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NF-κB p65 represses β-catenin-activated transcription of cyclin D1

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ABSTRACT

Signaling crosstalk between the β-catenin and NF-κB pathways represents a functional network. To test whether the crosstalk also occurs on their common target genes, the cyclin D1 promoter was used as a model because it contains binding sites for both proteins. β-Catenin activated transcription from the cyclin D1 promoter, while co-expression of NF-κB p65 reduced β-catenin-induced transcription. Chromatin immunoprecipitation revealed lithium chloride-induced binding of β-catenin on one of the T-cell activating factor binding sites. More interestingly, β-catenin binding was greatly reduced by NF-κB p65, possibly by the protein–protein interaction between the two proteins. Such a dynamic and complex binding of β-catenin and NF-κB on promoters might contribute to the regulated expression of their target genes.

1. Introduction

Dynamic and intricate binding of transcription factors on specific regions of DNA is a way to integrate diverse signaling pathways and to express target genes accordingly. Among the numerous known transcription factors, β-catenin and nuclear factor-kappa B (NF-κB) are important proteins mediating the expression of large numbers of target genes for Wnt signaling and inflammatory pathways, respectively [1,2]. Thus, abnormal activations of these factors might lead to tumorigenesis [3,4] so functional cross-talk of these pathways are suggested in some cancers [5,6]. However, how signaling cross-talk of β-catenin and NF-κB contributes to the regulated expression of their target genes is not fully understood.

β-catenin is a multifunctional protein that plays critical roles in cell adhesion as well as in Wnt-activated transcription [4]. In normal cells, the level of β-catenin is tightly regulated by glycogen synthase kinase 3 beta (GSK3β). Phosphorylation of β-catenin by GSK3β leads to its degradation by the ubiquitin dependent proteasome. However, lithium chloride (LiCl) treatment inactivates GSK3β mimicking Wnt-activation, leading to β-catenin accumulation and translocation to the nucleus. Nuclear β-catenin interacts with the T-cell activating factor (TCF) family of proteins to activate the transcription of various target genes that contain the TCF binding promoter sequence [5]. In tumors, the uncontrolled activation of the β-catenin/TCF complex contributes to the transcription of neoplastic transformation by inducing cancer-related genes such as cyclin D1, c-myc and matrix metalloproteinase-7 [7].

NF-κB is an essential transcription factor for the expression of a large number of genes that play pivotal roles in inflammation, immunity, stress response and apoptosis [8]. In the canonical pathway, dimers composed of p65 and p50 subunits are sequestered in the cytoplasm by an inhibitory protein, IκBα. Upon the appropriate signals, IκBα is phosphorylated and degraded. This allows NF-κB to enter the nucleus and bind to target genes containing a NF-κB binding promoter sequence [9]. Oncogenic and inflammation-induced NF-κB activation might contribute to leukemia and colorectal cancer [10,11]. Apart from such an induced NF-κB transcriptional activity, constitutive activity of NF-κB is also required for cell cycle progression in some tumors [11].

To understand how functional crosstalk of β-catenin and NF-κB signaling pathways converges on their target genes, the role of two proteins on the promoter of their common target gene has to be examined. Cyclin D1 might be a good model gene for that purpose, because its promoter contains many transcription factor binding sites including β-catenin/TCF and NF-κB binding sequences [12–14]. Cyclin D1 is a major regulator of cell cycle progression at the G1-to-S phase transition, which is a critical moment for tumor initiation. Even though β-catenin and NF-κB both have a tumor promoting function, how they are functionally related in the regulation of cyclin D1 expression remains to be established. The present results demonstrate that β-catenin and p65subunit bind to the cyclin D1 promoter, but only β-catenin is capable of activating transcription. More interestingly, p65 can repress β-catenin-induced cyclin D1 expression, probably by a dynamic and complex protein–protein interaction between the two proteins on the cyclin D1 promoter.

Abbreviations: NF-κB, nuclear factor-kappa B; TCF, T-cell activating factor.

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2. Materials and methods

2.1. Reagents and antibodies

LiCl, phosphatase inhibitor cocktail and protease inhibitor cocktail were purchased from Sigma–Aldrich (St. Louis, MO). Primers were synthesized by Bioneer (Daejeon, Republic of Korea). Anti-cyclin D1 antibody (#2261-1, EPR2241) was purchased from Epitomics (Burlingame, CA). Anti-p65 rabbit polyclonal antibody (sc-372, C-20) from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-catenin mouse monoclonal antibody (610,154) was purchased from BD Biosciences (Franklin Lakes, NJ). Anti-β-actin antibody (ac15) was purchased from Abcam (Cambridge, MA).

2.2. Plasmids

Expression clones for stable β-catenin (S37A) and p65/RelA were previously reported [15,16]. FLAG-tagged TCF-1 expression clone was generated by introducing mouse TCF-1 into the *Eco*RI site of the vector pcDNA3.1 and previously described [17]. Wild-type TCF reporter pGL3-OT and mutant pGL3-OF were kindly provided by Dr. Shivdasani (Dana-Farber Cancer Institute, Boston, MA). Wild-type cyclin D1 promoter reporter (−962 CD1/R) [18] was kindly donated by Drs. Tetsu and McCormick (University of California, San Francisco) [13].

2.3. Cell culture and transfection

Human embryonic kidney cell line HEK293T (American Type Culture Collection, Rockville, MD) was cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum. Cells were transfected with DNA using Lipofectamine (Invitrogen, Carlsbad, CA).

2.4. Real-time PCR analysis, Western blotting and co-immunoprecipitation

For real-time PCR, HEK293T cells were transfected with the p65/RelA or vector. After 24 h of incubation, cells were cultured under serum-free conditions for 3 h followed by treatment with 20 mM LiCl for 8 h. RNA was extracted with Trizol reagent (Invitrogen) and reverse transcribed. Samples were amplified with DyNAmo Flash master mix (Finnzyme, Espo, Finland) at 95 °C for 10 s, 62 °C for 10 s and 72 °C for 10 s for 40 cycles and were detected by a Step ONE™ real-time PCR kit (Applied Biosystems, Foster City, CA). The primers for cyclin D1 mRNA were CD1 E2 FOR (5′-CTACACCGACAACTCCATCC-3′) and CD1 A E5R REV (5′-ACTCCAGCAGGGCTTCGATC-3′). For Western blotting, cells were harvested and lysed with RIPA buffer. Whole cell extracts (30 μg) were prepared in 2× sample buffer, subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the proteins were transferred to polyvinylidene fluoride membranes. The membranes were incubated with blocking for 1 h at room temperature with appropriate antibodies for 16 h at 4 °C. The membranes were washed, incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody and detected by enhanced chemiluminescence. Anti-β-actin antibody was used for the controls. For co-immunoprecipitation, HEK293T cells were transfected with TCF-1-FLAG (5 μg), S37A-β-catenin, p65 or vector. After 24 h of incubation, cells were harvested with a cell lysis buffer comprised of 100 mM NaCl, 80 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5% NP-40. Prepared whole cell extracts (500 μg) were used for immunoprecipitation for 16–24 h with anti-β-catenin. Following immunoprecipitation, 30 μl protein A/G-Sepharose Bead (Roche, Basel, Switzerland) were added and incubated for 2 h at 4 °C. Sepharose beads were washed in lysis buffer five times. Immunoprecipitated protein was prepared in 2 × sample buffer and immunoblotting was performed sequentially.

2.5. Luciferase assay

For luciferase assays, cells were transiently transfected with Firefly luciferase reporter (50 ng) with the corresponding expression plasmids (100 ng) as indicated. Renilla luciferase reporter (10 ng) was co-transfected in each case to normalize transfection efficiency. Cells were harvested after 24 h, washed, and lysed in passive lysis buffer (Promega, Madison, WI). Luciferase activity was determined with a Dual luciferase assay system (Promega). Readings were made with a Glomax 20/20 luminolmeter (Promega), and relative firefly luciferase activities were obtained by normalizing with Renilla luciferase activity. Experiments were repeated at least three times and values are expressed as means and standard deviations.

2.6. Chromatin immunoprecipitation (ChIP)

Cells were cultured under serum-free conditions for 3 h followed by 20 mM LiCl treatment for 8 h. Cells were fixed in 1% formaldehyde in 125 mM glycine at room temperature to stop the cross-linking reaction. Cells were lysed with 600 μl ChIP RIPA buffer (50 mM Tris–HCl, pH 7.9, 140 mM NaCl, 1 mM EDTA, 0.5% Triton-X100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF and 20 μl protease

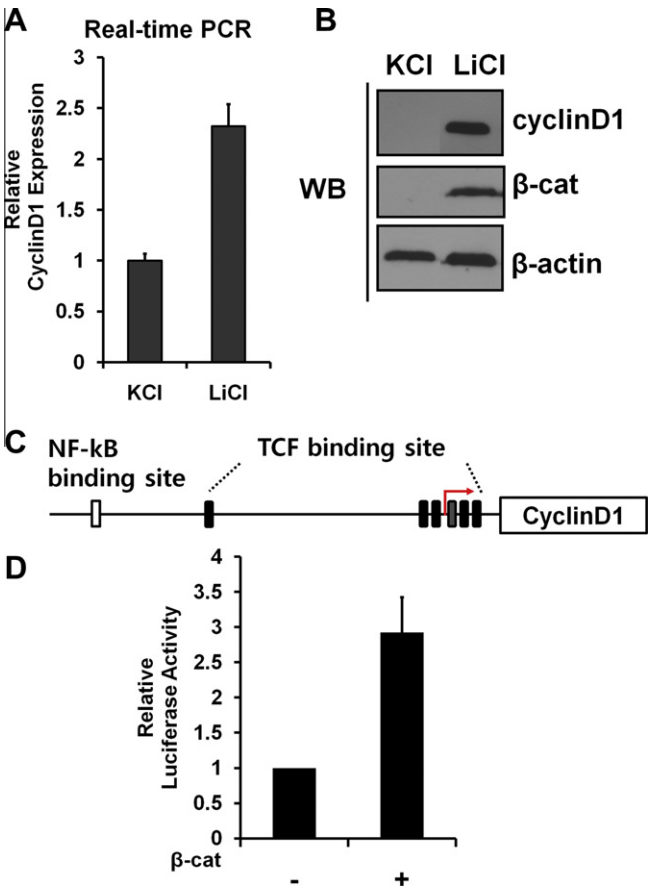

Fig. 1. Upregulation of cyclin D1 transcription by β-catenin in HEK293T cells. (A) Real-time PCR analysis of cyclin D1 mRNA. (B) Western blot analysis of cyclin D1 protein as well as β-catenin protein. β-Actin was also used as a control. (C) Schematic diagram of the −962 cyclin D1 promoter. The NF-κB binding site and TCF binding sites are shown. (D) Luciferase assay of −962 cyclin D1 promoter driven luciferase reporter (50 ng) with or without β-catenin (100 ng) coexpression. Three independent experiments were performed.

inhibitor cocktail) for 15 min on ice. Nonidet P-40 (5 μl of a 0.5% solution) was added and centrifuged. Resuspended cells were sonicated three times (Amp 40%, 20 s) using a model VCX130 sonifier (Sonics & Materials, Newtown, CT), centrifuged for 10 min and the supernatants were collected. Protein G-Sepharose (20 μl of a 50% slurry in 1 × phosphate buffered saline) beads blocked by tRNA (10 μg/ml) for 2 h at 4 °C. Immunoprecipitation was performed for 12–24 h with anti-β-catenin or anti-p65/RelA antibody. Control samples omitted the inclusion of antibody. Following immunoprecipitation, 20 μl of protein G-Sepharose beads were added and incubated for 2 h. The beads were collected and washed sequentially three times for 3 min (each buffer) in low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.0 and 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0 and 500 mM NaCl) and LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1 mM EDTA and 10 mM Tris–HCl, pH 8.0). Beads were washed three times with TE buffer and extracted with elution buffer (1% SDS, 0.1 M sodium bicarbonate). The eluant was heated at 65 °C for 6 h to reverse the formaldehyde cross-linking. DNA fragments were purified by phenol extraction and ethanol precipitation. Purified DNA fragments were amplified with real-time PCR as described above. Primers used for ChIP-quantitative PCR assay of the cyclin D1 promoter were described in a recent review on cyclin D1 transcription [19] and are listed in Supplementary Table 1.

3. Results and discussion

3.1. β-catenin transcriptionally activates cyclin D1 expression

Cyclin D1 promoter could be a good model as a common transcriptional target for β-catenin and NF-κB, based on its possession of TCF/β-catenin and NF-κB binding elements [13,14,20–23]. To more carefully map these elements, the cyclin D1 promoter region was analyzed from 1000 bp upstream to 200 bp downstream of the 5′ untranslated region (UTR) transcription start site (Supplementary Fig. 1, NCBI ID: NM_053056). Five TCF binding elements (TBE) were evident: TBE0 at −493 and four TBE1234 at −29 to +31 (Supple-

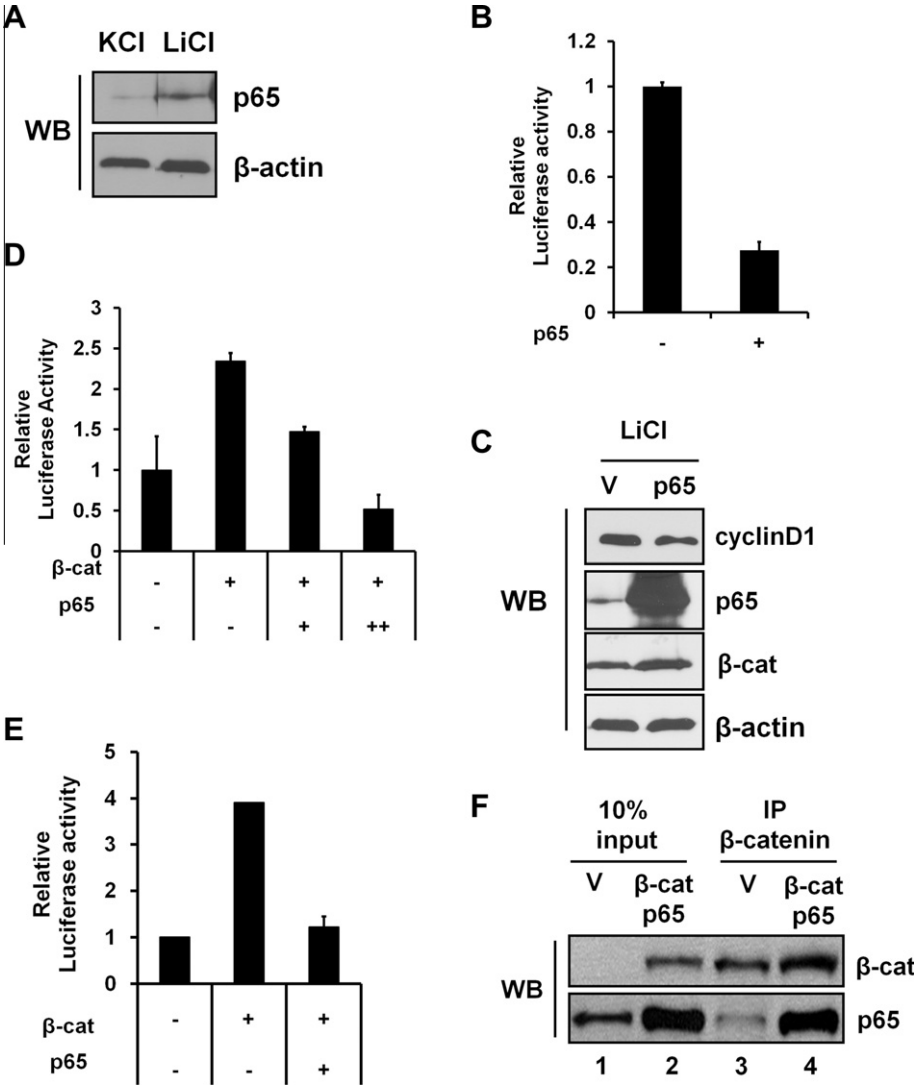

Fig. 2. Repression of cyclin D1 transcription by NF-κB p65 through protein–protein interaction between p65 and β-catenin in HEK293T cells. (A) Western blot analysis of p65 expression upon LiCl treatment. (B) Luciferase assay of −962 cyclin D1 promoter driven luciferase reporter (50 ng) with or without p65 (100 ng) coexpression. Three independent experiments were performed. (C) Western blot analysis of cyclin D1 expression after LiCl treatment with or without p65 expression. Anti-p65 and anti-β-catenin antibodies were used to detect protein levels. Anti-β-actin was used as a control. (D) Luciferase assay of −962 cyclin D1 promoter driven luciferase reporter (50 ng) together with β-catenin(100 ng) and/or p65 (10 ng and 100 ng). Three independent experiments were done. (E) Luciferase assay of OT reporter (50 ng) together with β-catenin(100 ng) and/or p65 (100 ng). Three independent experiments were done. (F) Co-immunoprecipitation of β-catenin and p65. HEK293T cells were transfected with β-catenin and p65 expression clones and immunoprecipitated with anti-β-catenin antibody. Immunoblot was performed with anti-p65 as well as anti-β-catenin antibodies.

mentary Fig. 2), exactly as reported previously [13]. In addition, two NF-κB binding sites were noted. To test whether β-catenin could activate cyclin D1 transcription, HEK293T cells were treated with LiCl to inactivate GSK-3β and increase β-catenin protein level in the nucleus. Cyclin D1 mRNA level was increased after LiCl treatment as shown by real-time PCR (Fig. 1A) in accordance with the increase of β-catenin protein level (Fig. 1B). The observation of five TCF sites within the luciferase reporter with −962 cyclin D1 promoter (Fig. 1C) prompted an experiment testing whether LiCl-activated β-catenin directly acted on the transcriptional activation of cyclin D1 promoter. As expected, overexpression of β-catenin significantly increased the luciferase activity from the −962 cyclin D1 promoter (Fig. 1D). The results confirmed that cyclin D1 transcription was directly activated by β-catenin in 293T cells.

3.2. Cyclin D1 transcription is repressed by NF-κB p65 at the transcriptional level

Since NF-κB consensus sites are in the cyclin D1 promoter (Supplementary Figs. 1 and 2), an experiment was designed to discern

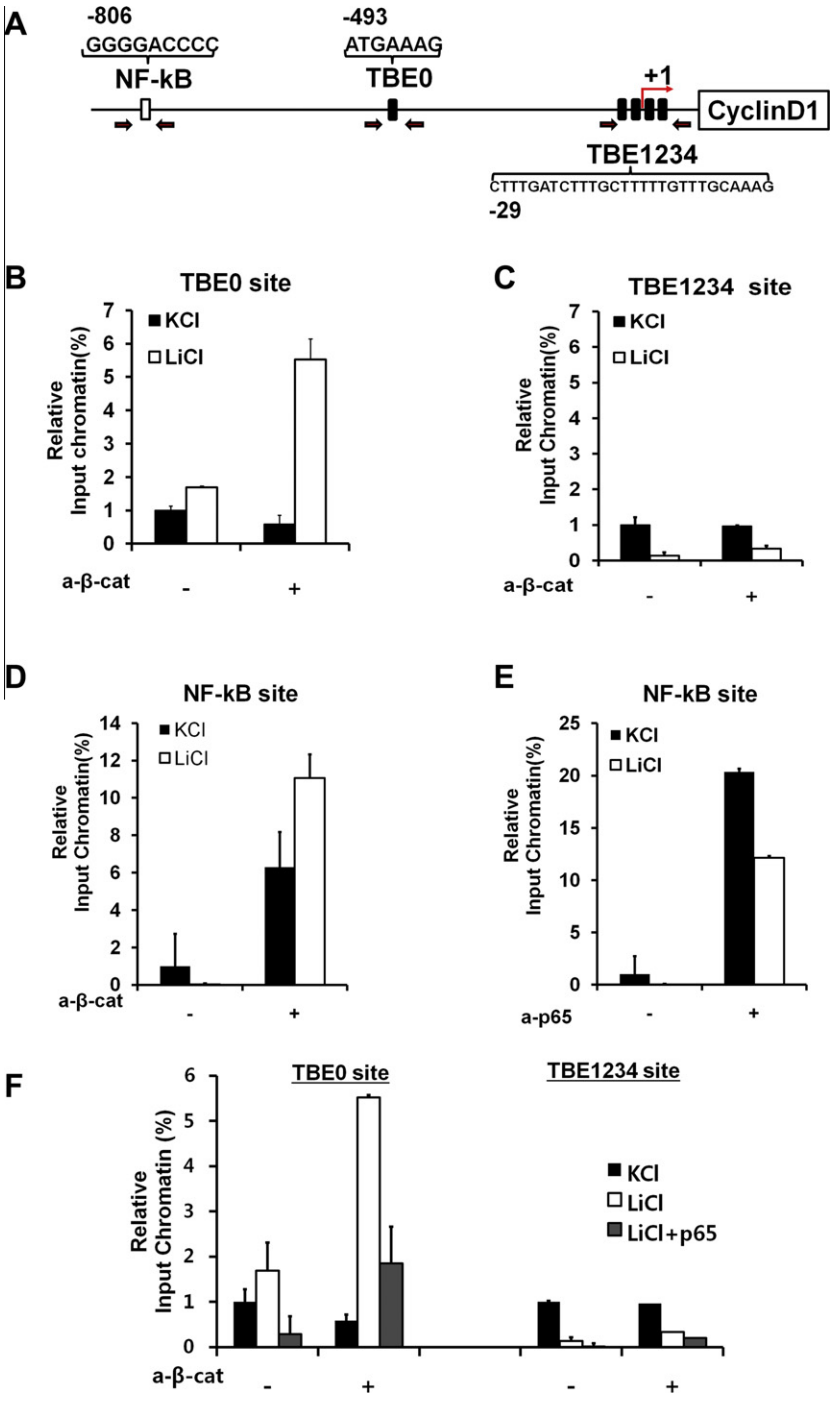

Fig. 3. Dynamic and combinatorial binding of β-catenin and NF-κB p65 on cyclin D1 promoter upon LiCl treatment in HEK293T cells. (A) Schematic representation of cyclin D1 promoter. NF-κB binding site (open box) and TCF binding sites (black boxes, TBE0 and TBE1234) and their sequences are shown. Transcription star site (+) is indicated with the arrow. Small arrows indicate the positions and directions of the PCR primers used for the ChIP assay. (B) ChIP assay with anti-β-catenin antibody on the TBE0 site. (C) ChIP assay with anti-β-catenin antibody on the TBE1234 site. (D) ChIP assay with anti-β-catenin antibody on the NF-κB site. (E) ChIP assay with anti-p65 antibody on the NF-κB site. (F) ChIP assay with anti-β-catenin antibody on the TBE0 and TBE1234 sites.

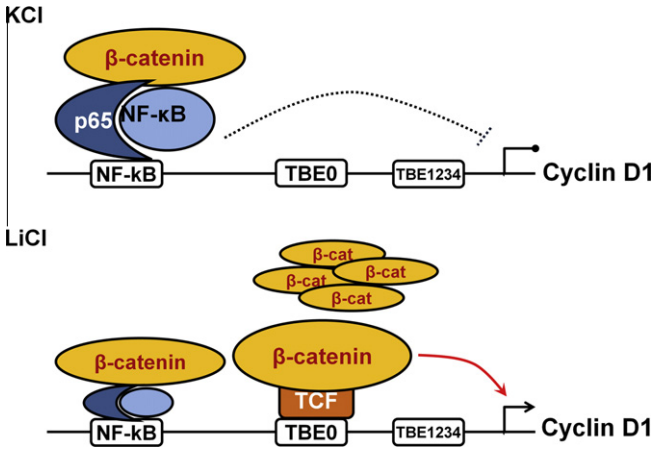

Fig. 4. Model for cyclinD1 transcription by NF-κB p65 and β-catenin. Constitutive binding of p65 and β-catenin to the NF-κB site in cyclin D1 promoter was observed in KCl-treated HEK 293T cells. LiCl induces the accumulation of β-catenin that leads to displacement of p65 with β-catenin, thereby relieving the transcriptional repression by p65.

whether p65 acted directly on cyclin D1 expression. Following LiCl treatment, a slight increase of p65 protein level was apparent (Fig. 2A), which might have reflected the cyclin D1 expression pattern noted in Fig. 1. Since GSK-3β can repress basal p65 transcriptional activity [24], it is possible that LiCl-mediated GSK-3β inhibition might activate the transcriptional activity as well. To rule out such an indirect effect by signaling crosstalk of GSK-3β and p65, the role of p65 on basal level cyclin D1 transcription was tested directly. p65 protein was overexpressed along with cyclin D1 promoter driven luciferase reporter in 293T cells. Surprisingly, overexpression of p65 greatly reduced luciferase activity, suggesting a repressive function of p65 in the basal level transcription from the cyclin D1 promoter (Fig. 2B). However, cyclin D1 mRNA and protein levels were reduced only marginally by p65 protein overexpression (data not shown), which might explain an additional layer of regulation on cyclin D1 expression by p65. In fact, it has been reported that p65 can regulate cyclin D1 stability post-translationally [25]. Intrigued by the observation concerning the basal transcriptional activity of p65 on cyclin D1 promoter, an experiment was devised to test whether p65 could also repress LiCl-activated cyclin D1 gene expression. Co-expression of p65 in the presence of LiCl treatment significantly reduced cyclin D1 protein (Fig. 2C). To test whether such a reduction in cyclin D1 expression was at the transcriptional level, −962 cyclin D1 luciferase reporter was used and the effects of co-transfected β-catenin and p65 on luciferase expression were analyzed (Fig. 2D). A luciferase assay showed that β-catenin activated cyclin D1 transcription was gradually reduced by an increasing amount of p65 protein. p65 also repressed activated cyclin D1 transcription as for constitutive cyclin D1 transcription. These results indicate that p65 can repress TCF/β-catenin complex driven transcriptional activity on the cyclin D1 promoter.

3.3. β-Catenin transcriptional activity is repressed by NF-κB p65 without κB sequences

Since the −962 cyclin D1 luciferase reporter contains NF-κB binding sites in addition to TCF/β-catenin binding sites, it was still possible that p65 could mediate the transcriptional repression on the cyclin D1 promoter only when it was bound nearby DNA elements. To directly test whether p65 could repress β-catenin activated transcription without DNA binding, the effects of p65 overexpression on an OT luciferase reporter containing four repeats of TCF binding sites only were examined. Cotransfection of 293T cells with p65 completely suppressed β-catenin-activated luciferase activity (Fig. 2E). One possible explanation for such p65-mediated β-catenin transcription is the involvement of protein–protein interaction between p65 and β-catenin. In fact, protein–protein interaction was evident between β-catenin and p65 (Fig. 2F), consistent with previous results [26,27]. These findings suggested that protein–protein interactions between β-catenin and NF-κB complex might be somehow involved in the p65-mediated repression of β-catenin activated transcription. In addition, additional proteins such as transcriptional regulators and nucleosome remodeling components might be involved in the p65-mediated repression of cyclin D1 transcription [28]. Recent report also suggested that p65 could repress basal transcriptional level of cyclin E2 for cell cycle regulation [29].

3.4. β-Catenin and NF-κB p65 dynamically bind on the cyclin D1 promoter upon signal induction

To understand more clearly the transcriptional regulation by β-catenin and p65, the promoter binding patterns of the two proteins on their respective binding elements in cyclin D1 promoter (Fig. 3A) were carefully monitored. ChIP analysis showed that β-catenin binding was not found when the cells are not activated in KCl condition (Fig. 3B and C). However, upon LiCl treatment, markedly enhanced binding was evident at TBE0 site (one site at −486 from the transcription start, Fig. 3B) but not at TBE1234 (four sites near transcription start, Fig. 3C), which are involved in cyclin D1 transcription [13]. Unexpectedly, β-catenin occupied the distal NF-κB site (−806 from the start, Fig. 3D) along with p65 (Fig. 3E) without any signal activation. More surprisingly, LiCl activation led to more enhancement of β-catenin binding on the NF-κB site that some p65 was dissociated from (Fig. 3D and E). These observations could be related to protein–protein interaction between β-catenin and NF-κB complex as shown in Fig. 2E and reported previously [26,27]. To test if the combinatorial and dynamic bindings of β-catenin and p65 could be related to p65-mediated suppression on β-catenin-activated cyclin D1 transcription, p65 was overexpressed in the presence of LiCl and ChIP was performed (Fig. 3F). As expected, β-catenin binding on TBE0 was completely abolished when p65 was overexpressed, which may be the result of β-catenin dissociation from TBE and subsequent protein–protein interaction with p65.

4. Conclusion

Cyclin D1 transcription is directly activated by LiCl-activated or overexpressed β-catenin in HEK293T cells, and one of five TCF binding elements in cyclin D1 promoter is responsible for β-catenin binding. Furthermore, one of the NF-κB binding elements is responsible for p65 and β-catenin binding, and such binding complexes are dynamically regulated by LiCl. Therefore, such a combinatorial binding of p65 and β-catenin might explain the regulation of cyclin D1 transcription by p65 in HEK293T cells. Based on the results shown in Fig. 3, a model summarized in Fig. 4 can be proposed. Constitutive binding of p65 to the cyclin D1 promoter may be responsible for p65-mediated repression of cyclin D1 transcription. LiCl induces the accumulation of β-catenin that leads to displacement of p65 with β-catenin, thereby relieving the transcriptional repression by p65. Further studies are needed to understand more precisely how such a p65-mediated transcriptional repression can occur. It has been proposed that p65 binding to the transcriptional co-repressor histone deacetylase 1 negatively regulates gene expression [30]. Therefore, it is conceivable that DNA-bound β-catenin activates transcription by displacing p65 from the DNA along with histone deacetylase 1 or by recruiting the co-activator histone acetyltransferase. More careful analysis

with other common genes of β-catenin and NF-κB, as well as understanding signaling pathways regulating the protein–protein interaction might help to fully elucidate the oncogenic transcriptional network [31,32].

Acknowledgments

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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.bbrc.2010.10.118.

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