THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 42, Issue of October 20, pp. 32592–32597, 2000

Printed in U.S.A.

Tumor Necrosis Factor α-induced Phosphorylation of RelA/p65 on Ser^{529} Is Controlled by Casein Kinase II*

Received for publication, February 18, 2000, and in revised form, August 9, 2000
Published, JBC Papers in Press, August 9, 2000, DOI 10.1074/jbc.M001358200

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Nuclear factor κB (NF-κB)/Rel transcription factors are key regulators of a variety of genes involved in immune and inflammatory responses, growth, differentiation, apoptosis, and development. In unstimulated cells, NF-κB/Rel proteins are sequestered in the cytoplasm by IκB inhibitor proteins. Many extracellular stimuli, such as tumor necrosis factor α (TNFα), cause rapid phosphorylation of IκB at N-terminal serine residues leading to ubiquitination and degradation of the inhibitor. Subsequently, NF-κB proteins translocate to the nucleus and activate gene expression through κB response elements. TNFα, as well as certain other stimuli, also induces the phosphorylation of the NF-κB proteins. Previously, we have shown that TNFα induces RelA/p65 phosphorylation at serine 529 and that this inducible phosphorylation increases NF-κB transcriptional activity on an exogenously supplied reporter (1). In this report, we demonstrate that casein kinase II (CKII) interacts with p65 *in vivo* and can phosphorylate p65 at serine 529 *in vitro*. A CKII inhibitor (PD144795) inhibited TNFα-induced p65 phosphorylation *in vivo*. Furthermore, our results indicate that the association between IκBα and p65 inhibits p65 phosphorylation by CKII and that degradation of IκBα allows CKII to phosphorylate p65 to increase NF-κB transactivation potential. These data may explain the ability of CKII to modulate cell growth and demonstrate a mechanism whereby CKII can function in an inducible manner.

The NF-κB[1]/Rel transcription factors play critical roles in regulating the expression of a variety of genes involved in immune and inflammatory responses, cell proliferation, and apoptosis (2, 3). NF-κB was first identified as a constitutively active transcription factor that binds to the immunoglobulin κ light chain enhancer in mature B cells (4). There are five members of the mammalian Rel family of proteins that have been cloned and characterized: RelA/p65, c-Rel, NF-κB1 (p50/p105), NF-κB2 (p52/p100), and RelB (2, 3, 5). Each of these proteins is characterized by a Rel homology domain (RHD), which is involved in dimerization, DNA binding, interactions with IκB, and nuclear localization. Several, but not all, of the NF-κB proteins contain transcriptional activation domains, which promote interactions with basal transcription components or with transcriptional co-activators. For example, RelA/p65 contains at least two transactivation domains in the C-terminal region and an element in the RHD involved with recruitment of co-activators (2, 3, 6).

In most unstimulated cells, NF-κB is sequestered in the cytoplasm by IκB proteins that mask the nuclear localization sequence of NF-κB (7–10). In response to various stimuli, the IκB kinase (IKK) signaling cascade is activated, leading to the phosphorylation of the N-terminal serine residues Ser^{32} and Ser^{36} of IκBα (11–15). This phosphorylation promotes ubiquitination of the IκB proteins, which are subsequently targeted for rapid degradation via the 26 S proteasome (2, 16). The degradation of IκB then promotes nuclear translocation of NF-κB. In the nucleus, NF-κB is a positive regulator of genes involved in immune and inflammatory processes and cell proliferation. Regarding the latter point, NF-κB is now known to regulate transcription of cyclin D1 and c-Myc, to be activated by a variety of oncoproteins, and to be required for oncogenesis or tumorigenesis in different settings (17).

Interestingly, nuclear translocation and DNA binding are apparently not sufficient to activate an NF-κB-dependent reporter. For example, it has been shown that inhibition of tyrosine kinase signaling inhibits the ability of interleukin-1 to activate an NF-κB-dependent reporter but does not block nuclear translocation and DNA binding of NF-κB (18). Additionally, it has been shown that inhibition of the p38 mitogen-activated protein kinase pathway by the use of a small molecule inhibitor blocks reporter activity but does not affect nuclear translocation of NF-κB (19). Consistent with these observations, it has been found that signals that activate NF-κB can also cause the phosphorylation of NF-κB molecules (6, 20–26). *In vitro* studies suggest that phosphorylation of p50 or p65 enhances NF-κB DNA binding ability (21, 23, 27). Work by Zhong *et al.* (6) demonstrated that LPS induces PKA-dependent phosphorylation of p65, increasing NF-κB transcriptional potential in B and T cells. This phosphorylation leads to the recruitment of the transcriptional coactivators CBP and p300 (28). In this case, the catalytic subunit of PKA is found associated with NF-κB, and PKA can phosphorylate p65 following IκB degradation. Another group has provided evidence that IKK can phosphorylate RelA/p65 on serine 536 (29). Recently, we have reported that TNFα treatment of fibroblasts and HeLa cells leads to phosphorylation of p65 on serine 529, which leads to increased transcriptional potential (1). Interestingly, the sequence surrounding serine 529 fits the consensus site for casein kinase II (CKII) phosphorylation.

CKII is a serine/threonine kinase, which is evolutionarily

* This work was supported by National Institutes of Health Grants AI35098 and CA72771 (to A. S. B.) and by an American Cancer Society postdoctoral fellowship (to S. D. W.). 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.

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[1] The abbreviations used are: NF-κB, nuclear factor κB; RHD, Rel homology domain; IκB, inhibitor of κB, TNFα, tumor necrosis factor α; IKK, IκB kinase; CKII, casein kinase II; PKA, protein kinase A; LPS, lipopolysaccharide; PAGE, polyacrylamide gel electrophoresis; CBP, CREB binding protein.

conserved from yeast to human (30). In most organisms, CKII is a tetramer composed of two α (and/or α') and two β subunits (31, 32). Disruption of both genes encoding α and α' subunits of CKII in yeast results in a complete loss of cell viability (33), indicating that CKII is essential. The consensus sequence for CKII phosphorylation is serine (or threonine)-*X*-*X*-acidic, where the acidic amino acid could be glutamic acid, aspartic acid, phosphoserine, or phosphotyrosine (30). To date, more than 100 proteins have been found to be substrates of CKII, including several transcription factors. Phosphorylation of transcription factors by CKII can affect their nuclear transport, DNA binding, or transactivation abilities (30). For example, phosphorylation of c-Jun by CKII negatively affects AP-1 DNA binding ability (34), while PU.1 phosphorylation by CKII enables its interaction with another transcription factor, NF-EM5, to activate transcription (35). Recently, it has been shown that overexpression of CKII enhances cell proliferation (36) and tumor formation in a p53 null setting (37), providing compelling evidence for a role for CKII in oncogenesis and cell growth.

CKII has been proposed to regulate NF-κB activity through modulation of IκB. Thus, several laboratories have reported that CKII constitutively phosphorylates the C-terminal PEST region of IκBα (38–41). It was proposed that this phosphorylation is required for the basal turnover of the IκBα protein (39, 41). However, others found that the C-terminal region of IκBα is dispensable for its degradation (42). CKII also causes inducible phosphorylation of IκBα, since it was found that CKII phosphorylates serine 32 of IκBα in response to TNFα or okadaic acid treatment of HeLa cells. Relevant to phosphorylation of RelA/p65, Bird *et al.* (44) have reported that a kinase with properties consistent with CKII activity associates with RelA/p65 in fibroblasts and hepatoma cells and that purified CKII can phosphorylate p65 *in vitro*.

In this study, we report that CKII phosphorylates p65 at serine 529 following TNFα stimulation, although overall CKII activity is only weakly increased in HeLa cells. We provide evidence that, in unstimulated cells, the association of IκBα with NF-κB inhibits CKII phosphorylation of p65. Signal-induced degradation of IκBα releases this inhibition and enables CKII to phosphorylate p65 on serine 529. These results provide a rationale to explain the involvement of CKII with oncogenesis and cell proliferation and offer a mechanism to explain inducible CKII activity.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells and the stable cells that either express F-p65 or F-529A (1) were grown in Dulbecco's modified Eagle's medium. All media were supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

Immunoprecipitation—Cell lysates were made in cold radioimmunoprecipitation assay buffer (1). Whole cell lysates were subjected to immunoprecipitation with Flag M2-conjugated beads or CKII antibody (Upstate Technologies) in protein A beads. The precipitated proteins were separated in SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell) for Western blot analysis. For the kinase assays, the precipitated proteins were boiled in the presence of 1% SDS and 10 mM Tris-HCl (pH 7.6) for 5 min and subjected to another round of immunoprecipitation before use in the kinase assay.

Electrophoretic Mobility Shift Assay and Western Blot Assay—Nuclear and cytoplasmic extracts were prepared as described previously (45). Electrophoretic mobility shift assay was performed as previously detailed (46). The DNA probe (1) contains the NF-κB binding site from the major histocompatibility complex class I H-2κ^b gene. Western blot analysis was performed by methods described elsewhere (1).

Transient Transfection and Luciferase Assay—HeLa cells were transfected using SuperFect (Promega). For each transfection, 5 μg of 3XκBluc plasmid was incubated with 30 μl of SuperFect. Six hours before harvest, TNFα (10 ng/ml) was added to the cells that have been pretreated with PD144795 (gift of Dr. S. Hunt, Parke-Davis Pharmaceuticals, Ann Arbor, MI) or Me_2SO for 1 h. Luciferase assays were performed as described previously (47).

Plasmid Construct and Purification of Bacterially Expressed p65—Plasmid pRSET-p65 was made by cloning the polymerase chain reaction product into the *Bam*HI site of pRSET B (Invitrogen). The template for the polymerase chain reaction is pCMV-p65. The primers are 5′-CCG GGA TCC GAC GAA CTG TTC CCC CTC ATC-3′ and 5′-GGC GGA TCC TTA GGA GCT GAT CTG ACT C-3′. His-tagged p65 was purified using His-Bind purification kits (Novagen).

In Vivo Labeling and Kinase Assay—For ^{32}P metabolic labeling, cells were grown in phosphate-free medium with 2% serum for 3 h. PD144795 and [^{32}P]H_3PO_4 were added 1 h before harvest. Cells were harvested by the methods described elsewhere (1).

For CKII assay, immunoprecipitated p65 or bacterially expressed His tagged p65 was incubated with CKII (Promega) at 37 °C for 10 min in 25 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 10 μM MgCl, 100 μM ATP, and 1 μCi of [γ-^{32}P]ATP. For PKA assay, the proteins were incubated with PKA catalytic subunit (Promega) at 30 °C for 5 min in 40 mM Tris-HCl (pH 7.4), 20 mM magnesium acetate, and 0.2 mM [γ-^{32}P]ATP. Phosphorylated p65 were resolved in SDS-PAGE. After exposure to x-ray film, the gels were dried and analyzed by Western blot or by GELCODE blue stain reagent (Pierce).

To assay for CKII activity, HeLa cells were either left untreated or treated with TNFα for various times. Cell pellets were resuspended in 0.25 M Tris-HCl (pH 7.6). After repeated freeze-thaw, whole cell lysates were collected by centrifuging. Whole cell lysates (50 μg) were used to assay for casein kinase II activity by using a casein kinase-2 assay kit (Upstate Biotechnology).

RESULTS

Casein Kinase II Interacts with p65 in Vivo—TNFα stimulation of HeLa cells or fibroblasts induces phosphorylation of p65 on serine 529 (1). Close examination of the sequence downstream of serine 529 (SGDE) revealed that it is a consensus CKII site (31, 32). Thus, the sequence matches the S-*X*-*X*-acidic consensus motif for CKII phosphorylation. It was therefore possible that CKII phosphorylates p65 in response to TNFα induction. To test this possibility, we first determined if CKII interacts with p65 *in vivo*. Flag-tagged p65 was immunoprecipitated from immortalized p65 knockout fibroblasts that have been restored with flag-tagged p65 (F-p65 cells described in Ref. 1), and the precipitated proteins were subjected to Western blot analysis using an anti-CKII antibody. The results of these experiments showed that the flag M2 antibody pulled down two peptides recognized by the anti-CKII antibody (Fig. 1*A*, *lane 1*). These two peptides represented the CKII catalytic subunits α and α', as the anti-CKII antibody was specifically made against these two subunits. As a positive control, the anti-CKII antibody precipitated the same molecular weight peptides (Fig. 1*A*, *lane 2*). The reciprocal co-immunoprecipitation, in which the cell lysates were immunoprecipitated with an anti-CKII antibody and the precipitated proteins were analyzed by an anti-p65 antibody, showed that the anti-CKII antibody co-immunoprecipitated p65 (Fig. 1*A*, *lane 4*).

The data shown in Fig. 1*A* support and extend the previous observation by Bird *et al.* (44), who showed that immunoprecipitated NF-κB complexes contained kinase activity that was indistinguishable from CKII, although direct blotting for CKII was not accomplished. Encouraged by this result, we then wanted to determine whether TNFα stimulation of cells affected the interaction between CKII and p65. To accomplish this, F-p65 cells were either left untreated or were treated with TNFα for various times. Whole cell lysates were subjected to immunoprecipitation with flag M2 antibody, and the precipitated proteins were analyzed by Western blot using the anti-CKII antibody. As shown in Fig. 1*B*, the p65-associated CKII level decreased upon TNFα induction (*lanes 1–4*). Interestingly, the interaction between CKII and a mutant p65 (529A) that cannot be phosphorylated following TNFα stimulation remained constant (Fig. 1*B*, *lanes 5–8*), suggesting that serine 529 of p65 may be a target for CKII and that phosphorylation of p65 may cause the release of CKII.

CKII Phosphorylates p65 at Serine 529—To determine

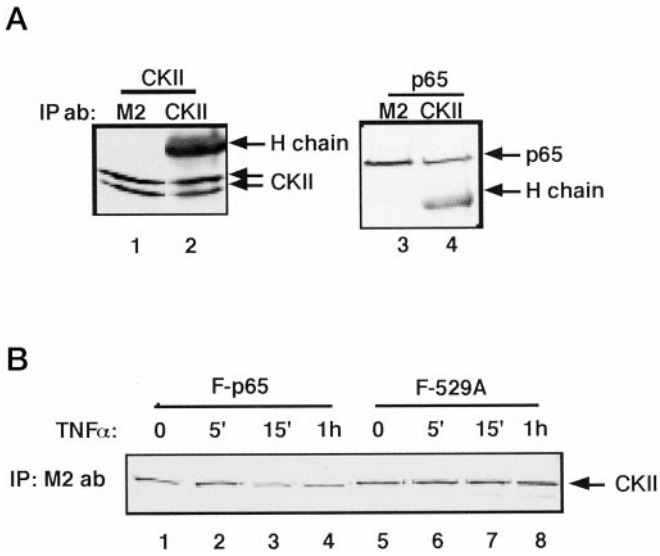

FIG. 1. **CKII interacts with p65 *in vivo*.** *A*, untreated cell lysates from the cells expressing F-p65 were subjected to immunoprecipitation (*IP*) with either anti-flag antibody (*ab*) (*lanes 1* and *3*) or CKII antibody (*lanes 2* and *4*). The precipitated proteins were further analyzed by Western blot using either CKII antibody (*left*) or p65 antibody (*right*). *B*, the F-p65 cells or F-529A cells were either left untreated or treated with TNFα for various times. The whole cell lysates were subjected to immunoprecipitation (*IP*) using anti-flag antibody (*ab*). The precipitated proteins were separated on SDS-PAGE and analyzed by Western blot with anti-CKII antibody.

whether CKII phosphorylates p65 at serine 529, both flag-tagged wild-type p65 and p65 529A were immunoprecipitated from the lysates of untreated cells and used for *in vitro* kinase assays with purified CKII. As shown in Fig. 2*A*, CKII phosphorylated wild-type p65 (*lanes 2* and *3*, *upper panel*), but not 529A (*lanes 5* and *6*, *upper panel*). Western blot analysis indicated that the loss of phosphorylation of 529A by CKII is not due to lower substrate protein levels (Fig. 2*A*, *lower panel*). Endogenous p65 from HeLa cells can also be phosphorylated by CKII (Fig. 2*A*, *lane 8*). Importantly, another serine/threonine kinase, PKA, which has been reported to phosphorylate p65 (6), phosphorylated the mutant protein as efficiently as the wild-type p65 (Fig. 2*A*, *lanes 10* and *12*). In addition, bacterially expressed His-tagged wild-type p65 but not 529A was phosphorylated by CKII, and this phosphorylation was inhibited by the CKII-specific inhibitor PD144795 (see below) (Fig. 2*B*, *left panel*), whereas PD144795 had no effect on PKA phosphorylation of p65 (Fig. 2*B*, *right panel*). These results demonstrated that CKII phosphorylates p65 *in vitro* and that phosphorylation occurs at serine 529.

We then wanted to determine if CKII phosphorylates p65 *in vivo*. To date, there are no reports in which CKII function can be inhibited *in vivo* by genetic approaches. The difficulties could result from the possibility that CKII α and α′ compensate for each other, with disruption of either one not affecting overall CKII activity, while knockout of both leads to loss of viability, as in yeast cells (33, 48). Therefore, we chose a pharmacological approach to determine if we could block TNFα-induced p65 phosphorylation. PD144795 is a benzothiophene, which was originally found to inhibit HIV expression (50). The selective target for inhibition was later found to be CKII, as this compound interacts with the kinase at the nucleotide binding site (51). Consistent with previous reports (50), we found that PD144795 did not affect TNFα-induced NF-κB nuclear translocation and DNA binding in HeLa cells (Fig. 3*A*). However, PD144795 inhibited κB-dependent transcription (Fig. 3*B*). Moreover, pretreatment of HeLa cells with PD144795 decreased TNFα-induced p65 phosphorylation (Fig. 3*C*). These results suggested that CKII phosphorylates p65 in response to TNFα induction in HeLa cells.

Evidence for a Requirement of IκBα Degradation for RelA/p65 Phosphorylation on Ser^{529}—Our original study utilized phosphopeptide mapping to identify serine 529 as the major site of TNF-induced phosphorylation (1). Recently, another group has reported that IKK can phosphorylate p65 both *in vivo* and *in vitro* on serine 536 (29). In order to confirm that serine 529 is phosphorylated in response to TNFα stimulation, we generated an antibody to a peptide with phosphoserine at position 529. Immunoblotting with this antibody confirms recognition of phosphoserine 529 following TNFα stimulation of HeLa cells (Fig. 4). Thus, it is clear that serine 529 is a major site of phosphorylation following TNFα treatment of HeLa cells or fibroblasts (see "Discussion").

We then utilized this antibody to determine if inducible phosphorylation of RelA/p65 on serine 529 requires release from IκBα. Potentially such a result could explain how CKII could be associated with p65 but not phosphorylate this substrate. In order to test this hypothesis, we treated cells with the proteasome inhibitor MG132 and then stimulated the cells with TNFα. Control cells were exposed to the diluent for MG132 but still received TNFα. As shown in Fig. 4, TNFα induced phosphorylation on Ser^{529}, with phosphorylation peaking at 10 min after stimulation. MG132 inhibited the induced phosphorylation, suggesting that IκB degradation is required for inducible phosphorylation. Additionally and consistent with this hypothesis, expression of the super-repressor (non-degradable) form of IκBα significantly blocked the TNFα-induced phosphorylation of p65 (data not shown).

IκBα Inhibits p65 Phosphorylation by CKII—Regulation of CKII activity has been difficult to explain. There have been reports that CKII activity is constitutive and is not subjected to regulation (31, 32, 52). However, other studies indicated that stimulation of CKII activity can occur in response to growth factors such as insulin-like growth factor 1 (53) and epidermal growth factor (54). It was important for us to determine if TNFα-induced p65 phosphorylation correlated with increased CKII activity. HeLa cells were treated with TNFα for various times, and cells were lysed by repeated freeze-thaw to avoid detergent that may interfere with the kinase assay. Whole cell lysates were then subjected to Western analysis with anti-CKII antibody (Fig. 5*A*), and CKII activity was measured using a synthetic CKII substrate (Fig. 5*B*). Unlike a recent report (43), we were not able to detect a significant increase in CKII activity in response to TNFα induction. Instead, the CKII protein level was unchanged and kinase activity was only slightly induced following TNFα stimulation.

In order to explain the inducible activation of CKII activity relevant to NF-κB phosphorylation and to explain the result showing that inhibition of IκB degradation inhibited inducible activity, we hypothesized that the association of CKII with the NF-κB complex leads to inhibition of kinase activity if IκB is present. To test our hypothesis, glutathione *S*-transferase-IκBα was added to the *in vitro* CKII kinase reaction. As shown in Fig. 5*C*, the addition of IκBα efficiently inhibited p65 phosphorylation by CKII. Therefore, these data indicate that when CKII is present in the NF-κB/IκB complex, it cannot phosphorylate p65. Induction of IκBα degradation would, therefore, lead to derepression of CKII activity, allowing it to phosphorylate p65.

DISCUSSION

The current understanding of the regulation of the transcription factor NF-κB indicates that NF-κB is activated by two mechanisms that appear to be interrelated. One mechanism

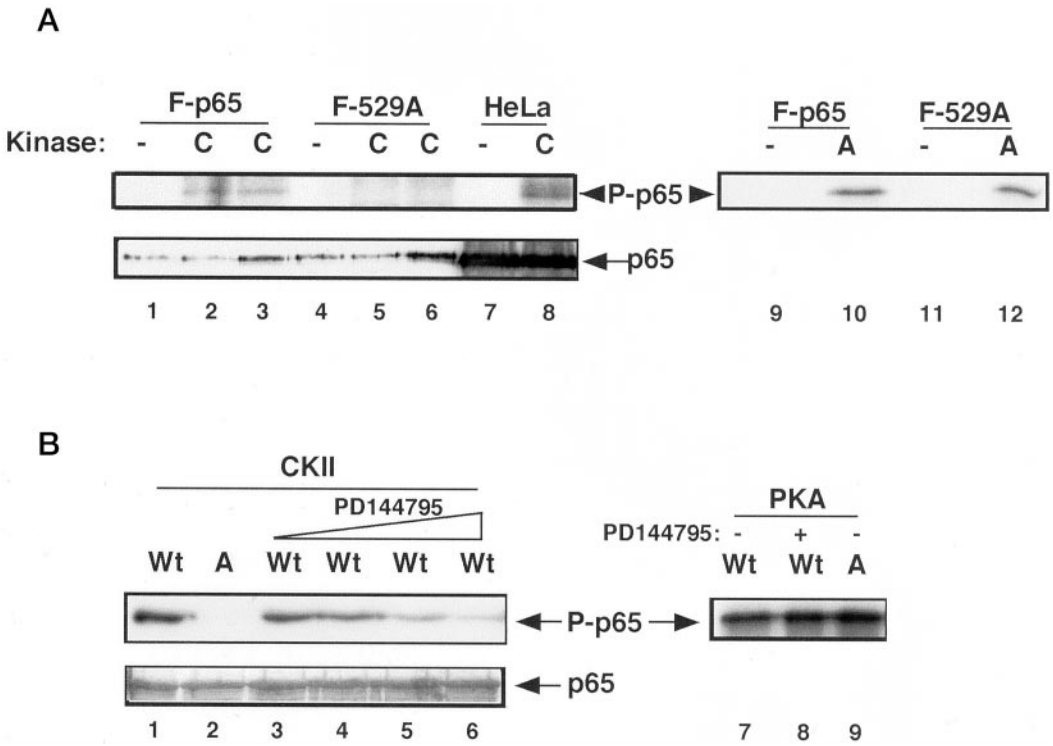

FIG. 2. **CKII phosphorylates p65 at serine 529.** *A*, p65 proteins were immunoprecipitated from F-p65 cells, F-529A cells, or HeLa cells. *In vitro* kinase assays were performed on the beads by adding [γ-^{32}P]ATP and purified CKII (*C*) (*lanes 2*, *3*, *5*, *6*, and *8*) or PKA (*A*) catalytic subunit (*lanes 10* and *12*). Phosphorylated p65 was resolved on SDS-PAGE. After exposure to x-ray film, the gel was re-hydrated and analyzed by Western blot with p65 antibody (*left lower panel*). *B*, bacterially expressed His-tagged wild type p65 (*Wt*) or 529A (*A*) was incubated with [γ-^{32}P]ATP and CKII (*left*) or PKA catalytic subunit (*right*). Increased amounts of PD144795 were added to the kinase reaction (*lanes 3–6*). Phosphorylated p65 was separated on SDS-PAGE. After exposure to x-ray film, the gel was re-hydrated and analyzed by GELCODE blue stain reagent (Pierce).

involves the induction of NF-κB to move into the nucleus following exposure of cells to a variety of stimuli. This mechanism is controlled by the activation of IKK activity, which subsequently phosphorylates IκB proteins on N-terminal serine residues leading to their ubiquitination and the subsequent degradation of IκB (3). A less well understood regulation of NF-κB involves induced phosphorylation of RelA/p65 in response to a variety of stimuli. Interestingly, it is speculated that phosphorylation may be required for transcriptional function of NF-κB. Thus, Zhong *et al.* (6, 28) showed convincingly that LPS induces phosphorylation of RelA/p65 on Ser^{276} in B and T cells and that this phosphorylation enhances the interaction with the transcriptional co-activators CBP and p300. The phosphorylation of Ser^{276} is controlled by the catalytic subunit of PKA associated with the NF-κB complex. Similar to the model proposed here for phosphorylation of p65 by CKII, it is proposed that degradation of IκB allows the PKA catalytic subunit to phosphorylate RelA/p65 on Ser^{276}. Sizemore *et al.* (26) showed that phosphorylation of p65 induced by interleukin-1 requires phosphatidylinositol 3-kinase and Akt and that this response activates the transcription function of NF-κB. Another group has presented data indicating that IKK itself may be involved directly in inducible phosphorylation of p65, showing that IKK can phosphorylate serine 536 *in vitro* and when overexpressed in cells (29). However, there are no data demonstrating that this phosphorylation event contributed to transcriptional potential. Previously, we have shown that phosphorylation of serine 529 contributes to the ability of p65 to activate a κB-dependent reporter. Others have provided evidence that phosphorylation of NF-κB may enhance DNA binding, although in our previous studies we did not see an effect of mutation of serine 529 on DNA binding affinity (1).

We have shown in this report that the serine/threonine kinase CKII interacts with p65 *in vivo*, and purified CKII phosphorylates p65 at serine 529 *in vitro*. A CKII inhibitor reduced TNFα-induced p65 phosphorylation, suggesting that CKII phosphorylates p65 in response to TNFα stimulation *in vivo*. The results presented in this paper also suggest an interesting mechanism by which the activity of CKII is regulated. Our data indicate that CKII is associated with the NF-κB/IκB complex but is unable to phosphorylate p65 unless IκBα is degraded. It is presently unknown whether CKII directly interacts with p65 or with IκB or possibly both.

CKII is a ubiquitous and highly conserved serine/threonine kinase which phosphorylates a number of nuclear proteins implicated in cell proliferation, such as c-Fos, c-Jun, Myc, Max, Myb, p53, adenovirus E1A protein, human papilloma virus E7 protein, and SV40 large T antigen (30). The effect of CKII phosphorylation varies depending on the protein. For example, CKII phosphorylates the transcriptional regulatory factor Max to inhibit the DNA binding activity of Max/Max homodimers (55), while phosphorylation of PU.1 by CKII promotes the interaction with NF-EM5 (35). We have shown that p65 is also a substrate for CKII and phosphorylation of p65 by CKII increases NF-κB transcriptional potential. RelA/p65 has been shown to be phosphorylated in response to interleukin-1 and LPS induction (20), and the putative p65 kinase that associates with both NF-κB and IκB has a molecular size that is very similar to the CKII α subunit (27). More recently, Bird *et al.* (44) demonstrated that a kinase with properties indistinguishable from CKII associates with p65 and phosphorylates it *in vitro.* As described above, it has been published recently that LPS-induced p65 phosphorylation by PKA at serine 276 enhances its interaction with the transcriptional coactivator CBP/p300 (6, 28). Why different signals target different phosphorylation sites on p65 is presently unknown, but may be explained by differences in cell types or differences in signal transduction pathways.

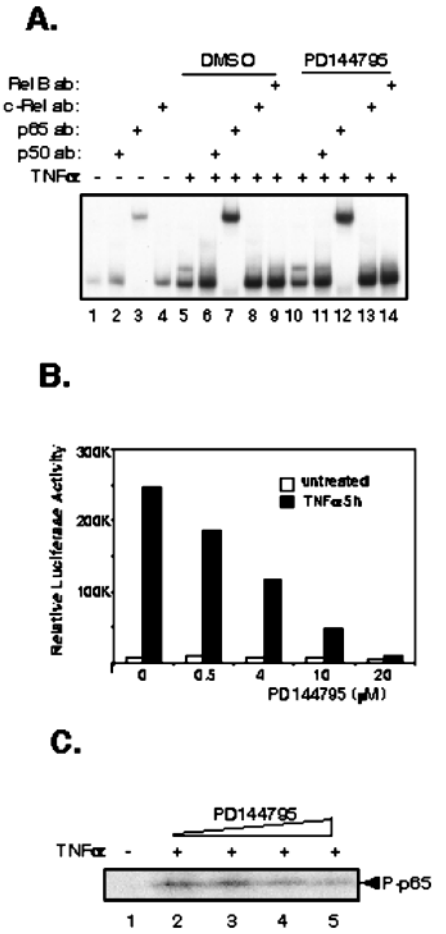

FIG. 3. **A CKII-specific inhibitor, PD144795, inhibits κB-dependent transcription and reduces TNFα-induced p65 phosphorylation.** *A*, HeLa cells were pretreated with Me_2SO (*DMSO*, *lanes 5–9*) or 20 μM PD144795 (*lanes 10–14*) before treatment with TNFα (10 ng/ml) for 10 min. The nuclear extracts were analyzed by electrophoretic mobility shift assay with a ^{32}P-labeled κB site containing DNA probe, and antibodies (*ab*) against different NF-κB subunits were added. The reaction mixtures were electrophoresed on a 5% non-denaturing gel. *B*, HeLa cells were transiently transfected with a 3XκBluc reporter that contains three copies of the κB binding site. Six hours before harvest, TNFα (10 ng/ml) was added to the transfected cells that had been pretreated with PD144795. Luciferase activities were measured using 100 μg of lysates. *C*, HeLa cells were labeled with $[^{32}P]H_3PO_4$, and either left untreated or treated with various amounts of PD144795. Ten minutes before harvest, TNFα (40 ng/ml) was added. The whole cell lysates were immunoprecipitated with anti-p65 antibody and separated on SDS-PAGE. Phosphorylated p65 was visualized by autoradiography.

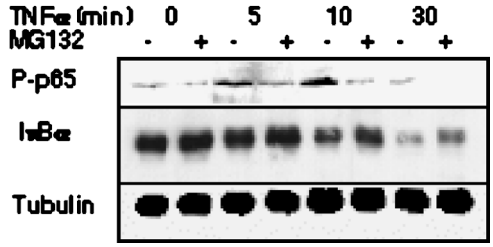

FIG. 4. **Proteasome inhibition abolishes inducible p65 phosphorylation at serine 529.** HeLa cells were treated with TNFα for the times indicated, in either the presence or absence of the proteasome inhibitor MG132. Whole cell protein extracts were isolated and resolved by SDS-PAGE. The gel was analyzed by Western blot with an antibody specific to p65 phosphorylated at serine 529. The gel was then stripped and reprobed with an antibody against IκBα to show that MG132 inhibits IκBα degradation. Anti-tubulin was used to verify equal protein loading.

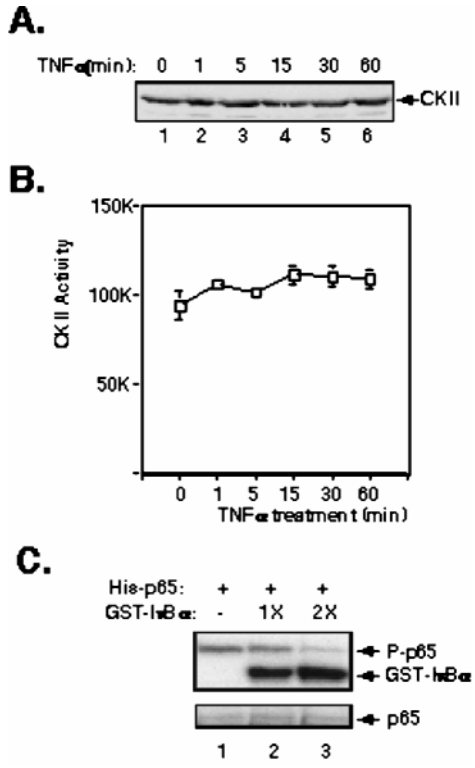

FIG. 5. **IκBα inhibits p65 phosphorylation by CKII.** HeLa cells were either left untreated or treated with TNFα for various times. Whole cell lysates were made, and 50 μg of lysates were analyzed by Western blot using anti-CKII antibody (*A*) and *in vitro* kinase assay using a CKII substrate peptide (*B*). *C*, His-tagged p65 was used for kinase assay with purified CKII. Different amounts of glutathione *S*-transferase (*GST*)-IκBα were added to the reaction. The phosphorylated proteins were resolved on SDS-PAGE and visualized by autoradiography (*upper panel*). The dried gel was re-hydrated and equal loading of His-p65 was shown by staining the gel with GELCODE blue stain reagent (Pierce) (*lower panel*).

It has been shown before that CKII phosphorylates IκBα at its C-terminal PEST region on several serine and threonine residues and the function of this modification is unknown, although some researchers suggested that the phosphorylation of the PEST region by CKII is required for the basal turnover of the free IκBα proteins (38–40, 42). CKII also phosphorylates the PEST domain of IκBβ (56, 57), which is required for IκBβ to associate with c-Rel or other NF-κB subunits to inhibit NF-κB DNA binding. Recently, CKII was shown to be present in the kinase activities that phosphorylated IκBα at serine 32 along with $p90^{rsk1}$ and IKKα/β (43) in the TNFα- or okadaic acid-stimulated HeLa cell extracts. In the *in vitro* kinase assay, CKII phosphorylates serine 32 of the IκBα-derived peptide much more efficiently than serine 36 or any of the other IκBβ or IκBε-derived peptides. CKII may act alone or cooperate with another kinase to induce the N-terminal serine phosphorylation and subsequent degradation of IκBα in response to some stimuli. Therefore, CKII appears to regulate NF-κB at two levels: 1) by phosphorylating IκBα to induce its degradation, and 2) by phosphorylating p65 to increase NF-κB transcriptional activity.

CKII has been shown to be necessary for cell cycle progression and for cell proliferation. CKII levels are elevated in rapidly proliferating non-neoplastic tissue and in solid tumors such as colorectal carcinomas, malignant melanomas, and bladder, kidney, gastric, and breast carcinomas (58). In transgenic mice, CKII cooperates with Tal-1 and c-Myc oncogenes to induce lymphoma (59, 60). NF-κB also plays an important role in cell proliferation. Elevated levels of NF-κB have been corre-

lated with cellular transformation (61, 62), and NF-κB has been shown to be required for transformation induced by oncogenic Ras and by the fusion oncoprotein BCR-ABL (49, 63). It will be interesting to determine if CKII cooperates with NF-κB to regulate cell transformation and tumorigenesis and if the phosphorylation of the p65 subunit by CKII is necessary for the cooperation.

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