THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 45, Issue of November 6, pp. 29411–29416, 1998
Printed in U.S.A.

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

(Received for publication, May 6, 1998, and in revised form, August 12, 1998)

Dan Wang and Albert S. Baldwin, Jr.‡

From the Lineberger Comprehensive Cancer Center and Department of Biology, CB 7295, University of North Carolina, Chapel Hill, North Carolina 27599-7295

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[1]/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, Bcl3, 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 IkBβ 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 ability (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 ^{32}P metabolic labeling, cells were grown in phosphate-free media with 2% serum for 3 h before ^{32}P H_3PO_4 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

* This work was supported by National Institutes of Health Grant AI35098 (to A. S. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 919-966-3652; Fax: 919-966-0444; E-mail: jhall@med.unc.edu.

[1] The abbreviations used are: NF-κB, nuclear factor-κB; TNFα, tumor necrosis factor α; IκB, 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 N 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:H_2O = 50:156:1794) followed by 12 min at 1.3 kV in pH 3.5 buffer (pyridine:acetic acid:H_2O = 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—F-p65, F539, F534, FΔ534, and F521 were made by cloning different PCR products into the *Hin*dIII and *Eco*RV sites of pFlag-CMV-2 expression vector. The template for the PCR reactions is CMV-p65. The 5′ primer is 1) 5′ GAC AAG CTT GAC GAA CTG TTC CCC CTC AT 3′. The 3′ primers are 2) GTC GAT ATC TTA GGA GCT GAT CTG ACT C (F-p65); 3) GAA GAT ATC GTC CGC AAT GGA GGA GAA GT (F539); 4) GCG GAT ATC GAA GTC TTC ATC TCC TGA AAG GA (F534); 5) GCG GAT ATC GAA GTC TTC ATC TCC TGC AAG G (FΔ534); and 6) GCG GAT ATC CGG GGC CCC CAG TGG AGC AG (F521).

F-529A, F-276A, and F-529E were made by site-directed mutagenesis using two-staged PCR. The template for the first round PCR is CMV-p65. The primers are: 1, 2, and 7) 5′ CTT CAT CTC C**TG C**AA GGA GGC C 3′; 8) 5′ GGC CTC CTT **GCA** GGA GAT GAA G 3′ (F-529A); 9) 5′ CTT CAT CTC C**TT C**AA GGC C 3′; 10) 5′ GGC CTC CTT **GAA** GAT GAA G 3′ (F-529E); 11) 5′ GCG GCG GCC T**GC C**GA CCG GGA G 3′; and 12) 5′ CTC CCG GTC **GGC** AGG CCG CCG C 3′(F-276A) (boldface type indicates the nucleotides that encode mutated amino acids). The products of second round PCR were cloned into the *Hin*dIII and *Eco*RV sites of the Flag-CMV-2 vector.

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′CAGGGCTGGGGATTCCCCATCTCCACAGTTTCACTTC 3′) contains the NF-κB binding site (underlined) from the MHC class I *H-2*κ^b gene. For Western blotting, a total of 50 μg of whole cell lysates or nuclear extracts were separated on a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose, and blots were blocked with 5% dry milk and then probed with anti-p65 antibodies (Rockland, Inc., Boyertown, PA). After the blots were incubated with secondary antibody, ECL reagents (Amersham) were used to detect the proteins.

Transient Transfection and Luciferase Assay—Cells were transfected using lipofectAMINE (Life Technologies, Inc.) (22). For TNFα induction, cells were grown in Dulbecco's modified Eagle's medium with 0.1% serum for 36 h after incubation with LipofectAMINE/DNA mixture. TNFα (10 ng/ml) was added to the cells 6–10 h before harvest. Luciferase assays were performed by methods described elsewhere (23).

For stable transfection, a vector (pHygro) encoding the hygromycin resistance protein was cotransfected with flag expression vectors. The individual stable clones were selected by adding 450 μg/ml hygromycin to the media. The expression of p65 proteins was quantified by Western blot method.

RESULTS

p65 Phosphorylation Is Rapidly Induced on Serine Residue(s) upon TNFα Treatment—Although inducible phosphorylation on IκBs 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 from ^{32}P metabolically labeled HeLa cells 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).

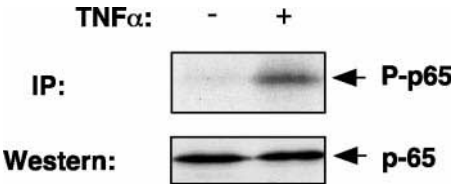

FIG. 1. **p65 phosphorylation is rapidly induced upon TNFα induction.** *Upper panel*, HeLa cells were labeled with [^{32}P]orthophosphate for 3 h and were either left untreated or were treated with TNFα for 10 min. After harvest, whole cell lysates were immunoprecipitated with anti-p65 antibody and 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.

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. 3*A*), 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. 3*A*, *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 to Arg-509 (Fig. 3*C*). The other phosphopeptides were likely to result from partial digestion, since their presence was dependent on the time of protease digestion (data not shown). Digestion of ^{32}P-labeled p65 from untreated cells and cells treated with V8 yielded a major phosphopeptide of approximately 4 kDa (Fig. 3*B*). 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. 3*C*). The overlapping region of the tryptic and V8 phosphopeptides is from Arg-509 to Glu-532, and in this region only one serine, Ser-529, is found (Fig. 3*C*). 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). These 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 phosphorylation site, we therefore made a number of expres-

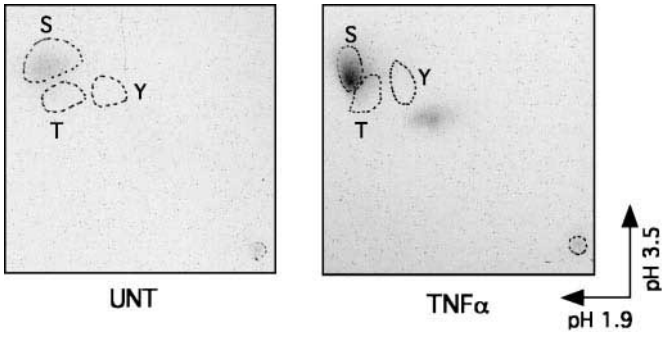

FIG. 2. **Inducible phosphorylation of p65 is on serine residue(s).** Immunoprecipitated p65 from untreated or TNFα-treated HeLa cells was hydrolyzed with 6 N HCl. The resulting amino acids were separated on two-dimensional thin layer cellulose plates. The *dotted areas* show the position of the nonradioactive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

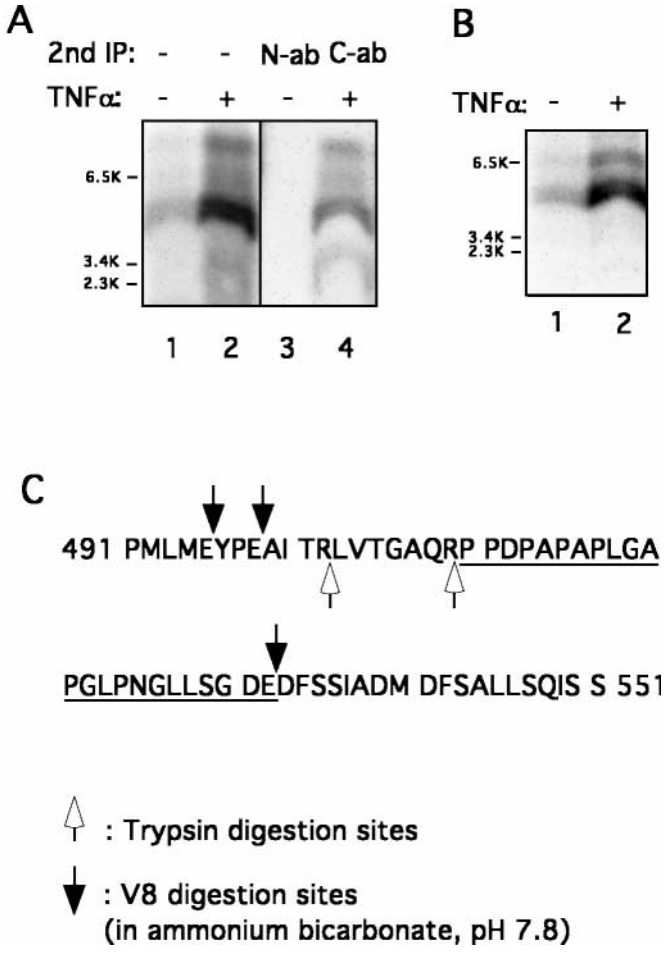

FIG. 3. **Mapping the inducible phosphorylation site of p65.** HeLa cells, labeled with ^{32}P for 3 h, were either left untreated or treated with TNFα for 10 min. After the cells were lysed, p65 was immunoprecipitated, separated on SDS-PAGE, and transferred to nitrocellulose membranes. Phosphorylated 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.

sion vectors encoding flag-tagged, C-terminal truncated p65 mutants (Fig. 4*A*) to determine if these proteins can be phosphorylated when expressed in Cos cells. After metabolically labeling the transfected cells with [^{32}P]orthophosphate, whole cell lysates were made and subjected to immunoprecipitation with the flag antibody. As expected, flag-tagged wild-type p65 (F-p65) was phosphorylated (Fig. 4*B*) as were F539 and F534 which express flag-tagged truncated p65 proteins from amino acids 2–539 and 534, respectively. FΔ534, which encodes the same length p65 protein as F534 except has a serine to alanine substitution at 529, cannot be phosphorylated. In addition, the mutant F521 lacks the last 30 amino acids of p65 and was incapable of being phosphorylated. Immunoblotting results (not shown) demonstrated that F521 expressed the highest amount of protein while F-p65 expressed the least. The higher levels of phosphorylation of F539 and F534 are due to higher levels of expression of these proteins relative to F-p65. It is obvious that whenever serine 529 was deleted or mutated, the mutant p65 was no longer phosphorylated, suggesting serine 529 of p65 (Fig. 4*A*, *underlined*) is the target for phosphorylation. Interestingly, the phosphorylation of p65 in Cos cells was largely constitutive, although some weak induction by TNFα was observed. This result suggests that the kinase that phosphorylates p65 has the potential to function constitutively, at least in Cos cells (see "Discussion").

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 [^{32}P]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. 4*B*.

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. 6*A*, *upper panel*) and Western blot analysis with p65 antibody (Fig. 6*A*, *lower panel*). The EMSA experiment identified two complexes that bind to the NF-κB site-containing probe. The lower complex can be totally supershifted by a p50 antibody, suggesting it is the p50·p50 homodimer. The upper complex (*asterisk*) can be almost totally supershifted with a p65 antibody, suggesting this complex contains the p65 subunit (Fig. 6*B*). 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

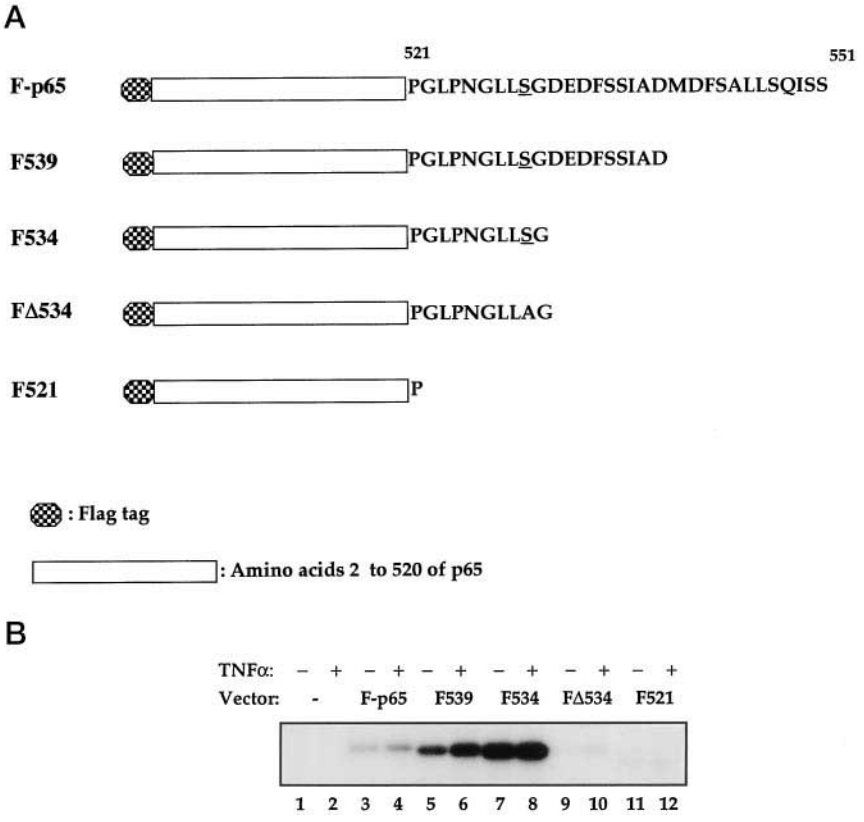

FIG. 4. **Mutant analysis of p65 phosphorylation.** The serial C-terminal deletion mutants of human p65 cDNA were cloned into the Flag-CMV2 vector. The C-terminal amino acid sequences of the resulting mutant proteins are shown in *A*. *B*, the flag-tagged vectors were transiently transfected into Cos cells. After the cells were labeled with ^{32}P for 3 h, TNFα was added. 10 min later, the cells were lysed and immunoprecipitated with the flag M2 antibody.

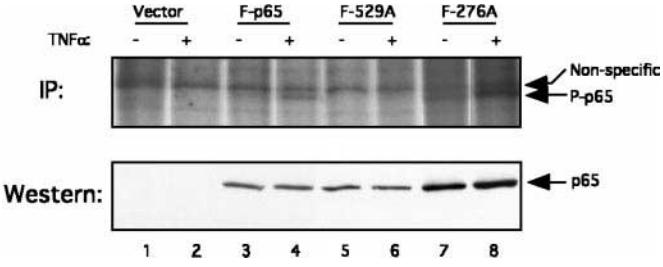

FIG. 5. **Serine 529 is a TNFα-inducible phosphorylation site of p65.** Flag empty vector, F-p65, F-529A, or F-276A was stably transfected into p65 null embryonic fibroblasts. The stable cells were labeled with [^{32}P]orthophosphate. TNFα was added 10 min before harvest. The cells were lysed in 600 μl of cold radioimmunoprecipitation assay buffer. *Upper panel*, 580 μl of the lysates were subjected to immunoprecipitation with flag antibody. *Lower panel*, 20 μl of the lysates were analyzed by Western blot with anti-p65 antibody.

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. 6*A*). 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 3XκBLuc reporter, which contains three copies of κB 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. 6*C*, TNFα activated 3XκBLuc 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-κB in the untreated cells. This result also excluded the possibility that F-529A acts as a dominant negative of other members of the NF-κB family. The NF-κB 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-κB-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 3XκBLuc significantly better than F-p65 and F-529A (Fig. 6*D*). 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-phosphorylated 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.

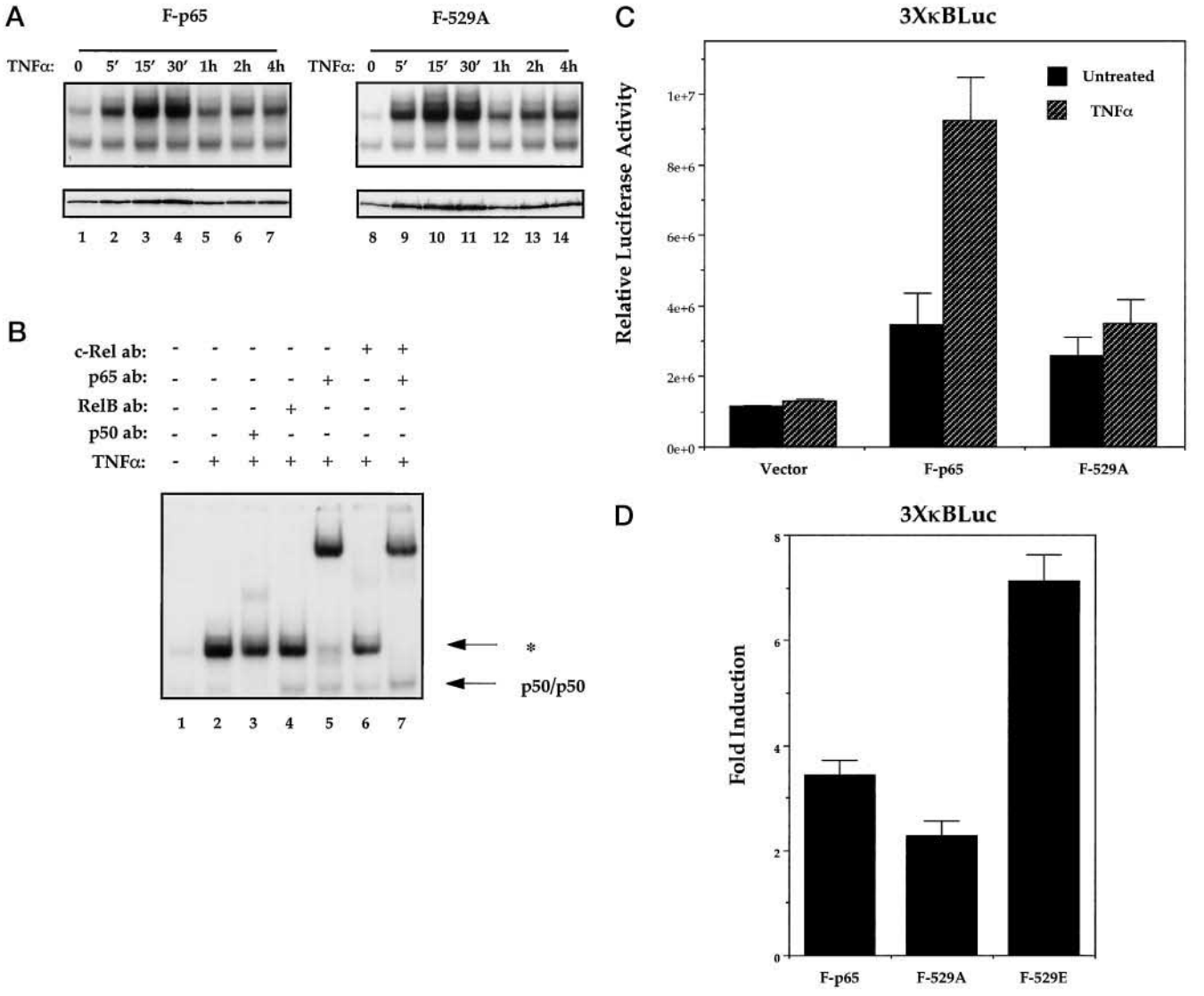

FIG. 6. **Transcriptional activity of p65 is stimulated by phosphorylation on serine 529.** *A*, the stable cells that express F-p65 (*left*) or F-529A (*right*) were either left untreated or treated with 10 ng/ml TNFα for varying times as indicated. *Upper*, the nuclear extracts were analyzed by EMSA with ^{32}P- labeled NF-κB site containing DNA probe. *Lower*, the nuclear extracts were analyzed by Western blot with p65 antibody. *B*, nuclear extracts from untreated and TNFα-treated cells were incubated with ^{32}P-labeled NF-κB site containing probe, and antibodies against different NF-κB subunits were added. The reaction mixtures were electrophoresed on 5% non-denaturing gel. *C*, the stable cells that express flag empty vector, F-p65, or F-529A were transiently transfected with 3XκBLuc reporter, which contains three copies of κB binding site. The cells were stimulated with 10 ng/ml TNFα for 6–10 h before harvest. After cell lysis, luciferase activities were measured. *D*, flag empty vector, F-p65, F-529A, or F-529E (10 ng of each) was cotransfected with 3 μg of 3XκBLuc reporter into p65 null cells. 36 h after transfection, the cells were harvested, and luciferase activities were measured. Fold induction was calculated relative to the luciferase activity when cells were cotransfected with flag empty vector and 3XκBLuc reporter.

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, 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 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 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-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-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-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. 6*D* 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. 4*B*), 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.

Acknowledgments—We thank Dr. Tim Finco for help in initiating this work and for encouragement, Dr. Shelley Earp and Ruth Dy for advice and technical assistance with the phosphoamino acid analysis, and Dr. Denis Guttridge for critical reading of the manuscript. We gratefully acknowledge Dr. Amer Beg for kindly providing p65 null fibroblasts.

REFERENCES

1. Baeuerle, P. A., and Henkel, T. (1994) *Annu. Rev. Immunol.* **12,** 141–179
2. Baldwin, A. S., Jr. (1996) *Annu. Rev. Immunol.* **14,** 649–681
3. Baeuerle, P. A., and Baltimore, D. (1996) *Cell* **87,** 13–20
4. Sen, R., and Baltimore, D. (1986) *Cell* **46,** 705–716
5. Beg, A. A., Ruben, S. M., Scheinman, R. I., Haskill, S., Rosen, C. A., and Baldwin, A. S., Jr. (1992) *Genes Dev.* **6,** 1899–1913
6. Whiteside, S. T., Epinat, J., Rice, N. R., and Israël, A. (1997) *EMBO J.* **16,** 1413–1426
7. Li, Z., and Nabel, G. J. (1997) *Mol. Cell. Biol.* **17,** 6184–6190
8. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) *Nature* **388,** 548–554
9. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J. W., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) *Science* **278,** 860–866
10. Régnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z., and Rothe, M. (1997) *Cell* **90,** 373–383
11. Woronicz, J. D., Gao, X., Cao, Z., Rothe, M., and Goeddel, D. V. (1997) *Science* **278,** 866–869
12. Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., and Karin, M. (1997) *Cell* **91,** 243–252
13. Naumann, M., and Scheidereit, C. (1994*) EMBO J.* **13,** 4597–4607
14. Li, C., Dai, R., Chen, E., and Longo, D. L. (1994) *J. Biol. Chem.* **269,** 30089–30092
15. Li, C. H., Korner, M., Ferris, D. K., Chen, E., Dai, R., and Longo, D. L. (1994) *Biochem. J.* **303,** 499–506
16. Diehl, J. A., Tong, W., Sun, G., and Hannink, M. (1995) *J. Biol. Chem.* **270,** 2703–2707
17. Schmitz, M. L., dos Santos Silva, M. A., and Baeuerle, P. A. (1995) *J. Biol. Chem.* **270,** 15576–15584
18. Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997) *Cell* **89,** 413–424
19. Schägger, H., and Von Jagow, G. (1987) *Anal. Biochem.* **166,** 368–379
20. Beg, A. A., Finco, T. S., Nantermet, P. V., and Baldwin, A. S., Jr. (1993) *Mol. Cell. Biol.* **13,** 3301–3310
21. Haskill, S., Beg, A. A., Tompkins, S. M., Morris, J. S., Yurochko, A. D., Sampson-Johannes, A., Mondal, K., Ralph, P., and Baldwin, A. S., Jr. (1991) *Cell* **65,** 1281–1289
22. Wang, D., Mayo, M. W., and Baldwin, A. S., Jr. (1997) *Oncogene* **14,** 2291–2299
23. Cogswell, P. C., Mayo, M. W., and Baldwin, A. S., Jr. (1997) *J. Exp. Med.* **185,** 491–497
24. Schmitz, M. L., and Baeuerle, P. A. (1991) *EMBO J.* **10,** 3805–3817
25. Moore, P. A., Ruben, S. M., and Rosen, C. A. (1993) *Mol. Cell. Biol.* **13,** 1666–1674
26. Schmitz, M. L., Stelzer, G., Altmann, H., Meisterernst, M., and Baeuerle, P. A. (1995) *J. Biol. Chem.* **270,** 7219–7226
27. Perkins, N. D., Felzien, L. K., Betts, J. C., Leung, K., Beach, D. H., and Nabel, G. J. (1997) *Science* **275,** 523–527
28. Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997) *Proc. Natl. Acad. Sci.* U. S. A. **94,** 2927–2932
29. Zhong, H., Voll, R., and Ghosh, S. (1998) *Mol. Cell* **1,** 661–671
30. Finco, T. S., Westwick, J. K., Norris, J. L., Beg, A. A., Der, C. J., and Baldwin, A. S., Jr. (1997) *J. Biol. Chem.* **272,** 24113–24116
31. Yoza, B. K., Hu, J. Y. Q., and McCall, C. E. (1996) *J. Biol. Chem.* **271,** 18306–18309
32. Meyer, C. F., Wang, X., Chang, C., Templeton, D., and Tan, T.-H. (1996*) J. Biol. Chem.* **271,** 8971–8976
33. Beyaert, R., Cuenda, A., Vanden Berghe, W., Plaisance, S., Lee, J. C., Haegeman, G., Cohen, P., and Fiers, W. (1996) *EMBO J.* **15,** 1914–1923
34. Vanden Berghe, W., Plaisance, S., Boone, E., De Bosscher, K., Schmits, M. L., Fiers, W., and Haegeman, G. (1998) *J. Biol. Chem.* **273,** 3285–3290
35. Bird, T. A., Schooley, K., Dower, S. K., Hagen, H., and Virca, G. D. (1997) *J. Biol. Chem.* **272,** 32606–32612