Phosphatidylinositol 3-Kinase in Interleukin 1 Signaling

PHYSICAL INTERACTION WITH THE INTERLEUKIN 1 RECEPTOR AND REQUIREMENT IN NFκB AND AP-1 ACTIVATION

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The signaling mechanisms utilized by the proinflammatory cytokine interleukin-1 (IL-1) to activate the trunscription factors NFκB and activator protein-1 (AP-1) are poorly defined. We present evidence here that IL-1 not only stimulates a dramatic increase in phosphatidylinositol 3-kinase (PI 3-kinase) activity but also induces the physical interaction of its type I receptor with the p85 regulatory subunit of PI 3-kinase. Furthermore, two PI 3-kinase-specific inhibitors, wortmannin and a dominant-negative mutant of the p85 subunit, inhibited IL-1-induced activation of both NFκB and AP-1. Transient transfection experiments indicated that whereas overexpression of PI 3-kinase may be sufficient to induce AP-1 and increase nuclear c-Fos protein levels, PI 3-kinase may need to cooperate with other IL-1-inducible signals to fully activate NFκB-dependent gene expression. In this regard, cotransfection studies suggested that PI 3-kinase may functionally interact with the recently-identified IL-1-receptor-associated kinase to activate NFκB. Our results thus indicate that PI 3-kinase is a novel signal transducer in IL-1 signaling and that it may differentially mediate the activation of NFκB and AP-1.

The biological processes of growth, differentiation, and immunity are dependent on the highly regulated action of transcription factor families such as NFκB and activator protein-1 (AP-1). These two transcription factors participate not only in normal physiology but also in disease conditions as well. Extracellular stimuli such as interleukin-1 (IL-1), tumor necrosis factor (TNF), viruses, and UV light are among the known potent inducers of NFκB (1). Of these, IL-1, which is a major proinflammatory cytokine, is responsible for mediating numerous host responses, including fever, activation of lymphocytes, and the induction of acute-phase proteins (2). Elevated levels of IL-1 have been associated with various pathological conditions, including rheumatoid arthritis (2). Although several biological activities of IL-1 have been characterized, the molecular mechanisms by which its signals are transduced from the plasma membrane to affect gene transcription in the nucleus remain to be elucidated.

One of the most prominent IL-1-inducible signals, one which requires the type I IL-1 receptor (IL-1RI), involves the rapid and dramatic activation of NFκB and AP-1 and results in the induction of discrete sets of genes (2). Since IL-1RI shares no significant homology with conserved protein kinase domains, it is unlikely to have any intrinsic protein kinase activity (3) and may need to recruit specific cytoplasmic proteins to transmit its signals. One such protein, recruited to the receptor in response to IL-1 stimulation, is the IL-1-receptor-associated protein kinase (IRAK), which bears significant homology to the Drosophila protein Pelle (4). Although there is no evidence linking IRAK directly to NFκB activation, studies on the function of Pelle have demonstrated its importance in the activation of Dorsal, the mammalian equivalent of NFκB. Recently, IRAK has been shown to physically associate with a protein belonging to the TNF receptor-associated factor family called TRAF6 (5). In contrast to TNF, which recruits TRAF2 to activate NFκB, IL-1-induced activation of NFκB is mediated by TRAF6 (5). However, the mechanisms by which the recruitment of TRAFs to the IL-1 and TNF receptors leads to the activation of NFκB are not understood.

NFκB is normally inactive and kept sequestered in the cytoplasm by its interaction with the inhibitory subunit IκB (1). Upon stimulation, IκB is rapidly phosphorylated, ubiquitinated, and then degraded, resulting in the release and subsequent nuclear translocation of active NFκB (1). In addition to the TRAFs, several other factors appear to play a role in NFκB activation. Although some interact with the TRAFs (6), others such as Raf kinase, tyrosine kinases, reactive oxygen intermediates, and sphingomyelins have also been reported (1).

AP-1 is predominantly a heterodimeric complex of c-Fos and c-Jun proteins, and its activation is mainly due to induction of c-Fos synthesis and the phosphorylation of both c-Jun and c-Fos (7). Recent studies have indicated that phosphatidylinositol 3-kinase (PI 3-kinase) may up-regulate c-Fos synthesis (8) and stimulate the Jun N-terminal kinase pathway (9), which might then lead to the phosphorylation and activation of c-Jun. Other studies have implicated PI 3-kinase in epidermal growth factor-induced AP-1 activation (10). PI 3-kinase consists of a catalytic subunit (p110) associated with a regulatory polypeptide (p85) (11). Ligand-dependent interactions between the SH2 domains of the p85 subunit and the phosphotyrosine-containing YXXM motif present on several cytokine/growth factor receptors have been reported (11). The phosphorylated lipid products generated by this enzyme may act as second messengers to activate protein kinases such as the Akt gene.

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29167
product (12) or certain isoforms of protein kinase C (13). It's reported interactions with Ras (14) and its potential ability to activate several signaling pathways (9) underscore the importance of PI 3-kinase in various cellular functions.

We have demonstrated that the acute-phase gene serum amyloid A 1 and 3 (15–17) are highly inducible by cytokines such as IL-1 and shown that NFκB is critical for their expression (16–18). In this study, we investigated the signaling events that lead to NFκB and AP-1 activation in IL-1-stimulated cells. Our results indicate a prominent role for PI 3-kinase in the activation of both NFκB and AP-1.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents**—HepG2 and KB cells were obtained from the American Type Culture Collection and maintained at 37 °C in modified Eagle's medium containing 10% fetal bovine serum and antibiotics. Wortmannin and phosphatidylinositol were from Sigma. PD98059 was obtained from Biomol Research Laboratories Inc., and staurosporine was obtained from Life Technologies, Inc.

**Electrophoretic Mobility Shift Assays**—Cells were serum-starved for 4 h before treatment with various inhibitors and IL-1. Nuclear extracts were prepared and electrophoretic mobility shift assays were performed as described earlier (19). Nuclear extracts of 2.5 μg were incubated with radiolabeled probe. Protein-DNA complexes were resolved on 5% polyacrylamide gels and visualized by autoradiography. The results presented here are representative of at least three independent experiments.

**PI 3-Kinase Assays and Coimmunoprecipitation**—KB or HepG2 cells were seeded in 60-mm culture dishes overnight. Cells were serum-starved for 4 h before treatment for the indicated times with or without IL-1. Whole cell lysates were prepared in ice-cold lysis buffer containing 50 mM HEPES, pH 7.5, 10% glycerol, 150 mM sodium chloride, 1% Nonidet P-40, 1.5 mM magnesium chloride, 1 mM EGTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 14 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 3 mM benzamidine. After 20 min on ice, the lysates were clarified by centrifugation at 14,000 × g. The supernatant was then utilized for PI 3-kinase assays as described earlier (20), and the labeled [32P]Phosphatidylinositol 3-phosphate was resolved by thin layer chromatography (21). For coimmunoprecipitation experiments, whole cell lysates were prepared as described above and incubated with anti-p85 PI 3-kinase antibodies (Santa Cruz) or anti-IL-1R type 1 antibodies (Santa Cruz) for 1 h before incubation with protein A-Sepharose beads (1 h). The immune complexes were boiled in Laemmli buffer and resolved on 10% denaturing polyacrylamide gels. After transfer to nitrocellulose membranes, proteins were probed with anti-IL-1R type antibodies or anti-p85 PI 3-kinase antibodies in accordance with the manufacturer's specifications. Proteins were visualized by ECL (Amerham) using goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies.

**Reporter Assays**—HepG2 cells were plated in 100-mm dishes and transfected the next day with the (NFκB)3/CAT or (AP-1)2/CAT reporter plasmids and various expression vectors. Transfections were done using the Polybrene method. Sixteen hours after transfection, cells were treated with cytokines or left untreated for 20 h. All other procedures were performed as described elsewhere (22).

**Construction of IRAK Expression Vector**—A forward primer 5′-GGCGGCAACCTGTCGCGG-3′ and a reverse primer 5′-TGTCCGCG-TACCAACACCTACGCTCTGGAAATC-3′ containing a KpnI restriction site were used to amplify IRAK cDNA from a HeLa cDNA library by the polymerase chain reaction. A 2.1-kilobase polymerase chain reaction product was cloned into the p85DN vector SRα (23) from which the p85DN cDNA had been excised after digestion with EcoRI and KpnI.

**Immunostaining**—Twenty hours after transfection, cells were washed with phosphate-buffered saline and fixed with 10% paraformaldehyde for 10 min before being permeabilized with 0.2% Nonidet P-40 for 2 min. Cells were washed five times with phosphate-buffered saline and then incubated with f-Fos polyclonal antibody (Santa Cruz) for 60 min. After washing, these cells were incubated with tetramethylrhodamine B isothiocyanate-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories) for 45 min. The coverslips were washed several times and then inverted on glass slides containing 90% glycerol and sealed. Cells were then observed under a Nikon Diaphot TMD-EF inverted microscope with epi-fluorescence attachment. All other procedures were performed as described in the Santa Cruz Research Applications (Santa Cruz).

**RESULTS**

Wortmannin Inhibits IL-1-induced Activation of NFκB and AP-1—To identify potential mediators of IL-1 signaling, we used gel mobility shift assays to examine the ability of various inhibitors to block the activation of NFκB and AP-1 by IL-1. Epidermoid carcinoma KB or hepatoma HepG2 cells were stimulated with IL-1 after pretreatment with either wortmannin, a potent PI 3-kinase inhibitor (24), PD98059, an inhibitor of the mitogen-activated protein kinase pathway (25), or staurosporine, a general protein kinase inhibitor. Whereas DNA binding activities of both NFκB and AP-1 were potently induced by IL-1, their activation was inhibited completely in cells pretreated with wortmannin (Fig. 1, A and B). Inhibition by wortmannin was observed in both KB and HepG2 cells, indicating that the wortmannin-sensitive mechanism in NFκB activation may be a general mechanism. PD98059, which inhibits the mitogen-activated protein kinase pathway, also had an inhibitory effect but was less effective than wortmannin (Fig. 1). Consistent with earlier reports (26), staurosporine had little or no effect on the activation of NFκB or AP-1 by IL-1.

**Rapid and Transient Activation of PI 3-Kinase Activity by IL-1**—The concentration of wortmannin that we used has been shown to selectively inhibit PI-3 kinase activity (24). To determine if PI 3-kinase activity is indeed regulated by IL-1, cytokine-treated cell extracts were assayed for PI 3-kinase activity...
Preincubated in the absence or presence of 100 nM wortmannin before B3-kinase activity in untreated (0 min) cell extracts. By about 27-fold (Fig. 2). Within 7 min, insulin stimulated PI 3-kinase activity elevation of PI-3 kinase activity in a time-dependent manner control. Treatment of KB cells with IL-1 or insulin resulted in pretreatment of cells with wortmannin prevented the stimulation of PI 3-kinase from that of any contaminating PI 4-kinase. The effect on PI 4-kinase activity and thus differentiates the activation kinetics (3 min) and to a greater magnitude (108-fold). In contrast, IL-1 stimulated PI 3-kinase with much faster induction of PI 3-kinase by IL-1 (Fig. 2A), which is comparable to the fold-activation reported by others (27). The insulin-stimulated increase in PI 3-kinase activity declined rapidly thereafter. Like insulin, the IL-1-stimulated increase in PI 3-kinase activity was also transient. Similar results were obtained in HepG2 cells (data not shown). IL-1-inducible PI 3-kinase activity was dependent on the presence of phosphatidylinositol as substrate. [32P]phosphatidylinositol 3-phosphate spots from one representative experiment is shown. Spot intensities were quantitated (PhosphorImager™, Molecular Dynamics) and normalized to PI 3-kinase activity in untreated (0 min) cell extracts. B, KB cells were preincubated in the absence or presence of 100 nM wortmannin before treatment with IL-1 (3 min). PI 3-kinase activity was quantitated as phosphorImager units and normalized to the activity in IL-1-treated extracts (without wortmannin) to which a value of 100 was assigned. PIP, [32P]phosphatidylinositol 3-phosphate.

with phosphatidylinositol as substrate. Insulin, a known potent activator of PI 3-kinase activity (11), was included as positive control. Treatment of KB cells with IL-1 or insulin resulted in elevation of PI-3 kinase activity in a time-dependent manner (Fig. 2). Within 7 min, insulin stimulated PI 3-kinase activity by about 27-fold (Fig. 2A), which is comparable to the fold-activation reported by others (27). The insulin-stimulated increase in PI 3-kinase activity declined rapidly thereafter. In contrast, IL-1 stimulated PI 3-kinase with much faster induction kinetics (3 min) and to a greater magnitude (108-fold). Like insulin, the IL-1-stimulated increase in PI 3-kinase activity was also transient. Similar results were obtained in HepG2 cells (data not shown). IL-1-inducible PI 3-kinase activity was dependent on the presence of phosphatidylinositol in the assays and was inhibited completely by 0.2% Nonidet P-40 (data not shown). The presence of detergent in these assays has little or no interaction was detected after 3 min (Fig. 3A). The physical coupling of these two proteins was further confirmed in converse experiments wherein anti-IL-1RI immune complexes coprecipitated p85 PI 3-kinase (Fig. 3B).

**PI 3-Kinase Is Necessary for NFκB Activation, but Overexpression of the p110 Catalytic Subunit Alone Is Insufficient to Activate NFκB-dependent Gene Expression**—Since wortmannin blocked the activation of both NFκB and AP-1, the role of PI-3 kinase in the activation of these two transcription factors was explored further by transient transfection assays. The functional importance of PI 3-kinase was evaluated by cotransfection of an (NFκB)3/CAT reporter plasmid, with either the dominant-negative mutant of the p85 regulatory subunit (p85DN) or p110 catalytic subunit of PI 3-kinase. To demonstrate that PI-3 kinase functions as a mediator in NFκB activation, we next employed a dominant-negative mutant of PI 3-kinase, p85DN (23) in these cotransfection assays. When p85DN was overexpressed, it inhibited IL-1-induced (NFκB)3/CAT expression by approximately 70% (Fig. 4A), implicating PI 3-kinase as an important mediator of NFκB activation. To ascertain the specificity of this inhibition, we tested its effect on the induction of a T1 kinogen/CAT reporter gene. We have previously shown that this reporter can be induced by a combination of IL-6 and Dex (22) and that the induction does not involve NFκB. In contrast to the (NFκB)3/CAT reporter, the induction of the T1 kinogen/CAT gene was unaffected by overexpression of p85DN, suggesting that its inhibition on NFκB-dependent gene expression was specific. Overexpression of p110 in-
duced CAT activity approximately 3-fold and in a dose-dependent manner (Fig. 4B). Interestingly, when p110-transfected cells were further stimulated with IL-1, synergistic activation of the (NFkB)3/CAT reporter was observed (Fig. 4C). Maximal synergism was observed at suboptimal concentrations of IL-1 (data not shown). In addition, this synergism between IL-1 and p110 was dependent on the dose of p110 used for transfection, the best effect being achieved with 5–10 μg of transfected p110 DNA. Induction of the (NFkB)3/CAT reporter by IL-1 and its synergistic activation by IL-1 and p110 can be blocked by IxB (Fig. 4C), the inhibitory subunit of NFkB. This indicates that induction of the reporter gene by these stimuli specifically involved NFkB activation. As a control, the mutant (NFkB)m/CAT reporter construct that lacks a functional NFkB binding site was nonresponsive regardless of the treatment.

**PI 3-Kinase**

**PI 3-kinase is necessary and sufficient for maximal activation of NFkB by IL-1.**

A, PI 3-kinase is required for maximal NFkB activation. The p85DN (dominant negative mutant of the p85 subunit of PI-3-kinase) or empty vector was cotransfected with either a CAT reporter gene containing three copies of wild-type NFkB binding sites or with a CAT reporter under control of the T1 kininogen promoter (22). B, effect of p110 overexpression on (NFkB)3/CAT reporter gene activity. Wild-type (αB) or mutant (mxB) CAT reporter genes were cotransfected into HepG2 cells with various doses of a p110 PI 3-kinase expression vector. C, synergistic activation of NFkB by p110 and IL-1. Cells were cotransfected with either 5 μg of p110 expression vector and the NFkB reporter or vector control. Wherever indicated, DNA mixtures also contained 1 μg of IxB-expression vector or empty vector. The averages and standard deviations from three independent experiments are shown.

**FIG. 4.**

**FIG. 5.** Overexpression of p110 PI 3-kinase induces c-Fos expression and activates an (AP-1)/CAT reporter gene. HepG2 cells grown on coverslips were transfected with p110-expression DNA (D) or the empty vector alone (C). Control cells were subjected to similar transfection conditions but without any DNA and were afterward either left untreated (A) or stimulated for 2 h with IL-1 (B). Cells were stained for c-Fos protein. For the cotransfection experiments (E), a (AP-1)/CAT reporter DNA that contained consensus AP-1 binding sites was cotransfected into HepG2 cells with the p110-expression vector or the p85DN mutant. Shown here are averages and standard deviations from two different experiments.

**PI 3-Kinase Is Necessary and Sufficient for AP-1-dependent Gene Expression**—The effect of p110 overexpression on an (AP-1)/CAT reporter was also examined by cotransfection studies. In contrast to its effects on the (NFkB)3/CAT reporter, overexpression of p110 alone induced the (AP-1)2/CAT reporter to the same extent as IL-1 treatment (Fig. 5E), and no additional increase was observed upon inclusion of IL-1, indicating that the reporter could be fully activated by p110 alone. Furthermore, p85DN blocked IL-1-induced AP-1 activation completely (Fig. 5E). Our data are therefore consistent with recent reports for a role of PI-3-kinase in epidermal growth factor-induced AP-1 activation (10) and further demonstrate the involvement of PI-3-kinase in IL-1-induced AP-1 activity. Activation of AP-1 involves a dramatic elevation of c-Fos protein that can then dimerize with Jun proteins to produce AP-1 complexes. There is evidence that PI-3-kinase is involved in the activation of c-Fos synthesis (8). Since IL-1 up-regulates c-Fos mRNA (2) and there is a dramatic induction of c-Fos protein in IL-1-treated cells (Fig. 5B), we examined the ability of overexpressed PI 3-kinase to induce c-Fos protein levels. Overexpress-
transfected IRAK expression plasmid (4) with the (NFκkCAT gene to test for its ability to activate NFκk by IL-1, and yet overexpression of the p110 catalytic subunit mains to be determined whether these effects can be accounted inflammatory or immunosuppressive effects (24, 29). It re-

by IL-1. Interestingly, wortmannin, which is a fungal metabo-

lules may cooperate in vivo to activate NFκB. We next evaluated the possibility that IRAK would synergize with IL-1. IRAK, like PI 3-kinase, synergized with IL-1 (Fig. 6B) to a comparable extent (compare Figs. 4C with 6B), and interest-

ingly, this effect was blocked by coexpression of the p85DN mutant of PI-3 kinase (Fig. 6B).

DISCUSSION

We have presented evidence to indicate that the interaction of PI 3-kinase with IL-1RI is one of the early events in IL-1-stimulated cells and that PI 3-kinase may be indispensable for the activation of NFκB and AP-1 by IL-1. Our results show that wortmannin blocked the activation of both transcription factors by IL-1. Interestingly, wortmannin, which is a fungal metabolite, has been shown in experimental animals to exert anti-

flammatory or immunosuppressive effects (24, 29). It re-

mains to be determined whether these effects can be accounted for, at least in part, by its inhibitory effects on NFκB activation.

Since wortmannin blocked the activation of NFκB and AP-1 by IL-1, it was predicted that IL-1 would stimulate PI 3-kinase activity. Indeed, IL-1 stimulated PI 3-kinase activity very po-
tently and with rapid kinetics of activation. The activation kinetics of NFκB and AP-1 have been well established and are slower in comparison to those for PI 3-kinase in IL-1-treated cells, consistent with a role for this enzyme in their activation. PI 3-kinase has been attributed with both lipid kinase and protein kinase activities, and since wortmannin inhibits both, it is difficult to assess at this time the relative importance of each activity in IL-1 signaling.

Wortmannin has recently been shown to inhibit phospho-
lipase A2 at concentrations that earlier were thought to be selective for PI 3-kinase (30). We therefore used a dominant negative mutant, p85DN, of the regulatory subunit of PI 3-kinase to verify the importance of this enzyme in the activation of NFκB and AP-1. p85DN has been used previously to confirm a role for PI 3-kinase in various cellular functions (23, 31). Consistent with our wortmannin experiments, overexpression of p85DN strongly inhibited the activation of NFκB- and AP-1-dependent gene expression by IL-1. PI 3-kinase therefore appears to be essential for their activation by IL-1.

The cytoplasmic domain of IL-1RI protein contains a se-

A A B B

sequence motifs are also found in the mouse IL-1RI and the Drosophila Toll protein, which is equivalent to human IL-1RI. 

Various studies have indicated that for the XXYM motifs to be capable of binding to p85 PI 3-kinase, the tyrosine residues must be phosphorylated (Ref. 33, see references in Ref. 34). Several receptor and nonreceptor tyrosine kinases that could potentially phosphorylate these sites and lie upstream of PI 3-kinase in various signaling pathways have been implicated (11). In one study, a sequence in the insulin receptor sub-

strate-1 containing a PI 3-kinase binding motif and several flanking amino acid residues was evaluated as a substrate for different nonreceptor tyrosine kinases (35). Using synthetic peptides in protein kinase assays, it was determined that the tyrosine kinases differed in their tolerance for various amino acid substitutions, and in particular, an aspartate immediately N-terminal to the tyrosine was indispensable. In this context, it may be noted that in addition to other acidic amino acids, there is an aspartic acid (Asp505) immediately adjacent to the Y496EKEM sequence on the IL-1RI. This suggests that the Y496EKEM sequence on the IL-1RI may be a good substrate for tyrosine kinases and may facilitate direct interaction between IL-1RI and PI 3-kinase. An IL-1-inducible tyrosine kinase activity has been reported but not identified (36). The involvement of tyrosine kinase activities in the steps leading to NFκB activation has been reported for various inducers (see references in Ref. 1).

Overexpression of p110 was sufficient to induce the AP-1/CAT reporter and to increase nuclear c-Fos protein levels as potently as IL-1. These results are consistent with recent re-

ports that epidermal growth factor-induced activation of AP-1 requires PI 3-kinase (10). In sharp contrast to the AP-1 re-

![Fig. 6. IRAK and PI 3-kinase can synergistically activate NFκB.](Image)
The mechanisms by which activated PI 3-kinase may ultimately result in the activation of NFkB and AP-1 are unclear. However, several signaling molecules have been shown to affect directly or indirectly the pathway leading to the activation of these transcription factors. For example, two atypical forms of protein kinase C, aPKCζ (39) and aPKCη (40), have been shown to be involved in NFkB and AP-1 activation, respectively. Since the phosphorylated lipid products of PI 3-kinase activate various isoforms of PKC in vitro, including aPKCζ (40), PKC may lie downstream of PI 3-kinase. aPKCζ activity was recently reported to be stimulated by IL-1 in rat renal mesangial cells (41). Small G-proteins and the protein kinase mitogen-activated protein kinase-extracellular signal-regulated kinase kinase 1 (MEKK1) are among the other signaling molecules that have been implicated in the activation of NFkB.

Physical interaction between the small GTPase Cdc42 and PI 3-kinase may result in the stimulation of PI 3-kinase activity (42, 43). Cdc42 appears to be required for the activation of NFkB by TNF (44), and our results suggest that the mechanism may involve interactions with PI 3-kinase. Finally, MEKK1, a key mediator of the Jun N-terminal kinase pathway, has recently been implicated as an upstream regulator of the 700-kDa IκB kinase (45). Since overexpression of PI 3-kinase activates the Jun N-terminal kinase pathway (9), it would be of interest to determine if it is involved in the up-regulation of MEKK1 activity in IL-1-stimulated cells. It is not known whether any of these proteins, Cdc42, PKCs, or MEKK1, interact with IRAK and if they do, whether those interactions would account for the synergism between PI 3-kinase and IRAK in NFkB activation.

Since our results suggested that PI 3-kinase may functionally interact with IRAK to activate NFkB, we are currently investigating the molecular basis of such interactions. It is hoped that in addition to providing greater insights into the mechanisms employed by IL-1 for NFkB activation, these studies would also reveal the identity of the downstream targets of both IRAK and PI 3-kinase.

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Activation of NFκB and AP-1 Requires PI 3-Kinase

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