Pim-1 Kinase-Dependent Phosphorylation of p21<sub>Cip1/WAF1</sub> Regulates Its Stability and Cellular Localization in H1299 Cells

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Abstract

Previous studies from our laboratory showed that p21<sub>Cip1/WAF1</sub> can be phosphorylated by Pim-1 kinase <i>in vitro</i>, implying that part of the function of Pim-1 might involve influencing the cell cycle. In the present study, site-directed mutagenesis and phosphorylation-specific antibodies were used as tools to identify the sites phosphorylated by Pim-1 and the consequences of this phosphorylation. What we found was that Pim-1 can efficiently phosphorylate p21 on Thr<sup>145</sup> <i>in vitro</i> using recombinant protein and <i>in vivo</i> in intact cells. Unexpectedly, we found that Ser<sup>146</sup> is a second site that is phosphorylated <i>in vivo</i>, but this phosphorylation event seems to be an indirect result of Pim-1 expression. More importantly, the consequences of phosphorylation of either Thr<sup>145</sup> or Ser<sup>146</sup> are distinct. When p21 is phosphorylated on Thr<sup>145</sup>, it localizes to the nucleus and results in the disruption of the association between proliferating cell nuclear antigen and p21. Furthermore, phosphorylation of Thr<sup>145</sup> promotes stabilization of p21. On the other hand, when p21 is phosphorylated on Ser<sup>146</sup>, it localizes primarily in the cytoplasm and the effect of phosphorylation on stability is minimal. Cotransfection of wild-type Pim-1 with p21 increases the rate of proliferation compared with cotransfection of p21 with kinase-dead Pim-1. Knocking down Pim-1 expression greatly decreases the rate of proliferation of H1299 cells and their ability to grow in soft agar. These data suggest that Pim-1 overexpression may contribute to tumorigenesis in part by influencing the cellular localization and stability of p21 and by promoting cell proliferation. (Mol Cancer Res 2007;5(9):909–22)

Introduction

Pim-1 was originally identified as a frequently activated oncogene because, when expressed from the Eμ enhancer in transgenic mice, it induced lymphomas, albeit at a low incidence and with a long latency (3). Pim-1, together with its two homologues, Pim-2 and Pim-3, belongs to a small group of kinases that do not require posttranslational modification to be activated because they are naturally constitutively active (4). This means that the level of kinase activity is dependent on the absolute amount of protein present in a cell. In fact, Pim-1 levels are tightly controlled at the transcriptional, posttranscriptional, translational, and posttranslational levels (5). Knocking out of all Pim kinases leads to reduced body size (6), indicating that Pim kinases may play very important roles in growth factor signaling. Up until now, substrates identified for Pim-1 kinase include BAD (7), NuMa (8), Socs (9, 10), Cdc25A (11), C-TAK1 (12), NFAc (13), HP-1 (14), PAP-1 (15), and p21<sub>Cip1/WAF1</sub> (hereafter referred to as p21; ref. 16), indicating that Pim-1 functions in a variety of cellular events, such as cell proliferation, differentiation, and cell survival (5). Most notably, Pim-1 can strongly synergize with c-Myc to rapidly cause B-cell lymphomas (3, 17, 18). The synergism is believed to originate from antiapoptotic activity promoted by Pim-1 (19), although the underlying mechanism for this remains unclear. With regards to tumor formation, recent findings showed that Pim-1 was highly expressed in tumors of prostate cancer patients (20, 21). Interestingly, in Myc-driven prostate cancers, Pim-1 was also shown to be up-regulated (20, 21), suggesting a synergism in cancer induction beyond the hematopoietic lineages.

The p53-inducible cell cycle inhibitor p21 is important in cell cycle control and cell survival (22–24). Its expression is both p53 dependent and p53 independent (25). Originally identified as a binding partner for cyclin D1 and cyclin-dependent kinase 2, p21 can form ternary complexes with cyclins D/E and related cyclin-dependent kinases (26). Therefore, it can inhibit cyclin-dependent kinases from phosphorylating the downstream targets, the retinoblastoma proteins. This event causes cell cycle arrest at G1-S phase. p21 has also been found to be involved in a variety of cellular events, such as proliferation (27), differentiation (22), senescence (28), cell motility and tumor metastasis (29), as well as cell survival (30). The involvement of p21 in multiple cellular functions underscores its importance and that its precise regulation is crucial to the maintenance of the normal cellular function.

The p21 protein level is mainly controlled at the transcriptional level by a variety of transcription factors (31). However, phosphorylation and association with other cellular proteins are also important factors that regulate p21 stability posttranscriptionally and, therefore, protein level. Normally, p21

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is a short-lived protein with a half-life of <30 min (32). The major mode of degradation involves ubiquitination and targeting to the proteasome, but it has also been shown that p21 can be directly targeted to the proteasome without ubiquitination. This occurs through binding of its COOH terminus with the C8 subunit of the 20S core of the proteasome (33). It was found that protein kinase Cζ (PKCζ) can phosphorylate Ser<sup>146</sup> in the COOH terminus, promoting p21 degradation (34). However, it was also found that Akt phosphorylation of the same site resulted in stabilization of p21 (35). In addition, it has been shown that p21 can bind to cyclin E through its COOH-terminal cyclin-binding site 2, leading to phosphorylation by cyclin-dependent kinase 2 at Thr<sup>130</sup>, which results in p21 degradation (36). On the other hand, binding of cyclin D1 to p21 stabilizes it (37). Most recently, it was shown that chaperones also play an important role in controlling the stability of p21. In this regard, data suggest that newly synthesized p21 binds to chaperon WISp3 and Hsp90 through its NH<sub>2</sub> terminus, leading to dramatic stabilization of p21 (38).

In the present study, we were surprised to find that phosphorylation of both Thr<sup>145</sup> and Ser<sup>146</sup> of p21 occurred when Pim-1 was expressed in vivo. However, in vitro with the full-length p21 protein, only Thr<sup>145</sup> is efficiently phosphorylated by Pim-1. This suggests that another kinase

Table 1. Consensus Sequences for Pim-1, PKC, and Akt as a Comparison with a Peptide in the COOH Terminus of p21

<table>
<thead>
<tr>
<th></th>
<th>Pim-1</th>
<th>PKC</th>
<th>Akt (PKB)</th>
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<tr>
<td>p21 peptide</td>
<td>R-K-R-R-Q-T&lt;sup&gt;145&lt;/sup&gt;-S&lt;sup&gt;146&lt;/sup&gt;-M</td>
<td>R-K-R-R-X-X-S/T</td>
<td>R-K-R-R-X-X-S/T</td>
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*NOTE: A p21 COOH-terminal peptide contains two potential sites (Thr<sup>145</sup> and Ser<sup>146</sup>) that could be phosphorylated by Pim-1 and other related kinases. As shown, PKC phosphorylates Ser<sup>146</sup> and Akt phosphorylates Thr<sup>145</sup>. The asterisk represents conserved basic residues.

FIGURE 1. Identification of phosphorylation sites of p21<sub>Cip1/WAF1</sub> by Pim-1 in vitro using site-directed mutagenesis and phosphorylated-specific antibodies. A, Site-directed mutagenesis was used to identify Thr<sup>145</sup> as the preferential site on p21 phosphorylated by Pim-1. WT, mutant GST-p21 (T/A, S/A, and AA), and GST were affinity purified by glutathione-Sepharose beads. Each GST substrate protein (2 μg) was incubated with 0.2 μg of Pim-1 (WT or KD) in the kinase buffer. After reactions, autoradiography was done as described in Materials and Methods. Top, GST proteins were subsequently stained by Coomassie blue; middle, 32P-labeled GST-p21 by autoradiography; bottom, densitometry was also done to quantify the 32P-labeled GST-p21. Columns, mean of three separate experiments; bars, SD. B, Western blot with two phosphorylated-specific antibodies (anti-pT145-p21 and anti-pS146-p21) shows only Thr145 of WT p21 is phosphorylated in vitro. Each of WT or mutant GST-p21 proteins (4 μg) was incubated with 0.5 μg of WT Pim-1 or KD Pim-1 kinase in 100 μL of kinase buffer containing 10 μM ATP. Reaction was done at room temperature for 20 min followed by analysis with the two phosphorylated-specific antibodies. Total p21 protein was detected by Coomassie blue staining as shown in the bottom blot. C, In vitro kinase assay with p21 peptides shows that phosphorylation on Thr<sup>145</sup> does not increase Ser<sup>146</sup> phosphorylation. The p21 peptide RKRRQTSM was synthesized on campus, and the phosphorylated p21 peptide RKRRQPSTM and phosphomimic peptide RKRRQDSM were synthesized by GenScript Corp. Kinase assays were done as described previously (40). Phosphorylation was quantified on a Packard 1900 TR liquid scintillation analyzer. Points, mean of triplicate; bars, SD.
might be involved in the phosphorylation of Ser\textsuperscript{146}. With LY294002 that inhibits Pim-1 kinase activity (39) and wortmannin that inhibits phosphatidylinositol 3-kinase/Akt pathway, we found that inhibition of Pim-1 activity transiently does not decrease the phosphorylation of Ser\textsuperscript{146}, suggesting that another kinase may be regulated by Pim-1 and responsible for the direct phosphorylation of Ser\textsuperscript{146}. Overall, we find that Pim-1 phosphorylation of p21 stabilizes it and results in a shift in the subcellular localization of p21 with a significant amount localizing in the cytoplasm in H1299 cells. With regard to functional consequences, the component of the phosphorylated p21 that remains nuclear promotes the dissociation of p21 from proliferating cell nuclear antigen (PCNA), which correlates with cell proliferation. In addition, we found that knocking down Pim-1 protein levels in this cell line significantly affects the rate of proliferation and growth in soft agar characteristics of malignant growth, providing further evidence for its contribution to promoting tumorigenesis.

**Results**

**Pim-1 Preferentially Phosphorylates Thr\textsuperscript{145} in p21**

A phosphorylation consensus sequence that Pim-1 has been shown to phosphorylate is R/K-R/K-R-R/K-X-S/T-X and is similar but not identical to the consensus sequences identified for PKC and Akt, which have also been shown to phosphorylate p21 (Table 1; ref. 40). The COOH terminus of p21 contains the peptide sequence RKRRQTSM, which we previously showed to be phosphorylated efficiently both as a peptide and full-length protein \textit{in vitro} (16). Although we previously showed that Pim-1 could phosphorylate p21 preferentially on Thr\textsuperscript{145} using a peptide assay, we were interested to further verify the phosphorylation site(s) using mutated p21 protein. Therefore, we generated a series of mutated glutathione S-transferase (GST)-p21 proteins, including Thr\textsuperscript{145}Ala (T/A), Ser\textsuperscript{146}Ala (S/A), and Thr\textsuperscript{145}Ala/Ser\textsuperscript{146}Ala (AA), and did an \textit{in vitro} kinase assay. As shown in Fig. 1A, \textsuperscript{32}P incorporation was clearly observed showing that Pim-1 phosphorylated p21 readily \textit{in vitro}, whereas the kinase-dead
(KD) form of Pim-1 did not. We found that, of the two potential target sites, Thr$^{145}$ and Ser$^{146}$, Thr$^{145}$ was efficiently phosphorylated relative to Ser$^{146}$. When both sites have been mutated to alanine, phosphorylation was essentially eliminated, indicating that there are no other sites in p21 phosphorylated by Pim-1. Further analysis by Western blot with two phosphorylated-specific antibodies confirmed this preference (Fig. 1B). In this experiment, the two phosphorylated-specific antibodies to p21 were anti-pS$^{146}$-p21 and anti-pT$^{145}$-p21. Results for the wild-type (WT) GST-p21 showed that only Thr$^{145}$ was phosphorylated, whereas Ser$^{146}$ phosphorylation was undetectable. However, when Thr$^{145}$ was mutated to alanine, detectable phosphorylation occurred on Ser$^{146}$ as shown in the lane with T/A. This indicates that Thr$^{145}$ is the preferential site for phosphorylation by Pim-1. To further determine if phosphorylation on Thr$^{145}$ had any effect on phosphorylation of Ser$^{146}$, we designed two peptides. The first one included a phosphate group on Thr$^{145}$ and the second peptide contained an aspartic acid to mimic phosphorylation. As illustrated in Fig. 1C, WT peptide was efficiently phosphorylated. However, if Thr$^{145}$ was already phosphorylated, incorporation of phosphate was dramatically reduced.

![FIGURE 3. Phosphorylation on Thr$^{145}$ of p21 by Pim-1 promotes its stabilization.](image-url)

A. H1299 cells were transiently cotransfected with pBK/CMV, pBK/CMV-WT-Pim-1, or pBK/CMV-KD-Pim-1 together with pBK/CMV-HA-p21 (WT). Following a previously published protocol to analyze protein half-life assay (35), 24 h after transfection, cells were treated with cycloheximide (CHX) at a concentration of 25 μg/mL for the indicated times. Cells were then harvested and analyzed by Western blot with anti-p21 antibody to detect p21. B. Quantitation of p21 amounts was determined using ImageJ software. The plot shows the degradation of p21 after cycloheximide addition. C. H1299 cells were transfected with equal amounts of HA-tagged p21 constructs, including WT, T/D, S/D, DD, T/A, S/A, and AA to determine the transfection efficiency, and cells were cotransfected with equal amount of PEGFP-C1, which allows visualization by fluorescence microscopy to check for transfection efficiency. Thirty-six hours after transfection, cells were harvested and normalized for evaluating the steady levels of these transfected proteins. The membrane was reprobed with anti-actin antibody to verify equal loading. D. H1299 cells were transfected with HA-tagged p21 plasmids (WT, T/D, S/D, DD, and AA). Twenty-four hours after transfection, cells were treated with cycloheximide (25 μg/mL). At the indicated time, the cells were lysed and 50 μg of cell lysates were used to detect the exogenous p21 degradation rates by Western blot using anti-p21 antibody. Membranes were reprobed with anti-actin to ensure equal loading. E. Quantitation of the p21 protein amounts in D and plotted as protein remaining after cycloheximide addition with time.
Our result also shows that a spartic acid at position 145 influences phosphorylation of Ser \(^{146}\) in a very similar manner to the peptide with the phosphorylated Thr\(^{145}\).

**Pim-1 Phosphorylates p21 on Thr\(^{145}\) and Indirectly Promotes Phosphorylation on Ser\(^{146}\) in vivo**

To identify the phosphorylation sites of p21 by Pim-1 in vivo, we cotransfected pBK/CMV-Pim-1 (both WT and KD) and the pBK/CMV-HA-p21 constructs into the p53-null H1299 cells. For phosphorylation controls, we also carried out cotransfection of the pBK/CMV-HA-p21 with pcDNA3-HA-PKC\(\zeta\) and pcDNA3-myristoylated-Akt because PKC\(\zeta\) and Akt are two known kinases that can phosphorylate p21 at either Thr\(^{145}\) or Ser\(^{146}\). An empty vector was used as a control. As illustrated in Fig. 2A, using phosphorylated-specific antibodies, we found that in the presence of Pim-1 both Thr\(^{145}\) and Ser\(^{146}\) are phosphorylated. On the other hand, Akt only phosphorylates Thr\(^{145}\), whereas PKC\(\zeta\) seems to phosphorylate neither Thr\(^{145}\) nor Ser\(^{146}\) at least under our experimental conditions. From the total p21 levels, it seems that overexpression of Pim-1 caused p21 levels to be increased. The fact that the presence of Pim-1 leads to phosphorylation of Ser\(^{146}\) in vivo was surprising because Ser\(^{146}\) is not an efficient phosphorylation site for Pim-1. To test this hypothesis, we transiently inhibited Pim-1 kinase activity with an inhibitor to determine if phosphorylation on Ser\(^{146}\) might be reduced. Currently, there is only one inhibitor called LY294002 that is reported to inhibit Pim-1 kinase. We pretreated the cells with LY294002 for 45 min at 37° C and then harvested the cells for analysis as shown in Fig. 2B. We observed that phosphorylation on Thr\(^{145}\) is diminished at 20 \(\mu\)mol/L, but phosphorylation on Ser\(^{146}\) is not affected at all. Rather, it seems that phosphorylation on Ser\(^{146}\) is increased a bit. This suggests that the mechanism for phosphorylation at each site is different. To eliminate the possibility of the involvement of phosphatidylinositols 3-kinase/Akt in the phosphorylation of either site, we also used wortmannin as an inhibitor because LY294002 inhibits not only Pim kinase but also Akt at certain concentrations. We found that wortmannin at 100 \(\mu\)mol/L, which inhibits phosphatidylinositols 3-kinase/Akt, does not influence phosphorylation of either Thr\(^{145}\) or Ser\(^{146}\) compared with cells without inhibitors. Therefore, most likely, Ser\(^{146}\) is phosphorylated by another kinase and not directly by Pim-1, although the presence of Pim-1 promotes the phosphorylation on Ser\(^{146}\), whereas KD Pim-1 does not.

The Association of Pim-1 with p21 Can Be Detected In vivo

Phosphorylation of p21 by Pim-1 means that there is an enzyme-substrate interaction. Coinmunoprecipitation was done on cotransfected Cos-7 cells with p21 and Pim-1 (Fig. 2C). In this case, hemagglutinin (HA)-tagged p21 expression vectors were used expressing either WT, T/A, S/A, or the double-mutant AA protein. These vectors were cotransfected with WT Pim-1. The KD Pim-1 was also cotransfected with WT HA-p21. Anti-p21 antibody was used to pull down Pim-1 along with p21. We found association between the two proteins even when the two sites were mutated. This indicates that binding between Pim-1 and p21 does not require a form of p21 that can be phosphorylated. We also observed association of the KD Pim-1 with p21.

**Phosphorylation of p21 by Pim-1 Promotes Stabilization of p21**

To determine whether elevated p21 levels are due to protein stabilization, we carried out a protein half-life assay in the presence of the protein translation inhibitor cycloheximide. As shown in Fig. 3A and B, transfection of WT Pim-1 into H1299 cells resulted in marked reduction in the degradation of p21. However, KD Pim-1 seemed to promote degradation of p21 compared with the empty vector control.

There has been an ongoing controversy about whether phosphorylation of Thr\(^{145}\) or Ser\(^{146}\) influences the stability of p21 (34, 35, 41, 42). To show the influence of phosphorylation on either Thr\(^{145}\) or Ser\(^{146}\) on the stability of p21, phosphomimics mutants of these two sites were used. As shown in Fig. 3C, phosphorylation on Thr\(^{145}\) site can increase the steady-state level of transfected p21 as does Thr\(^{145}\)Asp/Ser\(^{146}\)Asp (DD). On the other hand, Ser\(^{146}\)Asp (S/D) mutants showed no stabilizing effect compared with WT p21. Cells transfected with the control plasmids T/A, S/A, and AA had far less p21 remaining compared with the phosphomimetic mutants.

**FIGURE 4.** The cellular localization of p21 changes when Ser\(^{146}\) is phosphorylated by Pim-1. A. WT, T/D, S/D, DD, and AA mutants of HA-p21 were transfected into H1299 cells using standard transfection procedure. Cells were harvested after 36 h followed by separation into the cytoplasmic (C) and nuclear (N) fractions. The cellular fractions were then analyzed by anti-p21 antibody and then reprobed with the nuclear marker anti-lamin A antibody to verify that there was no cross-contamination between fractions and also anti-actin antibody to verify equal loading. Columns, mean of three independent experiments; bars, SD. B. Quantitation of the blots in A to show the different distribution of p21 subcellular for each mutant of HA-p21.
We also examined the protein half-life of these p21 mutants while inhibiting protein translation with cycloheximide. As shown in Fig. 3D and E, the phosphomimic mutant Thr145Asp (T/D) was more stable compared with the WT. The DD mutant was found to be similarly stable compared with the T/D mutant. The S/D mutant seems to be a little less stable than WT. These findings indicate that phosphorylation on Thr145 has an effect on the stability of p21. Taken together, our data show that phosphorylation by Pim-1 preferentially on Thr145 stabilizes p21.

Phosphorylation of p21 by Pim-1 Changes Its Subcellular Localization

The residue phosphorylated on p21 by Pim-1 occurs in the COOH terminus where the nuclear localization signal is located. Potentially, this phosphorylation could influence the subcellular localization of p21. In fact, there is some controversy over this issue and in some cases may be a result of cell type used in the experiments (27, 43). For example, in HER2/neu 3T3 cells in which Akt kinase was constitutively activated, the phosphorylation of Thr145 on p21 caused its translocation from the nucleus to the cytoplasm (43). However, in human umbilical vein endothelial cells, this did not occur (27). In our studies, phosphomimetic mutants of p21 at both sites were analyzed in H1299 cells. As shown in Fig. 4A and B, comparing the total distribution of p21 in cytosolic and nuclear fractions for WT and phosphomimic mutants of HA-p21, we found that phosphorylation of Thr145 generally does not change the nuclear localization of p21, which is what is observed similar to the WT HA-p21. In contrast, phosphorylation of Ser146 seems to cause the distribution of p21 to shift from the nucleus to the cytoplasm as shown by the decrease of the nuclear localization of p21. The DD mutant was distributed similarly to the S/D mutant but there seems to be overall higher p21 levels for the DD mutant presumably because of the increased stability promoted by the mimicked phosphorylation. We also examined the AA mutant and found that most of the p21 in these cells is observed in the nucleus as would be predicted, although the total amount is low as might also be predicted.

To confirm this finding, we examined each of the mutants and WT HA-p21 by confocal microscopy in H1299 cells. NIH3T3 cells were also examined for comparison. It has been reported that the T/D mutant of p21 has more cytoplasmic localization. In our studies, all mutants of HA-p21 were analyzed for nuclear and cytoplasmic localization as shown in Fig. 5.
staining in these cells (44). NIH3T3 cells are murine fibroblast cells, whereas H1299 cells are lung carcinoma cells. Different localization patterns of p21 in these two cell types may reflect the different metabolism of different cellular lineages. To show that a difference exists for T/D or S/D mutants, we carried out experiments with WT, T/D, S/D, DD, and AA mutants of p21 in H1299 and NIH3T3 cells. A typical field for each of the HA-p21 mutants is shown in Fig. 5. As reported for NIH3T3 cells, T/D causes nuclear p21 to redistribute in the cytoplasm. The S/D mutant seems to be less efficient in the translocation to the cytoplasm. However, for H1299 cells, the T/D mutant of p21 seems to be more localized in the nucleus than in the cytoplasm and overall has a stronger fluorescence signal compared with the WT HA-p21. The S/D mutant, in contrast, is mostly localized throughout the cells. DD is similar to SD, and AA mutant localized mostly in the nucleus. These observations support the subcellular distribution of p21 mutants revealed by Western blotting.

To determine whether Pim-1 promoted the translocation of p21 from the nucleus to the cytoplasm or vice versa, we fractionated the cells into cytosolic and nuclear fractions after cotransfection of Pim-1 with HA-p21. As shown in the blot of Fig. 6, from the total p21 levels, cells that were transfected with WT Pim-1 exhibited more p21 levels in the cytoplasm than in the nucleus, whereas more nuclear p21 was observed for the vector control. The KD Pim-1 caused p21 levels in both the nucleus and cytoplasm to be decreased dramatically. We also used phosphorylated-specific antibodies to determine the subcellular localization of the phosphorylated p21. We found that the phosphorylated form of p21 on Thr145 shows up only in the nucleus of cells that were transfected with WT Pim-1. However, the form of p21 phosphorylated on Ser146 occurs mostly in the cytoplasm. This observation agrees with the results of the localization of T/D and S/D mutants as shown in Fig. 4A.

Furthermore, we used confocal microscopy to examine the subcellular localization of p21 in H1299 cells. As shown in Fig. 7, with the empty vector and KD controls, most cells show only nuclear staining for HA-p21. With WT Pim-1, p21 can be clearly observed in the cytoplasm in most cells and the fluorescence signal is very strong most likely because of the stabilization induced by phosphorylation. This result provides strong evidence that Pim-1 is involved in promoting the phosphorylation of p21, which causes it to localize to the cytoplasm. However, for the KD Pim-1, the fluorescence signal is very weak in cells expressing p21 most likely because p21 is not stabilized by phosphorylation and is rapidly degraded. These findings show that phosphorylation of p21 induced by Pim-1 increases stability and results in a shift of p21 localization from the nucleus to the cytoplasm. As predicted, the KD Pim-1 did not have this effect.

**Phosphorylation of Thr145 on p21 by Pim-1 Influences the Association between p21 and PCNA**

PCNA is the processivity factor for DNA polymerase δ and ε. It is best known for its involvement in DNA synthesis and DNA repair but also plays a role in other cellular events as well (45). It has been reported that in vitro both Thr145 and Ser146 phosphorylation by Akt and PKC, respectively, leads to dissociation of PCNA from p21 (46). It was then shown that phosphorylation of p21 on Thr145 specifically by Akt causes dissociation of PCNA from p21 (27). This likely occurs because the phosphorylation site overlaps the PCNA-binding domain (27). To confirm previous published results, we firstly used the phosphomimic mutants to check if phosphorylation on either site could influence the association between PCNA and p21. As shown in Fig. 8A (lanes T/D and DD), T/D clearly caused dissociation of PCNA from p21. S/D seems to be somewhat less efficient in disrupting the association. As we have already shown that phosphorylated p21 on Thr145 stays exclusively in the nucleus, whereas the phosphorylated form of p21 on Ser146 mainly localizes to the cytoplasm, we hypothesized that, once p21 is phosphorylated by Pim-1 on Thr145, it localizes in the nucleus. This causes the disruption of the association between p21 and PCNA, whereas when p21 is phosphorylated on Ser146, it is translocated to the cytoplasm yet remains associated with PCNA although at a reduced level. To test this hypothesis, we carried out cotransfection of Pim-1 with HA-p21, with KD Pim-1 and empty vector for controls. Transfected cells were harvested and fractionated into the cytoplasmic and nuclear fractions. We found as expected that nuclear p21 dissociated from PCNA in the nucleus where Thr145 was phosphorylated. However, cytoplasmic p21 still associated with PCNA because in the cytoplasm Ser146 was phosphorylated but still results in the association with PCNA (Fig. 8B). This finding suggests that Pim-1 phosphorylation of p21 can result in dissociation of PCNA and p21 specifically in the nucleus but not for the whole-cell lysates as was originally predicted. However, the dissociation in the nucleus restores the function of the DNA synthesis machinery, which would be expected to allow cell proliferation.

![FIGURE 6](image-url) H1299 cells were cotransfected with pBK/CMV-HA-p21 and the empty pBK/CMV vector or pBK/CMV-Pim-1 (WT and KD). Thirty-six hours after transfection, cells were harvested and fractionated into the cytoplasm and the nuclear fractions followed by analysis of phosphorylated p21 as well as total p21 distribution in cells. Equal volumes of cytoplasmic and nuclear fractions were loaded.
Pim-1 Promotes Cell Proliferation through Phosphorylating p21

Nuclear localization is important for p21 to arrest cell cycle at G1-S because we found that Pim-1 causes p21 to localize to the cytoplasm and disrupt the association of p21 with PCNA, which is also an important contributor in DNA synthesis. We therefore hypothesized that phosphorylation of p21 by Pim-1 should contribute to cell proliferation. To test this hypothesis, H1299 cells were cotransfected with HA-p21 and Pim-1 (WT and KD) plasmids as before and assayed for cell proliferation. As controls, we also transfected the cells with empty vector only, WT Pim-1 only, as well as KD Pim-1 only. As shown in Fig. 9A and B, cells transfected with WT Pim-1 and WT HA-p21 exhibit a higher proliferation capacity than the controls. To verify that the proliferation is a direct result of p21 phosphorylation by Pim-1, we also did a [3H]thymidine incorporation assay with the different phosphomimic mutants of p21 (WT, T/D, S/D, DD, and AA). As shown in Fig. 9C, cells were transfected with equal amount of p21 plasmids; however, different proliferation rates were detected. Compared with WT p21, cells that were transfected with the S/D mutant had a marked increase in cell proliferation, whereas those transfected with T/D proliferated at a similar rate compared with WT p21.

Knockdown of Pim-1 Protein Levels Affects the Growth and Transforming Characteristic of H1299 Cells

To study the influence of knocking down endogenous Pim-1 protein in H1299 cells on proliferation and the transforming characteristic of growth in soft agar, we generated stable cell lines by knocking down Pim-1 protein levels using retroviral infection to deliver Pim-1 RNA interference. As shown in Fig. 10A, knocking down Pim-1 expression has almost a 2-fold reduction of Pim-1 protein expression. We found the reduction of Pim-1 to have a marked effect on the growth rate of H1299 cells as shown in Fig. 10B and was also manifested by marked reduction of the [3H]thymidine incorporation into DNA during proliferation as shown in Fig. 10C. Finally, we did the colony-forming assay to study whether Pim-1 protein expression in H1299 cells can influence the transforming characteristic of growth in soft agar. As shown in Fig. 10D, we found that, by knocking down Pim-1 protein, the ability of H1299 cells to grow in soft agar is markedly decreased. There seem to be much fewer and smaller colonies forming with these cells. These results strongly suggest that endogenous Pim-1 seems to play a very important role in proliferation and the malignant growth of H1299 cells.

Discussion

In the current report, we confirm that Pim-1 phosphorylates p21 protein in vitro and provide strong evidence that Pim-1 preferentially phosphorylates Thr145 in intact cells. We also find that one of the major consequences of the phosphorylation of p21 by Pim-1 is enhanced cell proliferation. We show that Pim-1 indirectly brings about the phosphorylation of Ser146 possibly through the activation of another protein kinase. However, phosphorylation on Thr145 and Ser146 residues has different consequences. The form of p21 phosphorylated on Thr145 is...
stabilized and localizes to the nucleus, which leads to disruption of the association between PCNA and p21. On the other hand, when p21 is phosphorylated on Ser\(^{146}\), this causes p21 to localize to the cytoplasm, yet maintaining its association with PCNA.

Our data show that a major increase in stability of p21 occurs when Thr\(^{145}\) is phosphorylated, whereas phosphorylation of Ser\(^{146}\) seems to have little effect on stability. The increase in stability of p21 when Thr\(^{145}\) is phosphorylated is consistent with a previous report in which the death-associated kinase Zip can phosphorylate p21 on Thr\(^{145}\) and cause its stabilization (41). The COOH terminus of p21 has previously been shown to be very important in controlling its stability. This occurs with the C8 subunit of proteasome binding to it, which then leads to ubiquitin-independent degradation (32). On the other hand, PCNA and cyclin D1 have also been shown to cause stabilization of p21 (37, 47). The molecular mechanism for the increased stability may involve the phosphate at Thr\(^{145}\) hindering the ubiquitination of Lys\(^{141}\) (48).

Contrary to what has been reported previously, we found that not Thr\(^{145}\) phosphorylation but rather Ser\(^{146}\) phosphorylation is what leads to p21 translocation to the cytoplasm from the nucleus. We found that p21 phosphorylated on Thr\(^{145}\) localizes in the nucleus. Although this is in contrast to what has been previously found with fibroblasts and cancer cells where phosphorylation of p21 by Akt on Thr\(^{145}\) changes its subcellular localization (43), it also suggests that the cell type could be an important factor in the results one obtains from such experiments. For example, another group did not observe the translocation effect with p21 in endothelial cells with Akt. Rather, it was observed that the T/D mutant tended to localize in the nucleus (27). Here, we present evidence that, in the lung carcinoma cell line H1299, phosphorylated p21 on Thr\(^{145}\) is localized to the nucleus, whereas the form of p21 phosphorylated on Ser\(^{146}\) mainly localizes in the cytoplasm. Our data with the phosphomimic mutants of p21 are consistent with this observation. Because both Thr\(^{145}\) and Ser\(^{146}\) are part of the nuclear localization signal sequence of p21, the different localization patterns might be a result of different binding partners that may vary from cell type to cell type and have a preference for one or the other phosphorylated forms of p21. Because we found that phosphorylation on Thr\(^{145}\) and Ser\(^{146}\) seems to occur by different pathways (Fig. 2B), the possibility exists that Pim-1 might activate another kinase in vivo and cause Ser\(^{146}\) to be phosphorylated or Pim-1 may influence the activity of phosphatases in vivo (11, 12, 49) and thereby affect the phosphorylation status on Ser\(^{146}\).

Although dissociation of p21 from PCNA should allow cell cycling to resume, apparently cellular localization and stability of p21 play more important roles for reasons that are not clear at this time. In vivo, we detected phosphorylation of both Thr\(^{145}\) and Ser\(^{146}\), and each of these forms of phosphorylated p21 localized differently. In addition to the DD mutant being very stable and localizing to both the nucleus and cytoplasm (Fig. 4A), it is not entirely surprising that proliferation promoted by the DD mutant of p21 seems to be similar to that of the WT p21. It is possible that the double charge results in canceling some of the effects observed for each of the T/D and S/D mutants. Because the AA mutant is not very stable (Fig. 4A), the proliferation observed for it was similar to that of the WT.

Taken together, our data show that overexpression of Pim-1 results in an increase in the total level of p21 with cytoplasmic localization of p21 in general. Through modulating p21 localization and its association with PCNA, Pim-1 promotes cell proliferation. Knocking down Pim-1 protein expression dramatically decreases the proliferation rate of H1299 cells and their ability to grow in soft agar. These results suggest that Pim-1 probably plays a very important role in the malignant growth of H1299 cells (54). Despite the many lines of evidence indicating the function of Pim-1 in prostate cancer cells, and overexpression of Pim-1 kinase dramatically enhances the growth of tumor cells (54).
cell proliferation, the detailed mechanism is largely unknown. One possible mediator is the phosphatase Cdc25A because phosphorylation by Pim-1 increases its activity (11), which is necessary for the progression from G1 to S phase of the cell cycle. In our report here, we provide a new line of evidence that Pim-1 enhances cell proliferation by phosphorylating the cell cycle regulator p21. This phosphorylation event influences proliferation by shifting the cellular localization of p21 between the nucleus and cytoplasm and by modulating the interaction of p21 with PCNA.

It is generally accepted that nuclear p21 and cytoplasmic p21 have distinct functions. Whereas nuclear p21 is important to arrest cell cycle at G1-S phase, cytoplasmic p21 seems more likely to promote tumorigenesis by playing a part in cell survival as well as cell motility (55). For example, it has been shown that, in the mitochondria, p21 can bind to procaspase-3 through its NH2 terminus, preventing cleavage into active caspase-3 and thereby inhibiting the onset of apoptosis (56). Cytoplasmic p21 can also bind to apoptosis signal-regulating kinase-1 (22), which is a general mediator for cell death. By binding to and inhibiting this kinase, p21 could contribute to cell survival. In terms of cell motility, cytoplasmic localization of cyclin-dependent kinase inhibitors, such as p21 and p27, has been shown to play a role (55). This occurs by disruption of actin stress fibers and focal adhesions. Cytoplasmic p21 has been shown to bind to ROCK kinase and thereby influence Rho kinase, which is important in assembly of the actin cytoskeleton (29). Thus, cytoplasmic p21 has been linked not only to cell survival and in promoting cell motility and tumor invasion but also to proliferation as we show here. Therefore, the significant effect of p21 subcellular distributions on the development of cancer is once again underscored here.

Materials and Methods

Cell Lines

Cos-7 and NIH3T3 cells were obtained from the American Type Culture Collection and cultured in DMEM (from Life Technologies) supplemented with 2 mmol/L L-glutamine, 10% (v/v) fetal bovine serum (FBS; Atlanta Biologicals), and 100 units/mL penicillin and 100 μg/mL streptomycin. H1299 cells were also obtained from the American Type Culture Collection and cultured in RPMI 1640 (Life Technologies) supplemented.
with 10% FBS and 100 units/mL penicillin and 100 µg/mL streptomycin. GP2-293 cells were purchased from Clontech and cultured in DMEM supplemented with 10% FBS and antibiotics as above. All cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

**FIGURE 10.** Knockdown of Pim-1 protein decreases the proliferation and transforming characteristic of H1299 cells. A. H1299 cells either infected with control retrovirus or retrovirus that knocks down Pim-1 protein expression and selected with 2 µg/mL puromycin for 7 d to set up stable Pim-1 knockout cell lines. Western blotting with Pim-1 antibody confirmed the correct sequences. B. Growth curves of H1299 cells after 6 d. Points, mean of the quadruplicates; bars, SD.

**Transfection and Stable Cell Line Generation**

Cos-7 and H1299 cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instruction. For NIH3T3 cells, transfectin from Bio-Rad was used following the manufacturer’s instructions. To generate retrovirus to infect target cells, GP2-293 cells were cotransfected with pVSV-G (from Clontech) and pSIREN (from Clontech), which contains an oligonucleotide coding for human Pim-1 small interfering RNA to knock down human pim-1 gene by the method of calcium phosphate precipitation (CalPhos Mammalian Transfection kit was purchased from Clontech). The sequence of the oligonucleotide was kindly provided by Dr. Xueke You (Cornell University, Ithaca, NY). It contains a sense 19-nucleotide strand (5'-GATCTCTTCGACTTCTACATCA) sequence followed by a spacer (5'-TCCAAGAGA) and its reverse complementary strand followed by five thymines as a RNA polymerase III transcriptional stop signal. In parallel, empty vector pSIREN was also cotransfected with pVSV-G to generate the control cell line. Forty-eight hours after transfection, supernatant was collected and filtered with 0.45-µm unit and target cells were infected in the presence of 8 µg/mL polybrene. Spinoculation was done at 750 × g for 2 h at 32°C to enhance the infection efficiency. Thirty-six hours after infection, cells were put under selection with 2 µg/mL puromycin for 7 days to establish the stable Pim-1 RNA interference–expressing cell line and the control cell line.

**Plasmids and Constructs**

Using standard PCR protocols, an NH₂-terminal HA-tag was added to the cDNA of WT human p21 and subcloned into a modified pBK/CMV vector in which LacZ promoter was deleted. To generate mutated p21, a PCR-based site-directed mutagenesis was carried out to generate T/A, S/A, and AA mutant and also T/D, S/D, and DD mutants. To generate GST-p21 with the desired mutations, p21 cDNA was subcloned into pGEX-2T and used as a template to generate mutated GST-p21 with T/A, S/A, and AA. COOH-terminal 6× His-tagged WT Pim-1 and KD Pim-1 were subcloned into pET30a digested with NdeI/KpnI double enzymes. Sequence analysis was done to confirm the correct sequences. pcDNA3-myristoylated-Akt was purchased from Addgene.

**Antibodies and Reagents**

Antibodies used included anti-p21 SXM30 monoclonal antibody (BD Pharmingen), anti-actin (Sigma), anti-HA antibody and anti-p-Akt-S473 (Cell Signaling), anti-pT145-p21 and anti-pS146-p21 (Santa Cruz Biotechnology), anti-PCNA (Transduction Laboratories), and anti-lamin A and anti-Akt (BioLegend). Anti-Pim-1 1140p polyclonal antibody was produced in our laboratory using full-length recombinant Pim-1 expressed in *Escherichia coli*. Reagents used include cycloheximide (Sigma), LA294002 (Calbiochem), wortmannin (Calbiochem), puromycin (Clontech), and noble agar (U.S. Biochemical).

**Recombinant Protein Purification**

Plasmids pGEX-2T-p21 (WT or mutants) were transformed into *E. coli* strain BL-21pLysS (DE3), and 0.1 mmol/L isopropyl-1-thio-B-D-galactopyranoside was used to induce the recombinant
protein expression. Cells were sonicated and centrifuged, and the supernatants were incubated with PBS-equilibrated Sepharose-glutathione 4B beads followed by extensive washing. Protein was then eluted with 20 mM glutathione in 100 mM Tris buffer followed by dialysis against Tris buffer without glutathione at 4°C overnight. To prepare recombinant Pim-1 kinases (WT and KD), pET30(a)-Pim-1 plasmids were transformed into E. coli strain BL-21pLysS (DE3), and 1 mM isopropyl-1-thio-B-D-galactopyranoside was used to carry out the induction. The protein was affinity purified with nickel-nitrilotriacetic acid beads and eluted with 300 mM imidazole. Protein was subsequently dialyzed against Tris buffer at 4°C overnight.

**Cycloheximide Treatment**

H1299 cells were plated on six-well tissue culture plates 1 day before transfection. Standard protocol was followed to transfect these cells using LipofectAMINE 2000 reagent. Twenty-four hours after transfection, cells were treated with cycloheximide with a final concentration of 25 μg/mL with the indicated time.

In vitro Kinase Assay

For kinase assay analyzed by 32P autoradiography, each GST substrate protein (2 μg) was incubated with 0.2 μg Pim-1 (WT or KD) in 50 μL kinase buffer [20 mM MOPS (pH 7.4), 150 mM NaCl, 12.5 mM MgCl₂, 1 mM MnCl₂, 1 mM EGTA, 1 mM DTT, 10 μM ATP] containing 20 μCi [γ-32P]ATP. The reactions were carried out at room temperature for 20 min and stopped with 2× Laemmli buffer, samples were boiled for 10 min, and then proteins were isolated in SDS-PAGE and transferred to polyvinylidene difluoride membrane until exposed to X-film. For the kinase assay without the radiolabel, each of WT or mutant GST-p21 proteins (4 μg) was incubated with 0.5 μg of WT Pim-1 or KD Pim-1 kinase in 100 μL of kinase buffer containing 10 mM unlabeled ATP, reactions were stopped as above, and samples were analyzed by Western blot with the indicated antibodies. For the kinase assay using the peptide substrates, reactions were carried in the same buffer but with 0.5 mM ATP as substrates. After reactions, 100 μg bovine serum albumin was added and then trichloroacetic acid was added to a final concentration of 2.5% to stop the reactions. The samples were kept in ice for 30 min and then centrifuged at 12,000 rpm at 4°C for 15 min. An aliquot of the supernatant was then spotted on a phosphocellulose filter (2.1 cm diameter, Whatman P-81) and washed with 15 mM/L phosphoric acid extensively. Filter papers were then air dried and put in scintillation counting vials for analysis.

Cell Lysate Preparation and Western Blotting

Cells were trypsinized and harvested, washed with PBS once, and resuspended in cell lysis buffer containing 25 mM Tris-HCl (pH 7.5), 1% (w/v) NP40, 1 mM EDTA, 1 mM activated sodium orthovanadate, 5 mM sodium fluoride, protease inhibitor cocktail set I (Calbiochem), and 150 mM/L NaCl. After brief sonication, cell lysates were centrifuged at 13,000 rpm for 10 min. Protein concentration was determined and equivalent amounts of lysate based on protein concentration were added to an equal volume of 2× Laemmli buffer and boiled for 10 min. For Western blot analysis, protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Membranes were subsequently blocked with 5% nonfat dry milk-PBS-Tween 20 for 1 h at room temperature and incubated with primary antibody at optimized dilution for 2 h at room temperature. Membranes were then washed, incubated with horseradish peroxidase–conjugated secondary antibody (Santa Cruz biotechnology) at 1:10,000 for 1 h, washed, treated with enhanced chemiluminescence reagent (Pierce), and exposed to hyperfilm. To quantitate the signals in Western blots, ImageJ software was downloaded from the Web site ¹ and used to carry out the densitometry for blots.

**Cell Fractionation**

Cytoplasmic/nuclear fractions were made using NE-PER from Pierce according to the manufacturer’s instructions.

**Immunoprecipitations**

Cells were harvested as described previously and then resuspended in the cell lysis buffer and incubated on ice for 10 min before centrifugation. The cell lysates were then precleared with protein G-agarose beads (Roche Diagnostics) followed by incubation with 2.5 μg of antibody at 4°C overnight. A volume of 20 μL of protein G beads equilibrated with PBS was then added and incubated at 4°C for an additional 2 h. Protein G beads were then centrifuged down at 5,000 rpm for 2 min followed by PBS washes.

**Confocal Microscopy**

NH3T3/H1299 cells were grown on cover slips in 24-well plates and then transfected with the appropriate plasmids. After treatment for 24 h, cells were fixed in 4% paraformaldehyde for 30 min, washed in three changes of PBS, and then permeabilized in 0.3% Triton X-100 for 20 min followed by three changes of PBS. Cells were then blocked for 30 min with 3% bovine serum albumin/PBS at room temperature and then incubated with primary antibody (1:100 anti-HA) for 2 h at room temperature. The cover slips were washed with three changes of PBS and then incubated with the secondary antibody (1:200 anti-mouse conjugated with Oregon Green) for 1 h at room temperature in the dark. The cells were washed with three changes of PBS and then mounted onto cover glass using mounting medium with propidium iodide and examined using confocal microscope after overnight staining.

**Cell Proliferation Assay**

H1299 cells were plated in 35-mm plate 1 day before transfection so that, on the day of transfection, cells were at about 60% to 70% confluence and transfected with the indicated plasmids. For the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

¹ http://rsb.info.nih.gov/ij/
assay (CellTiter96 Aqueous, Promega) that measures the number of viable cells, 36 h after transfection, cells were trypsinized and counted. An appropriate amount of cells was plated in triplicate into microtiter plate wells in 100 μL RPMI 1640. Controls using the same medium without cells were set up in parallel. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (20 μL) was added to the wells. Two hours after adding 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, the plates were read in a microplate autoreader at 490 nm wavelength. Alternatively, cell proliferation was analyzed by [3H]thymidine incorporation. Thirty-six hours after transfection, [3H]thymidine (methyl-) was added to a final concentration of 1 μCi/mL and cells were incubated for an additional 6 h. For analysis with the stable cell lines, an equal number of cells were seeded into 24-well plates and then serum starved for 24 h followed by addition of complete medium with 10% FBS. Cells were then pulsed with 1 μCi/mL [3H]thymidine (methyl-) and incubated for an additional 6 h. Cells were then washed with PBS once and treated with ice-cold 5% trichloroacetic acid at 4°C for 30 min. Cells were then washed with PBS once and DNA was extracted with 0.5 mol/L NaOH/0.5% SDS solution and added into scintillation counting vials containing scintillation fluid for counting.

Colony-Forming Assay

To study the transforming activity of H1299 cells, 1.0 × 10^4 cells were mixed in 2.0 mL 0.3% agar/1× RPMI 1640/10% FBS as the top agar and plated into six-well plates with 1.5 mL 0.6% agar/1× RPMI 1640/10% FBS as the base agar. Plates were incubated at 37°C and checked every 3 days and 0.5 mL of fresh complete RPMI 1640/10% FBS was added. Two weeks later, colonies were photographed.

Statistical Analysis

The statistical significance between the means of the unpaired values was determined by Student’s t test. Results were considered significant if P < 0.05.

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References


