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IKK- β /NF κ B p65 Mediates p27^{kip1} Protein Degradation in Arsenite Response

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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 IKK β on p27 expression following arsenite exposure. We found that although the basal level of p27 expression in the IKK β ^{-/-} cells is much lower than that in the IKK β ^{+/+} cells, the deletion of IKK β in the MEFs led to a marked increase in p27^{kip1} protein induction due to arsenite exposure in comparison to that in the IKK β ^{+/+} cells. The IKK β regulatory effect on p27 expression was also verified in the IKK β ^{-/-} and IKK β ^{-/-} cells with IKK β reconstituted expression, IKK β ^{-/-}(IKK β). Further studies indicated that IKK β -mediated p27 downregulation occurred at protein degradation level via p65-dependent and p50-independent manner. Moreover, the results obtained from the comparison of arsenite-induced GSK3 β activation among transfectants of WT, IKK β ^{-/-} and IKK β ^{-/-}(IKK β), and the utilization of GSK3 β shRNA, demonstrated that IKK β regulation of p27 protein degradation was mediated by GSK3 β following arsenite exposure.

Keywords

Arsenite; p27^{kip1} protein degradation; IKK β ; GSK; NF κ B

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

I kappa B kinase β (IKK β) is a major kinase that regulates nuclear factor κ B (NF- κ B) activation in cellular response to the pro-inflammatory and many stress stimuli by triggering the phosphorylation and degradation of the NF- κ B inhibitor I- κ B [12]. Several lines of evidence suggest that tumor cells show elevated activation of IKK β and NF- κ B [13,14]. Our published studies demonstrate that IKK β /NF- κ B activation plays an important role in the regulation of cell cycle transition and cell transformation due to arsenite exposure [15,16]. Does IKK β and NF- κ B plays 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. IKK β /p65 was found to be able to inhibit p27^{Kip1} protein accumulation through a GSK3 β -dependent manner following arsenite exposure.

MATERIALS AND METHODS

Plasmids, antibodies, and other reagents

The dominant active p65 mutant, p65^{res}, was a generous gift from Dr. Anna Bigas, Centre Oncologia Molecular, Institut de Recerca Oncologica [17]. The full-length HA-tagged IKK β (HA-IKK β) construct was described in our previous study [18]. The GSK3 β shRNA constructs were a kindly gift from Dr. Wancai Yang, Department of Pathology, University of Illinois at Chicago [19]. The antibody against-IKK β was purchased from Cell Signaling

Technology (Beverly, MA). The antibodies against p65, p50, p27, GSK3 β , p-GSK3 β 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).

Cell culture and transfections

IKK $\beta^{-/-}$, p50 $^{-/-}$, p65 $^{-/-}$, their reconstituted 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.

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 Na₃VO₄), and 40 μ g of proteins were subjected to Western blotting, as described in our previous reports[15].

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'-tcacttgaggttctgaagattctgt-3', 5'-tgcacccagattttgcccagt-3' respectively.

RESULTS

Deletion of IKK β resulted in a marked p27^{kip1} induction following arsenite exposure

To determine the potential role of IKK β in the regulation of p27^{kip1} expression, IKK $\beta^{-/-}$ 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 IKK β in MEFs (IKK $\beta^{-/-}$) 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 IKK β 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 IKK β into IKK $\beta^{-/-}$ MEFs, IKK $\beta^{-/-}$ (IKK β) (Fig. 1B). The results indicated that the restoration of IKK β expression in IKK $\beta^{-/-}$ eliminated the increased p27^{kip1} protein expression due to arsenite exposure (Fig. 1B).

IKK β impedes p27^{kip1} protein induction by arsenite through promoting its degradation

To determine whether IKK β regulates p27^{kip1} protein expression at the transcriptional level, p27^{kip1} mRNA expression was compared between WT, IKK $\beta^{-/-}$, and IKK $\beta^{-/-}$ (IKK β) 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 IKK β 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 IKK $\beta^{-/-}$ cells were pre-treated with MG132 for 12 hrs and the cells were then treated with DMEM containing cycloheximide (CHX) only or CHX together with arsenite for various time points for determination of p27^{kip1} protein expression. As shown in Fig. 2B, p27^{kip1} protein degradation was observed at 2 hr and thereafter in incubation of CHX in WT cells, whereas this degradation was delayed in IKK $\beta^{-/-}$ cells. Moreover, arsenite co-treatment dramatically impaired p27^{kip1} protein degradation in IKK $\beta^{-/-}$ 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 IKK β -regulated p27^{kip1} protein expression by arsenite occurred at protein degradation.

NF κ B p65, but not p50, is a downstream mediator for arsenite-induced p27^{kip1} protein up-regulation in IKK $\beta^{-/-}$ cells

Our previous studies demonstrated that NF- κ B p50 plays a key role in IKK β -mediated GADD45 α 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- κ B 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- κ B p65 was involved in the regulation of p27^{kip1} protein expression following arsenite treatment. As shown in Figs. 3C and 3D, knockout of NF- κ B p65 resulted in a significant increase in p27^{kip1} protein expression, indicating that NF- κ B p65 had a similar effect of IKK β on p27^{kip1} protein expression in arsenite responses.

NF κ B p65 nuclear-cytoplasmic shuttling and its subcellular localization are regulated by its NES domain [17]. To further investigate whether the export nuclear of NF- κ B p65 affects p27 protein degradation, NF κ B p65 mutant (p65^{nes}) with deletion of NES domain, which localizes exclusively in the nucleus, and keeps in an activated status, was stably transfected into IKK $\beta^{-/-}$ cells. The stable transfectant, IKK $\beta^{-/-}$ (p65^{nes}), was exposed to arsenite to determine its effect on p27^{kip1} protein expression. The results indicated that the reconstitutive expression of p65^{nes} in IKK $\beta^{-/-}$ 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 κ B p65 (p65^{nes}) could restore the effect of IKK β in regulation of p27^{kip1} protein expression (Fig. 3E), therefore suggesting that NF κ B p65, but not p50, acts as mediator in IKK β regulation of p27^{kip1} protein expression following arsenite treatment.

IKK β regulates GSK3 β phosphorylation and GSK3 β 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 GSK3 inhibition in myeloid/lymphoid or mixed lineage leukemia (MLL) cells [20]. Since GSK3 activation is negatively regulated by its phosphorylation and GSK3 exerts its regulatory effect by acting as a kinase and phosphorylating its targeted proteins, here we determine GSK3 β protein phosphorylation following arsenite treatment in WT, IKK $\beta^{-/-}$, and IKK $\beta^{-/-}$ (IKK β) cells. As shown in Figs. 4A and 4B, the basal level of GSK3 β phosphorylation was markedly elevated in IKK $\beta^{-/-}$ cells as compared with that in either WT or IKK $\beta^{-/-}$ (IKK β) cells. After arsenite treatment, GSK3 β phosphorylation was downregulated in IKK $\beta^{-/-}$ cells, while there was no observable effect on GSK protein phosphorylation in comparison to that in either WT or IKK $\beta^{-/-}$ (IKK β) cells (Figs. 4A and 4B), suggesting that IKK β -deletion resulted in an inhibition of GSK3 β phosphorylation, in turn leading to GSK3 β kinase activation. Moreover, co-treatment of cells with LiCl, a specific inhibitor of GSK3 β , showed an upregulation of GSK3 β phosphorylation (Fig. 4B). Consistent with alterations of GSK3 β activation status, p27^{kip1} protein expression and its phosphorylation was markedly upregulated in IKK $\beta^{-/-}$ cells in comparison to those in either WT or IKK $\beta^{-/-}$ (IKK β) cells, whereas LiCl treatment impaired the upregulation of p27^{kip1} protein expression in IKK $\beta^{-/-}$ cells following arsenite exposure (Fig. 4B). These results suggested that GSK3 β might be a mediator for IKK β regulation of p27^{kip1} protein expression following arsenite exposure.

To further explore the role of GSK3 β in the p27^{kip1} modulation, we transfected GSK3 β 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 GSK3 β knockdown cells compared with that in control transfectant (Fig. 4C). Moreover, we transfected the constitutive active mutant (GSK3 β S9A) and the constitutive inactive mutant (GSK3 β KD) constructs [21] into IKK $\beta^{-/-}$ cells and identified the transfectants, as shown in Fig. 4D. The results showed that exogenous expression of active mutant GSK3 β S9A in IKK $\beta^{-/-}$ cells led to an inhibition of p27^{kip1} protein induction by arsenite, whereas functional inactivation of GSK3 β by introduction of GSK3 β KD in IKK $\beta^{-/-}$ cells resulted in an increase in p27^{kip1} protein expression following arsenite exposure in comparison to that in vector control transfectants, IKK $\beta^{-/-}$ (pCDNA3) (Fig. 4E). These results strongly indicated that GSK3 β was essential for IKK β inhibition of p27^{kip1} protein expression.

DISCUSSION

Our present study revealed that IKK β deficiency led to remarkable increase in p27^{kip1} protein expression due to arsenite exposure in comparison to that observed in IKK $\beta^{+/+}$ cells. The IKK β regulatory effect on p27^{kip1} expression was also verified in the IKK $\beta^{-/-}$ and IKK β reconstituted expressed in IKK $\beta^{-/-}$ cells, IKK $\beta^{-/-}$ (IKK β). Further studies indicated that IKK β promoted p27^{kip1} protein degradation via p65- and GSK3 β -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 IKK β /NF κ B transcriptional-dependent pathway [16,18,22]. Those studies shed light on the important role of IKK β /NF κ B pathway in the arsenite biological functions. In the current study, we found that IKK β 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 IKK β ^{-/-} cells as compared to that in WT cells, and that exogenous expression of IKK β in IKK β ^{-/-} 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 (p65^{res}) in IKK β ^{-/-} MEFs inhibits arsenite-induced p27^{Kip1} protein expression, we found that IKK β 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 IKK β 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 IKK β regulation of p27^{Kip1} degradation following arsenite exposure (supplementary data).

GSK3 β 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 GSK3 β 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 (HMB) stimulation, GSK3 β expression in the nuclear is in accordance with the p27^{Kip1} induction [32]. Whether GSK3 β 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 GSK3 β knockdown. In addition, when GSK3 β functional active and negative constructs are transfected into IKK β ^{-/-} MEFs, only the GSK3 β constitutively active cells obtain lower p27^{Kip1} protein expression. We found that arsenite-induced GSK3 β phosphorylation was downregulated in IKK β ^{-/-} cells in comparison to those in either WT or IKK β ^{-/-} (IKK β) cells, whereas its basal level in IKK β ^{-/-} cells was higher than that in WT and IKK β ^{-/-} (IKK β) cells. It was noted that GSK3 β phosphorylation levels had a completely reversed association with p27^{Kip1} protein expression in WT, IKK β ^{-/-} and IKK β ^{-/-} (IKK β) cells, strongly indicating that GSK3 β might be implicated in IKK β regulation of p27^{Kip1} protein expression due to arsenite exposure. This notion was greatly supported by the results obtained from utilization of either GSK3 β chemical inhibitor LiCl or GSK3 β shRNA. Moreover, by ectopic expression of constitutive active mutant (GSK3 β S9A) and constitutive inactive mutant (GSK3 β KD) of

GSK3 β into IKK $\beta^{-/-}$ cells, we identified that GSK3 β was essential for IKK β inhibition of p27^{kip1} protein expression. Thus, our results indicate that GSK3 β phosphorylation and its inactivation plays an essential role in the IKK β /p65-mediated p27^{kip1} protein degradation following arsenite treatment.

NF κ B has been reported to be a downstream modulator of Gsk3 β activity [33]. However, Saegusa and colleagues report that NF κ B/p65 participates in the inhibition of β -catenin transcription, and thereby affecting GSK3 β expression [34]. Our current study shows that IKK β /NF κ B p65 acted as an upstream regulator for GSK3 β phosphorylation and inactivation. We anticipate that the crosstalk between NF κ B/p65 and GSK3 β pathways might play an important role in maintaining cell biological function, and abnormal of the crosstalk between NF κ B/p65 and GSK3 β pathways might contribute to cancer development upon carcinogenic exposure. Thus, the present identification of a substantial IKK β /NF κ B p65/GSK3 β regulation of p27^{kip1} protein degradation will provide a significant insight into understanding of biological effect of IKK β /NF κ B p65, as well as its crosstalk with the GSK3 β pathway, in arsenite-induced cellular responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The abbreviations used are

IKKβ	I κ B kinase β
NF-κB	nuclear factor κ B
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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As3+ induce p27 in $I\kappa\kappa\beta^{-/-}$ cells
 $I\kappa\kappa\beta^{-/-}$ /P65 impedes p27 induction
Gsk3 β participates

Figure 1. IKKβ inhibited p27^{kip1} protein expression due to arsenite exposure
 WT, IKKβ^{-/-}, and IKKβ^{-/(IKKβ)} cells were exposed to arsenite for 12h and 24h, 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.

Figure 2. IKK β regulated p27^{kip1} protein degradation following arsenite exposure (A), WT, IKK β ^{-/-}, and the reconstituted IKK β ^{-/-}(IKK β) cells were treated with arsenite, and the expression of p27^{kip1} mRNA was evaluated by PT-PCR. (B & C) WT and IKK β ^{-/-} cells were pre-treated with MG132 (20 μ M) for 12 hrs. The cell culture medium was replaced with 0.1% FBS DMEM containing CHX (10 μ M) (B) or 0.1% FBS DMEM containing CHX (10 μ M) and arsenite (10 μ M) (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.

Figure 3. p65 and not p50 mediated IKK β inhibition p27^{kip1} protein expression following arsenite exposure

(A & C) Identification of p50 protein expression and p65 protein expression in their specific gene knockout cells. (B, D, & E) p50^{+/+} and p50^{-/-} cells (B), p65^{+/+} and p65^{-/-} cells (D) WT, IKK β ^{-/-}, and IKK β ^{-/-}(p65^{Δnes}) 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.

Figure 4. GSK3β 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.