Stimulation of NFκB Activity by Multiple Signaling Pathways Requires PAK1*

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The p21-activated kinase (PAK1) is a serine-threonine protein kinase that is activated by binding to the Rho family small G proteins Rac and Cdc42hs. Both Rac and Cdc42hs have been shown to regulate the activity of the transcription factor NFκB. Here we show that expression of active Ras, Raf-1, or Rac1 in fibroblasts stimulates NFκB in a PAK1-dependent manner and that expression of active PAK1 can stimulate NFκB on its own. Similarly, in macrophages activation of NFκB as well as transcription from the tumor necrosis factor α promoter depends on PAK1. In these cells lipopolysaccharide is a potent activator of PAK1 kinase activity. We also demonstrate that expression of active PAK1 stimulates the nuclear translocation of the p65 subunit of NFκB but does not activate the inhibitor of κB kinases α or β. These data demonstrate that PAK1 is a crucial signaling molecule involved in NFκB activation by multiple stimuli.

NFκB is a transcription factor that is critically involved in cellular growth and transformation, the suppression of apoptosis, and the response to inflammatory stimuli (1–6). It consists of homo- and heterodimers of members of the Rel family of transcription factors (1, 2). The most frequently studied form of NFκB consists of two proteins, p50 (NFκB1) and p65 (RelA). In unstimulated cells, this heterodimer is retained in the cytoplasm by an inhibitory protein known as the inhibitor of κB (IκB). In response to most stimuli that activate NFκB, IκB becomes phosphorylated, ubiquitinated, and subsequently degraded by the 26 S proteosome (7, 8). Once free of IκB, NFκB translocates to the nucleus and activates the transcription of target genes. The transcriptional activity of NFκB is also controlled by phosphorylation of dimer subunits. For example, both p50 and p65 are phosphorylated in cells, and phosphorylation of p65 has been shown to positively regulate its transcriptional activity (9–14).

The phosphorylation of IκB that leads to its degradation occurs on two conserved serine residues within its N terminus (serines 32 and 36 in IκBα) (15). Two kinases have been identified and cloned that will phosphorylate both of these sites in cells, namely IκB kinase α and IκB kinase β (IKKα and IKKβ, respectively) (16–20). Based on knockout studies in mice, IKKβ seems to be more important than IKKα for controlling NFκB activity in response to cytokines and other ligands (21–25). Both of these kinases are present in a high molecular weight protein complex that contains at least two distinct scaffolding proteins (IκKγ and IκK complex-associated protein), as well as NFκB, IκB, and other proteins (26–29). The activities of IKKα and IKKβ are controlled by the related kinases NFκB-interacting kinase (NIK) and the mitogen-activated protein/extracellular signal-regulated kinase kinases 1–3 (MEKK1–3) (30–36). Other kinases that control IKK activity may yet be identified. Recent studies have shown that two upstream regulators of NIK are the transforming growth factor-β-activated kinase 1 and Cot/Tpl2, whereas MEKK1 has been shown to be regulated by the viral protein Tax (37–40). The relative importance of each of these pathways to the regulation of IKK activity in response to different stimuli remains unclear.

Pro-inflammatory cytokines such as interleukin 1β (IL1β) and tumor necrosis factor α (TNFα), as well as bacterial endotoxins such as lipopolysaccharide (LPS), signal to the IKK complex through the activation of specific receptors on the plasma membrane. In the case of IL1β, its receptor (IL1βRI) signals to an associated complex consisting of the IL1β-accessory protein, MyD88, and two related interleukin 1 receptor-associated kinase proteins (41, 42). The activated receptor complex signals to the IKK complex through a scaffolding protein called TRAF6 (43, 44). LPS induces signaling following binding to a glycosyl phosphatidylinositol-linked, membrane-associated protein called CD14. Recently, a member of the toll family of receptors known as toll receptor 4 was identified as a receptor for LPS (45). Another toll family member, toll receptor 2, has also been identified as possible LPS receptor (46). These receptors have significant homology to the IL1βRI and in fact require many of the known IL1βRI-associated molecules for efficient signaling (47–50).

Among the first cells to become activated following exposure to LPS are macrophages. Once activated, macrophages secrete pro-inflammatory cytokines such as IL1β, TNFα, and IL6. The signaling pathways required for these events are not well de-
fined but are known to result in the activation of three different MAPK cascades as well as an increase in NFκB activity (51). It is also clear that activation of NFκB is necessary for transcription of the genes encoding these cytokines (48).

Recent studies have shown that the Rho family small G proteins Rac1, Cdc42hrs, and RhoA are capable of activating NFκB in various cell types (52–55). In addition, expression of dominant negative forms of Rac1 and RhoA block NFκB activation by IκBα, TNFα, and bradykinin. These observations indicate that Rho family small G proteins are involved in regulating NFκB activation following cytokine stimulation.

All small G proteins mediate signaling through the activation of specific effector proteins. Both Rac1 and Cdc42hsa share a number of common effectors, including the p21-activated kinases (PAKs) (56). To date four PAKs have been cloned, and in the case of PAKs 1–3, binding of GTP-ligated Rac or Cdc42hsa promotes their activation by releasing an autoinhibitory constraint (56–59). Once activated, a number of cellular phenotypes have been attributed to PAK activity, including activation of the extracellular signal-regulated kinase (ERK), JNK, and p38 MAPK cascades (PAKs 1–4), regulation of cytoskeletal organization (PAKs 1, 2, and 4), and regulation of apoptosis (PAK2) (56, 58–60). A number of extracellular stimuli activate PAK1, including exposure to IκBα in epithelial cells and T cell receptor ligation in T cells (61, 62). This suggests that PAK1 may play a role in the immune response.

In this study we show that PAK1 mediates NFκB activation by Ras, Raf-1, and Rac1 and that expression of an active form of PAK1 is capable of stimulating NFκB activity on its own. In addition, we show that active PAK1 stimulates the nuclear translocation of the p65 subunit of NFκB in the apparent absence of IKKα or IKKβ activation. We also demonstrate that in mouse macrophages, PAK1 activity is stimulated by LPS and is required for efficient NFκB activation and TNFα transcription. These results identify PAK1 as an important regulator of NFκB in both fibroblasts and macrophages.

**MATERIALS AND METHODS**

**Plasmids, Reagents, and Expression of Recombinant Proteins—**Eukaryotic expression vectors for V9-H-Ras, Raf-BXB, V9-Rac1, PAK1, PAK1 165 K/A, PAK1 232 K/A, wild type MEKK1, MEKK1cat K/D, full-length MEKK1 D/A, full-length MEKK1 D/A, NIK, NIK K/Kα, IKKα, IKKβ S/S, IKKα, IKKβ K/M, and IκBα S/S/A have been described elsewhere (48, 59, 63). The NFκB-luciferase reporter vector contains two tandem repeats of the NFκB binding site from the β promoter 5′ to a minimal thymidine kinase promoter and is contained in pGLbasic (Amersham Pharmacia Biotech). The activator protein-1-luciferase reporter and the TNFα promoter chloramphenicol acetyltransferase reporter are as described elsewhere (51, 64).

Mouse anti-Myc and mouse anti-HA antibodies were from the Cell Culture Center and the Berkeley Antibody Company, respectively. Rabbit anti-NIK (H-248), rabbit anti-IKKα (M-280), rabbit anti-IKKβ (H-470), rabbit anti-p65 (H-286) and rabbit anti-PAK1 (N-20) were from Santa Cruz Biotechnology. *Escherichia coli* lipopolysaccharide from strain 0127:B8 was obtained from Difco.

GST-IκBα (1–54) was expressed in logically growing BL21DE3 E. coli by the addition of 400 μM isopropl-1-thio-β-galactopyranoside for 4 h. GST fusion proteins were purified essentially as described (59) and dialyzed overnight in buffer C (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM benzamidine, 10% glycerol). Hexa-histidine-tagged IKKα and IKKβ were expressed in SF9 cells and purified first by nickel-agarose affinity chromatography and then by MonoQ (anion exchange) fast protein liquid chromatography.

**Cell Culture and Transfections—**NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 100 units/ml penicillin/streptomycin. HEK 293 cells (293 cells) and REF52 cells were grown in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, and 100 units/ml penicillin/streptomycin. Prior to transfection, the cells were placed in fresh growth medium. Both cell lines were transfected by calcium phosphate precipitation (63). Twenty hours after transfection, the medium was replaced with DMEM plus 0.5% calf serum (NIH 3T3 cells) or DMEM without serum (293 cells). The cells were then allowed to incubate for another 24 h. RAW 264.7 cells were grown in DMEM containing 10% fetal bovine serum (endotoxin-free), 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM l-glutamine. These cells were transfected using the Profection DEAE-dextran transfection system (Promega). After the transfection, the cells were treated with LPS (1 μg/ml) or dexamethasone for 6 h and then harvested.

**Reporter Assays—**Transfected cells were washed once with cold phosphate-buffered saline (PBS) and scraped into luciferase lysis buffer (50 mM Tris- HCl (pH 8.0), 70 mM K2HPO4, 0.1% Nonidet P-40, 2 mM MgCl2, 1 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The lysates were rapidly mixed for 10 s, and insoluble material was pelleted by centrifugation at 4 °C. The supernatant was removed and either assayed immediately or flash frozen in liquid nitrogen and stored at −80 °C. Firefly luciferase assays were performed according to the manufacturer’s protocol using a Luciferase Assay kit (Promega) and a Turner luminometer. For promoter activation assays in NIH3T3 cells, transfection efficiency was monitored by assaying for β-galactosidase activity derived from a cotransfected, constitutively active β-galactosidase expression plasmid. β-Galactosidase activity was determined essentially as described (65). In RAW 264.7 cells, transfection efficiency was determined by measuring the activity of expressed Renilla firefly luciferase derived from the transfection of pRL-TK (Promega). Renilla luciferase assays were performed using the Dual Luciferase Assay kit (Promega) and the Turner luminometer. Chloramphenicol acetyltransferase assays were performed as described previously (51).

**Immunoprecipitation and Kinase Assays—**To assay the activity of transfected IKKα or IKKβ, 293 cells were washed once with cold PBS and lysed with Triton lysis buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5% Triton X-100, 80 mM β-glycerophosphate, 0.5 mM sodium orthovanadate, 1 mM EDTA, 20 μg/ml aprotinin, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Insoluble material was pelleted by centrifugation, and supernatants were removed, flash-frozen in liquid nitrogen, and stored at −80 °C. Equal amounts of epitope-tagged IKK proteins were used for immunoprecipitation based on prior immunoblotting. IKKs were immunoprecipitated by incubation for 2 h at 4 °C with antibodies directed against the appropriate epitope tags and protein A-Sepharose (Amersham Pharmacia Biotech). Each immunoprecipitate was washed three times with 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and once with 20 mM Tris-HCl (pH 8.0) and was divided in two parts. One-half was tested for immunoprecipitated IKKs or IKKβ protein by immunoblotting. The other half was assayed for kinase activity toward GST-IκBα (1–54). Kinase assays were performed at 30 °C for 30 min in buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM dithiothreitol, 100 μM ATP, 3 μM GST-IκBα (1–54), and 4500 cpn/mg of γ-32PAPTP. Reactions were stopped by adding Laemmli sample buffer and were resolved by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining. Phosphopeptide incorporated into GST-IκBα (1–54) was measured by excising the Coomassie Blue-stained bands and counting by liquid scintillation.

For assaying endogenous PAK1 activity in RAW 264.7 cells, the cells were stimulated with 1 μg/ml LPS for increasing amounts of time, washed once with cold PBS, and lysed in Triton lysis buffer. PAK1 was immunoprecipitated from 1 mg of cell lysate/sample by incubating with 1 μg of rabbit anti-PAK antibody (N20) and protein A-Sepharose for at least 2 h at 4 °C on a rotating platform. The immunoprecipitates were then pelleted by centrifugation and washed three times with 20 mM Tris-HCl (pH 7.5), 1 mM NaCl and once with 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 10 mM MgCl2. The immunoprecipitate was assayed for kinase activity using myelin basic protein (MBP) as a substrate. MBP kinase assays were carried out essentially as described (59). After the kinase reaction the pellet containing the immunoprecipitated PAK1 was solubilized with Laemmli sample buffer, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose. Immunoprecipitated PAK1 was detected by Western blotting using the rabbit anti-PAK1 antibody (N-20).

**Microinjection Assays—**REF52 cells were plated on glass coverslips in 10% fetal bovine serum and injected with expression vectors for p65 and Myc epitope-tagged versions of either V9-Rac1, PAK1 K/D, or wt NIK, or IκBα and expression vectors for the nucleolus at 0.25 mg/ml using an Eppendorf 5171 microinjector mounted on a Zeiss Axioptot S135 inverted microscope. Either 4 or 16 h after injection the cells were fixed with 3.7% formaldehyde in PBS for 5 min at 37 °C and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. Expressed p65 RelA was detected with rabbit anti-p65 diluted to 2 μg/ml in PBS + 0.05% Tween 20 (PBST). Myc-tagged proteins were detected with goat anti-Myc followed by Texas Red-labeled donkey anti-goat IgG.
detected with mouse anti-Myc diluted to 0.4 μg/ml in PBST. Fixed cells were incubated with these antibodies at 37 °C for 1 h followed by three 5-min washes with PBST. The cells were then incubated with rhodamine-conjugated donkey anti-rabbit (Jackson Labs) and fluorescein isothiocyanate-conjugated donkey anti-mouse for 1 h at 37 °C followed by three 5-min washes with PBST and once with distilled water. Epi-fluorescence was detected with a Zeiss Axiovert S135 microscope fitted with a Photometrics cooled CCD camera.

RESULTS

PAK1 Activity Is Required for NFκB Activity by Ras, Raf-1, and Rac1—Ras, Raf-1, and Rac1 have all been shown to stimulate NFκB activity (53, 66, 67). In the case of Ras and Raf-1, this activation may be critical to their ability to promote cell growth. On the other hand, NFκB activation by Rho family small G proteins has been suggested to be important for the response to pro-inflammatory cytokines. Because PAK1 is a Rac and Cdc42hs effector that is involved in the Ras-dependent activation of Raf-1, we tested whether PAK1 activity is required for activation of NFκB by these molecules. Thus, NIH 3T3 cells were cotransfected with an NFκB-luciferase reporter plasmid, active forms of Ras (V12H-Ras), Raf-1 (Raf BXB), or Rac1 (V12Rac1), and either a control vector or dominant negative (dn) PAK1. This dominant negative form of PAK1 consists of the catalytic domain of PAK1 (amino acids 232–544) containing a point mutation that renders it inactive (K298A). Because it lacks the N-terminal regulatory domain, it cannot bind to Rac1 or Cdc42hs. As shown in Fig. 1A, expression of active Ras, Raf-1, or Rac1 stimulated NFκB activity in these cells. In the case of Ras, NFκB activation was only partially blocked by dominant negative PAK1. Because Ras activates NFκB through multiple signaling pathways, this likely indicates that only one of these pathways relies on PAK1 (66). On the other hand, dominant negative PAK1 was more effective at blocking NFκB activation by active Rac1 and completely blocked activation by active Raf-1. This suggests that PAK1 plays a more important role in NFκB activation stimulated by Raf-1 and Rac1. For all three proteins, dominant negative PAK1 blocked NFκB activation slightly less well than dominant negative NIK, and coexpression of dominant negative NIK and dominant negative PAK1 did not result in a greater degree of inhibition of NFκB activation (data not shown). Because NIK phosphorylates and activates the IκB kinases IKKα and IKKβ, this suggests that NFκB activation by Ras, Raf-1, or Rac1 ultimately depends on the phosphorylation of IκB.

To test if PAK1 functions downstream of proteins known to mediate NFκB activation by cytokines, dominant negative PAK1 was coexpressed with increasing amounts of the adaptor proteins TRAF2 and TRAF6. TRAFs link cytokine receptor activation to the IκB kinase activation cascade, and overexpression of wild type TRAF proteins stimulates NFκB activity (43, 44, 68, 69). As shown in Fig. 1B, dominant negative PAK1 did not significantly inhibit NFκB activation by TRAF2 or TRAF6. This fits with the observation that expression of these TRAF proteins in cells does not activate PAK1. Consistent with previous observations, dominant negative NIK partially inhibited NFκB activation by TRAF2 (30, 70). Taken together, these data indicate that PAK1 activity is required for NFκB activation stimulated by active Raf-1 or Rac1 but is not involved in NFκB activation stimulated by TRAF2 or TRAF6 overexpression.

PAK1 Stimulates NFκB, but Does Not Activate Either IKKα or IKKβ—To test whether expression of active PAK1 can stimulate NFκB activity, NIH3T3 cells were cotransfected with the NFκB reporter and a constitutively active, N-terminal truncation mutant of PAK1 (PAK1 165) (59). As shown in Fig. 2A, the expression of active PAK1 stimulated NFκB activity to levels comparable to those stimulated by active Rac1 (Fig. 1A). This activation was specific for NFκB, because PAK1 165 did not significantly activate an activator protein-1-luciferase reporter construct (data not shown). NFκB activation depended on PAK1 kinase activity, because the kinase-inactive version of this protein did not stimulate NFκB activity (data not shown). This suggests that PAK1 selectively activates NFκB. In addition, activation of NFκB by active PAK1 was inhibited by coexpression of dominant negative forms of NIK, IKKα, and IKKβ, suggesting that PAK1-mediated NFκB activation depended on the phosphorylation of IκB. On the other hand, NFκB activation stimulated by active PAK1 was not inhibited by two different dominant negative forms of MEKK1 (Fig. 2A). These dominant negative forms of MEKK1 have been shown previously to inhibit NFκB activation by the viral protein Tax (40).

Because PAK1 does not appear to function downstream of

\[ J. A. Frost and M. H. Cobb, unpublished observations. \]
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TRAF2 or TRAF6 (Fig. 1B), we tested whether activation of NFκB by PAK1 represents a separate pathway leading to NFκB activation. If so, coexpression of active PAK1 with TRAF2 or TRAF6 should activate NFκB to a greater degree than expression of either protein alone. As shown in Fig. 2B, coexpression of constitutively active PAK1 with increasing amounts of TRAF2 or TRAF6 stimulated a higher level of NFκB activity than that observed with either TRAF protein alone. This is consistent with data showing that dominant negative PAK1 expression does not block NFκB activation stimulated by TRAF2 or TRAF6 (Fig. 1B) and suggests that PAK1 activates NFκB by a pathway distinct from that activated by TRAF2 or TRAF6.

Because NFκB activation by PAK1 was blocked by the expression of dominant negative forms of NIK, IKKα, and IKKβ, we examined whether PAK1 stimulated the kinase activity of either IKKα or IKKβ. Thus, 293 cells were cotransfected with epitope-tagged forms of IKKα or IKKβ and either a control vector, active PAK1 (PAK1 165), active Rac1 (V12Rac1), wild type NIK (wt NIK), or wild type MEKK1 (wt MEKK1). The next day the cells were placed in starvation medium, and after 24 h they were lysed. IKK proteins were then immunoprecipitated and tested for kinase activity using bacterially expressed GST-IκB-(1–54) as a substrate. As shown in Fig. 3, neither IKKα nor IKKβ were activated by coexpression of active Rac1 or active PAK1. Under these circumstances, NIK and MEKK1 were strong activators of IKKα and IKKβ activity, respectively. Similar results were found in mouse macrophages (data not shown). PAK1 165 expression also did not stimulate the activity of endogenous IKKα or IKKβ in 293 cells (data not shown). In separate experiments, coexpression of active Rac1 or PAK1 with epitope-tagged NIK did not increase the activity of the immunoprecipitated NIK toward recombinant IKKα or IKKβ (data not shown). Furthermore, recombinant PAK1 did not phosphorlyate recombinant IKKα or IKKβ purified from S9 cells and did not stimulate the phosphorylation in vivo of either wild type or kinase-inactive NIK expressed in 293 cells (data not shown). Thus, neither Rac1 nor PAK1 appear to stimulate the activity of NIK, IKKα, or IKKβ.

To test whether active PAK1 promotes nuclear translocation of NFκB by a mechanism other than activation of IKKα or IKKβ, we microinjected REF52 fibroblasts with expression vectors for p65 (RelA) and active Rac1 (V12Rac1), active PAK1 (PAK1 165), or wild type NIK (wt NIK). p65 is the transcriptional activation component of the most common form of the NFκB heterodimer (1, 2). Four or sixteen hours after injection, the cells were fixed, and the cellular localization of the expressed p65 was determined by indirect immunofluorescence. As shown in Fig. 4A, expression of either active Rac1, active PAK1, or wild type NIK for 4 h stimulated the translocation of p65 to the nucleus. In the cells expressing active PAK1, this was accompanied by a pronounced retraction of the cell membrane, consistent with previously observed effects of PAK1 on cell morphology (59). Determination of the percentage of cells showing nuclear staining for p65 showed that V12Rac1, PAK1 165, and wild type NIK were similarly capable of stimulating the nuclear translocation of p65 (Fig. 4C). At 16 h after injection, cells expressing active PAK1 or NIK still showed nuclear localization of p65 (Fig. 4, B and D). These data indicate that...

![Figure 2: PAK1 does not activate either IKKα or IKKβ](http://www.jbc.org/)

![Figure 3: IKKα and IKKβ](http://www.jbc.org/)

![Figure 4: Nuclear translocation of p65](http://www.jbc.org/)
PAK1 stimulates the nuclear translocation of NFκB, most likely in the absence of significant activation of IKKa or IKKβ activity. Translocation of NFκB to the nucleus in the absence of IKK activation has been observed previously (71–73). Thus, the finding that dominant negative forms of IKKa and IKKβ are capable of blocking NFκB activation by active PAK1 (Fig. 2A) may reflect the ability of these molecules to form tight complexes with NFκB and thereby preclude its nuclear localization stimulated by other mechanisms.

PAK1 Functions within the LPS Signaling Pathway to Activate NFκB—LPS is a potent activator of NFκB in macrophages. Because PAK1 is required for efficient NFκB activation in fibroblasts, we tested whether endogenous PAK1 is activated by LPS in the mouse macrophage cell line RAW 264.7. As shown in Fig. 5, exposure of these cells to LPS led to a time-dependent increase in PAK1 activity, as determined by the ability of immunoprecipitated PAK1 to phosphorylate MBP. This activity peaked at 30 min (22-fold) and was decreasing by 60 min (14-fold). These data indicate that LPS is an efficient activator of PAK1 in mouse macrophages.

To test whether PAK1 is required for stimulation of NFκB activity by LPS, RAW 264.7 cells were cotransfected with the NFκB-luciferase reporter and either a control vector, constitutively active PAK1 (PAK1 165), or dn PAK1. As shown in Fig. 6A, exposure to LPS stimulated NFκB activity 5-fold in vector-transfected cells. In nonstimulated cells, expression of PAK1 165 activated the NFκB reporter to levels equivalent to those in LPS-stimulated cells (5-fold), and in the presence of LPS this activation was much greater (15-fold). In addition, expression of dominant negative PAK1 reduced NFκB activation by LPS by half. These data indicate that PAK1 functions within the NFκB activation pathway stimulated by LPS in these cells.

Exposure of macrophages to LPS activates the transcription of many cytokine genes in an NFκB-dependent manner, including TNFα (51). To test whether PAK1 is involved in the regulation of a physiological promoter dependent on NFκB activity, RAW 264.7 cells were cotransfected with a murine TNFα promoter-chloramphenicol acetyltransferase reporter construct. This TNFα promoter contains five NFκB binding sites, one of which is similar to that found in our synthetic NFκB-luciferase promoter, as well as binding sites for other transcription factors. These cells were also transfected with a control vector, constitutively active PAK1 (PAK1 165), or dn PAK1. As shown in Fig. 6B, expression of dominant negative PAK1 almost completely blocked activation of this promoter by LPS. This indicating positive for nuclear p65 in cells expressing V12Rac1, PAK1 165, or wt NIK, four (C) or sixteen (D) hours after injection. Shown are the averages of at least three independent experiments. Error bars represent the standard error of the mean.
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FIG. 6. LPS activates NFκB in mouse macrophages in a PAK1-dependent manner. RAW 264.7 cells were cotransfected with the NFκB-luciferase reporter and either a control vector, dn PAK1, or constitutively active PAK1 (PAK1 165). The cells were then either stimulated or not with LPS (1 μg/ml) for 6 h. Shown is the average of three independent experiments. Error bars refer to the standard error of the mean. B, PAK1 activity is required for activation of the human TNFα promoter in mouse macrophages. RAW 264.7 cells were cotransfected with the TNFα-pro-chloramphenicol acetyltransferase reporter and a control vector, dn PAK1, or constitutively active PAK1 (PAK1 165). The cells were either stimulated or not with LPS (1 μg/ml) for 6 h, harvested, and assayed for chloramphenicol acetyltransferase activity. Shown is the average of five independent experiments. Error bars denote the standard error of the mean.

cates that LPS-stimulated TNFα transcription requires PAK1 activity. Interestingly, expression of constitutively active PAK1 did not activate this promoter nor did it potentiate activation by LPS. This suggests that active PAK1 expression alone is not sufficient for stimulation of TNFα transcription. This lack of activation may reflect the fact that this promoter contains a number of enhancers in addition to the characterized NFκB binding sites and that LPS-stimulated TNFα transcription requires the activation of one or more transcription factors in addition to NFκB (51).

DISCUSSION

The data presented in this study demonstrate that expression of constitutively active PAK1 stimulates NFκB activity in fibroblasts and macrophages. Furthermore, PAK1 activation is required for stimulation of NFκB activity by Rac1 and Raf-1 in fibroblasts and by LPS in mouse macrophages. PAK1 appears to stimulate NFκB activity independently of TRAF2 or TRAF6 and does not activate NIK, IKKα, or IKKβ. Nevertheless, in microinjection experiments PAK1 stimulates the nuclear translocation of the p65 subunit of NFκB. We have also shown LPS to be a potent activator of PAK1 in mouse macrophages and that PAK1 activation is necessary for full activation of the murine TNFα promoter. This indicates that PAK1 is an important regulator of NFκB activity in multiple cell types.

NFκB activity is controlled on multiple levels, including through regulation of its subcellular localization. In its inactive form, NFκB is sequestered in the cytoplasm by the IκB family of proteins. Most stimuli that activate NFκB do so by stimulating the phosphorylation of IκB on two sites within its N terminus that leads to its degradation, thereby allowing NFκB to translocate to the nucleus and activate transcription. The IκB kinases IKKα and IKKβ phosphorylate these sites in response to most ligands (15). Thus, the finding that PAK1 stimulates the activity of endogenous NFκB as well as the nuclear translocation of expressed p65 but does not activate either IKKα or IKKβ is unusual. The mechanism by which PAK1 does this is not clear. It may be that PAK1 stimulates the activity of an IκB kinase other than IKKα or IKKβ. It is also possible that PAK1 stimulates NFκB translocation through an IKK-independent mechanism. In this regard, both UV-C exposure and treatment with pervanadate have been shown to stimulate NFκB translocation in the absence of IKK activation (71–73). Additionally, it is possible that PAK1 functions in the NFκB activation pathway in a manner loosely analogous to that of its homolog in yeast, STE20. In Saccharomyces cerevisiae, STE20 regulates pheromone-dependent mitogen-activated protein kinase activation by a complex mechanism that depends on the scaffolding protein STE5 as well as the MAP3K STE11 (74). In a similar manner, PAK1 may regulate the association of NIK, the IKKs, IκB, or NFκB with the scaffolding proteins IKK complex-associated protein or IKKγ. In this regard, we have found that expression of active PAK1 reduces the coprecipitation of IKKβ with NIK from cells (5). Thus, perhaps PAK1 stimulates NFκB activity by altering the kinetics of association between NFκB-activating components in the cell. If this were the case, one could envision how expression of dominant interfering forms of NIK or an IKK might block NFκB activation by PAK1, because their expression would affect the association of the endogenous kinases with the NFκB scaffold. Future studies will be directed at determining the mechanism by which PAK1 stimulates NFκB activation.

The requirement for PAK1 activity in the activation of NFκB by Raf-1, and to a lesser degree Ras, may have implications for cellular transformation. An increasing body of evidence suggests that NFκB activation is crucial for cellular transformation. For example, Ras-dependent transformation of fibroblasts is inhibited by the expression of dominant negative IκBα (6, 75). Similarly, transformation of Rat-1 fibroblasts by Ras also requires PAK1 activity (76). Thus, activation of NFκB by PAK1 may be one way in which it contributes to cellular transformation.

The finding that PAK1 is an LPS-regulated kinase and that this activity is required for LPS-mediated NFκB activation is also potentially important. LPS causes septic shock in mammals following bacterial infection. The initial response to this type of infection occurs in macrophages, which react by producing pro-inflammatory cytokines such as TNFα and IL1β. The release of these cytokines then causes a massive immune response in the animal. The increase in transcription of both TNFα and IL1β in response to LPS is controlled in part by NFκB. LPS initiates signaling by binding to one or more toll family receptors. These receptors resemble the type 1 IL1β receptor in structure and appear to use some of the same signaling molecules. The observation that PAK1 controls NFκB activation in response to LPS defines another link in the signaling pathway leading to LPS-dependent transcriptional activation. Future efforts will be directed at understanding the mechanism by which LPS stimulates PAK1 activity and how PAK1, in turn, controls NFκB activation.
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