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 IκBαs. 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 IκBαs (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 IκBα, allowing the nuclear translocation of the transcription factor (1). There are seven members of the IκB family identified: IκBα, IκBβ, IκBγ, Bel3, p105, p100, and IκBε as well as Drosophila IκBα 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 IκBα 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 IκBα. 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 IκBαs 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 ability (13, 14). In vivo, the inducible phosphorylation on NF-κB subunits could also be correlated with dimerization, release from IκBαs, 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

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1The abbreviations used are: NF-κB, nuclear factor-κB; TNFα, tumor necrosis factor α; IκBα, inhibitor of kappa B; IKK, IκB kinase; LPS, lipopolysaccharide; EMSA, electrophoretic mobility shift assay; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase.
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 nitrocellulose was incubated with 6 M HCl at 110 °C for 1 h. The resulting amino acids were lyophilized and applied to thin-layer cellulose plates with cold PAA standards (1.0 mg/ml each phosphoserine, phosphothreonine, and phosphotyrosine). Two-dimensional electrophoresis was carried out for 25 min at 1.5 kV in pH 1.9 buffer (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 acids were visualized by exposing the plates to x-ray films at ~80 °C for a week.

**Plasmid Constructs**—For p56, F539, F534, F5354, and F521 were made by cloning different PCR products into the HindIII and EcoRV sites of pFlag-CMV-2 expression vector. The template for the PCR reactions was CMV-p65. The 5′ primer is 1) 5′ GAG AAG CTT GAC GAA CTG TCC CTC AT 3′. The 3′ primers are 2) GTC GAT TTC TTA GGA GCT GTG ACT C (F-p65); 3) GAA GAT ATC GTC CGG AAT (F539); 4) GCG GAT GAA GTC TTC ATC TCC TGC (F534); 5) GCG GAT TTC AGG GAT ACG TCC CAG GCC CAG TGC AG (F5354); and 6) GCG GAT TTC AGG GAT ACG TCC CAG GCC CAG TGC AG (F521).

**Electrophoretic Mobility Shift Assay and Western Blot Assay**—Nuclear and cytoplasmic extracts were prepared as described previously (20). EMSA were performed as previously detailed (21). The DNA probe (5′-CTGGGGAGATTCCCTCCATCGACCTTCACGCT-3′) contains the NF-κB binding site (underlined) from the MHC class I H-2k β2m plays an important role in NF-κB activation; some NF-κB/Rel proteins are also inducibly phosphorylated (13–18). Although 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 trypsin 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 phosphate is approximately 5 kDa, it was reasonable to propose that the target of phosphorylation was in the last 42 amino acids, C-terminal to Arg-509, and that its presence was dependent on the time of protease digestion (data not shown). Digestion of 32P-labeled p65 from untreated cells and cells treated with TNFα yielded a major phosphopeptide of approximately 4 kDa (Fig. 3B). This peptide could also be recognized by a C-terminal p65 antibody (data not shown). V8 cleaves the peptide bond after Glu; therefore, the only way this protease can generate a 4-kDa peptide at the C terminus of p65 was by cleaving at Glu-498 and Glu-532 (Fig. 3C). The overlapping region of the trypsin and V8 phosphopeptides is from Arg-509 to Glu-532, and in this region only one serine, Ser-529, is found (Fig. 3C). This result suggests that inducible phosphorylation occurs on the same Serine residue as occurs in basal phosphorylation, and possibly on the same serine residue(s). To determine the relative position of the phosphopeptide in p65, the trypsin 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 phosphate is approximately 5 kDa, it was reasonable to propose that the target of phosphorylation was in the last 42 amino acids, C-terminal to Arg-509, and that its presence was dependent on the time of protease digestion (data not shown). Digestion of 32P-labeled p65 from untreated cells and cells treated with V8 yielded a major phosphopeptide of approximately 4 kDa (Fig. 3B). This peptide could also be recognized by a C-terminal p65 antibody (data not shown). V8 cleaves the peptide bond after Glu; therefore, the only way this protease can generate a 4-kDa peptide at the C terminus of p65 was by cleaving at Glu-498 and Glu-532 (Fig. 3C). The overlapping region of the trypsin and V8 phosphopeptides is from Arg-509 to Glu-532, and in this region only one serine, Ser-529, is found (Fig. 3C). The results of phosphopeptide mapping also indicated that the TNFα-inducible phosphorylation site of p65 is the same as the basal phosphorylation site (data not shown). This data indicate that serine 529 is the major site of phosphorylation on p65 in response to TNFα stimulation.

**Mutation Analysis Confirms Serine 529 as the Major Site of p65 Phosphorylation**—To confirm that serine 529 of p65 is the phosphosites, we therefore made a number of expres-
with TNFα.

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 acids 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 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.

A

2nd IP: - - N-ab C-ab

TNFα: - - - +

B

6.5K

3.4K

2.3K

3

4

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.

C

491 PMLMEYPEAI TRLVTGAQRP PDPAPAPLG

PGLPNGLLSG DEDFSSIADM DFSSALLSQIS S 551

: Trypsin digestion sites

: V8 digestion sites (in ammonium bicarbonate, pH 7.8)

To demonstrate that serine 529 is the TNFα-induced phosphorylation site, we made another flag-tagged p65 mutant (F-529A). F-529A expresses full-length p65 but has an alanine to serine substitution at 529. Since in Cos cells, transiently transfected p65 has high basal phosphorylation (Fig. 4), we stably expressed F-p65 and F-529A into p65 −/− embryonic fibroblasts. The stable cells that contain the flag empty vector, F-p65 or F-529A, were labeled with [32P]orthophosphate and stimulated with TNFα for 10 min. After cells were harvested, the whole cell extracts were subjected to immunoprecipitation with flag antibody. The proteins were then separated by SDS-PAGE and visualized by autoradiography (Fig. 5, upper panel). The results demonstrate that TNFα-induced phosphorylation only occurred with wild-type p65 (Fig. 5, lanes 3 and 4, lower bands, upper panel), while F-529A exhibited no detectable phosphorylation in response to TNFα induction (Fig. 5, lanes 5 and 6, upper panel). Immunoblotting analysis indicated that the lack of phosphorylation of F-529A was not due to reduced protein levels (Fig. 5, lower panel). Recently, it was reported that serine 276 of p65 was phosphorylated by protein kinase A after LPS induction (18). It was therefore possible that different inducers target distinct sites on p65 for phosphorylation. To test this hypothesis, we made a flag-tagged p65 mutant (F-276A) that has an alanine to serine substitution at 276. When stably expressed in p65 −/− cells, this mutant still can be phosphorylated in response to TNFα induction (Fig. 5, lanes 7 and 8, upper panel), suggesting that TNFα- and LPS-induced phosphorylation sites on p65 are distinct. It is noted that high levels of expression of the alanine 276 mutant leads to enhanced basal phosphorylation, similar to results obtained in Fig. 4B.

TNFα-induced Phosphorylation on Serine 529 Increases p65 Transcriptional Activity—To determine the possible role for TNFα-induced phosphorylation relative to p65 function, we utilized the stable cell lines expressing F-p65 or F-529A. The cells were treated with TNFα for various times, and nuclear extracts were collected for EMSA (Fig. 6A, upper panel) and Western blot analysis with p65 antibody (Fig. 6A, lower panel). The EMSA experiment identified two complexes that bind to the NF-κB site-containing probe. The lower 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 alanine 529 were transiently transfected with a 3xGLuc reporter, which contains three copies of kB binding site. To assay transcription function, TNFα was added to the cells, and whole cell lysates were made for luciferase assays. As shown in Fig. 6C, TNFα activated 3xGLuc activity in the cells expressing wild-type p65 but had little or no effect in the cells expressing mutant (alanine 529) p65, indicating that serine 529 of p65 is the target for TNFα and that the inducible phosphorylation on this site increases p65 transcriptional activity. The higher basal transcription in the cells expressing F-p65 or F-529A compared with the cells expressing flag empty vector may be due to nuclear NF-kB in the untreated cells. This result also excluded the possibility that F-529A acts as a dominant negative of other members of the NF-kB family. The NF-kB responsive human immunodeficiency virus-long terminal repeat fused to a chloramphenicol acetyltransferase reporter was activated by TNFα in the F-p65 cells but not in the F-529A-expressing cells (data not shown). Thus, mutation of serine 529 significantly inhibits the ability of TNFα to activate transient NF-kB-dependent transcription.

To further confirm that phosphorylation on serine 529 increases p65 transcription 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 3xGLuc 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 IkBα and to subsequent translocation of NF-kB 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-kB. Phosphopeptide mapping of TNFα-induced phosphorylated p65 indicates that phosphorylation occurs exclusively on serine 529. By the use of p65 Δ/Δ 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 its transcriptional potential. The C-terminal transcriptional activation domain of p65 interacts with TBP, TFIIIB, 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 involve interaction 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 IκBs 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 IκBs 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 transactivation 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-κB, 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-κB regulation. It has been reported that JNK can physically associate with c-Rel and activate human immunodeficiency virus-1 long terminal repeat and IL-2R 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-κB while having no effect on nuclear translocation or DNA binding of NF-κB. 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-κB activation by modulation of transcription 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 transactivation 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 κB-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 IκB 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 IκB (for example, following IκB 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-κB activity. Such a kinase activity may prove to be a useful target in treating diseases associated with dysregulation of NF-κB activity.

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