Regulation of IKK ϵ Expression by Akt2 Isoform

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Abstract

The inhibitor of κ B kinase- ϵ (IKK ϵ), a breast cancer oncogene, functions as a transforming kinase by activating NF- κ B. IKK ϵ is often elevated in breast cancers in the absence of any gene amplification. Because Akt-mediated transformation was shown to require IKK ϵ , we examined if Akt regulates IKK ϵ level in breast cancer cells. Knockdown of Akt2, but not other Akt isoforms, decreased the basal and TNF-induced IKK ϵ protein and mRNA level, and overexpression of Akt2 in MDA-MB-231 cells increased IKK ϵ level. The decrease in IKK ϵ level by Akt2 knockdown was not only restricted to MDA-MB-231 cells but was also observed in several other breast cancer cells, including HCC1937 and MCF-10CA1a cells. Knockdown of p65/RelA subunit of NF- κ B decreased IKK ϵ level and attenuated the increase in IKK ϵ caused by Akt2 overexpression, suggesting that Akt2-mediated induction of IKK ϵ involves NF- κ B activation. Silencing of IKK ϵ also decreased long-term clonogenic survival of Akt2-overexpressing MDA-MB-231 cells. Taken together, these results demonstrate for the first time that IKK ϵ functions downstream of Akt2 to promote breast cancer cell survival.

Keywords

IKK ϵ , Akt2, NF- κ B, TNF, breast cancer

Introduction

The transcription factor nuclear factor- κ B (NF- κ B) is an important regulator of critical cellular processes such as cell growth, proliferation, and survival.^{1–3} Aberration in NF- κ B-associated pathways in various breast cancer cell lines and primary tumors has been associated with neoplastic transformation.^{3–5} NF- κ B is primarily activated by the inhibitor of the κ B kinase (IKK) family of proteins, which phosphorylate the inhibitor of NF- κ B, I κ B. I κ B is subsequently degraded, releasing NF- κ B to translocate into the nucleus to initiate its transcriptional activities.^{1–3} Apart from the canonical IKK α , IKK β , and the adaptor protein IKK γ , the noncanonical members of the IKK family, IKK ϵ and TANK-binding kinase-1 (TBK1), also activate NF- κ B.^{6,7} IKK ϵ was reported to function as a transforming kinase by activating NF- κ B.⁸ It was shown to phosphorylate I κ B α preferentially at the Ser36 residue, leading to its degradation and NF- κ B activation.^{9,10} It can also contribute to NF- κ B activation by phosphorylating different NF- κ B subunits.^{11–14}

Integrative genomic approaches established *IKBKE* as a novel breast cancer oncogene,⁸ although subsequent studies have demonstrated the oncogenic properties of IKK ϵ in other forms of cancer.^{15–18} Elevated levels of IKK ϵ have been detected in primary human breast cancer specimens and in a mouse model of breast cancer,¹⁹ and suppression of IKK ϵ compromised cell proliferation and viability in MCF-7 and ZR-75-1 cells.⁸ IKK ϵ was shown to substitute for Akt in inducing transformation. Two recent reports suggested that IKK ϵ directly phosphorylates Akt,^{20,21} placing it upstream of

the Akt signaling pathway. However, Akt-driven transformation required IKK ϵ , indicating that IKK ϵ may act downstream of Akt.^{8,22}

IKK ϵ is frequently elevated in breast cancers in the absence of any increase in gene copy number or somatic mutation.⁸ It is an inducible kinase and was shown to be induced by lipopolysaccharide in mouse macrophages.¹⁰ It is also induced by tumor-promoting phorbol esters as well as cytokines such as interleukin-1 and -6 and tumor necrosis factor- α (TNF).¹⁰ Thus, a defect in the signaling pathways that causes an induction of IKK ϵ may also contribute to breast cancer in the absence of any genetic changes in IKK ϵ . Because IKK ϵ was shown to functionally substitute for Akt, we examined if IKK ϵ acts downstream of Akt. We made a novel observation that Akt2, but not Akt1 or Akt3, positively regulates basal and TNF-mediated induction of IKK ϵ in several breast cancer cells, including MDA-MB-231 breast cancer cells. In addition, induction of IKK ϵ by Akt2 involves activation of NF- κ B. Moreover, our results demonstrate for the first time that IKK ϵ promotes breast cancer cell survival by acting downstream of Akt2.

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Figure 1. Knockdown of Akt2 decreased TNF-induced IKK ϵ levels in MDA-MB-231 cells. **(A)** Cells were transfected with Akt1, Akt2, Akt3, IKK ϵ , or nontargeting SMARTpool siRNA. **(B)** Cells were transfected with Akt1, Akt2, or nontargeting siRNA and then treated with or without 1 nM TNF, as indicated in the Materials and Methods. Western blot analyses were carried out with indicated antibodies. **(C)** The intensity of IKK ϵ was determined by densitometry using ImageJ software and normalized with actin. The data represent the fold change in IKK ϵ level compared with control siRNA-transfected cells. Each bar represents the mean $\pm$ standard error of 4 independent experiments. The open bar represents the untreated cells, and the hatched bar represents TNF treatment. * $P < 0.01$ and *** $P < 0.005$, using paired Student *t* test.

Results

Akt2 positively regulates IKK ϵ expression. IKK ϵ is an inducible kinase, but little is known about how its level is regulated. Because Akt-mediated transformation required IKK ϵ in breast cancer cells,⁸ we wanted to know if IKK ϵ level is regulated by Akt. Although most of the studies have focused on Akt1, there are 3 isoforms of Akt.²³ Because MDA-MB-231 cells express high levels of IKK ϵ , we silenced each Akt isoform with the specific siRNA and monitored IKK ϵ level. Figure 1 shows that while Akt1 and Akt3 knockdown had little effect on IKK ϵ , Akt2 knockdown attenuated IKK ϵ level (Fig. 1A). In contrast, knockdown of Akt isoforms had no effect on IKK β level.

Because IKK ϵ is induced by cytokines, such as TNF,^{10,24} we examined if Akt2 regulates induction of IKK ϵ by TNF. As shown in Figure 1B, TNF caused an increase in IKK ϵ but not IKK β , and knockdown of Akt2, but not Akt1, decreased TNF-induced IKK ϵ level. Based on densitometric scanning of 4 independent experiments, TNF caused a 1.5-fold increase in IKK ϵ level (Fig. 1C). Upon Akt2 depletion, the basal level of IKK ϵ was decreased by 2-fold, and the TNF-induced IKK ϵ level was decreased by 2.2-fold. To determine if Akt2 regulates IKK ϵ level in other cell lines besides MDA-MB-231 cells, we extended our study to include several other breast cancer cell lines. Figure 2 shows that silencing of Akt2 by siRNA also attenuated

Figure 2. Knockdown of Akt2 decreased IKK ϵ level in breast cancer cells. HCC1937 **(A)** or MCF-10CA1a **(B)** cells were transfected with indicated siRNAs, treated with or without 1 nM TNF, and Western blot analyses were carried out with the indicated antibodies, as described in the Materials and Methods. The results are representative of 2 individual experiments.

basal and TNF-induced IKK ϵ level in HCC1937 and MCF-10CA1a cells. Based on the densitometric quantification, Akt1 knockdown had little effect on IKK ϵ level when corrected for loading.

To determine if Akt2 affects IKK ϵ expression at the transcriptional level, we depleted Akt2 using siRNA and monitored IKK ϵ mRNA using RT-PCR. As shown in Figure 3A, the knockdown of Akt2 in MDA-MB-231 cells caused a substantial decrease in IKK ϵ mRNA. The densitometric scanning of 3 independent experiments revealed a significant decrease in IKK ϵ mRNA (~2.8 fold) upon Akt2 depletion (Fig. 3B).

Because knockdown of Akt2 decreased IKK ϵ level, we examined if overexpression of Akt2 increases IKK ϵ level. Figure 4A shows that both basal and TNF-induced IKK ϵ levels are higher in Akt2-overexpressing MDA-MB-231 cells compared to vector-transfected cells (Fig. 4B). Taken

Figure 3. Depletion of Akt2 decreased IKKε mRNA level. **(A)** MDA-MB-231 cells were transfected with Akt2 siRNA. Twenty-four hours after transfection, RT-PCR analysis was performed to detect IKKε mRNA level. **(B)** Densitometric quantification of the mRNA from 3 independent experiments normalized with actin. Data are the mean $\pm$ standard error. *** $P < 0.005$, using paired Student *t* test.

together, our results demonstrate that IKKε level is regulated by the Akt2 isoform.

Akt2 induces IKKε via activation of NF-κB. Although IKKε is known to activate NF-κB,⁸⁻¹⁴ it also has a NF-κB binding site in its promoter region.²⁴ To determine if Akt2 induces IKKε via activation of NF-κB, we first examined if Akt2 regulates NF-κB activity in MDA-MB-231 cells. Figure 4A shows that overexpression of Akt2 was associated with an increase in phosphorylation of p65/RelA, indicative of NF-κB activation.²⁵ Figure 5A shows that TNF increased NF-κB activity as determined by the NF-κB luciferase reporter assay. Knockdown of Akt2 decreased, and overexpression of Akt2 increased both basal and TNF-induced NF-κB activity in MDA-MB-231 cells (Fig. 5A and 5B).

To determine if Akt2 regulates IKKε level via NF-κB, we depleted p65/RelA subunit of NF-κB in cells transfected with an empty vector (pBabe) or a vector containing Akt2 construct (pBabe-Akt2) and monitored IKKε level. Figure 5C shows that knockdown of p65/RelA in cells transfected with the empty vector decreased both basal and TNF-induced IKKε levels. While overexpression of Akt2 enhanced basal and TNF-induced increase in IKKε levels, knockdown of p65/RelA attenuated the increase in IKKε level caused by Akt2 overexpression. These results demonstrate that Akt2 upregulates IKKε via NF-κB.

Akt2 promotes MDA-MB-231 cell survival via IKKε. To determine the functional significance of Akt2-mediated induction of IKKε, we performed a long-term clonogenic cell survival assay. Figure 6A shows that knockdown of either Akt2 or IKKε caused a decrease in the clonogenicity of MDA-MB-231 cells. Figure 6B represents the quantification of the number of colonies. While overexpression of

Akt2 enhanced the number of colonies, knockdown of IKKε decreased the number of colonies not only in MDA-MB-231 cells but also in Akt2-overexpressing cells. These results suggest that IKKε acts downstream of Akt2 to promote cell survival.

Discussion

IKKε is an inducible kinase and is amplified and overexpressed in breast cancers,⁸ but little is known about how its level is upregulated in these breast cancers. The results of our present study demonstrate for the first time that IKKε level is regulated by the Akt signaling pathway in highly aggressive breast cancer cells and that Akt2, but not the Akt1 or Akt3 isoform, is responsible for IKKε induction. In addition, Akt2 enhanced IKKε expression by activating the transcription factor NF-κB. Moreover, IKKε functions downstream of Akt2 to promote the survival of MDA-MB-231 breast cancer cells.

The functional relationship between Akt and IKKε was explored by Boehm *et al.*⁸ IKKε was shown to substitute for Akt in promoting transformation.⁸ Because Akt-driven transformation required IKKε, it was proposed that IKKε functions downstream of the Akt signaling pathway.^{8,22} Two recent studies, however, demonstrated that IKKε could directly phosphorylate Akt *in vitro*.^{20,21} While Xie *et al.* suggested that PI3K-dependent membrane association of Akt is required for its phosphorylation by IKKε,²⁰ Guo *et al.* showed PI3K-independent phosphorylation of Akt by IKKε.²¹ However, these studies do not explain how IKKε level is elevated in breast cancers.

Although IKKε is often amplified or overexpressed in breast cancers, there was no evidence of somatic mutations.⁸ It was speculated that IKKε is required for the proliferation and survival of cells in which IKKε is amplified.⁸ These studies were conducted in MCF-7 and ZR-75-1 cells, which harbored 5 and 10 copies of IKKε, respectively. However, IKKε was also required for the anchorage-independent growth of breast cancer Hs578t cells¹⁹ and for the survival of cervical cancer HeLa cells,¹³ which overexpressed IKKε without any gene amplification, suggesting additional mechanisms leading to IKKε upregulation.⁸ Because IKKε is an inducible kinase, an elevation in IKKε level could be caused by a deregulation in the signaling pathway that leads to IKKε induction.

Our observation that the Akt signaling pathway is responsible for the induction of IKKε is extremely important because the Akt signaling pathway is frequently deregulated in breast cancer and plays a central role in the development and progression of breast cancer. We have utilized both a loss-of-function and gain-of-function approach to demonstrate that Akt2 positively regulates IKKε level. It was reported that Akt2 phosphorylates and interacts with

Figure 4. Overexpression of Akt2 increased IKK ϵ level. (A) MDA-MB-231 cells stably transfected with pBabe or pBabe-Akt2 were treated with or without 1 nM TNF, and Western blot analyses were carried out with the indicated antibodies. (B) Densitometric quantification of IKK ϵ level from 3 independent experiments normalized with actin. The data represent the fold change in IKK ϵ level compared with untreated vector-transfected cells.

Figure 5. Akt2 induced IKK ϵ by activating NF- κ B. (A) MDA-MB-231 cells transfected with Akt2 or control nontargeting siRNA were transfected with 3X- κ B construct and Renilla luciferase and treated with or without 1 nM TNF, as described in the Materials and Methods. Luciferase activity was determined, and the relative luciferase units were normalized and represented as fold change. * $P < 0.05$. The results are representative of 3 independent experiments. (B) The MDA-MB-231/pBabe and MDA-MB-231/Akt2 cells were transfected with 3X- κ B construct and Renilla luciferase and treated with or without 1 nM TNF, as indicated in the Materials and Methods. The results are representative of 2 independent experiments. (C) The MDA-MB-231/pBabe and MDA-MB-231/Akt2 cells were transfected with p65/RelA siRNA or nontargeting control siRNA and treated with 1 nM of TNF for 24 hours and processed. Western blot analyses were carried out with the indicated antibodies. The results are representative of 2 independent experiments.

IKK α in ovarian cancer and HEK293 cells in which myr-Akt2 was overexpressed.²⁶ We, however, found that silencing of endogenous Akt2, but not Akt1 or Akt3, by siRNA decreased, whereas ectopic expression of Akt2 increased both the basal and TNF-induced IKK ϵ levels in MDA-MB-231 breast cancer cells. The regulation of IKK ϵ level by Akt2 was not unique to MDA-MB-231 cells because knockdown of Akt2 also attenuated IKK ϵ levels in HCC1937, MCF-10CA1a, HCC1395, and MCF-10CA1d cells (Fig. 2 and data not shown). However, knockdown of Akt2 did not decrease IKK ϵ levels in MCF-7 and ZR-75-1 cells in which IKK ϵ was amplified (data not shown). It was reported that overexpression of the protein kinase CK2 increased IKK ϵ level in murine fibroblasts and MCF-10F breast epithelial cells and that pharmacological inhibition of CK2 or ectopic expression of kinase-inactive CK2 decreased IKK ϵ expression in MDA-MB-231 and Hs578t cells, respectively.¹⁹ We also found that the CK2 inhibitor DMAT (2-dimethylamino-4,5,6,7-tetra-bromo-1H-benzimidazole) inhibited Akt2 level in MDA-MB-231 cells (data not shown). Because CK2 was shown to phosphorylate Akt,²⁷ it is conceivable that CK2 regulates IKK ϵ expression via Akt2.

We also explored the mechanism by which Akt2 upregulates IKK ϵ in breast cancer cells. Depletion of Akt2 decreased IKK ϵ not only at the protein level but also at the mRNA level, suggesting that Akt2 transcriptionally regulates IKK ϵ expression. The promoter region of IKK ϵ and the transcription factors required for IKK ϵ induction were extensively characterized by Wang *et al.*, who demonstrated that IKK ϵ is transcriptionally regulated by p65/RelA upon induction by TNF.²⁴ Our results show that Akt2 regulates IKK ϵ expression via NF- κ B in MDA-MB-231 breast cancer cells. First, knockdown of

Figure 6. Akt2 promotes cell survival via IKK ϵ . **(A)** MDA-MB-231 cells were transfected with Akt2, IKK ϵ , or nontargeting siRNA, and a clonogenic assay was performed, as detailed in the Materials and Methods. **(B)** Quantification of the number of colonies as determined by the clonogenic assay in MDA-MB-231 and MDA-MB-231/Akt2 cells transfected with control nontargeting or IKK ϵ siRNA. The results are representative of 3 independent experiments. ** $P < 0.01$ and *** $P < 0.005$, using paired Student t test.

Akt2 decreased both basal and TNF-induced NF- κ B activity. Second, overexpression of Akt2 enhanced constitutive and TNF-induced activation of NF- κ B. Third, knockdown of p65 decreased IKK ϵ level. Finally, knockdown of p65/RelA attenuated the ability of Akt2 to induce IKK ϵ level. Although Akt1 is well known to activate NF- κ B,²⁸⁻³⁰ reports of Akt2 activating NF- κ B have been emerging.^{26,31,32} Knockdown of Akt2 was shown to inhibit NF- κ B activity by decreasing the phosphorylation of p65/RelA at the Ser536 residue in PC3 prostate cancer cells.³¹ These results are consistent with our observation that Akt2 activates NF- κ B in MDA-MB-231 cells.

It has been proposed that IKK ϵ is required for breast cancer cell proliferation and survival.⁸ Although the Akt signaling pathway is also believed to promote breast cancer cell proliferation and survival,³³ a recent study demonstrated that introduction of myristoylated Akt1 and Akt2 inhibited proliferation of MDA-MB-231 cells.³⁴ In contrast, Santi *et al.* used siRNA-mediated silencing of Akt2 to demonstrate that Akt2 facilitates cell cycle progression and cell proliferation in these cells.³⁵ We have used both ectopic expression and siRNA-mediated knockdown of Akt2 and a long-term clonogenic assay to establish the importance of Akt2 in mediating the survival of MDA-MB-231 cells. In addition, we show that IKK ϵ functions downstream of Akt2 to promote the survival of MDA-MB-231 cells.

In summary, we made a novel observation that the Akt2 isoform positively regulates IKK ϵ expression via NF- κ B to promote the survival of MDA-MB-231 cells. Because the Akt signaling pathway is activated in more than 50% of breast cancers, and there is a close correlation between IKK ϵ upregulation and pAkt in breast cancer tissue specimens,²¹ this study underscores the importance of targeting the Akt2/IKK ϵ axis for the treatment of breast cancer.

Materials and Methods

Materials. Rabbit monoclonal antibodies to IKK ϵ , IKK β , Akt1, Akt2, Akt3, and S536-p65 were obtained from Cell Signaling Technology (Danvers, MA). Mouse monoclonal antibodies to p65 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma-Aldrich (St. Louis, MO), respectively. The Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI). Horseradish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from Jackson ImmunoResearch Laboratories (Bar Harbor, ME). Control nontargeting siRNA and siRNA SMARTpool specific to AKT1, AKT2, AKT3, IKBKE, and RELA were purchased from Dharmacon (Lafayette, CO). The polyvinylidene fluoride membrane was from Millipore (Billerica, MA), and the enhanced chemiluminescence detection kit was from Amersham (Piscataway, NJ). Puromycin was obtained from Sigma-Aldrich. The 3X- κ B construct was kindly provided by Dr. Zhijian Chen (University of Texas Southwestern Medical Center, Dallas, TX). pBabe-Akt2 (plasmid 14551) was obtained from Addgene (Cambridge, MA).³⁶

Cell culture and transfection. MDA-MB-231 and HCC1937 cells were maintained in RPMI medium supplemented with 10% FBS and 2 mmol/L glutamine. MCF-10CA1a cells were maintained in DMEM-F12 medium supplemented with 5% horse serum. Cells were kept in a humidified incubator at 37°C with 95% air and 5% CO₂. The cells were authenticated by DNA fingerprinting at the Department of Forensic Genetics at the University of North Texas Health Science Center. siRNA was transfected using Lipofectamine RNAiMax according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Immunoblot analysis. Equivalent amounts of total cellular extracts were electrophoresed by SDS-PAGE and transferred electrophoretically to the polyvinylidene fluoride membrane. Immunoblot analyses were carried out as described before.³⁷

RT-PCR analysis. Total RNA was isolated using TRI reagent RT from Molecular Research Center (Cincinnati, OH) as per the manufacturer's protocol and subjected to RT reaction using reverse transcriptase enzyme from Promega. PCR amplifications were performed using the following primers: IKBKE: forward, 5'-GAA GTT CGT CTC GGT CTA TG-3'; reverse, 5'-CGG TAC ATG ATC TCC TTG TT-3'; and ACTB: forward, 5'-TAC AAT GAG CTG CGT GTG GCT-3'; reverse, 5'-ATC CAC ATC TGC TGG AAG GTG GA-3'. Cycle conditions for all PCRs were set up for 35 cycles as follows: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. The products were resolved on 1% agarose gel containing ethidium bromide.

NF- κ B luciferase reporter assay. Cells were plated or transfected with the siRNA using RNAiMax reagent per the manufacturer's protocol (Invitrogen). After 24 hours, the cells were transfected with the 3X- κ B construct and Renilla luciferase construct using Lipofectamine 2000 (Invitrogen). Cells were harvested after 48 hours of transfection and lysed in the passive lysis buffer (Promega). Luciferase assay was measured with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA), and Renilla luciferase was measured using the Stop n' Glo reagent (Promega).

Clonogenic assay. Cells transfected with or without control nontargeting, Akt2, or IKK ϵ siRNA were cultured at 37°C in a humidified incubator with 5% CO₂ until there were at least 50 cells per colony. At the end of the incubation, the cells were washed with PBS and incubated with 0.25% crystal violet solution for 15 minutes. Colonies were counted using ImageJ software (<http://rsbweb.nih.gov/ij/>), and the plate was photographed using the BioChem System (BioImaging System, UVP, Upland, CA).

Statistical analysis. Statistical significance was determined by a paired Student *t* test using the GraphPad Prism software (La Jolla, CA). *P* < 0.05 was considered statistically significant.

Declaration of Conflicting Interests

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