Jab1 co-activation of c-Jun is abrogated by the serine 10 phosphorylated form of p27\textsuperscript{Kip1}.

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Running Title: p27\textsuperscript{Kip1} inhibits Jab1-dependent transcription
Abstract

The cyclin-dependent kinase (cdk) inhibitor p27Kip1 is a central mediator in the imposition and maintenance of quiescence through the sequestration of G1-specific cyclin/cdk complexes. Previous studies have implicated the c-Jun co-activator protein Jab1 as a regulator of intracellular p27Kip1 levels. Jab1 has been reported to interact with p27Kip1 and cause its translocation to the cytoplasm as a prelude to the degradation of the cdk inhibitor. Here we describe experiments that showing phosphorylation of p27Kip1 at serine 10 leads to the suppression of Jab1 levels with the concomitant inhibition of c-Jun-dependent transcription. This repression is minimised upon quiescence exit through the rapid and preferential loss of the serine 10 phosphorylated form of p27Kip1 following serum stimulation. Our results, therefore, demonstrate an additional role for p27Kip1 in the modulation of c-Jun-dependent transcription via Jab1.

Introduction

The cyclin-dependent kinase (cdk) inhibitor p27Kip1 is a potent intracellular mediator of many anti-mitogenic signals and is closely involved in the imposition and maintenance of the quiescent state (reviewed in 1,2). Elevated levels of p27Kip1 enforce the maintenance of the quiescent state whereas down-regulation of p27Kip1 allows cells to recommence proliferation (3,4). The major function attributed to p27Kip1 is the stiochiometric inhibition of the G1-specific cyclin/cdk complexes (reviewed in 1,5).

p27Kip1 is regulated by a complex series of transcriptional and post-transcriptional mechanisms with protein degradation being a major determinant of intracellular p27Kip1 levels. Degradation of p27Kip1 can be promoted by the cyclin E/cdk2-dependent phosphorylation of threonine 187 (6-8). This modification activates ubiquitin-mediated proteolysis dependent on the ubiquitin ligase SCF^Skp2 (9,10).
In addition to binding to cyclin/cdk complexes, p27\textsuperscript{Kip1} has been reported to interact with other intracellular molecules including the c-Jun co-activator Jab1 (11, 12). Endogenous Jab1 (also known as CSN5) is also found incorporated into the COP9 signalosome, a multi-protein complex involved in modulating signal transduction, gene transcription and protein stability (reviewed in 12-16). The binding of p27\textsuperscript{Kip1} with Jab1 has been proposed to cause nuclear export of the resulting complex prior to ubiquitin-mediated degradation of the cdk inhibitor (11).

Here we describe experiments that cast a different light on the p27\textsuperscript{Kip1}/Jab1 relationship. Our data demonstrate that p27\textsuperscript{Kip1} can negatively regulate Jab1-stimulated transcription of an AP-1 reporter gene via c-Jun. This regulation is enforced by phosphorylation of p27\textsuperscript{Kip1} on serine 10, a modification that is readily detectable in quiescent cells. The serine 10 phospho-form of p27\textsuperscript{Kip1} is rapidly and preferentially lost on quiescence exit, providing a means for abolishing this negative regulation when conditions are favourable for proliferation, thus facilitating c-Jun-dependent transcription.

**Experimental Procedures**

NIH 3T3 cells were routinely cultured and made quiescent by maintenance in 0.5% normal calf serum as described (17). Immunoblotting was performed as described (18). The following antibodies were used: anti-HA (12CA5) from Boehringer Mannheim, anti-c-Jun (sc-1694), anti-p27\textsuperscript{Kip1} C terminus (sc-528) and anti-cdk4 (sc-260) from Santa Cruz, anti-α-tubulin (TAT-1) from the Imperial Cancer Research Fund, anti-FLAG (M2) from Sigma and anti-cdk2 (06-148) was from Upstate Biotech. Immunoblots were quantified with a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Immunoprecipitation/kinase assays were performed as described (17,19). Phosphatase treatment was performed by incubating 100μg of total cell extract with 60 units of calf...
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intestinal alkaline phosphatase (Boehringer Mannheim) at 37°C for 30 minutes prior to immunoblotting.

p27\textsuperscript{Kip1} in pcDNA3 (18) was N-terminally HA-epitope-tagged by the insertion of a double stranded oligonucleotide. S10A, S10E, S177A and T187A mutations were introduced into this construct using the polymerase chain reaction as described (17). All constructs generated by polymerase chain reaction were sequenced. Flag-tagged Jab1 in pcDNA3 was a gift from Dr W. Breitwieser. Other plasmids have been described (20-22).

Asynchronous NIH 3T3 or U2-OS cells were transfected by calcium phosphate co-precipitation (23). Promoter activity of the transfected reporter genes was measured using the Dual-Luciferase Reporter Assay System following the instructions of the manufacturer (Promega, UK) and values corrected for Renilla Luciferase activity from the co-transfected pRL-TK plasmid (Promega, UK). An E2F-Luciferase reporter (pGL2 3XWT E2F) was used as a control (22). Cell cycle analysis of transfected cells was performed as described (23).

Results and Discussion

Phosphorylation of p27\textsuperscript{Kip1} in vivo

Immunoblot analyses of p27\textsuperscript{Kip1} from quiescent cells using 10% polyacrylamide gels were used to resolve two distinct protein species, with a faster migrating form comprising 60-70% of the total p27\textsuperscript{Kip1} protein (Figure 1). The appearance of such a doublet is often indicative of a phospho-form of the target protein. In order to assess p27\textsuperscript{Kip1} phosphorylation, cell extracts were treated with calf intestinal alkaline phosphatase and analysed by immunoblotting (Figure 1A). Dephosphorylation of the cell extracts resulted in the loss of the slower migrating p27\textsuperscript{Kip1} species, demonstrating that this upper form is a phosphoprotein. p27\textsuperscript{Kip1} has been shown to be a target for cyclin/cdk-mediated phosphorylation (6-8). In order to identify the site(s) of phosphorylation within p27\textsuperscript{Kip1}, site-directed mutagenesis was used to replace the
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phosphoacceptor residues in the three minimal cyclin/cdk consensus motifs (S/T-P) with alanines. Epitope-tagged versions of these mutants were transfected into NIH 3T3 cells and extracts were immunoblotted for the exogenous p27\textsuperscript{kip1} using the anti-HA antibody. The p27\textsuperscript{kip1} double mutant in which serine 177 and threonine 187 were altered to alanines (p27\textsuperscript{kip1}\textsubscript{(S177A/T187A)}) was present as a doublet, indistinguishable from the epitope-tagged wild type protein, whereas mutation of serine 10 to alanine (p27\textsuperscript{kip1}\textsubscript{(S10A)}) lead to the loss of the upper band (Figure 1B). Taken together, these data indicate that p27\textsuperscript{kip1} is phosphorylated on serine 10 \textit{in vivo} by a kinase that is active in quiescent cells, a finding in agreement with recent observations (24,25).

In order to characterise the role of p27\textsuperscript{kip1} phosphorylated on serine 10, immunoblot analysis was used to examine the abundance of the two p27\textsuperscript{kip1} species as quiescent cells re-enter the cell cycle after serum stimulation. Serum treatment of G0 cells resulted in the degradation of both forms of p27\textsuperscript{kip1}, over the 16h time course, corresponding with an increase in cdk activity as the cells re-enter the cell cycle (Figure 2A). More detailed kinetic analysis revealed the preferential loss of the serine 10 phosphorylated form of p27\textsuperscript{kip1}; the levels of the serine 10 phosphorylated form of p27\textsuperscript{kip1} was reduced by 60% within 30 minutes of serum stimulation whereas the faster migrating species was largely unchanged (Figure 2B and 2C). This loss of the serine 10 phosphorylated p27\textsuperscript{kip1} occurred markedly earlier than the activation of either cdk4 or cdk2 kinases (Figure 2A). Quantitative analysis of these data indicated that within four hours of serum treatment, the serine 10 phosphorylated p27\textsuperscript{kip1} was preferentially eliminated (Figure 2C). When cells were cultured under conditions of reduced growth factors, the serine 10 phospho-form of p27\textsuperscript{kip1} accumulated with similar kinetics to total p27\textsuperscript{kip1} (Figure 2D). The accumulation of serine 10 phosphorylated p27\textsuperscript{kip1} as cells enter quiescence and the specific and rapid loss of this species, whether through degradation or dephosphorylation, indicates that this form of the cdk inhibitor may play a role in the regulation of early events in the exit from quiescence prior to cyclin/cdk activation.
Serine 10 phosphorylated p27\textsuperscript{kip1} inhibits Jab1-mediated activation of c-Jun

During exit from quiescence, AP-1-dependent transcription of immediate early genes occurs, this transcription being associated with cell proliferation (reviewed in (26)). The c-Jun co-activator Jab1 has been shown to interact with p27\textsuperscript{kip1} \textit{in vivo}, resulting in the nuclear export of the complex and enhanced p27\textsuperscript{kip1} degradation (11). Recent studies have demonstrated that the serine 10 phospho-form of p27\textsuperscript{kip1} is readily exported from the nucleus (24,25). Thus, we wondered whether this phosphorylated form of p27\textsuperscript{kip1} may regulate AP-1-dependent transcription via Jab1 rather than be a target for Jab1-dependent control.

In order to address this hypothesis, we performed reporter assays in NIH 3T3 cells using transient transfection. We employed a Luciferase cDNA under the control of an AP-1-dependent promoter in the absence and presence of c-Jun and/or Jab1 and analysed the effect of co-expressing wild type, S10A or S10E mutants of p27\textsuperscript{kip1} on reporter gene expression (Figure 3A). The introduction of Jab1 resulted in a 6-fold increase in c-Jun-dependent promoter activity. Co-expression of wild type p27\textsuperscript{kip1} suppressed this activation by approximately two thirds. Immunoblot analysis of wild type p27\textsuperscript{kip1} indicated that a portion of the ectopic wild type p27\textsuperscript{kip1} is readily phosphorylated on serine 10 (Figures 1 and 3C). The phospho-mimetic S10E mutant of p27\textsuperscript{kip1} was even more potent than the wild type protein in inhibiting reporter gene expression, completely abrogating the Jab1-dependent transactivation. Importantly, p27\textsuperscript{kip1} with the alanine replacement at amino acid 10 (which could not be phosphorylated) caused no repression of reporter gene expression (Figure 3A). This reciprocal regulation of reporter gene expression by the phospho-mimetic and the unmodified alanine mutant demonstrates the dependency of this mode of regulation of Jab1 on phosphorylation of p27\textsuperscript{kip1} on serine 10. Transfection experiments employing an E2F-driven Luciferase construct indicated that the regulation of the c-Jun transcription by the variant p27\textsuperscript{kip1} constructs was a specific effect; the p27\textsuperscript{kip1} variants had similar effects on E2F-reporter activity (Figure 3B). In addition, expression of each of the p27\textsuperscript{kip1} constructs \textit{in vivo} caused the imposition of cell cycle
arrest in the G1 phase (Figure 3D). Thus, the affects on c-Jun-dependent transcription are unlikely to be due to specific changes in proliferation imposed by the wild type, S10A or S10E variants of p27^Kip1.

In order to begin to address the mechanism by which the serine 10 phosphorylated form of p27^Kip1 regulates c-Jun dependent transcription via Jab1, we performed immunoblot analysis of the ectopically expressed proteins (Figure 3C). Levels of endogenous c-Jun protein are high in NIH 3T3 cells making it difficult to detect the exogenous protein. However, transfection of either Jab1 or the p27^Kip1 variants had no effects on the level of total c-Jun protein. Each of the transfected p27^Kip1 constructs directed expression of HA-tagged p27^Kip1, with the wild type migrating as a doublet, the S10A form co-migrating with the smallest species and the S10E mutant migrating as a single band intermediate in molecular weight between the non-phosphorylated and serine 10 phosphorylated species. Analysis of the expression of the Jab1 protein indicated that the phosphorylation status of p27^Kip1 had a profound affect on the level of the co-activator. In the absence of co-transfected p27^Kip1, Flag-tagged Jab1 was readily detectable in cell lysates. The S10A mutant of p27^Kip1 caused minimal changes in the level of Jab1. However, co-transfection of the wild type p27^Kip1 or, even more strikingly, the S10E mutant caused a dramatic reduction in the level of Jab1 expression. Thus, there is a direct correlation between the levels of Jab1 and AP-1-dependent transcription dependent on phosphorylation status of the p27^Kip1 at serine 10. It should be noted that this assay relies on the over-expression of the transfected components. Experiments to confirm this data in a physiological setting are underway.

The data presented indicate that p27^Kip1 may modulate the function of Jab1 at least partially through the regulation of Jab1 levels. Jab1 is also a component of the COP9 signalosome and p27^Kip1 is known to be able to interact with Jab1 in the setting of the signalosome (12). This raises the intriguing possibility that p27^Kip1 may to modulate some of the many functions of the COP9 signalosome.
Recently, Boehm et al (27) have reported that hKIS can phosphorylate p27\textsuperscript{Kip1} on serine 10 in a mitogen-dependent manner. Since we detect significant phosphorylation of p27\textsuperscript{Kip1} on serine 10 in quiescent cells, it is unclear at present what relevance hKIS-mediated phosphorylation of p27\textsuperscript{Kip1} has to the regulation of Jun transcription through p27\textsuperscript{Kip1} via Jab1.

Our data are consistent with a model in which p27\textsuperscript{Kip1} plays two roles in the enforcement and maintenance of quiescence. Firstly, it sequesters cyclin/cdk complexes to block their mitogenic activity (28-30). Secondly, p27\textsuperscript{Kip1} phosphorylated on serine 10 by a kinase active in quiescent cells inhibits AP-1-dependent immediate early gene expression through repression of Jab1 transcription activity. Our data shows that this is mediated at least partially through the destruction of Jab1. Serine 10 phosphorylated p27\textsuperscript{Kip1} is specifically lost from the cell as an early event following serum stimulation, thus relieving its inhibitory effects on Jab1-activated transcription and facilitating immediate early gene expression. Thus, the anti-proliferative role of p27\textsuperscript{Kip1} as a cdk inhibitor may be augmented by its ability to repress the pro-mitogenic activity of c-Jun through Jab1.

**Figure Legends**

Figure 1: p27\textsuperscript{Kip1} is phosphorylated on serine 10 in vivo. A. Lysates of quiescent NIH 3T3 cell were incubated in the presence and absence of calf intestinal alkaline phosphatase prior to immunoblotting for p27\textsuperscript{Kip1}. B. NIH 3T3 cells were transfected with cDNAs encoding HA-tagged wild type or phosphosite mutant p27\textsuperscript{Kip1}. Cells were lysed and extracts were subject to immunoblotting through the epitope tag.

Figure 2: The serine 10 phosphorylated form of p27\textsuperscript{Kip1} is preferentially degraded upon quiescence exit. A. NIH 3T3 cells were made quiescent by serum deprivation for 72 hours and subsequently stimulated by addition of normal calf serum to 10%. Cells were then harvested at the indicated times and subject to either immunoblot for
p27^Kip1 or α-tubulin or immunoprecipitated through the indicated cdk subunit prior to assay of kinase activity against the Retinoblastoma protein. B. NIH 3T3 cells were made quiescent by serum deprivation for 72 hours and subsequently stimulated by addition of normal calf serum to 10%. Cells were then harvested at the indicated times and the lysates subject to immunoblot for p27^Kip1. C. p27^Kip1 levels from immunoblots generated as described in part B were quantified using NIH Image. The data represent the means and standard deviations of three separate experiments, each performed in duplicate. Serine 10 phosphorylated p27^Kip1 is shown in white with the non-phosphorylated species in black. D. Asynchronous cultures of NIH 3T3 cells were washed in PBS and cultured for the indicated time in 0.5% normal calf serum. Cells were then harvested at the indicated times and the lysates subject to immunoblot for p27^Kip1. In C. and D. equal amounts of protein levels were loaded in each sample as verified via α-tubulin immunoblots (not shown).

Figure 3: Serine 10 phosphorylated p27^Kip1 can modulate Jab1-mediated c-Jun transcription. Asynchronous NIH 3T3 cells were transfected with an AP-1 (A.) or and E2F (B.) driven Luciferase reporter gene and the indicated constructs. Cells were lysed and assayed for Luciferase activity. Results were normalised to the activity of co-transfected pRL-TK plasmid encoding Renilla Luciferase. C. NIH 3T3 cells were transfected as described above. Cells were then harvested and subject to immunoblot for c-Jun, Flag-tagged Jab1 and p27^Kip1. D. U2-OS cells were transfected with 5µg of the indicated p27 variant and 0.5µg EGFP-F (Clontech) as a marker for transfected cells. After 72 hours, cells were harvested and subject to flow cytometry (23). Results show the cell cycle distribution of the transfected cells and are representative of three separate experiments. At least 10 000 cells were gated for each sample. Control cells were transfected with EGFP-F alone. Similar data were obtained with NIH 3T3 cells (not shown).

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References
Figure 1

A. wild type p27

alkaline phosphatase

p27

B. wild type S177A/T187A S10A

anti-HA

p27Kip1 Inhibits Jab1-dependent transcription
Figure 2

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B.  

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Figure 2, cont.

C.

D.
Figure 3

A.

- Luciferase activity (fold increase)

- c-Jun
- Jab1
- p27
- p27 S10A
- p27 S10E

B.

- Luciferase activity (fold increase)

- c-Jun
- Jab1
- p27
- p27 S10A
- p27 S10E
Figure 3

C. + + + + + + c-Jun
    + + + + + + Jab1
    + + + + + + p27
    + + + + + + p27S10A
    + + + + + + p27S10E

c-Jun
Jab1
p27

D.

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Percentage of cells
Jab1 co-activation of c-Jun is abrogated by the serine 10 phosphorylated form of p27Kip1

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