Activation of Nuclear Factor-κB-dependent Transcription by Tumor Necrosis Factor-α Is Mediated through Phosphorylation of RelA/p65 on Serine 529*

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Nuclear factor-κB (NF-κB) is an essential transcription factor in the control of expression of genes involved in immune and inflammatory responses. In unstimulated cells, NF-κB complexes are sequestered in the cytoplasm through interactions with IκBα and other IκB proteins. Extracellular stimuli that activate NF-κB, such as tumor necrosis factor α (TNFα), cause rapid phosphorylation of IκBα at serines 32 and 36. The inducible phosphorylation of IκBα is followed by its ubiquitination and degradation, allowing NF-κB complexes to translocate into the nucleus and to activate gene expression. Previously, it has been shown that TNFα as well as other stimuli also lead to the phosphorylation of the RelA/p65 subunit of NF-κB. In this report, we demonstrate that the TNFα-induced phosphorylation of the RelA/p65 subunit occurs on serine 529, which is in the C-terminal (TA1) transactivation domain. Accordingly, the TNFα-induced phosphorylation of Rel/p65 increases NF-κB transcriptional activity but does not affect nuclear translocation or DNA binding affinity.

NF-κB/Rel transcription factors are key regulators of transcription of a variety of genes involved in immune and inflammatory responses, growth, differentiation, development, and cell death (1–3). NF-κB was originally identified as a nuclear factor that binds to the enhancer element of the immunoglobulin kappa light chain gene (4). To date, eight members of the NF-κB/Rel proteins have been cloned and characterized. They are c-Rel, NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and the Drosophila proteins Dorsal, Dif, and Relish (2). These proteins can form homo- or heterodimers through their N-terminal Rel homology domains, which also function in DNA binding and interaction with inhibitor proteins known as IκBs. The prototypical, inducible NF-κB complex is a heterodimer containing p50 and p65. The C-terminal region of p65 contains a potent transactivation domain that is lacking in p50 (1, 2).

In most cells, NF-κB is inactive due to its cytoplasmic sequestration through interactions with inhibitor proteins IκBs (1, 2). The activation of NF-κB by a wide variety of stimuli such as mitogens, cytokines, bacterial lipopolysaccharide, viral infection, double-stranded RNA, and UV light involves the dissociation of NF-κB from IκB, allowing the nuclear translocation of the transcription factor (1). There are seven members of the IκB family identified: IκBα, IκBβ, IκBγ, Rel3, p105, p100, and IκBε as well as Drosophila IκB protein cactus (2, 6, 7), each of which contains multiple copies of the ankyrin repeat.

Stimulation of cells with inducers such as TNFα leads to rapid phosphorylation, ubiquitination, and degradation of IκBα. NF-κB is therefore released and translocates into the nucleus to activate the expression of target genes (2). Early studies implicated IκB phosphorylation as a crucial step for NF-κB activation, and much attention has been focused on the signal transduction pathway involved with induced phosphorylation of IκB. Recently, it was shown that two highly related serine kinases, IKKα and IKKβ, are induced in response to TNFα treatment and phosphorylate IκBα and IκBβ on critical serine residues known to be required for NF-κB activation (8–12).

Signals that induce phosphorylation of IκBs can also cause the phosphorylation of NF-κB proteins (13–18). For example, p50 is hyperphosphorylated in response to phorbol myristate acetate in Jurkat cells (15). In vitro studies suggest that phosphorylation of p50 and p65 enhances NF-κB DNA binding activity (13, 14). In vivo, the inducible phosphorylation on NF-κB subunits could also be correlated with dimerization, release from IκBs, nuclear translocation, or activation of transcription function of NF-κB. Recent work by Zhong et al. (18) demonstrated that LPS induced the phosphorylation of the p65/RelA subunit on serine 276 and increased its transactivating potential (18). Consistent with reports of others (16), we report here that p65 phosphorylation is rapidly induced upon TNFα stimulation. Using phosphopeptide mapping and site-directed mutagenesis, we identified the inducible phosphorylation site as serine 529 in the C-terminal region of p65. A mutant p65 protein that has a serine 529 to alanine substitution cannot be phosphorylated in response to TNFα stimulation when stably expressed in fibroblasts from p65−/− mice. Our data also demonstrate that inducible phosphorylation on p65 does not affect nuclear translocation or DNA binding ability but functions to increase its transcriptional activity.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells were grown in Dulbecco’s modified Eagle’s medium. Cos cells were grown in Iscove’s minimal essential medium. All media were supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Stable cell lines that express flag-empty, flag-p65, flag-p65(529A), or flag-p65(276A) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and hygromycin (450 µg/ml).

In Vivo Labeling, Phosphoamino Acid Analysis, and Phosphopeptide Mapping—For 32P metabolic labeling, cells were grown in phosphate-free media with 2% serum for 3 h before 32P H2PO4 was added. After 3 h of labeling, the cells were stimulated with TNFα (30 ng/ml) and harvested in cold radioimmunoprecipitation assay buffer (25 mM Tris, pH...
7.6, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) supplemented with phosphatase and protease inhibitors. Whole cell lysates were subjected to immunoprecipitation with p65 antibody, and the precipitated proteins were separated on SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell). Phosphorylated p65 was excised after autoradiography and digested with trypsin or V8 protease (Sigma). The resulting peptides were resuspended in Laemmli SDS loading buffer and resolved on three layer Tris-Tricine gels (19). After electrophoresis, the gel was dried and exposed to x-ray film at −80 °C for 3–10 days.

For phosphoamino acid analysis, excised phosphorylated p65 from the gels was incubated with the appropriate phosphatase and phosphoamino acids were visualized by exposing the plates to x-ray film. Formic acid:acetic acid:H2O = 50:156:1794) followed by 12 min at 1.3 kV in pH 3.5 buffer (pyridine:acetic acid:H2O = 1:10:189). Cold phosphoamino acids were detected by spraying the plates with 0.25% ninhydrin. Hot phosphoamino acid analysis. The inducible p65 phosphorylation was exclusively on serine residues (Fig. 2).

RESULTS

p65 Phosphorylation Is Rapidly Induced on Serine Residue(s) upon TNFα Induction—Although inducible phosphorylation on Leu and Thr plays an important role in NF-κB activation, some NF-κB/Rel proteins are also inducibly phosphorylated (13–18). Consistent with results of others (16), we found that p65 phosphorylation is rapidly induced upon TNFα stimulation in HeLa cells. After treatment with TNFα for 10 min, whole cell lysates were generated and immunoprecipitated with anti-p65 antibodies. A TNFα-induced phosphoprotein of 65 kDa was detected following electrophoresis (Fig. 1, upper panel). The identity of this protein as p65 was confirmed by competition with the peptide against which the anti-p65 antibody was raised (data not shown). The levels of p65 protein did not change in response to TNFα (Fig. 1, lower panel); therefore, quantitative changes in p65 cannot explain the increase in phosphorylation. Additionally, a level of basal phosphorylation of p65 in untreated cells was also detected. Immunoprecipitated p65 from untreated cells or cells treated with TNFα for 10 min was isolated, hydrolyzed with 6 N HCl, and subjected to two-dimensional phosphoamino acid analysis. The inducible p65 phosphorylation was exclusively on serine residues (Fig. 2).

Mapping of the TNFα-induced p65 Phosphorylation Site—To determine a potential function for inducible phosphorylation of p65 in NF-κB activation, it was important for us to identify the site of phosphorylation. We used high resolution Tris-Tricine gel electrophoresis to fractionate the proteolyzed phospho-p65 from untreated HeLa cells or from cells treated with TNFα for 10 min. Trypsin digestion generated a major phosphopeptide of approximately 5 kDa in both untreated and TNFα-treated cells (Fig. 3A), suggesting that inducible phosphorylation occurs on the same peptide as occurs in basal phosphorylation, and possibly on the same serine residue(s). To determine the relative position of the phosphopeptide in p65, the tryptic digestion product was subjected to another round of immunoprecipitation with either C-terminal or N-terminal p65 antibody. The 5-kDa peptide could be specifically recognized by a C-terminal p65 antibody but not by an N-terminal p65 antibody (Fig. 3A, lanes 3 and 4). Therefore, the phosphopeptide is at the C terminus of p65. Since trypsin cleaves after Lys and Arg residues and since the phosphopeptide is approximately 5 kDa, it was reasonable to propose that the target of phosphorylation was in the last 42 amino acids, C-terminal p65 antibody but not by an N-terminal p65 antibody (Fig. 3A, lanes 3 and 4). Therefore, the phosphopeptide is at the C terminus of p65. Since trypsin cleaves after Lys and Arg residues and since the phosphopeptide is approximately 5 kDa, it was reasonable to propose that the target of phosphorylation was in the last 42 amino acids, C-terminal p65 antibody and separated on SDS-PAGE. The phosphorylated proteins were visualized by autoradiography. Lower panel, HeLa cells were treated with TNFα for 10 min. Whole cell extracts were analyzed by Western blot using the anti-p65 antibody.
with TNFα-treated HeLa cells was hydrolyzed with 6 N HCl. The resulting amino acids were separated on two-dimensional thin layer cellulose plates. The dotted areas show the position of the nonradioactive phosphoamino acid standards. The 32P-labeled phosphoamino acids were visualized by autoradiography.

**FIG. 2. Inducible phosphorylation of p65 is on serine residue(s).** Immunoprecipitated p65 from untreated or TNFα-treated HeLa cells was hydrolyzed with 6 N HCl. The resulting amino acids were separated on two-dimensional thin layer cellulose plates. The dotted areas show the position of the nonradioactive phosphoamino acid standards. The 32P-labeled phosphoamino acids were visualized by autoradiography.

**FIG. 3. Mapping the inducible phosphorylation site of p65.** HeLa cells, labeled with 32P for 3 h, were either left untreated or treated with TNFα for 10 min. After the cells were lysed, p65 was immunoprecipitated, separated on SDS-PAGE, and transferred to nitrocellulose membranes. Phosphorylated p65 was excised after autoradiography and digested with trypsin (A) or V8 (B). The resulting phosphopeptide was either separated on high resolution Tris-Tricine gel (A, lanes 1 and 2, and B) or were subjected to another round of immunoprecipitation with C- or N-terminal p65 antibodies before loading on Tris-Tricine gel. C, trypsin and V8 digestion sites in the C-terminal region of p65.

The EMSA experiment identified two complexes that bind to the NF-κB site-containing probe. The lower complex can be almost totally supershifted with a p65 antibody, suggesting this complex contains the p65 subunit (Fig. 6A, lower panel). The upper complex (asterisk) can be almost totally supershifted with a p65 antibody, suggesting this complex contains the p65 subunit (Fig. 6B). The relatively weak supershift by the p50 antibody is likely due to the poor ability of this antibody to recognize the p50-p65 heterodimer. It
is obvious that both wild-type and mutant p65 rapidly translocated to nucleus after TNFα induction and remained there for at least 4 h (Fig. 6A). Additionally, there was no defect in DNA binding for TNFα-induced F-529A. These results demonstrate that the phosphorylation of p65 on serine 529 does not control nuclear translocation or DNA binding affinity.

Previously, it has been shown that the C terminus of p65, which contains serine 529, functions as a strong transactivation domain when fused to heterologous DNA binding domains (24). Phosphorylation on serine 529 may, therefore, regulate the transcriptional activity of p65. To test this possibility, the stable cells expressing flag empty vector, wild-type p65, or F-529A were transiently transfected into Cos cells. After 10 min later, the cells were lysed and immunoprecipitated with the flag M2 antibody.

To further confirm that phosphorylation on serine 529 increases p65 transcriptional activity, we made F-529E, which has a glutamic acid substitution at position 529 to mimic constitutively phosphorylated p65. For unknown reasons, we were unable to stably express F-529E in the p65 null cell line. In transient transfection assays, F-529E activated 3XκBLuc significantly better than F-p65 and F-529A (Fig. 6D). This result indicates that a mutation that mimics phosphorylation at position 529 leads to constitutively enhanced p65 transcriptional activity.

DISCUSSION
Stimulation of cells with TNFα leads to phosphorylation and degradation of IκBα and to subsequent translocation of NF-κB to the nucleus to activate gene-specific transcription. In this paper, and consistent with previous reports (16), we have shown that TNFα also induces phosphorylation on the p65 subunit of NF-κB. Phosphopeptide mapping of TNFα-induced phosphorylated p65 indicates that phosphorylation occurs exclusively on serine 529. By the use of p65 −/− embryonic fibroblasts stably expressing wild-type p65 or the non-phosphorylatable mutant p65, we conclude that TNFα-induced phosphorylation on p65 does not affect its nuclear translocation or DNA binding abilities but increases its transcriptional potential.
Transient transfection experiments with F-529E also indicated that phosphorylation on serine 529 increases p65 transactivation ability.

RelA/p65 contains at least two strong transactivation domains in its C-terminal region (24, 25). Serine 529 is within the TA1 domain, which comprises the last 30 amino acids of p65. TA1 belongs to the class of acidic activators; thus, it is not surprising that the additional negative charge by the phosphate group at serine 529 increases transcriptional potential. The C-terminal transcriptional activation domain of p65 interacts with TBP, TFIIB, and coactivators such as CBP and p300 (26–28). It will be interesting to determine if the phosphorylation on serine 529 potentiates any of these interactions. Also, it remains possible that phosphorylation may interact with other transcription factors and with the ability of NF-κB to disrupt chromatin. Thus, the inducible phosphorylation of p65 may have different effects on different promoters.

The most widely studied mechanism for inducible NF-κB activation is the phosphorylation of IκB on serines located in the N-terminal region of the proteins (2). Different inducers converge on this step that involves IKK activation, which subsequently causes degradation of IκB and nuclear translocation of NF-κB (2). Our data and those of others show that there is a second level of regulation on NF-κB activity: modulation of p65 transactivation potential by additional phosphorylation events. Schmits et al. (17) showed that phosphorylation and transcriptional activity of a defined region within the TA2 domain (90 amino acids adjacent to TA1 domain) were stimulated by phorbol myristate acetate treatment of HeLa cells. Recently, Zhong et al. (18) observed that upon LPS stimulation, the transcriptional activity of p65 was increased after phosphorylation on serine 276, which is in the Rel homology domain of p65. Importantly, we found that a mutant p65 protein with an alanine to serine substitution at position 276 can still be phosphoryl-
ated upon TNFα treatment (Fig. 5). Therefore, it is possible that different inducers can activate different kinases to phosphorylate p65 at distinct sites to modulate its transcriptional activity. Phosphorylation of p65 at serine 276 enhances the ability of this transcription factor to interact with the transcriptional coactivator CBP/p300 (29). Whether these phosphorylation events have the same functional outcome or whether they may lead to distinct functions is presently unclear. Recent data from our laboratory (30) and others (31) demonstrate that several inducers can control the transcriptional function of NF-xB, independent of induced nuclear translocation.

How does TNFα induce phosphorylation of p65? TNFα activates JNK and p38 MAP kinases and previous studies indicated that both SEK/JNK and p38 MAP kinase pathways are involved in NF-xB regulation. It has been reported that JNK can physically associate with c-Rel and activate human immunodeficiency virus-1 long terminal repeat and IL-2Rα promoters (32). However, whether JNK causes p65 phosphorylation is yet to be investigated. Bayaert et al. (33) found that p38 MAP kinase pathway was required for transcriptional induction mediated by NF-xB while having no effect on nuclear translocation or DNA binding of NF-xB. Recently, Vanden Berghe and co-workers (34) showed that p38 and ERK pathways target the transactivation domain of p65 in response to TNFα. All these data suggest that JNK or p38 MAP kinase pathways constitute a second level of regulation of NF-xB activation by modulation of transcriptional function. Whether JNK or p38 can phosphorylate p65 directly or whether they control other kinases to phosphorylate p65 remains unknown. In our studies, we found that SB203580, a p38 inhibitor, did not inhibit TNFα-induced p65 phosphorylation (data not shown). Therefore, JNK or p38 pathways may cause phosphorylation of a distinct component of the transcription pathway to enhance p65 transcription function. Recently, it was reported that casein kinase II can phosphorylate the p65 subunit and that casein kinase II is associated with p65 in vivo (35). How casein kinase II could be modulated to induce the potential phosphorylation of p65 is presently unknown. Transiently transfected p65 activates xB-dependent gene expression without TNFα induction (Fig. 6D and data not shown). One explanation for this is that in transient transfection experiments, cells cannot make enough IxB protein to keep p65 in cytoplasm. Also considering that transiently transfected p65 has high basal phosphorylation on serine 529 (Fig. 4B), it is possible that the kinase that phosphorylates p65 is constitutively active. This kinase may phosphorylate p65 only when it is released from IxB (for example, following IxB degradation or when p65 is overexpressed), appearing to function as an inducible kinase. The identification of the kinase that directly phosphorylates serine 529 of p65 will provide new insight into mechanisms whereby TNFα controls NF-xB activity. Such a kinase activity may prove to be a useful target in treating diseases associated with dysregulation of NF-xB activity.

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