{28}, Opa_{29}, Opa_{30}, lipopolysaccharide type L1) has been described (8). The adherent but noninvasive P^+ strain N138 (P^+ $PilE_{F3}$) and the P^- Opa^- control strain N300 ($PilE_{B1}$, *opa*C_{30}::*cat*, pTH7) are derivatives of strain MS11 (34, 35). Gonococci were routinely grown on GC phosphate agar as described previously (36). Strain N242 was also grown in RPMI 1640 containing 25 mM Hepes, pH 7.2, for 2 h at 37°C before infection.

Transfections and Luciferase Reporter Assays. To measure the transactivating activity of AP-1, HeLa cells at 50–70% confluence were cotransfected by cationic liposomes (Promega) with 1 μg of a luciferase expression plasmid containing three repeats of the AP-1 binding site as an enhancer element and expression constructs: dominant negative MEKK1 (K432M), dominant negative (DN) MKK4 (K116R, DNJNKK), DNPAK1 (K299R), PAK2 (K278R) or PAK1 (H83L, H86L, and K299R), or wild-type PAK2 or PAK1. 24 h after transfection, cells were left untreated, infected with *Ngo* strains, treated with 50 nM PMA, or preincubated with Toxin B. Luciferase assays were performed at the indicated time points as recommended by the manufacturer's instructions (Promega). The results were recorded on a Wallac 409 β-counter (Berthold-Wallac, Bad Wildbad, Germany), and given as fold induction against untreated cells. The data presented are representative of more than three separate experiments.

Immunoprecipitation, Immunoblotting, and Protein Kinase Assays. To assess the kinase activity of JNK and p38, cells were lysed in

RIPA buffer as described previously (37). For PAK, cells were lysed in 50 mM Tris, pH 7.5, 1 mM EGTA, 5 mM $MgCl_2$, 1% NP-40, 2.5% glycerol, 150 mM NaCl, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_4$, 50 mM NaF, 100 μM PMSF, 10 μM Pepstatin A, and 4 μM Aprotinin. The cells were disrupted by passing the lysate several times through a 21-gauge syringe. The cell debris was collected by centrifugation and the supernatant was subsequently incubated with appropriate antisera (Santa Cruz Biotechnology, Santa Cruz, CA) coupled to protein A– or G–Sepharose (Amersham Biotechnology Inc., Piscataway, NJ). The antisera were coupled to the protein A or G beads in PBS for 1 h at 4°C. In a first preadsorption step the supernatants were incubated with protein A or G beads alone for 1 h at 4°C. After this preadsorption the supernatants were incubated with the appropriate antisera for 2–3 h at 4°C. Immunocomplexes were recovered and washed several times with RIPA buffer and twice with kinase buffer. Immunoprecipitates of JNK were resuspended in 30 μl of kinase buffer containing 100 mK KCl, 0.1 mM $CaCl_2$, 6 mM $MgCl_2$, 30 mM Tris, pH 7.5, 0.1 mM Na_3VO_4, 10 mM K_2HPO_4, 10 mM NaF, 1 mM DTT, and 1 μg of the substrate (GST–c-Jun for JNK, GST-ATF2 for p38; Santa Cruz Biotechnology). Immunoprecipitates of PAK were resuspended in 30 μl of kinase buffer containing 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM dithiothreitol, 0.1 mM Na_3VO_4, 14 μM ATP, and 1 μg of myelin basic protein (MBP; Sigma Chemical Co.). The kinase reactions were initiated by addition of 10 μCi γ-[^{32}P]ATP (5,000 Ci/mmol; Amersham Pharmacia Biotechnology Inc.). After 30 min incubation at 30°C, reactions were terminated by adding SDS loading buffer. Samples were separated by SDS-PAGE, blotted, and developed as described (38) to show similar protein amounts in all lanes. After immunostaining the blots were analyzed further by autoradiography or PhosphorImager analysis.

Results

Activation of JNK in Response to Ngo–Epithelial Cell Contact. In our previous report, we showed that the early response transcription factor AP-1 and inflammatory cytokine gene expression are coordinately induced in *Ngo* infected epithelial cells (3). Activation of the dimeric sequence specific enhancer factor AP-1 is mediated through phosphorylation of c-Jun by members of the MAPK family. Therefore, we examined whether the JNKs and/or p38, also known as stress activated kinases (39–41) are involved in the signaling leading to *Ngo*-induced AP-1 activation and inflammatory cytokine gene expression. Cell lysates from *Ngo*-infected epithelial cells were used at different time points after infection for immunoprecipitation of the endogenous kinases and solid-phase kinase assays using appropriate substrates were performed.

The *Ngo* strains tested differed with regard to the infection of epithelial cells. Strain N138 (P^+ Opa^-) expresses pili that confer adherence to epithelial cells, but does not express any Opa proteins and therefore is not invasive (42). Strain N242 (Opa^+) enters epithelial cells by binding to heparan sulfate containing surface proteoglycan receptor proteins (8, 9). Strain N300 (P^- Opa^-) is neither piliated, nor does it express Opa proteins (35) and, hence, is a very weak adherent. Infection with *Ngo* strains was routinely monitored by light microscopy.

The activity of JNK1 was induced several fold in subconfluent monolayers of HeLa cells by adherence of *Ngo* strain N138 (P^+ Opa^-; Fig. 1, *top*) within 15 min after infection. The immediate JNK1 induction was followed by a sustained JNK1 kinase activation for at least 90 min. Activation of JNK1 in Chang epithelial cells was at least 10 times weaker than in HeLa cells, probably due to lower adherence levels of *Ngo* to Chang cells. To test whether cellular JNK1 activation is at variance in epithelial cells infected with adherent versus invasive *Ngo* strains, we compared JNK1 kinase activity in HeLa cells and Chang cells infected either with the P^+ or Opa^+ strain. At a MOI of 50, the invasive Opa^+ strain induced a slightly stronger activation of JNK1 activity than the P^+ strain, whereas weak JNK1 activation was observed in cells treated with the nonpathogenic strain N300 (P^- Opa^-) or LPS. The activation of JNK1 in response to *Ngo* was delayed compared to the JNK1 induction in TNF-α–treated cells, but showed a similar potential to induce JNK1 activity. The lower panel shows a Western blot probed with an anti-JNK1 antibody, to show similar protein amounts in all lanes. Weak activation of JNK2 was observed in HeLa cells in response to *Ngo* infection (data not shown). In contrast to JNK1, we did not detect any p38 induction paralleled with JNK1 activation (data not shown).

DNMKK4 Inhibits Ngo-mediated Induction of AP-1. Besides the various stimuli (growth factors, UV light irradiation, cytokines, protein synthesis inhibitors, etc.) that could induce the JNK cascade (19), pathogens represent new po-

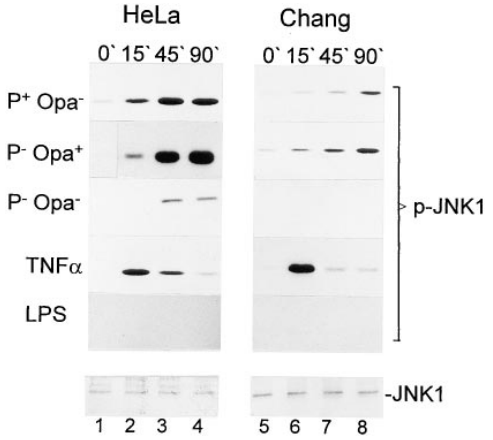

Figure 1. *Ngo*-induced JNK1 activation of epithelial cells. HeLa (lanes *1–4*) or Chang (lanes *5–8*) cells were infected either with different *Ngo* strains (*P^+ Opa^-*, *P^- Opa^+*, and *P^- Opa^-*) at a MOI of 50, or stimulated with 10 ng/ml TNF-α or 10 μg/ml LPS at 37°C. JNK1 was immunoprecipitated with an anti-JNK1 (Santa Cruz, sc-474) antibody and immunocomplex kinase activity (p-JNK1) was determined by phosphorylation of the substrate GST-c-Jun (amino acids 1–79). First, the samples were separated by SDS-PAGE, blotted and the Western blots probed with the anti-JNK1 antibody, to show similar protein amounts in all lanes. One representative Western blot for each cell line is shown (*bottom*). The JNK1 protein is indicated. In the subsequent autoradiography the JNK1 kinase activity (*p-JNK1*) measured by c-Jun substrate phosphorylation is indicated (*top*). The data are representative for at least three independent experiments.

tent inducers in this regard. Thus it is a challenge to understand whether pathogens inducing inflammation use similar or different signaling pathways compared to known stimuli. For JNK, MKK4/SEK1 (stress-activated protein kinase/extracellular-regulated kinase 1 [SAPK/ERK]) has been identified as a direct activator, phosphorylating JNK at threonine and tyrosine residue in the motif TPY (43–45). In addition, recent studies using sek1$^{-/-}$ cells indicated that there are MKK4/SEK1-dependent and MKK4/SEK1-independent signaling pathways for JNK activation (46, 47).

To address the possible role of MKK4 in *Ngo*-induced transcriptional activity of AP-1, we examined the effect of inhibitory DNMKK4 on the transcription of an AP-1–dependent reporter construct (Fig. 2). In transient transfection assays the overexpressed DNMKK4 significantly inhibited P$^+$ Opa$^-$ and P$^-$ Opa$^+$ induced AP-1–dependent reporter gene expression (Fig. 2, *A* and *B*), but not of an NF-κB–dependent reporter gene (data not shown). This is in agreement with the earlier observation that the JNK activation is not linked to NF-κB activation (48, 49). Similarly, PMA induced AP-1 activation was blocked by DNMKK4 (Fig. 2 *D*). *Ngo* strain P$^-$ Opa$^-$ did not induce AP-1 transactivation activity (Fig. 2 *C*). The strong inhibitory effect of DNMKK4 on pathogenic *Ngo* and PMA induced AP-1 activation indicates a major role of MKK4 for the JNK-MAPK signal transduction pathway. DNMKK4 might inhibit *Ngo*-induced signal transduction pathways, in which MKK4 is intimately involved by strongly interacting with its upstream activators and/or its downstream elements.

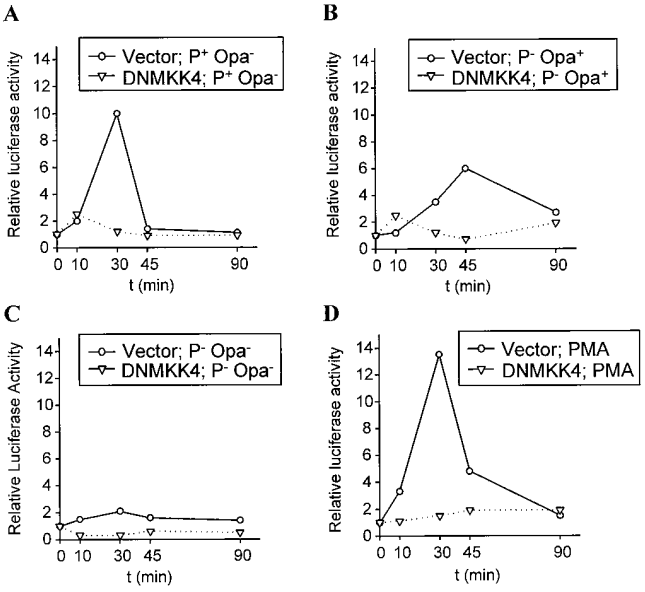

Figure 2. Inhibition of AP-1 transactivation activity by dominant inhibitory MKK4. HeLa cells were transfected with 1 μg reporter construct containing three repeats of the AP-1 binding site and either 1 μg dominant inhibitory MKK4 (*DNMKK4*) or empty vector. After overnight incubation with the DNA, cells were infected with different *Ngo* strains (*P$^+$ Opa$^-$*, *P$^-$ Opa$^+$*, and *P$^-$ Opa$^-$*; *A–C*) at a MOI of 50 or treated with 50 nM PMA (*D*) for the indicated periods of time. The results of a representative experiment are expressed as fold induction compared with the activity observed with transfection of the reporter vector and empty vector, and in the absence of *Ngo* strains or PMA. Similar results were obtained in more than three independent experiments.

Toxin B Inhibits AP-1 and JNK Activation in Ngo-stimulated Cells. Attachment of epithelial cells by *Ngo* suggests the involvement of cytoskeletal rearrangements (36) as shown for *Helicobacter pylori* (50) and other pathogenic microbes (51). Cytoskeletal rearrangements are known to be regulated by small GTPases of the Rho family (52). Therefore, we studied whether GTPases were involved in *Ngo*-induced signaling pathways leading to JNK and AP-1 activation. The GTPases Rac1 and Cdc42 have been identified as important intermediates to the JNK signaling cascades (20, 27, 53). The activity of Rho GTPases but not of Ras is specifically inhibited by enterotoxin Toxin B from *Clostridium difficile*, which glycosylates and inactivates Cdc42, Rac1, and Rho (54, 55). Pretreatment of HeLa cells for 15 min with 10 ng/ml Toxin B is sufficient to reduce AP-1 transactivation activity in the presence of adherent *Ngo* strain (P$^+$ Opa$^-$) to the baseline levels (Fig. 3, compare right and left panel) indicating the involvement of Rho-GTPases in *Ngo*-induced AP-1 activation. As a control we studied the PMA-induced AP-1–dependent reporter activity, which appears not to be affected by Toxin B (Fig. 3). Similarly to AP-1, JNK activation (assayed in an in vitro kinase assay) in response to the adherent *Ngo* strain (P$^+$ Opa$^-$) was inhibited in the presence of Toxin B (Fig. 4, lanes *1* and *3* versus *4* and *6*). Treatment of HeLa cells with Toxin B already induces JNK activity (Fig. 4, lanes *1* and *4*). In contrast to *Ngo*, PMA-stimulated cells could induce JNK activity in Toxin B–treated cells (Fig. 4, lanes *1*, *2*, *4*, and *5*).

AP-1 Activation in Ngo-stimulated Epithelial Cells Involves PAK Signaling. There are potentially many different kinases that could contribute to small GTPase-mediated JNK and AP-1 activation. The most prominent kinases acting downstream of Rho-GTPases are the PAKs (p21-activated kinases) and the MEKK (56). The PAKs show homology to STE20p (28), which mediate specific responses involving Cdc42p in *S. cerevisiae* (57). In analogy to *S. cerevisiae*, it was suggested that PAKs are intermediate in Cdc42/

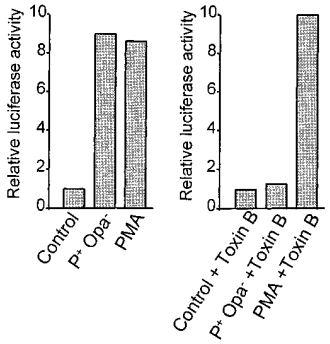

Figure 3. Toxin B inhibits *Ngo*-stimulated AP-1 activation. HeLa cells were transfected with 1 μg reporter construct and after overnight incubation, cells were treated with 10 ng/ml Toxin B 15 min before the infection with *Ngo* strain P$^+$ Opa$^-$ at a MOI of 50, or with 50 nM PMA for 30 min, respectively (*right*). As a control, cells were left without Toxin B (*left*). The results from a representative experiment are expressed as fold induction compared with the activity observed with transfection of the reporter vector and in the absence of Toxin B, *Ngo* or PMA. Similar results were obtained in more than three independent experiments.

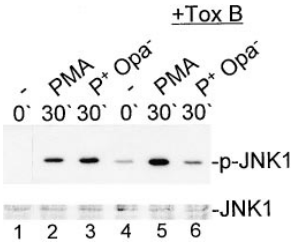

Figure 4. Toxin B inhibits *Ngo*-stimulated JNK activation. HeLa cells were treated with Toxin B (50 ng/ml) 15 min before the infection with *Ngo* strain P^+ Opa^- at a MOI of 50, with 50 nM PMA for 30 min or left untreated, respectively (lanes *3–6*). As a control, cells were left without Toxin B (lanes *1–3*). JNK1 was immunoprecipitated with an anti-JNK1 (sc-474; Santa Cruz Biotechnology) antibody and immunocomplex kinase activity (*p-JNK1*) was determined by phosphorylation of the substrate GST-c-Jun (amino acids 1–79). First, the Western blots were probed with the anti-JNK1 antibody, to show similar protein amounts in all lanes (*bottom*). In the subsequent autoradiography the JNK1 kinase activity (*p-JNK1*) measured by c-Jun substrate phosphorylation is indicated (*top*). The data are representative for at least three independent experiments.

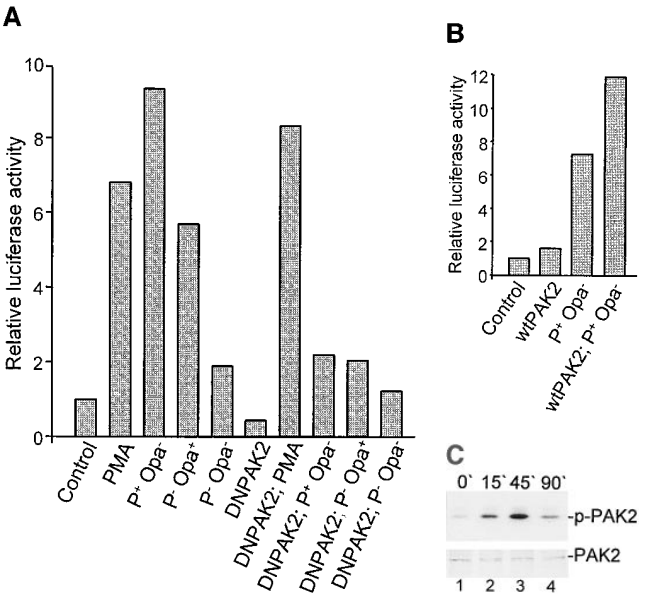

Figure 5. (*A*) PAK is involved in *Ngo*-induced AP-1–dependent transcription. HeLa cells were transfected with 1 μg reporter construct and either 0.75 μg dominant inhibitory PAK2 (*DNPAK2*) or empty vector. After overnight incubation with the DNA, cells were infected with different *Ngo* strains (P^+ Opa^-, P^- Opa^+, and P^- Opa^-) at a MOI of 50 or treated with 50 nM PMA for 30 min. The results of a representative experiment are expressed as fold induction over the activity observed with transfection of the reporter vector and empty vector and in the absence of *Ngo* strains or PMA. Similar results were obtained in more than three independent experiments. (*B*) HeLa cells were transfected as described above using 0.5 μg wild-type PAK2 (*wtPAK2*) instead of DNPAK2 and incubated with the *Ngo* strain P^+ Opa^- at a MOI of 50 for 30 min. The results of a representative experiment are expressed as fold induction compared with the activity observed with transfection of the reporter vector and empty vector and in the absence of *Ngo*. Similar results were obtained in more than three independent experiments. (*C*) PAK2 activation in response to *Ngo*-epithelial cell contact was studied for the indicated periods of time in HeLa cells treated with *Ngo* strain P^+ Opa^- at a MOI of 100 before preparation (lanes *1–4*). PAK2 was immunoprecipitated with an anti-PAK2 antibody (Santa Cruz, sc-1872) and immunocomplex kinase activity (*p-PAK2*) was determined by phosphorylation of 1 μg of the substrate MBP. The samples were separated by SDS-PAGE and blotted. First, the Western blot was probed with an anti-PAK2 antibody, to show similar protein amounts in all lanes (*bottom*). The PAK2 protein is indicated. In the subsequent autoradiography the PAK2 kinase activity (*PAK2*) measured by MBP substrate phosphorylation is indicated (*top*). The data are representative for at least three independent experiments.

Rac1-mediated activation of MEKK (26, 27). Recent results show that MEKK1 directly interacts with Rac1 and Cdc42 (30), indicating that PAK and MEKK1 independently associate with Rho-GTPases. To examine whether PAK or MEKK1 are involved in transcriptional responses characteristic of *Ngo*-infected epithelial cells, we compared their capacity to contribute to *Ngo*-induced AP-1 activation.

In transient transfection assays we used PAK2 and PAK1 dominant inhibitory mutant kinases to investigate whether PAK might be involved upstream in the *Ngo*-induced signaling cascade leading to MKK4, JNK, and AP-1 activation. The PAK2 mutant contains a K278R and PAK1 a K299R mutation that inactivate the catalytic activity of the kinase domain. The mutations in the amino acids arginine 278 and 299 have been demonstrated to be important for the kinase activity of PAKs (58). HeLa cells transfected with DNPAK2 and treated either with adherent (P^+ Opa^-) or invasive (P^- Opa^+) *Ngo* strains for 30 min strongly reduced the AP-1 transactivation activity, compared to mock transfected cells (Fig. 5 *A*). Similar to PAK2 we observed reduced transactivation activity of AP-1 in transfected DNPAK1 and *Ngo*-stimulated cells (data not shown). The experiments were performed after careful titration of the cDNAs to allow specific inhibition of the AP-1 transactivation activity. Since mammalian PAKs (PAK1, 2 and 3) exert >90% homology in their COOH-terminal kinase domain, they will putatively inhibit downstream signaling of all three PAK isoenzymes. To exclude potential effects on the putative upstream components due to titration of Rho-GTPases, we performed experiments with a PAK1 construct mutated in histidine 83 and 86, which prevent the binding of GTPases. Using such construct, similar results as described above were obtained (data not shown). Therefore, these results indicate that PAK could lie upstream of MKK4 and JNK leading to AP-1 activation. As already shown in Fig. 2 *C*, nonpathogenic *Ngo* strain P^- Opa^- slightly induces the transactivation activity of AP-1, which is reduced in DNPAK2-transfected cells. In PMA-treated cells, AP-1 transactivation activity was substantially increased but unaffected in DNPAK2-transfected cells (Fig. 5 *A*) suggesting that PMA induces JNK/AP-1 activation via a PAK-independent pathway, as previously shown by Rudel et al. (33).

To address the possible role of wild-type PAK in transcriptional activity of AP-1 in response to *Ngo* (P^+ Opa^-), we examined the effect of expression of PAK on the transcription of an AP-1–dependent reporter construct. Overexpression of PAK2 (wtPAK2) slightly enhanced transcription of an AP-1–dependent reporter gene in the absence of *Ngo* (Fig. 5 *B*). Similar data were obtained using a wild-type PAK1 construct (data not shown). This may be in agreement with the recent observation that the PAK activation depends on active GTPases (28) and confirms previous data showing small JNK protein kinase activity of wild-

type PAK3 overexpression (20). In the presence of *Ngo* P^+ Opa^- we observed an increased and additive effect on the AP-1 transactivation activity compared to mock transfected cells (Fig. 5 *B*).

To study the JNK/AP-1 activation by the kinase activity of PAK2, we immunoprecipitated PAK2 from *Ngo* (P^+ Opa^-)-stimulated HeLa cells after the indicated periods of time. The activity of PAK2 (p-PAK2) was induced several fold in HeLa cells by the *Ngo* strain N138 (P^+ Opa^-; Fig. 5 *C*) within 15 min after infection measuring phosphorylation activity of PAK2 with MBP as a substrate. The immediate PAK2 induction further increased within 45 min and declined to a reduced activity within 90 min. The lower panel of Fig. 5 shows the Western blot probed with an anti-PAK2 antibody, to show similar protein amounts in all lanes.

In an attempt to explore whether MEKK1 is involved when *Ngo* bacteria induce MKK4/JNK/AP-1 activity, we tested if inhibitory DNMEKK1 blocks this pathway. In contrast to kinase-inactive inhibitory mutants of PAKs, DNMEKK1 does not block *Ngo*-induced AP-1 transcriptional activity (Fig. 6, *A* and *B*). In contrast to *Ngo*, PMA-induced AP-1 transactivation activity clearly appears inhibited by DNMEKK1 (Fig. 6 *C*). A role for PMA-activated PKC in the activation of MEKK1 was recently described (59).

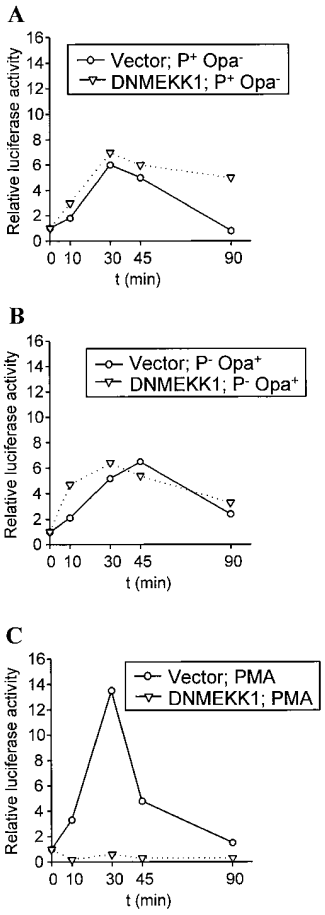

Figure 6. *Ngo*-induced AP-1 transcription activity does not involve MEKK1. AP-1–dependent transcription was analyzed using HeLa cells transfected with a reporter construct (1 μg) and either 1 μg dominant inhibitory MEKK1 or empty vector. After overnight incubation with the DNA, cells were infected with *Ngo* strain P^+ Opa^- (*A*) or *Ngo* strain P^- Opa^+ (*B*) at a MOI of 50, or treated with 50 nM PMA (*C*) for the indicated periods of time. The results of a representative experiment are expressed as fold induction compared with the activity observed with transfection of the reporter vector and empty vector and in the absence of *Ngo* strains or PMA. Similar results were obtained in more than three independent experiments.

Therefore, these results indicate that PAKs do not lie upstream of MEKK1 in a pathway leading to AP-1 activation. Rather, the data suggest that PAKs independently mediate JNK/AP-1 activation in a *Ngo*-induced signaling pathway.

Discussion

In most pathogen infections, epithelial cells represent the first barrier for the microorganisms in the host tissue and could function as an early warning system that activates the immune response using signals that lead to the attraction of phagocytic cells (1). The activatory signaling leading to posttranslational modification and activation of the transcription factor AP-1 plays a critical role in the regulation of proinflammatory cytokine genes and other gene promoters whose gene products have immunomodulatory functions (60). The ability to respond appropriately to such signaling events is essential for the survival of the eukaryotic cell. Perturbations in the normal regulation of these specific responses can result in pathogenic events that lead to acute or chronic disease. AP-1 transcriptional activity and subsequent upregulation of proinflammatory cytokines is induced upon the initial contact of *Neisseria gonorrhoeae* with epithelial cells, which influence the immune surveillance. Adherent P^+ *Ngo* or invasive Opa^+ *Ngo* induce a rapid and specific activation of AP-1 and subsequently a panel of cytokine genes. The physical invasion of epithelial cells by *Ngo* is not a prerequisite of AP-1 activation and cytokine upregulation (3). Thus, it appears that the *Ngo*-eukaryotic cell contact is already sufficient to induce an efficient downstream signaling. To a better understanding of the crucial outcome of the *Ngo* infection, we studied the upstream components involved in AP-1 activation.

In this study we analyzed the capability of adherent and invasive *Ngo* strains to induce stress response kinase pathways leading to AP-1 activation in epithelial cells. Components of kinase cascades modulate AP-1 activation at different control points including regulation at the transcriptional and posttranslational level. Exposure of mammalian cells to a variety of physiological stress stimuli, including *Ngo* cell contact (3), activate the transcription factor AP-1 and many target genes (19). Moreover, overexpression of AP-1 in response to a variety of stress factors has been implicated in a number of immunoinflammatory and proliferative diseases (60). c-Jun is the central component of all AP-1 dimeric protein complexes and phosphorylation of c-Jun in the activation domain is exerted only by the JNKs (19). We found that *Ngo* stimulates JNK in epithelial cells (Fig. 1), which phosphorylates c-Jun at serines 63/73 (61), and AP-1–dependent transcriptional activity (Fig. 2). The JNK kinase activity in response to adherent (P^+ Opa^-) or invasive (P^- Opa^+) Ngo strains, which induce a variety of proinflammatory cytokines, was observed rapidly within 15 min after infection at a MOI of 50 and further increased within 90 min, whereas the nonpathogenic strain (P^- Opa^-) or LPS induced weak and no JNK activity, respectively. Our data indicate that contact between *Ngo* and epithelial cells is sufficient to induce an efficient downstream signaling leading

to the activation of cytokine genes. The TNF-α–treated cells showed JNK activation to a similar extent, but with a maximum within 15 min. Activation of JNK was observed in both cell lines studied, but was at least 10 times less effective and delayed in Chang cells compared to HeLa cells. This points to the assumption that epithelial cells are capable of responding differently to external stimuli. For example, the opa-dependent invasion of *Ngo* in Chang conjunctiva cells occurs irrespective of the presence of serum but involves PLC and ASM (12). In many other epithelial cells, this signaling pathway appears to be less prominent, whereas the serum factor vitronectin was shown to allow HSPR-dependent invasion (10). The observation that JNK, but not p38 kinase was activated by *Ngo* suggests that the activatory mechanisms of these kinases by this stimulus are at least partially different. Our finding that *Ngo* induced stress response kinase JNK contributing in the activation of cytokine genes in epithelial cells is consistent with another report on *Salmonella typhimurium* (62), which supports the notion that epithelial cells are an integral component of the host immune system. LPS does not effectively induce cytokines AP-1 and JNK in epithelial cells, which could be explained in part by lack of CD14 on epithelial cells. In contrast to *Ngo* epithelial cell contact, infection of macrophages by *Yersinia enterocolitica* suppresses the LPS-mediated activation of JNK as well as p38 kinase activation (63). Thus, different pathogen stimulants trigger multiple signal transduction pathways in the target cell.

Stimulation of cells with epidermal growth factor (EGF; references 43–45) or IL-1 (64) leads to JNK activation by a pathway that involves the activation of MKK4. Like other physiologic stress inducers (e.g. UV light and osmotic shock; reference 2), the human pathogenic adherent (P^+ Opa^-) or invasive (P^- Opa^+) *Ngo* strains also induce MKK4, and overexpression of the kinase negative MKK4 inhibits activation of AP-1 (Fig. 2, *A* and *B*). The dominant inhibitory effect of MKK4 on *Ngo*-induced AP-1 activation indicates that JNK is required to mediate full transcriptional activation of AP-1 in response to *Ngo* and suggests either the formation of a stable JNK/MKK4 complex or sequestration of crucial elements immediately upstream of MKK4. A number of kinases have been postulated to regulate the JNK/AP-1 activation. These include the MEKKs 1, 2, and 4 (65–67) and tumor progression locus 2 (Tpl-2; reference 24), which phosphorylate and activate MKK4. Germinal center kinase (21), the mixed lineage kinases (MLK), MLK2 and 3/SPRK, DLK/MUK (23, 68–70), and TGF-β–activated kinase (22) show selectivity for the activation of JNK and phosphorylate MKK4 in vitro. In contrast to other stimuli like EGF, we could exclude that MEKK1 is involved in *Ngo*-induced JNK/AP-1 activation. DNMEKK1 does not affect the pathogen-induced (P^+ Opa^- or P^- Opa^+) activation of AP-1, whereas PMA-induced AP-1 activity, which presumably involves activated PKC (59), is inhibited (Fig. 6, *A–C*). Similar to a number of other kinases, PAKs are shown to be activators of JNK signaling cascades (20), although PAKs do not directly phosphorylate MKK4 (53). In *Ngo*-stimulated cells, PAK mediates its effect on the JNK/AP-1 pathway independent of MEKK1. The ability of DNPAKs (DNPAK2 and DNPAK1) to block AP-1 activation by *Ngo* (P^+ Opa^- or P^- Opa^+), and the activation of AP-1 by PAK (wtPAK2 and wtPAK1) overexpression and activation of endogenous PAK2 in response to *Ngo* (P^+ Opa^-) indicate that PAK may function as a component of the same signaling transduction pathway that leads to MKK4/JNK and AP-1 activation (Fig. 5). In the future it will be necessary to address which components downstream of PAKs lead to JNK/AP-1 activation. A suggested candidate kinase that might play such a role could be SPRK, a mixed lineage kinase (23). p21-activated kinase is the most upstream component of the Rac1/Cdc42 pathway linking these GTPases to JNK (56). By studying the involvement of GTPases in *Ngo*-induced signaling, we used Toxin B from *Clostridium difficile*, which specifically inhibits the activity of the members of the Rho family (54). AP-1 transcriptional activity and JNK activation is strongly reduced in the presence of Toxin B (Fig. 3). These data indicate that GTPases and PAKs may function as components of the same signal transduction pathway that leads to JNK protein kinase and the activation of the transcription factor AP-1.

From these findings, we suggest a pathway, depicted in Fig. 7, through which human pathogenic *Ngo* stimulate the JNK/AP-1 activity. Activation of GTPases by upstream signals leads to increased activity of PAK kinase(s). PAK does not directly phosphorylate JNK. Activation of JNK by dual phosphorylation is mediated by MKK4, which becomes phosphorylated by MKKK. Thus PAKs presumably regulate the activity of MKKK. The signaling pathway through GTPases, PAK, MKKK, MKK4, JNK, and AP-1 are orchestrated to yield a coordinated response of cytoskeletal changes and transcriptional activity leading to the release of immune response mediators.

Neisseria strains used in our experiments produce either pili (P^+) or Opa proteins *(*Opa^+) capable of interacting with cells, or neither pili nor Opa proteins. In phagocytes the Opa-CD66 receptor interaction could be important for the invasion-dependent downstream signaling involving Src-kinases and Rac1 (16). Internalization of Opa-expressing *Ngo* in epithelial cells has been shown to be HSP-dependent (9) and occur by at least two different mechanisms. Invasion in Chang cells depends on the activation of ASM and the second messengers diacylglycerol (DAG) and

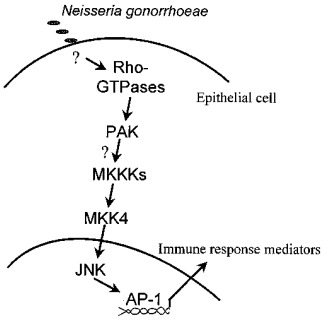

Figure 7. Schematic presentation of the signaling leading to JNK and AP-1 activation in response to *Ngo*-epithelial cell contact. The binding of *Ngo* to epithelial cells leads to the activation of low molecular mass GTPases and sequential protein kinase pathways controlling AP-1 activation. PAK activates the JNK/AP-1 pathway involving unknown MKKK and MKK4, but not MEKK1.

ceramide (12). In other epithelial cells including HeLa, another pathway depending on the serum factor vitronectin allows HSP-dependent invasion. Vitronectin-dependent invasion of Opa^+ *Ngo* (10) involves integrin receptors (11). Vitronectin also possesses a HSP-binding site and is generally known to interact with certain integrins (71). In this respect internalization of *Ngo* by epithelial cells could be mediated by integrin-dependent endocytosis of vitronectin, which results from interaction with heparan-sulfate proteoglycans (HSPGs). Since *Ngo*-induced cytokines, the activation of AP-1 (3) and stress response kinases are already induced by noninvasive *Ngo* (P^+ Opa^-), we suggest that *Ngo* adhesion activates signals leading to the activation of immune response mediators. Thus it seems, that *Ngo* trigger a number of signaling pathways, which are either essential for the pathogen infection, or represent the host reaction in response to the pathogenic microbes.

Cytokines produced from epithelial cells represent specific modulators of the immune response and could be an advantage in the eradication of the parasite, but could also be a disadvantage by leading to chronic inflammatory diseases instead. The ability to respond appropriately to such signaling events is essential for the survival of the cell and ultimately the whole organism. A chronic inflammatory state, induction of adhesion molecules, chemotaxis and activation of leukocytes, the production of mediators of inflammation and continuous T cell activation leads to self-perpetuating destruction of the epithelial structure. All these events are controlled at the level of transcription of genes necessary for the production of new proteins such as growth factors, interleukins, proteinases and cytokines. Regulating this process, e.g., by disrupting the signal transduction pathways of transcription factors, has the potential to attenuate the production of immune response mediators, thereby halting or reversing the course of the disease.

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Address correspondence to Dr. Michael Naumann, Max-Planck-Institut für Infektionsbiologie, Abt. Molekulare Biologie, Monbijoustr. 2, 10117 Berlin, Germany. Phone: 49-30-28026317; Fax: 49-30-28026611; E-mail: naumann@mpiib-berlin.mpg.de

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