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Complex formation of p65/*RelA* with nuclear Akt1 for enhanced transcriptional activation of NF-κB

Osong Kwon [a], Kyung A Kim [a], Long He [a], Mira Jung [b], Sook Jung Jeong [a], Jong Seog Ahn [a,*], Bo Yeon Kim [a,*]

[a] *Functional Metabolomics Research Center, KRIBB, Yuseong, Daejeon, Republic of Korea*
[b] *Department of Radiation Medicine, Georgetown University School of Medicine, Washington, DC 20057-1482, USA*

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Abstract

Akt1 was revealed to interact with Ki-Ras in the cytoplasm of Ki-Ras-transformed human prostate epithelial cells, 267B1/K-ras. Moreover, p65/RelA in the nucleus was found to interact with both Ki-Ras and Akt1, suggesting the nuclear translocation of Akt1:Ki-Ras complex for NF- κB activation. In support of this, compared with wild type Akt1, the dominant negative Akt1 mutant was decreased in its nuclear expression, reducing the Ki-Ras-induced NF-κB transcriptional activation. Moreover, inhibitors of Ras (sulindac sulfide and farnesyltransferase inhibitor I) or PI3K/Akt (wortmannin), reduced the amounts of Akt1 and Ki-Ras in the nucleus as well as partial NF-κB activity. The complete inhibition of Ki-Ras-induced NF-κB activation, however, could only be obtained by combined treatment with wortmannin and proteasome inhibitor-1. Accordingly, clonogenic assay showed Akt1 contribution to IκBα-mediated NF-κB activation for oncogenic cell growth by Ki-Ras. Our data suggest a crucial role of Ki-Ras:Akt1 complex in NF-κB transcriptional activation and enhancement of cell survival.

Keywords: Ki-Ras; Akt; NF-κB; Proteasome

Akt has emerged as a key regulatory element in a wide range of cellular processes, including proliferation, differentiation, development, and diabetes-mellitus. Recently, Akt1 and Akt2 were found to translocate to the plasma membrane in response to agonist stimulation [1,2], and after phosphorylation by its upstream kinase PDK-1 [3], Akt translocates to the nucleus. In this respect, nuclear translocation of Akt/PKB in response to IGF-I and PDGF was reported in osteoblast-like MC3T3-E1 cells [4]. In addition, TCL1 proto-oncogene family was reported to bind to and mediate nuclear translocation of Akt1 [5,6], and oncogenic Ras induces Akt activation [7]. However, until now, the questions of how Akt is translocated to the nucleus and of the mechanistic role of its translocation for NF-κB activation have remained unanswered.

Ras is known to activate NF-κB through either IKK phosphorylation and IκBα degradation or Akt-mediated pathway [8,9]. TC21, a fourth member of the *ras* supergene family that exhibits oncogenic activation in human cancer cells [10,11], mediates transformation and cell survival via Akt mediated NF-κB activation [12]. Previously, we reported that IKKβ and IκBα are responsible for Ki-Ras-induced NF-κB activation, and Akt1 was suggested to be an alternative enzyme concerned in the transcriptional activity of NF-κB by Ki-Ras [13].

In the present study, we found that Akt1 interacts with Ki-Ras in the cytosol, their levels being abundant in the nucleus of Ki-Ras-overexpressing 267B1/Ki-Ras cells [14]. Furthermore, p65/RelA was found to bind to both Akt1 and Ki-Ras in the nucleus, thus leading to the enhancement of the transcriptional activity of NF-κB and

* Corresponding authors. Fax: +82 42 860 4595.
E-mail addresses: jsahn@kribb.re.kr (J.S. Ahn), bykim@kribb.re.kr (B.Y. Kim).

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growth rate of the cells. Hence, Ki-Ras-induced NF-κB activation is suggested to be enhanced by nuclear translocation of Akt1 and its subsequent interaction with p65/RelA.

Materials and methods

Chemicals, antibodies, and plasmids. T4 polynucleotide kinase, lipofectin, and lipofectamine plus were purchased from GIBCO-BRL, wortmannin, sulindac sulfide, and farneysyltransferase inhibitor I were obtained from Calbiochem, antibodies to p65, Akt1, HA, actin, and proliferating cell nuclear antigen (PCNA) were from Santa Cruz Biotechnology, antibody to phospho-Akt1 was purchased from Cell Signaling, antibody to Ki-Ras was from Oncogene, NF-κB oligonucleotide was purchased from Promega, Sephadex G-25 column was obtained from Pharmacia Biotechnology, enhanced chemiluminescence reagent was from Amersham, [γ-^{32}P]ATP was purchased from NEN, Dupont, plasmids encoding mutant IKKβ (pCMV2-IKK2-S177/181A), IKKα (pFlag-IKK1-S176/180A) and NIK (pMyc-NIK-K429/430A) were obtained as described [13], and expression vectors for the HA-tagged Akt (wt and kd) were kindly provided by Dr. Thomas Frank at Columbia University.

Cell culture and transfection. 267B1 and 267B1/Ki-ras cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 0.5 μg ml^{-1} hydrocortisone, and 10% heat-inactivated fetal bovine serum. Cells were deprived of serum for 24 h prior to use.

Subcellular fractionation, immunoprecipitation, and immunoblot analysis. Cells were suspended in a solution containing 10 mM Hepes–NaOH (pH 7.9), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM NaF, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, 100 μM Na_3VO_4, and 1 mM dithiothreitol (DTT). After 15 min, NP-40 was added to a final concentration of 0.1% and the cell lysate was centrifuged at 12,000*g* for 1 min at 4 °C. For preparation of a nuclear fraction, the pellet obtained after centrifugation of the cell lysate was resuspended in nuclear lysis buffer [50 mM Hepes–NaOH (pH 7.9), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 0.2 mM NaF, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, 0.4 mM PMSF, 100 μM Na_3VO_4, 1 mM DTT, and 10% glycerol] and incubated for 30 min on ice. For preparation of a cytosolic fraction, the supernatant obtained after centrifugation of the cell lysate was recentrifuged at 12,000*g* for 30 min at 4 °C. Immunoblot analysis was performed with the indicated specific antibodies, and immune complexes were detected with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech). For immunoprecipitation, nuclear or cytoplasmic lysate was mixed with a specific antibody depending on the experiments and incubated at 4 °C overnight with a gentle rotation. Secondary antibody conjugated to a magnetic bead (Dynal) was added to the protein-antibody complex for another 1 h at 4 °C and washed four times in lysis buffer. Samples were mixed with gel loading dye, boiled, and applied to SDS-PAGE. Proteins were transferred to a PVDF membrane, immunoblotted with specific antibodies, and detected by enhanced chemiluminescence reagent.

EMSA analysis. EMSA was performed as described [15]. In brief, the nuclear lysate was used for EMSA analysis. The κB oligonucleotide (3.5 pmol; Promega) was labeled with 10 μCi of [γ-^{32}P]ATP [16] in the presence of 5 U of T4 polynucleotide kinase (Gibco-BRL) by incubation for 30 min at 37 °C in 10 μl of kinase buffer. The reaction was terminated by the addition of 50 mM EDTA and centrifugation through a Sephadex G-25 column (Amersham Pharmacia Biotech) to remove unincorporated ^{32}P. For the EMSA reaction, nuclear protein extract (5–10 μg) was incubated for 30 min at room temperature in a final volume of 10 μl containing 0.03 pmol of the ^{32}P-labeled κB oligonucleotide, 40 mM Hepes–KOH (pH 7.8), 10% glycerol, 1 mM $MgCl_2$, 0.1 mM DTT, and 1 μg of poly(dI-dC). For "supershift" analysis, the nuclear extract was incubated with specific antibodies for 30 min at room temperature before exposure to the ^{32}P-labeled oligonucleotide. The reaction was stopped by the addition of gel loading dye, and the samples were subjected to electrophoresis on a nondenaturing 6% polyacrylamide gel in 0.5× Tris–borate–EDTA buffer followed by autoradiography.

Clonogenic assay. Cells at semiconfluency in 60 mm dishes were exposed to wortmannin or Pro1 at appropriate concentrations and immediately trypsinized to be transferred to 6-well plates at 1 x 10^3 cells/well in complete RPMI1640 medium. After 7-10 days of incubation, the medium was removed and 200 μl of 0.3% crystal violet solution (dissolved in 1:1 mixture of methanol and H_2O) was added into each of the wells for 2 min. The cells were then washed once with PBS buffer, air dried, counted, and photographed.

Results

Phosphorylation of Akt1 is required for its nuclear accumulation

In a determination of whether phosphorylation of Akt1 is required for Ki-Ras-induced NF-κB activation, Akt1 was found to be significantly phosphorylated in the cytoplasm of 267B1/K-ras cells and was reduced by Ras inhibitors, farnesyltransferase inhibitor I (FTI, inhibitor of membrane attachment of Ras) and sulindac sulfide (SS, inhibitor of Ras signaling) (Fig. 1A). Unexpectedly, nuclear levels of Akt1 and Ki-Ras were also higher in the transformed cells than in the normal cells (Fig. 1B), and were found to be reduced by the Ras inhibitors (Fig. 1B). These results raise a possibility that that Ki-Ras-induced phosphorylation of Akt1 is required for its enhanced nuclear accumulation or translocation as supported by an experiment in which 267B1/K-ras cells were treated with wortmannin, a PI3K inhibitor. As shown in Fig. 1C, wortmannin drastically reduced the phosphorylation of Akt1 in the cytoplasm but without any effect on the protein level. Accordingly, nuclear level of Akt1 as well as of its phosphorylated form was greatly diminished by wortmannin. Moreover, the amount of Ki-Ras in the nucleus was also diminished by the compound while p65/RelA did not change. Thus, all these results suggested that increased phosphorylation of Akt1 might be required for its nuclear accumulation. A supportive observation could be that significant amount of the exogenously expressed wild type Akt1 (wt) was found in the nucleus while the level of the kinase dead form (kd) was very low (Fig. 1D).

Interaction of Akt1 with Ki-Ras and p65

Tcl1 protooncogene was reported to bind to Akt1 and mediate its nuclear translocation [6]. To see if Akt1 interacts with Ki-Ras in the cytoplasm, cytoplasmic or nuclear lysate prepared from 267B1/Ki-Ras cells transfected with the HA-tagged Akt1 plasmids was subjected to an immunoprecipitation analysis. It was revealed that only the wild type but not the mutant Akt1 interacted with Ki-Ras in the cytoplasm (Fig. 2, first panel). On the other hand, unexpectedly, interaction of Ki-Ras with p65 was enhanced by transfection with the wild type Akt1 in the nucleus of the cells (Fig. 2, second panel). However, any interaction of Ki-Ras with p65 could not be found in the cytoplasm (data not shown). Hence,