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Role of PI3-Kinase/Akt Pathway in the Activation of Etoposide-Induced NF-κB Transcription Factor

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Abstract NF-κB is a transcription factor involved in the innate immunity against bacterial infection and inflammation. It is also known to render cells resistant to the apoptosis caused by some anticancer drugs. Such a chemoresistance of cancer cells may be related to the activation of NF-κB transcription factor; however, the mechanism of activation is not well understood. Here, we demonstrate that a chemotherapeutic agent, etoposide, independently stimulates the IκBα degradation pathway and PI3-kinase/Akt signaling pathway: The classical IκBα degradation pathway leads to the nuclear translocation and DNA binding of p65 subunit through IKKβ kinase, whereas the PI3-kinase/Akt pathway plays a distinct role in activating this transcription factor. The PI3-kinase/Akt pathway acts on the p50 subunit of the NF-κB transcription factor and enhances the DNA binding affinity of the p50 protein. It may also explain the role of the PI3-kinase/Akt pathway in the anti-apoptotic function of NF-κB during chemoresistance of cancer cells.

Key words: NF-κB, IκBα, PI3-kinase, Akt, p65, p50

The transcription factor NF-κB is essential for inflammation, innate immune response against bacterial infection, as well as apoptosis regulation [7]. Ordinarily, NF-κB exists as a dimer composed of p65 and p50 subunits that are sequestered in the cytoplasm by an inhibitory protein, IκBα [4]. In response to appropriate signals, IκBα is phosphorylated and degraded. This allows NF-κB to enter the nucleus and bind to target DNA. A variety of signals, such as LPS, TNF-α, IL-1, UV, and DNA damaging drugs, activate NF-κB by causing the degradation of IκBα. Activation of NFκB is under several types of control, probably because of the importance of maintaining a tight control of its activity [8]. In addition to the phosphorylation and degradation of IκBα, several critical events are required for full activity. These include its nuclear translocation, binding of its component subunits to DNA, and an increase in its transcriptional potential due either to binding of co-activators or release of co-repressors. It is not clear at present how these critical steps are regulated by the various NF-κB agonists.

Although IκBα degradation may be the major pathway of NF-κB activation, accumulating lines of evidence suggest that the networks regulating activation are more complex than previously envisioned [6]. Thus, recent studies indicate that NF-κB can be activated by the PI3-kinase signaling pathway in response to a variety of stimuli [3, 11, 15, 22, 28, 29, 33]. However, many aspects of the activation process remain unexplained. It is not known how the separate steps in activation, such as translocation, DNA binding, and modulation of transcriptional potential of NF-κB, are regulated by these distinct pathways. Nor is it known whether the two subunits of the NF-κB complex are regulated by the same or separate pathways. In particular, the effect of the PI3-kinase pathway on each subunit requires clarification. In general, LPS, TNF-α, and IL-1 cause strong and rapid activation of NF-κB, whereas DNA damaging agents and UV have a weaker and more delayed effect [2, 13, 17, 23]. Furthermore the pathway by which DNA damage acts has yet to be identified, and this is of some importance as DNA damage-induced NF-κB activation is believed to suppress apoptosis and to reduce the efficacy of chemotherapeutic agents [2, 5, 13, 23, 31, 34].

Here, we have examined the role of the PI3-kinase pathway and the degradation of IκBα in the various steps leading to NF-κB activation in response to the DNA damaging drug, etoposide. We have shown that PI3-kinase/Akt operates independently of IκBα degradation, nuclear translocation, and DNA binding of the p65 subunit of NF-κB. In fact, the PI3-kinase/Akt pathway is critical for the other subunit of NF-κB transcription factor, such as enhancing the DNA-binding affinity of the p50 subunit. The finding

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provides insight into how NF-κB activation can increase chemoresistance, and may help extend the effectiveness of chemotherapeutic drugs by reducing the associated anti-apoptotic action of NF-κB.

MATERIALS AND METHODS

Antibodies, Plasmids, and Reagents

Anti-p65 C-20 rabbit polyclonal antibody (sc-372), anti-p50 rabbit polyclonal antibody (sc-8414), anti-IκBα rabbit polyclonal antibody (sc-371), and NF-κB consensus oligonucleotide were purchased from Santa Cruz Biotechnology. Anti-phospho-Akt (ser473) 4E2 monoclonal antibody and anti-Akt1 antibody were from Cell Signaling Technology. Anti-FLAG M2 monoclonal antibody as well as etoposide, wortmannin, LY-294002, phosphatase inhibitor cocktail, and protease inhibitor cocktail were purchased from Sigma-Aldrich. 3X-κB-Luc, 3X-mut κB-Luc reporters [18], and p65/RelA expression clone were kindly provided by Dr. Jung Chan Park (Hankook University of Foreign Studies), and DN-IKKα, DN-IKKβ, and IκB-SR (S32A and S36A mutations) were kind gifts of Drs. Soo Young Lee (Ewha Womans University) and Yong Keun Jung (Seoul National University) [19]. Myc-wt-PDK1 and Myc-DN-PDK1 expressing vectors were from Dr. Hyun Jeong Ha (Chungbuk University). Mouse and human p50 expression clones were kindly provided by Drs. Kyung Hee Choi (Chung Ang University) and Young Mee Kim (Asan Medical Center). DN-Akt expressing vector (Upstate Biotechnology) was a kind gift of Dr. Yoon Soo Bae (Ewha Woman's University). pCMV-β was purchased from Clonetech, BD Sciences. Radioisotopes and poly(dI-dC) were from Amersham Pharmacia Biotech, and restriction and modifying enzymes were from Roche Molecular Biochemicals.

Cell Culture, Transfection, and Luciferase Assay

293T and HeLa cells were cultured in DMEM containing 10% fetal bovine serum (BRL Life Technology, Inc). Cells were transiently transfected with the 3X-κB-Luc reporter (200 ng) and the plasmids, indicated using LipofectAmine (Promega). pCMV-β (50 ng) was co-transfected in each case to normalize transfection efficiency. Cells were incubated for 24 h, serum-depleted for 3 h, and stimulated with 50 μM etoposide for the times indicated. In some cases, the cells were preincubated for 1 h with 10 nM wortmannin, 20 μM LY-294002, or 10 μg/ml ALLN before adding the etoposide [14]. They were washed once with phosphate-buffered saline (PBS) and incubated with 200 μl of lysis buffer (Promega) for 10 min at room temperature, and cleared by centrifugation at 13,000 rpm. Luciferase activity was measured with the Luciferase Assay System (Promega) using a luminometer (TD-20/20 Luminometer, Turner Designs). Experiments were repeated at least three times, and values are expressed as means and standard deviations [12].

Preparation of Nuclear Extracts

293T cells were washed twice with ice-cold PBS, harvested, and lysed in 200 μl of buffer A [20 mM HEPES (pH 7.9), 10 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluroride, phosphatase inhibitor cocktail, and protease inhibitor cocktail] for 15 min on ice. Nonidet P-40 was added to 0.5%, and after centrifugation, the pellets were washed with buffer A and resuspended in ice-cold buffer C [20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 300 mM phenylmethylsulfonyl fluroride, 10% glycerol, 1% Triton X-100, phosphatase inhibitor cocktail, and protease inhibitor cocktail] and incubated for 15 min at 4°C. Supernatants obtained by centrifugation at 20,000 ×g for 5 min were stored at -70°C.

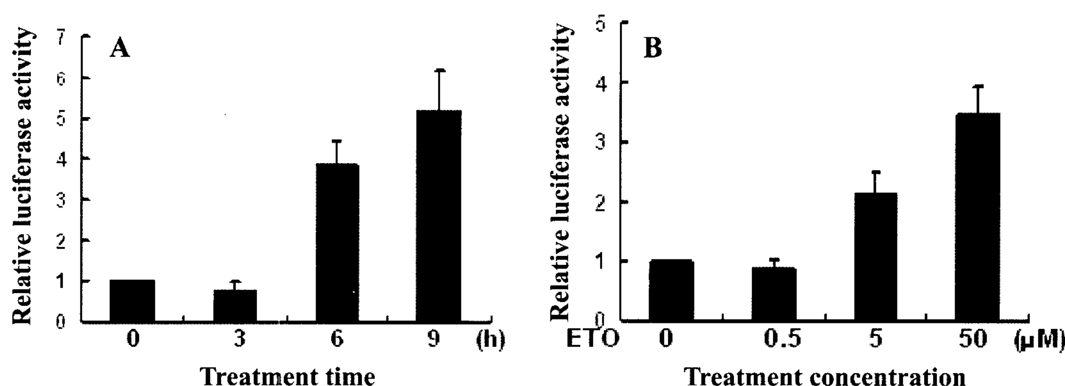

Fig. 1. Etoposide activated NF-κB transcription factor. 293T cells were transfected with the 3X-κB-Luc reporter and treated with etoposide for the times (**A**) and concentrations (**B**) indicated.

Luciferase activity was determined, normalized with β-gal activity, and presented as Relative Luciferase Activity. At least five experiments were performed for each case.

Electrophoretic Mobility Shift Assays

NF-κB consensus oligonucleotide was end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. Nuclear extracts were incubated with 2 μg of poly(dI-dC) in binding buffer (5 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol) for 10 min at 37°C. The end-labeled probe was added, the mixtures incubated for 10 min at 37°C, and aliquots run on 4% native polyacrylamide gels in 1× TAE buffer. The gels were dried and subjected to autoradiography.

RESULTS

Etoposide Causes IκBα Degradation

To test if exposure to etoposide activates NF-κB, we performed luciferase reporter assays designed to detect

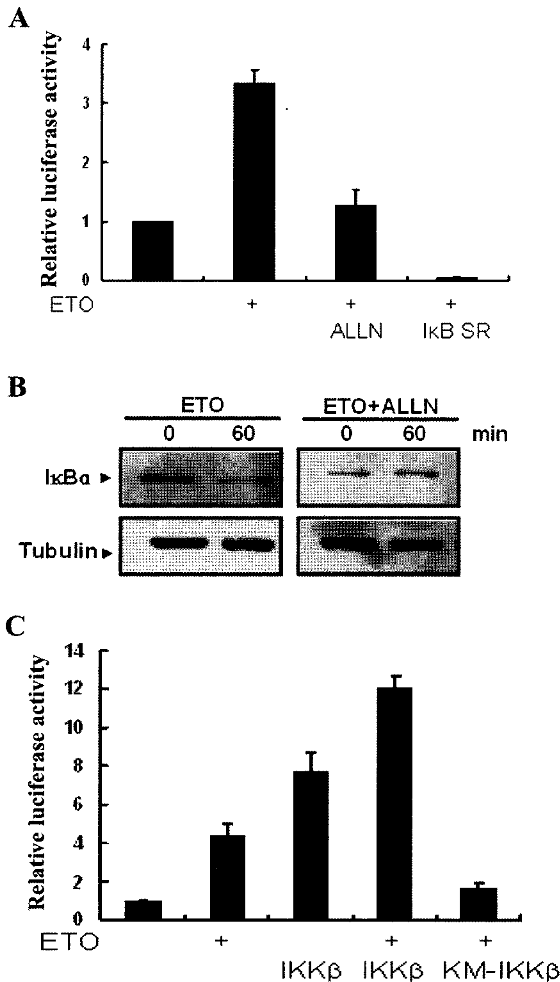

Fig. 2. Etoposide induces IκBα degradation.
A. 293T cells were transfected with the 3X-κB-Luc reporter with or without co-transfecting IκBα super-repressor expression vector (IκBα SR). After transfection, the cells were serum-depleted for 3 h and pre-incubated with ALLN for 1 h, followed by etoposide treatment for 9 h. Relative luciferase activities were determined as described above. **B.** 293T cells were pre-incubated with ALLN for 1 h, treated with etoposide for 9 h, and cell extracts fractionated on a 10% PAGE-SDS gel (25 μg of protein/lane). Western blot analysis was performed using an anti-IκBα antibody, and anti-tubulin antibody was used to provide a loading control. **C.** 293T cells were transfected with the 3X-κB-Luc reporter in the presence of wild-type or KM (kinase mutant) IKKβ expression vectors. After transfection, the cells were serum-depleted for 3 h, followed by etoposide treatment for 9 h. Relative luciferase activities were determined as described above. At least three independent experiments were performed in each case.

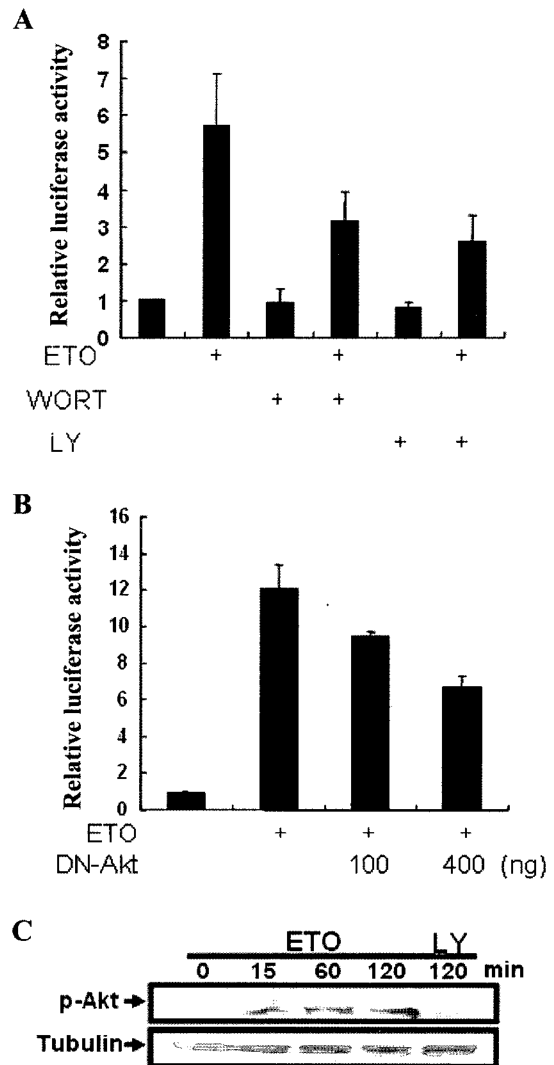

Fig. 3. Etoposide-induced NF-κB activation is mediated by the PI3-kinase/Akt-dependent pathway.
A. Luciferase assay of wortmannin or LY-294002 treated 293T cells. Cells were transfected with the 3X-κB-Luc reporter and pre-incubated with wortmannin or with LY-294002 for 4 h in serum-free medium and stimulated with etoposide for 9 h. Relative luciferase activities were determined as described above. **B.** 293T cells were transfected with a DN-Akt expression plasmid and treated with etoposide. **C.** 293T cells were treated with etoposide for the times indicated, and one sample was pre-incubated with LY-294002. Western blot analysis was performed with anti-phospho-Akt antibody. Anti-tubulin antibody was used to provide a loading control.

NF-κB activation with the 3X-κB-Luc reporter. As shown in Fig. 1A, the transcriptional activity of NF-κB was enhanced 5–6-fold after 9 h of etoposide treatment. Dose-dependent activation was also observed (Fig. 1B). Gel mobility shift assays also showed that etoposide treatment stimulated NF-κB binding to its consensus DNA sequence (data not shown). Activation of NF-κB is known to result from IKK-dependent phosphorylation and degradation of IκBα. Therefore, to test whether etoposide-induced NF-κB activation was also caused by IκBα degradation, we used a proteasome-blocking agent, ALLN, to inhibit IκBα degradation. As expected, pretreatment with ALLN inhibited activation of NF-κB (Fig. 2A) and prevented etoposide-induced IκBα degradation (Fig. 2B). The role of IκBα was confirmed by the observation that an IκBα Super Repressor (SR) mutant also completely blocked NF-κB activation (Fig. 2A). Hence, like other NF-κB activators, etoposide activates NF-κB by causing the phosphorylation and proteolysis of IκBα. We further tested which IKK was responsible for the phosphorylation and degradation of IκBα. As shown in Fig. 2C, the kinase activity of IKKβ was responsible for the etoposide-induced NF-κB activation.

PI3-Kinase and Akt-Dependent NF-κB Activation

Since other NF-κB-inducing chemicals suppress apoptosis by activating Akt kinase, it was important to test if etoposide activates the Akt pathway [24]. Since Akt kinase is activated by PI3-kinase, we first tested if inhibitors of the important kinase reduced the NF-κB activation. Pretreatment with wortmannin or LY-294002, well known PI3-kinase inhibitors, substantially inhibited etoposide-induced NF-κB activation in both 293T and HeLa cells (Fig. 3A),

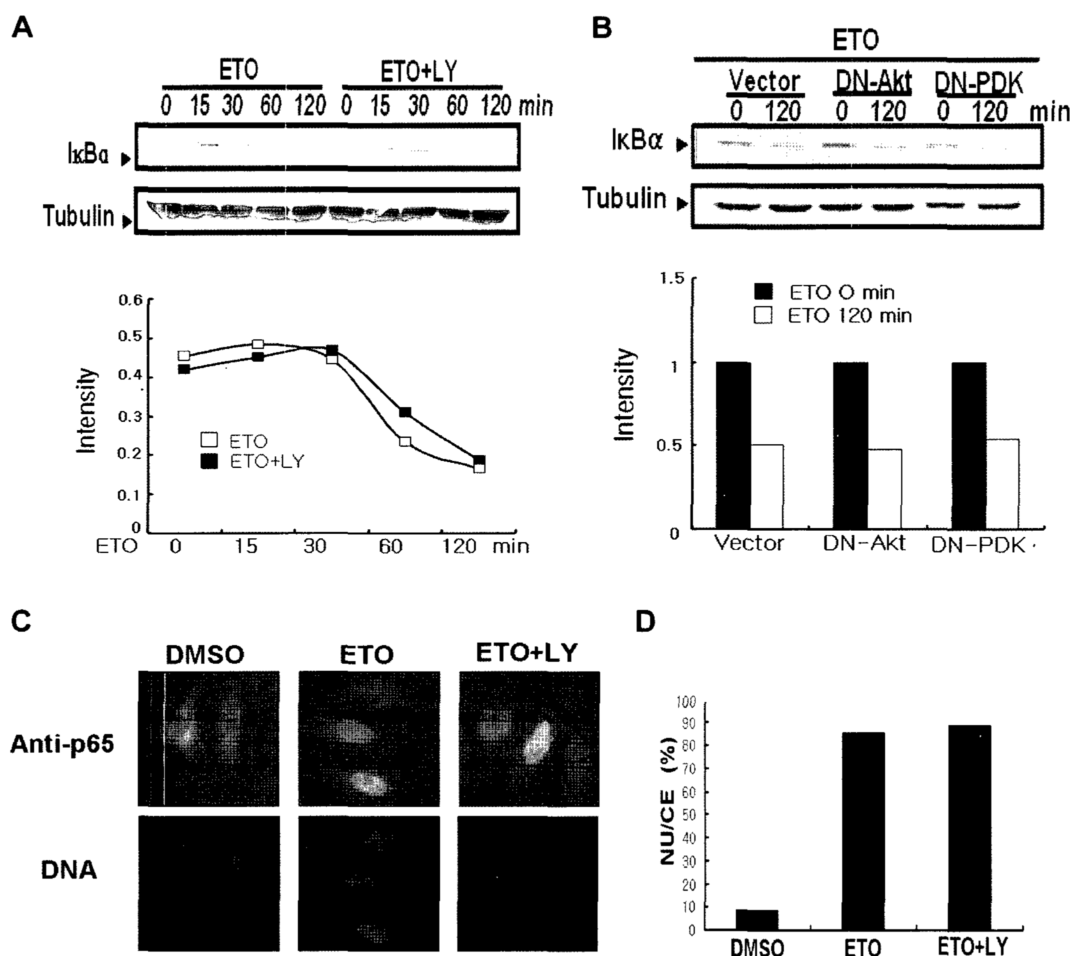

Fig. 4. The PI3-kinase/PDK1/Akt pathway is not required for etoposide-induced IκBα degradation.
A. IκBα degradation assay. 293T cells were pre-incubated with LY-294002 and stimulated with etoposide for the times indicated. Western blot analysis was performed with anti-IκBα antibody. Anti-tubulin antibody was used as a control. Quantification of the Western blot is shown as the bar graph. **B.** 293T cells were transfected with DN-Akt or DN-PDK1 expression clones and treated with etoposide. Western blot analysis was performed as above. **C.** HeLa cells were treated with etoposide as well as LY-294002, as above. The cells were stained with the anti-p65 antibody and immunofluorescence detected. Nuclei were identified by Hoechst staining. **D.** The percentage of cells showing prominent nuclear localization of p65 protein is presented in the bar graph. The numbers of examined cells are at least 150 for each sample.

which strongly suggests that etoposide at least in part activates NF-κB through the PI3-kinase.

Since the kinase activities of PI3-kinase proved to be critical for NF-κB activation, we questioned whether their downstream effector, Akt, was also involved. Etoposide-induced NF-κB activation was significantly reduced, when a Dominant Negative (DN)-Akt protein was expressed (Fig. 3B). To detect a more specific effect on Akt activation, we also measured the phosphorylation status of Akt kinase. Akt was phosphorylated on Ser 473 by etoposide treatment, and this process was inhibited by LY-294002 (Fig. 3C). These results show that Akt kinase activated by PI3-kinase is involved in NF-κB activation by etoposide, as reported for other NF-κB stimulators such as IL-1 and TNF cytokines, as well as UV [13, 23, 31].

The PI3-Kinase/Akt Pathway is Not Responsible for IκBα Degradation

The above experiments indicate that etoposide causes IκBα degradation, induces the PI3-kinase/Akt pathway, and activates NF-κB. To clarify whether the PI3-kinase pathway acts upstream of IκBα degradation, we examined the effect of etoposide on IκBα degradation, after pretreatment with LY-294002. Western blot analysis with anti-IκBα antibody showed that LY-294002 had no effect on etoposide-induced IκBα degradation (Fig. 4A). The lack of a requirement for the PI3-kinase pathway for IκBα degradation was confirmed by showing that IκBα degradation was induced by etoposide in cells expressing dominant negative forms of Akt and PDK1 (Fig. 4B). This is consistent with the fact that the PI3-kinase inhibitor LY-294002 and the tumor

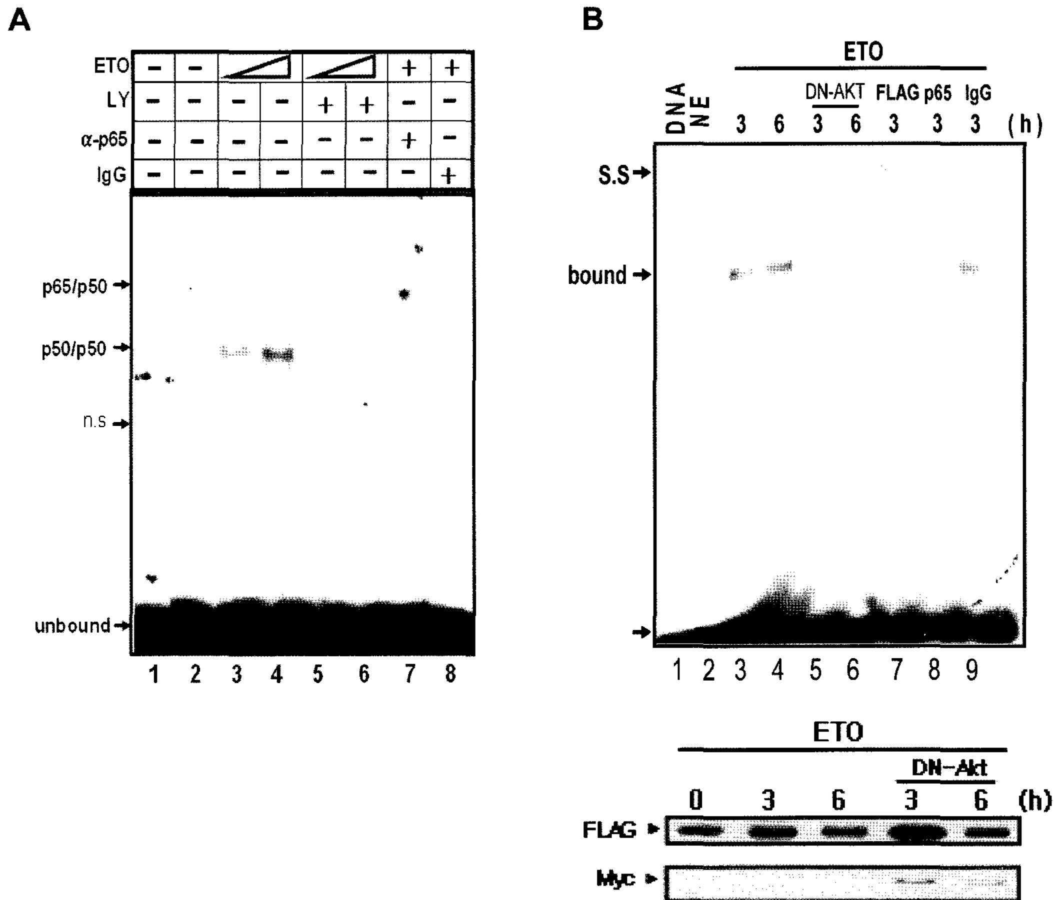

Fig. 5. The PI3-kinase pathway regulates p50/p50 protein binding to DNA.
A. EMSAs were performed with a radiolabeled consensus NF-κB oligonucleotide (lane 1). The probe was incubated with nuclear extracts of 293T cells treated with DMSO for 3 h (lane 2), etoposide for 30 min (lanes 3 and 5) or 3 h (lanes 4 and 6), as well as pretreated with LY-294002 (lanes 5 and 6). Identity of each band was confirmed by supershifting with anti-p65 antibody (lane 7) or with normal rabbit IgG (lane 8). Expression of FLAG-p50 and Myc-DN-Akt was confirmed by Western blot analysis with anti-FLAG and anti-Myc antibodies. **B.** 293T cells were transfected with FLAG-tagged p50 plasmid in the presence or absence of DN-Akt plasmid and treated with etoposide for the times indicated. Nuclear extracts were prepared and EMSAs were performed as described above. The probe was incubated with a nuclear extract of 293T cells (NE, lane 2), or of etoposide-treated FLAG-p50 transfected cells (lanes 3–4), and of cells co-transfected with FLAG-p50 and DN-Akt proteins (lanes 5–6). Identities of the bands were confirmed by supershifting with anti-FLAG antibody (lane 7), anti-p65 antibody (lane 8), or rabbit IgG (lane 9). Expression of FLAG-p50 and Myc-DN-Akt was confirmed by Western blot analysis with anti-FLAG and anti-Myc antibodies.

suppressor PTEN inhibit NF-κB activation without interfering with IκB degradation in cytokine-induced NF-κB activation [16, 23]. Since IκBα degradation is generally thought to be responsible for nuclear translocation of the p65 subunit of NF-κB, we tested if etoposide-induced p65 translocation requires the PI3-kinase pathway. As shown in Figs. 4C and 4D, etoposide caused p65 nuclear translocation, but the translocation was not inhibited by LY-294002. This suggests that nuclear translocation of p65 is also dependent on IκBα degradation in this situation, and that neither IκBα degradation nor translocation of p65 is dependent on the PI3-kinase/PDK1/Akt pathway.

The PI3-Kinase/Akt Pathway Controls p50 Binding to DNA

The increased DNA binding activity of p65 brought about by a number of NF-κB agonists is dependent on IκBα proteolysis. Since the activation of NF-κB by etoposide required both IκBα proteolysis and the PI3-kinase/Akt pathway, we tested if the DNA binding activity of NF-κB was regulated by both pathways. As shown by EMSA in Fig. 5A, etoposide stimulated DNA binding of p50/p50 homodimers as well as of p65/p50 heterodimers. Interestingly, binding by the p65/p50 heterodimer was not inhibited by LY-294002, whereas binding by the p50/p50 homodimer was, strongly suggesting that the PI3-kinase pathway regulates the binding of p50/p50 to DNA, but not that of p65/p50. The identities of the bound bands were confirmed by supershifting with antibodies. To confirm the effect of the PI3 kinase/Akt pathway on DNA binding by p50, p50 was overexpressed as a FLAG-tagged protein and the EMSA assays were repeated (Fig. 5B). The FLAG-p50 protein produced a single prominent DNA-bound band that was substantially reduced by expressing DN-Akt. Evidently, binding of the p50/p50 homodimer is regulated by the PI3 kinase/Akt pathway. Since we have shown that p65 binding was inhibited by the proteasome inhibitor ALLN, and the binding of p65/p50 was not controlled by the PI3-kinase/Akt pathway, our findings strongly suggest that the binding of p50 and p65 is regulated by distinct pathways. Most importantly, DNA binding by the p50 subunit is controlled by the PI3-kinase/Akt pathway.

DISCUSSION

NF-κB is a complex transcription factor that requires several steps for full activation. Hence, it is not surprising to find that a number of parallel signaling pathways turned on by a single "signal" regulate distinct steps of its activation [4, 7, 8, 26]. In contrast to the IκBα degradation pathway, which is important for high-level activation of NF-κB, it seemed unclear how the PI3-kinase/Akt pathway might activate NF-κB [2, 11, 22, 28, 29, 33]. We have now shown that these two pathways play distinct roles in activating the NF-κB complex in response to DNA damage by the drug etoposide. The nuclear localization and DNA binding activities of the p65 and p50 subunits of NF-κB are separately controlled. The IκBα proteolysis pathway is important for p65 translocation, as expected, but it is not critical for other steps of NF-κB activation. We propose that the PI3-kinase/Akt pathway is critical for enhancing the basal level of NF-κB transcription in the following ways [21]. It is also critical for promoting nuclear translocation and DNA binding of the p50 subunit of NF-κB. Nuclear translocation and DNA binding of p50 is thought to be important for activation of NF-κB, because it is a prerequisite for NF-κB activation. Since the PI3-kinase/Akt pathway has a basic role in maintaining cell survival, it makes sense that it mediates the basal mode of activation of NF-κB transcription, thereby eliciting anti-apoptotic gene expression.

The role of the PI3-kinase pathway in activating NF-κB binding is still not clear. In some studies, the PI3-kinase inhibitor LY-294002 did not interfere with NF-κB binding to consensus DNA in response to TNF-α or IL-1 [22, 28, 29]. On the other hand, the tumor suppressor PTEN that inhibits PI3-kinase was reported to reduce the DNA binding of p50/NF-κB in response to TNF-α treatment [16, 35]: These contradictory results can be reconciled if one assumes that the pathways regulating the DNA binding of the p50/p50 homodimer and the p65/p50 heterodimer are distinct and that PI3-kinase regulates only p50 binding. In fact, it has been shown that the p50/p50 homodimer and the p65/p50 heterodimer differ in their HDAC binding and DNA binding behaviors [1, 35]. The p50/p50 homodimers bound to κB consensus DNA when associated with HDAC in resting cells, whereas NF-κB activation in response to cytokines resulted in the formation of p65/p50 heterodimers without associated HDAC. Therefore, our finding that LY-294002 and DN-Akt expression inhibited p50 homodimer binding (Fig. 5) can be explained if PI3-kinase/Akt signaling releases HDAC1 protein from the NF-κB complex. In other words, the etoposide-induced PI3-kinase pathway could release HDAC1 from DNA-bound HDAC/p50, thereby specifically reducing the amount of p50/p50 bound to DNA without altering the number of p65/p50 complexes on κB consensus DNA. A number of kinases have been shown to phosphorylate p65 [20, 25, 32, 35]. However, the phosphorylation status of p50 was not known nor the identity of possible kinases, except for the phosphorylation critical for the DNA binding [10]. We have preliminary data that p50 is phosphorylated on the serine residues in a PI3-kinase-dependent way (Choi and Jeong, manuscript in preparation).

The role of NF-κB in apoptotic cell death induced by topoisomerase poisons and its suppression is the subject of intensive study [27, 30]. We propose that etoposide-induced

resistance to apoptosis could be due to the action of PI3-kinase/PDK1/Akt in inducing DNA binding of p50. This could induce a low level of NF-κB activity and might provide a clue to missing pieces of the puzzle of the cellular NF-κB regulatory network [9].

Acknowledgments

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