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Akt Stimulates the Transactivation Potential of the RelA/p65 Subunit of NF-κB through Utilization of the IκB Kinase and Activation of the Mitogen-activated Protein Kinase p38*

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The serine/threonine kinase Akt/PKB is a potent regulator of cell survival and has oncogenic transformation potential. Previously, it has been shown that Akt can activate the transcription factor NF-κB and that this functions to block apoptosis induced by certain stimuli. The mechanism whereby Akt activates NF-κB has been controversial, with evidence supporting induction of nuclear translocation of NF-κB via activation of IκB kinase activity and/or the stimulation of the transcription function of NF-κB. Here we demonstrate that Akt targets the transactivation function of NF-κB by stimulating the transactivation domain of RelA/p65 in a manner that is dependent on IκB kinase β activity and on the mitogen-activated protein kinase p38 (p38). Activation of RelA/p65 transactivation function requires serines 529 and 536, sites shown previously to be inducibly phosphorylated. Consistent with the requirement of p38 in the activation of NF-κB transcriptional function, expression of activated Akt induces p38 activity. Furthermore, the ability of IL-1β to activate NF-κB is known to involve Akt, and we show here that IL-1β induces p38 activity in manner dependent on Akt and IκB kinase activation. Interestingly, activated Akt and the transcriptional co-activators CBP/p300 synergize in the activation of the RelA/p65 transactivation domain, and this synergy is blocked by p38 inhibitors. These studies demonstrate that Akt, functioning through IκB kinase and p38, induces the transcription function of NF-κB by stimulating the RelA/p65 transactivation subunit of NF-κB.

Akt/PKB is a serine/threonine kinase that is activated in response to certain growth factors and cytokines (1, 2). Consistent with its activation by growth factors, Akt has transforming potential (3). Akt isoforms have been shown to be overexpressed in breast cancer cell lines, in ovarian and pancreatic cancers, and amplified in gastric adenoma (4, 5). Additionally, Akt is a downstream activator for oncogenic Ras and Src and for the proto-oncoprotein HER-2/neu (6–8). Importantly, Akt provides a potent cell survival signal that is likely involved in its transformation and growth-promoting properties (3). Mechanisms associated with the ability of Akt to suppress apoptosis include the phosphorylation and inactivation of many proapoptotic proteins (9–15). Additionally, Akt has been shown to activate the transcription factor NF-κB to provide cell survival functions (7, 16–19).

Classic nuclear factor-κB (NF-κB) is a heterodimer composed of the p50 and the RelA/p65 subunits. NF-κB is activated by a variety of stimuli including cytokines and oncoproteins (20, 21). In unstimulated cells, the majority of NF-κB is found in the cytoplasm associated with a family of inhibitory molecules known as the IκBs. The canonical NF-κB activation mechanism involves the phosphorylation of IκB on two critical serine residues by the IκB kinase (IKK)[1] signalsome complex (22–25). Phosphorylated IκB is then targeted for ubiquitination and subsequent degradation by the 26 S proteosome, which allows liberated NF-κB to translocate to the nucleus, where it activates transcription of NF-κB-responsive genes (20, 21).

Although the induced nuclear translocation of NF-κB has been highly regarded as the principal method to activate NF-κB-dependent gene expression, an alternate mechanism of NF-κB activation is emerging that involves the phosphorylation of the RelA/p65 transactivation subunit. For example, it has been shown that the proinflammatory cytokines tumor necrosis factor and IL-1β lead to the phosphorylation of RelA/p65 and the subsequent stimulation of NF-κB transactivation through pathways distinct from induced nuclear translocation (18, 26, 27). The catalytic subunit of protein kinase A (PKAc) has also been shown to phosphorylate RelA/p65, which leads to the association of RelA/p65 with the CREB-binding protein/p300 (CBP/p300) transcriptional co-activator (28, 29). Recently, the generation of GSK3 and T2K (TBK1) knockout mice has highlighted the physiological importance of modulating transactivation functions of NF-κB, because cells generated from these animals are capable of inducing NF-κB nuclear translocation but are deficient in their ability to stimulate transactivation functions of NF-κB (30, 31). In addition, evidence has been presented that the stress-activated kinase p38 is involved in the regulation of NF-κB transcription function at a level distinct from the induction of nuclear translocation (32, 33). These studies indicate that dual controls exist for NF-κB with mechanisms controlling induction of nuclear translocation of NF-κB as well as regulating the inherent transcriptional activity of NF-κB.

Reports describing processes whereby Akt stimulates NF-κB activity have indicated different mechanisms whereby this

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[1] The abbreviations used are: IKK, IκB kinase; IL, interleukin; CBP, CREB-binding protein; MEF, mouse embryo fibroblasts; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; HA, hemagglutinin.

process may occur. Two studies indicated that Akt, either in the context of tumor necrosis factor signaling or in response to growth factor stimulation, stimulated NF-κB nuclear translocation via the activation of the IKK complex (16, 17). Another study indicated that Akt alone could not induce nuclear translocation of NF-κB but synergized with PMA to induce this response (19). Arguments against the involvement of Akt in controlling nuclear accumulation of NF-κB have been recently reported (34). Others, including ourselves, have provided evidence that Akt signaling involves the stimulation of the transcription function of NF-κB (7, 18). Thus, we showed that the ability of oncogenic Ras to activate NF-κB transcriptional activity is dependent on Akt activity (7). In a separate study, Sizemore *et al.* showed that IL-1β induces phosphorylation of the RelA/p65 subunit in an Akt-dependent manner and that IL-1β activated the RelA/p65 transcriptional activation domain (18).

In this study, we show that the ability of Akt to stimulate the transactivation potential of the RelA/p65 subunit of NF-κB requires IKK and p38. Expression of activated Akt in IKKβ null mouse embryo fibroblasts (MEFs) significantly reduces transcriptional activity of NF-κB. In addition, mutation of serine 529 and the IKKβ phosphorylation site serine 536 (24, 35, 36) within the RelA/p65 transactivation domain, sites previously shown to be inducibly phosphorylated, decreases the activation of NF-κB-mediated transcription in response to Akt. Since p38 has been determined to activate NF-κB by targeting the transactivation function of NF-κB, we examined the role of p38 in Akt-mediated NF-κB transactivation. Treatment of cells with IL-1β or expression of an activated form of Akt stimulates the phosphorylation and activation of p38. Interestingly, IL-1β-induced phosphorylation of p38 requires Akt and IKK. Furthermore, inhibition of p38 kinase activity by the p38 inhibitor SB203580 significantly reduces Akt- and IL-1β-induced NF-κB activation. The stimulation of the RelA/p65 transactivation domain is most likely not due to a direct phosphorylation event on RelA/p65 by p38 (32); rather, we provide evidence that p38 activates RelA/p65 in response to Akt through cooperation with the CBP/p300 transcriptional co-activator. These studies define new mechanisms for Akt-mediated NF-κB transactivation and demonstrate that Akt utilizes IKK and the p38 MAPK to stimulate the transactivation potential of the RelA/p65 subunit of NF-κB.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Murine NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% calf serum (Hyclone Laboratories, Logan, UT) and penicillin/streptomycin unless otherwise indicated. Human 293T kidney cells and IKK null mouse embryo fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and penicillin/streptomycin unless otherwise indicated. The specific p38 MAPK pharmacological inhibitor SB203580 and the MEK inhibitor PD98059 (Calbiochem) were used at a working concentration of 600 nM and 2 μM, respectively, unless otherwise indicated. Recombinant human IL-1β (Promega Corp., Madison, WI) or recombinant mouse IL-1β (Life Technologies) was used at a concentration of 5 ng/ml.

Plasmid Constructs and Mutagenesis—3x-κB luciferase reporter constructs contain 4 κB DNA binding consensus sites from the MHC class I promoter fused upstream to firefly luciferase. The Gal4 luciferase constructs (Gal4-Luc) contain five Gal4 DNA consensus binding sites derived from the yeast GAL4 gene upstream of luciferase, and Gal4-p65 constructs have the yeast Gal4 DNA binding domain fused to the carboxyl-terminal transactivation domain of p65 (37). Activated Akt as well as dominant negative constructs have been described previously (38, 39). Wild type, dominant negative p38, MAPK kinase 6 (MKK6), and IKK constructs have been described previously (25, 40). Mutagenesis was performed per the manufacturer's suggestions (Stratagene, La Jolla, CA). Briefly, oligonucleotides were made corresponding to the sequence in RelA/p65 overlapping the serines 529 and 536. PCR was performed, and products were digested with *Dpn*I and subsequently transformed into the XL-1 Blue strain of *Escherichia coli*. All mutants were verified by sequencing.

Transfection and Luciferase Reporter Assays—NIH3T3 cells at 60–80% confluency were transiently transfected using the Superfect reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, plasmid constructs (1–2 μg of DNA total) were diluted in serum-free medium and mixed with the Superfect reagent. Complexes were allowed to form for 10 min before serum-containing medium was added to the mixture. The cells were washed once with 1× phosphate-buffered saline, and Superfect-DNA complexes were added to the cells and placed in a humidified incubator at 37 °C with 5% CO_2. Three hours after the addition, cells were washed with 1× phosphate-buffered saline and replenished with fresh serum-containing medium. Human 293T cells at 70–80% confluency were transfected by the calcium phosphate protocol. 24–48 h post-transfection, cells were washed once with 1× phosphate-buffered saline and lysed in Reporter Lysis Buffer (Promega, Madison, WI) for 10 min at room temperature. Extracts were collected and cleared by centrifugation at high speed. Protein concentration was determined with the Bio-Rad protein assay dye reagent. D-Luciferin was used as a substrate, and relative light units were measured using an AutoLumat LB953 luminometer (Berthold Analytical Instruments). Results were normalized to an internal β-galactosidase-expressing plasmid (PCMV-LacZ) by a β-galactosidase colorimetric assay followed by spectrophotometric quantitation (Promega, Madison, WI). In addition, cells transfected with Akt, p38, or IKK mutant constructs were co-transfected with pCMV-LacZ and assayed for transfection efficiency and/or cell death by counting β-galactosidase-positive cells as previously described (41).

Western Blot Analysis and Kinase Assays—Western blot analysis was performed by preparing whole cell extracts in the presence of protease inhibitors or by lysing cells in 2× SDS sample buffer. The indicated primary antibodies were incubated, washed, and visualized by incubation with horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI) and ECL chemiluminescent reagents (Amersham Pharmacia Biotech). Western blotting antibodies were obtained from the following companies: Akt and p38 antibodies (New England Biolabs, Beverly, MA); M2 FLAG epitope and α-tubulin antibodies (Sigma), and HA epitope antibody (Babco, Berkeley, CA). The p38 kinase assays were performed as per the manufacturer's instructions (Cell Signaling Technology, Inc., Beverly, MA).

RESULTS

Akt Stimulates the Transactivation Domain I of RelA/p65 by Utilizing the IκB Kinase β—Various cellular stimuli can activate NF-κB-dependent transcription, at least in part, through mechanisms independent of signaling pathways that influence nuclear translocation. These signaling pathways stimulate the transactivation domain of the RelA/p65 subunit of NF-κB, presumably by targeting basal or induced levels of NF-κB in the nucleus (7, 26). Previous work in our laboratory has established that Akt activates NF-κB by stimulating the carboxyl-terminal transactivation domain I of RelA/p65. In addition, expression of a dominant negative mutant of IKKβ inhibited the ability of Akt to activate NF-κB (7). Our results imply that Akt requires IKK to efficiently stimulate the transactivation function of NF-κB. To further address this question, we were interested in determining if Akt could activate NF-κB-dependent transcription in a setting where endogenous IKK is absent. The multisubunit IKK complex contains two primary catalytic subunits, IKKα and IKKβ, and each has been shown to be involved in NF-κB activation at the level of nuclear accumulation (20). We utilized nullizygous IKKα and IKKβ mouse embryo fibroblasts (MEFs) and performed transient transfection experiments with M-Akt or full-length wild type RelA/p65 and an NF-κB reporter construct (3X-κB Luc). As seen in Fig. 1*A*, wild type RelA/p65 activated the NF-κB-responsive promoter equally in the wild type, IKKα−/−, or IKKβ−/− cells as expected. However, in response to activated Akt, the IKKβ−/− cells were deficient in NF-κB-dependent transcriptional activation, and, furthermore, reintroduction of IKKβ by transfection restored this activity. Consistent with the role of Akt in regulating NF-κB transcriptional activity, IKKβ−/− cells were inhibited in their ability to activate a Gal4-dependent luciferase reporter when transfected

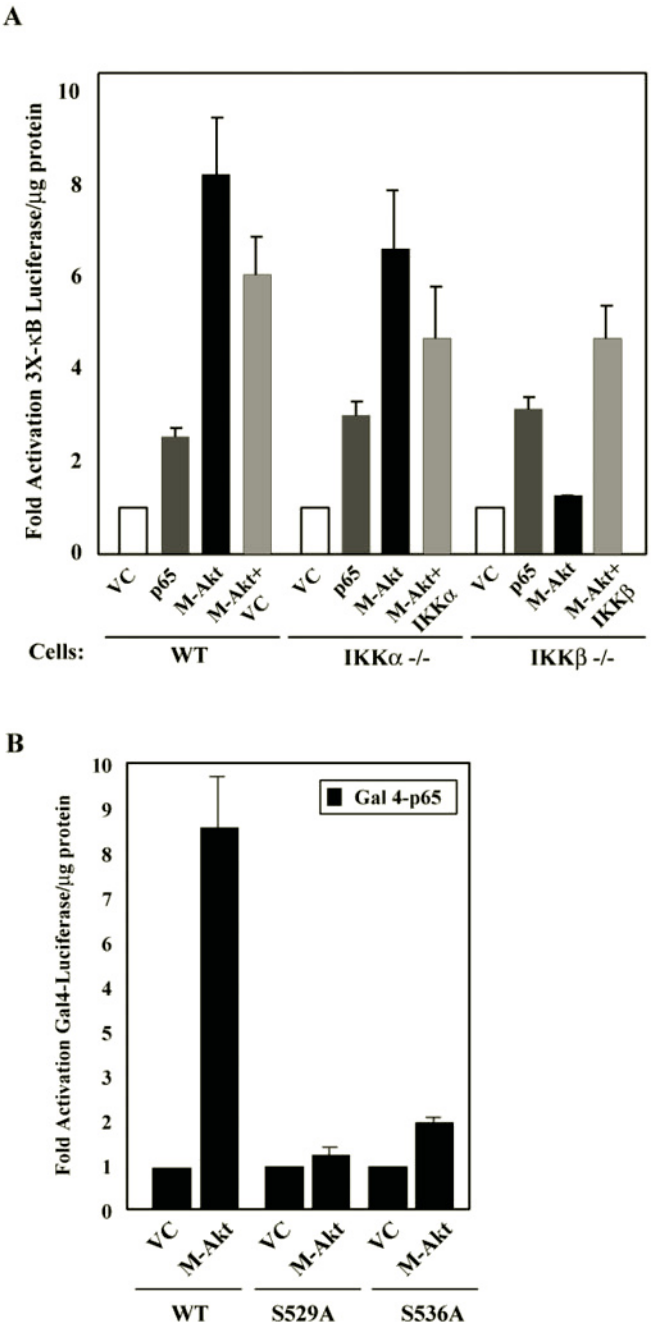

FIG. 1. **Akt-mediated NF-κB activation requires IKKβ and serine 536 of RelA/p65.** *A*, wild type, IKKα, and IKKβ nullizygous mouse embryo fibroblasts were transfected with 3X-κB luciferase, vector control, M-Akt (2 μg each), p65 (100 ng), or wild type IKKα and IKKβ (for reintroduction experiments). *B*, NIH 3T3 cells were transiently co-transfected with Gal4-Luc, Gal4-p65 mutants S529A and S536A (100 ng each), and activated myristylated Akt (M-Akt) or a vector control plasmid (VC) (2 μg each). 48 h post-transfection, luciferase levels were assayed, normalized, and expressed as -fold induction over the vector control ± S.D. of three independent experiments.

with activated Akt and a fusion (Gal4-p65) between the Gal4 DNA binding domain and the transactivation domain I of RelA/p65 (data not shown). To determine if the reduction of NF-κB activity was due to aberrant expression of these proteins, the extracts were reanalyzed for M-Akt, IKKα, and IKKβ expression by Western blot, and both the IKKα−/− and IKKβ−/− MEFs show equal levels of M-Akt protein (data not shown). This result demonstrates that Akt requires endogenous IKKβ for efficient stimulation of NF-κB-dependent transcription in a manner distinct from the induction of nuclear translocation of NF-κB.

Since Akt expression alone does not induce nuclear localization of NF-κB and because Akt requires IKK for activation of NF-κB (7, 16, 17, 19), we were interested in determining whether IKK utilizes a specific site in RelA/p65 for Akt-mediated stimulation of RelA/p65. Since IKKβ has been reported to phosphorylate RelA/p65 on serine 536 *in vitro* and *in vivo*, it was important to determine if this site is essential for Akt-mediated RelA/p65 transactivation (24, 36). To address this question, we utilized a plasmid encoding the Gal4-p65 fusion protein, where sequences encoding the DNA-binding domain of Gal4 have been joined with sequences encoding the transactivation domain of RelA/p65 (37). This plasmid, when co-transfected with a Gal4-Luc reporter, allows us to determine whether cellular signals up-regulate gene expression by specifically targeting the transactivation domain of the RelA/p65 subunit of NF-κB. To examine the importance of the reported IKKβ phosphorylation site, Gal4-p65 was mutated at serine 536 and for control purposes at serine 529 (Gal4-p65 S536A and S529A). The Gal4-p65 S529A mutant was examined, because serine 529 has been previously shown to be important for transactivation of NF-κB in response to tumor necrosis factor (26). NIH 3T3 cells were transiently co-transfected with Gal4-Luc, wild type Gal4-p65, Gal4-p65 S529A, or Gal4-p65 S536A in the presence (M-Akt) or absence (VC) of activated Akt. As shown in Fig. 1*B*, M-Akt stimulates the transactivation function of RelA/p65 but has little or no effect on gene expression when serine 529 or serine 536 are mutated to alanine. In addition, stimulation of cells with IL-1β also required serines 529 and 536 for efficient activation of NF-κB transactivation function (data not shown). These results demonstrate that a known IKKβ phosphorylation site, serine 536, and a known tumor necrosis factor-inducible site, serine 529, are each required for Akt to efficiently stimulate the transactivation domain of the RelA/p65 subunit of NF-κB.

IL-1β Requires Akt and IKK for Efficient Stimulation of p38 Activity—Previous data from our laboratory and from others have suggested that the stress-activated kinase p38 is required for activation of NF-κB by expression of oncogenic Ras or by IL-1β treatment (33, 42). Additionally, p38 and IL-1β have been shown to modulate the transactivation potential of NF-κB independent of signals that induce nuclear accumulation and DNA binding (18, 33, 43). Therefore, we were interested in determining if p38 was required by IL-1β and Akt to modulate the transactivation potential of NF-κB, since it has also been demonstrated that Akt modulates the transactivation functions of NF-κB (7, 18). First, it was important to determine if Akt can stimulate the phosphorylation of p38 and therefore lead to its activation. To address this question, FLAG-tagged wild type p38 (FLAG-p38) and activated Akt were transiently transfected into human 293T cells. 48 h post-transfection, extracts were prepared and analyzed for the presence of phosphorylated p38 using a p38 phosphospecific antibody. As shown in Fig. 2*A*, activated Akt induces the phosphorylation of FLAG-p38, suggesting that p38 is a downstream target of Akt signaling. The activity of p38 in response to M-Akt was also measured by p38 kinase assay, and, as shown in Fig. 2*A*, M-Akt stimulated the activity of p38 to phosphorylate the transcription factor ATF-2, a known p38 substrate. These extracts were also analyzed for equivalent FLAG-p38 and α-tubulin (to control for loading) expression (Fig. 2*A*, *bottom panels*). Interestingly, IL-1β has been shown to activate both Akt and p38; however, it has not been demonstrated that p38 is a target of IL-1β-Akt signaling (18, 44). Fig. 2*B* demonstrates that stimulation of cells with IL-1β leads to the activation of p38 as previously reported (44). Interestingly, this activation requires functional Akt because concomitant expression of a dominant

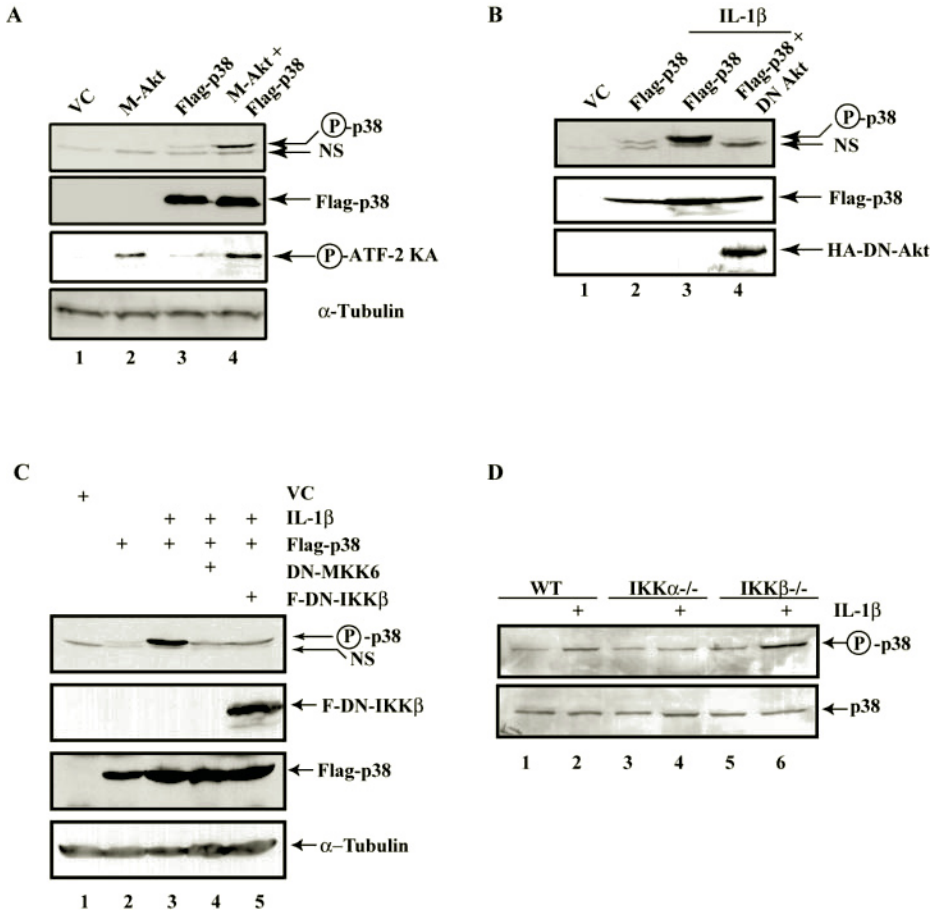

FIG. 2. **IL-1β requires Akt and IKK for efficient stimulation of p38 activity.** *A*, human 293T cells were transiently transfected with constructs encoding VC, M-Akt, or FLAG-tagged p38 (FLAG-p38) (2 μg each). 48 h post-transfection whole cell extracts were isolated and assayed for phosphorylated p38 using a phosphospecific p38 antibody or p38 kinase activity by assaying phosphorylated ATF-2. Extracts were reanalyzed for equivalent expression of FLAG-p38 and α-tubulin for control purposes. *NS*, nonspecific bands. *B*, 293T cells were transfected with constructs encoding FLAG-p38 and/or a dominant negative Akt mutant, Akt K179A (HA-DN-Akt) (2 μg each). 48 h post-transfection, cells were stimulated with recombinant human IL-1β (5 ng/ml) for 30 min and assayed for p38 phosphorylation status. Extracts were reanalyzed for expression of FLAG-p38 and HA-DN-Akt (*NS* indicates nonspecific products). *C*, 293T cells were transfected with vector control (*VC*), FLAG-p38, DN-MKK6, or FLAG-DN-IKKβ (FLAG-IKKβ SS-AA) by the calcium phosphate transfection protocol. 48 h post-transfection, cells were stimulated with recombinant murine IL-1β (5 ng/ml) for 30 min and assayed for p38 phosphorylation status. The *arrows* indicate proteins detected, FLAG-p38 and α-tubulin as a loading control and nonspecific products (*NS*). *D*, wild type (*WT*) and IKKα (IKKα−/−) and IKKβ (IKKβ−/−) null MEFs were plated in equal cell numbers, stimulated with IL-1β (5 ng/ml) for 30 min, and assayed for p38 phosphorylation status. *Bottom panel*, blots were stripped and reprobed for total endogenous p38. Results are representative of three independent experiments.

negative mutant of Akt (DN-Akt) effectively abolished the ability of IL-1β to activate p38. To control for equivalent expression of FLAG-p38 and DN-Akt, we reanalyzed these extracts with FLAG- and HA-specific antibodies, respectively (Fig. 2*B*, *bottom panels*). Taken as a whole, these results suggest that Akt signals to activate p38 and that IL-1β requires Akt for efficient stimulation of p38.

Since IKKβ is required for Akt-mediated NF-κB activity (Fig. 1*A*), we were also interested in determining if IL-1β stimulation of p38 required the IKK pathway. To address this question, 293T cells were transiently transfected with constructs encoding a vector control plasmid (VC), FLAG-p38, dominant negative MKK6 (DN-MKK6), and FLAG-tagged dominant negative IKKβ (F-DN-IKKβ (SS-AA)). The dominant negative IKKβ construct affects both endogenous IKKα and IKKβ function, because the dominant negative IKKβ dimerizes with and inactivates both IKK subunits (23). 24 h post-transfection, the cells were stimulated with IL-1β for 30 min and assayed for p38 phosphorylation status. As shown in Fig. 2*C*, DN-MKK6 blocked the ability of IL-1β to stimulate p38 phosphorylation, as expected (*lane 4*). Interestingly, expression of DN-IKKβ also blocked IL-1β-mediated stimulation of p38 phosphorylation (*lane 5*). In addition, Western analysis was performed to control for proper expression of the transfected constructs and equal loading (Fig. 2*C*, *bottom panels*). However, these results do not distinguish between the IKK subunits relative to which one may be required for this process. To address this question, we performed similar experiments utilizing the IKKα and IKKβ null MEFs. These cells were treated with IL-1β for 30 min and assayed for phosphorylated endogenous p38 as described above. As shown in Fig. 2*D*, cells expressing wild type IKKα and IKKβ (*WT*) and cells lacking the IKKβ subunit (IKKβ−/−) retain the ability of IL-1β to stimulate phosphorylation of p38 (*lanes 1* and *2* and *lanes 5* and *6*), but interestingly, the IKKα null MEFs are defective in IL-1β-mediated p38 phosphorylation (*lanes 3* and *4*). This observation suggests that IL-1β signaling requires IKKα, but not IKKβ, for stimulation of p38 and potentially places p38 downstream of the IL-1β-Akt-IKK pathway (see "Discussion").

IL-1β and Akt Utilize the Mitogen-activated Protein Kinase p38 for Transactivation of NF-κB—To test whether p38 mediates the stimulation of NF-κB transcriptional activity by Akt and IL-1β, we suppressed p38 activity in cells expressing M-Akt and determined the effect on NF-κB transactivation. NIH3T3 cells were co-transfected with plasmids encoding M-Akt, Gal4-Luc, and Gal4-p65 and then were treated with the

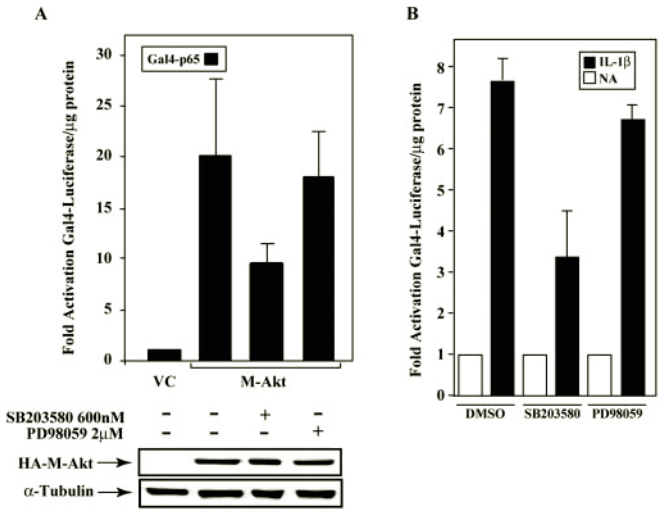

FIG. 3. **Akt requires the p38 MAPK for efficient stimulation of NF-κB transactivation.** *A*, NIH 3T3 cells were transiently transfected with plasmids encoding Gal4-Luc and Gal4-p65 (100 ng each) and VC or M-Akt (2 μg each). 24 h post-transfection, SB203580 (600 nM) and PD98059 (2 μM) were added for an additional 24 h. *B*, human 293T cells were transiently transfected with plasmids encoding Gal4-Luc and Gal4-p65 (1 μg each). 24 h post-transfection, SB203580 (600 nM) and PD98059 (2 μM) were pretreated for 90 min followed by human recombinant IL-1β (5 ng/ml) or vehicle (*NA*) for an additional 20 h. Luciferase levels were assayed, normalized, and expressed as -fold induction over vector control ± S.D. of three experiments.

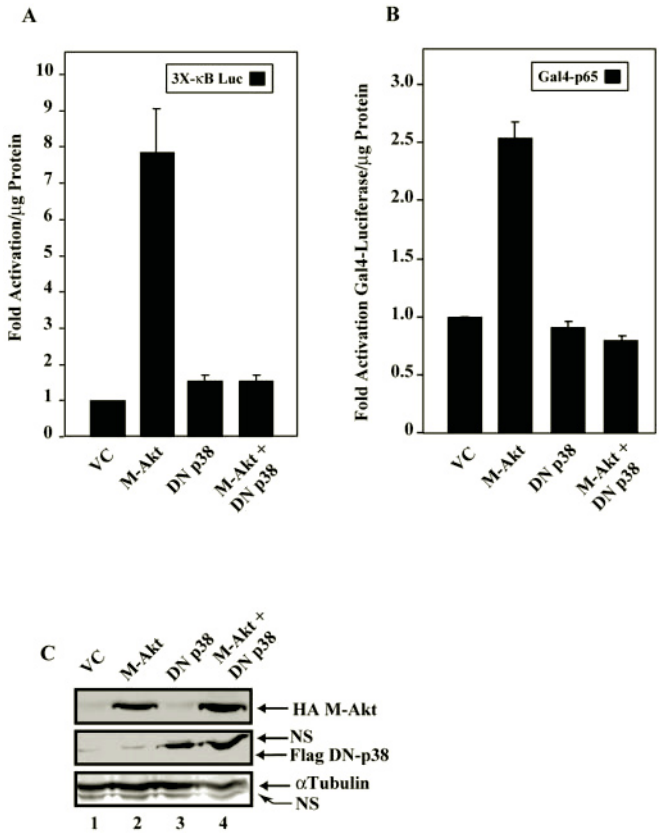

FIG. 4. **Dominant negative p38 mutants block Akt-mediated NF-κB transactivation.** *A*, NIH 3T3 cells were transiently transfected with plasmids encoding 3X-κB Luc (0.5 μg) or VC or M-Akt (2 μg each). *B*, NIH 3T3 cells were transiently transfected with plasmids encoding Gal4-Luc and Gal4-p65 (100 ng each) and VC, M-Akt, or dominant negative p38 (DN-p38) (2 μg each). 48 h post-transfection, whole cell extracts were isolated. Luciferase was normalized to total protein, and β-galatosidase staining was performed to ensure equal transfection efficiency and to ensure equal cell viability between conditions. Data is presented as -fold activation, where the values obtained for the vector control group were normalized to 1. Results represent the mean ± S.D. of three independent experiments. *C*, whole cell extracts were reanalyzed for equivalent protein expression by Western blot. Activated HA-tagged Akt (*HA M-Akt*), FLAG-tagged DN p38, and α-tubulin-specific antibodies were used as described under "Experimental Procedures."

pharmacological inhibitor of p38 (SB203580), or, for control purposes, the pharmacological inhibitor of the MEK pathway (PD98059) was added for 24 h. Fig. 3*A* shows that in the presence of the p38 inhibitor, the ability of Akt to activate Gal4-p65 was reduced, while the MEK inhibitor showed only a marginal reduction in Gal4-Luc activity. In addition, Fig. 3*B* shows that IL-1β stimulation of 293T cells similarly showed a reduction in NF-κB transactivation potential in the presence of SB203580. The SB203580 compound has been shown to inactivate additional MAPK signaling pathways and has been suggested to inhibit Akt kinase activity at high concentrations (45). The effects of the p38 inhibitor shown in Fig. 3, *A* and *B*, are unlikely to be due to reduced Akt kinase activity or other nonspecific effects, since the concentration of SB203580 used in our experiments (600 nM) is considerably lower than reported concentrations (3–5 μM) affecting Akt or other signaling pathways (45). Additionally, analysis of extracts from cells treated with SB203580 or PD98059 shows that these inhibitors do not affect M-Akt expression levels as determined by Western blot (Fig. 3*A*, *bottom panel*). Nevertheless, additional experiments were performed to confirm the results obtained with the SB203580 compound. Constructs encoding a dominant negative mutant of p38 (DN p38) were transiently transfected into NIH 3T3 cells with activated Akt and assayed for 3X-κB Luc and Gal4-Luc/Gal4-p65 activity. The dominant inhibitory mutant of p38 blocked the ability of Akt to stimulate NF-κB dependent transcription and showed a similar inhibition on Gal4-Luc/Gal4-p65 activity corroborating the effects seen with the SB203580 compound (Fig. 4, *A* and *B*). Importantly, these effects were not due to aberrant expression of M-Akt or p38 as assayed by Western blot (Fig. 4*C*). These results taken in whole demonstrate that IL-1β requires Akt and IKK to stimulate p38 and that p38 activity is required for IL-1β and Akt to activate the transcription function of NF-κB. Interestingly, the activation of p38 alone is apparently insufficient for the activation of p65 transactivation function, since MKK3, an upstream inducer of p38 function, was unable to activate Gal4-p65 (data not shown).

Akt Utilizes p38 and CBP/p300 for Efficient Stimulation of the RelA/p65 Subunit of NF-κB—The mechanism of p38-mediated NF-κB activation is most likely not directly at the level of NF-κB, because p38 does not activate RelA/p65 by a direct phosphorylation event (32). In addition, RelA/p65 does not contain a consensus MAPK/p38 phosphorylation site.[2] However, NF-κB interacts with the basal transcription machinery and requires co-activators for efficient stimulation of transcriptional activity (46, 47). One co-activator that is essential for NF-κB-dependent transcription is CBP/p300 (28). Therefore, we hypothesized that in response to Akt, p38 may utilize CBP/p300 to stimulate NF-κB transcription. To answer this question, constructs encoding activated Akt, CBP, or p300 were transiently co-transfected into NIH 3T3 cells along with Gal4-Luc and Gal4-p65. As shown in Fig. 5*A*, CBP and p300 synergistically activate RelA/p65 in conjunction with activated Akt. Importantly, these effects were not due to elevated levels of HA-M-Akt in the transfections (Fig. 5*A*, *bottom panel*). Interestingly, as shown in Fig. 5*B*, the SB203580 compound blocked this synergy by approximately 2-fold, consistent with the data presented in Fig. 3*A*. This effect is specific to p38, since the

[2] L. V. Madrid and A. S. Baldwin Jr., unpublished observations.

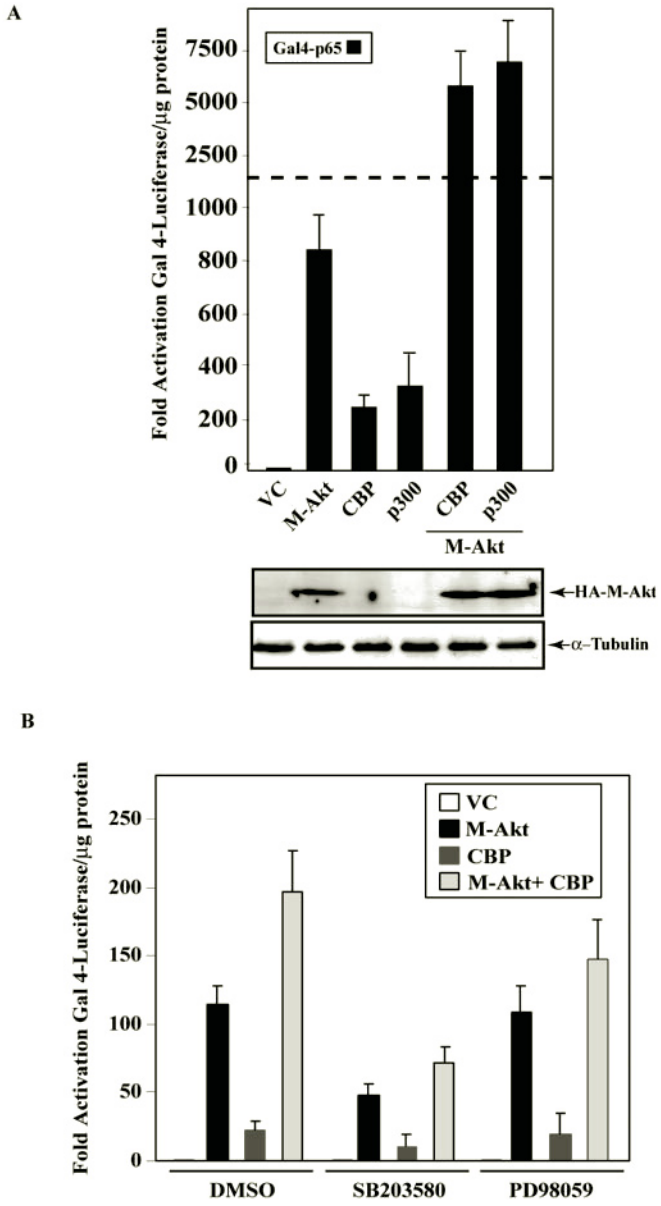

FIG. 5. **Akt and p38 require the co-activator CBP/p300 for efficient stimulation of the RelA/p65 transactivation domain.** *A*, NIH 3T3 cells were transiently co-transfected with constructs encoding Gal4-Luc and Gal4-p65 (100 ng of each) and VC, M-Akt, CBP, or p300 (2 μg of each). *B*, NIH 3T3 cells were transiently transfected with plasmids encoding Gal4-Luc and Gal4-p65 (100 ng each) and VC, M-Akt, or CPB (2 μg of each). 24 h post-transfection, SB203580 (600 nM), PD98059 (2 μM), or Me_2SO (*DMSO*; vehicle control) were added for an additional 24 h. Luciferase levels were assayed, normalized, and expressed as -fold induction over vector control ± S.D. of three experiments.

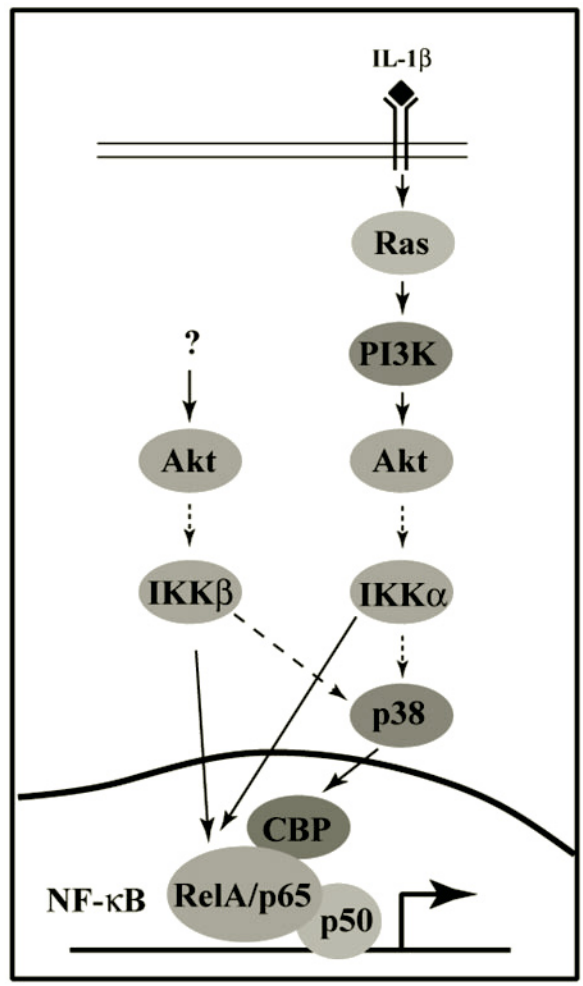

FIG. 6. **Model: IL-1β/Akt stimulates the transactivation potential of RelA/p65 by targeting IKK and p38.** In this model, we propose that 1) Akt expression alone utilizes IKKβ in a p38-dependent manner that requires serines 529 and 536 of RelA/p65 to directly stimulate NF-κB activity and 2) Akt signaling in response to IL-1 exposure stimulates NF-κB by activating p38 in a manner dependent on IKKα.

MEK inhibitor PD98059 does not appreciably reduce this synergy (Fig. 5*B*). Together, these data support the notion that Akt activates p38 to indirectly stimulate the transactivation domain I of the RelA/p65 subunit of NF-κB through a functional interaction with the co-activator CBP/p300.

DISCUSSION

The results presented here are consistent with the idea that IL-1β and Akt activate NF-κB by stimulating the transactivation domain I of RelA/p65. In Fig. 6, we present a model to explain how Akt transcriptionally activates the RelA/p65 subunit of NF-κB. We propose two mechanisms for Akt-mediated NF-κB activation: 1) Akt expression alone utilizes IKKβ in a p38-dependent manner, which requires serines 529 and 536 of RelA/p65 to directly stimulate NF-κB activity, and 2) Akt signaling in response to IL-1 exposure stimulates NF-κB by activating p38 in a manner dependent on IKKα. It is presently unclear why different Akt-dependent events differentially target the two forms of IKK. Additionally, how Akt may activate p38 is presently unclear, but it may involve utilization of the p38-activating kinases known as the MKKs, or the activation of p38 by Akt may involve MKK-independent mechanisms. In addition, Ras expression or integrin ligation of cells has been shown to activate p38 by a linear pathway requiring Rac, Pak, and MKK3 (48, 49). Interestingly, Ras-induced activation of Pak required Akt kinase activity, indicating that Akt may be a Pak kinase leading to p38 phosphorylation. However, in a separate study, IL-1 stimulation of cells activated p38 by a Ras-dependent, but Rac-independent mechanism, indicating that IL-1 stimulation may not require the Rac/MKK pathway (50). Recent data suggest that MKK6 interacts with IKKβ, suggesting that p38 may be activated by the MKK6·IKKβ complex (51). However, our results presented here demonstrate that IKKβ is dispensable for the ability of IL-1β to activate p38. IL-1 signaling is known to involve many pathways in addition to the described Akt-IKK pathway, and this report highlights the complexity of IL-1 signaling. This observation and the data presented in Fig. 2, *C* and *D*, suggest that p38 lies downstream of IKK activity. However, these observations do not rule out the

possibility that IKK and p38 are on separate but parallel pathways for NF-κB activation and that the inhibition of IKK inhibits a parallel but not upstream event required for p38 induction. Regulation of p38 activation is complex, and the mechanisms whereby Akt and IL-1 regulate this kinase require further investigation.

In contrast to the canonical role of IKKβ to induce phosphorylation of IκB and to subsequently induce nuclear translocation of NF-κB, we find that IKKβ can also modulate the transactivation function of NF-κB in response to Akt. The function of IKKβ in the activation of NF-κB by Akt expression alone is probably associated with activation of NF-κB transcriptional function and may not be associated with its well defined role in transient NF-κB activation. However, we cannot rule out the possibility that Akt functions to induce nuclear translocation in response to certain physiological stimuli. Additionally, our findings provide a function for the direct phosphorylation event seen on RelA/p65 by IKKβ that has been previously reported (24, 36), and they likely explain, at least partly, previous reports showing that Akt utilizes IKK for activation of NF-κB.

Our findings also demonstrate that p38 is activated by Akt to utilize the CBP/p300 co-activator for transactivation of NF-κB. The p38 pathway is well established in stimulating the transactivation properties of NF-κB independent of signals that induce the nuclear accumulation and DNA binding. Our work suggests that p38 utilizes co-activators to stimulate NF-κB, and it corroborates previously published reports demonstrating that p38 modulates NF-κB by an indirect mechanism (32, 42). Together, these findings represent novel functions associated with IKKα, IKKβ, and p38 in IL-1β- and Akt-mediated NF-κB activation.

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