Biochemical and Biophysical Research Communications 447 (2014) 563–568

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

IKK-β/NF-κB p65 mediates p27^{Kip1} protein degradation in arsenite response

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ARTICLE INFO

Article history:
Received 3 April 2014
Available online 18 April 2014

Keywords:
Arsenite
p27^{Kip1} protein degradation
IKKβ
GSK
NF-κB

ABSTRACT

p27^{Kip1} is a potent inhibitor of the cyclin-dependent kinases that drive G1 to S phase transition. Since deregulation of p27^{Kip1} is found in many malignancies and is associated with the poor prognosis, elucidation of the molecular bases for regulation of p27^{Kip1} expression is of great significance, not only in providing insight into the understanding of biological p27^{Kip1}, but also in the development of new cancer therapeutic tactics. We here explored the inhibitory regulation of IKKβ on p27^{Kip1} expression following arsenite exposure. We found that although the basal level of p27^{Kip1} expression in the IKKβ$^{-/-}$ cells is much lower than that in the IKKβ$^{+/+}$ cells, the deletion of IKKβ in the MEFs led to a marked increase in p27^{Kip1} protein induction due to arsenite exposure in comparison to that in the IKKβ$^{+/+}$ cells. The IKKβ regulatory effect on p27^{Kip1} expression was also verified in the IKKβ$^{-/-}$ and IKKβ$^{-/-}$ cells with IKKβ reconstitutional expression, IKKβ$^{-/-}$ (IKKβ). Further studies indicated that IKKβ-mediated p27^{Kip1} downregulation occurred at protein degradation level via p65-dependent and p50-independent manner. Moreover, the results obtained from the comparison of arsenite-induced GSK3β activation among transfectants of WT, IKKβ$^{-/-}$ and IKKβ$^{-/-}$ (IKKβ), and the utilization of GSKβ shRNA, demonstrated that IKKβ regulation of p27 protein degradation was mediated by GSK3β following arsenite exposure.

1. Introduction

p27^{Kip1} protein, an inhibitor of G1 cyclin dependent kinase (CDK) plays a fundamental role in the regulation of key cellular processes, such as proliferation, differentiation, apoptosis, and motility [1,2]. The abundance of p27^{Kip1} protein, but not the mutation of the gene, plays an important role in tumor pathogenesis. Loss of expression or dysfunction of p27^{Kip1} promotes the G1→S transition, which has been reported in many human cancers, such as breast, colon, and lung carcinomas [3,4]. Furthermore, low expression of p27^{Kip1} protein is also associated with poor responses to cancer therapies and poor prognostic outcomes [3]. Thus, upregulation of p27^{Kip1} is thought to be one of the most promising new cancer therapeutic strategies. Indeed, several anticancer compounds including Src inhibitors, BCR-ABL inhibitors, HDAC inhibitors, and proteasome inhibitors that have been reported to upregulate p27^{Kip1} expression have been tested in either laboratory or clinical trials [5]. But the mechanisms of p27^{Kip1} expression are not fully elucidated.

Arsenic is well recognized as a therapeutic agent, for leukemia as well as for solid tumors [6,7]. However, the molecular mechanisms concerning the anti-cancer effect of arsenic are yet to be further clarified. Several studies reported that arsenic exposure results in cell cycle alteration, whereas the p27^{Kip1} regulatory effect on arsenic-induced cell cycles is paradoxical depending on the cell type and duration of the exposure. For an example, Park and his colleagues report that p27^{Kip1} expression is not altered in arsenic trioxide-induced cell cycle arrest and apoptosis in myeloma cells [8]. While another study demonstrates that p27^{Kip1} mRNA level is increased after a 4-month arsenic exposure [9]. Recently, arsenic trioxide is also reported to induce p27^{Kip1} expression in breast cancer cell line and hepatocellular carcinoma (HCC) cells [10,11]. However, the mechanisms underlying the regulation

Abbreviations: IKKβ, IκB kinase β; NF-κB, nuclear factor κB; Skp2, S-phase kinase associated protein 2; Kpc, Kip1 ubiquitination-promoting complex; CHX, cyclohexamide; GSK3, glycogen synthase kinase 3; Cul4a, Culin 4A, a core component for a ubiquitin ligase; Ro52, a member of the RING finger B-box coiled-coil (RBCC) motif family; NES, nuclear export signal.

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http://dx.doi.org/10.1016/j.bbrc.2014.04.055
0006-291X/

of p27^{Kip1} expression following arsenic exposure has not been explored yet.

I kappa B kinase β (IKKβ) is a major kinase that regulates nuclear factor κB (NF-κB) activation in cellular response to the pro-inflammatory and many stress stimuli by triggering the phosphorylation and degradation of the NF-κB inhibitor I-κB [12]. Several lines of evidence suggest that tumor cells show elevated activation of IKKβ and NF-κB [13,14]. Our published studies demonstrate that IKKβ/NF-κB activation plays an important role in the regulation of cell cycle transition and cell transformation due to arsenite exposure [15,16]. Does IKKβ and NF-κB play a role in p27^{Kip1} expression? In this study, we are trying to find out the regulatory mechanism of p27^{Kip1} in response to arsenite exposure. IKKβ/p65 was found to be able to inhibit p27^{Kip1} protein accumulation through a GSK3β-dependent manner following arsenite exposure.

2. Materials and methods

2.1. Plasmids, antibodies, and other reagents

The dominant active p65 mutant, p65Δnes, was a generous gift from Dr. Anna Bigas, Centre Oncologia Molecular, Institut de Recerca Oncologica [17]. The full-length HA-tagged IKKβ (HA-IKKβ) construct was described in our previous study [18]. The GSK3β shRNA constructs were a kindly gift from Dr. Wancai Yang, Department of Pathology, University of Illinois at Chicago [19]. The antibody against-IKKβ was purchased from Cell Signaling Technology (Beverly, MA). The antibodies against p65, p50, p27, GSK3β, p-GSK3β were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Anti-β-actin antibody and LiCl were purchased from Sigma–Aldrich (St. Louis, MO). CHX was purchased from Calbiochem (San Diego, CA).

2.2. Cell culture and transfections

IKKβ$^{-/-}$, p50$^{-/-}$, p65$^{-/-}$, their reconstitutional expression MEFs, and their corresponding wild-type (WT) MEFs were established, as described, in our previous studies [18]. All MEFs were maintained in a cell culture incubator with DMEM (Calbiochem, San Diego, CA) supplemented with 10% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine (Life Technologies). Cell transfections were performed with FuGENE® HD Transfection Reagent (Roche Applied Science) according to the manufacturer's instruction. For stable transfection, cells were subjected to hygromycin B selection, and the cells that survived from the drug selection were pooled as a stable mass culture. These stable transfectants were cultured in the hygromycin B-free medium for at least two passages prior to experiments.

2.3. Western blotting

Cells were exposed to arsenite for various time periods, and whole cell extracts were prepared using the cell lysis buffer (10 mM Tris–HCl, pH 7.4, 1% SDS, and 1 mM Na_3VO_4), and 40 μg of proteins were subjected to Western blotting, as described in our previous reports[15].

2.4. RT-PCR

Total RNA was extracted with Trizol reagent (Invitrogen), and cDNAs were synthesized with ThermoScript RT-PCR system (Invitrogen). The mouse β-actin and p27^{Kip1} cDNA fragment were amplified by primers 5′-gacgatgatattgccgcact-3′, 5′-gataccacgcttgctctgag-3′ and 5′-tcacttggaggttctgaagattctgt-3′, 5′-tgcaccccagatttttgcccagt-3′ respectively.

3. Results

3.1. Deletion of IKKβ resulted in a marked p27^{Kip1} induction following arsenite exposure

To determine the potential role of IKKβ in the regulation of p27^{Kip1} expression, IKKβ$^{-/-}$ and its corresponding WT cells were exposed to arsenite, and the p27^{Kip1} protein expression levels were compared between the two cell lines. The results showed that deletion of IKKβ in MEFs (IKKβ$^{-/-}$) resulted in a marked increased induction of p27^{Kip1} protein expression in comparison to those in WT MEFs following arsenite treatment (Fig. 1A), suggesting that IKKβ expression may provide an inhibitory effect on p27^{Kip1} protein induction by arsenite. This notion was further verified by the results obtained from stably transfection of IKKβ into IKKβ$^{-/-}$ MEFs, IKKβ$^{-/-}$ (IKKβ) (Fig. 1B). The results indicated that the restoration of IKKβ expression in IKKβ$^{-/-}$ eliminated the increased p27^{Kip1} protein expression due to arsenite exposure (Fig. 1B).

3.2. IKKβ impedes p27^{Kip1} protein induction by arsenite through promoting its degradation

To determine whether IKKβ regulates p27^{Kip1} protein expression at the transcriptional level, p27^{Kip1} mRNA expression was compared between WT, IKKβ$^{-/-}$, and IKKβ$^{-/-}$ (IKKβ) cells. The results showed that p27^{Kip1} mRNA from either non-treated or arsenite-treated cells did not show observable differences (Fig. 2A). These results excluded the possibility of IKKβ having a regulatory effect on p27^{Kip1} expression by changing its transcription or mRNA stability upon arsenite exposure.

To test whether such regulation occurred at post-translational level, both WT and IKKβ$^{-/-}$ cells were pre-treated with MG132 for 12 h and the cells was then treated with DMEM containing cycloheximide (CHX) only or CHX together with arsenite for various time points for determination of p27^{Kip1} protein expression.

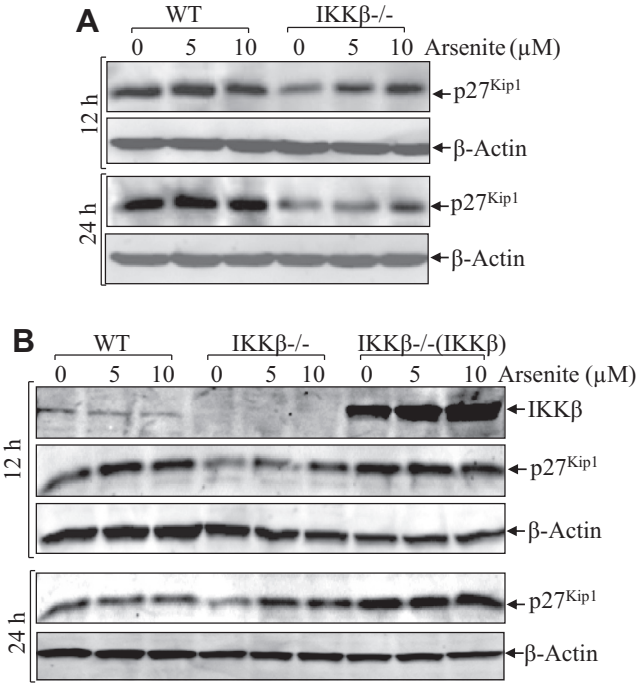

Fig. 1. IKKβ inhibited p27^{Kip1} protein expression due to arsenite exposure. WT, IKKβ$^{-/-}$, and IKKβ$^{-/-}$ (IKKβ) cells were exposed to arsenite for 12 h and 24 h, and the cell extracts were subjected to Western blotting to determine protein expression of p27^{Kip1} and IKKβ. β-Actin was used as a protein loading control.

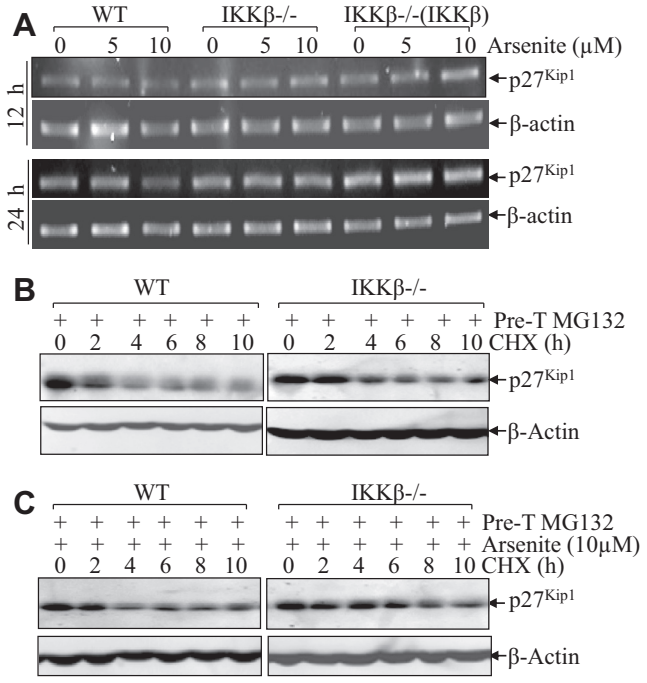

Fig. 2. IKKβ regulated $p27^{Kip1}$ protein degradation following arsenite exposure. (A) WT, IKKβ$^{-/-}$, and the reconstituted IKKβ$^{-/-}$ (IKKβ) cells were treated with arsenite, and the expression of $p27^{Kip1}$ mRNA was evaluated by PT-PCR. (B and C) WT and IKKβ$^{-/-}$ cells were pre-treated with MG132 (20 μM) for 12 h. The cell culture medium was replaced with 0.1% FBS DMEM containing CHX (10 μM) (B) or 0.1% FBS DMEM containing CHX (10 μM) and arsenite (10 μM) (C) for time period as indicated. The cells were extracted and subjected to Western blotting for determination of $p27^{Kip1}$ protein expression. β-Actin was used as a protein loading control.

As shown in Fig. 2B, $p27^{Kip1}$ protein degradation was observed at 2 h and thereafter in incubation of CHX in WT cells, whereas this degradation was delayed in IKKβ$^{-/-}$ cells. Moreover, arsenite co-treatment dramatically impaired $p27^{Kip1}$ protein degradation in IKKβ$^{-/-}$ cells, whereas it only showed a slightly inhibition of the $p27^{Kip1}$protein degradation in WT cells (Fig. 2C). Collectively, our results strongly suggested that IKKβ-regulated $p27^{Kip1}$ protein expression by arsenite occurred at protein degradation.

3.3. NF-κB p65, but not p50, is a downstream mediator for arsenite-induced $p27^{Kip1}$ protein up-regulation in IKKβ$^{-/-}$ cells

Our previous studies demonstrated that NF-κB p50 plays a key role in IKKβ-mediated GADD45α protein de-ubiquitination and accumulation [18]. To test whether p50 also plays a role in regulation of $p27^{Kip1}$ protein expression due to arsenite exposure, NF-κB p50 knockout MEFs (p50$^{-/-}$) were employed. The results indicated that p50 deficiency, identified by Western blotting, as shown in Fig. 3A, showed a slight inhibitory effect on $p27^{Kip1}$ protein expression following arsenite exposure as compared with that in p50$^{+/+}$ cells (Fig. 3B). We next determined whether NF-κB p65 was involved in the regulation of $p27^{Kip1}$ protein expression following arsenite treatment. As shown in Fig. 3C and D, knockout of NF-κB p65 resulted in a significant increase in $p27^{Kip1}$ protein expression, indicating that NF-κB p65 had a similar effect of IKKβ on $p27^{Kip1}$ protein expression in arsenite responses.

NF-κB p65 nuclear-cytoplasmic shuttling and its subcellular localization are regulated by its NES domain [17]. To further investigate whether the export nuclear of NF-κB p65 affects p27 protein degradation, NF-κB p65 mutant (p65Δnes) with deletion of NES domain, which localizes exclusively in the nucleus, and keeps in an activated status, was stably transfected into IKKβ$^{-/-}$ cells. The stable transfectant, IKKβ$^{-/-}$ (p65Δnes), was exposed to arsenite to determine its effect on $p27^{Kip1}$ protein expression. The results indicated that the reconstitutional expression of p65Δnes in IKKβ$^{-/-}$ cells blocked the $p27^{Kip1}$ protein induction by arsenite even though the basal level of $p27^{Kip1}$ protein was increased. These results suggested that the expression activated NF-κB p65 (p65Δnes) could restore the effect of IKKβ in regulation of $p27^{Kip1}$ protein expression (Fig. 3E), therefore suggesting that NF-κB p65, but not p50, acts as mediator in IKKβ regulation of $p27^{Kip1}$ protein expression following arsenite treatment.

3.4. IKKβ regulates GSK3β phosphorylation and GSK3β was essential for $p27^{Kip1}$ induction following arsenite treatment

$p27^{Kip1}$ is reported as being a critical downstream mediator of cell cycle arrest associated with GSK3β inhibition in myeloid/lymphoid or mixed lineage leukemia (MLL) cells [20]. Since GSK3β activation is negatively regulated by its phosphorylation and GSK3β exerts its regulatory effect by acting as a kinase and phosphorylating its targeted proteins, here we determine GSK3β protein phosphorylation following arsenite treatment in WT, IKKβ$^{-/-}$, and IKKβ$^{-/-}$ (IKKβ) cells. As shown in Fig. 4A and B, the basal level of GSK3β phosphorylation was markedly elevated in IKKβ$^{-/-}$ cells as compared with that in either WT or IKKβ$^{-/-}$ (IKKβ) cells. After arsenite treatment, GSK3β phosphorylation was downregulated in IKKβ$^{-/-}$ cells, while there was no observable effect on GSK3β protein phosphorylation in comparison to that in either WT or IKKβ$^{-/-}$ (IKKβ) cells (Fig. 4A and B), suggesting that IKKβ-deletion resulted in an inhibition of GSK3β phosphorylation, in turn leading to GSK3β kinase activation. Moreover, co-treatment of cells with LiCl, a specific inhibitor of GSK3β, showed an upregulation of GSK3β phosphorylation (Fig. 4B). Consistent with alterations of GSK3β activation status, $p27^{Kip1}$ protein expression and its phosphorylation was markedly upregulated in IKKβ$^{-/-}$ cells in comparison to those in either WT or IKKβ$^{-/-}$ (IKKβ) cells, whereas LiCl treatment impaired the upregulation of $p27^{Kip1}$ protein expression in IKKβ$^{-/-}$ cells following arsenite exposure (Fig. 4B). These results suggested that GSK3β might be a mediator for IKKβ regulation of $p27^{Kip1}$ protein expression following arsenite exposure.

To further explore the role of GSK3β in the $p27^{Kip1}$ modulation, we transfected GSK3β shRNA into HeLa cells, and detected its regulatory effect on $p27^{Kip1}$ expression following arsenite exposure. As expected, $p27^{Kip1}$ protein induction by arsenite was remarkably induced in GSK3β knockdown cells compared with that in control transfectant (Fig. 4C). Moreover, we transfected the constitutive active mutant (GSK3βS9A) and the constitutive inactive mutant (GSK3βKD) constructs [21] into IKKβ$^{-/-}$ cells and identified the transfectants, as shown in Fig. 4D. The results showed that exogenous expression of active mutant GSK3βS9A in IKKβ$^{-/-}$ cells led to an inhibition of $p27^{Kip1}$ protein induction by arsenite, whereas functional inactivation of GSK3β by introduction of GSK3β KD in IKKβ$^{-/-}$ cells resulted in an increase in $p27^{Kip1}$ protein expression following arsenite exposure in comparison to that in vector control transfectants, IKKβ$^{-/-}$ (pCDNA3) (Fig. 4E). These results strongly indicated that GSK3β was essential for IKKβ inhibition of $p27^{Kip1}$ protein expression.

4. Discussion

Our present study revealed that IKKβ deficiency led to remarkable increase in $p27^{Kip1}$ protein expression due to arsenite exposure in comparison to that observed in IKKβ$^{+/+}$ cells. The IKKβ regulatory effect on $p27^{Kip1}$ expression was also verified in the IKKβ$^{-/-}$ and IKKβ reconstitutional expressed in IKKβ$^{-/-}$ cells, IKKβ$^{-/-}$ (IKKβ). Further studies indicated that IKKβ promoted

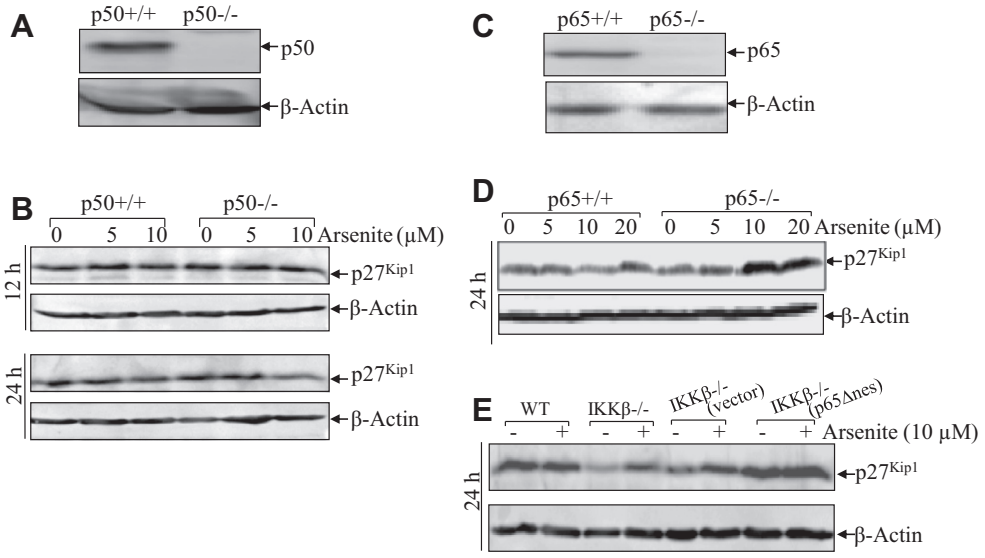

Fig. 3. p65 and not p50 mediated IKKβ inhibition $p27^{Kip1}$ protein expression following arsenite exposure. (A and C) Identification of p50 protein expression and p65 protein expression in their specific gene knockout cells. (B, D and E) $p50^{+/+}$ and $p50^{-/-}$ cells (B), $p65^{+/+}$ and $p65^{-/-}$ cells (D) WT, IKKβ$^{-/-}$, and IKKβ$^{-/-}$ (p65Δnes) cells (E) were exposed to arsenite and cell extracts were subjected to Western blotting as indicated. β-Actin was used as a protein loading control.

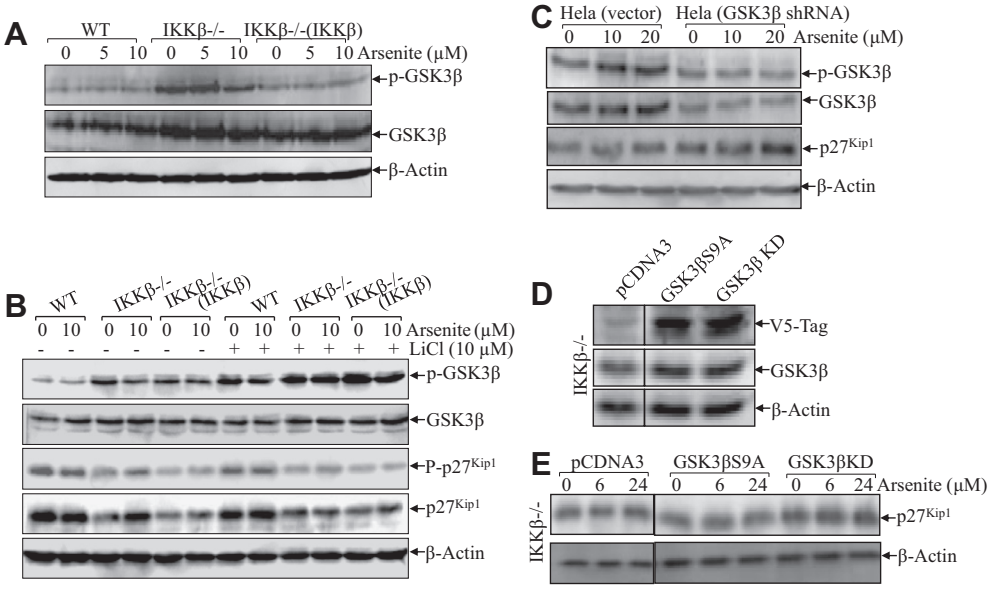

Fig. 4. GSKβ was a mediator for IKKβ inhibition $p27^{Kip1}$ protein expression following arsenite exposure. (A & B) WT, IKKβ$^{-/-}$, and IKKβ$^{-/-}$ (IKKβ) cells were exposed to 10 μM arsenite (A) or arsenite combining 10 μM LiCl (B) and cell extracts were subjected to Western blotting as indicated. β-Actin was used as a protein loading control. (C) HeLa cells were transfected with either GSK3β ShRNA or control vector, and then followed by arsenite treatment, as indicated. The cell extracts were subjected to Western blotting as indicated. β-Actin was used as a protein loading control. (D) The identification of transfection of different GSK3β constructs into IKKβ$^{-/-}$ cells. (E) IKKβ$^{-/-}$ (pcDNA3), IKKβ$^{-/-}$ (GSK3βS9A), and IKKβ$^{-/-}$ (GSK3βKD) were exposed to 10 μM arsenite treatment and cell extracts were subjected to Western blotting as indicated. β-Actin was used as a protein loading control.

$p27^{Kip1}$ protein degradation via p65- and GSK3β-dependent manner.

In the previous studies, arsenite has been indicated as affecting the expression of cell cycle related proteins in several cells. However, the mechanisms underlying the regulation of $p27^{Kip1}$ expression following arsenite exposure have not been well explored. Our previous study indicates that arsenite exposure leads to cell cycle progression and cell transformation in IKKβ/NF-κB transcriptional-dependent pathway [16,18,22]. Those studies shed light on the important role of IKKβ/NF-κB pathway in the arsenite biological functions. In the current study, we found that IKKβ plays a negative regulatory role in arsenite-induced $p27^{Kip1}$ protein expression, evidenced by the results that arsenite only induces high level of $p27^{Kip1}$ induction in IKKβ$^{-/-}$ cells as compared to that in WT cells, and that exogenous expression of IKKβ in IKKβ$^{-/-}$ cells reduces $p27^{Kip1}$ protein expression. By comparison of $p27^{Kip1}$ protein expression between $p50^{+/+}$ and $p50^{-/-}$ cells or $p65^{+/+}$ and $p65^{-/-}$ cells following arsenite treatment, we found that p65 but not p50 is able to inhibit $p27^{Kip1}$ protein expression. By observation that exogenous expression of dominant active p65 (p65Δnes) in IKKβ$^{-/-}$ MEFs inhibits arsenite-induced $p27^{Kip1}$ protein expression, we found that IKKβ inhibits $p27^{Kip1}$ protein expression by arsenite is via a p65-dependent manner. Although several transcription factors act on the CDKN1B promoter [23,24], and several proteins and microRNAs regulate CDKN1B translation [25], our further studies demonstrate that IKKβ modulates $p27^{Kip1}$ protein expression via

promoting $p27^{Kip1}$ protein degradation, rather than inhibiting its mRNA transcription or protein translation following arsenite exposure.

$p27^{Kip1}$ protein degradation is mainly regulated through a ubiquitination-proteasome dependent pathway [3]. Several $p27^{Kip1}$ protein degradation mechanisms have been defined, and the related proteins such as the S-phase kinase-associated protein 2 (Skp2) [26], Cullin 4A (CUL4A) [27], Ro52 [28], and the ubiquitin ligase Kip1 ubiquitination-promoting complex (KPC) [29] were reported to be involved in the respective $p27^{Kip1}$ degradation pathway. In this study, we excluded the possibility of the involvement of the above proteins in IKKβ regulation of $p27^{Kip1}$ degradation following arsenite exposure (Supplementary data).

GSK3β was initially identified as a protein kinase participates in the regulation of glycogen biosynthesis. Recently it has been recognized as a key component of a diverse range of cellular functions essential for survival. Several investigators report that inactivation of GSK3β results in tumor cell cycle arrest and apoptosis, and the both are due to the increase of CDK inhibitors including $p27^{Kip1}$ [30,31]. On the contrary, in hexamethylene bisacetamide (HMBA) stimulation, GSK3β expression in the nuclear is in accordance with the $p27^{Kip1}$ induction [32]. Whether GSK3β plays a positive or negative role in $p27^{Kip1}$ expression seems to depend on the specific study circumstances. In the present study, $p27^{Kip1}$ can be induced by arsenite only in the HeLa cells with GSK3β knockdown. In addition, when GSK3β functional active and negative constructs are transfected into $IKK\beta^{-/-}$ MEFs, only the GSK3β constitutively active cells obtain lower $p27^{Kip1}$ protein expression. We found that arsenite-induced GSK3β phosphorylation was downregulated in $IKK\beta^{-/-}$ cells in comparison to those in either WT or $IKK\beta^{-/-}$ (IKKβ) cells, whereas its basal level in $IKK\beta^{-/-}$ cells was higher than that in WT and $IKK\beta^{-/-}$ (IKKβ) cells. It was noted that GSK3β phosphorylation levels had a completely reversed association with $p27^{Kip1}$ protein expression in WT, $IKK\beta^{-/-}$ and $IKK\beta^{-/-}$ (IKKβ) cells, strongly indicating that GSK3β might be implicated in IKKβ regulation of $p27^{Kip1}$ protein expression due to arsenite exposure. This notion was greatly supported by the results obtained from utilization of either GSK3β chemical inhibitor LiCl or GSK3β shRNA. Moreover, by ectopic expression of constitutive active mutant (GSK3βS9A) and constitutive inactive mutant (GSK3βKD) of GSK3β into $IKK\beta^{-/-}$ cells, we identified that GSK3β was essential for IKKβ inhibition of $p27^{Kip1}$ protein expression. Thus, our results indicate that GSK3β phosphorylation and its inactivation plays an essential role in the IKKβ/p65-mediated $p27^{Kip1}$ protein degradation following arsenite treatment.

NF-κB has been reported to be a downstream modulator of GSK3β activity [33]. However, Saegusa and colleagues report that NF-κB/p65 participates in the inhibition of β-catenin transcription, and thereby affecting GSK3β expression [34]. Our current study shows that IKKβ/NF-κB p65 acted as an upstream regulator for GSK3β phosphorylation and inactivation. We anticipate that the crosstalk between NF-κB/p65 and GSK3β pathways might play an important role in maintaining cell biological function, and abnormal of the crosstalk between NF-κB/p65 and GSK3β pathways might contribute to cancer development upon carcinogenic exposure. Thus, the present identification of a substantial IKKβ/NF-κB p65/GSK3β regulation of $p27^{Kip1}$ protein degradation will provide a significant insight into understanding of biological effect of IKKβ/NF-κB p65, as well as its crosstalk with the GSK3β pathway, in arsenite-induced cellular responses.

Acknowledgments

This work was partially supported by grants from NIH/NCI RO1 CA112557 and RO1 CA177665; NIH/NIEHS ES000260; and NSFC81229002.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.055.

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