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Identification of a p65 Peptide That Selectively Inhibits NF-κB Activation Induced by Various Inflammatory Stimuli and Its Role in Down-regulation of NF-κB-mediated Gene Expression and Up-regulation of Apoptosis*

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Because of the critical role of the nuclear transcription factor NF-κB in inflammation, viral replication, carcinogenesis, antiapoptosis, invasion, and metastasis, specific inhibitors of this nuclear factor are being sought and tested as treatments. NF-κB activation is known to require p65 phosphorylation at serine residues 276, 529, and 536 before it undergoes nuclear translocation. Small protein domains, termed protein transduction domains (PTDs), which are able to penetrate cell membranes can be used to transport other proteins across the cell membrane. We have identified two peptides from the p65 subunit of NF-κB (P1 and P6 were from amino acid residues 271–282 and 525–537, respectively) that, when linked with a PTD derived from the third helix sequence of antennapedia, inhibited tumor necrosis factor (TNF)-induced NF-κB activation *in vivo*. Linkage to the PTD was not, however, required to suppress the binding of the p50-p65-heterodimer to the DNA *in vitro*. PTD-p65-P1 had no effect on TNF-induced AP-1 activation. PTD-p65-P1 suppressed NF-κB activation induced by lipopolysaccharide, interleukin-1, okadaic acid, phorbol 12-myristate 13-acetate, H_2O_2, and cigarette smoke condensate as well as that induced by TNF. PTD-p65-P1 had no effect on TNF-induced inhibitory subunit of NF-κB (IκBα) phosphorylation, IκBα degradation, or IκBα kinase activation, but it blocked TNF-induced p65 phosphorylation and nuclear translocation. NF-κB-regulated reporter gene expression induced by TNF, TNF receptor 1, TNF receptor-associated death domain, TNF receptor-associated factor-2, NF-κB-inducing kinase, IκBα kinase, and p65 was also suppressed by these peptides. Suppression of NF-κB by PTD-p65-P1 enhanced the apoptosis induced by TNF and chemotherapeutic agents. Overall, our results demonstrate the identification of a p65 peptide that can selectively inhibit NF-κB activation induced by various inflammatory stimuli, down-regulate NF-κB-mediated gene expression, and up-regulate apoptosis.

NF-κB represents a group of five proteins, namely c-Rel, RelA (p65), RelB, NF-κB1 (p50 and p105), and NF-κB2 (p52) (1). Ways to modulate NF-κB expression therapeutically have focused on its active-inactive state transition mechanisms. NF-κB is regulated by a family of inhibitors, called IκB[1] (2). In an inactive state, NF-κB is present in the cytoplasm as a heterotrimer consisting of p50, p65, and IκBα subunits. In response to an activation signal, the IκBα subunit is phosphorylated at serine residues 32 and 36, ubiquitinated at lysine residues 21 and 22, and degraded through the proteosomal pathway, thus exposing the nuclear localization signals on the p50-p65 heterodimer. The p65 is then phosphorylated, leading to the nuclear translocation and binding to a specific sequence in DNA, which in turn results in transcriptions of various genes including cyclin D1, cyclooxygenase (COX)-2, and matrix metalloproteinase (MMP)-9.

NF-κB has been shown to regulate the expression of a number of genes whose products are involved in inflammation, viral replication, carcinogenesis, antiapoptosis, invasion, and metastasis (3, 4). Specific adhesion molecule, chemokine, inflammatory cytokine, and cell cycle regulatory gene are affected. Thus, agents that can suppress NF-κB activation have the potential to be treatments for inflammatory diseases and cancer (4–7).

Most proteins enter the cell through their specific cell surface receptors. Recent studies, however, indicate that certain short protein sequences can enter the cells without any receptors, and these have been named protein transduction domain (PTD) peptides (10, 11). Importantly, proteins, peptides, and antisense oligonucleotides conjugated to these PTD have been shown to deliver these cargoes effectively, as demonstrated in several cell and animal models (10, 11). Peptides derived from the third helix of the antennapedia homeodomain, herpes virus structural protein, and human immunodeficiency virus Tat protein have been used to deliver both small and large peptides of interest to the cells through an energy- and receptor-independent mechanism (12–14). Most of the PTD are arginine-rich peptides (15).

Using these PTD, several peptides based on protein-protein interaction domains have been delivered to the cells to suppress cell signaling. These include Grb2-binding peptide (16), mitogen-activated protein kinase (17), STAT3 (18), NEMO-

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[1] The abbreviations used are: IκB, inhibitory subunit of NF-κB; TNF, tumor necrosis factor; TNFR1, TNF receptor 1; IKK, IκBα kinase; TRAF2, TNF receptor-associated factor-2; TRADD, TNF receptor-associated death domain; EMSA, electrophoretic mobility shift assay(s); SEAP, secretory alkaline phosphatase; PMA, phorbol myristate acetate; PTD, protein transduction domain(s); MMP, matrix metalloproteinase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; COX, cyclooxygenase.

IKK-interacting peptide (19), and peptides carrying nuclear localization sequences (20–23). Besides peptides, PTD have also been used to deliver larger full-length polypeptides, including IκBα (24), cyclin-dependent kinase inhibitory protein p27 (25), antiapoptotic protein Bcl-x_L (26), and proapoptotic proteins (27).

The p65 subunit of NF-κB, which contains at least two strong transactivation domains (TAD) within the C terminus (TA1, 30 amino acids; TA2, 90 amino acids), when activated has been shown to undergo phosphorylation (28, 29). Where the phosphorylation occurs and by which kinase has been controversial. For instance, phosphorylation at Ser^{276} by protein kinase A (30, 31), at Ser^{529} by casein kinase II (32–34), at Ser^{536} by IKK-β (35–38), and at Ser^{471} by PKC-ϵ (39–41) have been demonstrated. In addition, phosphorylation of p65-TAD by glycogen synthase kinase-3β (42) and by Ca^{2+}/calmodulin-dependent protein kinase IV (43) has been demonstrated.

We designed specific inhibitors of NF-κB activation based on the phosphorylation sites present in the p65. To permit the inhibitor to cross the cell membrane, the synthetic p65 peptide was linked with a protein transduction peptide, a short protein sequence that can enter cells without any receptors (10, 11). Specifically, we linked the peptide to an antennapedia-derived PTD and investigated its ability to inhibit NF-κB activation *in vitro* and *in vivo* and explored its mechanism of action. We report the identification of a novel peptide that suppressed NF-κB activation induced by a variety of inflammatory stimuli and NF-κB-mediated gene transcription and enhanced apoptosis induced by TNF and other apoptotic stimuli.

MATERIALS AND METHODS

Reagents—Bacteria-derived human recombinant TNF, purified to homogeneity with a specific activity of 5×10^7 units/mg, was kindly provided by Genentech (South San Francisco, CA). Penicillin, streptomycin, Iscove's modified Dulbecco's medium, and fetal bovine serum were obtained from Invitrogen. Lipopolysaccharide, phorbol 12-myristate 13-acetate (PMA), okadaic acid, H_2O_2, and anti-β-actin antibody were obtained from Sigma. The cigarette smoke condensate was kindly provided by Dr. C. Gary Gariola (University of Kentucky, Lexington, KY). The polyclonal antibodies anti-p65, anti-p50, anti-IκBα, anti-cyclin D1, and MMP-9 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phosphospecific anti-IκBα (Ser^{32}) antibody was purchased from Cell Signaling (Beverly, MA). Phosphospecific anti-p65 antibody was kindly provided by Rockland Laboratory (Rockland, MD). Anti-IKK-α and anti-IKK-β antibodies were kindly provided by Imgenex (San Diego, CA). Anti-COX-2 antibody was obtained from BD Biosciences Pharmingen (San Diego, CA). Purified recombinant NF-κB p65 protein expressed in bacteria (lot 00903001) was kindly provided by Active Motif (Carlsbad, CA).

Cell Lines—We used the leukemic cell line KBM-5, which is phenotypically myeloid with monocytic differentiation and A 293 embryonic kidney cells. KBM-5 cells were maintained in Iscove's modified Dulbecco's medium supplemented with 15% fetal bovine serum, and A 293 cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Peptide Synthesis—All peptides (see Fig. 1) were synthesized using an automated peptide synthesizer (Symphony Multiplex, Rainin Instruments, MA). The peptides were purified to more than 90% purity using high pressure liquid chromatography.

Electrophoretic Mobility Shift Assays (EMSA)—To measure NF-κB activation, we performed EMSA as described previously (44, 45). Briefly, nuclear extracts prepared from TNF-treated cells (1×10^6/ml) were incubated with ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotides (10 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat, 5′-TTGTTACAA**GGGACTTTC**CGCTG**GGGACTTTC**CAGGGAGGCGTGG-3′ (boldface type indicates NF-κB binding sites) for 30 min at 37 °C, and the DNA-protein complex formed was separated from free oligonucleotides on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5′-TTGTTACAA**CTCACTTTC**CGCTG**CTCACTTTC**CAGGGAGGCGTGG-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotides. To investigate the binding of recombinant p65 to DNA, poly(dI-dC) was excluded from the binding reaction buffer. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with antibodies against either the p50 or p65 subunits of NF-κB for 30 min at 37 °C, and then the complex was analyzed by EMSA. Antibodies against cyclin D1 and preimmune serum were included as negative controls. The dried gels were visualized, and radioactive bands were quantitated by a PhosphorImager (Amersham Biosciences) using ImageQuant software.

Western Blot Analysis—To determine the levels of protein expression in the cytoplasm or the nucleus, we prepared extracts (46) from TNF-treated cells and fractionated them by SDS-PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with each antibody, and detected by ECL regent (Amersham Biosciences).

IKK Assay—The IKK assay was performed by a method described previously (47). Briefly, IKK complex from whole-cell extract was precipitated with antibody against IKK-α, followed by treatment with protein A/G-Sepharose beads (Pierce). After a 2-h incubation, the beads were washed with lysis buffer and then assayed in kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 2 mM dithiothreitol, 20 μCi of [γ-^{32}P]ATP, 10 μM unlabeled ATP, and 2 μg of substrate GST-IκBα (residues 1–54). After incubation at 30 °C for 30 min, the reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized by a PhosphorImager. To determine the total amounts of IKK-α and IKK-β in each sample, 30 μg of the whole-cell protein was resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK-α or anti-IKK-β antibodies.

Cell-free phosphorylation of peptide by IKK was also determined using 10 μg of peptides as a substrate in the kinase reaction mixture as described above and then fractionated on 20% SDS-PAGE in 2× SDS electrophoresis buffer.

NF-κB-dependent Reporter Secretory Alkaline Phosphatase (SEAP) Expression Assay—The effect of inhibitory peptides on TNF-, TNFR-, TRADD-, TRAF2-, NF-κB-inducing kinase-, IKK-, and p65-induced NF-κB-dependent reporter gene transcription was analyzed by SEAP assay as previously described (48). Briefly, A 293 cells (5×10^5 cells/well) were plated in 6-well plates and transiently transfected by the calcium phosphate method with pNF-κB-SEAP (0.5 μg). To examine TNF-induced reporter gene expression, we transfected the cells with 0.5 μg of the SEAP expression plasmid and 2 μg of the control plasmid pCMVFLAG1 DNA for 24 h. Thereafter, the cells were treated for 24 h with 150 μM peptides and then stimulated with 1 nM TNF for 24 h. The cell culture medium was then harvested and analyzed for alkaline phosphatase (SEAP) activity according to the protocol essentially as described by the manufacturer (Clontech, Palo Alto, CA) using a 96-well fluorescence plate reader (Fluoroscan II, Labsystems, Chicago, IL) with excitation set at 360 nm and emission at 460 nm.

Cytotoxicity Assay (MTT Assay)—The cytotoxic effects of TNF were determined by the MTT uptake method as described (47). Briefly, 5000 cells were incubated with synthetic peptides for 1 h in triplicate in 96-well plates and then treated with various concentrations of TNF for 72 h at 37 °C. Thereafter, MTT solution was added to each well. After a 2-h incubation at 37 °C, extraction buffer (20% SDS, 50% dimethylformamide) was added, the cells were incubated overnight at 37 °C, and then the optical density was measured at 570 nm using a 96-well multiscanner (MRX Revelation; Dynex Technologies, Chantilly, VA).

Cytotoxicity Assay (Live/Dead® Assay)—The cytotoxic effects of TNF were also determined by the Live/Dead® assay (Molecular Probes, Inc., Eugene, OR). Briefly, 1×10^5 cells were incubated with 100 μM PTD-p65-P1 for 1 h and then treated with 1 nM TNF for 16 h at 37 °C. Cells were stained with Live/Dead® reagent (5 μM ethidium homodimer, 5 μM calcein-AM) and then incubated at 37 °C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2, Nikon, Tokyo, Japan).

TUNEL Assay—The TNF-induced apoptosis was determined by TUNEL assay using *In Situ* Cell Death Detection reagent (Roche Applied Science). Briefly, 1×10^5 cells were incubated with PTD-p65-P1 for 1 h and then treated with 1 nM TNF for 16 h at 37 °C. Thereafter, cells were plated on a poly-L-lysine-coated glass slide by centrifugation using a Cytospin 4 centrifuge (Thermoshendon, Pittsburgh, PA), air-dried, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After washing, cells were incubated with reaction mixture for 60 min in 37 °C. Stained cells were mounted with mounting medium purchased from Sigma and analyzed under a fluorescence microscope (Labophot-2).

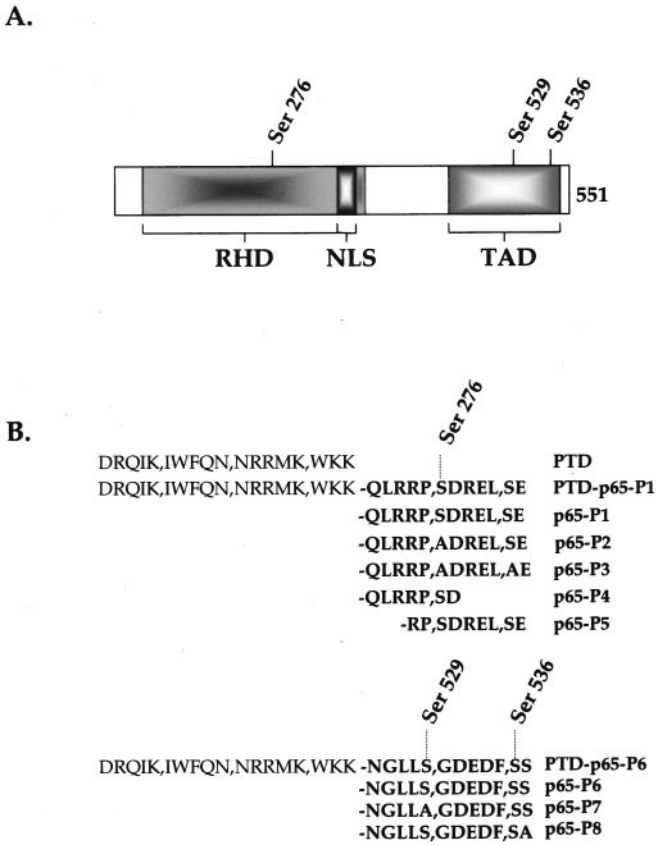

FIG. 1. **Structure of the p65 subunit of NF-κB and amino acid sequence of the p65-derived peptides.** *A*, the p65 consists of a DNA-binding and dimerization domain (*RHD*), nuclear localization domain (*NLS*), and transactivation domain (*TAD*). The dimerization domain is located between residues 191 and 290, and the nuclear localization domain is between residues 290 and 325. The p65 phosphorylation sites are indicated. *B*, sequence of cell-permeable peptide and the p65 peptides. The membrane-translocating peptide sequence derived from antennapedia (PTD) was conjugated with p65-P1 or p65-P6 for *in vivo* study. Other p65 peptides (p65-P1 and -P8) without the antennapedia segment were used in *in vitro* study.

RESULTS

To design a peptide that can suppress NF-κB activation, we targeted the phosphorylation sites of the p65 subunit of NF-κB. The p65 consists of a DNA-binding and dimerization domain (*RHD* in Fig. 1; residues 191–290), nuclear localization domain (*NLS*; residues 291–325), and transactivation domain (*TAD*). The phosphorylation residue Ser^{276} present in the DNA-binding and dimerization domain and Ser^{529} and Ser^{536} present in the transactivation domain were targeted (see Fig. 1*A*). The p65 peptides listed in Fig. 1 were linked with the PTD derived from the third helix of the antennapedia homeodomain (Fig. 1*B*). These peptides were tested for their ability to suppress NF-κB activation induced by various proinflammatory stimuli.

Specific Amino Acid Sequence Is Required for Suppression of NF-κB Activation in Vitro—We used the *in vitro* assay to determine the amino acid sequence needed for NF-κB activation (Fig. 2*A*). The p65-derived peptides in which Ser^{276} was mutated (p65-P2) did not inhibit NF-κB binding to the DNA. Peptides in which 5 amino acid residues from the C terminus (QLRRP, SD; p65-P4) or QLR-deleted (RP, SDREL, SE; p65-P5), were also inactive. The minimum peptide required for suppression of NF-κB activation was QLRRPSDRELSE (p65-P1).

Whether p65-P6 can suppress the p50-p65 binding to the DNA was examined. Peptides in which Ser^{529} was mutated (p65-P7) or Ser^{536} was mutated (p65-P8) did not inhibit NF-κB binding to the DNA (Fig. 2*A*). These results suggest that the inhibition of TNF-induced NF-κB activation by p65-P6 requires the presence of both of the phosphorylation sites, Ser^{529} and Ser^{536}. In contrast, p65-P1 contains a single phosphorylation site, and it is needed to inhibit NF-κB activity. All subsequent studies were performed with PTD-p65-P1.

Whether the p65-derived peptides also inhibit the binding of recombinant p65 protein to the DNA was investigated. To determine this, the recombinant purified p65 protein was incubated with various peptides and then examined for DNA binding. The p65-P1 and p65-P6 peptides, whether linked with or without PTD, inhibited the binding of recombinant p65 to the DNA in the same manner as natural p65/p50-NF-κB complex (Fig. 2*B*).

The dose-dependent effects of PTD-p65-P1 and p65-P1 on the p50-p65 binding to the DNA were investigated. Nuclear extracts from TNF-treated cells were incubated with different concentrations of the peptide and then examined for DNA binding. The p65-P1 inhibited NF-κB binding in a dose-dependent manner, and maximum inhibition occurred at 50 μM (Fig. 2*C*). PTD-p65-P1 also inhibited NF-κB binding at the same concentration. PTD alone had no effect.

PTD-p65-P1 Inhibits TNF-induced NF-κB Activation in Vivo—To determine the effect of the peptide containing Ser^{276} (PTD-p65-P1), we preincubated KBM-5 cells with the peptides for 1 h and then treated them with 0.1 nM TNF for the indicated times. Nuclear extracts were prepared, and we then analyzed NF-κB activation by EMSA (Fig. 3*A*). TNF induced NF-κB activation in a time-dependent manner, and pretreatment with PTD-p65-P1 completely abolished the TNF-induced NF-κB activation. Neither PTD nor p65-P1 alone had any effect on TNF-induced NF-κB activation, indicating that p65-P1 must be attached to a PTD for it to enter the membrane. We also investigated the minimum dose of PTD-p65-P1 required to suppress NF-κB activation. PTD-p65-P1 suppressed TNF-induced NF-κB activation by 25% at 100 μM and completely at 150 μM (Fig. 3*B*).

PTD-p65-P6 Also Inhibits TNF-induced NF-κB Activation in Vivo—To determine the effect of the peptide containing Ser^{529} and Ser^{536} (PTD-p65-P6), we preincubated KBM-5 cells with various concentrations of peptides for 1 h and then treated them with 0.1 nM TNF for 30 min. PTD-p65-P6 inhibited TNF-induced NF-κB activation in a dose-dependent manner. Neither PTD nor p65-P6 alone had any effect on TNF-induced NF-κB activation (Fig. 3*C*). These results indicated that both Ser^{539} and Ser^{536} were important for inhibiting NF-κB activation.

Specificity of PTD-p65-P1 Inhibiting Activity—Various combinations of Rel/NF-κB protein can constitute an active NF-κB heterodimer that binds to a specific sequence in the DNA (1). To show that the retarded band visualized by EMSA in TNF-treated cells was indeed NF-κB, we incubated nuclear extracts from TNF-stimulated cells with antibodies to either the p50 (NF-κB1) or the p65 (RelA) subunit of NF-κB. Both shifted the band to a higher molecular mass (Fig. 3*D*), thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. Furthermore, 50 μM p65-P1 inhibited the formation of the higher molecular mass band. Neither preimmune serum nor the irrelevant antibody anti-cyclin D1 had any effect. Excess unlabeled NF-κB (100-fold; competitor) caused complete disappearance of the band, but mutant oligonucleotides did not.

TNF-induced AP-1 Activation Is Not Inhibited by PTD-p65-P1—Like NF-κB, TNF is a potent activator of AP-1 (49). Whether PTD-p65-P1 affects TNF-induced AP-1 activation was also investigated. Cells were treated with 0.1 nM TNF for the indicated times, and nuclear extracts were prepared and assayed for AP-1 activation by EMSA (Fig. 4*A*). TNF activated AP-1, but PTD-p65-P1 had no effect on the activation of AP-1.

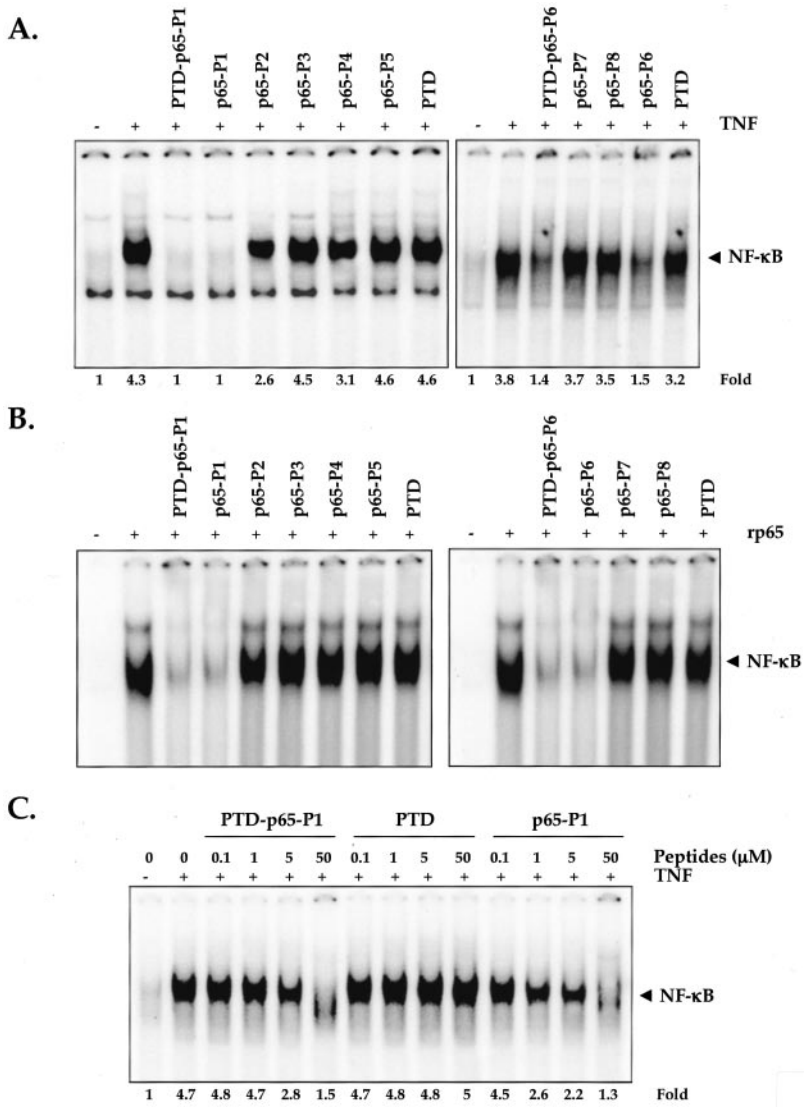

FIG. 2. *A*, effect of various peptides containing the phosphorylation site on p65 *in vitro*. KBM-5 cells were treated with 0.1 nM TNF for 30 min, and the nuclear extracts were prepared, incubated for 30 min with 50 μM of various peptides, and then assayed for NF-κB activation by EMSA. *B*, effect of various p65-inhibitory peptides on binding of purified recombinant p65 protein to the DNA *in vitro*. Recombinant p65 protein (100 ng/sample) was incubated for 30 min with 50 μM of various peptides in 0.025 ml and then assayed for DNA binding activity by EMSA. *C*, dose-dependent effect of p65-P1 peptide on NF-κB binding to DNA *in vitro*. Nuclear extracts were prepared from TNF-treated cells, incubated for 30 min with various concentrations of peptides, and then assayed for NF-κB activation by EMSA.

PTD-p65-P1 Inhibits NF-κB Activation Induced by Different Activators—Lipopolysaccharide, interleukin-1, okadaic acid, PMA, H_2O_2, and cigarette smoke condensate are potent activators of NF-κB, but the mechanisms differ (3, 4). We investigated whether PTD-p65-P1 could suppress NF-κB activated by these agents. Cells were preincubated with 150 μM PTD-p65-P1 for 1 h, treated with 0.1 nM TNF, 1 μg/ml lipopolysaccharide, 100 ng/ml interleukin-1, 500 nM okadaic acid, 10 ng/ml PMA, 500 μM H_2O_2, or 1 μg/ml cigarette smoke condensate and then analyzed for NF-κB activation by EMSA. PTD-p65-P1 suppressed the activation of NF-κB induced by all of these agents (Fig. 4*B*), suggesting that the PTD-p65-P1 acts at a step common to all of these agents.

PTD-p65-P1 and PTD-p65-P6 Have No Effect on IκBα Phosphorylation or Degradation—The translocation of NF-κB to the nucleus is proceeded by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα (1). To determine whether PTD-p65-P1 inhibits TNF-induced NF-κB activation by inhibiting IκBα degradation and phosphorylation, we pretreated cells with the peptide for 1 h and then exposed them to 0.1 nM TNF for the indicated times. We then examined the cells for IκBα status in the cytoplasm by Western blot analysis. TNF induced phosphorylation and degradation of IκBα, and pretreatment of cells with PTD-p65-P1 had no effect on either phosphorylation or degradation of IκBα (Fig. 5*A*, *upper panel*). Similarly, PTD-p65-P6 also did not affect either phosphorylation or degradation of IκBα (Fig. 5*B*, *upper panel*).

PTD-p65-P1 and PTD-p65-P6 Inhibit Cytoplasmic p65 Phosphorylation and Nuclear Translocation—Western blot analysis showed that TNF induced nuclear translocation of p65 in a time-dependent manner. As early as 5 min after TNF stimulation, p65 was translocated to the nucleus, and the amount remained constant for 30 min (Fig. 5, *A* and *B*, *middle panel*), whereas cells treated with either PTD-p65-P1 or PTD-p65-P6 did not show p65 nuclear translocation. Our results also show that TNF induced the phosphorylation of cytoplasmic p65 in a time-dependent manner, whereas PTD-p65-P1 or PTD-p65-P6 suppressed it almost completely (*bottom* of Fig. 5, *A* and *B*). These results suggest that PTD-p65-P1 and PTD-p65-P6 suppressed TNF-induced NF-κB activation by inhibiting phosphorylation and nuclear translocation of p65.

PTD-p65-P1 Has No Effect on the TNF-induced IKK Activation—Since IKK is required for TNF-induced NF-κB activation, we next determined the effect of PTD-p65-P1 on TNF-induced IKK activation. Immune complex kinase assays showed that TNF activated IKK as early as 5 min after TNF treatment, and PTD-p65-P1 had no effect on this activation (Fig. 5*C*).

IKK Phosphorylates the p65 Peptides in a Cell-free System—p65-P1 or p65-P6 has one or two serine residues, respectively. Whether these residues could be phosphorylated by IKK was investigated. Whole-cell extracts from TNF-treated cells were immunoprecipitated with antibody against IKK and then sub-

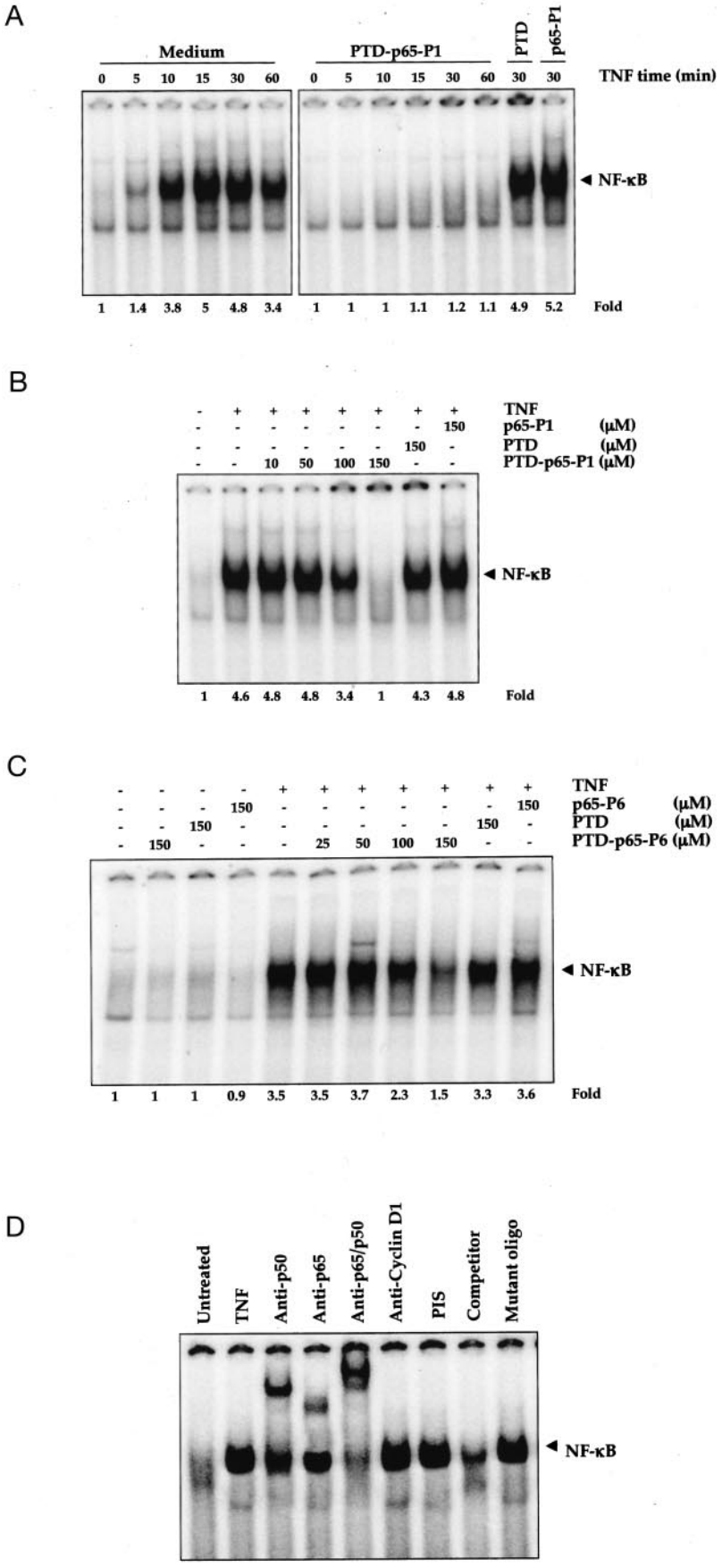

FIG. 3. **PTD-p65-P1 peptide inhibits TNF-induced NF-κB activation.** *A*, KBM-5 cells were incubated with 150 μM peptides for 1 h and treated with 0.1 nM TNF for the indicated times. Nuclear extracts were prepared, and then NF-κB activation was analyzed by EMSA. *B*, KBM-5 cells were incubated with various concentrations of peptides for 1 h and treated with 0.1 nM TNF for 30 min. Nuclear extracts were prepared, and then NF-κB activation was analyzed by EMSA. *C*, PTD-p65-P6 peptide inhibits TNF-induced NF-κB activation. KBM-5 cells were incubated with various concentrations of peptides for 1 h and treated with 0.1 nM TNF for 30 min. Nuclear extracts were prepared, and then NF-κB activation was analyzed by EMSA. *D*, PTD-p65 peptides specifically inhibit TNF-induced NF-κB activation. KBM-5 cells were treated with 0.1 nM TNF for 30 min. Nuclear extracts were prepared; incubated for 30 min with different antibodies, p65-P1 peptide, preimmune serum (*PIS*), unlabeled NF-κB oligonucleotide probes (*Competitor*), or mutant NF-κB oligonucleotide probe; and then assayed for NF-κB activation by EMSA.

jected to the immunocomplex kinase assay using p65 peptides as a substrate. After reaction, samples were fractionated on 20% SDS-PAGE with 2-fold electrophoresis buffer. Fig. 5*D* shows that precipitated IKK complex phosphorylated p65-P1 and p65-P6, suggesting that synthetic peptides from the p65 subunit of NF-κB can be phosphorylated by IKK complex. Our

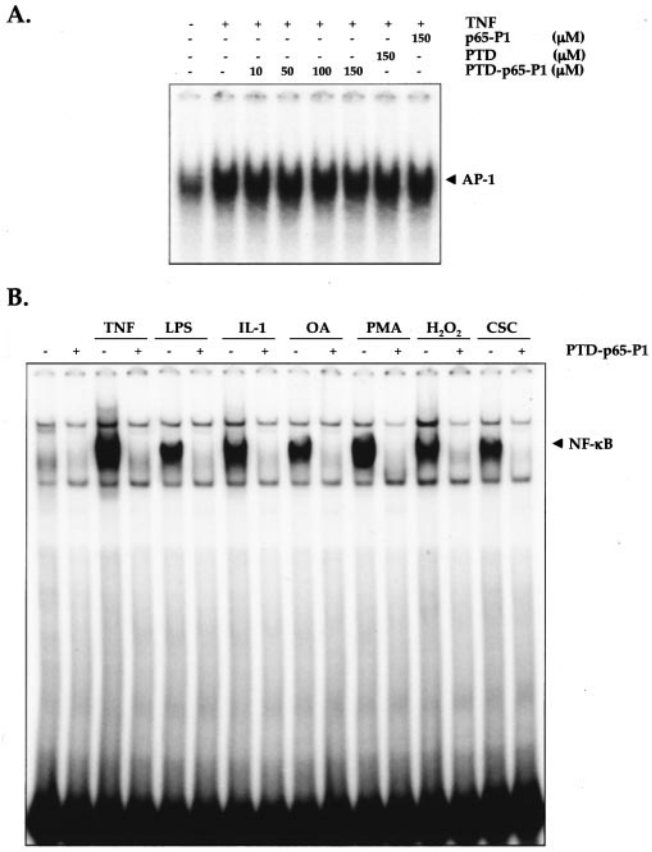

FIG. 4. *A*, effect of PTD-p65-P1 on TNF-induced AP-1 activity. KBM-5 cells were incubated with various concentrations of peptides for 1 h and treated with 0.1 nM TNF for 30 min. Nuclear extracts were prepared, and then NF-κB activation was analyzed by EMSA. *B*, PTD-p65-P1 peptide inhibits NF-κB activation induced by different activators. KBM-5 cells were incubated with 150 μM peptide for 1 h, treated with 0.1 nM TNF, 1 μg/ml lipopolysaccharide, 100 ng/ml interleukin-1 (*IL-1*), 500 nM okadaic acid (*OA*), 10 ng/ml PMA, 500 μM H_2O_2, or 1 μg/ml cigarette smoke condensate (*CSC*), and then analyzed for NF-κB by EMSA.

results also showed that p65 peptides in which Ser^{276}, Ser^{529}, or Ser^{536} residues were mutated into alanine did not undergo phosphorylation by the IKK complex. These results suggested that Ser^{276}, Ser^{529}, and Ser^{536} are necessary for p65 to be phosphorylated by IKK.

PTD-p65-P1 Inhibits TNF-induced NF-κB-dependent Reporter Gene Expression—Although we had shown by EMSA that PTD-p65-P1 blocked NF-κB activation, DNA binding does not always correlate with NF-κB-dependent gene transcription, suggesting that there are additional regulatory steps (50). To determine the effect of PTD-p65-P1 on TNF-induced NF-κB-dependent reporter gene expression, we transiently transfected the cells with the NF-κB-regulated SEAP reporter construct, incubated them with the peptide, and then stimulated the cells with TNF. An almost 4-fold increase in SEAP activity over the vector control occurred upon stimulation with TNF, and peptide PTD-p65-P1 completely suppressed the TNF-induced increase. PTD or p65-P1 alone failed to suppress it (Fig. 6*A*). These results demonstrate that PTD-p65-P1 also repressed NF-κB-dependent reporter gene expression induced by TNF.

TNF-induced NF-κB activation is mediated through sequential interaction of the TNF receptor with TRADD, TRAF2, NF-κB-inducing kinase, and IKK, resulting in phosphorylation of IκBα (50, 51). To delineate the site of action of PTD-p65-P1 in the TNF-signaling pathway leading to NF-κB activation, we transfected cells with TNFR1-, TRADD-, TRAF2-, NF-κB-inducing kinase-, IKK-, and p65-expressing plasmids and then monitored NF-κB-dependent SEAP expression. As shown in Fig. 6*B*, NF-κB-SEAP gene expression was induced in all of the plasmid-transfected cells, and PTD-p65-P1 suppressed NF-κB reporter gene expression in every case. These results suggest that PTD-p65-P1 affected NF-κB activation at the terminal step.

PTD-p65-P1 Inhibits TNF-induced NF-κB-dependent Cyclin D1, COX-2, and MMP-9 Gene Expression—Because TNF treatment induces cyclin D1, COX-2, and MMP-9, which have NF-κB binding sites in their promoters (52–55), we next examined whether PTD-p65-P1 inhibits TNF-induced cyclin D1, COX-2, and MMP-9. Cells were pretreated with PTD-p65-P1 for 1 h and then treated with TNF for the indicated times, and whole-cell extracts were prepared and analyzed by Western blot analysis for the expression of cyclin D1, COX-2, and MMP-9 (Fig. 6*C*). TNF induced cyclin D1, COX-2, and MMP-9 expressions in a time-dependent manner, and PTD-p65-P1 blocked TNF-induced expression of these gene products.

PTD-p65-P1 Potentiates TNF-induced Cytotoxicity—Activation of NF-κB has been shown to inhibit TNF-induced apoptosis (56–59). Whether suppression of NF-κB by PTD-p65-P1 affects TNF-induced cytotoxicity was investigated by an MTT assay. As shown in Fig. 7*A*, TNF was cytotoxic to KBM-5 cells, and PTD-p65-P1 enhanced the TNF-induced cytotoxicity. PTD or p65-P1 by themselves had no effect on TNF-induced cytotoxicity.

Whether suppression of NF-κB by PTD-p65-P1 affects TNF-induced apoptosis was also investigated by the Live/Dead® assay (Fig. 7*B*) and TUNEL staining (Fig. 7*C*). As shown, TNF induced apoptosis in KBM-5 cells, and PTD-p65-P1 potentiated the TNF-induced apoptosis from 4 to 45% (see *red staining* in Fig. 7*B*).

PTD-p65-P1 Potentiates Chemotherapy-induced Cytotoxicity—Chemotherapeutic agents are also known to activate NF-κB and mediate chemoresistance (60). Whether suppression of NF-κB by PTD-p65-P1 affects chemotherapy-induced cytotoxicity was investigated by the MTT assay. As shown in Fig. 7*D*, doxorubicin-induced (*top panel*) and cisplatin-induced (*bottom panel*) cytotoxicity was potentiated by PTD-p65-P1. These results suggest that PTD-p65-P1 increases the therapeutic effects of chemotherapeutic agents.

DISCUSSION

The nuclear transcription factor NF-κB mediates inflammation, viral replication, carcinogenesis, antiapoptosis, invasion, and metastasis, so specific inhibitors of this factor should have therapeutic potential. In the present report, we identified a peptide from the p65 subunit of NF-κB that contains phosphorylation sites (amino acids 271–282 or 524–537) and that suppresses TNF-induced NF-κB activation *in vivo*. We found that a membrane-penetrating peptide, specifically a PTD, was required to suppress NF-κB activation *in vivo* but not *in vitro*. PTD-p65-P1 had no effect on TNF-induced AP-1 activation. The p65 peptide in which either of the serines was mutated or deleted did not inhibit DNA binding of NF-κB. PTD-p65-P1 had no effect on TNF-induced IκBα phosphorylation, IκBα degradation, or IKK activation, but it blocked cytoplasmic p65 phosphorylation and nuclear translocation. *In vitro* PTD-p65-P1 underwent phosphorylation when incubated with IKK. PTD-p65-P1 was not specific in suppressing TNF-induced NF-κB activation; it also suppressed activation induced by all of the seven inflammatory stimuli tested. NF-κB-regulated reporter gene expression induced by TNF, TNFR1, TRADD, TRAF2, NF-κB-inducing kinase, IKK, and p65 was also suppressed by PTD-p65-P1. Suppression of NF-κB by PTD-p65-P1 enhanced the apoptosis induced by TNF and chemotherapeutic agents.

Our results indicate that the p65-P1 (QLRRPSDRELSE) amino acids sequence when linked with the cell delivery PTD can suppress NF-κB activation. Deletion of residues either from the C terminus or N terminus abolished the NF-κB sup-

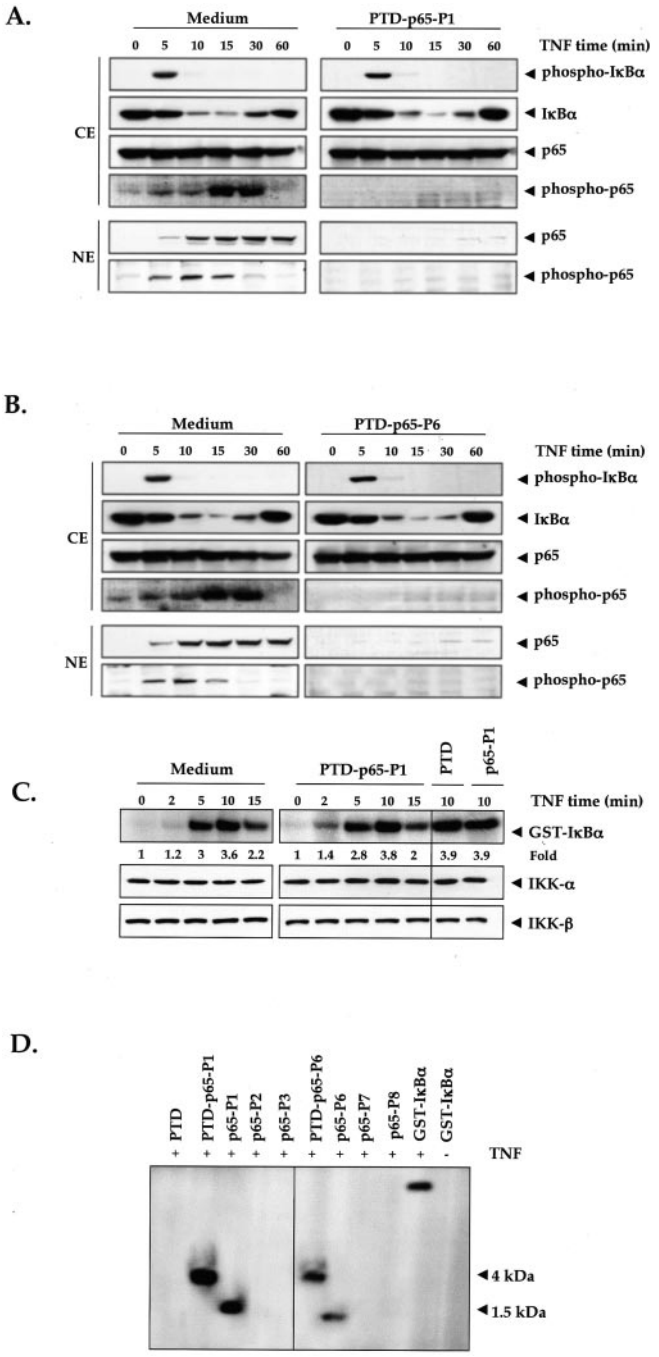

FIG. 5. *A*, PTD-p65-P1 has no effect on the TNF-induced IκBα phosphorylation or degradation but inhibits cyoplasmic p65 phosphorylation and nuclear translocation. Cells were incubated with 150 μM PTD-p65-P1 for 1 h and treated with 0.1 nM TNF for the indicated times. Nuclear and cytoplasmic extracts were prepared and then fractionated on 10% SDS-PAGE. Western blot analysis was performed using with phospho-specific anti-IκBα, anti-IκBα, phospho-specific anti-p65, anti-p65, and β-actin. *B*, PTD-p65-P6 has no effect on the TNF-induced IκBα phosphorylation or degradation but inhibits p65 phosphorylation and nuclear translocation. Cells were incubated with 150 μM PTD-p65-P6 for 1 h and treated with 0.1 nM TNF for the indicated times. Nuclear and cytoplasmic extracts were prepared and then fractionated on 10% SDS-PAGE. Western blot analysis was performed using phospho-specific anti-IκBα, anti-IκBα, phospho-specific anti-p65, anti-p65, and β-actin. *C*, PTD-p65-P1 peptide has no effect on the TNF-induced IKK activation. Cells were incubated with 150 μM PTD-p65-P1 for 1 h and treated with 0.1 nM TNF for the indicated times. Whole-cell extracts were prepared, incubated with anti-IKK-α antibody, and then immunoprecipitated using protein A/G-Sepharose beads. Immunocomplex kinase reaction was performed as described under "Materials and Methods." Whole-cell extracts were fractionated on 7.5% SDS-PAGE and immunoblotted using anti-IKK-α and anti-IKK-β antibodies. *D*, IKK phosphorylates p65 peptides in a cell-free system. Whole-cell extracts were prepared from TNF-treated cells and immunoprecipitated with antibody against IKK-α. Thereafter, an immunocomplex kinase assay was performed in the presence of the peptides as a substrate.

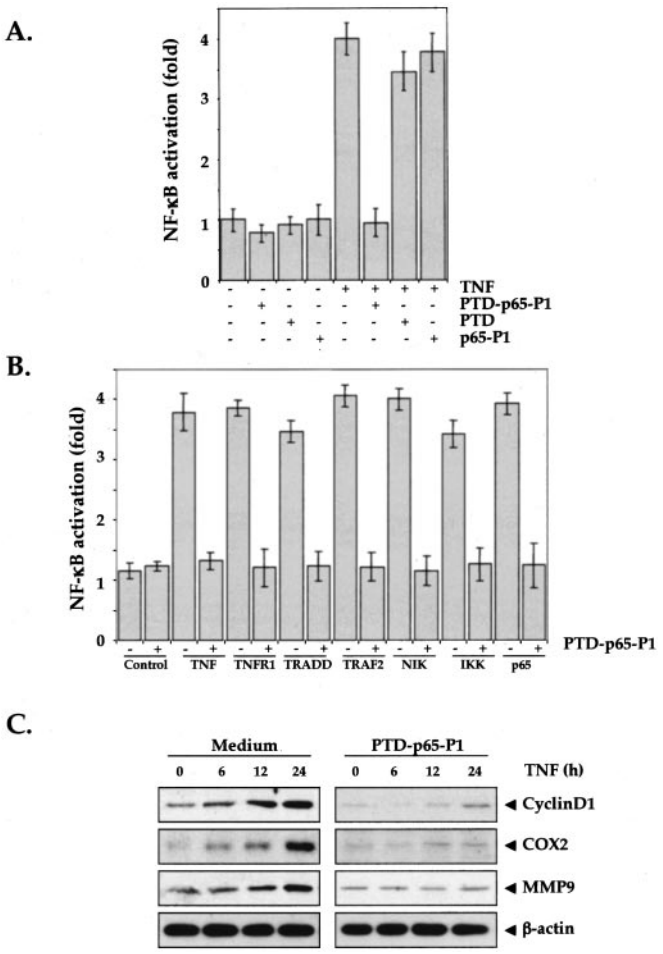

FIG. 6. **PTD-p65-P1 inhibits TNF-induced expression of NF-κB-dependent gene.** *A*, A 293 cells were transiently transfected with NF-κB-containing plasmid linked to the SEAP gene and incubated with 150 μM PTD-p65-P1. Cells were treated with 1 nM TNF, and supernatants were collected and assayed for SEAP. *B*, A293 cells were transiently transfected with a NF-κB-containing plasmid along with the indicated plasmids and then incubated with 150 μM PTD-p65-P1. Cells were exposed to TNF, and supernatants of the culture medium were assayed for SEAP. Results are expressed as -fold activity of the vector control. *C*, KBM-5 cells were incubated with 150 μM PTD-p65-P1 for 1 h and treated with 0.1 nM TNF for the indicated times. Nuclear and cytoplasmic extracts were prepared and then fractionated on 10% SDS-PAGE.

pressive activity of this peptide. Substitution of serine with an alanine residue also abolished the activity of the peptide. Previously, a sequence representing the nuclear localization of sequence of p50 has been reported to suppress NF-κB activated by lipopolysaccharide and TNF (20–23). A NEMO-binding domain peptide based on the carboxyl-terminal segment of IKK-α and IKK-β also has been reported to block NF-κB activation (19). We showed that 150 μM peptide is required to suppress NF-κB activation. May *et al.* (19) showed complete suppression of NF-κB at a 200 μM concentration of their peptide. Interestingly, we found that the p65 peptide without the delivery peptide was quite effective in inhibiting binding of NF-κB to the DNA *in vitro* and at a concentration lower than that required to suppress *in vivo*.

The p65-P1 (QLRRPSDRELSE) had no effect on AP-1 and

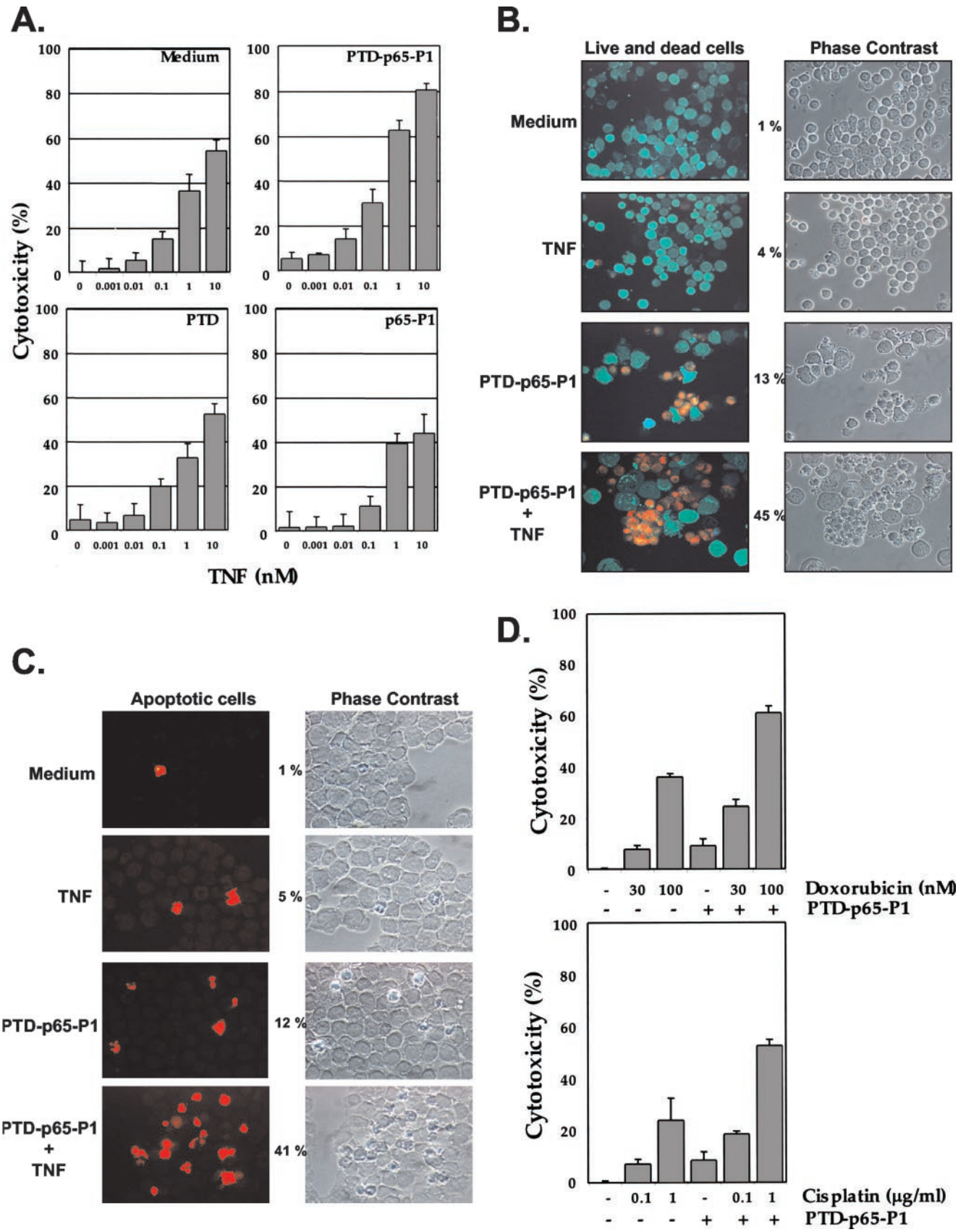

FIG. 7. **PTD-p65-P1 enhances TNF-induced cytotoxicity.** *A*, 5000 KBM-5 cells were seeded in triplicate in 96-well plates. Cells were pretreated with 100 μM PTD-p65-P1 and then incubated with the indicated concentrations of TNF for 72 h. Thereafter, cell viability was analyzed by an MTT assay. *B*, 1×10^5 cells were pretreated with 100 μM PTD-p65-P1 and then incubated with 1 nM TNF for 16 h. Cells were stained with Live/Dead® assay reagent for 30 min and then analyzed under a fluorescence microscope. *C*, 1×10^5 cells were pretreated with 100 μM PTD-p65-P1 and then incubated with 1 nM TNF for 16 h. Cells were fixed stained with TUNEL assay reagent and then analyzed under a fluorescence microscope. *D*, PTD-p65-P1 enhances chemotherapy-induced cytotoxicity. 5000 cells were seeded in triplicate in 96-well plates. Cells were pretreated with 100 μM PTD-p65-P1 and then incubated with the indicated concentrations of doxorubicin or cisplatin for 72 h. Thereafter, cell viability was analyzed by the MTT method.

did not affect IKK or IκBα phosphorylation or degradation. It did suppress cytoplasmic p65 phosphorylation and nuclear translocation. Whether p65 phosphorylation is needed for nuclear translocation is not fully understood. We also found that the peptide itself undergoes phosphorylation upon treatment with IKK. These results are consistent with previous reports that IKK can induce the phosphorylation of p65 (36–38). Sakurai *et al.* (36) have shown that IKK phosphorylates p65 at serine residue 536.

We also found that peptide from 525–536 amino acids of p65 (p65-P6) suppressed TNF-induced NF-κB activity, and substitution of serine with alanine residue at 529 or 536 also abolished the activity of the peptide, the same as p65-P1. However, both serine 529 and 536 amino acids were necessary to inhibit NF-κB activity in p65-P6. These results showed different regulation mechanisms between p65-P1 and p65-P6 on NF-κB activity. Why mutation of the serine phosphorylation sites to alanines should abolish the inhibitory activity of the peptides is not clear. It is possible that mutated peptide has low affinity for binding to p65 and thus is a poor decoy. These results are in agreement with a previous report showing that a point mutation within the NEMO-binding peptide from aspartic acid 738, tryptophan 739, or tryptophan 741 to alanine prevented the association with NEMO and thus lost the ability to inhibit NF-κB activation (19). The presence of the dimerization domain of p65 between residues 191 and 290 also raises the possibility that mutation of Ser^{276} altered the ability of the native peptide to modulate p65 dimerization. Zhong *et al.* (31) have shown that p65 phosphorylation enhances its binding to the DNA. Thus, if p65 phosphorylation is critical for binding to the DNA, a peptide with a mutated phosphorylation site may not inhibit the p65 phosphorylation. However, the latter possibility is less likely, because our experiments show that the binding of the recombinant p65 protein to the DNA is also inhibited by the native peptide and not by the mutated analogue (Fig. 2*B*).

Numerous reports suggest that NF-κB mediates suppression of apoptosis. We showed that p65-P1 (QLRRPSDRELSE) could sensitize the cells to apoptosis induced by TNF, doxorubicin, and cisplatin. We showed that p65-P1 (QLRRPSDRELSE) suppresses TNF-induced NF-κB-mediated reporter gene expression. Several genes that are involved in suppression of apoptosis are regulated by NF-κB. These include cIAP, TRAF1, TRAF2, cFLIP, survivin, Bcl-x_L, and XIAP. It is possible that p65-P1 (QLRRPSDRELSE) potentiates apoptosis by suppressing the expression of one or more of these genes. Genes involved in tumorigenesis, metastasis, angiogenesis, and inflammation are all regulated by NF-κB. These genes may also be target for p65-P1 (QLRRPSDRELSE). Indeed we found that TNF-induced cyclin D1, COX-2, and MMP-9 expression is down-regulated by this peptide. Thus, the peptide p65-P1 (QLRRPSDRELSE) reported here has a wide ranging therapeutic potential.

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