Jab1 Co-activation of c-Jun Is Abrogated by the Serine 10-phosphorylated Form of p27Kip1*

Received for publication, Received, May 20, 2002, and in revised form, July 12, 2002
Published, JBC Papers in Press, July 15, 2002, DOI 10.1074/mcp.C2000511200

Shalu Chopra‡, Silvia Fernandez de Mattos§‡, Eric W.-F. Lam§, and David J. Mann¶

From the ‡Department of Biological Sciences, Imperial College of Science, Technology and Medicine, Exhibition Road, South Kensington, London SW7 2AY and §Department of Cancer Medicine, Cancer Research United Kingdom Laboratories and Section of Cancer Cell Biology, MRC Cyclotron Building, Imperial College School of Medicine at Hammersmith Hospital, Du Cane Road, London W12 0NN, United Kingdom

Accelerated Publication

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 show 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 minimized 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.

The cyclin-dependent kinase (cdk) inhibitor p27Kip1 is a potent intracellular mediator of many anti-mitogenic signals and is involved closely in the imposition and maintenance of the quiescent state (reviewed in Refs. 1 and 2). Elevated levels of p27Kip1 enforce the maintenance of the quiescent state whereas down-regulation of p27Kip1 allows cells to recommit proliferation (3, 4). The major function attributed to p27Kip1 is the stoichiometric inhibition of the G1-specific cyclin-cdk complexes (reviewed in Refs. 1 and 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 SCFβTrCP (9, 10).

In addition to binding to cyclin-cdk complexes, p27Kip1 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 multiprotein complex involved in modulating signal transduction, gene transcription, and protein stability (reviewed in Refs. 12–16). The binding of p27Kip1 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 p27Kip1/Jab1 relationship. Our data demonstrate that p27Kip1 can negatively regulate Jab1-stimulated transcription of an AP-1 reporter gene via c-Jun. This regulation is enforced by phosphorylation of p27Kip1 on serine 10, a modification that is readily detectable in quiescent cells. The serine 10-phospho-form of p27Kip1 is rapidly and preferentially lost on quiescence exit, providing a means for abolishing this negative regulation when conditions are favorable for proliferation, thus facilitating c-Jun-dependent transcription.

EXPERIMENTAL PROCEDURES

NIH 3T3 cells were cultured routinely 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 Roche Molecular Biochemicals, anti-c-Jun (sc-1694), anti-p27Kip1 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) from Upstate Biotech. Immunoblotting were quantified with a Macintosh computer using the public domain NIH Image program (developed at the United States National Institutes of Health and available on the Internet at rsb.info.nih.gov/nih-image/). Immunoprecipitation/kinase assays were performed as described (17, 19). Phosphatase treatment was performed by incubating 100 μg of total cell extract with 60 units of calf intestinal alkaline phosphatase (Roche Molecular Biochemicals) at 37 °C for 30 min prior to immunoblotting.

p27Kip1 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), and values were corrected for Renilla luciferase activity from the co-transfected pRL-TK plasmid (Promega). 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 p27Kip1 in Vivo—Immunoblot analyses of p27Kip1 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 p27Kip1 protein (Fig. 1). The appearance of such a doublet is often indicative of a phospho-form of the target protein. To assess p27Kip1 phosphorylation, cell extracts were treated with calf intestinal alkaline phosphatase and analyzed by immunoblotting (Fig. 1A). Diphosphorylation of the cell extracts resulted in the loss of the...
slower migrating p27^Kip1 species, demonstrating that this upper form is a phosphoprotein. p27^Kip1 has been shown to be a target for cyclin/cdk-mediated phosphorylation (6–8). To identify the site(s) of phosphorylation within p27^Kip1, site-directed mutagenesis was used to replace the phosphoacceptor residues in the three minimal cyclin/cdk consensus motifs (Ser-Pro or Thr-Pro) with alanines. Epitope-tagged versions of these mutants were transfected into NIH 3T3 cells, and extracts were subject to immunoblotting through the epitope tag.

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

FIG. 2. The serine 10-phosphorylated form of p27^Kip1 is preferentially degraded upon quiescence exit. A, NIH 3T3 cells were made quiescent by serum deprivation for 72 h 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 h and subsequently stimulated by addition of normal calf serum to 10%. Cells were then harvested at the indicated times, and the lysates were subject to immunoblot for p27^Kip1. C, p27^Kip1 levels from immunoblots generated as described in B were quantified using NIH Image. The data represent the means and S.D. 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 NIH 3T3 cells were transfected with p27^Kip1. C, p27^Kip1 levels from immunoblots generated as described in B were quantified using NIH Image. The data represent the means and S.D. 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 NIH 3T3 cells were transfected with p27^Kip1. C, p27^Kip1 levels from immunoblots generated as described in B were quantified using NIH Image. The data represent the means and S.D. 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 NIH 3T3 cells were transfected with p27^Kip1. C, p27^Kip1 levels from immunoblots generated as described in B were quantified using NIH Image. The data represent the means and S.D. of three separate experiments, each performed in duplicate. Serine 10-phosphorylated p27^Kip1 is shown in white with the non-phosphorylated species in black.

FIG. 3. Serine 10-phosphorylated p27^Kip1 can modulate Jab1-mediated c-Jun transcription. Asynchronous NIH 3T3 cells were transfected with an AP-1 (A) and/or E2F (B)-driven luciferase reporter gene and the indicated constructs. Cells were lysed and assayed for luciferase activity. Results were normalized 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 5 μg of the indicated p27 variant and 0.5 μg of EGFP-F (CLONTECH) as a marker for transfected cells. After 72 h, 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).
studies have demonstrated that the serine 10 phospho-form of p27Kip1 is phosphorylated on serine 10 in vivo by a kinase that is active in quiescent cells, a finding in agreement with recent observations (24, 25).

To characterize the role of p27Kip1 phosphorylated on serine 10, immunoblot analysis was used to examine the abundance of the two p27Kip1 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 p27Kip1, over the 16h time course, corresponding with an increase in cdk activity as the cells re-enter the cell cycle (Fig. 2A). More detailed kinetic analysis revealed the preferential loss of the serine 10-phosphorylated form of p27Kip1; the levels of the serine 10-phosphorylated form of p27Kip1 was reduced by 60% within 30 min of serum stimulation whereas the faster migrating species was largely unchanged (Fig. 2, B and C). This loss of the serine 10-phosphorylated p27Kip1 occurred markedly earlier than the activation of either cdk4 or cdk2 kinases (Fig. 2A). Quantitative analysis of these data indicated that within 4 h of serum treatment, the serine 10-phosphorylated p27Kip1 was preferentially eliminated (Fig. 2C). When cells were cultured under conditions of reduced growth factors, the serine 10 phospho-form of p27Kip1 accumulated with similar kinetics to total p27Kip1 (Fig. 2D). The accumulation of serine 10-phosphorylated p27Kip1 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 p27Kip1 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 Ref. 26). The c-Jun co-activator Jab1 has been shown to interact with p27Kip1 in vivo, resulting in the nuclear export of the complex and enhanced p27Kip1 degradation (11). Recent studies have demonstrated that the serine 10 phospho-form of p27Kip1 is exported readily from the nucleus (24, 25). Thus, we wondered whether this phosphorylated form of p27Kip1 may regulate AP-1-dependent transcription via Jab1 rather than be a target for Jab1-dependent control.

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 analyzed the effect of co-expressing wild type, S10A, or S10E mutants of p27Kip1 on reporter gene expression (Fig. 3A). The introduction of Jab1 resulted in a 6-fold increase in c-Jun-dependent promoter activity. Co-expression of wild type p27Kip1 suppressed this activation by approximately two-thirds. Immunoblot analysis of wild type p27Kip1 indicated that a portion of the ectopic wild type p27Kip1 is phosphorylated readily on serine 10 (see Fig. 1 and Fig. 3C). The phospho-mimetic S10E mutant of p27Kip1 was even more potent that the wild type protein in inhibiting reporter gene expression, completely abrogating the Jab1-dependent transactivation. Importantly, p27Kip1 with the alanine replacement at amino acid 10 (which could not be phosphorylated) caused no repression of reporter gene expression (Fig. 3A). This reciprocal regulation of reporter gene expression by the phospho-mimetic and the unmodified alanine mutant demonstrates the dependence of this mode of regulation of Jab1 on phosphorylation of p27Kip1 on serine 10. Transfection experiments employing an E2F-driven luciferase construct indicated that the regulation of the c-Jun transcription by the variant p27Kip1 constructs was a specific effect; the p27Kip1 variants had similar effects on E2F reporter activity (Fig. 3B). In addition, expression of each of the p27Kip1 constructs in vivo caused the imposition of cell cycle arrest in the G1 phase (Fig. 3D). Thus, the effects on c-jun-dependent transcription are unlikely to be because of specific changes in proliferation imposed by the wild type, S10A, or S10E variants of p27Kip1.

To begin to address the mechanism by which the serine 10-phosphorylated form of p27Kip1 regulates c-jun-dependent transcription via Jab1, we performed immunoblot analysis of the ectopically expressed proteins (Fig. 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 p27Kip1 variants had no effects on the level of total c-Jun protein. Each of the transfected p27Kip1 constructs directed expression of HA-tagged p27Kip1, 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 p27Kip1 had a profound affect on the level of the co-activator. In the absence of co-transfected p27Kip1, FLAG-tagged Jab1 was readily detectable in cell lysates. The S10A mutant of p27Kip1 caused minimal changes in the level of Jab1. However, co-transfection of the wild type p27Kip1 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 p27Kip1 at serine 10. It should be noted that this assay relies on the overexpression of the transfected components. Experiments to confirm this data in a physiological setting are underway.

The data presented indicate that p27Kip1 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 p27Kip1 is known to be able to interact with Jab1 in the setting of the signalosome (12). This raises the intriguing possibility that p27Kip1 may modulate some of the many functions of the COP9 signalosome.

Recently, Boehm et al. (27) have reported that hKIS can phosphorylate p27Kip1 on serine 10 in a mitogen-dependent manner. Because we detect significant phosphorylation of p27Kip1 on serine 10 in quiescent cells, it is unclear at present what relevance hKIS-mediated phosphorylation of p27Kip1 has to the regulation of Jun transcription through p27Kip1 via Jab1.

Our data are consistent with a model in which p27Kip1 plays two roles in the enforcement and maintenance of quiescence. First, it sequesters cyclin-cdk complexes to block their mitogenic activity (28–30). Second, p27Kip1 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 show that this is mediated at least partially through the destruction of Jab1. Serine 10-phosphorylated p27Kip1 is specifically lost from the cell as an early event following serum stimulation, thus relaxing its inhibitory effects on Jab1-activated transcription and facilitating immediate early gene expression. Thus, the anti-proliferative role of p27Kip1 as a cdk inhibitor may be augmented by its ability to repress the pro-mitogenic activity of c-jun through Jab1.

Acknowledgments—We thank Drs. A. Armesilla, E. Black, W. Breitwieser, D. Gillespie, N. Jones, and M. Rincon for the generous gift of plasmids and A. Rae for assistance with the flow cytometry.

REFERENCES
1. Slingerland, J., and Pagano, M. (2000) J. Cell. Physiol. 183, 10–17
2. Sherr, C. J. (2000) Cancer Res. 60, 3689–3695
3. Ladha, M. H., Lee, K. Y., Upton, T. M., Reed, M. F., and Ewen, M. E. (1998) Mol. Cell. Biol. 18, 6605–6615
4. Rivard, N., L’Allemain, G., Bartek, J., and Pouyssegur, J. (1996) J. Biol. Chem. 271, 18337–18341
5. Sherr, C. J., and Roberts, J. M. (1996) Genes Dev. 10, 1501–1512
6. Sheaff, R. J., Groudine, M., Gordon, M., Roberts, J. M., and Clurman, B. E. (1997) Genes Dev. 11, 1464–1478
7. Vlach, J., Hennecke, S., and Amati, B. (1997) EMBO J. 16, 5334–5344
8. Muller, D., Bouchard, C., Rudolph, E., Steiner, F., Stuckmann, I., Safrisch, R., Ansorge, W., Huttner, W., and Ehlers, M. (1997) Oncogene 15, 2561–2576
9. Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. (1999) Nat. Cell Biol. 1, 193–199
10. Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H., and Zhang, H. (1999) Curr. Biol. 9, 661–664
11. Tomoda, K., Kubota, Y., and Kato, J. (1999) Nature 398, 160–165
12. Tomoda, K., Kubota, Y., Arata, Y., Mori, S., Maeda, M., Tanaka, T., Yoshida, M., Yoneda-Kato, N., and Kato, J. (2002) J. Biol. Chem. 277, 2302–2310
13. Schwechheimer, C., and Deng, X. W. (2001) Trends Cell Biol. 11, 420–426
14. Chamovitz, D. A., and Segal, D. (2002) EMBO Rep. 2, 96–101
15. Bech-Otschir, D., Seeger, M., and Dabiel, W. (2002) J. Cell Sci. 115, 467–473
16. Chamovitz, D. A., and Glickman, M. (2002) Curr. Biol. 12, R232
17. Child, E. S., and Mann, D. J. (2001) Oncogene 20, 3311–3322
18. Mann, D. J., Child, E. S., Swanton, C., Laman, H., and Jones, N. (1999) EMBO J. 18, 654–663
19. Mann, D. J., Higgins, T., Jones, N. C., and Rozengurt, E. (1997) Oncogene 14, 1759–1766
20. Rincon, M., and Flavell, R. A. (1994) EMBO J. 13, 4370–4381
21. Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., and Woodgett, J. R. (1991) Nature 353, 670–674
22. Joss, K., Lam, E. W., Bybee, A., Girling, R., Muller, R., and La Thangue, N. B. (1995) Oncogene 10, 1529–1536
23. Mann, D. J., and Jones, N. C. (1996) Curr. Biol. 6, 474–483
24. Ishida, N., Hara, T., Kamura, T., Yoshida, M., Nakayama, K., and Nakayama, K. I. (2002) J. Biol. Chem. 277, 14355–14359
25. Rodier, G., Montagnoli, A., Di Marcotullio, L., Coulombe, P., Draetta, G. F., Pagano, M., and Melchior, S. (2001) EMBO J. 20, 6672–6682
26. Shaulian, E., and Karin, M. (2002) Nat. Cell Biol. 4, E131–E136
27. Boehm, M., Yoshimoto, T., Crook, M. F., Nallathamshetty, S., True, A., Nabel, G. J., and Nabel, E. (2002) EMBO J. 21, 3390–3401
28. Polyak, K., Kato, J. Y., Solomon, M. J., Sherr, C. J., Massague, J., Roberts, J. M., and Koff, A. (1994) Genes Dev. 8, 9–22
29. Toyoshima, H., and Hunter, T. (1994) Cell 78, 67–74
30. Poon, R. Y., Toyoshima, H., and Hunter, T. (1995) Mol. Biol. Cell 6, 1197–1213

p27Kip1 Inhibits Jab1-dependent Transcription