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Glycogen Synthase Kinase-3β Regulates ΔNp63 Gene Transcription Through the β-Catenin Signaling Pathway

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ABSTRACT

Overexpression of ΔNp63 has been observed in a number of human cancers, suggesting a role for ΔNp63 in carcinogenesis. In the present study, we show that inhibition of glycogen synthase kinase-3β (GSK-3β) by lithium chloride (LiCl) elicited a stimulatory effect on ΔNp63 promoter activity in HEK 293T cells. Exposure to LiCl induced ΔNp63 promoter activation as well as ΔNp63 protein expression in the cells. The effect of GSK-3β on ΔNp63 expression was further confirmed by the use of two highly specific GSK-3β inhibitors, SB216763 and SB415286. Further study showed the presence of a putative β-catenin responsive element (β-catenin-RE) in the ΔNp63 promoter region, and the stimulation of ΔNp63 promoter activity by GSK-3β inhibitor is markedly abolished by mutation or deletion of the putative β-catenin-RE. Data are also presented to show that β-catenin acts together with Lef-1 to influence ΔNp63 promoter activity and protein expression. J. Cell. Biochem. 105: 447–453, 2008.

KEY WORDS: β-CATENIN; GSK-3β; ΔNp63; PROMOTER

The discovery of the transcription factor p63 was followed by back-to-back reports showing that p63 is essential for the development of a number of epithelial structures like the skin, breast, prostate, urothelia, and others [Mills et al., 1999]. High levels of p63 expression were found in the basal cells of many stratified epithelial tissues, from which a majority of human neoplasms develop [Mills et al., 1999]. A number of studies contended that p63 identifies keratinocyte stem cells [Pellegrini et al., 2001]; however, subsequent work by others found that p63 expression is not limited to stem cells in the epithelial tissues that express this gene [Wang et al., 2003]. p63 is a member of the p53 gene family. The p63 gene contains two distinct promoters encoding proteins containing (TAp63) or lacking (ΔNp63) the N-terminal transactivation domain. The TAp63 promoter is located upstream to exon 1 (P1). In contrast, ΔNp63 are generated by a second internal promoter (P2) located upstream to exon 3′. ΔNp63 isoforms are thought to antagonize the transcriptional regulation of the p53 and TAp63 target genes [Yang et al., 1998]. Although ΔNp63 can antagonize transcription by TAp63, there has been no evidence shown that expression of ΔNp63 is associated with TAp63. ΔNp63 is predominantly expressed in the basal cells of various stratified epithelia, and it is overexpressed in human cancers like bladder carcinoma, non-small cell lung cancers (NSCLCs), nasopharyngeal carcinoma (NPC), and liver cancers. These observations suggest that overexpression of ΔNp63 may have important implications for carcinogenesis [Crook et al., 2000; Park et al., 2000; Ratovitski et al., 2001; Massion et al., 2003; Westfall and Pietenpol, 2004; Chu et al., 2006, 2008].

β-Catenin has been shown to link the cytoplasmic side of cadherin-mediated cell–cell contacts to the actin cytoskeleton, and it also regulates gene transcription by binding to Lef/Tcf transcription factors [Korinek et al., 1997]. β-Catenin-mediated transactivation depends on its accumulation in the nucleus. The level of β-catenin in the cell is regulated by its association with adenomatous polyposis coli (APC), axin, glycogen synthase kinase 3β (GSK-3β), conductin, and ΔNp63 [Polakis, 2000; Patturajan et al., 2002]. Active GSK-3β can phosphorylate β-catenin, which results in degradation of β-catenin by the ubiquitin–proteosome system [Polakis, 2000]. GSK-3β activity can be suppressed by a variety of stimuli, such as activity of the Wnt signaling pathway; suppression stabilizes β-catenin and causes an accumulation of β-catenin in the nucleus. It has been reported that cytosolic β-catenin levels are stabilized due to a mutational inactivation of APC or β-catenin during early stages of tumor development [Korinek et al., 1997; Morin et al., 1997]. Elevated β-catenin levels in colon cancer and melanoma have been suggested to confer uncontrolled activation of gene transcription by the β-catenin/LEF-1 complex and may have implications in tumor progression.

ΔNp63 expressing kidney epithelial cell line (HEK 293T) has been shown to have intact Wnt/Gsk-3/β-catenin signaling pathway, and harbors no defect in the inhibitory functions of APC [Sadot et al.,

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2002]. HEK 293T cell has been the most commonly used cell line to study β-catenin accumulation and its subsequent transactivation of target genes by LiCl and other GSK-3 inhibitors [Shtutman et al., 1999; Jho et al., 2002; Le Floch et al., 2005; Steinbrecher et al., 2005; Bachar-Dahan et al., 2006]. Previous report also indicated that amplification of ΔNp63 but not TAp63 was observed in several human cancers [Crook et al., 2000; Park et al., 2000; Massion et al., 2003; Westfall and Pietenpol, 2004]. In addition, Ratovitski et al. also showed that ΔNp63 was the unique isoform detected in HEK 293T cells. The reports suggest that ΔNp63 and TAp63 expression maybe differentially regulated.

We show that GSK-3 inhibitors exert a positive regulatory effect on the activity of the ΔNp63 promoter in HEK 293T cells and β-catenin is critical for such effect. Moreover, we identify a putative β-catenin responsive element (β-catenin-RE) in the ΔNp63 promoter region at −231 to −215. We show that the regulation of ΔNp63 promoter activity by the GSK-3β/β-catenin signaling pathway is dependent on the presence of this putative β-catenin-RE.

MATERIALS AND METHODS

CELL LINE

Human embryonic kidney cells (HEK) 293T cells were maintained in basal medium (DMEM/F-12 at 3:1, v/v; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator at 37°C under 5% CO_2/95% air.

CONSTRUCTION OF ΔNp63 PROMOTER-LUCIFERASE CONSTRUCTS AND EXPRESSING VECTORS

A human ΔNp63 promoter fragment from −823 to +262 relative to the transcriptional start site was subcloned by PCR amplification. The PCR reaction was carried out with the sequence specific primer pairs, these primers were designed to contain a *Xho*I site and a *Hin*dIII site for the subsequent cloning reactions. The primers used were: forward, 5′-CCCCTCGAGGGCCAGATTCTACATGAATGTTGG-3′; reverse, 5′-CCCAAGCTTGTTAGCTGTAAGATTGATCAATG-3′. Desired DNA fragments were PCR amplified and inserted into luciferase reporter vector pGL3-Basic, a promoter- and enhancer-less vector (Promega, Madison, WI). The inserts were positioned in sense orientation relative to the luciferase coding sequence between *Xho*I and *Hin*dIII sites. Proper insertion was verified by direct DNA sequencing. The nomenclature of pGL3-823-Luc was based on the length of the insert upstream to the transcriptional start site of the ΔNp63 promoter. The β-catenin luciferase reporter pGL3-β-cateninRE-Luc was constructed with five tandem copies of the β-catenin-RE (TCAGTTACAAAGAGTAA) from the 5′-flanking region of the ΔNp63 gene followed by one copy of a minimal TATA box. The construct was subcloned into pGL3-basic. A full-length human β-catenin cDNA fragment lacking the stop codon was generated by oligo-dT-primed reverse transcription-PCR using total RNA isolated from the human colon cancer cell line SW48. β-Catenin of the SW48 cell contains a tyrosine substitution in place of the normal serine at residue 33 (S33Y). The primers used were: forward, 5′-gtggacaatggctactcaagctg-3′; reverse, 5′-caggtcagtatcaaaccaggccag-3′. Lef-1 cDNA was generated by RT-PCR. The primers used were: forward, 5′-gggatgccccaactctccggaggag-3′; reverse, 5′-gatgtaggcagctgtcattcttgga-3′. The PCR products were cloned into a pcDNA3.1/V5-HisTOPO vector (Invitrogen) to generate expressing plasmids pCMV-β-catenin-S33Y and pCMV-Lef-1.

TRANSIENT TRANSFECTION AND LUCIFERASE ASSAY

Transient transfection of luciferase reporter plasmids or expressing vectors was performed using Lipofectamine 2000 (Invitrogen) according to the protocol recommended by the supplier. Cells were seeded in 12-well tissue culture plates at 2×10^5 per well in 1 ml OPTI-MEM (Invitrogen) 24 h before transfection. On the day of transfection, the cells were exposed to DNA-lipofectamine 2000 mixtures containing 0.5 μg of ΔNp63-luciferase reporter plasmid and 0.5 μg of pSV-β-galactosidase control vector (Promega). To analyze the ΔNp63 promoter activity in response to pCMV-β-catenin-S33Y and pCMV-Lef-1 vectors, 0.2 μg/ml of either one or both pCMV-expressing vectors or pCMV-empty vector (Invitrogen) was/were added to the DNA-lipofectamine mixtures. Cells were incubated for 24 h, rinsed with PBS, and lysed in 150 μl 1× reporter lysis buffer (Promega). Lysates were used directly for the Luciferase activity assay (Promega), which was performed according to the manufacturer's protocols. A β-Galactosidase enzyme assay (Promega) was also performed with the same lysates to standardize the transcription efficiency. All experiments were performed at least three times to obtain the mean relative luciferase activity.

PREPARATION OF CELL LYSATE AND WESTERN BLOT ANALYSIS

The cells (2×10^6) were seeded into 100 mm tissue culture plates 1 day before transfection, and they were subsequently transfected with an appropriate amount of the expression plasmid or pCMV-empty vector using Lipofectamine 2000. To prepare the total cell lysate, cells were washed with ice-cold PBS and lysed in Mammalian Protein Extraction Reagent (M-PER; Pierce, Rockford, IL). Nuclear and cytoplasmic extracts were prepared using the Nuclear and Cytoplasmic Extraction Kits (NE-PER™ Nuclear and Cytoplasmic Extraction Reagents; Pierce), respectively. All lysis buffers contained 10 mM NaF, 10 mM sodium orthovanadate, 1 mM PMSF, and a 1× cocktail protease inhibitor (Sigma, Milwaukee, WI). Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Protein samples were fractionated on a 10% SDS–polyacrylamide gel and blotted onto Immobilon(TM)-P membranes (Millipore, Bedford, MA). Membranes were blocked in TTBS and probed with primary antibodies (1:2,000 for β-catenin, Lef-1, p63, and V5 and 1:10,000 for β-actin) overnight at 4°C. Membranes were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5,000 in TTBS). Immunoreactive protein bands were visualized by Enhanced Chemiluminescence (ECL) (Amersham Pharmacia Biotech, Freiburg, Germany). Primary antibodies used included Lef-1 monoclonal antibody, β-catenin monoclonal antibody (Upstate, Lake Placid, NY), p63 (4A4) monoclonal antibody, β-actin monoclonal antibody (Chemicon, Temecula, CA), and V5 polyclonal antibody (Novus, Littleton, CO).

SITE-DIRECTED MUTAGENESIS OF THE pGL3-823-LUC VECTOR

To construct a pGL3-823-Luc vector containing a deletion mutation in the putative β-catenin responsive element (pGL3-823Δβ-cat-

Luc), the consensus Lef-1 binding site (−231 to −215 relative to the transcriptional start site of ΔNp63) was deleted. The pGL3-823mutβ-cat-Luc vector containing a substitution mutation in the β-catenin-RE was mutated from TCAGTTACAAAGAGTAA to TCAGTTACACGTAGTAA. Site-directed mutagenesis of the pGL3–823-Luc vector was carried out using PCR methods. Briefly, the PCR mixture contained 1 μg pGL3-823-Luc vector, 12.5 μl 2× Extensor Hi-fidelity PCR Master Mix (ABgene) (consisting 350 μM dNTP, 2.25 μM $MgCl_2$, and 2.5 U extensor PCR enzyme), and 0.1 μg of each primers. The synthetic oligonucleotide primers were: 5′-GTTTTCC-TGAAGTTTACTTTAATAACTTTCTGAAATGCC-3′(sense) and 5′-GGCATTTCAGAAAGTTATTAAAGTAAACTTCAGGAAAAC-3′(anti-(antisense) for pGL3-823Δβ-cat-Luc and 5′-CTGAAGTTTACTTTT-CAGTTACACGTAGTAAAATAACTTTCT-3′(sense) and 5′-AGAAAG-TTATTTTACTACGTGTAACTGAAAAGTAAACTTCAG-3′(antisense) for pGL3-823mutβ-cat-Luc. PCR was performed at three-temperature cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 8 min for a total of 12 cycles. After PCR, the methylated parental DNA template was digested with 20 U *Dpn*I (NEB, Schwalbach, Germany) at 37°C overnight. The DNA fragment containing the desired mutation was transformed into competent *E. coli* JM109 cells, which are capable of repairing the nicked DNA. The presence of the desired mutation was verified by direct DNA sequencing.

RESULTS

INHIBITION OF GSK-3 UP-REGULATES ΔNp63 EXPRESSION

Inhibition of GSK-3 activity has been documented to stabilize cellular β-catenin, which binds with Tcf/Lef to form a transcriptional complex to promote the expression of downstream genes. In the present study, we treated cells with several GSK-3 inhibitors to examine a possible effect of the GSK-3β/β-catenin pathway on ΔNp63 promoter activity. We cotransfected a pGL3-823-Luc reporter and pSV-β-galactosidase vector into HEK 293T cells and exposed the transfected cells to LiCl. As shown in Figure 1A, pGL3-823-Luc reporter activity was dose-dependently stimulated by LiCl; the reporter activity was stimulated up to 4.3-fold compared to that of the untreated control cells. The reporter activity was also slightly stimulated by the same concentration range of NaCl. The effect of LiCl on ΔNp63 protein expression was further confirmed by Western blotting. As shown in Figure 1B, LiCl exposure prominently increased the β-catenin protein level from that of the untreated and NaCl treated control cells. Consistent with the reporter activity assay, increased ΔNp63 protein levels were also detected in cells treated with LiCl. ΔNp63 protein was upregulated by approximately 1.6- and 3.5-fold in cells treated with 10 mM and 30 mM LiCl, respectively (Fig. 1B).

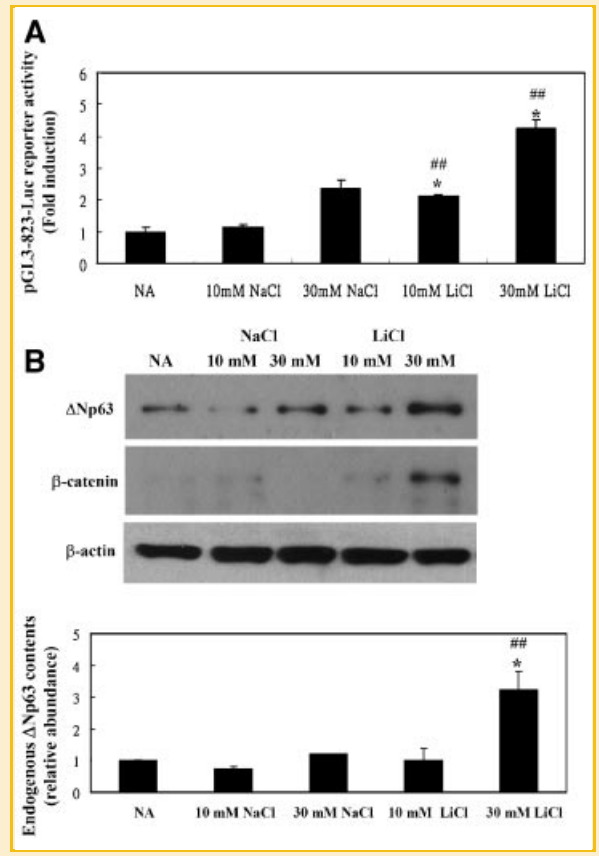

Fig. 1. Lithium ions induce ΔNp63 transcriptional activity. A: Cells were cotransfected with pGL3-823-Luc and pSV-β-galactosidase vectors (0.5 μg/ml each), and the β-galactosidase activity was used to normalize the luciferase activity. After 24 h, the transfected cells were incubated with LiCl or NaCl at the indicated concentrations. Five hours after incubation, the cells were harvested and the luciferase and β-galactosidase activities were measured. Data are mean ± SD from three independent analyses. Luciferase activity is presented as the fold induction relative to untreated cells (NA). Significance was tested using one-way ANOVA with Holm–Sidak method post hoc test, where * denotes $P < 0.05$ versus NA; ## denotes $P < 0.05$ versus NaCl at the same concentration. B: HEK 293T cells were treated with NaCl or LiCl at the concentrations indicated. Cell lysates were prepared 24 h after treatment and were subjected to Western Blot analysis with anti-p63, anti-β-catenin, and anti-β-actin antibodies (upper panel). The immunoreactive bands were analyzed with a densitometer, and the ratio of the endogenous ΔNp63 to β-actin signal was calculated (lower panel). The ΔNp63 to β-actin ratio in the absence of treatment was set as 1. Significance was tested using one-way ANOVA with Holm–Sidak method post hoc test, where * denotes $P < 0.05$ versus NA; ## denotes $P < 0.05$ versus NaCl at 30 mM.

The effect of GSK-3 activity on cellular β-catenin and ΔNp63 protein levels was further examined by treating the cells with SB214763 and SB415286. These two compounds are cell-permeable maleimide compounds that selectively inhibit GSK-3 [Coghlan et al., 2000]. Inhibition of GSK-3β by SB214763 and SB415286 dose-dependently increased nuclear β-catenin protein levels (Fig. 2). As shown in Figure 2A, the protein levels of β-catenin and ΔNp63 were dose-dependently upregulated by approximately 3.7- and 2.6-fold, respectively in the cell treated with 5 μM of SB216763, and by approximately 30- and 4.7-fold, respectively, in the cell treated with 10 μM of SB216763. Moreover, Figure 2B also shows that treatment with SB415286 leads to upregulation of β-catenin and ΔNp63 expression in a manner comparable to that of SB216763 treated cells. The increased β-catenin protein level was correlated with a simultaneous increase of the ΔNp63 protein level.

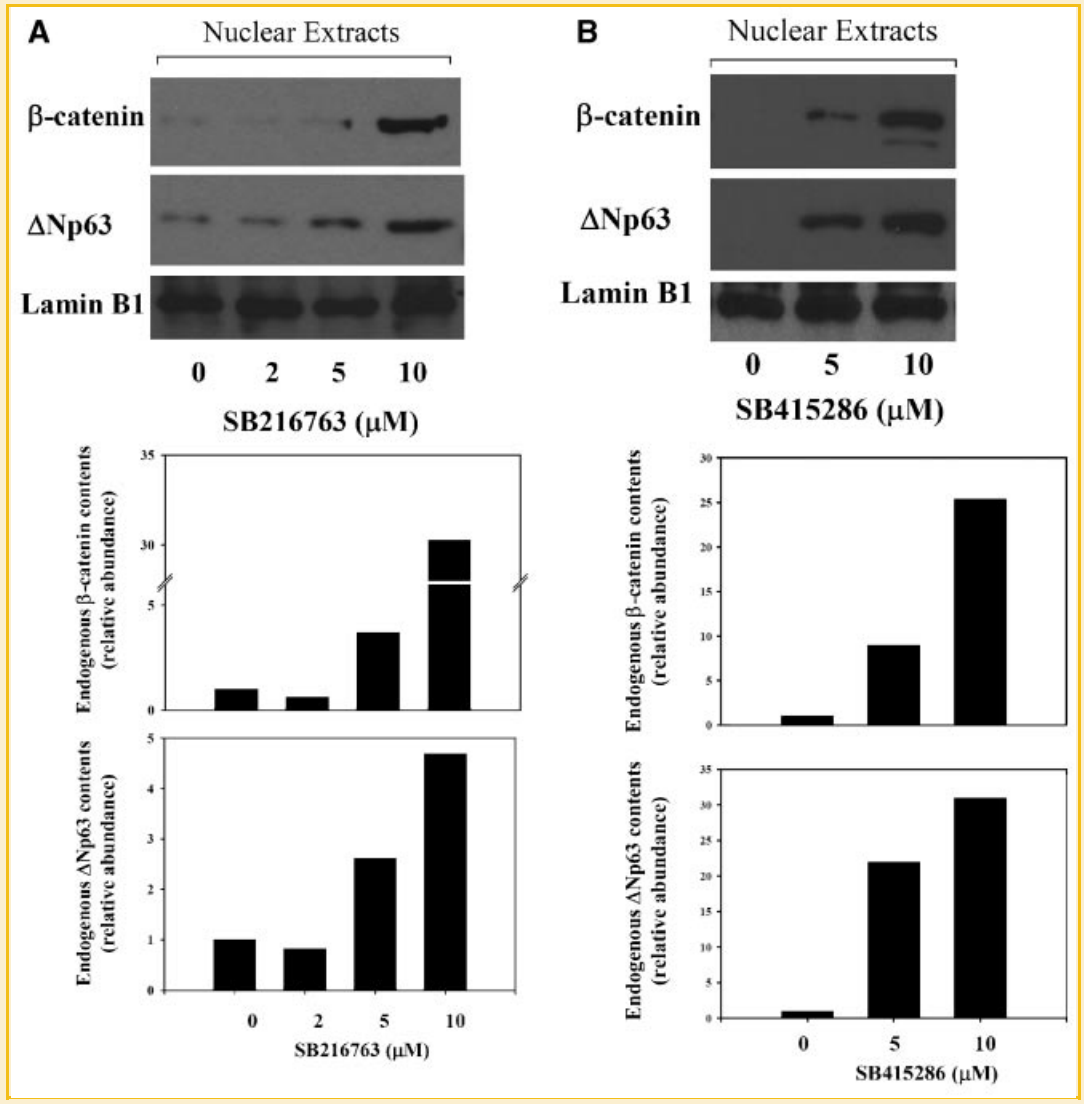

Fig. 2. Inhibition of GSK-3β increases nuclear β-catenin and ΔNp63 levels. Cells were treated with various concentrations of SB216763 or SB415286 as indicated. Twenty-four hours after treatment, nuclear extracts were prepared. Ten micrograms of nuclear extracts were subjected to Western blotting analysis. ΔNp63 and β-catenin proteins in the nuclear extracts were visualized by Western blotting analysis using antibody against ΔNp63 and β-catenin, respectively. Lamin B1 was also blotted to indicate that an equal amount of protein was applied to each lane. To compare the relative band intensities, all bands were normalized against Lamin B1 by densitometry. The ratio in the untreated cells was set as 1.

β-CATENIN RESPONSIVE ELEMENT IS INVOLVED IN THE UPREGULATION OF ΔNp63 EXPRESSION

For β-catenin to transactivate ΔNp63 gene transcription, it is assumed that at least a β-catenin responsive element (RE) has to be present in the ΔNp63 promoter region. We searched the TRANSFAC database using a MatInspector program, and we identified a putative β-catenin binding site in the 5′-flanking region at −231 to −215.

To see if the putative β-catenin-RE responds to LiCl for transcriptional regulation, the cells were transfected with a pGL3-β-catenin-RE-Luc vector containing five copies of a β-catenin-RE followed by a minimal TATA box and a luciferase gene. In the cells transfected with the pGL3-β-catenin-RE-Luc vector, LiCl treatment elicited a greater than sixfold stimulation of the reporter activity (Fig. 3A). To examine the functional significance of this putative β-catenin-RE for ΔNp63 promoter activity, we introduced a deletion (pGL3-823Δβ-cat-Luc) or a point mutation (pGL3-823mutβ-cat-Luc) in the β-catenin-RE of the pGL3-823-Luc reporter. The cells were transfected with either the point or deletion mutation ΔNp63 reporter, and the effect of LiCl on the reporter activity was examined. LiCl-induced stimulation of the transcriptional activity of the ΔNp63 reporter was suppressed (compared with data shown in Fig. 1) when the putative β-catenin-RE was either deleted (upper panel) or mutated (lower panel) (Fig. 3B). As shown in Figure 3B, the stimulation of the deletion reporter (2-fold) and mutation reporter (2-fold) activities by 30 mM NaCl is much weaker than by the same concentration of LiCl (4.3-fold) (Fig. 1A). Part of the LiCl effect is probably nonspecific, as that observed in NaCl treated cell. The possible complication by NaCl is discussed in Discussion Section. The results clearly suggest that the β-catenin-RE is involved in GSK-3 inhibitor induced upregulation of ΔNp63.

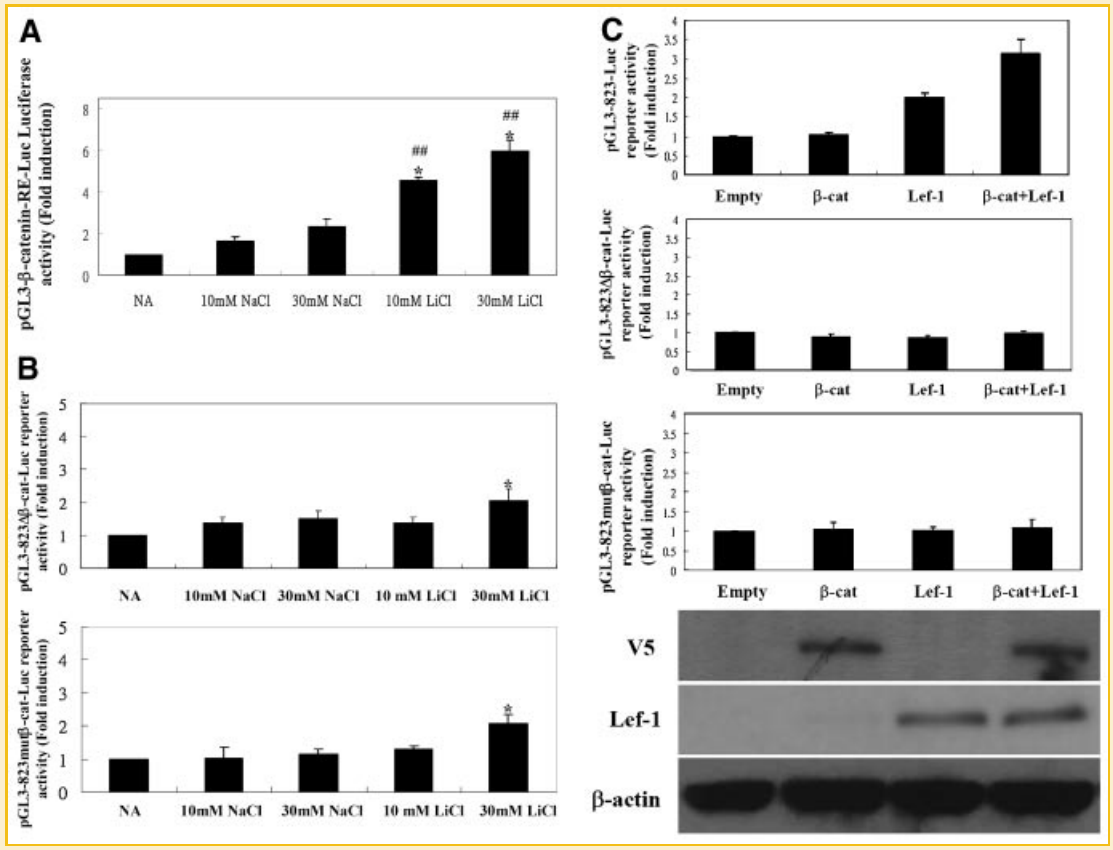

Fig. 3. The putative β-catenin-RE is required for the regulation of ΔNp63 promoter activity by β-catenin. A: Cells were cotransfected with 0.5 μg/ml each of pGL3-β-catenin-RE-Luc and pSV-β-galactosidase vectors for 24 h. After transfection, cells were treated with LiCl at the concentrations indicated for 5 h. Luciferase activity is presented as the fold induction relative to untreated cells. Data are mean ± SD from three independent analyses. Significance was tested using one-way ANOVA with Holm–Sidak method post hoc test, where * denotes $P < 0.05$ versus NA; ## denotes $P < 0.05$ versus NaCl at the same concentration. B: Cells were cotransfected with 0.5 μg/ml of β-catenin-RE deleted (pGL3-823Δβ-cat-Luc) or mutated (pGL3-823mutβ-cat-Luc) luciferase reporter and pSV-β-galactosidase vector for 24 h. After transfection, cells were treated with LiCl at the concentrations indicated for 5 h. Luciferase activity is presented as the fold induction relative to untreated cells. Data are means ± SD from triplicate analysis. Significance was tested using one-way ANOVA with Holm–Sidak method post hoc test, where * denotes $P < 0.05$ versus NA. C: The Cells were cotransfected with either pGL3-823-Luc, pGL3-823Δβ-cat-Luc or pGL3-823mutβ-cat-Luc with pCMV-β-catenin-S33Y or pCMV-Lef-1 or together with pCMV-β-catenin-S33Y and pCMV-Lef-1 for 24 h. The pCMV-empty vector was added when necessary to keep the total plasmid concentration constant. The cells were also cotransfected with a pSV-β-galactosidase vector, and the β-galactosidase activity was used to normalize the luciferase activity. Luciferase activity is presented as the fold induction relative to pCMV-empty transfected cells. Data are mean ± SD from three independent analyses. The translational activity of pCMV-β-catenin-S33Y and pCMV-Lef-1 in the transfected cells was examined by Western blot analysis. β-catenin expression was probed by V5 protein expression. Equal amounts of protein lysate were subjected to Western blotting analysis using antibodies against V5, Lef-1, or β-actin.

β-CATENIN TRANSACTIVATES ΔNp63 GENE PROMOTER

To further confirm that the putative β-catenin-RE in the ΔNp63 promoter region plays a role in the regulation of ΔNp63 expression, we examined the effect of elevated β-catenin and Lef-1 expression on the transcriptional activities of the pGL3-823-Luc, pGL3-823Δβ-cat-Luc, and pGL3-823mutβ-cat-Luc luciferase reporter constructs. The transcriptional activities of the ΔNp63 reporters were examined by transfection with either pCMV-Lef-1 alone or pCMV-Lef-1 together with pCMV-β-catenin-S33Y. Transfection of the former elicited a 2.2-fold stimulation, and the latter elicited a 3.5-fold stimulation on pGL3-823-Luc reporter activity. Interestingly, transfection of pCMV-β-catenin-S33Y alone showed no obvious effect on pGL3-823-Luc reporter activity (Fig. 3C). Deletion or mutation of the β-catenin-RE abolished the stimulation of ΔNp63 promoter by β-catenin or/and Lef-1 compared to that of the wild type (pGL3-823-Luc) control. The result suggests that β-catenin acts together with Lef-1 to regulate ΔNp63 expression.

DISCUSSION

ΔNp63 is overexpressed in a number of epithelial cancers, suggesting that the aberrant expression of ΔNp63 may be oncogenic. Therefore, understanding the transcriptional regulation of the ΔNp63 gene may have great biological significance. Recent reports indicate that impaired ΔNp63 expression is associated with reduced β-catenin expression and is suggested to have implications in the progression of urothelial neoplasms [Koga et al., 2003]. Furthermore, it has been shown that the activation of β-catenin signaling in basal mammary epithelial cells in pregnant mice

produces an upregulation of Myc and cyclin D1 gene transcription and a shift of p63 variant expression toward the ΔNp63 form [Teuliere et al., 2005]. These observations strongly suggest the possible existence of a cross-regulation mechanism underlying the expression of β-catenin and ΔNp63.

β-Catenin is a multifunctional protein and an important component of the Wnt/Gsk-3 signaling pathway. Accumulation of β-catenin by Wnt/Gsk-3β pathway signaling is believed to play a critical role in cell proliferation during embryogenesis [Haegel et al., 1995; Miller and Moon, 1996], and mutations of β-catenin constitute an important event in carcinogenesis [Morin et al., 1997]. β-Catenin is also known to associate with the Lef/Tcf transcription factor family and promote the expression of several genes, including cyclin D1, c-MYC, and matrilysin (MMP-7) [Shtutman et al., 1999; Polakis, 2000; Stambolic, 2002]. Elevated MMP-7 activity is believed to be implicated in both the degradation of the extracellular matrix and the uncontrolled cell proliferation and differentiation observed in some cancer cells [Miyata et al., 2006; Liu et al., 2007].

In the present study, we investigate the possible linkage between the expression of the ΔNp63 gene and the activity of the GSK-3β/β-catenin signaling pathway. We found that a GSK-3 inhibitor (LiCl) stimulated ΔNp63 promoter activity in HEK 293T cells. The promoter activity simulated by NaCl was about 2.3-fold, which is much weaker than by LiCl (4.3-fold) at the same concentration (Fig. 1A). The stimulation by NaCl is probably mediated through a different pathway; PI3K seemed a likely candidate because high NaCl (Hypertonicity) has been shown to activates its downstream target AKT/PKB [Irarrazabal et al., 2006; Perez-Pinera et al., 2006]. In a recent report, Barbieri and colleagues showed that ΔNp63α is a target of the PI3K pathway downstream to the epidermal growth factor (EGF) receptor in keratinocytes [Barbieri et al., 2003]. They showed that treatment of keratinocytes with EGF resulted in an increased ΔNp63α expression at the mRNA level, and inhibition of PI3K abrogated the EGF stimulation.

To clarify the possible interference by NaCl, two selective GSK3 inhibitors, SB216763 and SB415286, were used to confirm the results. As shown in Figure 2, treatment with SB216763 or SB415286 resulted in an increased expression of β-catenin and ΔNp63 as that observed in the LiCl treated cell.

The suppression of GSK-3β activity resulted in the accumulation of β-catenin in the nucleus and a concomitant increase in ΔNp63 gene expression. Our results clearly demonstrate that suppression of GSK-3β activity positively regulates ΔNp63 transcription. Importantly, this effect was abolished by deletion or mutation of the putative β-catenin-RE in the ΔNp63 promoter region (Fig. 3B). The sequence of the regulatory events appears to inhibit GSK-3β. This GSK-3β inhibition leads to increased β-catenin nuclear translocation, and β-catenin then acts through the putative β-catenin-RE to upregulate ΔNp63 expression. Our study further indicates that the effect of β-catenin is dependent on the simultaneous transfection of the Lef-1 expression vector (Fig. 3C). This finding suggests that β-catenin cooperates with Lef-1 to activate the putative β-catenin-RE. Although the exact function of ΔNp63 in carcinogenesis has not been fully elucidated, our study clearly shows that β-catenin/Tcf signaling is involved in the regulation of ΔNp63 gene expression.

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