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Glycogen synthase kinase 3-β phosphorylates novel S/T-P-S/T domains in Notch1 intracellular domain and induces its nuclear localization

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ABSTRACT

We identified two S/T-P-S/T domains (2122–2124, 2126–2128) inducing Notch intracellular domain (NICD) nuclear localization. The GFP-NICD (1963–2145) fusion protein deletion mutant without classical NLS was localized in the nucleus like the full length GFP-NICD. However, quadruple substitution mutant (T2122A T2124A S2126A T2128A) showed increased cytoplasmic localization. GSK-3β enhanced nuclear localization and transcriptional activity of WT NICD but not of quadruple substitution mutant. *In vitro* kinase assays revealed that GSK-3β phosphorylated S and T residues in NICD S/T-P-S/T domains. These results suggest that the novel S/T-P-S/T domain, phosphorylated by GSK-3β is also involved in the nuclear localization of NICD as well as classical NLS.

1. Introduction

Notch proteins, ligand-activated single-pass transmembrane receptors are involved in cell fate determination and differentiation [1,2]. Notch signaling is initiated by cell–cell interaction leading to Notch cleavage by a γ-secretase, resulting in release of the Notch intracellular domain (NICD) [3,4]. The NICD translocates into the nucleus and binds the transcription factor CBF1 to transactivate target genes through recruitment of transcriptional coactivators such as histone acetyl transferases and CREB-binding protein/p300 [3,5,6]. Glycogen synthase kinase-3β (GSK-3β) was originally identified as an enzyme that regulates glycogen synthesis in response to insulin [7]. Previous studies showed that GSK-3β can phosphorylate NICD and up-regulate NICD transcriptional activity by controlling NICD protein stability [8,9].

The control of subcellular protein localization is a necessary requirement for the regulation of enzyme function in various biological processes including cell proliferation, transformation and tumorigenesis [10]. The translocation of macromolecules larger than 40 kDa into and out of the nucleus is an active, energy-dependent process that is mediated by specific sequence motifs, nuclear localization signal (NLS) and nuclear export signal (NES). However, these mechanisms do not always induce precise regulation of nuclear processes and mechanism(s) responsible for reversible NLS-independent translocation of proteins needs more experimental challenges [11,12]. A recent study has shown that in addition to classical NLS, phosphorylated S/T-P-S/T can also act as a general nuclear translocation signal (NTS) for NLS-lacking proteins that shuttle to the nucleus upon stimulation [13].

In this study, we identified two novel S/T-P-S/T domains (379TP381T, 383SP385T) that are also involved in the nuclear localization of NICD as well as classical NLS. We also demonstrated that GSK-3β kinase could up-regulate the transcriptional activity of NICD by phosphorylation the S/T-P-S/T domains.

2. Materials and methods

2.1. Plasmids and vectors

Mouse NICD (corresponds to amino acid residues 1744–2184 of Notch1), and deletion mutants NICD (1744–1987, 1963–2184 and 1963–2145) were cloned into the pEGFP-C1 (Clontech). Various point mutations were introduced by site-directed mutagenesis and confirmed by DNA sequencing. HA-tagged wild type GSK-3β (HA-GSK-3β) and kinase-dead dominant negative GSK-3β (HA-dnGSK-3β) 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, at 37 °C under 5% CO_2 in a humidified incubator. The cells were transfected by calcium phosphate in HEPES buffered saline as described previously [9].

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2.3. Reporter assays

293T cells (12-well plates) were transfected with 1 μg of total DNA containing 500 ng of reporter constructs 6 × Notch response elements (6 × NRE-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. Subcellular fractionation and western blots

Cell lysates were prepared as described [14] and cleared by centrifugation at 12,000×*g* for 15 min. Sub-cellular fractionation was performed as previously described [9]. The samples were separated by SDS–PAGE and transferred onto nitrocellulose membranes. The membranes were then incubated with GFP antibody (Clontech), or actin antibody (Santa Cruz). The antigen-antibody complexes were visualized by chemiluminescence (iNtRON).

2.5. Immunostaining

Cell fixation and immunostaining were performed as described previously [14,15]. Briefly, cells were washed with PBS, fixed with 3.7% 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. Anti-HA was used at 1:200 dilution in 1% skim milk. The cells were washed three times with PBS then incubated with anti-rabbit IgG-Cy3 (Zymed) in 1% skim milk in PBS. Next, the cells were washed again three times for 10 min with PBS. Nuclei were stained with Hoechst 33258. The coverslips were mounted on slide glasses and observed by fluorescence microscopy (Olympus BX50).

2.6. Expression and purification of GST-tagged proteins

The GST-NICD WT, GST-NICD T2122A T2124A, GST-NICD S2126A T2128A and GST-NICD T2122 T2124A S2126A T2128A expression plasmids were transformed into *Escherichia coli* BL21 (DE3) *pLys*S. Transformed cells were inoculated into 100 ml of LB medium and incubated at 37 °C with vigorous shaking until an A_{600} value of 0.6 was reached. Expression of recombinant protein was induced by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside. After 4 h, the cells were lysed with a sonicator and centrifuged to remove cell debris. The supernatants were loaded onto a Glutathione Sepharose 4B column (MicroSpin GST Purification Module; GE Healthcare) and GST-tagged proteins were eluted in elution buffer (50 mM Tris–HCl pH 9.5, 10 mM reduced glutathione). An aliquot of each eluate was analyzed using SDS–PAGE and the fractions containing the protein were quickly frozen in small aliquots using liquid nitrogen.

2.7. GSK-3β in vitro kinase assay

293T cells seeded at the density of 1×10^6 cells in 100 mm dish were transfected with 10 μg of HA-GSK-3β and harvested at 48 h post-transfection. One milligram of whole cell lysates was immunoprecipitated with HA-antibody for 2 h and subsequently treated with protein A-Sepharose CL-4B. The immunocomplexes were incubated with 1.5 μg of pre-purified GST-NICD 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.

3. Results

3.1. Identification of potential S/T-P-S/T domain in NICD

It is well known that endogenous NICD is localized predominantly in the nucleus via NLS-dependent nuclear localization [16]. However, a recent study [13] showing that novel phosphorylated S/T-P-S/T domain could act as a general nuclear translocation signal (NTS) prompted us to investigate the possible involvement of a novel S/T-P-S/T domain in NICD nuclear localization. We searched for the domain in the NICD and identified two potential S/T-P-S/T motifs (2122TP2124T and 2126SP2128T) (Fig. 1A). To determine whether these S/T-P-S/T domains alone can direct nuclear localization, plasmids expressing the series of deletion mutants of NICD fused to the C-terminus of GFP were constructed (Fig. 1A). We transfected 293T cells with GFP-NICD (1744–2184), GFP-NICD (1744–1987), GFP-NICD (1963–2184) (1744–2184, 1744–1987 and 1963–2184 contained NLS) or GFP-NICD (1963–2145) and performed fluorescence microscopy to observe subcellular localization of the fusion proteins. As shown in Fig. 1B and C, GFP-NICD (1963–2145) fusion protein deletion mutant which do not contain any classical NLS could also be localized in the nucleus like GFP-NICD (1744–2184) with four NLS, GFP-NICD (1744–1987) with two NLS and GFP-NICD (1963–2184) with two NLS. Western blot analysis of the nuclear and cytoplasmic fractions of 293T cells transfected with the same set of constructs further supported the results observed by fluorescence microscopy (Fig. 1D). Thus, the S/T-P-S/T domain of NICD alone was sufficient to induce nuclear localization of the fusion protein. Similar results were also obtained in COS-7 cells (data not shown).

3.2. S and T residues are essential for nuclear localization of NICD via S/T-P-S/T domain dependent mechanism

To investigate whether the S and/or T residues in S/T-P-S/T domain is responsible for nuclear localization of NICD via S/T-P-S/T domain-dependent mechanism, point mutants of GFP-NICD within 1963–2145 in which two or four S/T residues were replaced with alanine (T2122A T2124A, S2126A T2128A, T2122A T2124A S2126A T2128A) were generated. As shown in Fig. 2A and B, GFP-NICD (1963–2145) WT, GFP-NICD (1963–2145) T2122A T2124A and GFP-NICD (1963–214) S2126A T2128A were predominantly localized in the nucleus. In contrast, GFP-NICD (1963–2145) T2122A T2124A S2126A T2128A was localized in both nucleus and cytoplasm. Western blot analysis of the nuclear and cytoplasmic fractions of 293T cells transfected with the same set of constructs further supported the results observed by fluorescence microscopy (Fig. 2C). Collectively, these results suggest that phosphorylations of S/T-P-S/T domains might be crucial for the nuclear transport of NICD via S/T-P-S/T domain-dependent mechanism.

3.3. GSK-3β could increase the nuclear localization of GFP-NICD (1963–2145) and GFP-NICD full length

Recently, Jin et al. [17] reported that GSK-3β can down-regulate the expression of NICD and showed that T1851, T2122 and T2124 of NICD are critical for its process. This report led us to test whether GSK-3β activity could regulate the nuclear localization of NICD through the interaction with S/T-P-S/T domain. 293T cells were co-transfected with GFP-NICD (1963–2145) WT and HA-GSK-3β-WT/kinase dead (KD), or GFP-NICD (1963–2145) T2122A T2124A S2126A T2128A and HA-GSK-3β. At 48 h post-transfection, indirect immunostaining of HA was used to detect the ectopically expressed GSK-3β. As illustrated in Fig. 3A and quantitated

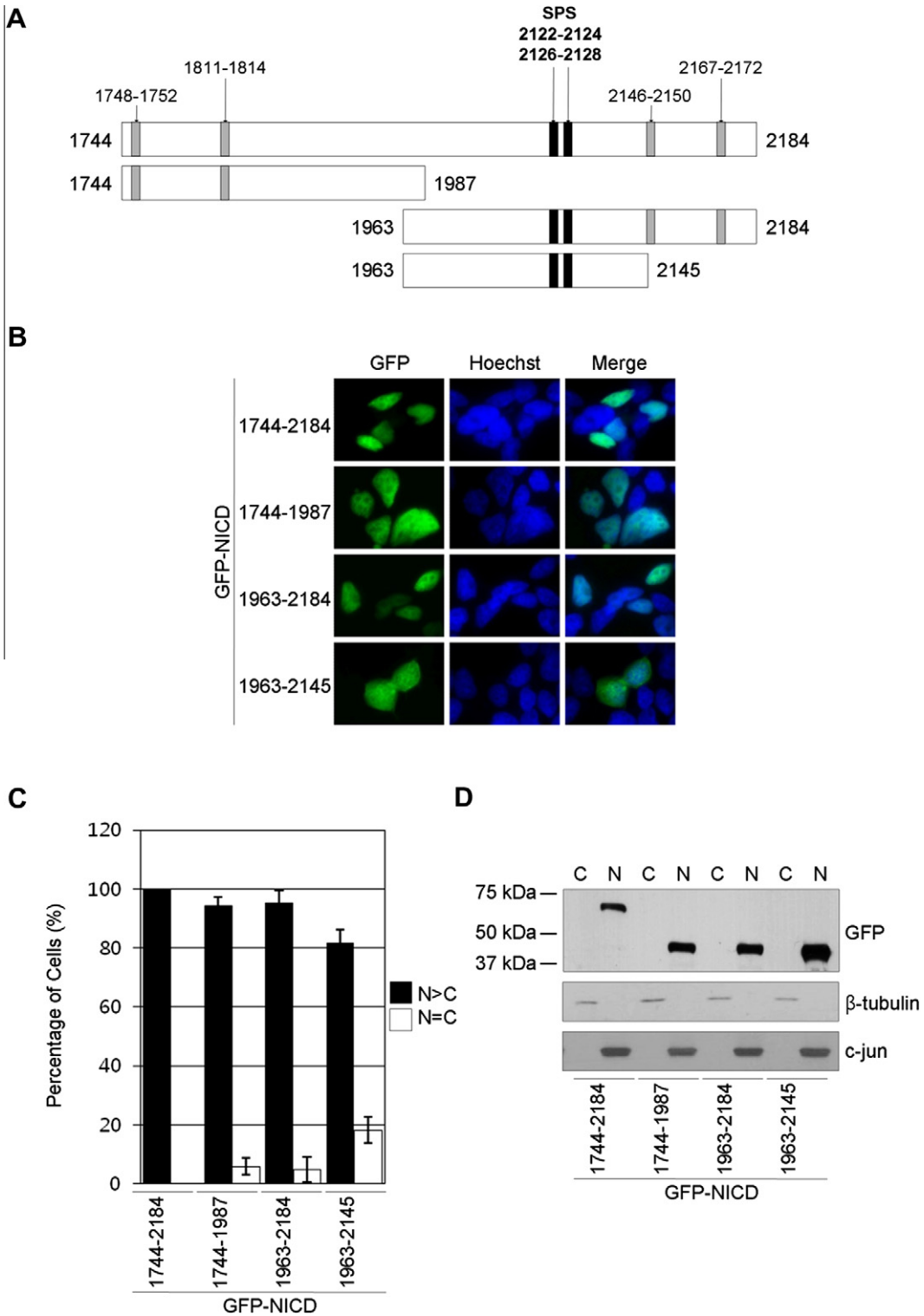

Fig. 1. Identification of S/T-P-S/T domain in the NICD. (A) Schematic representation of NICD, including the NLS and the S/T-P-S/T domains. (B) 293T cells were transfected with expression plasmids for GFP-NICD (1744–2184), GFP-NICD (1744–1987), GFP-NICD (1963–2184) or GFP-NICD (1963–2145). At 48 h post-transfection, the cells were fixed and their nuclei were stained with Hoechst dye. The GFP-fluorescence were then monitored using a fluorescence microscope. (C) Quantitation of the results in (B). The bar graphs show the mean values ± SD from at least three independent experiments. More than 50 cells from each slides were scored as N > C (predominantly nuclear localization of GFP-NICD) or N = C (both nuclear and cytoplasmic localization of GFP-NICD). (D) Western blot analysis of the nuclear (N) and cytoplasmic (C) fractions of 293T cells transfected with the GFP-NICD constructs. At 48 h post-transfection, the cell lysates were subjected to subcellular fractionation and subjected to western blot analyses.

in Fig. 3B, more than 90% of the cells co-transfected with GFP-NICD (1963–2145) WT and HA-GSK-3β-WT exhibited nuclear localization of ectopic NICD. In contrast, approximately 50% of cells co-transfected cells with GFP-NICD (1963–2145) WT and HA-GSK-3β-KD showed nuclear localization of ectopic NICD. The remaining 50% of the cells exhibited both cytosol and nuclear localization of ectopic NICD. In the cells co-transfected with GFP-NICD (1963–2145) T2122A T2124A S2126A T2128A and

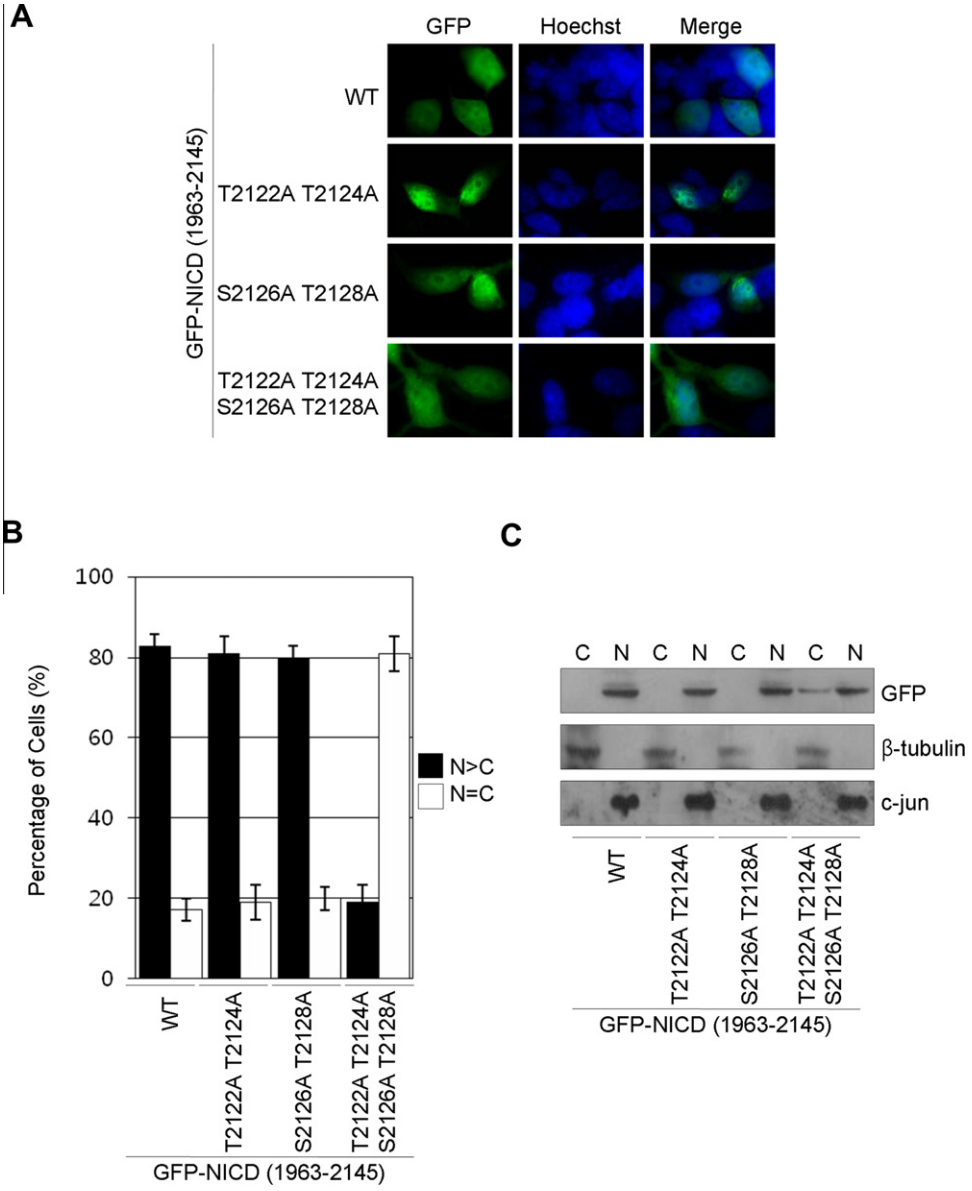

Fig. 2. S/T-P-S/T phosphorylation is essential for nuclear localization of NICD. (A) 293T cells were transfected with the constructs as indicated. At 48 h post-transfection, the cells were fixed and counter-stained with Hoechst. The cells were then observed using a fluorescence microscope. (B) Quantitation of the results in (A). The bar graphs show the mean values ± SD from at least three independent experiments. More than 50 cells from each slides were scored as N > C (predominantly nuclear localization of GFP-NICD) or N = C (both nuclear and cytoplasmic localization of GFP-NICD). (C) 293T cells were transfected with the plasmids as indicated, harvested after 48 h and fractionated into cytoplasmic (C) and nuclear (N) fractions. Each fraction was subjected to western blot analyses.

HA-GSK-3β-WT/KD, GFP fusion protein was mostly localized both in the cytoplasm and the nucleus. In contrast, the cells co-transfected with HA-GSK-3β-WT/KD and GFP-NICD (1744–2184) WT or T2122A T2124A S2126A T2128A containing classical NLS exhibited predominantly nuclear localization of GFP fusion proteins. These results suggest that GSK-3β can assist the nuclear localization of NICD possibly by phosphorylation of S and/or T residues in the 2122–2128 of NICD and classical NLS-dependent nuclear localization of NICD may occur in GSK-3β activity-independent manner. Western blot analyses of the nuclear and cytoplasmic fractions of 293T cells co-transfected GFP-NICD (1963–2145)/(1744–2184) WT and HA-GSK3β-WT/KD, or GFP-NICD (1963–2145)/(1744–2184) T2122A T2124A S2126A T2128A and HA-GSK3β-WT/KD further supported the results observed by fluorescence microscopy (Fig. 3C).

3.4. GSK-3β could up-regulate NICD transcriptional activity by the phosphorylation of S/T-P-S/T domain of NICD

Previous studies showed that GSK-3β can phosphorylate NICD and up-regulate NICD transcriptional activity by controlling NICD protein stability [8,9]. But the exact phosphorylation residues are still remained to be precisely mapped. To examine the effect of GSK-3β on transcriptional activities of NICD constructs, 293T cells were transfected with the 6 × NRE-luc reporter construct and GFP-NICD (1744–2184) WT, T2122A T2124A, S2126A T2128A or T2122A T2124A S2126A T2128A, and HA-GSK-3β-WT/KD and subjected to dual luciferase assays. Consistent with the previous reports, data in Fig. 4A revealed that GSK-3β could up-regulate NICD transcriptional activity except for the NICD (1744–2184) T2122A T2124A, S2126A T2128A in which all four S/T residues

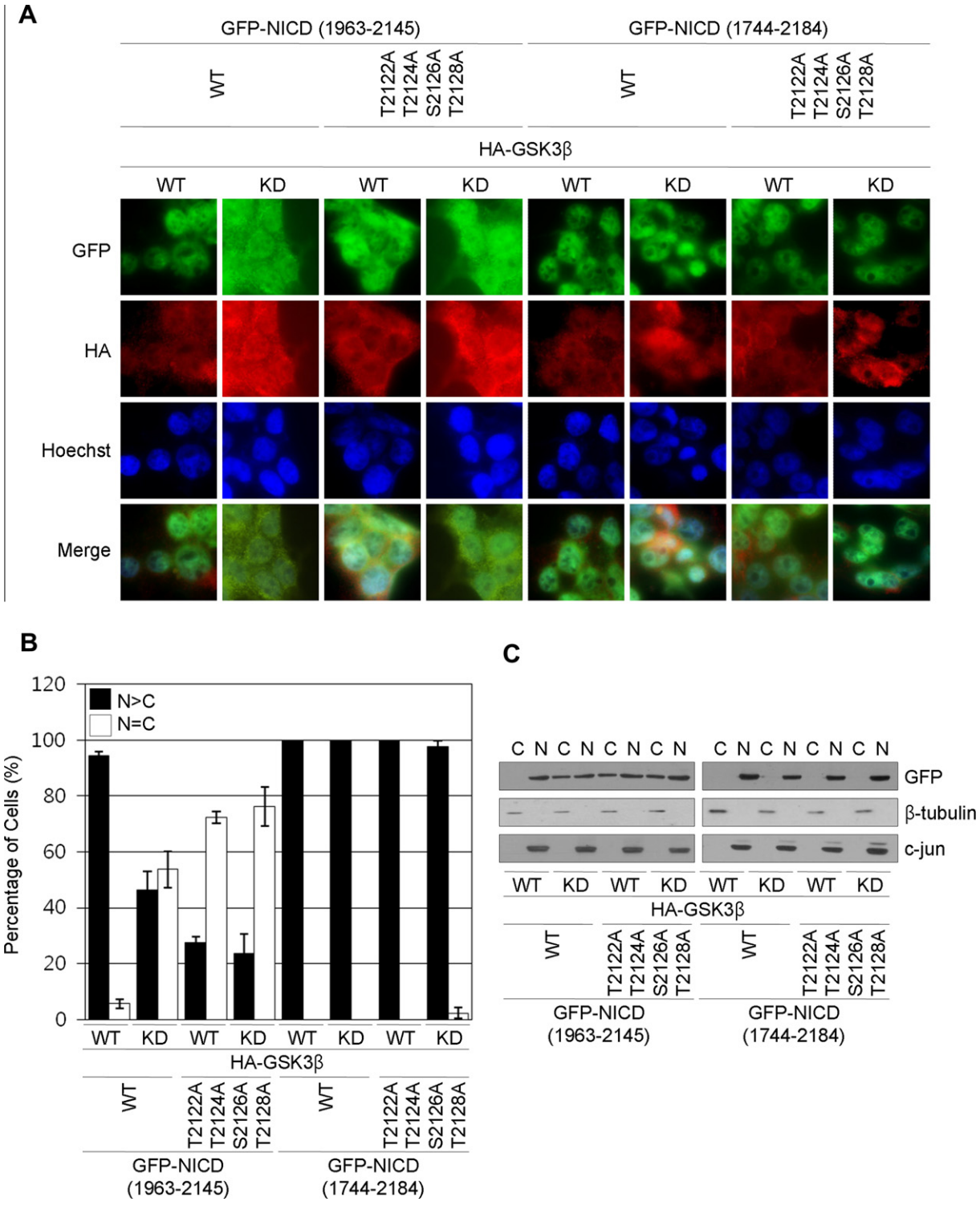

Fig. 3. The S/T-P-S/T domain-dependent nuclear localization of GFP-NICD (1963–2145) occurs in GSK-3β activity-dependent manner. (A) 293T cells were co-transfected with the plasmids as indicated. At 48 h post-transfection, the cells were fixed and stained with anti-HA to detect GSK-3β and Hoechst for nuclear counter-staining. NICD was detected by its GFP fluorescence. (B) Quantitation of the results in (A). The bar graphs show the mean values ± SD from at least three independent experiments. More than 50 cells from each slides were scored as N > C (predominantly nuclear localization of GFP-NICD) or N = C (both nuclear and cytoplasmic localization of GFP-NICD). (C) 293T cells were transfected with the plasmids as indicated, harvested after 48 h and fractionated into cytoplasmic (C) and nuclear (N) fractions. Each fraction was subjected to western blot analyses.

were replaced with alanines (Fig. 4A, 1st bar to 5th bar vs. 6th bar to 10th bar). It was also revealed that GSK-3β kinase activity is essential for the transcriptional activity regulation since HA-GSK-3β-KD rather decreased NICD transcriptional activities (Fig. 4A, 1st bar to 5th bar vs. Fig. 4A 11th bar to 15th bar). We further confirmed the requirement of kinase activity of GSK-3β by using LiCl, a specific GSK-3β inhibitor [18]. The LiCl treated cells exhibited much lower NICD transcriptional activity than control cells (Fig. 4A, 1st bar to 5th bar vs. Fig. 4A 16th bar to 20th bar). These results may suggest that GSK-3β increased the transcription

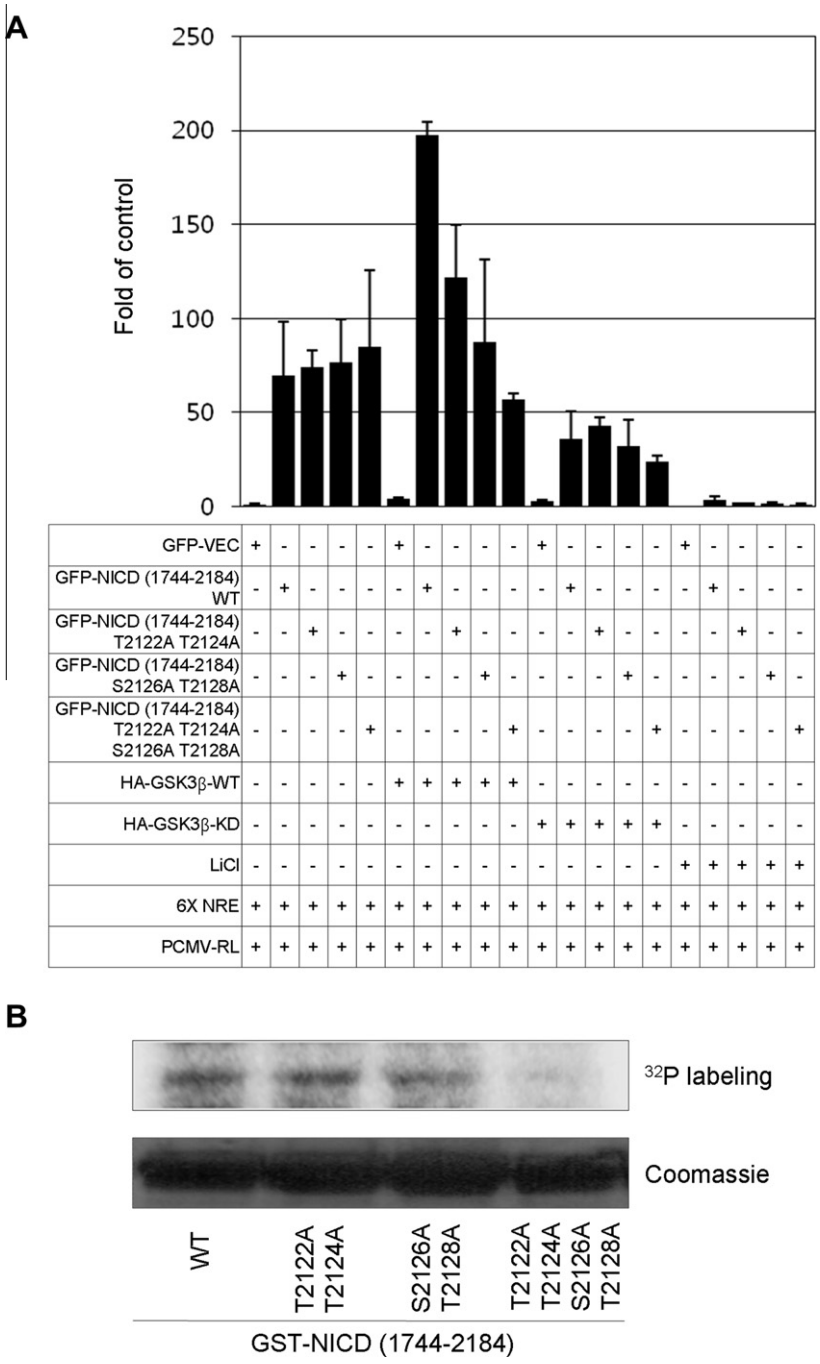

Fig. 4. GSK-3β could up-regulate the NICD transcriptional activity by through phosphorylation of S/T-P-S/T domain of NICD. (A) The 293T cells were co-transfected with the indicated plasmids. The cells were harvested at 48 h post-transfection and subjected to dual luciferase assays. LiCl was treated to the cells at the concentration of 20 mM for 24 h. (B) Lysate from 293T cells transfected with HA-GSK-3β was immunoprecipitated with anti-HA. The immunoprecipitates were incubated with purified GST-tagged NICD WT, NICD T2122A T2124A, NICD S2126A T2128A and NICD T2122A T2124A S2126A T2128A and ^{32}P-γATP. Reaction products were subjected to SDS–PAGE and subsequent autoradiography.

activity of NICD possibly through phosphorylation on S/T residues on the S/T-P-S/T domain of NICD and its subsequent nuclear translocation.

Having confirmed that intact S and T residues on S/T-P-S/T domain were required for nuclear localization of GFP-NICD (1963–2145) (Fig. 2A and B) and GSK-3β-mediated up-regulation of NICD transcriptional activity (Fig. 4A), we investigated whether GSK-3β could directly phosphorylate the S and T residues on S/T-P-S/T domain of NICD. *In vitro* kinase assays shown in Fig. 4B indicates that GSK-3β effectively phosphorylated GST-NICD (1744–2184) WT, GFP-NICD (1744–2184) T2122A T2124A and GFP-NICD (1744–2184) S2126A T2128A *in vitro*, but not GST-NICD (1744–2184) T2122A T2124A S2126A T2128A. These results suggest that NICD transcriptional activity as well as nuclear localization of NICD via S/T-P-S/T domain dependent mechanism are regulated through phosphorylation of T2122, T2124, S2126 and/or T2128 residues on S/T-P-S/T domain of NICD.

4. Discussion

Trafficking between cytoplasm and the nucleus is crucial for the signaling process: events as diverse as Notch-mediated gene

expression, proliferation, all require the entrance of the activated NICD into the nucleus [19,20]. In addition to canonical NLS, we report new novel S/T-P-S/T domains mediating nuclear localization of NICD in a GSK-3β phosphorylation-dependent manner. Nuclear import of signaling molecules like extracellular signal-regulated kinase, mitogen-activated protein kinase, Smad3 and RNase III enzyme Drosha are regulated via S/T-P-S/T domain phosphorylation-dependent fashion [13,21]. It was suggested that the existence of two acidic residues next to P also promotes nuclear localization of target molecules [13]. The association of phosphorylated S/T-P-S/T domain with importin 7 was shown to facilitate nuclear trafficking of cargo proteins [13]. Although the importance of phosphorylated S/T-P-S/T domain in stimulation-dependent nuclear localization of signaling molecules has been addressed, the kinase responsible for the phosphorylation of S/T-P-S/T domain has not been clearly elucidated.

Glycogen synthase kinase 3 beta (GSK-3β) is known as a major component of Wnt signaling pathway [22,23]. GSK-3β can negatively regulate various signaling proteins involved in cell proliferation and survival, such as β-catenin, cyclin D1, c-jun and c-myc [24]. It was reported that GSK-3β could phosphorylate and up-regulate NICD protein level [8]. However, the exact phosphorylation site on Notch1 by GSK-3β has not been clearly indicated. According to previous reports, GSK-3β consensus phosphorylation site was identified as (S/T)XXX(S/T)PO_4 or SPXXS in which first S or T is phosphorylated [24,25]. Based on these, we presume that T2122 or T2124 might be the best candidates for GSK-3β phosphorylations residues in NICD. But we also have evidence that S2126 and T2128 could also be phosphorylated by GSK-3β since GST-NICD T2122A T2124A could also be efficiently phosphorylated as GST-NICD or GST-NICD S2126A T2128A. It is unlikely that all four residues are simultaneously phosphorylated by GSK-3β since they are too close to each other hence phosphorylation on one of the residues may not preferably lead to another phosphorylation on adjacent residues due to the repulsion between negatively charged phosphate groups.

Effect of GSK-3β on Notch transcriptional activity and protein levels has been a matter of extensive debate. Jin et al. reported that GSK-3αβ can decrease Notch1 protein level and its transcriptional activity [17]. It was also reported that GSK-3β phosphorylates and down-regulates transcriptional activity of Notch2 [25]. Conversely, GSK-3β mediated phosphorylation on Notch1 could stabilize and activate Notch1 transcriptional activity via inhibition of proteasomal degradation of Notch1 [8]. We have also previously reported that Akt and protein kinase Cδ down-regulates Notch1 transcriptional activity while up-regulating its protein level [9,26]. We showed that nuclear accumulated NICD may not efficiently associate with its target gene promoter by chromatin immunoprecipitation assays [26]. Based on our data herein and previous findings, we speculate that nuclear localization and accumulation of NICD is not sufficient to increase its transcriptional activity and phosphorylation on S/T-P-S/T domains by GSK-3β is required for NICD transcriptional activity as well as its nuclear targeting.

Acknowledgments

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