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Involvement of PKA and Sp1 in the induction of $p27^{Kip1}$ by tamoxifen

Te-Hsiu Lee[a], Hui-Chiu Chang[b], Lea-Yea Chuang[c], Wen-Chun Hung[d,*]

[a]*Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan, ROC*
[b]*Department of Physiology, Kaohsiung Medical University, Kaohsiung 807, Taiwan, ROC*
[c]*Department of Biochemistry, Kaohsiung Medical University, Kaohsiung 807, Taiwan, ROC*
[d]*School of Technology for Medical Sciences, Kaohsiung Medical University, No. 100, Shih-Chuan 1st Road, Kaohsiung 807, Taiwan, ROC*

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Abstract

We have previously shown that tamoxifen (Tam) inhibits proliferation of estrogen receptor-negative human non-small cell lung cancer cells and this inhibition is associated with induction of $p27^{Kip1}$. In this study, we investigated the mechanism by which Tam increases $p27^{Kip1}$ expression. Because intracellular $p27^{Kip1}$ protein level is mainly controlled *via* posttranslational regulation, we first tested whether Tam might affect protein stability of $p27^{Kip1}$. Metabolic labeling and pulse chase assays showed that Tam did not affect the half-life of this protein. We next examined whether Tam enhanced $p27^{Kip1}$ expression through transcriptional activation. Our results demonstrated that Tam directly stimulated the $p27^{Kip1}$ promoter in lung cancer cells. Deletion and mutation analysis revealed that two Sp1 consensus sites located between −545 and −532 bp from the transcription start site were crucial for the induction of $p27^{Kip1}$ by Tam. Conversely, mutation in a CTF site (−525/−520) nearby these two Sp1 sites had little effect. Electromobility shift assays showed that Sp1 transcription factor bound to these consensus sites and the DNA binding activity of Sp1 was enhanced by Tam. Our data also demonstrated that induction of $p27^{Kip1}$ by Tam was inhibited by protein kinase A inhibitor H89, but not by protein kinase C inhibitor calphostin C and mitogen-activated kinase kinase inhibitor PD98059. Taken together, our results suggest that Tam transcriptionally activates $p27^{Kip1}$ expression *via* the Sp1 consensus sites in the $p27^{Kip1}$ promoter and PKA is involved in this process.

Keywords: Tamoxifen; $p27^{Kip1}$; Protein kinase A; Sp1; Lung cancer

1. Introduction

Tam is not only an effective drug used for the treatment of breast cancer but also a potent agent used for the prevention of invasive breast cancer in women at increased risk for the disease [1]. Accumulating evidences support the notion that Tam exerts its biological effect by competitive inhibition of estradiol binding to estrogen receptors (ERs) [2]. However, this drug has also been reported to inhibit growth of ER-negative cancer cells including lung cancer, melanoma and brain tumor [3–5]. The existence of a non-ER mediated anti-cancer mechanism could be clinically important because recent studies demonstrated that a high proportion of ER-negative patients that respond to adjuvant Tam. However, the molecular mechanism of such ER-independent action of Tam is largely unknown.

Our previous results have demonstrated that Tam effectively inhibited growth of ER-negative human non-small cell lung cancer (NSCLC) cells [6]. We found that Tam treatment reduced enzymatic activity of cyclin E-associated kinases and induced G1 growth arrest in these cells. Our results also demonstrated that Tam did not affect the expression of G1-acting cyclins or cyclin-dependent kinases (CDKs) in these cells. On the contrary, Tam potently up-regulated the expression of CDK inhibitors (CDKIs) $p21^{Waf1}$ and $p27^{Kip1}$ and increased the formation of CDKI–CDK–cyclin complexes in lung cancer cells. Our results suggest that induction of CDKIs may be a possible mechanism responsible for Tam-induced growth inhibition in ER-negative cancer cells.

* Corresponding author. Tel.: +886-7-3121101x2199; fax: +886-7-2259573.
E-mail address: hung1228@ms10.hinet.net (W.-C. Hung).

Abbreviations: PKA, protein kinase A; PKC, protein kinase C; Tam, tamoxifen; ER, estrogen receptor; NSCLC, non-small cell lung cancer; EMSA, electromobility shift assay; MEK, mitogen-activated kinase kinase.

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$p27^{Kip1}$ was cloned as a binding protein with cyclin E-CDK2 [7] or cyclin D-CDK4 [8]. This CDKI acts during the transition from G1 to S phase of the cell cycle to inhibit G1 cyclin/CDK activities [9]. Overexpression of $p27^{Kip1}$ blocks cell cycle progression in G1 phase and this CDKI appears to be involved in G1 growth arrest induced by a number of extracellular stimuli such as transforming growth factor-β1 (TGF-β1), cyclic AMP (cAMP) and retinoic acid [10–12]. Recent studies have shown that $p27^{Kip1}$ is involved in the tumorigenesis of many types of cancer [13]. Although mutations of this CDKI are found rarely in human cancers [14], abnormally low level of the $p27^{Kip1}$ protein are observed frequently in human cancers. Moreover, down-regulation of $p27^{Kip1}$ correlates with reduced survival among patients with lung [15], breast [16], prostate [17] and gastric carcinoma [18].

Recent studies have shown that the protein level of $p27^{Kip1}$ in cells is mainly regulated posttranslationally by the ubiquitin-proteasome pathway [19,20]. The $p27^{Kip1}$ protein is phosphorylated by the cyclin E/CDK2 complex, which leads to subsequent ubiquitination [21]. Ligation of ubiquitin and protein requires the sequential action of three enzymes. Activation of ubiquitin is achieved by a specific activating enzyme, E1, and activated ubiquitin is transferred to E2, a ubiquitin carrier protein. Finally, ubiquitin is linked to a protein substrate by a ubiquitin-protein ligase (E3) and polyubiquitinated proteins are degraded by the 26S proteasome [22]. Conversely, little is known about the transcriptional control of the $p27^{Kip1}$ gene. In this study, we attempt to elucidate the mechanism by which Tam activates the expression of $p27^{Kip1}$. Our data suggest that Tam directly induces $p27^{Kip1}$ expression *via* transcriptional activation and protein kinase A (PKA) and Sp1 transcription factor are involved in this process.

2. Materials and methods

2.1. Cell culture and reagents

H358 human lung cancer cells were cultured in Dulbecco's modified Eagle's medium and F12 nutrition mixture (DMEM/F12) supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics in a 5% CO_2 incubator. Tam was purchased from Sigma. $p27^{Kip1}$ antibody and protein A/G-agarose were purchased from Santa Cruz Biotechnology. Luciferase assay system was obtained from Promega.

2.2. Plasmids

The promoter region of human $p27^{Kip1}$ gene was subcloned into the *Xho*I site of the pGL2 basic vector (Promega) to create the p27PF luciferase reporter plasmid. Deletion and point mutation constructs of p27PF including p27KpnI, p27ApaI, p27MB-435, p27SacII, p27mSp1-1, p27mSp1-2 and p27mCTF were generated as described previously [23,24] and were kindly provided by Dr. Sakai.

2.3. Luciferase assays

Cells were plated onto 6-well plates at a density of 100,000 cells/well and grown overnight. Cells were transfected with 2 μg of plasmids by the LipofectAMINE method [25]. After transfection, cells were incubated with vehicle (0.2% ethanol) or Tam (10 μM) for 24 hr and luciferase activities were assayed. Luciferase activities were normalized for the amount of proteins in cell lysates and were calculated as an average of three independent experiments.

2.4. Metabolic labeling, pulse chase and immunoprecipitation

Cells (1×10^6) were plated in 75 cm^2 flasks and grown overnight. Cells were incubated in the absence or presence of Tam (10 μM) in 10% FCS medium for 24 hr. After incubation, cells were washed with PBS and metabolically labeled with ^{35}S-methionine (100 μCi/mL) in methionine-free medium for 1 hr and then chased with 10% FCS medium containing 100 μg/mL unlabeled methionine for 1, 3 or 6 hr. After incubation, cells were rinsed twice with ice-cold PBS and harvested in a lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 1 mg/mL aprotinin, 2 μg/mL pepstatin A and 2 μg/mL leupeptin) for 20 min on ice. Equal amount of proteins was subjected to immunoprecipitation with anti-$p27^{Kip1}$ antibody for 3 hr at 4°. Protein A/G-agarose was added to collect the antigen–antibody complexes at 4° for another 1 hr and the immunoprecipitates were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Radiolabeled $p27^{Kip1}$ was visualized by autoradiography and the intensity of the signals was analyzed by a densitometer.

2.5. Oligonucleotide labeling, nuclear extract preparation and electromobility shift assay (EMSA)

A double-stranded 20-bp oligonucleotide corresponding to bases −548 to −529 of wild type $p27^{Kip1}$ promoter (5′-GCGGGGCGGCTCCCGCCGCC-3′ and 3′-CGCCCCGCCGAGGGCGGCGG-5′) and a mutant oligonucleotide in which two Sp1 binding sites were simultaneously mutated (5′-GCGGGATGGCTCCTACCGCC-3′ and 3′-CGCCCTACCGAGGATGGCGG-5′) were synthesized. Two hundred nanograms of wild type oligonucleotide was end-labeled with γ^{32}P-ATP by using a 5′-end labeling system (Promega). Preparation of nuclear extracts from control or Tam-treated H358 cells were carried out as described previously [26]. In brief, H358 cells were plated in

75 cm^2 flasks and grown to 70–80% confluence. Cells were incubated in the absence or presence of 10 μM of Tam for 24 hr. Cells were then washed with cold PBS and lysed in cold buffer A (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 0.5 mM dithiothreitol, 1.5 mM $MgCl_2$, and 0.2 mM PMSF). Cells were spun down and extracted in cold buffer B (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM PMSF) on ice for 20 min. Cellular debris was clarified and protein concentrations were determined. EMSAs were performed at 4°. The reaction mixture for EMSA contained 20 mM Tris–HCl, pH 7.6, 1 mM dithiothreitol, 2 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 μg of poly(dI-dC) and 5–8 μg of nuclear extract. Non-labeled wild type or mutant oligonucleotides or antibodies were added and incubated for 10 min. ^{32}P-Labeled oligonucleotide (50,000 cpm) was added to the mixture and the binding reaction was allowed to proceed at 4° for another 20 min. The mixtures were resolved on 0.5× TBE, 6% polyacrylamide gel at 200 V for 2 hr. Gels were dried and autoradiography was performed.

3. Results

3.1. Tam treatment on the half-life of $p27^{Kip1}$ protein

Recent studies suggest that regulation of $p27^{Kip1}$ is generally controlled by posttranslational modification. Therefore, we first examined whether Tam treatment might change the half-life of $p27^{Kip1}$. Cells were metabolically labeled with ^{35}S-methionine in methionine-free medium and then chased with 10% FCS medium containing 100 μg/mL of unlabeled methionine for different times. Figure 1 indicated that the turnover rate of $p27^{Kip1}$ was similar in cells treated with vehicle or Tam and the estimated half-life is about 2.5 hr. These data suggest that Tam does not modulate the expression of $p27^{Kip1}$ at posttranslational level.

3.2. Up-regulation of $p27^{Kip1}$ by Tam via Sp1 concensus sites in the promoter region

We next investigated whether Tam might directly activate $p27^{Kip1}$ gene expression. The effect of Tam on the $p27^{Kip1}$ promoter activity was investigated by using the promoter-luciferase constructs. The human $p27^{Kip1}$ promoter (3.6 kb) construct p27PF was transfected into H358 cells and Tam-induced luciferase activity was determined. We found that a 3.4-fold increase of luciferase activity was induced by Tam (Fig. 2A). To determine the regions in the $p27^{Kip1}$ promoter responsible for the induction by Tam, a series of 5′-deletion mutants based on the p27PF construct were used (Fig. 2B) and Tam-induced luciferase activity was measured. Figure 2A showed that deletion constructs (p27KpnI and p27ApaI) consisting of 1797 or 774 bp

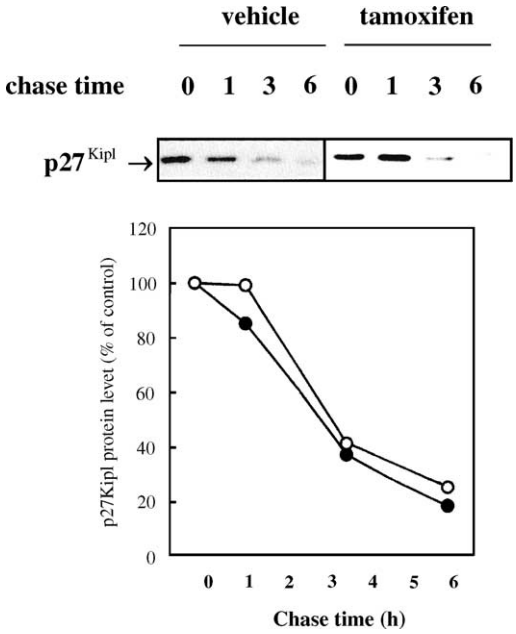

Fig. 1. Effect of Tam on protein stability of $p27^{Kip1}$ in lung cancer cells. Cells were treated with vehicle (0.2% ethanol, ●) or Tam (10 μM, ○) in 10% FCS medium for 24 hr and metabolically labeled with ^{35}S-methionine in methionine-free medium for 1 hr and then chased with 10% FCS medium containing 100 μg/mL methionine for different times. Cells were harvested with a lysis buffer and equal amount of cellular proteins was immunoprecipitated with $p27^{Kip1}$ antibody. Radiolabeled $p27^{Kip1}$ was resolved by SDS–PAGE and autoradiography was performed. The intensity of signals was determined by a densitometer and graphical representation of the quantitative data for the radiolabeled $p27^{Kip1}$ remaining in each sample of a typical experiment was shown. Another three independent experiments showed similar results.

proximal to the transcription start site were responsive to Tam. In contrast, Tam could not activate deletion constructs p27MB-435 and p27SacII which containing 435 and 311 bp proximal to the transcription start site. Therefore, the Tam-responsive element is located between −774 and −435 bp in the $p27^{Kip1}$ promoter. Previous study has shown that several transcription factor binding sites including Sp1, Ap2, CTF and ATF site were found in this region [23]. Furthermore, two Sp1 sites (−544 and −534) and a CTF site (−522) located within this region were identified to be the elements for basal promoter activity and were found to mediate the effect of Vitamin D_3 on the induction of $p27^{Kip1}$ [24]. Therefore, we tested whether these transcription factor binding sites might play a role in the induction of $p27^{Kip1}$ by Tam. Three mutant constructs p27mSp1-1, p27mSp1-2 and p27mCTF with mutations in the Sp1-1 site (−544), the Sp1-2 site (−534) and the CTF site (−522) were transfected into H358 cells and Tam-induced luciferase activity was measured. We found that Tam-activated p27mCTF to a level similar to that of p27PF (Fig. 3). On the contrary, mutation in the Sp1-1 site (−544) or in the Sp1-2 site (−534) significantly reduced the luciferase activity induced by Tam. In addition, reduction of Tam-induced promoter activity is more significant in p27mSp1-1-transfected cells. Our results suggest that two

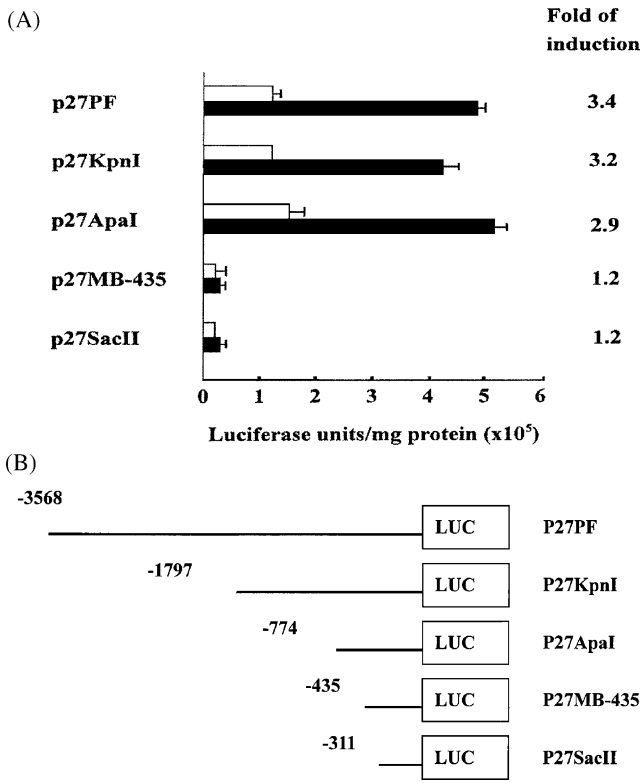

Fig. 2. Deletion analysis of the human $p27^{Kip1}$ promoter. (A) $p27^{Kip1}$ full length or deletion promoter-reporter constructs were transfected into H358 cells and cells were incubated with (0.2% ethanol, □) or Tam (10 μM, ■) for 24 hr. Luciferase activity was determined by a luciferase assay system and normalized for the amount of protein in cell lysates. Fold induction was calculated by comparing the luciferase activity of cells treated with Tam and vehicle. (B) Diagram showing the deletion mutants used in the experiments. Results from three independent experiments were shown as mean ± SD. Standard errors were expressed as error bars in the figure.

Sp1 sites located between −545 and −532 bp in the promoter region of $p27^{Kip1}$ are necessary for the induction by Tam. We also attempted to elucidate the nuclear factors that interacted with the Sp1 sites located between −545 and −532 bp in the $p27^{Kip1}$ promoter. Oligonucleotides corresponding to this region of the promoter were synthesized and EMSAs were performed. One major DNA–protein complex was detected in the autoradiograms and the intensity of this band was increased by Tam treatment (Fig. 4A). These data suggest that Tam enhances the DNA binding activity of nuclear proteins. Additionally, binding of the nuclear factors to the Sp1 sites located between −545 and −532 bp is sequence-specific because the major band could be competed away by an excess of unlabeled wild type oligonucleotides, but not by an excess of mutant oligonucleotides in which the two Sp1 sites were mutated simultaneously. We next tested whether the nuclear factors that bound to this region are Sp1-related transcription factors. As shown in Fig. 4B, in the presence of anti-Sp1 antibody but not anti-Sp3 antibody, the complex was supershifted. We conclude that Sp1 binds to the Sp1 sites located between −545 and −532 bp in the $p27^{Kip1}$ promoter.

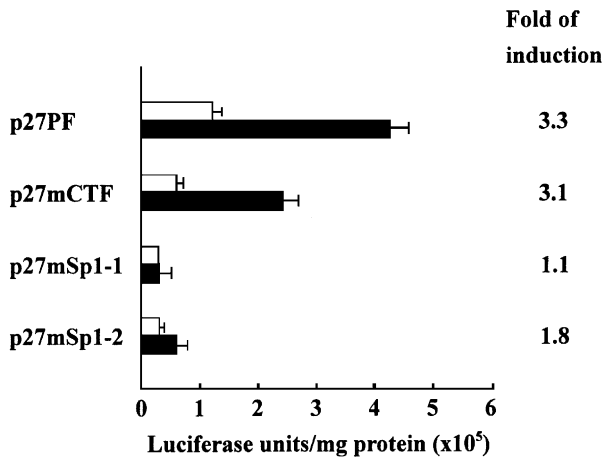

Fig. 3. Mutation analysis of the human $p27^{Kip1}$ promoter. p27PF or mutant constructs p27mSp1-1, p27mSp1-2 and p27mCTF containing mutations in Sp1 sites or a CTF site in the $p27^{Kip1}$ promoter were transfected into H358 cells and cells were incubated with vehicle (0.2% ethanol, □) or Tam (10 μM, ■) for 24 hr. Luciferase activity was determined by a luciferase assay system and normalized for the amount of protein in cell lysates. Fold induction was calculated by comparing the luciferase activity of cells treated with Tam and vehicle. Results from three independent experiments were shown as mean ± SD. Standard errors were expressed as error bars in the figure.

3.3. Involvement of PKA in the induction of $p27^{Kip1}$ by Tam

To clarify the signaling pathway that mediated the effect of Tam on $p27^{Kip1}$ induction, cells were pretreated with

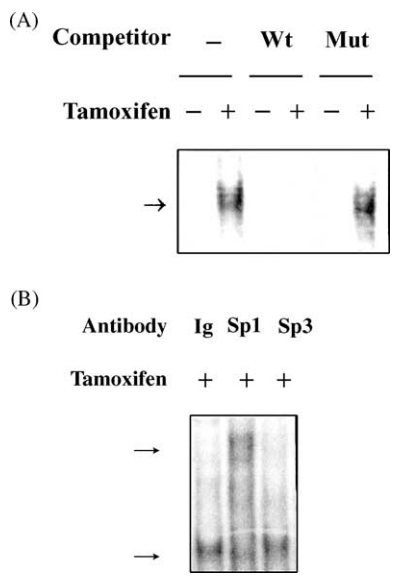

Fig. 4. Identification of the nuclear proteins that interact with the Sp1 consensus sites located between −545 and −532 bp in the $p27^{Kip1}$ promoter. (A) EMSA was carried out with nuclear extracts prepared from control or Tam-treated H358 cells. Nuclear extracts were incubated with ^{32}P-labeled oligonucleotides corresponding to bases −548 to −529 of wild type $p27^{Kip1}$ promoter in the absence (−) or presence (+) of 20-fold amounts of wild type (Wt) or mutant (Mut) oligonucleotides. Arrow indicated the major complex formed in EMSA experiments. (B) Effects of preimmune immunoglobulin (Ig), Sp1 or Sp3 antibody on the formation of the DNA–protein complex. Arrow indicated the major complex and the supershifted complex in EMSA experiments.

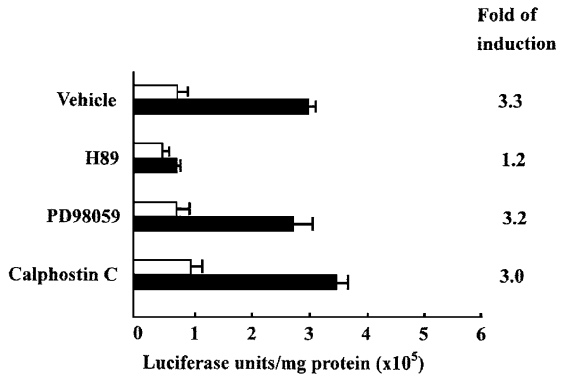

Fig. 5. Effect of selective kinase inhibitors on Tam-induced $p27^{Kip1}$ promoter activity. Cells were transfected with p27PF plasmid and were preincubated with H89 (PKA inhibitor, 10 μM) or PD98059 (mitogen-activated kinase kinase (MEK) inhibitor, 10 μM) or calphostin C (protein kinase C (PKC) inhibitor, 50 nM) for 1 hr and then stimulated with vehicle (0.2% ethanol, □) or Tam (10 μM, ■) for 24 hr. Luciferase activity was determined by a luciferase assay system and normalized for the amount of protein in cell lysates. Fold induction was calculated by comparing the luciferase activity of cells treated with Tam and vehicle. Results from three independent experiments were shown as mean ± SD. Standard errors were expressed as error bars in the figure.

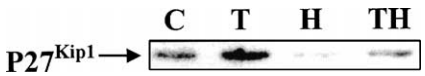

Fig. 6. Inhibition of Tam-induced $p27^{Kip1}$ protein expression by H89. Cells cultured in 10% FCS medium were pretreated with H89 (10 μM) for 1 hr and stimulated with Tam (10 μM) for 24 hr. Cellular proteins were extracted and $p27^{Kip1}$ protein level was detected by immunoblotting as described in Section 2. C: control; T: tamoxifen; H: H89; TH: tamoxifen + H89.

different kinase inhibitors and stimulated with 10 μM Tam. Tam-induced $p27^{Kip1}$ promoter activity was investigated. Our results showed that only H89, a specific PKA inhibitor, could suppress $p27^{Kip1}$ promoter activity activated by Tam (Fig. 5). In accordance with this result, Tam-induced increase of $p27^{Kip1}$ protein was inhibited by H89 (Fig. 6). H89 alone had minor effect on basal $p27^{Kip1}$ expression. These results suggest that PKA is involved in the induction of $p27^{Kip1}$ by Tam.

4. Discussion

In this study, we show that Tam transcriptionally activates $p27^{Kip1}$ expression through the Sp1 consensus sites in the promoter region. Recently, regulation of $p27^{Kip1}$ protein level *via* posttranslational modification has been studied intensively. Phosphorylation of $p27^{Kip1}$ on a single threonine residue by the cyclin E/CDK2 complex stimulate ubiquitination and degradation of this protein *via* the ubiquitin-proteasome pathway [19,20]. Additionally, translational control of $p27^{Kip1}$ has also been reported. Increase of $p27^{Kip1}$ protein synthesis has been described in HL60 cells arrested in G0 by TPA [27] or in fibroblasts arrested by contact inhibition [28]. However, little is known about the transcriptional regulation of the $p27^{Kip1}$ gene. Kamiyama *et al.* [29] indicated that a CTF site, which is bound by the ubiquitous transcription factor NF-Y, plays an important role in the regulation of $p27^{Kip1}$ basal promoter activity. Moro *et al.* [30] demonstrated that interferon-α 2b induced $p27^{Kip1}$ expression through two interferon regulatory factor (IRF) binding sites in the $p27^{Kip1}$ promoter. Another work showed that granulocyte colony stimulating factor stimulated $p27^{Kip1}$ expression in a STAT-3-dependent manner [31]. Furthermore, transcriptional activation of $p27^{Kip1}$ can be induced by the forkhead transcription factors [32]. Conversely, c-*myc* may inhibit $p27^{Kip1}$ expression through transcriptional repression [33]. Our results reveal a new mechanism of regulation of $p27^{Kip1}$ gene transcription *via* Sp1 consensus sites. Interestingly, Inoue *et al.* [24] reported recently that these two Sp1 sites, but not Vitamin D receptor, are involved in the induction of $p27^{Kip1}$ by Vitamin D_3 during U397 cell differentiation. Study of the functional role of Sp1 in the regulation of $p27^{Kip1}$ is helpful for the understanding of the alteration of $p27^{Kip1}$ expression under various physiological or pathological circumstances.

Our previous data has demonstrated that H358 cells do not express ER-α and -β [6]. In addition, our recent data also showed that estrogen could not modulate the expression of cyclin D1, a ER-regulated gene, in H358 cells (data not shown). Therefore, induction of $p27^{Kip1}$ by Tam in H358 cells is independent of ER. Results of this work suggest that Tam may activate PKA to increase Sp1 transcriptional activity and stimulate $p27^{Kip1}$ expression *via* Sp1 sites. Lines of evidence support this notion. First, a recent study has demonstrated that PKA could phosphorylate Sp1 and stimulate its DNA binding and transcriptional activity *in vitro* and *in vivo* [34]. We also found that Tam stimulated Sp1 phosphorylation in a PKA-dependent manner in H358 cells (unpublished results). Second, our results demonstrated that PKA inhibitor H89 specifically inhibited $p27^{Kip1}$ expression in H358 cells. Thus, these data suggest that Tam activates $p27^{Kip1}$ expression *via* the PKA/Sp1 pathway and this work provides a molecular basis for the anti-proliferative effect of Tam on ER-negative cancer cells.

To date, a number of studies have shown that loss of $p27^{Kip1}$ in many types of cancer is linked with high tumor grades and poor patient outcome [15–18,20]. Recent studies also demonstrated that high proportion of NSCLC tumor tissues exhibited significantly reduced $p27^{Kip1}$ immunoreactivity and the expression of this CDKI was found to be a favorable prognostic factor for patient survival [35,36]. It is rational to speculate that natural or synthetic agents which can activate $p27^{Kip1}$ expression may be helpful for the treatment of lung cancer. Indeed, two recent studies demonstrated that Tam in combination with chemotherapeutic drugs is useful for the treatment of NSCLC [37,38]. These results suggest that Tam is a useful drug for treatment or prevention of lung cancer.

Acknowledgments

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