RESEARCH ARTICLE

8-60hIPP5<sup>m</sup>-Induced G2/M Cell Cycle Arrest Involves Activation of ATM/p53/p21<sup>cip1/waf1</sup> Pathways and Delayed Cyclin B1 Nuclear Translocation

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Abstract

Protein phosphatase 1 (PP1) is a major serine/threonine phosphatase that controls gene expression and cell cycle progression. The active mutant IPP5 (8-60hIPP5<sup>m</sup>), the latest member of the inhibitory molecules for PP1, has been shown to inhibit the growth of human cervix carcinoma cells (HeLa). In order to elucidate the underlying mechanisms, the present study assessed overexpression of 8-60hIPP5<sup>m</sup> in HeLa cells. Flow cytometric and biochemical analyses showed that overexpression of 8-60hIPP5<sup>m</sup> induced G2/M-phase arrest, which was accompanied by the upregulation of cyclin B1 and phosphorylation of G2/M-phase proteins ATM, p53, p21<sup>cip1/waf1</sup> and Cdc2, suggesting that 8-60hIPP5<sup>m</sup> induces G2/M arrest through activation of the ATM/p53/p21<sup>cip1/waf1</sup>/Cdc2/cyclin B1 pathways. We further showed that overexpression of 8-60hIPP5m led to delayed nuclear translocation of cyclin B1. 8-60hIPP5<sup>m</sup> also could translocate to the nucleus in G2/M phase and interact with pp1α and Cdc2 as demonstrated by co-precipitation assay. Taken together, our data demonstrate a novel role for 8-60hIPP5m in regulation of cell cycle in HeLa cells, possibly contributing to the development of new therapeutic strategies for cervix carcinoma.

Keywords: G2/M arrest - HeLa cell - IPP5 - nuclear translocation - signal transduction

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Introduction

Reversible protein phosphorylation regulates the biological activity of many protein complexes, which is regarded as a major mechanism of the control of cell cycle progression. It has been reported that the semi selective inhibitors of PPase, such as okadaic acid, cantharidin, and fostriecin, influence several aspects of cell cycle progression (Cohen, 2002). An important Ser/Thr protein phosphatase, protein phosphatase-1 (PP1), regulates a series of physiological events, such as cell cycle, gene expression, protein synthesis, glycolipid metabolism and memory formation (Ceulemans and Bollen, 2004). Its critical function in mitosis is evidenced by the occurrence of metaphase arrest in various eukaryotic cells after PP1 mutation or inhibition (Booher and Beach, 1989; Ohkura et al., 1989; Kinoshita et al., 1990). The genetic and microtubule dynamics experiments also indicate that PP1 activity is necessary for the completion of mitosis (Cheng et al., 2000). Furthermore, abnormally high expression of PP1 was observed in some tumor cells, indicating that PP1 might speed up the growth of malignant tumors (Sogawa et al., 1996).

Cervical cancer is the leading cause of cancer-related death among women in most countries. It has been known that the high prevalence of HPV infection occurs in cervical cancer, and the two commonest HPV genotypes in cervical cancer were HPV 16 and 18 (Li et al., 2013; Raub et al., 2014). Viral DNA integration into the host genome thereby deregulating host tumor suppressor proteins p53 and pRb via E6 and E7 proteins (Cheah et al., 2012). It is known that the E6 and E7 genes of oncogenic HPV-16 and -18 play important roles in the early stages of malignant transformation and immortalization of cervical epithelial cells (Woodworth et al., 1989). The inactivation of p53 and pRb by the E6 and E7 proteins, respectively, is an important step in maintaining abnormal cell proliferation through the disruption of normal cell cycle checkpoints (Boyer et al., 1996). HPV-E6 protein forms a complex with E6-AP and subsequently interacts with p53, leading to ubiquitin dependent proteasomal degradation (Subramanian and Chinnappan, 2013). E7 oncoprotein binds with pRb which controls the G1-S transition in cell cycle (Zhang and Tang, 2012).

Protein phosphatase inhibitor-1 (PPI-1) is the first endogenous molecule found to inhibit PP1 activity,...
when phosphorylated by protein kinase A (PKA) at Thr-35 (Nimmo and Cohen, 1978). However, when Thr-35 is mutated to Asp, PPI-1 could inhibit the activity of PP1 without phosphorylation by PKA. The active mutant of IPP5 (8-60hIPP5m), the latest member of the inhibitory molecules for PP1, has been demonstrated to inhibit the activity of PP1 in vitro with a similar IC50 as PPI-1 (Wang et al., 2008). Previous studies from our laboratory have shown that 8-60hIPP5+m significantly inhibited the growth of human cervix carcinoma cells (HeLa) by inducing G2/M arrest (Zeng et al., 2012). Now, we report that 8-60hIPP5+m-induced G2/M arrest was accompanied by the upregulation and phosphorylation of G2/M-phase proteins ATM, p53, p21<sup>WAF1</sup>, Cdc2 and cyclin B1. These findings suggest that active mutant IPP5 induces G2/M arrest through activation of ATM/<sup>p34</sup> and cyclin B1 pathways. We also found that the overexpression of 8-60hIPP5+m led to cyclin B1 delayed nuclear translocation. These results suggest that 8-60hIPP5+m might be a functional growth inhibitor for cervix cancer cells. The mechanism for its inhibition could involve cell cycle regulation.

**Materials and Methods**

**Reagents and cell culture**

Human cervix carcinoma cells (HeLa) (ATCC, Manassas, VA, USA) were grown in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (HyClone, Logan, UT, USA), 4.5 g/liter D-glucose, nonessential amino acids (100 μM each), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine at 37°C in a 5% CO2 incubator. The wild type human IPP5 plasmid, pcDNA3.1/myc-His(-)-B-hIPP5 (referred as phIPP5-B later), and active mutant hIPP5 plasmid (amino acids 8-60 of hIPP5, Thr→Asp-40), pcDNA3.1/myc-His(-)-B-p8-60hIPP5m (referred as p8-60hIPP5m-B later) were kindly provided by Dr. Xiao-jian Wang at Zhejiang University (Zhejiang, China).

**Cell transfection**

The expression vectors phIPP5-B and p8-60hIPP5m-B were transfected into HeLa cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) with pcDNA3.1/myc-His(-)-B as a mock control. Stable cell lines overexpressing hIPP5 or 8-60hIPP5m were selected by 600-1000 μg/ml G418 for 2-3 weeks, and cloned by limiting dilution. These stable cell lines were designated by 600-1000 μg/ml G418 for 2-3 weeks, and cloned by limiting dilution. These stable cell lines were designated (Xia et al., 2002). Briefly, 24 h after transfection, cells were serum-starved for 36 h, and refed with 10% FCS for 12 h. Cells were then harvested, washed with PBS, fixed with 70% ethanol at 4°C overnight. Fixed cells were washed with cold PBS and incubated in 1 ml of PBS solution containing 100 μg of RNase A (Sigma Chemical Co., St. Louis, MO, USA) and 40 μg of propidium iodide (PI) (Sigma Chemical Co., St. Louis, MO, USA) for 30 min at 37°C. The DNA profiles were examined with a FACScan flow cytometer machine using the CellQuest program (Becton Dickinson, Mountain View, CA, USA).

For stable HeLa cells, cell synchronization was carried out by plating cells onto 6-well plates with a density that cells reached 50% confluence on the following day. Cells were then treated with 2 mM thymidine in complete medium (DMEM with 10% FBS) for 16 h, washed twice with DMEM-0% FBS, incubated for 8 h in complete medium, and treated again for 16 h with 2 mM thymidine in complete medium. The resulting G1/S-enriched cells were washed twice with DMEM-0% FBS and release into the cell cycle in the presence of complete medium for up to 26 h. Cells were harvested at indicated time, and treated as described above for FACS analysis. To observe mitosis progression, these cells were also examined under a microscope equipped with a CoolSNAPcf digital camera system (Photometrics, Roper Scientific, Tucson, AZ, USA), and stained with Giemsa.

**Immunoprecipitation and nuclear translocation assay**

Non-transfected and transfected HeLa cells were lysed in lysis buffer (Cell Signaling Technology Inc., Beverly, MA, USA). After mixing with Ni-NTA beads (Qiagen, Valencia, CA, USA), cell lysates were agitated gently overnight at 4°C. The beads were then washed four times in lysis buffer containing 10 mM amidazole and 0.1% TritonX-100, and Western blot performed. To examine the nuclear translocation of proteins, nuclear proteins were extracted using NE-PERTM nuclear reagents (Pierce, Biotechnology, Rockford, IL, USA), and analyzed by Western blot.

**Western blot analysis**

The protein concentrations were determined using the BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA). Samples containing equal amounts of protein were separated by 12% SDS-PAGE, and electrophoretically transferred to Protran nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked with TBST containing 10% nonfat milk before incubating with primary antibodies followed by secondary antibodies conjugated to horseradish peroxidase (Pierce, Biotechnology, Rockford, IL, USA). Membranes were then treated with ECL reagent (GE Healthcare, Life Sciences, Piscataway, NJ, USA). Membrane bands were quantified using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The fold changes of IPP5 expression were calculated as the ratio of IPP5 to β-actin.

**Cell cycle analysis**

HeLa cells transiently transfected with phIPP5-B, p8-60hIPP5m-B or control vector were synchronized as described (Xia et al., 2002). Briefly, 24 h after transfection, cells were serum-starved for 36 h, and refed with 10% FCS for 12 h. Cells were then harvested, washed with PBS, fixed with 70% ethanol at 4°C overnight. Fixed cells were analyzed by Western blot with anti-Myc polyclonal antibody.
antibodies specific for cyclinA1, cyclinB1, (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), Myc, p21, PP1α, p-ATM, p-p53, p-p21, p-cdc2 (Cell Signaling Technology Inc., Beverly, MA, USA). After washing, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) (Cell Signaling Technology Inc., Beverly, MA, USA). The HRP activity was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL, USA).

Statistical analysis
Pairwise comparisons were conducted using Student’s t test. p values of less than 0.05 were considered statistically significant.

Results
Transfected HeLa cells express hIPP5 or 8-60hIPP5m gene and related Myc fusion proteins
To investigate the role of IPP5 in cell cycle regulation, human cervical carcinoma HeLa cells were transfected with hIPP5 or 8-60hIPP5m expression vector. RT-PCR showed that hIPP5 or 8-60hIPP5m mRNA was expressed in the transfected cells (Figure 1A). Western blotting analysis further confirmed the expression of hIPP5 or 8-60hIPP5m-Myc fusion protein in the transfected HeLa cells, but not in the non-transfected HeLa cells or the mock-transfected ones (Figure 1B). These results indicated that hIPP5 or 8-60hIPP5m gene had been efficiently transfected into HeLa cells.

8-60hIPP5m overexpression blocks the cell cycle at G2/M phase in human cervix carcinoma cells
Cell cycle distribution analysis showed that HeLa cells transfected with p8-60hIPP5m exhibited a higher percentage of G2/M cells compared to other three groups, suggesting that 8-60hIPP5m induced G2/M cell cycle arrest (Figure 2A). We also treated HeLa cells stably expressing IPP5 with thymidine to obtain the G1/S phase-synchronous cells. Flow cytometry analysis of DNA content showed that the majority of control cells entered G2/M with 4N DNA at 6 h and returned to G1 at 9 h after thymidine withdrawal. In contrast HeLa-8-60hIPP5m cells showed a significant delay in its return to 2N, which was most apparent at 12 h after thymidine withdrawal (Figure 2B). These results further demonstrated that 8-60hIPP5m induced G2/M arrest in HeLa cells.

8-60hIPP5m inhibits early and late mitotic progression
The role of 8-60hIPP5m in regulating mitotic progression was further characterized by examining the cell cycle distribution of HeLa cells transfected with hIPP5 or 8-60hIPP5m. Cells were harvested, then fixed and stained with propidium iodide (PI) for DNA content analysis by FACS. A, Transiently transfected or parental HeLa cells were synchronized as described in Materials and Methods. B, Cell synchronization and effects of hIPP5-B or 8-60hIPP5m on S/G2/M progression. Stably transfected HeLa cells and parental HeLa cells were synchronized by thymidine arrest. The medium was then replaced with DMEM–10% FBS, and cells were analyzed at indicated time points by flow cytometry. (a) Asynchronous (Asynch) cells showed peak fluorescence of 2N and 4N cells (arrows). Synchronized cells sampled before release showed arrest in G1/S (b to e). Comparison of synchronized HeLa-hIPP5 cells (b), HeLa-mock and parental HeLa cells (d and e), HeLa-8-60hIPP5m cells (c) showed delay in progression of cells from 4N to 2N.
mitotic cells under a microscope for chromatin staining in synchronized cells. Within 6 h after release from thymidine treatment, there was no significant difference in the number of mitotic cells among the four groups. At 7.5 h and 9 h, the amount of mitotic cells in HeLa-8-60hIPP5m group was significantly lower than those of other groups. At 12 h, HeLa-8-60hIPP5m cells were enriched in mitosis while most cells in the control groups returned to G0/G1 (data not shown). Similar results were obtained by Giemsa staining. Cells released from thymidine arrest were examined for distributions among mitotic stages. Based on the appearance of condensed chromatin, the largest fraction of control cells in metaphase occurred at 7.5 and 9 h for parental HeLa cells and mock-HeLa cells respectively, while the number of mitotic cells at all stages was reduced in HeLa-8-60hIPP5m cells, indicating a delay in mitotic entry. However by 10.5 to 12 h, HeLa-8-60hIPP5m cells showed significant enrichment in metaphase when most of the control cells returned to G1 (Figure 3).

8-60hIPP5m changes the phosphorylation status and nuclear translocation of several cell cycle-regulatory proteins.

To investigate the mechanism involved in 8-60hIPP5m-induced G2/M cell cycle arrest, we examined the phosphorylation status of some cell cycle-regulatory factors. It was discovered that there was an increase in phosphorylation of ATM, p53, and p21cip1/waf1, proteins in HeLa-8-60hIPP5m cells after release from thymidine arrest (Figure 4A). Increased phosphorylation of ATM Ser1981 indicates activation of G2 checkpoint kinase. It has been reported that ATM is normally an inactive multimer, and activated by autophosphorylation at Ser1981 after double strand breaks or changes in the chromatin structure (Lavin and Kozlov, 2007; Luo et al., 2007).

Moreover, phosphorylation of p53 Ser15, a target of ATM kinase, was noticed at 6 h after released from thymidine arrest. This is consistent with the known phenomenon that p53 Ser15 phosphorylation is dependent on ATM (Lavin and Kozlov, 2007). Phosphorylation of p53 results in the stabilization and accumulation of this protein (Shirata et al., 2005), which is important for its role in regulating downstream targets such as p21cip1/waf1.

To further explore the molecular mechanism of 8-60hIPP5m-induced cell cycle arrest at G2/M phase, the expression of cyclins A1, B1 and Cdc2, which are regulators of the cell cycle G2-M transition (Dash et al., 2005), were analyzed. As shown in Figure 4A, Cyclin B1, cyclin A1, and Tyr15-phosphorylated Cdc2, were still expressed in HeLa-8-60hIPP5m cells at 12 h after release from thymidine arrest.

Cyclin B1 is a mitotic cyclin that accumulates in the cytosol during late S phase and G2 phases to form the inactive mitosis-promoting factor (MPF) with Cdc2. It enters the nucleus at the onset of mitosis and associates with condensed chromosomes in prophase and metaphase (Nurse, 1990; Pines and Hunter, 1991; Bailly et al., 1992; Roberts et al., 2002). Considering that mitotic entry is related to cyclin B1 nuclear translocation, we assessed its expression in the nucleus. As shown in Figure 4B, in HeLa-8-60hIPP5m cells, not until 7.5 h after release from thymidine arrest, did the nuclear level of cyclin B1 reach to that of other three groups at 6 h. This suggests that the nuclear translocation of cyclin B1 delayed for about 1.5 hour in HeLa-8-60hIPP5m cells. This result is consistent with the Giemsa staining result in which fewer mitotic cells were found at 7.5 h in HeLa-8-60hIPP5m cells. These findings lend further support to the notion that 8-60hIPP5m induces a delay in mitotic entry.

8-60hIPP5m Delays Early Mitotic Progression. Stably transfected HeLa cells and parental HeLa cells grown on coverslips were synchronized by thymidine arrest, released, and stained with Giemsa at 7.5, 9, 10.5, 12h and scored by coverslips were synchronized by thymidine arrest, released and harvested at the indicated times. The nuclear proteins were then extracted from thymidine arrest.

Figure 3. 8-60hIPP5m Delays Early Mitotic Progression. Stably transfected HeLa cells and parental HeLa cells grown on coverslips were synchronized by thymidine arrest, released, and stained with Giemsa at 7.5, 9, 10.5, 12h and scored by Giemsa staining. Cells released from thymidine arrest. This is consistent with the known phenomenon that p53 Ser15 phosphorylation is dependent on ATM (Lavin and Kozlov, 2007). Phosphorylation of p53 results in the stabilization and accumulation of this protein (Shirata et al., 2005), which is important for its role in regulating downstream targets such as p21cip1/waf1.

Figure 4. 8-60hIPP5m Changes the Phosphorylation Status and Nuclear Translocation of Several Cell Cycle-Regulatory Proteins. A) Stably transfected HeLa cells and parental HeLa cells were synchronized by thymidine arrest. Then the medium was replaced with DMEM–10% FBS, and cells were collected at the indicated times. Several cell cycle regulatory proteins including ATM, p53, p21cip1/waf1, cyclin A1, cyclin B1 and Cdc2 were analyzed by Western blot. B) Delayed nuclear translocation of cyclinB1 induced by 8-60hIPP5m during cell cycle in HeLa cells. Stably transfected HeLa cells and parental HeLa cells were synchronized by thymidine arrest, released and harvested at the indicated times. The nuclear proteins were then extracted for Western blot.

Figure 4. 8-60hIPP5m Changes the Phosphorylation Status and Nuclear Translocation of Several Cell Cycle-Regulatory Proteins. A) Stably transfected HeLa cells and parental HeLa cells were synchronized by thymidine arrest. Then the medium was replaced with DMEM–10% FBS, and cells were collected at the indicated times. Several cell cycle regulatory proteins including ATM, p53, p21cip1/waf1, cyclin A1, cyclin B1 and Cdc2 were analyzed by Western blot. B) Delayed nuclear translocation of cyclinB1 induced by 8-60hIPP5m during cell cycle in HeLa cells. Stably transfected HeLa cells and parental HeLa cells were synchronized by thymidine arrest, released and harvested at the indicated times. The nuclear proteins were then extracted for Western blot.

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8-60hIPP5m translocates into the nucleus at G2/M phase and interacts with PP1α and cdc2

It has been reported that the PP1 catalytic subunit, PP1α, translocates into the nucleus in G2/M, and dephosphorylates a variety of nuclear structural proteins that are substrates of the cyclin B/cdc2 kinase (Mumby and Walter, 1993), thus promotes the completion of mitosis. To understand the effect of 8-60hIPP5m on mitosis, we assessed its expression in the nucleus, and analyzed the proteins that 8-60hIPP5m interacted with. At 6 h after release from thymidine arrest, 8-60hIPP5m level was significantly enhanced in the nucleus of HeLa-8-60hIPP5m cells (Figure 5A). Immunoprecipitation assay revealed that 8-60hIPP5m interacted with PP1α and cdc2 (Figure 5B). These results suggest that 8-60hIPP5m might translocate into the nucleus at G2/M phase where it interacts with PP1α and inhibits its activity, resulting in the delay of mitotic exit. 8-60hIPP5m may also interact with cdc2 and regulate its activity, through which it influences mitosis progression.

Discussion

In this study, we showed that overexpression of IPP5 resulted in G2/M arrest through inhibiting early and late mitotic progression. This study is an extension of our earlier work in which it was discovered that 8-60hIPP5m caused cell growth retardation in vitro (Zeng et al., 2012). In eukaryotic organisms, cell cycle progression is regulated to a large extent by the reversible phosphorylation of various proteins. PP1 plays an important role during cell cycle progression, especially during mitosis. There is a lot of evidence demonstrating the involvement of both phosphorylation and dephosphorylation in cell cycle control. Neutralizing PP1 by anti-PP1 antibodies, mutation of PP1, or treatment with various natural phosphatase inhibitors such as okadaic acid, calyculin-A, tautomycin, microcystin-LR, fostriecin and cantharidin have been shown to cause abnormalities in cell cycle progression and checkpoint abrogation (Axton et al., 1990; Kinoshita et al., 1990; Edelson et al., 2011; Honkanen et al., 2012; Rubiolo et al., 2012). In particular, fostriecin and cantharidin have been shown to force cells to go through the cycle prematurely and into mitosis, resulting in multiple aberrant mitotic spindles and apoptotic cell death (Verma et al., 2012; Theobald et al., 2013). In human cells, PP1 could act as a histone H1 phosphatase, which is required for chromatin decondensation during the exit from mitosis (Paulson et al., 1996). PP1 recruitment to the spindle pole body (SPB) component Cut12 sets a threshold for Polo’s feedback-loop activity that locks the cell in interphase until Cdc25 pushes MPF activity through this barrier to initiate mitosis (Grallert et al., 2013). Subcellular localizations of PP1 isoforms are different at mitosis. PP1α is located at the centrosome, while PP1γ and PP1β are associated with mitotic spindles and chromosomes, respectively (Andreassen et al., 1998). This might explain why PP1 mutations and inhibitors cause complex abnormal phenotypes, including delayed transition of metaphase to anaphase, condensed chromosomes, formation of abnormal spindles, microtubule dynamics and chromosome separation malfunction, and defect of cytokinesis. Our results also demonstrated that 8-60hIPP5m could suppress cell cycle progression in human cervix carcinoma HeLa cells.

To investigate the mechanism how IPP5 regulate cell cycle in HeLa cells, we analyzed the cell cycle progression by FACS in both transiently and stably 8-60hIPP5m transfected cells. After synchronized with thymidine, the mitotic entry of HeLa-p8-60hIPP5m cells was significantly delayed. This observation was confirmed by Giemsa staining.

Cells progress through each phase in a tightly controlled manner. This process is regulated by cyclins and cyclin-dependent kinases. Disruption of tumor suppressor protein p53 is a common event in cervical cancer (Zhou et al., 2012). 8-60hIPP5m stimulates cellular responses through the activation of ATM/p53/p21cip/waf1 signal pathways. Accumulation of phosphorylated p53 and p21cip/waf1 proteins, which may have been preceded by the increased expression of their upstream activators ATM, MDM2, and Chk1/2, is accompanied by reduced hyperphosphorylation of Cdc25C that is required to promote Cdc2 dephosphorylation during G2 arrest.
In summary, we report a novel role for 8-60hIPP5m in the control of G2/M cell cycle progression, a process that involves the activation of ATM/p53/p21cip1/waf1/Cdc2/cyclin B1 pathways. It was discovered that 8-60hIPP5m translocated into the nucleus at G2/M phase, where it interacted with Cdc2 and PP1α, and disrupted the dephosphorylation of Cdc2. In addition, 8-60hIPP5m-induced G2/M arrest of HeLa cells involved the delayed nuclear translocation of cyclin B1. These results suggest that 8-60hIPP5m might be explored as a therapeutic strategy, either alone or in combination with chemotherapy.

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