Tumor Suppressor MMAC/PTEN Inhibits Cytokine-induced NFκB Activation without Interfering with the IκB Degradation Pathway

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The phosphoinositide 3-kinase (PI 3-kinase) pathway has been implicated in the activation of the proinflammatory transcription factor nuclear factor κB (NFκB). To investigate the role of this pathway in NFκB activation, we employed mutated in multiple advanced cancers/phosphatase and tensin homologue (MMAC/PTEN), a natural antagonist of PI 3-kinase activity. Our results show that cytokine-induced DNA binding and transcriptional activities of NFκB were both inhibited in a glioma cell line that was stably transfectected with MMAC/PTEN. The ability of interleukin-1 (IL-1) to induce inhibitor (IκB) degradation or nuclear translocation of NFκB was, however, unaffected by MMAC/PTEN expression, suggesting that PI 3-kinase utilizes another equally important mechanism to control NFκB activation. It is conceivable that NFκB is directly phosphorylated through such a mechanism because treatment with protein phosphatase 2A significantly reduced its DNA binding activity. Moreover, IL-1-induced phosphorylation of p50 NFκB was potentely inhibited in MMAC/PTEN-expressing cells. Whereas the mediators of NFκB phosphorylation remain to be identified, IL-1 was found to induce physical interactions between the PI 3-kinase target Akt kinase and the IκB-IκB complex kinase. Physical interactions between these proteins were antagonized by MMAC/PTEN consistent with their potential involvement in NFκB activation. Taken together, our observations suggest that PI 3-kinase regulates NFκB activation through a novel phosphorylation-dependent mechanism.

The transcription factor NFκB is activated by interleukin-1 (IL-1), tumor necrosis factor (TNF), and a variety of other stress-inducing stimuli (1–3). In addition to its role in inflammation, NFκB has also been implicated in cellular survival, transformation, and oncogenesis (1–3). Predominantly a heterodimeric complex of two polypeptides (p65/RelA and p50), NFκB is physically confined to the cytoplasm through its interactions with inhibitors belonging to the IκB family of proteins (1–3). When phosphorylated on serine 32 and serine 36, IκBα is marked and degraded by the ubiquitin/26 S proteasome pathway liberating the NFκB heterodimer so that it may translocate to the nucleus. The signaling cascade that induces IκB degradation and thus leads to NFκB activation has recently been delineated (3). There is compelling evidence that phosphorylation of the regulatory serines on IκBα is mediated by a 300–500-kDa multisubunit IκB protein kinase (IKK) (4–10). This kinase complex was purified to apparent homogeneity and shown to be composed primarily of the protein kinases IKKα and IKKβ as well as a protein that lacks a catalytic kinase domain known as IKKγ (4–10).

The phosphorylation and degradation of IκB may not, however, be sufficient to activate NFκB. Using two different phosphoinositide 3-kinase (PI 3-kinase) inhibitors, we have previously shown that the PI 3-kinase signaling pathway is also required for NFκB activation (11). Whereas wortmannin efficiently blocked IL-1-induced increases in the DNA binding activity of NFκB, a dominant-negative mutant of the p85 regulatory subunit of PI 3-kinase inhibited the ability of IL-1 to induce an NFκB-dependent reporter gene (11). More recently, Marmiroli et al. (12) have shown that tyrosine 479 on the type I IL-1 receptor (IL-1RI) is required for receptor interaction with PI 3-kinase. When tyrosine 479 was replaced with phenylalanine, the mutant IL-1RI lost its ability to interact with PI 3-kinase and was deficient in signaling for the activation of both PI 3-kinase and NFκB.

Our recent studies have shown that TNF-induced NFκB activation also requires PI 3-kinase and that, when inhibited, PI 3-kinase potentiates TNF-induced apoptosis (13). Consistent with a role for PI 3-kinase in NFκB activation and the antiapoptotic properties of NFκB, p65/RelA protected cells from apoptosis induced by TNF in combination with wortmannin (13). Various other studies have shown the involvement of PI 3-kinase and/or its mediator Akt kinase in NFκB activation induced by IL-1, TNF, phorbol myristate acetate, platelet-derived growth factor, bradykinin, hypoxia, oncogenic ras, and SV40 small t antigen (14–21). The precise role of PI 3-kinase in NFκB activation is, however, still uncertain as there is evidence in support of (15, 16) and against (14, 20) a role for it in IκB degradation and NFκB nuclear translocation. Evidence has been presented that, rather than being involved in DNA binding, PI 3-kinase and Akt are instead critical for the transcriptional activity of NFκB (p65/RelA) (14). The reasons for the discrepancies between the various studies are not clear, but they could be related to the inhibitors used and/or the molecular characteristics of the cell lines employed.

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Although these studies have implicated the PI 3-kinase/Akt pathway in NFκB activation, much ambiguity about its role remains. Indeed, there is a need for approaches that would more clearly reveal the function of PI 3-kinase in NFκB activation. One approach would be to analyze mice with targeted deficiencies in the relevant individual molecules, and another would be to study somatic cell lines with lesions in the PI 3-kinase/Akt pathway. In this study, we utilized a glioma cell line that lacks MMAC/PTEN, a natural antagonist of the PI 3-kinase/Akt pathway, to investigate the function of PI 3-kinase in cytokine-induced NFκB activation. The lipid products of PI 3-kinase that are critical for the activation of downstream protein kinases such as Akt are specifically dephosphorylated at the 3' OH position by the lipid phosphatase activity of MMAC/PTEN (22, 23). This function of MMAC/PTEN, which appears to be responsible for its tumor suppressor properties, is important for regulating PI 3-kinase activity in vivo (24, 25).

The use of MMAC/PTEN as a specific inhibitor could therefore be advantageous for studying the role of PI 3-kinase in NFκB activation. Our results indicate that PI 3-kinase is involved in the regulation of DNA binding activity and trans-activation potential of NFκB through a phosphorylation-dependent mechanism that is parallel but distinct from the IκB degradation pathway.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—U251 human glioblastoma cells (ATCC) were cultured in Dulbecco's modified Eagle's medium/F12 medium containing 5% fetal bovine serum and antibiotics in a humidified atmosphere containing 5% CO2 at 37 °C. Antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA (MMAC/PTEN, p65/RelA, Jun N-terminal kinase (JNK), and IκBα antibodies), New England Biolabs, Beverly, MA (anti-phospho-Akt/protein kinase B serine 473), or Ingexen, San Diego, CA (anti-IκKα). LY294002 was obtained from Biomol Research Laboratories Inc., Plymouth Meeting, PA.

Retrovirus Gene Construction—pLNCX retroviral vector (CLONTECH) derived from Moloney murine leukemia virus was utilized for retroviral gene delivery and expression. A full-length MMAC/PTEN retroviral construct was generated by ligating a NorI-SalI fragment from pBluescript-MMAC/PTEN into the multiple cloning site of pLNCX.

Stable Expression of MMAC/PTEN in the U251 Glioma Cell Line—PT67 retrovirus producer cells were grown in Dulbecco's modified Eagle's medium/F12 containing 10% fetal calf serum, 2 mM glutamine and transfected with the wild-type MMAC/PTEN construct by calcium phosphate precipitation. The human glioma cell line U251 was infected with 48-h supernatants from the transfected PT67 cells. After 14 h of incubation, infected cells were selected with 400 μg/ml G418. Drug-resistant colonies were expanded to generate clonal cell lines and screened for MMAC/PTEN expression by immunoblotting.

Immunoblotting—Cells were washed with ice-cold phosphate-buffered saline and lysed in a buffer containing 50 mM HEPES, pH 7.5, 1.5 mM MgCl2, 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 10 mM Na4P2O7 (sodium pyrophosphate), 10% glycerol, 1% Triton X-100, 3 mM benzamidine, 1 mM Na3VO4 (sodium orthovanadate), 1 mM pepstatin, 10 μg/ml aprotinin, 5 mM iodoacetamide, and 2 μg/ml leupeptin to prepare whole-cell lysates. Lysates were clarified by centrifugation at 14,000 χ g for 5 min. Proteins were resolved by SDS-PAGE and electroblotted to polyvinylidene difluoride membranes (Millipore), and then they were probed with various primary antibodies (MMAC/PTEN, IκB, IκK, and phospho-Akt). For the detection of p65/RelA, nuclear extracts were used instead of whole-cell lysates. Specific proteins were detected by chemiluminescence (ECL) (Amersham Pharmacia Biotech) following incubation with horseradish peroxidase-conjugated secondary antibodies.

Electrophoretic Mobility Shift Assay—Parental U251 or MMAC/PTEN-expressing U251(MMAC) cells were treated with IL-1 (1 nM) or TNF (1 nM) for various periods of time. 2.5 μg of nuclear extracts that were prepared as described previously (11) were incubated for 15 min at room temperature with radiolabeled NFκB-binding probe. For supershift assays, anti-p65/RelA antibody or IgG was added to the incubation mixtures for 5 min before the addition of the radiolabeled probe. Where indicated, IL-1-treated U251 nuclear extracts were incubated with 10 units/ml of the catalytic subunit of protein phosphatase 2A (homogeneity determined by silver staining, a gift of Dr. Zahi Damuni) for 10 min at 37 °C before incubation with the radiolabeled probe. The protein-DNA complexes were resolved on 5% non-denaturing polyacrylamide gels and visualized by autoradiography.

Reporter Assays—U251 and U251(MMAC) cells were plated in 6-well tissue culture plates and transfected the following day with an NFκB/Luciferase reporter along with an IκBα expression plasmid or an empty vector control using the FuGeneTM reagent (Roche Molecular Biochemicals) according to the manufacturer's protocol. After 24 h, cells were treated with IL-1, lysed, and assayed with the enhanced luciferase assay kit (PharMingen). HEK 293 and Hep3B cells were treated in an identical manner except that the (NFκB/Luciferase) reporter was cotransfected with empty pCMV-Flag2 vector or with pCMV-Flag2-MMAC/PTEN.

Immunoprecipitations—Whole-cell lysates were prepared as described earlier and incubated for 1 h with appropriate antibodies to immunoprecipitate either Akt or phospho-Akt (serine 473). Immunocomplexes were precipitated with a 50% slurry of protein A-Sepharose beads (Pierce), washed, and eluted by boiling in SDS sample buffer. Eluted proteins were then resolved by SDS-PAGE and probed by Western blotting analysis with anti-IκKα or anti-IκBα antibodies. Proteins were visualized by ECL (Amersham Pharmacia Biotech).

JNK Assay—Whole-cell lysates prepared from IL-1-treated cells were incubated with anti-JNK antibodies. Immune complexes were precipitated with protein G-Sepharose and assayed for enzymatic activity with 2 μg of GST-c-Jun(-1-79) as substrate. Assay mixtures, which included 0.2 μmol [32P]ATP and 10 μM MgCl2, were incubated for 5 min at 30 °C, after which reactions were stopped by adding SDS sample buffer. Protein phosphorylation was visualized by autoradiography.

Immunoprecipitations—Whole-cell lysates prepared from the transfected U251 cell lines and control cells were incubated with anti-IκKα or anti-IκBα antibodies. Proteins were immunoprecipitated with protein A-Sepharose beads, precipitated with 50 μg of IκKα or IκBα antibodies, and resolved by SDS-PAGE. Radiolabeled protein bands were visualized by autoradiography.

RESULTS

Stable Expression of MMAC/PTEN in U251 Glioma Cells Inhibits Serum and IL-1-induced Akt Phosphorylation—MMAC/PTEN is frequently mutated or deleted in a wide variety of human cancers (26, 27). We examined various cell lines for MMAC/PTEN expression to identify those cells that might be suitable for studying the role of PI 3-kinase in NFκB activation. Among others, the U251 glioma cell line lacked MMAC/PTEN expression and was highly responsive to IL-1 and TNF stimulation. These cells were previously reported to have a mutated MMAC/PTEN gene (27). Both IL-1 and TNF were able to stimulate the activation of PI 3-kinase and NFκB very potently in U251 cells (data not shown) with kinetics that were similar to those induced by them in other cell lines (11, 13). We proceeded to generate stable MMAC/PTEN-expressing clones by infecting parental U251 cells with supernatants from retrovirus producer cells transfected with wild-type MMAC/PTEN. Drug-resistant U251(MMAC), but not parental U251 cells, expressed MMAC/PTEN as confirmed by immunoblotting analysis (Fig. 1).

The lipid products of PI 3-kinase are known to target the serine/threonine protein kinase Akt to the plasma membrane, where it is fully activated through phosphorylation on serine 473 and threonine 308 (28–30). MMAC/PTEN phosphorylates the lipid products of PI 3-kinase at the 3'-OH position and prevents the phosphorylation and activation of Akt (22, 24). We therefore examined U251 and U251(MMAC) cells to compare and contrast the phosphorylation status of Akt. The basal levels of phosphorylated Akt, which were clearly detectable in U251 cells, were significantly higher than those in the MMAC/PTEN-expressing U251(MMAC) cell line.
PTEN-expressing cell line (Fig. 1). In addition, whereas serum- and IL-1-inducible levels of Akt phosphorylation were profound in U251 cells, they were barely detectable in the U251(MMAC) cells (Fig. 1). Because MMAC/PTEN did not affect the protein levels of Akt (Fig. 1), its effect on Akt phosphorylation is presumably attributable to its lipid phosphatase function in the PI 3-kinase pathway as demonstrated previously (for example, see Refs. 24, 31, 32). We conclude that when MMAC/PTEN is reintroduced into U251 cells, it is able to block IL-1-induced phosphorylation and activation of Akt through the inhibition of PI 3-kinase-generated signals.

**MMAC/PTEN Inhibits IL-1- and TNF-induced NFκB Activation**—Both PI 3-kinase and Akt have been shown to be required for the activation of NFκB (11–21). Because IL-1-induced Akt phosphorylation was efficiently inhibited in U251(MMAC) cells, we investigated whether MMAC/PTEN expression would also affect NFκB activation. Both IL-1 and TNF strongly induced the DNA binding activity of NFκB (30–120 min) in parental U251 cells as determined by gel shift assays (Fig. 2). Cytokine-inducible DNA-protein complexes contained NFκB (p65/p50 heterodimer) because they could be supershifted with anti-p65/RelA antibodies but not by nonspecific IgG. The ability of IL-1 and TNF to activate NFκB was, however, inhibited in U251(MMAC) cells, suggesting that MMAC/PTEN had the potential to regulate the DNA binding activity of NFκB (Fig. 2). It is interesting to note that the cytokine-mediated induction of a second DNA-protein band, which was probably the p50/p50 homodimeric complex of NFκB, was also inhibited in U251(MMAC) cells (Fig. 2). To confirm that MMAC/PTEN inhibits cytokine-induced NFκB activation, we examined its effects on the induction of an NFκB-luciferase reporter gene. The expression of this gene, which could be strongly induced (10-fold) in IL-1-treated U251 cells (Fig. 3A), required NFκB activation because it was inhibited in the presence of the IκB inhibitor. Consistent with the gel mobility shift assays (Fig. 2A), IL-1-induced expression of the NFκB-luciferase reporter gene was inhibited in U251(MMAC) cells (Fig. 3A). Furthermore, transient transfection of MMAC/PTEN into human embryonic kidney 293 and hepatoma Hep3B cells also sufficed to inhibit NFκB-dependent gene expression (Fig. 3B). Taken together, these results would strongly support a role for the PI 3-kinase pathway in the DNA binding and transcriptional activities of NFκB.

**PI 3-Kinase Is Not Required for IL-1-induced JNK Activation**—Because expression of MMAC/PTEN in U251 cells blocked the activation of both Akt and NFκB (Figs. 1–3), we investigated whether it generally inhibited various other IL-1-induced signals as well. To evaluate this possibility, we examined the effects of MMAC/PTEN on the ability of IL-1 to stimulate the serine/threonine protein kinase JNK. JNK was immunoprecipitated from IL-1-treated U251 and U251(MMAC) cell extracts and assayed for activity with GST-Jun as the substrate (Fig. 4). The stimulation of JNK activity was observed within 15 min of treatment of U251 cells with IL-1 and persisted for about 2 h. Interestingly, the pattern of activation and the level of induction of JNK in these cells were similar to those observed in the U251(MMAC) cells (Fig. 4). It is therefore unlikely that MMAC/PTEN and, by inference, the PI 3-kinase pathway are involved in regulating JNK activation in IL-1 signaling.

In a manner similar to IL-1, TNF was also able to stimulate JNK activity equally well in both U251 and U251(MMAC) cells (data not shown). Consistent with these results, two other PI 3-kinase inhibitors, wortmannin and a dominant-negative mutant of PI 3-kinase (p85DN), did not affect cytokine-induced JNK activation either (data not shown). PI 3-kinase-independent pathways have previously been shown to be involved in the activation of JNK, such as in platelet-derived growth factor signal transduction (33). On analysis, these results would therefore strongly argue that the inhibitory effects of MMAC/PTEN on Akt and NFκB activation are relatively specific.

**MMAC/PTEN Does Not Interfere with IL-1-induced IκBα Gene Expression**—Expression of MMAC/PTEN in U251 cells blocks the activation of both Akt and NFκB (Figs. 1–3), we investigated whether it generally inhibited various other IL-1-induced signals as well. To evaluate this possibility, we examined the effects of MMAC/PTEN on the ability of IL-1 to stimulate the serine/threonine protein kinase JNK. JNK was immunoprecipitated from IL-1-treated U251 and U251(MMAC) cell extracts and assayed for activity with GST-Jun as the substrate (Fig. 4). The stimulation of JNK activity was observed within 15 min of treatment of U251 cells with IL-1 and persisted for about 2 h. Interestingly, the pattern of activation and the level of induction of JNK in these cells were similar to those observed in the U251(MMAC) cells (Fig. 4). It is therefore unlikely that MMAC/PTEN and, by inference, the PI 3-kinase pathway are involved in regulating JNK activation in IL-1 signaling.

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**MMAC/PTEN Does Not Interfere with IL-1-induced IκBα Gene Expression**
MMAC/PTEN Regulates NFκB Activation

**FIG. 4.** JNK activity is induced by IL-1 through a PI 3-kinase-independent mechanism. Anti-JNK antibodies were used to immunoprecipitate JNK from whole-cell extracts that were prepared from IL-1-treated U251 and U251(MMAC) cells. Immune complexes were incubated with GST-Jun in protein kinase assays for 5 min. Phosphorylated protein was resolved by SDS-PAGE and visualized by autoradiography.

**FIG. 5.** PI 3-kinase and Akt are not required for IκB degradation and NFκB nuclear translocation. Whole-cell or nuclear extracts were prepared from IL-1-treated U251 and U251(MMAC) cells. Whole-cell extracts were analyzed by Western blotting with anti-IκB antibodies (A), whereas nuclear extracts were probed with anti-p65/RelA (B) antibodies. Specific bands were detected by ECL (Amersham Pharmacia Biotech).

Degradation or the Nuclear Translocation of p65/RelA—The site-specific phosphorylation of IκBα induces its degradation and facilitates the nuclear translocation of the p65/p50 heterodimer. Because IL-1- and TNF-induced NFκB activation was inhibited in U251(MMAC) cells (Figs. 2 and 3), we investigated whether this occurred because MMAC/PTEN interfered with either of these obligatory steps in NFκB activation. To our surprise immunoblotting analysis revealed that IL-1 induced the degradation of IκBα efficiently and with nearly identical kinetics in both U251 and U251(MMAC) cells (Fig. 5A). Furthermore, NFκB (p65/RelA) was found to translocate normally to the nucleus after cytokine treatment with no apparent differences between parental and MMAC/PTEN-expressing cells (Fig. 5B). Similar results were observed when the PI 3-kinase inhibitors wortmannin and LY294002 were employed (data not shown). These data would suggest that MMAC/PTEN does not regulate any of the steps that lead to IκB degradation and NFκB nuclear translocation, and this indicates that the PI 3-kinase pathway modulates the DNA binding activity of NFκB through an alternative mechanism.

**Physical Interaction of Akt with IκBα and IKK**—Our results suggest that the PI 3-kinase/Akt pathway regulates the DNA binding activity of NFκB. To identify the underlying mechanisms, we first tested the possibility that the signaling proteins of the PI 3-kinase pathway might physically interact with proteins that are known to function in IL-1-induced NFκB activation. Physical interactions between Akt and IKK were recently reported to be important in TNF- and platelet-derived growth factor-induced NFκB activation (15, 17). We therefore used specific anti-Akt antibodies to immunoprecipitate Akt and any interacting proteins from IL-1-treated U251 and U251(MMAC) cell extracts. Western blotting analysis of the immune complexes from IL-1-treated U251 cell extracts showed that IκBα coprecipitated with Akt and suggested that the two proteins physically interact with each other (Fig. 6A). Consistent with its degradation in stimulated cells, little or no IκBα was detectable in complex with Akt about 15 min after treatment with IL-1. IκBα did, however, reappear in Akt immune complexes after about 60 min of IL-1 treatment. In addition to IκBα, the IκB kinase IKKα was also detectable in Akt immune complexes, and interactions between the three proteins were observed even when anti-phospho-Akt (serine 473) antibodies were used for immunoprecipitation (Fig. 6B). The association of Akt with IκBα and IKKα was found to be inducible in a time- and IL-1-dependent manner (Fig. 6B).

Interestingly, physical interactions between Akt and IκBα (Fig. 6A and B) or Akt and IKKα (Fig. 6B) were inhibited in
the U251(MMAC) cells. Because IL-1-induced Akt phosphorylation was also strongly inhibited in these cells (Fig. 1), it is possible that phosphorylation of Akt is required for its interactions with IκBα and IKKα. Consistent with this possibility, IL-1-induced interactions between Akt and IκBα were also inhibited in U251 cells that were pretreated with the PI 3-kinase inhibitor LY294002 (Fig. 6C).

**IL-1-induced Phosphorylation of NFκB Might Be Required for Its DNA Binding Activity and Can Be Inhibited by MMAC/PTEN.**—The p50 and p65/RelA subunits of NFκB have been shown to be phosphorylated (34–38). Because our results suggested that PI 3-kinase regulates the DNA binding activity of NFκB without involving the IκB degradation pathway, we assessed the possibility that the underpinning mechanism could involve NFκB phosphorylation. IL-1-treated U251 nuclear extracts were incubated with near homogeneous preparations (purity determined by silver staining, data not shown) of the serine/threonine protein phosphatase 2A (PP2A). Phosphatase treatment of nuclear extracts resulted in a significant reduction of IL-1-induced DNA binding activity of NFκB (Fig. 7A), as determined by gel mobility shift assays. Immunoblotting analyses confirmed that the PP2A preparations did not contain any IκB and that the inhibitory effect of PP2A was not attributable to any degradation of the NFκB proteins (data not shown). The effect of PP2A on NFκB is therefore similar to that observed with alkaline phosphatase (23), and it supports a role for phosphorylation in the DNA binding activity of NFκB.

It is possible that MMAC/PTEN inhibits the DNA binding activity of NFκB by interfering with the steps that lead to NFκB phosphorylation. To assess this possibility, we examined the effect of MMAC/PTEN on the in vitro phosphorylation of p50, which is primarily responsible for the DNA binding activity of NFκB. IL-1 was found to strongly induce the phosphorylation of a 50-kDa polypeptide immunoprecipitated from radiolabeled U251 cells with anti-p50 antibodies (Fig. 7B). The phosphorylated 50-kDa band was judged to be p50 NFκB based on Western blotting analyses, in parallel, of immune complexes from unlabeled cells and through the use of a nonspecific control antibody for immunoprecipitation (data not shown). Although still detectable, phosphorylation of p50 NFκB was significa-

nificantly inhibited in U251(MMAC) cells. This observation would support the possibility that MMAC inhibits the DNA binding activity of NFκB by interfering with its phosphorylation on specific IL-1-inducible sites.

**DISCUSSION**

We have investigated the role of PI 3-kinase in cytokine-induced NFκB activation. Our results show that both IL-1- and TNF-induced DNA binding and the transcriptional activities of NFκB were potently inhibited in a glioma cell line that stably expressed MMAC/PTEN. The inhibition of NFκB activation was deemed to be relatively specific because MMAC/PTEN did not interfere with other IL-1-induced signals, such as those that lead to IκBα degradation, NFκB nuclear translocation, or JNK activation. Consistent with its role as a PI 3-kinase antagonist, MMAC/PTEN inhibited the ability of IL-1 to induce Akt phosphorylation in the stable cell line. These observations would suggest that the PI 3-kinase pathway is essential for the activation of NFκB. Various PI 3-kinase inhibitors such as wortmannin, LY294002, and dominant-negative mutants of PI 3-kinase and/or Akt have been used previously to implicate PI 3-kinase in NFκB activation (11, 14, 15, 17, 20).

Whereas PI 3-kinase and Akt are required for NFκB activation, they induce NFκB-dependent reporter gene expression poorly when compared with IL-1 or TNF (11, 13, 14). Our previous studies showed, however, that NFκB-dependent gene expression was synergistically activated when PI 3-kinase-overexpressing cells were stimulated with IL-1 or TNF (11, 13). We had therefore suggested that PI 3-kinase must synergize with other IL-1- or TNF-inducible signals to activate NFκB (11, 13). The results presented in this report and elsewhere (14) indicate that PI 3-kinase does not participate in the pathways that lead to IκB degradation, which may explain its failure to activate NFκB by itself. Furthermore, the synergism between PI 3-kinase and IL-1 or TNF for the induction of NFκB-dependent gene expression could be attributable to the ability of PI 3-kinase-generated signals to cooperate with the IκB degradation pathway. A mechanism of this type, involving the convergence of two or more signals, is unlikely to be involved in the IL-1 signaling pathway for the activation of other transcription factors such as AP-1 (11).

Using two different assays, we have shown that MMAC/PTEN can prevent IL-1 and TNF from activating NFκB. Whereas gel mobility shift assays revealed that MMAC/PTEN blocks IL-1- and TNF-induced increases in the DNA binding activity of NFκB, transient transfection assays showed that MMAC/PTEN inhibited NFκB-dependent gene expression. Because the expression of the reporter gene used in our transient transfection experiments is driven by the NFκB-binding consensus sequences, it cannot be trans-activated in the absence of NFκB or when NFκB binds poorly. Indeed, a mutant reporter
gene that did not bind NFκB was unresponsive to IL-1 in our previous studies (11). We are, therefore, unable to evaluate those mechanisms that are sensitive to inhibition by MMAC/PTEN but that regulate only the trans-activation potential of NFκB. The phosphorylation of serine 529 on p65/RelA is an example of a mechanism that exclusively regulates the transcriptional activity of NFκB (36).

Although MMAC/PTEN inhibited the cytokine-induced activation of NFκB in our studies, it did not interfere with the degradation of IκB nor with the nuclear translocation of p65/RelA, which are two obligatory steps in NFκB activation. These results would underscore the insufficiency of the IκB degradation pathway and indicate that additional PI 3-kinase-dependent signals are required for the ability of NFκB to bind DNA and to trans-activate genes. The identity of such signals, which cooperate with the IκB degradation pathway for NFκB activation, is not entirely clear. There is evidence to suggest, however, that PI 3-kinase-mediated signals might induce site-specific phosphorylation of the p65 and/or p50 NFκB proteins. First, IL-1 and TNF have both been shown to induce the phosphorylation of NFκB (14, 35, 36). Second, the IL-1-induced phosphorylation of p65/RelA and the expression of an NFκB-dependent reporter gene are both induced by the PI 3-kinase inhibitor LY294002 (14). Third, the mutation of serine residues 276 and 529, which are inductibly phosphorylated on p65/RelA, caused the inhibition of NFκB-dependent gene expression (36–38). Whereas serine 529 was shown to be required only for the transcriptional activity of NFκB, the phosphorylation of serine 276 by protein kinase A greatly enhanced the DNA binding affinity of NFκB in vitro experiments (38). The phosphorylation of serine 276 also facilitates the physical interaction of p65/RelA with CREB-binding protein and constitutes a mechanism by which the phosphorylation of NFκB regulates transcriptional activity (38).

In this study, the treatment of nuclear extracts from IL-1-treated cells with the serine/threonine protein phosphatase PP2A drastically reduced the DNA binding activity of NFκB. A similar effect was reported by Naumann and Scheidereit (35), who showed that alkaline phosphatase treatment abolished the DNA binding activity of NFκB. Because recombinant NFκB is capable of binding DNA without modification (for example, see Ref. 38), phosphorylation might serve to enhance the affinity of NFκB for the binding site. Indeed, the phosphorylation of serine 276 greatly increases the binding affinity of NFκB for DNA (38). The incomplete inhibition that we observed in cytokine-induced U251(MAC) cells (Fig. 2) might therefore be reflective of the DNA binding activity of NFκB in its unphosphorylated or hypophosphorylated state.

The possibility that a phosphorylation-dependent mechanism is induced by PI 3-kinase/Akt to regulate NFκB activation is therefore well supported and deserves further investigation. We have shown that the IL-1-induced phosphorylation of p50 NFκB was inhibited in MMAC/PTEN-expressing cells, because LY294002 inhibits the phosphorylation of p65/RelA (14), our data would suggest that the PI 3-kinase pathway induces the phosphorylation of both subunits of NFκB. Although our studies have suggested that the phosphorylation of p50 is involved in the DNA binding activity of NFκB, a supporting role cannot be ruled out for p65/RelA phosphorylation. So far, however, the evidence has linked p65/RelA phosphorylation only to the transcriptional activity of NFκB (14, 20, 36, 37). The logical next step would be to identify the phosphorylation sites on p65/p50 that are regulated by PI 3-kinase and that are involved in the DNA binding and transcriptional activities of NFκB.

The PI 3-kinase/Akt-mediated signals that could lead to NFκB phosphorylation have not been identified so far. Two different studies have recently shown that Akt interacts with IκK. Ozes et al. (15) have noted that whereas the interaction of Akt with IκK is constitutive, the phosphorylation of Akt in IκK immunoprecipitates increases with TNF stimulation. Romashkova and Makarov (17) have also reported interactions between Akt and IκK, although only in cells stimulated with platelet-derived growth factor. Akt phosphorylates threonine 23 on IκKα in vitro, and when overexpressed, an IκKα mutant with alanine at position 23 was able to inhibit the TNF-induced DNA binding activity of NFκB (15). The interaction of IκK with Akt might be important for its activation (17). Our communoprecipitation studies revealed that phosphorylated Akt physically interacted with IκB as well as with IκK and that these interactions were inhibited by MMAC/PTEN. It remains to be determined if Akt directly interacts with either protein and whether these interactions are critical for NFκB phosphorylation and activation.

Targeted gene disruption studies have shown that IκKβ, but not IκKα, is largely responsible for cytokine-induced IκB degradation and for the nuclear translocation of NFκB (39–42). However, when fibroblasts from mice lacking the IκKα gene were stimulated with IL-1 or TNF, there was a significant decrease in the DNA binding activity of NFκB and in the ability of TNF to induce IL-6 and macrophage colony-stimulating factor mRNA (42). These observations raise the interesting possibility that whereas IκKα is dispensable for the IκB degradation pathway, it might be required at another regulatory step in NFκB activation, possibly in cooperation with Akt.

In addition to providing clues about the mechanisms that might be induced by PI 3-kinase/Akt for NFκB activation, these studies have implications for understanding how tumor suppressors, such as MMAC/PTEN, and oncogenes, such as Akt or PI 3-kinase, play a decisive role in cellular survival and oncogenesis.

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