Akt Is a Downstream Target of NF-κB*

Fanyin Meng, Li Liu, Paul C. Chin, and Santosh R. D’Mello‡

From the Department of Molecular and Cell Biology, University of Texas at Dallas, Richardson, Texas 75083

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The ubiquitously expressed transcription factor NF-κB and the serine-threonine kinase Akt both are involved in the promotion of cell survival. Although initially believed to operate as components of distinct signaling pathways, several studies have demonstrated that the NF-κB and Akt signaling pathways can converge. Indeed, IκB kinase, the kinase involved in NF-κB activation, is a substrate of Akt, and activation of Akt therefore stimulates NF-κB activity. Although these results place Akt upstream of NF-κB activation in the sequence of signaling events, we report that this may not necessarily be the case and that Akt is a downstream target of NF-κB. Treatment of NIH3T3 cells with the NF-κB activators, tumor necrosis factor (TNF) α and lipopolysaccharide, results in the stimulation of Akt phosphorylation. The stimulation of Akt is, however, detected only after IκB-α degradation is induced by these agents. The nuclear translocation of p65 and increased DNA binding activity of NF-κB also precede Akt phosphorylation. Treatment with two pharmacological inhibitors of NF-κB, SN50 and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), blocks TNF-induced Akt activation. On the other hand TNF-mediated NF-κB activation is not reduced by the phosphoinositide-3 kinase inhibitors wortmannin and LY294002, although these inhibitors completely block the activation of Akt. These results suggest that NF-κB is required for TNF-mediated Akt activation and that it lies upstream of the stimulation of Akt. Consistent with this conclusion is the finding that overexpression of p65/RelA leads to Akt phosphorylation in the absence of extracellular stimulatory factors, whereas overexpression of IκB-α reduces Akt phosphorylation below basal levels. Interestingly, in addition to stimulated Akt, overexpression of p65 causes an increase in the expression of Akt mRNA and protein.

NF-κB plays a critical role in regulating inducible gene expression in immunity, stress responses, and inflammation (reviewed in Refs. 1–3). Moreover, NF-κB is involved in the regulation of cell survival (1–3). Although found to cause cell death in some cases, in a majority of systems NF-κB activation provides a survival-promoting signal (4). Classical NF-κB is a heterodimer composed of the p50 and p65/RelA subunits, which exists in the cytoplasm in an inactive complex bound by IκB proteins (1–3). Generally, the activation of NF-κB involves the phosphorylation of IκB, which then targets IκB for ubiquitination and degradation. This permits NF-κB to translocate to the nucleus, where it activates gene transcription. A wide variety of NF-κB-responsive genes have been identified. A key regulatory step in this pathway of NF-κB activation is the activation of a high molecular weight IκB kinase (IKK) complex in which catalysis is thought to be performed by kinases including IKKα and IKKβ. Exactly how these IKKs are activated is the subject of much investigation. One well studied pathway that leads to NF-κB activation and which is activated by the cytokine TNFα involves the intracellular signaling molecules TNF receptor-associated factors (TRAF2 and TRAF6) and leads to the activation of NF-κB-inducing kinase (NIK), which phosphorylates the IKKs (1–3). More recent studies have shown that IKKα and IKKβ can be phosphorylated by an alternative pathway, which involves Akt. Indeed TNFα- and platelet-derived growth factor (PDGF)-induced NF-κB activation has been reported to require Akt (5–8), although this relationship is not always observed (9, 10). In addition to TNFα and PDGF, the activation of NF-κB by interleukin-1, bradykinin, interferon α/β, and bacterial proteins also has been reported to involve Akt activation (11–14).

Akt/PKB is a serine-threonine kinase that is best known for its ability to inhibit cell death pathways (reviewed in Refs. 15–17). It does so by directly phosphorylating and inactivating proteins involved in apoptosis including GSK3, Bad, Forkhead, and procaspase-9. Activation of Akt by growth factor and cytokine treatment generally occurs via the phosphoinositide 3-kinase (PI-3 kinase) pathway. Upon stimulation, PI-3 kinase phosphorylates specific phosphoinositide lipids, which accumulate in the plasma membrane, creating docking sites for Akt. At the plasma membrane Akt undergoes phosphorylation at two residues, Thr208 and Ser473, leading to its activation.

Although the evidence for the involvement of Akt in NF-κB activation is compelling, whether NF-κB can affect Akt activity has not been examined. We report here that the expression and activity of Akt is regulated by NF-κB.

MATERIALS AND METHODS

Reagents and Plasmids—Recombinant human IGF-1 was purchased from Roche Molecular Biochemicals (Indianapolis, IN), recombinant human TNFα was purchased from Promega (Madison, WI), and lipopolysaccharide (LPS) was purchased from Sigma. Actinomycin D, cycloheximide, SN-50, wortmannin, and LY294002 were purchased from Calbiochem (La Jolla, CA), and TPCK was purchased from Sigma. The plasmids encoding p65-FLAG (CMV-p65) and IκB-α (CMV-IκBα) were gift from A. S. Baldwin (University of North Carolina) and R. Gaynor (University of Texas Southwestern Medical Center), respectively. The CMV-Akt-Myc was purchased from Upstate Biotechnology (Lake Placid, NY).

Rabbit polyclonal antibodies against IκB-α and p65 and the mouse monoclonal Myc antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against total Akt,

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‡ To whom correspondence should be addressed: Dept. of Molecular and Cell Biology, University of Texas at Dallas, 2601 N. Floyd Rd., Richardson, TX 75083. Tel.: 972-883-2520; Fax: 972-883-2409; E-mail: dmello@utdallas.edu.

The abbreviations used are: IKK, IκB kinase; IGF-1, insulin-like growth factor-1; LPS, lipopolysaccharide; PI-3 kinase, phosphoinositide-3 kinase; RT-PCR, reverse transcriptase-PCR; TNF, tumor necrosis factor; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; TRAF, TNF receptor-associated factor; NIK, NF-κB-inducing kinase; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay.
Ser(P)177 Akt, Akt2, Akt3, and phospho-GSK3 were from Cell Signaling, Inc. (Beverly, MA). Polyclonal β-tubulin antibody was purchased from Sigma. Peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology.

**Cell Lines and Neuronal Cultures—**NIH3T3 cells and HEK293 T cells were obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml), which were all obtained from Invitrogen.

Cerebellar granule neurons were cultured from dissociated cerebella of 7–8-day-old rats as described previously (18). Cells were plated in basal Eagle’s medium with Earle’s salts (Invitrogen) supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine (Invitrogen), and 100 μg/ml gentamicin on dishes coated with poly-L-lysine at a density of 1.0 × 10⁵ cells/well, 1.2 × 10⁶ cells/60-mm dish, or 3.0 × 10⁷ cells/100-mm dish. Cytosine arabinofuranoside (10 μM) was added to the culture medium 18–22 h after plating to prevent replication of non-neuronal cells. The cultures were maintained for 6–7 days prior to experimental treatments.

**Treatment with Stimulators and Inhibitors—**When NIH3T3 cells were treated with TNFα or LPS, cultures at about 80% confluency on 60-mm dishes were used. Prior to treatment, the cells were maintained in Dulbecco’s modified Eagle’s medium containing 0.5% fetal calf serum for 24 h. When used, SN-50 (10 μM) and TPCK (10 μM) were added to the medium 1 h prior to the addition of TNFα or LPS when where warranted. SN-50 was added 2 h prior to the addition. Pretreatment with actinomycin D and cycloheximide was performed for 1 h. For treatment with IGF-1, cerebellar granule neuron cultures were switched to serum-free Basal Eagle’s medium with Earle’s salts for 3 h prior to the addition of IGF-1 (25 ng/ml). When used, SN-50 and TPCK were added 2 h prior to stimulation with IGF-1.

**Transient Transfections—**NIH3T3 cells and HEK293 T cells were plated at a density of 6 × 10⁶ per 60-mm dish in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Transfections were carried out the following day using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s guidelines. 5–6 μg of plasmid DNA was used per dish, and the transfections were carried out for 4–6 h in Opti-MEM (Invitrogen) medium. The transfection medium was then replaced for 48 h by serum-containing Dulbecco’s modified Eagle’s medium (unless specified otherwise).

**Western Blots—**After treatment, confluent cell monolayers in 60-mm dishes were washed twice with ice-cold phosphate-buffered saline and lysed by incubation for 20 min in 1 ml of ice-cold cell lysis buffer (1% Nonidet P-40, 50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 1 mM sodium fluoride, and 1× protease mixture) and stored at −70 °C. Protein concentrations were measured using a Bradford protein assay kit (Bio-Rad). Equivalent amounts of protein were resolved and mixed with 6× SDS-PAGE sample buffer and then subjected to SDS-PAGE in a 4–20% linear gradient Tris-Page-ready gel (Bio-Rad, Hercules, CA). The resolved proteins were then transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% nonfat dry milk (except for phospho-Akt Western blots in which 5% bovine serum albumin was used) in Tris-buffered saline, pH 7.4, containing 0.05% Tween 20 and were incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies according to the manufacturer’s instructions. The protein of interest was visualized with enhanced chemiluminescent (ECL, Amershams Biosciences) reagents. Images of the bands were scanned by reflectance densitometry, and the intensity of the bands was quantified using NIH Image software.

**Akt Activity Assays—**Akt assays were performed on Akt that was immunoprecipitated from neuronal cultures or cell lines using a kit from Cell Signaling Technologies. Following treatment, the cultures were placed on ice and harvested using non-denaturating lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, and protease inhibitors). The lysates were centrifuged for 10 min at 10,000 × g, and the protein Akt was immunoprecipitated from 200 μl of cell-free extracts. The immunocomplexes were washed twice with kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium vanadate, and 10 mM MgCl₂). In vitro kinase assays were performed for 30 min at 30 °C in 40 μl of reaction volume containing the 30 μl of immunoprecipitates in kinase buffer, 200 μM ATP. GSK3 fusion protein (Cell Signaling Technology) was used as a substrate for Akt kinase activity. The reactions were terminated with 20 μl of 3× SDS sample buffer and subjected to Western blotting using a phospho-GSK3 antibody (Cell Signaling Technology).

**Preparation of Nuclear and Cytoplasmic Extracts—**To obtain cytoplasmic proteins, cells were washed with cold phosphate-buffered saline (PBS; pH 7.4) and resuspended in buffer containing 10 mM HEPES pH 7.6, 1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol, 50 mM sodium fluoride, 50 mM β-glycerophosphate, 5% glycerol, and 1× protease inhibitor mixture (Roche Molecular Biochemicals), and incubated on ice for 15 min. At the end of incubation, 0.05 volumes of 10% Nonidet-P-40 was added. Cells were vortexed for 30 s and then subjected to micro-centrifugation for 30 s. Supernatants were collected as cytoplasmic extracts. The protein concentrations of the cytoplasmic extracts were determined using Bio-Rad reagent.

Nuclei from NIH3T3 cells were resuspended in buffer containing 20 mM HEPES pH 7.6, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 1× protease inhibitor mixture and extracted on ice for 30 min followed by micro-centrifugation at 14,000 rpm for 10 min. The supernatants were collected as nuclear extracts. Concentrations of these nuclear extracts were determined by the Bradford method using reagents from Bio-Rad.

**Gel Electrophoresis Mobility Shift Assay—**10 μg of each nuclear extract sample was incubated with 0.1 pmol of 32P-labeled double-stranded DNA binding oligonucleotide (5′-GCTGGGGACTTTC-3′) or SP1 binding oligonucleotide (5′-ATTGATCGGCGCGGCGGACG-3′) in buffer containing 1 μg of poly(dI-c)1, 1 mg of bovine serum albumin, 10 mM HEPES pH 7.6, 0.5 mM dithiothreitol, 0.1 mM EDTA, 60 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM MgCl₂, and 12% glycerol at room temperature for 30 min. Samples were analyzed by 5% native PAGE followed by autoradiography. For competition and antibody-mediated supershift experiments, antibodies or oligonucleotides were added to the reaction for 15 min at room temperature before the addition of radiolabeled oligonucleotide probe.

**Isolation of RNA and Semi-quantitative RT-PCR—**The mRNA levels of Akt in different plasmid-transfected cells were analyzed by semi-quantitative RT-PCR. 1 μg of total RNA, isolated using an RNA isolation kit (Invitrogen), was used in reverse transcription reactions as described by the manufacturer. The resulting total cDNA was then used in the PCR to measure the mRNA levels of Akt. The mRNA level of β-actin was used as internal control. PCR was carried out with Taq polymerase, and conditions were as follows: pre-denaturing at 94 °C for 3 min and 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. The sequences of the primers used were upstream primer (5′-ATCGT-GCCAAAAGTGGAGGT-3′) and downstream primer (5′-CTCTGGTGT-CCGGTTGTGATG-3′) for Akt and upstream primer (5′-TCAACCACACTG-GACGACATG-3′) and downstream primer (5′-AGTCCCTGTGGAC- CACGAA-3′) for β-actin.

**RESULTS**

TNFα is a commonly used activator of NF-κB. Treatment with TNFα causes the rapid degradation of IκB proteins, permitting the nuclear translocation of NF-κB and resulting in increased DNA binding activity of NF-κB (1–3). As shown in Fig. 1A, besides stimulating IκB breakdown, treatment of NIH3T3 cells with TNFα increases Akt phosphorylation. Stimulation of Akt by TNFα has been observed by other laboratories (5–8) and has led to the conclusion that Akt can stimulate NF-κB activity. As shown in Fig. 1, A and B, however, following TNFα treatment the reduction in IκB-α levels is detectable after 5 min, whereas the increase in Akt phosphorylation occurs only after 15 min. As shown in Fig. 2, the translocation of p65/RelA to the nucleus and the resulting increase in DNA binding activity of p65 can be observed within 10 min of TNFα treatment, which is also before the phosphorylation of Akt can be detected.

Another agent that is commonly used to activate NF-κB in vivo and in culture is LPS. As shown in Fig. 1C, LPS treatment also stimulates Akt phosphorylation. The increase in Akt phosphorylation occurs at 1 h whereas the decrease in IκB-α levels is detectable after 30 min (Fig. 1, C and D). Taken together, these results show that the stimulation of Akt phosphorylation by TNFα and LPS begins after NF-κB activation has occurred,
FIG. 1. TNFα and LPS treatment stimulates IκB-α degradation and Akt phosphorylation. NIH3T3 cells were treated with 10 ng/ml TNFα (A and B) or 100 nM LPS (C and D) for various time periods as indicated above the panels. Following treatment whole cell lysates were prepared, and the lysates were analyzed by Western blot using an IκB-α antibody. The same blot was stripped and reprobed with antibodies against Ser(P)473 Akt (P-AKT) and total Akt (Akt). A and C, protein levels after treatment with TNFα and LPS, respectively. Densitometric analysis of the blots from three separate experiments was performed, and the relative levels of IκB-α and phospho-Akt after TNFα and LPS treatments are shown in B and D, respectively. With TNFα treatment (A and B) reduced IκB-α occurred at 5 min whereas stimulation of Akt phosphorylation was seen at 15 min. With LPS treatment (C and D) IκB-α degradation occurred at 30 min whereas increased Akt phosphorylation was detected at 1 h.

FIG. 2. Nuclear translocation and DNA binding activity of p65 following TNFα treatment. NIH3T3 cells were treated with 10 ng/ml TNFα for various time periods as indicated above the panels. Following treatment the cells were lysed, and nuclear extracts were prepared. An aliquot of the extracts was used for Western blotting while the rest was used in EMSA assays. A, results of Western blot analysis using an antibody against p65. B, results of EMSA using a radiolabeled oligonucleotide containing the consensus NF-κB binding site as probe. Lanes marked αB and SPI are control lanes that demonstrate specificity of binding in which a 100-fold excess of unlabeled NF-κB oligonucleotide or a nucleotide containing the SP1 binding site was included in the binding reaction. In the lanes marked p65-Ab and Control-Ab, a preincubation of the extract with p65 antibody or preimmune serum was performed. NF-κB binding activity was disrupted by p65 antibody. Similar supershift assays using antibodies against p50, c-rel, and relB failed to affect binding activity appreciably (data not shown).

raising the possibility that NF-κB may be involved in stimulating Akt phosphorylation.

To examine whether the stimulation of Akt is in fact a consequence of NF-κB activation, we used SN-50, a cell-permeable peptide that inhibits NF-κB by blocking its translocation to the nucleus (19). Previous studies have demonstrated that SN-50 is highly selective against NF-κB and has no effect on the activities of any other signaling molecule (20). Treatment with SN-50 reduced Akt phosphorylation and activation by TNFα (Fig. 3A). As shown in Fig. 3A, a similar reduction of Akt phosphorylation and activity is seen with TPCK, another commonly used pharmacological inhibitor of NF-κB that acts by inhibiting IκB degradation (21–23). To examine whether the inhibitory effect of SN-50 and TPCK occurred in other cell
typical autophagy-related genes, and by stimuli other than TNFα, we used primary cultures of cerebellar granule neurons. Treatment of cerebellar neurons with IGF-1 causes Akt phosphorylation and activation (24, 25). As shown in Fig. 3B, pretreatment with SN-50 or TPCK substantially reduces Akt phosphorylation induced by IGF-1 treatment, consistent with the involvement of NF-κB in

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by an NF-κB-independent mechanism cannot be completely excluded. To more directly determine whether NF-κB affects Akt, we transfected NIH3T3 cells with p65/RelA and examined its effect on Akt phosphorylation. Transfection using a p65 expression vector resulted in a dose-dependent increase in total p65 levels (Fig. 4A). The increased p65 levels resulted in a robust increase in NF-κB binding activity but not in the binding activity of another transcription factor, SP1 (Fig. 4B). As shown in Fig. 4, A and C and consistent with the idea that Akt is a downstream target of NF-κB, overexpression of p65 caused an increase in Akt phosphorylation. Overexpression of IκB-α on the other hand, which would lead to inhibition of NF-κB translocation, showed reduced Akt phosphorylation compared with control cells that overexpressed lacZ (Fig. 4C). To rule out the possibility that the stimulatory effect of p65 on Akt was restricted to NIH3T3 cells, we performed a similar experiment using the HEK293 cell line. As shown in Fig. 4D, stimulation of Akt phosphorylation by p65 was seen in HEK293 cells also. When TNFα was applied to cells overexpressing p65, the induction of Akt phosphorylation was not higher than that seen with p65 alone, suggesting that similar mechanisms were involved in the stimulatory effects of p65 and TNFα (Fig. 4E).

Interestingly, in addition to inducing Akt phosphorylation, overexpression of p65 caused an increase in the level of total Akt expression in both NIH3T3 and HEK293 cells (Fig. 4A, C, and D). The overexpression of IκB-α reduced Akt expression to a level slightly below that seen in lacZ-expressing cells (Fig. 4, C and D). In addition to increasing the level of Akt protein, overexpression of p65 caused an increase in Akt mRNA, ruling out post-translational mechanisms as responsible for the stimulatory effect of p65 (Fig. 5A). As a step toward determining whether the increase in Akt expression occurred at the transcriptional level as opposed to increasing mRNA stability, we expressed Akt using a CMV-driven promoter. As shown in Fig. 5B, although the expression of endogenously produced Akt is increased by p65 overexpression, the level of Akt produced via a CMV promoter is unchanged by p65, arguing against increased mRNA as the mechanism by which Akt expression is stimulated.

To examine the time course of Akt induction following p65 expression, we transfected NIH3T3 cells with CMV-p65 and examined the effect on Akt expression and phosphorylation. As shown in Fig. 6, A and B, although the increase in p65 expression is seen at 12 h, the stimulation of Akt expression occurs at

### Fig. 5. p65-mediated increase of Akt expression is transcriptionally mediated.

A, analysis of Akt mRNA. NIH3T3 cells were transfected with CMV-lacZ or CMV-p65. Twenty fours hours after transfection the cultures were lysed, and total RNA was prepared. Equal amounts of RNA (1 μg) were used in RT-PCR reactions using primers specific for Akt and β-actin. The lane marked control shows the result of amplification in the absence of RNA. A robust elevation of Akt mRNA expression is seen in cultures overexpressing p65. In contrast, p65 had no effect on β-actin mRNA. RT-PCR analysis was performed using two other primer pairs that amplified different regions of Akt mRNA. Although the data is not shown, a similar increase of Akt mRNA was observed. B, NIH3T3 cells were transfected with the following plasmids: CMV-lacZ (LacZ), CMV-Akt-Myc (−Myc Akt), CMV-p65 (P65), CMV-lacZ + CMV-Akt-Myc (LacZ + Akt), or CMV-p65 + CMV-Akt-Myc (P65 + Akt). The expression and phosphorylation of endogenous Akt is increased by p65. Expression of Akt driven off the CMV promoter was detected using an antibody against the Myc epitope tag. No change was seen in the levels of transfected Akt as judged by the similar intensity of the Myc immunoreactive bands. The same blot was reprobed with antibodies against p65 (to confirm expression of transfected p65) and β-tubulin (to control for equal loading of proteins).

### Fig. 6. Time course of p65-mediated induction of Akt expression and phosphorylation.

NIH3T3 cells were transfected with CMV-p65. Lysates were prepared at various time points ranging from 0–24 h after transfection as indicated above the lanes. The lysates were subjected to Western blot analysis using antibodies against Ser(P)473 Akt and total Akt. The same blot was reprobed with an antibody against p65 and β-tubulin. Whereas A shows the results of a representative experiment, B shows the data obtained from three independent experiments. Although high p65 expression is seen within 12 h, the increase in Akt expression is clearly evident only at 18 h after transfection. Stimulation of Akt phosphorylation is seen at 24 h.

the stimulation of Akt phosphorylation.

Although both SN-50 and TPCK are commonly used NF-κB inhibitors, the possibility that these compounds may affect Akt...
The increased phosphorylation of Akt follows the increased level of expression and is detectable at 24 h. Following treatment with a variety of growth factors and cytokines, the phosphorylation of Akt is mediated by a PI-3 kinase-dependent mechanism (15–17). Not surprisingly, therefore, treatment with the PI-3 kinase inhibitor wortmannin inhibits TNF-induced Akt phosphorylation and activity (Fig. 7A). However, the increased DNA binding activity of NF-κB following TNFα treatment is not substantially reduced by wortmannin or by LY294002, two specific inhibitors of PI-3 kinase, even when used at relatively high concentrations (Fig. 7B). Similarly, translocation of p65 to the nucleus and reduction of IκB-α levels in the cytoplasm is not affected by wortmannin (Fig. 7C). This observation is consistent with Akt being downstream of NF-κB in the sequence of events activated by TNFα, and it places PI-3 kinase in between NF-κB and Akt. To gain insight into the mechanism by which TNFα-mediated NF-κB activation stimulates Akt phosphorylation, we examined the effect of the transcriptional inhibitor actinomycin D and the translational inhibitor cycloheximide. As shown in Fig. 8, both actinomycin D and cycloheximide prevented the increase in Akt phosphorylation by TNFα, suggesting the need for new gene expression.

Akt generally refers to Akt1, one of three known members of the Akt gene family (15–17). Although it is likely that the three Akt proteins have at least some non-overlapping functions, these have yet to be fully understood. It is known that mice lacking Akt1 have defects in the induction of apoptosis and are growth-retarded (27), whereas Akt2-lacking mice display defects in the capacity of insulin to reduce blood glucose (32). To examine whether p65 also increases expression of Akt2 and Akt3, we used antibodies specific for these two forms in Western blot analysis. In contrast to its effect on Akt, p65 had no discernible effect on the expression of Akt2 and Akt3 (Fig. 9).
Our results indicate that NF-κB is involved in the stimulation of Akt activity. NF-κB-activating stimuli such as TNFα and LPS also stimulate Akt phosphorylation and activity. Events necessary for the activation of NF-κB (such as IκB degradation, nuclear translocation, and increase in NF-κB DNA binding) all occur before the increase in phosphorylation of Akt is detectable. TNFα-mediated phosphorylation and activation of Akt is blocked by two independent pharmacological inhibitors of NF-κB. Finally, overexpression of p65 leads to higher Akt phosphorylation, whereas similar overexpression of IκB-α inhibits it. The stimulatory effect of NF-κB on Akt is not restricted to cell lines and is seen in primary cultures of cerebellar granule neurons. The mechanism by which NF-κB activates Akt phosphorylation is unclear. Our finding that the stimulation of Akt by TNFα can be blocked by inhibitors of gene transcription and translation suggest the requirement for the synthesis of new proteins, an event likely regulated by the activation of NF-κB.

Several recent reports have described that NF-κB activation by TNFα is mediated by Akt. Contrary to the findings of Ozes et al. (5) and consistent with the findings of other groups (6, 13, 26, 28), we find that wortmannin, even when used at high doses, does not affect TNF-mediated translocation of NF-κB to the nucleus or its DNA binding activity. It does, however, inhibit Akt phosphorylation by TNFα efficiently, as reported previously (5–7, 13, 28). Assuming that the three molecules are part of a common TNF-activated signaling cascade, our results place PI3 kinase upstream of Akt but downstream of NF-κB. Other studies have shown that the expression of dominant-negative Akt fails to reduce NF-κB translocation or DNA binding activity (7, 13) and that Akt overexpression by itself is unable to activate NF-κB translocation in the absence of TNFα (7). In contrast, we find that overexpression of p65 in the absence of any extracellular signals is capable of robustly stimulating Akt phosphorylation. While not ruling out the possibility that Akt activates NF-κB as reported previously (5–7), our results indicate that Akt can be a downstream target of NF-κB.

Interestingly, in addition to stimulating its phosphorylation and activity, overexpression of p65 increases the expression of Akt mRNA and protein. The fact that the level of Akt produced following p65 overexpression in response to sustained p65 overexpression is in contrast to transient transfection, which produces large quantities of p65, TNFα treatment results in a relatively modest translocation of p65. Moreover, in contrast to the sustained production of p65 by transfection, TNF-mediated stimulation of NF-κB is transient. The physiological relevance of increased Akt production in response to sustained p65 overexpression is therefore unclear. It is noteworthy, however, that sustained up-regulation of NF-κB has been observed to occur in some cancers as discussed below. It would be of interest to examine whether in such cancers the expression and activity of Akt are also elevated.

NF-κB can directly activate proto-oncogenes such as cyclinD1 and c-Myc (29–31) and has thus been implicated in the development or progression of human cancers. Moreover, chromosomal amplification, overexpression, and rearrangement of genes coding for NF-κB proteins have been found to occur in many human hematopoietic and solid tumors, and the high constitutive activity of NF-κB noted in Hodgkin’s lymphoma has been attributed to a mutation in the IκB-α gene (reviewed in Ref. 31). In addition to providing a direct stimulus toward proliferation, NF-κB can contribute to the development of cancer by suppressing apoptosis, and several anti-apoptotic genes whose expression is activated by NF-κB have been identified, including those encoding cellular inhibitors of apoptosis proteins, manganese superoxide dismutase, A20, and the anti-apoptotic Bcl-2 proteins Bfl-1/A1 and Bcl-CL (reviewed in Ref. 4). We now show that another downstream target of NF-κB is Akt. Given the established role of Akt in the inhibition of apoptosis and its direct involvement in certain cancers such as ovarian cancer, prostate cancer, and gastric adenocarcinomas (reviewed in Ref. 17), the activation of Akt by NF-κB may serve as a powerful stimulus in the development or progression of cancer.

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REFERENCES
1. Karin, M., and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663.
2. Howard, T., Malek, S., and Ghosh, G. (1989) Cold Spring Harbor Symp. Quant. Biol. 64, 533–540.
3. Baldwin, A. S., Jr. (1996) Annu. Rev. Immunol. 14, 649–683.
4. Bouris, V., Bentires-Alj, M., Helin, A. C., Viotour, P., Robe, P., Delhalle, S., Benoist, Y., and Merville, M. P. (2000) Biochem. Pharmacol. 60, 1085–1089.
5. Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. (1999) Nature 401, 82–85.
6. Komashkov, J. A., and Makrides, S. S. (1999) Nature 401, 86–90.
7. Madrid, L. V., Wang, C. Y., Guttridge, D. C., Schottelius, A. J., Baldwin, A. S., Jr., and Mayo, M. W. (2000) Mol. Cell. Biol. 20, 1638–1638.
8. Burness, M. E., Weldon, C. H., Meltz, L. I., Duong, B. N., Collins-Burrell, B. M., Beckman, B. S., and McElnay, J. A. (2000) Biochem. Biophys. Res. Commun. 271, 342–345.
9. Mudge, A. L., and Pober, J. S. (2000) J. Biol. Chem. 275, 15458–15465.
10. Rauch, B. H., Weber, A., Braun, M., Zimmermann, N., and Schror, K. (2000) FEBS Lett. 481, 3–7.
11. Xie, P., Browning, D. D., Hay, N., Mackman, N., and Ye, R. D. (2000) J. Biol. Chem. 275, 24907–24914.
12. Mansell, A., Kheleif, N., Cossart, P., and O’Neill, L. A. (2001) J. Biol. Chem. 276, 43597–43603.
13. Sizemore, N., Leung, S., and Stark, G. R. (1999) Mol. Cell. Biol. 19, 4788–4805.
14. Yang, C. H., Murti, A., Pfeffer, S. R., Kim, J. G., Donner, D. B., and Pfeffer, L. M. (2001) J. Biol. Chem. 276, 13756–13761.
15. Kandel, E. S., and Hay, N. (1999) Exp. Cell Res. 233, 210–229.
16. Brazel, D. P., and Hemmings, B. A. (2001) Trends Biochem. Sci. 26, 657–664.
17. Scheid, M. P., and Woodgett, J. R. (2001) Nat. Rev. Mol. Cell. Biol. 2, 760–768.
18. D’Mello, S. R., Galli, C., Ciotti, T., and Calissano, P. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 10899–10903.
19. Lin, Y. Z., Yao, S. Y., Veach, R. A., Torgerson, T. R., and Hawiger, J. (1995) J. Biol. Chem. 270, 14455–14458.
20. Maggiarini, S. B., Sarniere, P. D., Dewhurst, S., and Freeman, R. S. (1998) J. Neurosci. 18, 10356–10365.
21. Henkel, T., Machleidt, T., Alkalay, I., Kronke, M., Ben-Neriah, Y., and Baueerle, P. A. (1993) Nature 365, 182–185.
22. Wang, M., Lee, H., Schauer, T. S., Arurma, M., Katz, D., Fitzgerald, M. J., Rothstein, T. L., Sherr, D. H., and Sonenshein, G. E. (1996) EMBO J. 15, 4682–4690.
23. Phillips, R. J., and Ghosh, S. (1997) Mol. Cell. Biol. 17, 4380–4386.
24. Dork, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997) Science. 275, 661–665.
25. Kumari, S., Liu, X., Nguyen, T., Zhang, X., and D’Mello, S. R. (2001) Brain Res. Mol. Brain Res. 96, 157–162.
26. Reddy, S. A., Huang, J. H., and Liao, W. S. (2000) J. Immunol. 164, 1355–1363.
27. Guttridge, D. C., Albanese, C., Reutter, J. Y., Postell, R. G., and Baldwin, A. S., Jr. (1999) Mol. Cell. Biol. 19, 5785–5799.
28. Hinz, M., Krappmann, D., Eichten, A., Heder, A., Scheidereit, C., and Strauss, M. (1999) Mol. Cell. Biol. 19, 2060–2068.
29. Cho, H., Mu, J., Kim, J. K., Thorvaldsen, J. L., Chu, Q., Crenshaw, B. E., Ill, Kaestner, K. H., Bartolomaei, M. S., Shulman, G. I., and Birnbaum, M. J. (2001) Science 292, 1728–1731.