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14-3-3 Sigma and 14-3-3 Zeta Plays an Opposite Role in Cell Growth Inhibition Mediated by Transforming Growth Factor-Beta 1

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The expression of 14-3-3 proteins is dysregulated in various types of cancer. This study was undertaken to investigate the effects of 14-3-3 ζ and 14-3-3 σ on cell growth inhibition mediated by transforming growth factor-beta 1 (TGF-β1). Mouse mammary epithelial cells (Eph4) that are transformed with oncogenic c-H-Ras (EpRas) and no longer sensitive to TGF-β1-mediated growth inhibition displayed increased expression of 14-3-3 ζ and decreased expression of 14-3-3 σ compared with parental Eph4 cells. Using small interfering RNA-mediated knockdown and overexpression of 14-3-3 σ or 14-3-3 ζ, we showed that 14-3-3 σ is required for TGF-β1-mediated growth inhibition whereas 14-3-3 ζ negatively modulates this growth inhibitory response. Notably, overexpression of 14-3-3 ζ increased the level of Smad3 protein that is phosphorylated at linker regions and cannot mediate the TGF-β1 growth inhibitory response. Consistent with this finding, mutation of the 14-3-3 ζ phosphorylation sites in Smad3 markedly reduced the 14-3-3 ζ-mediated inhibition of TGF-β1-induced p15 promoter-reporter activity and cell cycle arrest, suggesting that these residues are critical targets of 14-3-3 ζ in the suppression of TGF-β1-mediated growth. Taken together, our findings indicate that dysregulation of 14-3-3 σ or 14-3-3 ζ contributes to TGF-β1 resistance in cancer cells.

INTRODUCTION

Transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine that signals through heteromeric complexes of TGF-β type I (TβRI) and type II (TβRII) transmembrane Ser-Thr kinase receptors (Derynck et al., 2003; Massagué et al., 2000). One important function of TGF-β1 is the regulation of cell growth. In epithelial and lymphoid cells, TGF-β1 inhibits cell proliferation by inducing cell cycle arrest at the G_1 phase (Bhowmick et al., 2003), and functions as a tumor suppressor during the early phases of carcinogenesis (Wakefield et al., 2002). This cytostatic activity is dependent on the ability of TGF-β1 to increase expression of the cyclin-dependent kinase inhibitors $p15^{INK4B}$ and $p21^{cip1/waf1}$ and repress expression of the growth promoting factors c-Myc and Id family members, and is mainly controlled by Smad3-dependent signals (Kowalik, 2002; Robson et al., 1999; Siegel et al., 2003). Indeed, Smad3-deficient primary cells fail to respond to the growth inhibitory effects of TGF-β1 (Datto et al., 1999; Rich et al., 1999; Yang et al., 1999). Moreover, phosphorylation of the regulatory linker regions of Smad3 by cyclin-dependent kinases (CDKs) CDK2 and CDK4 or extracellular signal-regulated kinase 1/2 (ERK1/2) inhibits its transcriptional activity, abolishing the anti-proliferative effect of TGF-β1 and eventually facilitating cell cycle progression from G_1 to S phase (Matsuura et al., 2004; 2005). Thus, Smad3 plays a prominent role in mediating the TGF-β1 growth inhibitory signal.

The 14-3-3 family of proteins contains seven isoforms, β, ε, η, γ, τ, ζ, and σ, which are highly conserved and ubiquitously expressed in all eukaryotic organisms (Morrison, 2009; Takahashi, 2003). 14-3-3 proteins regulate fundamental cellular functions such as apoptosis, migration, cell cycle progression, and cytoskeletal integrity. Dysregulation of 14-3-3 protein expression has been implicated in the development of cancer; for example, expression of 14-3-3 σ, a tumor suppressor that is induced by p53 in response to DNA damage, is lost in breast cancer (Hermeking et al., 1997; Moreira et al., 2005) whereas expression of 14-3-3 ζ, which plays a dominant role in promoting survival of cancer cells, is highly elevated in several types of cancer (Li et al., 2008; Maxwell et al., 2009).

Here, we investigated the role of 14-3-3 σ and 14-3-3 ζ in the antiproliferative response induced by TGF-β1. Our results indicate that 14-3-3 ζ and 14-3-3 σ are important opposing regulators of the TGF-β1 growth inhibitory response, and suggest that their dysregulation confers resistance to the growth inhibitory effects of TGF-β1 in cancers.

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MATERIALS AND METHODS

Cell culture

Eph4 mouse mammary epithelial cells were obtained from Dr. Anita B. Roberts of the National Cancer Institute (USA). The amphotropic retroviral packaging cell line Phoenix E was obtained from Dr. Rik Derynck (University of California-San Francisco, USA). Cells were grown in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, SUA), 100 U/ml penicillin, and streptomycin (100 μg/ml) at 37°C under a humidified 95/5% (v/v) mixture of air and CO_2. Mv1Lu cells were maintained in minimum essential medium (Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum.

Plasmid DNAs and cell lines

The expression plasmid DNA encoding wild type form of the Smad3 (pCMV-Smad3) and triple mutant form of the Smad3 (pCMV-Smad3 (T178V/S204A/S208A)) were kindly provided by Dr. Fang Liu (The State University of New Jersey, USA). V12-Has-Ras-transformed derivative EpRas cells were generated by retroviral gene transfer using retroviral vector of V12-Ras in pBabe-puro (addgene), allowing for selection in puromycin. To generate retroviruses, Phoenix E cells (2×10^6 cells/100-mm tissue culture plates) were transfected by the calcium phosphate method (Pear et al., 1993), using 10 μg DNA/plate. Thirty-six h after transfection, the supernatant containing recombinant retroviruses was collected and filtered through 0.45-μM sterilization filters. 5 ml of these supernatants were applied immediately to Eph4 cells (5×10^5 cells/100 mm plate) with addition of Polybrene (Sigma, USA) at a final concentration of 8 μg/ml. Stably transfected clones were selected with 2 μg/ml puromycin (Invitrogen, USA). After 2 weeks of selection, puromycin-resistant colonies were analyzed Ras expression by immunoblotting.

Antibodies and other reagents

The recombinant TGF-β1 was purchased from R&D Systems (Minneapolis, USA). Small interfering RNAs for control, mouse 14-3-3 σ and mouse 14-3-3 ζ were purchased from Santa Cruz Biotechnology (USA). Smad3 phosphopeptide antibodies against Ser^{208}, Ser^{213}, and Thr^{179} were kindly provided by Dr. Fang Liu (The State University of New Jersey, USA).

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the phenol-guanidinium isothiocyanate method (Chomczynski et al., 1987). RT-PCR was performed by Acess RT-PCR system (Promega, USA) according to the manufacturer's instructions using the following mouse 14-3-3 σ specific primers: forward, 5′-CCTGCTGGACTCGCACCTCA-3′, and reverse, 5′-TGTCGGCTGTCCACAGCGTC-3′. The mouse 14-3-3 ζ gene was amplified using the following primers: forward, 5′- GCTGGTGCAGAAGGCCAAGC-3′, reverse, 5′-TCACCAGCAGCAACCTCGGC-3′. The mouse glyceroldehydes-3-phosphate dehydrogenase (GAPDH) gene was amplified as a control, using the following primers: forward, 5′-TGAAGGTCGGTGTGAACGCATTTGGC-3′, reverse, 5′-TTCTGGGTGGCAGTGATGGC-3′. PCR amplified bands (14-3-3 σ, 398 bp; 14-3-3 ζ, 350bp; GAPDH, 600 bp) were visualized on 1.2% agarose gels stained with ethidium bromide using Bio-Doc-ItTM system (Ultraviolet Products, USA).

DNA transfection and luciferase assay

Cells were transfected with FuGENE 6 (Roche, Germany) according to the manufacturer's instructions. To control for variation in transfection efficiency, cells were co-transfected with 0.2 μg of CMV-β-GAL, a eukaryotic expression vector in which *Escherichia coli* β-galactosidase (Lac Z) structural gene is under the transcriptional control of the CMV promoter. Luciferase reporter activity was assessed on a luminometer with a luciferase assay system (Promega, USA) according to the manufacturer's protocol. Transfection experiments were performed in triplicate with two independently isolated sets, and the results were averaged.

Immunoblot analysis

Cytosolic extracts were obtained in 1% Triton X-100 lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Western blotting was performed using anti-14-3-3 σ (C-18; Santa Cruz Biotechnology, USA), anti-14-3-3 ζ (C-16; Santa Cruz Biotechnology, USA), anti-p15 (C-20; Santa Cruz Biotechnology, USA), anti-p27 (F-8; Santa Cruz Biotechnology, USA), anti-cyclin D1 (C-20; Santa Cruz Biotechnology, USA), and anti-β-actin (AC-15; Sigma, USA) antibodies. Protein samples were heated at 95°C for 5 min and analyzed by SDS-PAGE. Immunoblot signals were developed using Super Signal Ultra chemiluminescence detection reagents (Pierce Biotechnology, USA).

Flow cytometric analysis

For flow cytometric assay (Chelli et al., 2004), cells were grown in six-well plates and incubated for 24 h at 37°C, and then treated with TGF-β1. After 24 h, cells were harvested and washed twice with PBS (pH 7.4). After fixing in 80% ethanol for 30 min, cells were washed twice and resuspended in PBS (pH 7.4) containing 0.1% Triton X-100, 5 μg/ml propodium iodide (PI), and then analyzed by a FACScan cytometer (Program Cell-Quest, BD Biosciences).

Statistical analysis

All data presented are expressed as means ± SD, and a representative of three or more independent experiments. Statistical analyses were by Student's *t*-test for paired data. Results were considered significant at $p < 0.05$.

RESULTS

Expression of 14-3-3 σ and 14-3-3 ζ in V12-Ras-transformed mammary epithelial cells

As a first step in evaluating the role of 14-3-3 family proteins in TGF-β1-induced cell growth inhibition, we generated EpRas, a transformed Eph4 mammary epithelial cell line that expresses oncogenic Ha-RasV12. Cell cycle profiling by FACS analysis revealed an increase in the number of Eph4 cells with G_0/G_1 DNA content after 12 h TGF-β1 treatment, whereas EpRas cells were refractory to the growth inhibitory effect of TGF-β1 (Fig. 1A). Consistent with these findings, TGF-β1 induced an increase in the level of cyclin-dependent kinase inhibitor $p27^{KIP1}$ in Eph4 cells that was completely abolished in EpRas cells (Fig. 1B). Because cell cycle dysregulation caused by changes in 14-3-3 protein expression has been implicated in cancer development, we next compared the expression levels of 14-3-3 ζ and 14-3-3 σ in Eph4 and EpRas cell lines. As shown in Figs. 1C and 1D, expression of 14-3-3 ζ was up-regulated and that of 14-3-3 σ was down-regulated at both mRNA and protein levels in EpRas cells compared with parental Eph4 cells. These results suggest that aberrant expression of 14-3-3 σ or 14-3-3 ζ

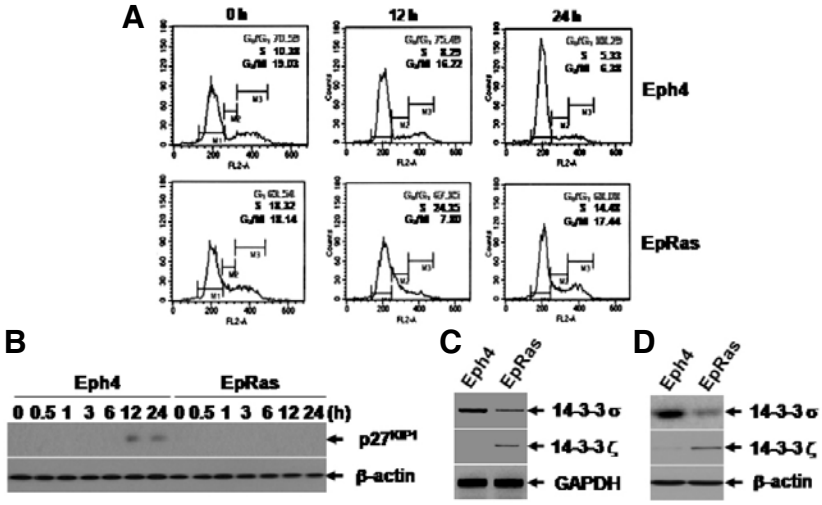

Fig. 1. Susceptibility to the TGF-β1 antiproliferative response and expression levels of endogenous 14-3-3 σ and 14-3-3 ζ in Eph4 and EpRas cells. (A) Eph4 and EpRas cells were incubated with TGF-β1 (5 ng/ml) for the indicated times. Cells were fixed, stained, and subjected to fluorescence-activated cell sorting analysis. The percent distribution in different stages of the cell cycle is shown. (B) Eph4 and EpRas cells were incubated with TGF-β1 (5 ng/ml) for the indicated times. Immunoblots of whole cell lysates were probed with antibody against p27, then stripped and reprobed with an anti-β-actin antibody. (C) Total RNA was prepared from Eph4 and EpRas cells and subjected to RT-PCR to measure 14-3-3 σ and 14-3-3 ζ expression levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed as a control. Expression levels of 14-3-3 σ, 14-3-3 ζ, and β-actin protein were analyzed by immunoblotting (lower panel).

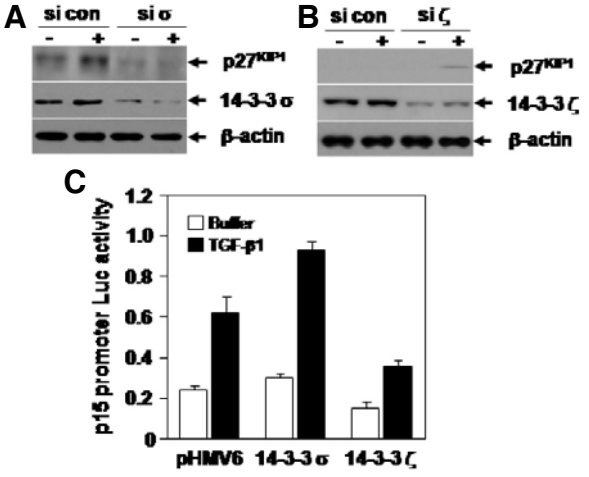

Fig. 2. 14-3-3 σ and 14-3-3 ζ have opposite effects on TGF-β1-induced p27 expression and p15-Luc activity. Eph4 (A) or EpRas (B) cells were transfected with control, 14-3-3 σ (A), or 14-3-3 ζ (B) siRNA oligonucleotides. After transfection, cells were incubated for 18 h before addition of TGF-β1 (5 ng/ml) for 24 h. The expression level of endogenous p27, 14-3-3 σ, 14-3-3 ζ, and β-actin was confirmed by immunoblot analysis of cell lysates using antibodies against p27, 14-3-3 σ, 14-3-3 ζ, and β-actin. (C) HepG2 cells were co-transfected with the p15-Luciferase reporter gene and vector control, 14-3-3 σ, or 14-3-3 ζ as indicated. After 16 h, cells were incubated with or without TGF-β1 (5 ng/ml) for a further 24 h before performing the luciferase assay. Luciferase activity represents the average of four independent transfection experiments.

may contribute to Ras-induced resistance to TGF-β1-mediated growth inhibitory effects in EpRas cells.

Roles of 14-3-3 σ and 14-3-3 ζ in TGF-β1-induced cell growth inhibition

To determine whether dysregulation of 14-3-3 σ or 14-3-3 ζ in EpRas cells causes loss of sensitivity to TGF-β1-mediated cell growth inhibition, we silenced the expression of each gene using small interfering RNA (siRNA) oligonucleotides specific to 14-3-3 σ or 14-3-3 ζ. TGF-β1-induced expression of $p27^{KIP1}$ was completely abolished in Eph4 cells transfected with siRNA 14-3-3 σ (Fig. 2A). In contrast, knock-down of 14-3-3 ζ in EpRas cells significantly rescued the suppression of $p27^{KIP1}$ induced by treatment with TGF-β1 (Fig. 2B). Consistent with these results, transient transfection of Eph4 cells with 14-3-3 σ markedly enhanced TGF-β1-induced $p15^{INK4B}$ reporter gene activity, whereas transient transfection of Eph4 cells with 14-3-3 ζ significantly reduced it (Fig. 2C).

14-3-3 ζ induces phosphorylation of Smad3 linker sites

Recent studies indicated that CDK phosphorylates Smad3 at regulatory linker regions, thus decreasing Smad3 activity and the antiproliferative function of TGF-β1 (Matsuura et al., 2004; 2005). We therefore examined the phosphorylation state of the regulatory linker regions of Smad3 in Eph4 and EpRas cells. As expected, cyclin D1 expression was much higher in EpRas cells than in Eph4 cells, whereas expression of p15 was completely blocked in EpRas cells (Fig. 3A). Under these conditions, phosphorylation of endogenous Smad3 at the linker sites Ser209 and Ser213 was markedly induced in EpRas cells compared with control Eph4 cells (Fig. 3A). We next examined whether phosphorylation of the Smad3 linker is induced by 14-3-3 ζ. As shown in Fig. 3B, transient transfection of Eph4 cells with 14-3-3 ζ induced phosphorylation at Ser209 and Ser213 residues in the Smad3 linker region. To further elucidate the role of 14-3-3 ζ, we transfected EpRas cells with siRNA specific for 14-3-3 ζ. Western blot analysis revealed that the phosphorylation of Smad3 at Ser209 and Ser213 in EpRas cells was substantially reduced by treatment with 14-3-3 ζ siRNA, but not by scrambled siRNA (Fig. 3C). These results indicate that Ras-induced phosphorylation of Smad3 linker regions is mediated by signaling events involving 14-3-3 ζ.

Mutation of Smad3 linker phosphorylation sites reduces inhibition of TGF-β1-induced cell growth inhibition by 14-3-3 ζ

To confirm that the inhibitory effect of 14-3-3 ζ on the antiproliferative function of TGF-β1 is mediated through induction of phosphorylation in the Smad3 linker region, we examined the effect of mutating the Smad3 14-3-3 ζ phosphorylation sites on expression of a p15 reporter gene. As shown in Fig. 4A, TGF-β1 induced p15 reporter gene activity and this effect was significantly abolished by 14-3-3 ζ. However, co-transfection of

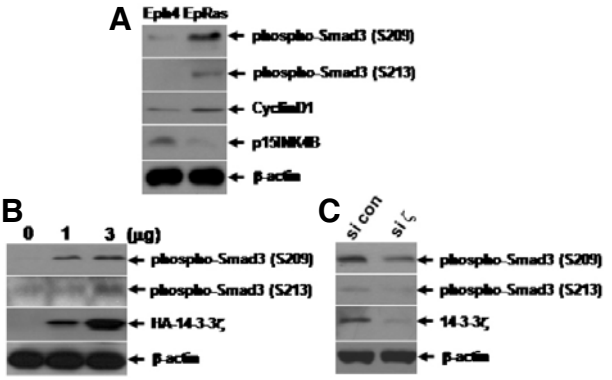

Fig. 3. 14-3-3 ζ-dependent phosphorylation of Smad3 linker sites in EpRas cells. (A) Total cell extracts were prepared from Eph4 and EpRas cells and phosphorylation of Ser^{209}, Ser^{213}, and $Ser^{423/425}$ was analyzed by immunoblotting with specific phosphopeptide antibodies. Cyclin D1 and p15 expression levels were also analyzed by immunoblotting. Eph4 cells were transiently transfected with the indicated quantities of 14-3-3 ζ (B), whereas EpRas cells were transiently transfected with control or 14-3-3 ζ siRNA oligonucleotide (C). Cells were harvested 30 h after transfection and phosphorylation of Ser^{209} and Ser^{213} was analyzed by immunoblotting with specific phosphopeptide antibodies. Expression levels of HA-tagged and endogenous 14-3-3 ζ were confirmed by immunoblotting analysis using anti-HA and anti-14-3-3 ζ antibodies respectively.

Mv1Lu cells with the Smad3 Thr179A/Ser204A/Ser209A triple mutant significantly blocked the 14-3-3 ζ-mediated inhibition of TGF-β1-induced p15 reporter gene activity (Fig. 4A). Likewise, FACS analysis showed that the significant increase in the G1 fraction in TGF-β1-stimulated Mv1Lu cells was markedly inhibited by 14-3-3 ζ and that the Smad3 triple mutant significantly reversed this effect of 14-3-3 ζ (Fig. 4B), indicating that phosphorylation of the Smad3 linker region by 14-3-3 ζ leads to a loss of susceptibility to TGF-β1-induced cell growth inhibition.

DISCUSSION

The high incidence of up- or down-regulation of 14-3-3 family proteins in a variety of cancers indicates that dysregulation of these proteins plays an important role in tumorigenesis. However, the roles of 14-3-3 proteins in the signaling pathway induced by the tumor suppressor TGF-β1 have never been addressed. Here, we show that 14-3-3 σ and 14-3-3 ζ perform opposite roles in the anti-proliferative activity of TGF-β1. Compared with parental Eph4 cells, EpRas cells that are resistant to the growth inhibitory effects of TGF-β1 showed decreased expression of 14-3-3 σ and increased expression of 14-3-3 ζ (Fig. 1C). Knockdown of 14-3-3 σ or overexpression of 14-3-3 ζ in Eph4 cells decreased the expression level of p27 and p15, whereas knockdown of 14-3-3 ζ in EpRas cells or overexpression of 14-3-3 σ in Eph4 cells had an opposite effect (Fig. 2), suggesting that 14-3-3 σ and 14-3-3 ζ play positive and negative roles respectively in the antiproliferative TGF-β1 signaling pathway.

Smad3 performs a key function in the growth inhibitory response of TGF-β1. The Akt-mTOR pathway inhibits the TGF-β signal by blocking phospho-activation of Smad3 (Song et al., 2006). 14-3-3 σ, which relays signals downstream of p53 in response to DNA damage, inhibits Akt-mediated cell growth, survival, and transformation (Yang et al., 2006). These reports strongly support our data suggesting that 14-3-3 σ positively mediates the inhibition of cell growth by TGF-β.

The Ras protein exhibits transforming properties in a variety of cells. It shifts TGF-β signaling from tumor suppression to oncogenesis (Sekimoto et al., 2007). Increasing evidences suggest that linker domain of Smad3 undergoes regulatory phosphorylation by Ras-associated kinases, including ERK1/2, cyclin-dependent kinase (CDK) 4, and Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) (Matsuura et al., 2004; 2005; Wicks et al., 2000). Linker phosphorylation of Smad3 indirectly inhibits its COOH-terminal phosphorylation (Matsuzaki et al., 2009) and abrogates both the transcriptional activity and the growth inhibitory function of Smad3 (Matsuura et al., 2004; 2005; Wicks et al., 2000). Cancer cells often express high levels of 14-3-3 ζ (Bajpai et al., 2005). Our data also show the increased level of 14-3-3 ζ in EpRas cells. In line with this, 14-3-3 ζ is known to associate with proteins involved in Ras signal, including Raf and phosphatidylinositol 3-kinase (PI3K) (Chang and Rubin, 1997; Munday et al., 2000), and regulate signaling mediated by Ras (Chang and Rubin, 1997). 14-3-3 ζ also control cell cycle

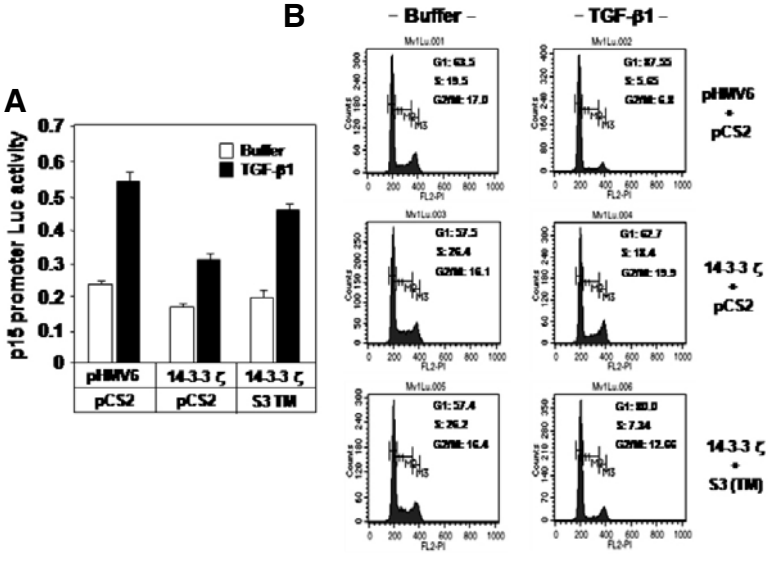

Fig. 4. Phosphorylation of Smad3 linker is required for inhibition of TGF-β1-induced expression of p15 and cell cycle G_1 arrest by 14-3-3 ζ. (A) HepG2 cells were co-transfected with p15-Luciferase reporter gene and the vector control, 14-3-3 ζ, or 14-3-3 ζ and the phosphorylation mutant of Smad3 as indicated. At 16 h after transfection, cells were incubated with or without TGF-β1 (5 ng/ml) for a further 24 h before performing the luciferase assay. Luciferase activity represents the average of four independent transfection experiments. (B) Eph4 cells were transfected with vector control, 14-3-3 ζ WT, or 14-3-3 ζ WT and the triple mutant of Smad3, S3(TM). S3(TM); Smad3 triple (Thr179A/Ser204A/Ser209A) mutant. At 16 h after transfection, cells were incubated with or without TGF-β1 (5 ng/ml) for a further 24 h. Cells were fixed, stained, and subjected to fluorescence-activated cell sorting analysis. The percent distribution in different stages of the cell cycle is shown.

progression by modulating CDK activity through interaction with Cdc25B (Mils et al., 2000). These reports indicate that 14-3-3 ζ function as a scaffold for and regulator of signaling proteins such as protein kinases, and raise a possibility that 14-3-3 ζ may functions as a negative regulator of Smad3 in TGF-β signaling. In further evidence supporting this hypothesis, our data show that the linker domain of Smad3 is phosphorylated by 14-3-3 ζ and mutation of the 14-3-3 ζ phosphorylation sites of Smad3 significantly abrogated the ability of 14-3-3 ζ to inhibit the growth inhibitory effects of TGF-β1. Although further studies are needed to clarify the mechanism by which 14-3-3 ζ induces phosphorylation of Smad3 linker sites, it is thought to be promoting via an indirect action of 14-3-3 ζ.

In summary, the data presented here provide the first evidence that 14-3-3 σ and 14-3-3 ζ plays an opposing role in TGF-β1-induced growth inhibition. Our findings suggest that 14-3-3 σ may be required for Smad3 to mediate the antiproliferative response of TGF-β1, whereas 14-3-3 ζ performs a negative role in the TGF-β1 growth inhibitory response by inducing phosphorylation of Smad3 at linker regions, inhibiting its transcriptional activity, decreasing expression of p15 and p27, and eventually facilitating cell cycle progression from G_1 to S phase. Our findings have important implications with respect to the molecular mechanisms underlying resistance to TGF-β1-mediated growth inhibition in cancer cells.

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