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Down-regulation of Notch-dependent transcription by Akt in vitro

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Abstract The effect of Akt on Notch intracellular domain (NICD)-mediated transcription was investigated. Transfection experiments revealed that constitutively active Akt down-regulates NICD-dependent transcription. Kinase inactive dominant negative Akt did not affect NICD transcriptional activity, indicating that Akt kinase activity is responsible for the down-regulation. Studies using histone deacetylase (HDAC) and silencing mediator of retinoid and thyroid hormone receptor (SMRT) revealed that modulation of NICD transcriptional activity is not mediated by an HDAC-dependent mechanism or recruitment of the co-repressor SMRT. Akt inhibited proper nuclear localization of NICD, and phosphorylated NICD both in vitro and caused its hyperphosphorylation in vivo. These results may suggest possible regulation of NICD transcriptional activity by Akt-mediated phosphorylation and subsequent inhibition of proper nuclear localization of NICD.
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Keywords: Akt; NICD; Notch; Transcription

1. Introduction

Notch-mediated intracellular signal transduction is an evolutionary conserved pathway, reported to play seminal roles in cell fate control during embryonic development, cell growth, differentiation and apoptosis [1]. Notch is a single transmembrane protein containing multiple epidermal growth factor (EGF)-like repeats in their extracellular domain and cdc10/ankyrin repeats in their intracellular domain. Upon ligand binding, proteolytic cleavage of Notch near the transmembrane domain occurs, resulting in release of the Notch intracellular domain (NICD) [2,3]. NICD is then translocated to the nucleus, forming a complex with the transcription factor CBF1/RBP-Jκ, and activates transcription of its target genes including Hes1 and Hes5 [1,4–6].

CBF1/RBP-Jκ functions as a bifunctional transcriptional regulatory protein with a strong repressive effect in co-transfection assays using Gal4-CBF1/ RBP-Jκ and reporters with Gal4 binding sites, while functioning as a transcriptional activator in the presence of Notch [7]. Prior to Notch activation, CBF1/RBP-Jκ interacts with a co-repressor complex containing silencing mediator of retinoid and thyroid hormone receptors (SMRT) and histone deacetylase-1 (HDAC-1) [8]. Upon activation by Notch, the repressor complex dissociates and NICD can form a complex with histone acetyltransferase P/CAF, GCN5, p300 and Mastermind [9–11] to activate downstream transcription.

Serine threonine kinase Akt/protein kinase B has been reported to play very important roles in cell survival, proliferation and other physiological functions [12,13] Akt1 was first found as a homolog of the transforming oncogene of the AKT8 retrovirus [14]. Two additional Akt isoforms have also been identified, Akt2 and Akt3 [15,16]. All three Akt family proteins have a central kinase domain [17] and a pleckstrin homology domain responsible for lipid–protein and protein–protein interactions in the amino terminus [18].

Accumulating evidence suggest that Notch signaling interacts with other pathways, such as the mitogen activated protein kinase (MAPK) [19] and phosphatidylinositol 3-kinase (PI3K)/Akt pathways [20]. Sade et al. [21] showed that Notch1 associates with tyrosine kinase $p56^{lck}$ and PI3K, and PI3K-dependent activation of Akt plays a role in regulation of Notch-mediated anti-apoptotic function. In human T-ALL cells, pharmacological inhibition of PI3K/Akt causes modulation of Notch1 protein, suggesting an interplay between Notch and Akt signaling [20]. Very recently, it was reported that Notch-1 negatively regulates phosphatase and tensin homolog (PTEN) expression via the downstream Hes1 in leukemic T cells [22]. The authors also showed that Notch signaling and the PI3K/Akt pathway synergize in a fly model of Notch-induced tumorigenesis, indicating the evolutionary conserved interplay between the two signaling pathways. These investigations prompted us to examine the direct effect of Akt on Notch-induced signaling. In this report, we used transfection experiments to examine the effect of constitutive active myristoylated Akt (myr-Akt) co-expression on Notch-dependent transcription using reporter assays.

2. Materials and methods

2.1. Plasmids and vectors

HA-tagged myr-Akt1, a kinase-dead (K179M) dominant negative Akt1 (dnAkt), myr-Akt2 and myr-Akt3 were described previously [23,24]. The dnAkt2 (K181A) and dnAkt3 (K177M) mutants were constructed using the QuikChange kit according to the manufacturer's instruction (Stratagene). The dnAkt2 mutagenesis primer sequence was 5′-CCG CTA CTA CGC CAT GGC GAT CCT GCG AAA GG-3′ and the dnAkt3 mutagenesis primer sequence was 5′-CGA GAG AAG GCA AGT GGA AAA TAC TAT GCT ATG ATG ATT CTG AAG-3′. Flag tagged CBF1 and co-repressor SMRT were kindly donated from Dr. Walmsley (University of Pennsylvania,

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Abbreviations: NICD, Notch intracellular domain; dnAkt, dominant negative Akt; HDAC, histone deacetylase; TSA, trichostatin A; SMRT, silencing mediator of retinoid and thyroid hormone receptors; GSK3β, glycogen synthase kinase 3β

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Philadelphia, PA, USA). The plasmid pBJ5-HDAC1-Flag was from Dr. Jongbok Yoon (Yonsei University, Seoul, Korea). The plasmid pcDNA3-Flag-HDAC1 (H141A) was a gift from Dr. Sung Hee Baek (Seoul National University, Seoul, Korea). HA-tagged wild type GSK3β (HA-GSK3β) and kinase-dead dominant negative GSK3β (HA-dnGSK3β) were gifts from Dr. Jho E.H. (University of Seoul).

2.2. Cell culture and transfection

293T cells and Cos7 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin at 37 °C under 5% CO_2 in a humidified incubator. The cells were transfected by calcium phosphate in HEPES buffered saline as described [25].

2.3. Reporter assays

293T cells or Cos7 cells (12-well plates) were transfected with 1 μg of total DNA containing 500 ng of reporter constructs (4 × CSL-Luc; 6 × Notch response elements (6 × NRE-Luc); Hes1-Luc) and analyzed 48 h after transfection. Dual luciferase assays were performed using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's instruction.

2.4. Western blot and immunoprecipitation

All cells were lysed and processed 48 h post-transfection. Transfected cells were washed once with phosphate-buffered saline (PBS) and lysed with a lysis buffer (20 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% Triton X-100, 0.1% SDS, 20 mM NaF, 1 mM Na_3VO_4, 1 × protease inhibitor (Roche)). All protein samples were resolved by 10% SDS–PAGE after boiling 5 min in SDS sample buffer. For Western blot analysis, proteins were transferred onto nitrocellulose in Tris-glycine-methanol buffer and visualized with appropriate primary antibody followed by HRP-conjugated secondary antibody and WEST-ZOL® plus Western Blot Detection System (iNtRON).

For immunoprecipitations (IP), 1 mg of total proteins were incubated overnight in 1 ml of lysis buffer with appropriate primary antibody at 4 °C and then with 30 μl protein A-Sepharose CL-4B (Amersham) for 2 h. The beads were washed three times with lysis buffer. After complete removal of supernatant, beads were resuspended in SDS sample buffer, boiled 10 min and analyzed by SDS–PAGE and subsequent Western blots.

The antibodies used are mouse Myc-Tag (9B11) (Cell Signaling), rabbit HA-probe (Y-11) (Santa Cruz Biotechnology), rabbit β-Tubulin (H-235) (Santa Cruz Biotechnology), mouse anti-FLAG (Sigma), goat SMRT (N-20) (Santa Cruz Biotechnology), and rabbit c-Jun (60A8) (Cell Signaling).

2.5. Immunostaining

Cos7 cells (210^5) seeded on glass coverslips in 6-well plates, were transfected with 4 μg of total DNA and processed for immunostaining 48 h after transfection. Cells were washed with PBS, fixed with 3.8% paraformaldehyde in PBS for 10 min and permeabilized with 0.05% Triton X-100 in PBS for 10 min at ambient temperature. The fixed cells were washed three times with PBS for 5 min each and blocked with 3% skim milk in PBS for 30 min. Eighty microliters of primary antibody diluted in 1% skim milk was added per coverslip and incubated for 1 h. Cells were washed 3 times with PBS, incubated with 80 μl of secondary antibody solution (anti-rabbitt IgG-Oregon Green (Molecular Probe) or anti-rabbit IgG-Cy3 (Zymed) in 1% skim milk in PBS). Cells were washed three times for 10 min with PBS, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) in first wash. Coverslips were mounted on slide glasses and observed by fluorescence microscopy (Olympus BX50).

2.6. Cell fractionation

Cells seeded at the density of 110^6 cells in 100 mm dish were transfected with 6 μg of total DNA. Forty-eight hours post-transfection, the cells were washed with ice cold PBS, and harvested in cytoplasmic extraction buffer (10 mM HEPES; pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and agitated for 10 min at 4 °C. After addition of NP-40 (final 0.5%), the samples were further agitated for 10 min at 4 °C. The samples were then subjected to centrifugation at 13 000 rpm in a microcentrifuge (Eppendorf) for 5 min. The supernatant was collected as cytosolic fraction. The nuclear pellets were washed two times with cold PBS and resuspended in a nuclear extraction buffer (20 mM HEPES; pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). The nuclear extracts were agitated for 10 min at 4 °C and centrifuged at 13 000 rpm at 4 °C and the supernatants were collected as nuclear fraction.

2.7. Expression and purification of the recombinant protein

Mouse Notch1 NICD was excised by EcoRI/BamH1 digestion of myc-NICD. The insert was then ligated into EcoRI and BamHI predigested the bacterial expression vector pGEX4T-3 (Amersham Pharmacia). The recombinant glutathione S-transferase (GST)-NICD fusion construct was transformed into *Escherichia coli* BL21. Expression of GST-NICD fusion protein was induced by the addition 2 mM IPTG (Amersham Pharmacia). GST-NICD was purified with glutathione (GSH)-agarose (Sigma) beads according to the manufacturer's instruction.

2.8. Immunocomplex kinase assay

293T cells seeded at the density of 110^6 cells in 100 mm dish were transfected with 10 μg of total DNA, harvested 48 h after transfection. The 1 mg of whole cell lysates was incubated with HA-antibody and subsequently treated with protein A-Sepharose CL-4B. The immunocomplexes were incubated with 1.5 μg of pre-purified GST-NICD and in 20 μl kinase reaction buffer (20 mM MOPS, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 112.5 μM ATP, 17 mM magnesium chloride) and 10 μCi [γ^{32}-P]ATP (Amersham) for 10 min at 37 °C. The samples containing ^{32}P-labeled proteins were analyzed by SDS–PAGE and subsequent autoradiography.

2.9. In vivo labeling

293T cells seeded at a density of 110^6 cells in a 100 mm dish were transfected with 10 μg of total DNA. Forty-eight hours after transfection, the medium was changed to phosphate free DMEM supplemented with 10% dialyzed FBS and 250 μCi/ml [^{32}P]-orthophosphate (Amersham). The labeled cells were harvested 12 h after radioisotope-labeling. Myc-tag antibody was added to 1 mg of whole cell lysates and incubated for overnight. Myc-tagged ^{32}P-labeled proteins were precipitated by adding protein A-Sepharose CL-4B and analyzed by SDS–PAGE and subsequent autoradiography.

3. Results

3.1. Akt down-regulates NICD-dependent transcription

To investigate the effect of Akt on Notch dependent transcription, reporter assays were performed in 293T cells and Cos7 cells using luciferase reporter constructs. In these studies, three types of different luciferase reporter constructs including 4 × CSL-Luc with four copies of CSL synthetic binding sites, 6 × NRE-Luc with six copies of Notch response elements, and Hes1-Luc containing the promoter region of Hes-1 were used to investigate the effect of Akt on Notch dependent transcriptional activity. NICD-dependent transcriptional activity was down-regulated by all Akt isoforms in both cell lines using all three different reporter constructs (Fig. 1A).

To investigate whether this decrease in NICD-dependent transcriptional activity was attributable to a reduction in NICD protein levels by Akt, we performed Western blot analyses in 293T cells and Cos7 cells transfected with myc-NICD and HA-myr-Akt isoforms (Fig. 1B). We found that protein levels of myc-NICD did not decrease markedly in cells co-expressing myr-Akt, suggesting that Notch protein levels can not account for the effect on NICD dependent transcriptional activity.

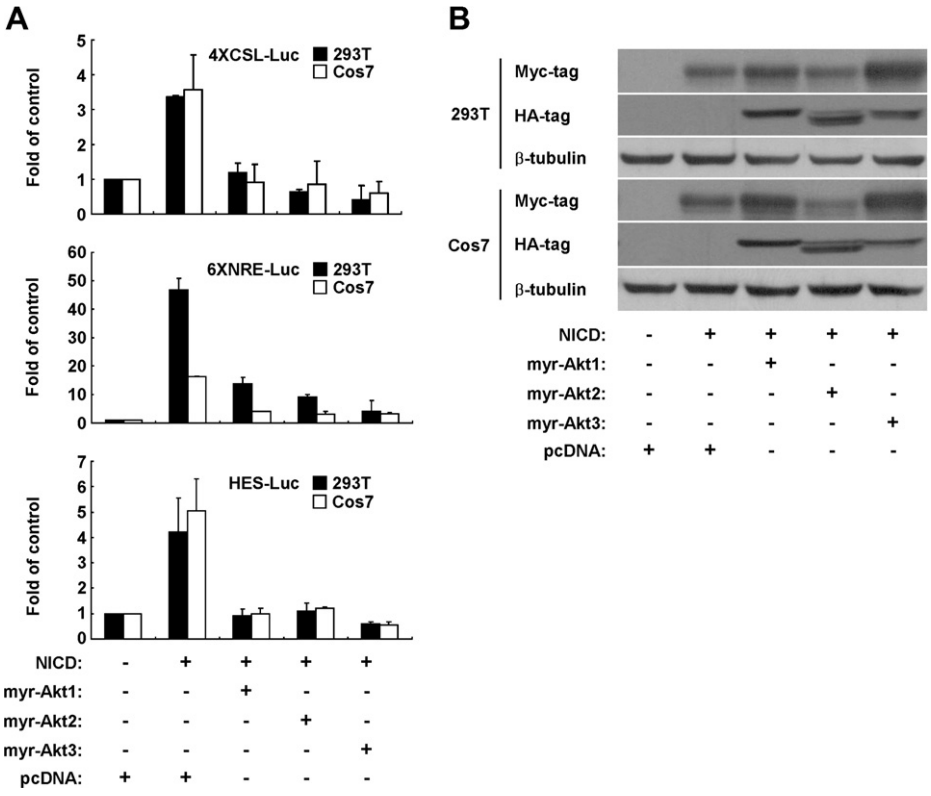

Fig. 1. Myr-Akt isoforms down-regulate NICD-dependent transcription. (A) The cells were transfected with NICD, myr-Akt isoforms, and luciferase reporter constructs, harvested after 48 h and analyzed by dual luciferase assay (Section 2). (B) The cells were transfected with myc-NICD and myr-Akt isoforms, harvested after 48 h, then analyzed for myc-tagged NICD levels by Western blot analyses. HA-tagged Akt levels were also shown by Western blot using HA antibody.

3.2. Akt kinase activity is necessary for down-regulation of NICD-dependent transcriptional activity

Since Akt is known to regulate its target proteins by phosphorylation, we set out to investigate whether the kinase activities of Akt isoforms are required for down-regulation of NICD-dependent transcription. Experiments using kinase-dead dnAkt isoforms, where lysine residues at the active site have been replaced with methionine (K179M for Akt1, K177M for Akt3) or alanine (K181A for Akt2), revealed that the kinase activity of Akt is indispensable for down-regulation of NICD-dependent transcription (Fig. 2A). Data in Fig. 2B confirm again that the inhibitory effect of myr-Akt isoforms on transcriptional activity of NICD does not result from Akt-mediated down-regulation of NICD protein levels.

3.3. The regulation of NICD-dependent transcription is not mediated by an HDAC dependent mechanism or recruitment of co-repressors

HDACs remove an acetyl group from histones, which promotes the formation of repressive chromatin, to cause inhibition of gene transcription [8,26,27]. To investigate whether the Akt-mediated inhibitory effect of Akt on NICD-dependent transcription is mediated by a mechanism involving HDAC activity, we treated cells with trichostatin A (TSA) (Calbiochem), an HDAC inhibitor, at 5 nM for 24 h after transfection and performed reporter assays (Fig. 3A). NICD-dependent transcriptional activity was slightly up-regulated by TSA treatment, however, TSA treatment failed to prevent down-regulation caused by myr-Akt isoforms (Fig. 3A, 5th bar vs. 6th bar; 7th bar vs. 8th bar; and 9th bar vs. 10th bar). These results indicated that regulation of Notch-dependent transcription by myr-Akt is likely not mediated by an HDAC dependent mechanism. Experiments using HDAC expression constructs also confirmed that myr-Akt-mediated repression of NICD transcriptional activity is independent from HDAC activity (Fig. 3B). Co-expression of dominant negative HDAC did not prevent down-regulation of Notch-dependent transcriptional activity by myr-Akt.

CBF1 belongs to a family of CSL proteins with homologs in *Drosophila* [Suppressor of Hairless, Su(H)] [28] and in *Caenorhabditis elegans* (Lag-1) [29]. CBF1 binds to DNA and functions as a transcriptional repressor [7,30]. After binding to DNA, CBF1 recruits a co-repressor complex that includes SMRT and HDAC1 [8] to exert its inhibitory effect on transcription. To determine whether NICD-dependent transcription is down-regulated by modulating binding of CBF1 and the co-repressors, co-immunoprecipitation was performed in 293T cells transiently transfected with Flag-CBF1 and SMRT. Myr-Akt did not increase the association between CBF1 and SMRT (Fig. 3C), suggesting that recruitment of co-repressor SMRT is likely not the mechanism responsible for myr-Akt-mediated down-regulation of NICD-dependent transcription.

3.4. Akt inhibits proper nuclear localization of NICD

In an attempt to find the mechanism by which Akt could down-regulate transcriptional activity of NICD, the effect of Akt on subcellular distribution of NICD was examined. As shown in Fig. 4, myr-Akt isoforms effectively inhibited proper

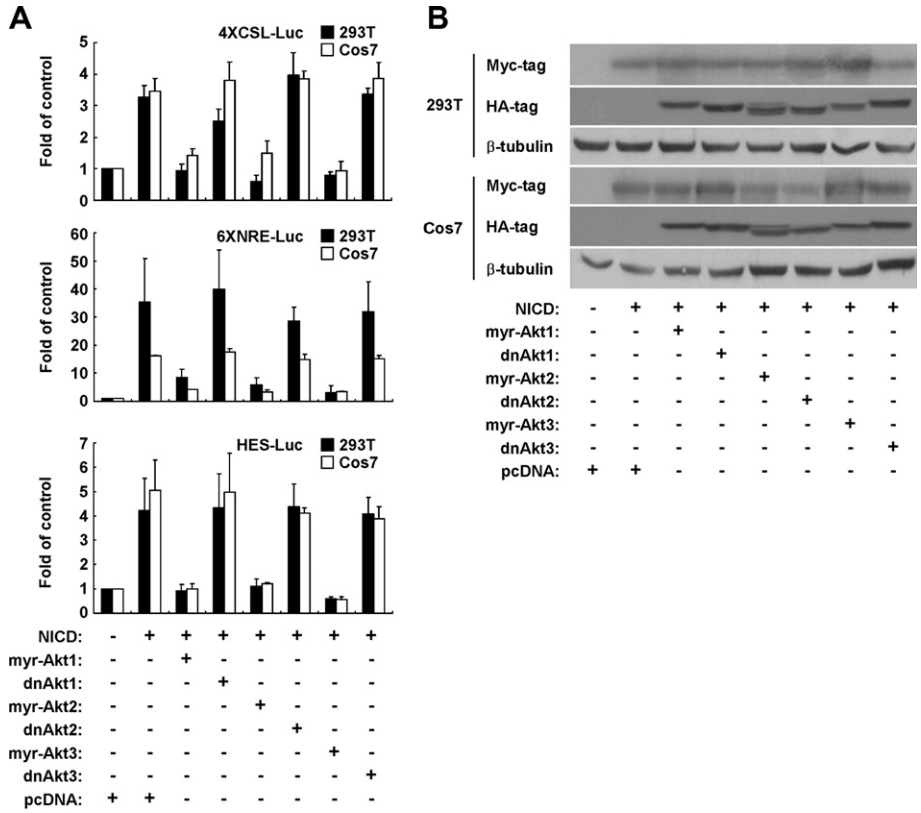

Fig. 2. Kinase activities of Akt isoforms are required for inhibition of NICD transcriptional activity. (A) The cells were transfected with NICD, myr-Akt/dnAkt isoforms, and luciferase reporter constructs, harvested after 48 h and analyzed by dual luciferase assay. (B) The cells were transfected with myc-NICD and myr-Akt/dnAkt, harvested after 48 h, then analyzed for myc-tagged NICD levels by Western blots. HA-tagged Akt levels were also shown by Western blot using HA antibody.

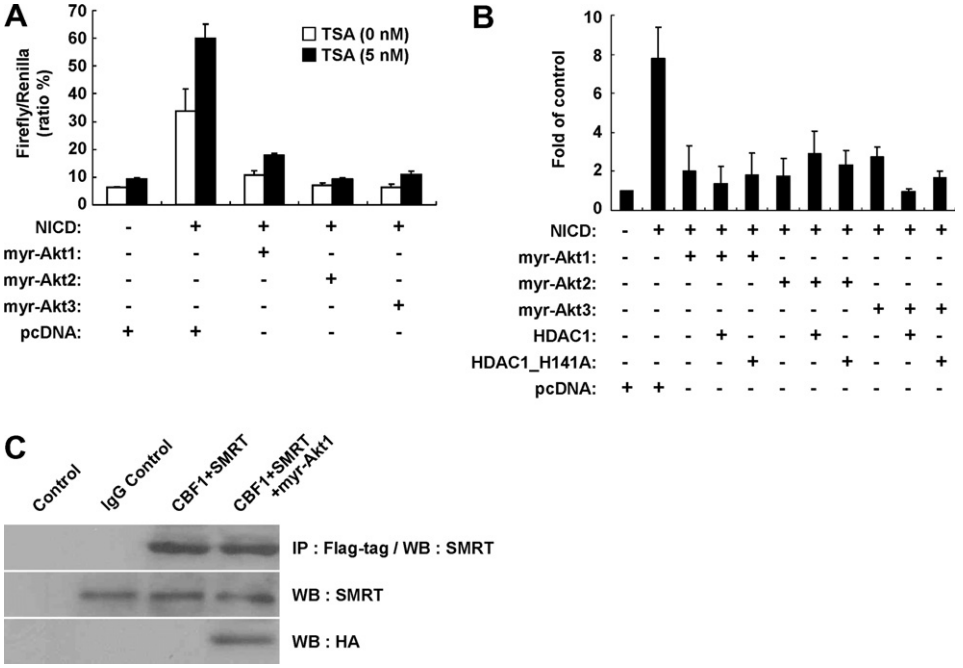

Fig. 3. Inhibition of NICD-dependent transcription by Akt is not mediated by recruitment of co-repressors. (A) 293T cells were transfected with 4 × CSL-Luc, NICD and Akt isoforms for 48 h, treated with 5 nM TSA 24 h post-transfection for an additional 24 h and subjected to dual luciferase assays. (B) 293T cells were transfected with 4 × CSL-Luc, HDAC1 or HDAC1_H141A, and with various combination of myr-Akt and dnAkt isoforms as indicated, harvested after 48 h and analyzed by dual luciferase assays. (C) 293T cells were transfected with Flag-CBF1, SMRT, and myr-Akt1 for 48 h, immunoprecipitated with Flag-tag and subjected to Western blot with SMRT antibody (upper panel), Western blot with SMRT (lower panel).

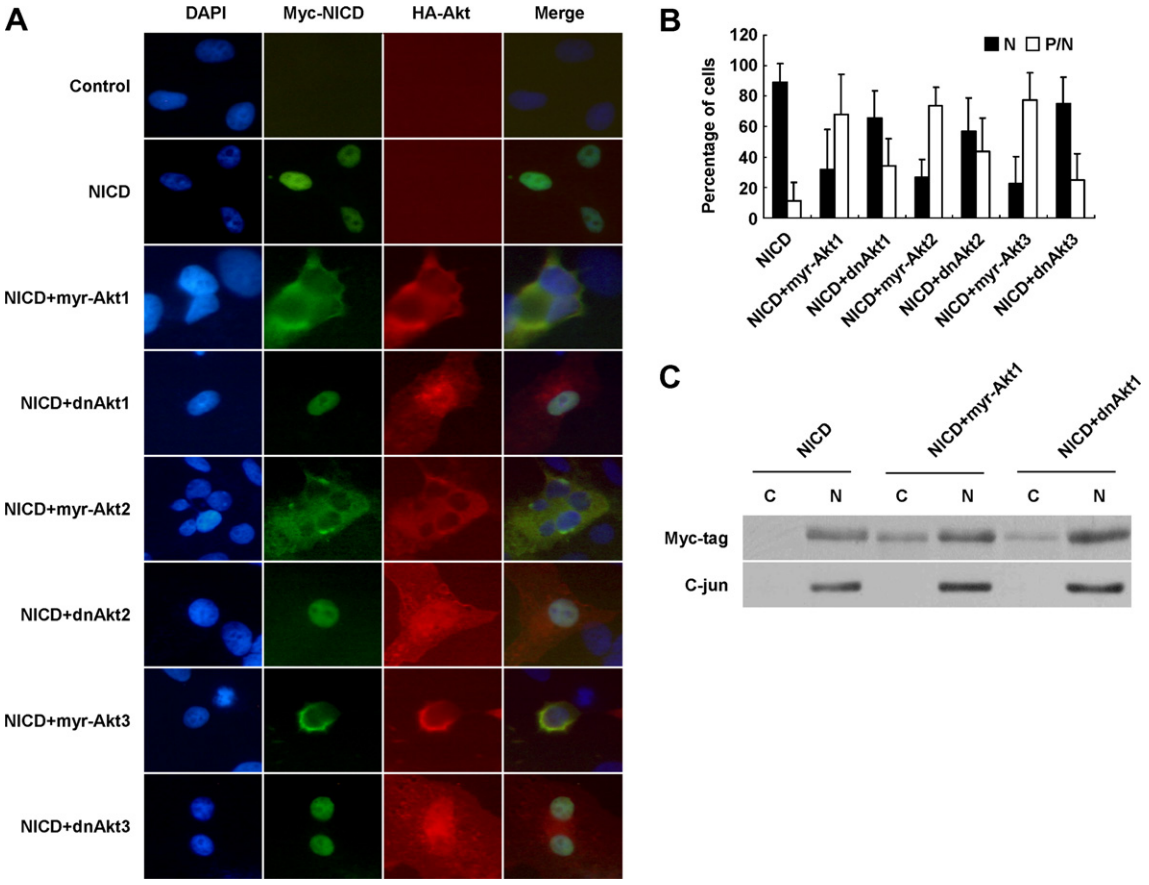

Fig. 4. Active Akt isoforms inhibit proper nuclear localization of NICD. (A) Cos7 cells were transfected with myc-NICD and Akt/dnAkt, harvested after 48 h, then immunostained using myc and HA antibodies (Section 2). All three Akt isoforms effectively inhibited proper nuclear localization of NICD. (B) The graph shows the percentage of cells where NICD was localized the in nucleus (N, black bars) or perinuclear cytoplasm (P/N, white bars). More than 100 myc-positive cells were counted per sample in independent experiments performed triplicate. (C) Cos7 cells were transfected with myc-NICD and myr-Akt1, harvested after 48 h and fractionated into cytoplasmic (C) and nuclear (N) fraction (Section 2). Each fraction was subjected to Western blot analyses.

nuclear localization of NICD in Cos7 cells. Furthermore, co-expression of dnAkt isoforms rescued the mislocalization of NICD in the perinuclear cytoplasm. The representative immunostaining pictures are shown in Fig. 4A and quantification of the results are indicated in Fig. 4B. In the absence of myr-Akt, around 90% of exogenous NICD was found in the nucleus. However, upon co-expression of myr-Akt isoforms, NICD was shown to be mislocalized to the perinuclear cytoplasm in more than 60% of myc-positive cells. Interestingly, myr-Akt isoforms were found to be colocalized with NICD predominantly in the cytoplasm while dnAkt isoforms were found both in the nucleus and cytoplasm. Fractionation of cells into cytosolic and nuclear fractions essentially produced similar results (Fig. 4C), indicating that Akt kinase activity may play a major role for mislocalization of NICD. The results may indicate that Akt causes down-regulation of NICD transcriptional activity by interfering with proper nuclear localization of NICD and blocking the association of NICD with DNA. The experiments using 293T cells showed identical results (data not shown).

3.5. The regulation of NICD transcriptional activity might be mediated by phosphorylation of NICD by Akt

Having shown that Akt kinase activity is responsible for mislocalization of NICD in the perinuclear cytoplasm, we investigated whether Akt can directly phosphorylate NICD. In in vitro kinase assays shown in Fig. 5A, myr-Akt1 effectively phosphorylated GST-NICD in vitro, suggesting that NICD-dependent transcriptional activity may be regulated through phosphorylation of NICD by Akt. Glycogen synthase kinase 3β (GSK3β), which was reported to phosphorylate NICD in vitro and in vivo [31], could also phosphorylate NICD. In vivo [^{32}P]-orthophosphate labeling experiments also indicated that Akt can cause phosphorylation of NICD in vivo. As quantified in Fig. 5B (right panel) co-expression of myr-Akt1 increased ^{32}P-phosphate incorporation into NICD more than 2-fold, while dnAkt1 did not. These results may suggest that Akt can modulate the subcellular localization of NICD and effect transcriptional activity through direct phosphorylation.

4. Discussion

We have investigated the effect of Akt isoforms on NICD-dependent transcription. All three myr-Akt isoforms effectively inhibited NICD-dependent transcriptional activity, but not dnAkts, indicating that kinase activity of Akt regulates NICD transcriptional function (Fig. 2). We have also shown that co-

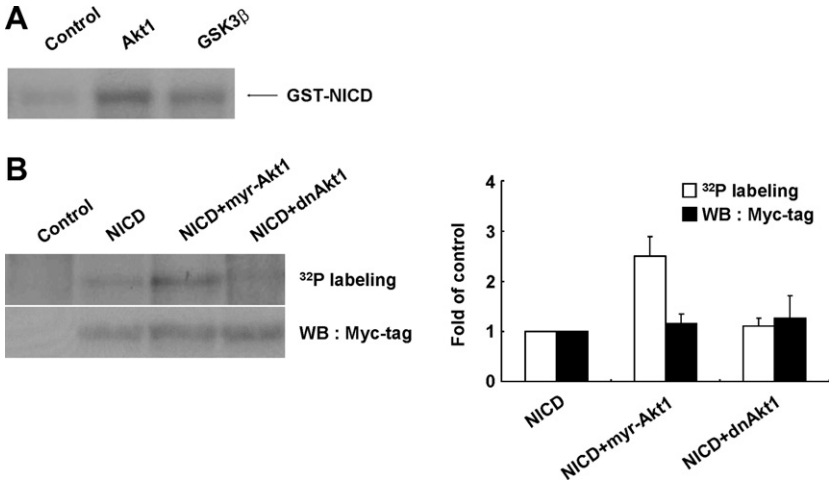

Fig. 5. Akt phosphorylates NICD both in vitro and in vivo. (A). 293T cells were transfected with 10 μg of myr-Akt1 or HA-GSK3β, harvested after 48 h and immunoprecipitated with HA antibody. Immunocomplex kinase assays were performed as described in Section 2. The [^{32}P]-labeled samples were subjected to SDS–PAGE and subsequent autoradiography. (B) 293T cells were transfected with myc-NICD and Akt1 or pcDNA, labeled with [^{32}P]-orthophosphate in vivo. The cells were harvested 12 h after radioisotope-labeling. The whole cell lysates were immunoprecipitated using myc antibody and [^{32}P]-labeled myc-NICD was analyzed by SDS–PAGE and subsequent autoradiography.

expression of myr-Akt isoforms cause cytoplasmic mislocalization of NICD (Fig. 4). Interestingly, dnAkt isoform co-expressing cells showed moderately increased cytoplasmic misolocalization as compared to control cells expressing NICD alone, but the percentage of cells showing cytoplasmic mislocalization in dnAkt co-expressing cells is still lower than myr-Akt co-expressing cells (Fig. 4). This might suggest a kinase activity-independent effect of Akt on NICD translocation. However, since dnAkt isoform co-expressing cells showed reporter activities almost comparable to cells expressing NICD alone (Fig. 2A), and Akt could phosphorylate NICD both in vitro and cause hyperphosphorylation in vivo, we conclude that phosphorylation-dependent cytosolic mislocalization of NICD may be the major mechanism by which Akt causes down-regulation of NICD transcriptional activity. Indeed, Akt has been shown to modulate subcellular distribution of cell cycle regulatory proteins like p21 [32] and p27 [24] and FOXO transcription factors [33,34] by direct phosphorylation. Nuclear exclusion of FOXO transcription factors by Akt-mediated phosphorylation was reported to be the major mechanism by which Akt down-regulates FOXO-dependent transcription.

Though we showed here that Akt could phosphorylate NICD (Fig. 5) and the kinase activity of Akt isoforms are required for down-regulation of NICD transcriptional activity (Fig. 2), we can not completely rule out the possibility that Akt-mediated phosphorylation of NICD and Akt-induced NICD transcriptional repression are not directly coupled. While preparing this manuscript, Baek et al. [35] reported that Zinc-induced down-regulation of NICD transcriptional activity is associated with cytoplasmic retention of NICD via PI3K-Akt signaling pathway. Their report is in agreement with our findings here that Akt activity is responsible for down-regulation of NICD transcriptional activity by nuclear exclusion of NICD. It was also demonstrated using fractionated *Drosophila* embryos and cultured mammalian cells that phosphorylated NICD localized in the nuclear fraction [36,37].

GSK3β acts downstream of Akt, and is negatively regulated by Akt-mediated phosphorylation [38]. Foltz et al. [31] showed that GSK3β can phosphorylate NICD and protect NICD from proteasome-mediated degradation, indicating that GSK3β could up-regulate NICD transcriptional activity by controlling NICD protein stability. We also showed that GSK3β could phosphorylate NICD (Fig. 5A) and inhibition of GSK3β activity by co-expression of dnGSK3β or inhibition by LiCl treatment resulted in a moderate decrease of NICD transcriptional activity (Supplementary Fig. S1). This result may suggest that the inhibitory effect of Akt on NICD transcriptional activity may in part be mediated by inhibition of the downstream kinase GSK3β. However, the effect of Akt on cytoplasmic mislocalization of NICD and subsequent inhibition of transcriptional activity seemed to be directly mediated by Akt, since co-expression of dnGSK3β did not modulate subcellular distribution of NICD (Supplementary Fig. S2). We also have shown that treatment with LiCl did not significantly further reduce NICD transcriptional activity in Akt expressing cells (Fig. S3), indicating that Akt could effectively inhibit the LiCl-sensitive endogenous GSK3β pool. Moreover, GSK3β was reported to modulate Notch signaling by protecting degradation of NICD in proteasomes [31], and therefore it is not likely that inhibition of NICD transcriptional activity is mediated through GSK3β. However, the effect of GSK3β on Notch transcriptional activity remains controversial since another other report [39] indicates that GSK3β can phosphorylate Notch2 and inhibit the transcriptional activation of Notch target genes.

In summary, we have demonstrated here that myr-Akt isoforms cause inhibition of NICD transcriptional activity mainly by modulation of intracellular distribution, and that phosphorylation of NICD by Akt might the main mechanism responsible for this inhibitory effect.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.04.024.

References

[1] Artavanis-Tsakonas, S., Rand, M.D. and Lake, R.J. (1999) Notch signaling: cell fate control and signal integration in development. Science 284, 770–776.

[2] Blair, S.S. (2000) Notch signaling: fringe really is a glycosyltransferase. Curr. Biol. 10, R608–R612.

[3] Blaumueller, C.M., Qi, H., Zagouras, P. and Artavanis-Tsakonas, S. (1997) Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. Cell 90, 281–291.

[4] Fortini, M.E. and Artavanis-Tsakonas, S. (1994) The suppressor of hairless protein participates in Notch receptor signaling. Cell 79, 273–282.

[5] Jouve, C., Palmeirim, I., Henrique, D., Beckers, J., Gossler, A., Ish-Horowicz, D. and Pourquie, O. (2000) Notch signalling is required for cyclic expression of the hairy-like gene HES1 in the presomitic mesoderm. Development 127, 1421–1429.

[6] Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F. and Kageyama, R. (1999) Hes1 and Hes5 as Notch effectors in mammalian neuronal differentiation. Embo J. 18, 2196–2207.

[7] Hsieh, J.J. and Hayward, S.D. (1995) Masking of the CBF1/RBPJ kappa transcriptional repression domain by Epstein-Barr virus EBNA2. Science 268, 560–563.

[8] Kao, H.Y., Ordentlich, P., Koyano-Nakagawa, N., Tang, Z., Downes, M., Kintner, C.R., Evans, R.M. and Kadesch, T. (1998) A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. Genes Dev. 12, 2269–2277.

[9] Kurooka, H. and Honjo, T. (2000) Functional interaction between the mouse Notch1 intracellular region and histone acetyltransferases PCAF and GCN5. J. Biol. Chem. 275, 17211–17220.

[10] Oswald, F., Tauber, B., Dobner, T., Bourteele, S., Kostezka, U., Adler, G., Liptay, S. and Schmid, R.M. (2001) p300 acts as a transcriptional coactivator for mammalian Notch-1. Mol. Cell Biol. 21, 7761–7774.

[11] Fryer, C.J., Lamar, E., Turbachova, I., Kintner, C. and Jones, K.A. (2002) Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. Genes Dev. 16, 1397–1411.

[12] Manning, B.D. and Cantley, L.C. (2007) AKT/PKB signaling: navigating downstream. Cell 129, 1261–1274.

[13] Testa, J.R. and Tsichlis, P.N. (2005) AKT signaling in normal and malignant cells. Oncogene 24, 7391–7393.

[14] Staal, S.P. (1987) Molecular cloning of the Akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci USA 84, 5034–5037.

[15] Cheng, J.Q., Ruggeri, B., Klein, W.M., Sonoda, G., Altomare, D.A., Watson, D.K. and Testa, J.R. (1996) Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. Proc Natl Acad Sci USA 93, 3636–3641.

[16] Brodbeck, D., Cron, P. and Hemmings, B.A. (1999) A human protein kinase B gamma with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain. J. Biol. Chem. 274, 9133–9136.

[17] Coffer, P.J. and Woodgett, J.R. (1991) Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. Eur. J. Biochem. 201, 475–481.

[18] Musacchio, A., Gibson, T., Rice, P., Thompson, J. and Saraste, M. (1993) The PH domain: a common piece in the structural patchwork of signalling proteins. Trends Biochem. Sci. 18, 343–348.

[19] Fitzgerald, K., Harrington, A. and Leder, P. (2000) Ras pathway signals are required for Notch-mediated oncogenesis. Oncogene 19, 4191–4198.

[20] Calzavara, E., Chiaramonte, R., Cesana, D., Basile, A., Sherbet, G.V. and Comi, P. (2007) Reciprocal regulation of Notch and PI3K/Akt signalling in T-ALL cells in vitro. J. Cell Biochem..

[21] Sade, H., Krishna, S. and Sarin, A. (2004) The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells. J. Biol. Chem. 279, 2937–2944.

[22] Palomero, T. et al. (2007) Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nat. Med. 13, 1203–1210.

[23] Shin, I., Edl, J., Biswas, S., Lin, P.C., Mernaugh, R. and Arteaga, C.L. (2005) Proapoptotic activity of cell-permeable anti-Akt single-chain antibodies. Cancer Res. 65, 2815–2824.

[24] Shin, I., Rotty, J., Wu, F.Y. and Arteaga, C.L. (2005) Phosphorylation of p27Kip1 at Thr-157 interferes with its association with importing alpha during G1 and prevents nuclear re-entry. J. Biol. Chem. 280, 6055–6063.

[25] Graham, F.L. and van der Eb, A.J. (1973) Transformation of rat cells by DNA of human adenovirus 5. Virology 54, 536–539.

[26] Heinzel, T. et al. (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. Nature 387, 43–48.

[27] Alland, L., Muhle, R., Hou Jr., H., Potes, J., Chin, L., Schreiber-Agus, N. and DePinho, R.A. (1997) Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. Nature 387, 49–55.

[28] Schweisguth, F. and Posakony, J.W. (1992) Suppressor of Hairless, the Drosophila homolog of the mouse recombination signal-binding protein gene, controls sensory organ cell fates. Cell 69, 1199–1212.

[29] Christensen, S., Kodoyianni, V., Bosenberg, M., Friedman, L. and Kimble, J. (1996) lag-1, a gene required for lin-12 and glp-1 signaling in *Caenorhabditis elegans,* is homologous to human CBF1 and Drosophila Su(H). Development 122, 1373–1383.

[30] Dou, S., Zeng, X., Cortes, P., Erdjument-Bromage, H., Tempst, P., Honjo, T. and Vales, L.D. (1994) The recombination signal sequence-binding protein RBP-2N functions as a transcriptional repressor. Mol. Cell Biol. 14, 3310–3319.

[31] Foltz, D.R., Santiago, M.C., Berechid, B.E. and Nye, J.S. (2002) Glycogen synthase kinase-3beta modulates Notch signaling and stability. Curr. Biol. 12, 1006–1011.

[32] Zhou, B.P., Liao, Y., Xia, W., Spohn, B., Lee, M.H. and Hung, M.C. (2001) Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat. Cell Biol. 3, 245–252.

[33] Brunet, A. et al. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96, 857–868.

[34] Guo, S., Rena, G., Cichy, S., He, X., Cohen, P. and Unterman, T. (1999) Phosphorylation of serine 256 by protein kinase B disrupts transactivation by FKHR and mediates effects of insulin on insulin-like growth factor-binding protein-1 promoter activity through a conserved insulin response sequence. J. Biol. Chem. 274, 17184–17192.

[35] Baek, S.H. et al. (2007) Zinc-induced downregulation of Notch signaling is associated with cytoplasmic retention of Notch1-IC and RBP-Jk via PI3k-Akt signaling pathway. Cancer Lett. 255, 117–126.

[36] Kidd, S., Lieber, T. and Young, M.W. (1998) Ligand-induced cleavage and regulation of nuclear entry of Notch in Drosophila melanogaster embryos. Genes Dev. 12, 3728–3740.

[37] Shimizu, K. et al. (2000) Binding of Delta1, Jagged1, and Jagged2 to Notch2 rapidly induces cleavage, nuclear translocation, and hyperphosphorylation of Notch2. Mol. Cell Biol. 20, 6913–6922.

[38] Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M. and Hemmings, B.A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785–789.

[39] Espinosa, L., Ingles-Esteve, J., Aguilera, C. and Bigas, A. (2003) Phosphorylation by glycogen synthase kinase-3 beta down-regulates Notch activity, a link for Notch and Wnt pathways. J. Biol. Chem. 278, 32227–32235.