AD_____

Award Number: DAMD17-01-1-0404

TITLE: Activation of P13K/PKB Signaling in Breast Cancer may Inhibit TGFB-Induced G1 Arrest through Changes in p27 Function

PRINCIPAL INVESTIGATOR: Jiyong Liang, M.D.

CONTRACTING ORGANIZATION: Sunnybrook and Women's College
Health Science Center
Toronto, Ontario, Canada M4N 3M5

REPORT DATE: October 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2004	Annual Summary(1 Oct 2001 - 30 Sep 2004)

4. TITLE AND SUBTITLE
Activation of P13K/PKB Signaling in Breast Cancer may Inhibit TGFB-Induced G1 Arrest through Changes in p27 Function

5. FUNDING NUMBERS
DAMD17-01-1-0404

6. AUTHOR(S)

Jiyong Liang, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Sunnybrook and Women's College
Health Science Center
Toronto, Ontario, Canada M4N 3M5

E-Mail: jliang@srcl.sunnybrook,utoronto.ca

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Transforming growth factor beta (TGF-β)-induced cell cycle arrest in G1 phase is a mechanism that prevents abnormal cell proliferation. Resistance the growth inhibitory effect of TGF-β, often acquired during oncogenesis, may in turn contribute to malignant progression. This grant set out to investigate the mechanisms of loss of TGF-β sensitivity in breast cancers. We showed that PKB over-activation in TGF-β resistant mammary epithelial cells and inhibition of PI3K/PKB restores TGF-β sensitivity. We further demonstrated that PKB phosphorylates the dual cdk regulator $p27^{KIP1}$. PKB-dependent phosphorylation of $p27^{Kip1}$ not only impairs the cdk inhibitory function of $p27^{Kip1}$ by impairing its nuclear localization; it also participates in activating the function of $p27^{Kip1}$ to mediate cyclin D1-cdk4 assembly. Our findings suggest that PI3K/PKB positively regulates cell proliferation via regulation of $p27^{Kip1}$, contributing to loss of sensitivity to TGF-β-induced cell cycle arrest and tumor progression, thus providing a rationale for targeting the PI3K/PKB pathway in breast cancer therapy.

14. SUBJECT TERMS
Breast cancer, TGF-β, p27Kip1, PI3K, PKB/Akt, phosphorylation, cell cycle, G1 arrest, proliferation, subcellular localization

15. NUMBER OF PAGES
61

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Final Progress Report

Title: **Activation of PI3K/PKB signaling may inhibit TGF-β-induced G1 arrest in breast cancer through changes in p27 function**

Jiyong Liang

INTRODUCTION

TGF-β1 plays a pivotal role in maintaining homeostasis of normal tissues including mammary epithelial cell growth [1,2], in which TGF-β1 induces cell cycle arrest through activating cdk inhibitors including $p15^{INK4B}$ and $p27^{Kip1}$ [3,4,5]. Loss of $p27^{Kip1}$, seminally described in breast cancers, has been found in a plethora of human malignancies [6]. The Slingerland (my mentor) lab found that p27 is subject to complex post-translational regulation, e.g. phosphorylation.

To elucidate the mechanisms underlying TGF-β resistance in breast cancer cells, this proposal set out to address the hypothesis that ***changes in phosphorylation serve to switch the cdk inhibitor p27 from an active state that binds avidly to cyclin E/cdk2 in TGF-β arrest and in G0, to an inactive state in late G1 and mitogenic activation of the PI3K/PKB pathways leads to phosphorylation of p27, reducing its affinity for cyclin E/cdk2 and abrogating its inhibitory function.***

We proposed 3 specific Aims to pursue this hypothesis. **Aim1)** Functional difference in p27 will be examined during cell cycle progression in TGF-β S and R lines by i) assaying differences in p27's affinity for and inhibition of recombinant cyclin E/cdk2 and ii) correlating changes in p27 function with shifts in its intracellular localization. **Aim2)** The role of phosphorylation in modulation of p27 function will be assayed by 2DIEF of p27 in different cyclin/cdk complexes from S

and R lines. In **Aim3)**, effects of constitutive activation of the PI3K/PKB signal transduction pathways on p27 activity and TGF-β sensitivity will be assayed by transforming HMEC with activated PI3K or PKB. These studies may link TGF-β resistance to oncogenic activation of mitogenic signaling pathways commonly seen in breast cancers. It is hoped that elucidation of the molecular mechanisms of cell cycle deregulation and TGF-β resistance in breast cancers may open up new therapeutic avenues for breast cancer patients.

In the **first report period (2001-2002),** we addressed **Aim3** and **Aim1-ii** by showing that 1) Increased activation of PKB in lines resistant to G1 arrest by TGF-β; 2) Constitutive PKB activation inhibits responsiveness to TGF-β. 3) A PKB-dependent pathway causes cytoplasmic mislocalization of p27 in TGF-β resistant cells; 4) PKB/Akt binds and phosphorylates cellular $p27^{Kip1}$. In the **second report period (2002-2003),** we addressed **Aim1-i** and **Aim2** by showing that 1) T157 phosphorylation of p27 is dependent on PI3K/PKB; 2) The association of p27-cyclin D1-cdk4 is increased in cells with PKB/Akt over-activation; 3) PKB activation precedes assembly of p27-cylcin D1-cdk4 in G1; 4) PI3K/PKB activation is required for association of p27-cyclin D1-cdk4; 5) Cyclin D1-asociated p27 is hyper-phosphorylated.

My first and second reports covered all 3 Aims and the majority of the tasks have been completed. Our data indeed support our original hypothesis that p27 function and localization are critically regulated by phosphorylation. To further elucidate the mechanisms we focus our attention on identifying the phosphorylation sites that are critical in regulating p27 function and better characterizing the role of PKB and PKB-

dependent phosphorylation. The current report will summarize the progress made in the **final report period (2003-2004)** of this grant.

1. Multi-site phosphorylation activates the assembly function of p27

As described in the second report we showed that cyclin D1-associated p27 is hyperphosphorylated. p27 has been shown to be phosphorylated at T187 [8,9], S10 [10,11,12], S178 [10], T157 [7,14,15], and T198 [13]. To identify the sites that are differentially phosphorylated in cyclin D1-bound and cdk2-bound p27, FLAG-tagged p27S7A, S10A, S12D, T157A, T198A were generated by converting the respective serine (S) or threonine (T) residues to either alanine (A) or aspartic acid (D) and two-dimensional phosphopeptide mapping of phosphate labeled FLAG p27 precipitates was performed following transfection of these different alleles into MCF-7 cells.

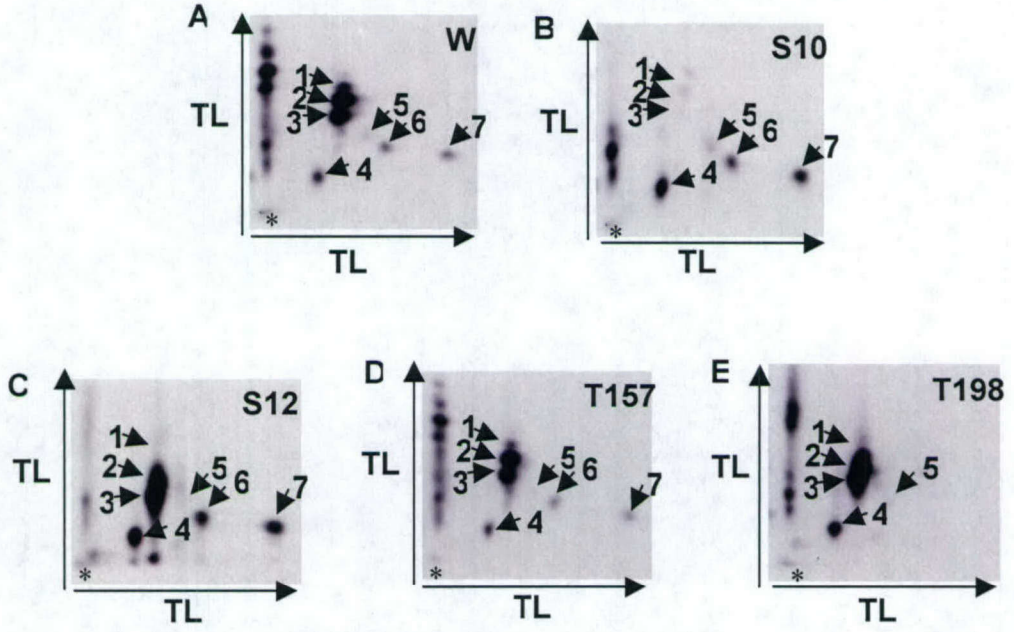


Figure 1. 2-D phosphopeptide mapping of p27 phosphorylation sites. FLAG-tagged wild-type p27 and the indicated mutant p27 alleles were transfected into MCF-7 cells and 24 hours post-transfection, cells were phosphate labeled and p27 immunoprecipitated and phosphopeptide mapping carried out as described in Methods. Phosphopeptides are numbered or labeled with letters and indicated by arrows. The directions of chromatography (TLC) and electrophoresis (TLE) separation are indicated by arrows and the origin of each map is marked as an asterisk.

Spots 1 to 7 were detected in wild-type FLAG-p27 (F-p27WT) (Fig 1A). As noted above, spots 2 and 3 were more strongly phosphorylated in cyclin D1-bound than in cdk2-bound p27 (See progress report 2, 2002-2003.). Ishida et al showed that these spots were influenced by mutation of S10 to alanine [10]. Consistent with prior reports, spots 1, 2, and 3 were barely detectable in p27S10A (Fig 1B). The tryptic peptide containing S10 has three putative phosphorylation sites, S7, S10 and S12. Of the three S10 related spots, spot 1, migrating closest to the anode and with greater hydrophobicity could represent a mono-phosphorylated peptide and spots 2 and 3 could represent the S10-containing peptide phosphorylated at more than one of 3 potential sites. However, S7A mutation did not affect any of the numbered spots on phosphopeptide mapping or the mobility of the mutant protein on SDS-PAGE (Fig 1C). Since S10A mutation abolished all of spots 1-3, the phosphorylation of S10 and S12 appear to be interdependent. The effect of the phosphomimetic mutatant S12D was assayed. S12D mutation abolished spot 1 (Fig 1D). Thus, peptide 1 may reflect mono-phosphorylation of the peptide containing S10/S12. The detection of two doubly phosphorylated peptide spots (2 and 3) may result from an oxidation side effect of performic acid treatment [10].

2. PKB activates the assembly function of p27 for cyclin D-cdk4

We also examined the role of PKB-dependent phosphorylation in modulating cyclin D1-cdk4 assembly by p27 *in vitro* by using recombinant proteins. Recombinant bacculoviral produced cyclin D1 and cdk4 were mixed with or without recombinant p27 and their assembly was detected by immunoprecipitating cyclin D1 followed by immunoblotting cyclin D1 and cdk4. While purified cyclin D1 and cdk4

did not form detectable complexes without added p27 (Fig 2, lane 1), the addition of purified recombinant p27 (Rec-p27) mediated assembly of cyclin D1 and cdk4 (Fig 2, lane 2), consistent with previous studies [16]. Treatment of recombinant p27 with PKB kinase for 30 min prior to the assembly reaction markedly increased the ability of p27 to mediate cyclin D1-cdk4 assembly (Fig 2, lane 3).

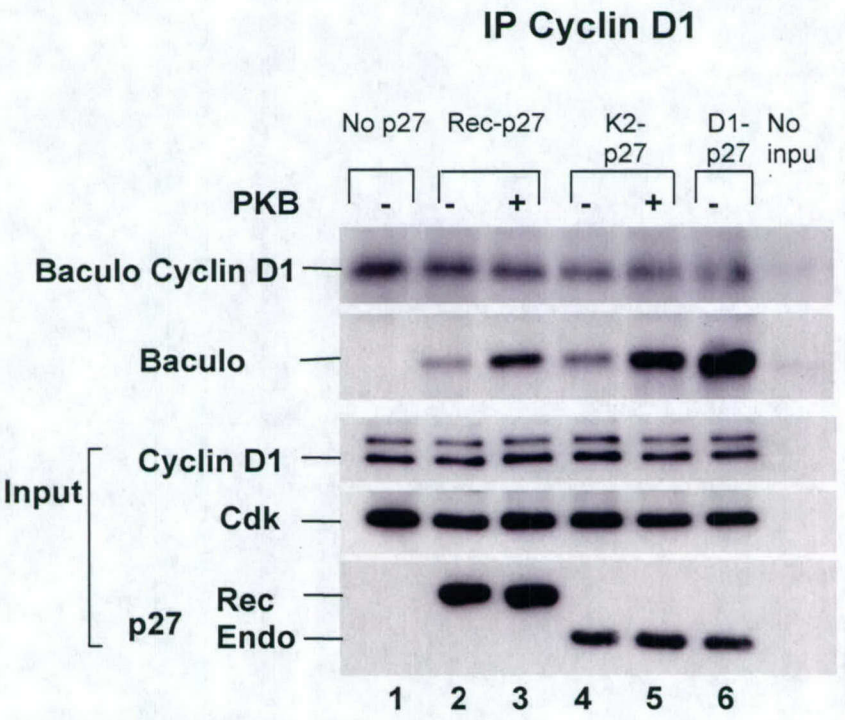


Figure 2. PKB increases p27-mediated assembly of cyclin D1-cdk4. Recombinant human cyclin D1 and cdk4 were incubated for 20 min at 25 °C without (No p27) or with recombinant (Rec-p27) or with cellular p27 released by boiling from cdk2 (K2-p27) and cyclin D1 (D1-p27) immunecomplexes from MCF-7 cells. Where indicated (+ or -), p27 was incubated with PKB under kinase reaction conditions for 30 min prior addition of p27 to recombinant cyclin D1 and cdk4 for p27-cyclin D1-cdk4 assembly assays. Cyclin D1 was then immunoprecipitated and cdk4 binding was detected by Western blot. The bottom panel shows Western blots of the input proteins.

The assembly activity of p27 from different cellular cyclin-cdk complexes was similarly assayed. Immunoprecipitations of cdk2 and cyclin D1 were performed. Each

of these complexes was boiled and the heat-stable cellular p27 from these immune complexes was recovered. Cdk2-associated cellular p27 was able to mediate the assembly of cyclin D1 and cdk4, but did so less effectively than equivalent amounts of p27 isolated from cellular cyclin D1 complexes (Fig2, lane 4 *vs* 6). As for recombinant p27, the cyclin D1-cdk4 assembly activity of p27 recovered from cdk2 precipitates was also increased following pretreatment with PKB kinase (Fig 2, lane 4 and 5). These data suggest that PKB-dependent phosphorylation of p27 up regulates p27 assembly function for cyclin D1 and cdk4 in a manner independent of the subcellular localization of p27.

KEY RESEARCH ACCOMPLISHMENTS

- Major phosphorylation sites of p27 were mapped.
- Preferentially phosphorylated sites of cyclin D1-associated p27 were identified.
- The activity of p27 to mediate the assembly of cyclin D1 and cdk4 was shown.
- Cdk2-associated p27 is less effective in mediating cyclin D1/cdk4 assembly than cyclin D1-associated p27.
- PKB-dependent p27 phosphorylation increases the assembly function of p27.

REPORTABLE OUTCOMES

1. **Liang J**, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catzavelos C, Beniston R, Franssen E, and Slingerland JM (2002) *Nature Med.* 8, 1153-1160. *Original article, first author.*
2. **Liang J**, Slingerland JM. Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. Cell Cycle. 2003; 2(4): 339-45. *Invited review, first author.*
3. Sheng W, Wang G, Wang Y, **Liang J**, Wen J, Zheng PS, Wu Y, Lee V, Slingerland J, Dumont D, Yang BB. The roles of versican V1 and V2 isoforms in cell proliferation and apoptosis. Mol Biol Cell. 2005. 16(3): 1330-1340. *Collaborator.*
4. **Jiyong Liang**, Shan H. Shao, Kathy Han, Joyce M. Slingerland. Regulation of p27Kip1-mediated cyclin D1-cdk4 assembly by PKB through multisite phosphorylation. 2004 Miami Nature Biotechnology Symposium. February 2004, Miami, FL, US. *Abstract.*
5. **Jiyong Liang**, Shan H. Shao, Kathy Han, Dan Dumont, Joyce M. Slingerland. Multisite phosphorylation activates p27-mediated cyclin D1/Cdk4 assembly. The American Society for Cell Biology, 2003 Annual Meeting. *Invited speaker.*
6. Ph. D., March 2004, University of Toronto. *Degree obtained and supported by this award.*
7. 2004-2007, MD Anderson Cancer Center Odyssey Fellowship. *Funding applied based on work supported by this award.*

CONCLUSIONS

TGF-β is a potent growth inhibitory cytokine that is secreted by normal and tumor cells. To varying degrees, most cancer cells exhibit resistance to growth arrest by TGF-β, a molecular mechanism that normally maintains tissue homeostasis. Re-sensitizing cancer cells to TGF-β may therefore subject tumors to this physiological growth control mechanism. We showed that PKB phosphorylates the dual cdk regulator $p27^{KIP1}$. PKB-dependent phosphorylation of p27 not only impairs the cdk inhibitory function of p27 by impairing its nuclear localization; it also participates in activating the function of p27 to mediate cyclin D1-cdk4 assembly. These mechanisms link PI3K/PKB activation to cell proliferation via regulation of p27, contributing to loss of sensitivity to TGF-β-induced cell cycle arrest and tumor progression.

The finding that TGF-β resistant cancer cell lines can be rendered sensitive to TGF-β-induced proliferation arrest via inhibiting the PI3K pathway might be further exploited therapeutically.

In addition, our data also support the notion that p27 may represent a signaling convergence point that responds to multiple mitogen signals and differentially regulates cyclin D- and cyclin E-dependent kinases. Cytoplasmic mislocalization and functional alteration of p27 are among the mechanisms that abrogate the tumor suppressive activity of p27. We have shown that inhibition of PI3K pathway can restore not only the physiological localization of p27 but also its cyclin E-cdk2 inhibitory function to mediate cell cycle arrest. Since the p27 gene is rarely inactivated by mutation in human cancer, the intrinsic tumor suppressive action of

p27 has the potential to be re-activated by inhibiting the oncogenic pathways that disrupt its function in cancer cells.

References

1. Kingsley, DM. The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes and Development, 8:133-146, 1994.
2. Alexandrow, MG and Moses, H. Transforming growth factor and TGF-β and cell cycle regulation. Cancer Res., 55: 1452-1457, 1995.
3. Reynisdottir, I., Polyak, K., Iavarone, A., and Massague, J. (1995) *Genes Dev.* **9,** 1831-1845.
4. Sandhu, C., Garbe, J., Daksis, J., Pan, C.-H., Bhattacharya, N., Yaswen, P., Koh, J., Slingerland, J., and Stampfer, M. R. (1997) *Mol.Cell Biol.* **17,** 2458-2467.
5. Donovan, J. and Slingerland, J. (2000) *Breast Cancer Res.* **2,** 116-124.
6. Alkarain A, Slingerland J. Deregulation of p27 by oncogenic signaling and its prognostic significance in breast cancer. Breast Cancer Res. 2004; 6(1): 13-21.
7. Liang J, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catzavelos C, Beniston R, Franssen E, and Slingerland JM (2002) *Nature Med.* **8,** 1153-1160.
8. Sheaff, R. J., Groudine, M., Gordon, M., Roberts, J. M., and Clurman, B. E. (1997) *Genes & Dev.* **11,** 1464-1478.
9. Vlach, J., Hennecke, S., and Amati, B. (1997) *EMBO J.* **16,** 5334-5344.
10. Ishida, N., Kitagawa, M., Hatakeyama, S., and Nakayama, K. (2000) *J.Biol.Chem.* **275,** 25146-25154.

11. Rodier, G., Montagnoli, A., Di Marcotullio, L., Coulombe, P., Draetta, G. F., Pagano, M., and Meloche, S. (2001) *EMBO J* **20,** 6672-6682.

12. Boehm, M., Yoshimoto, T., Crook, M. F., Nallamshetty, S., True, A., Nabel, G. J., and Nabel, E. G. (2002) *EMBO J.* **21,** 3390-3401.

13. Fujita N, Sato S, Katayama K, and Tsuruo T (2002) *J Biol Chem* **277,** 28706-28713.

14. Viglietto, G., Motti, M. L., Bruni, P., Melillo, R. M., D'Alessio, A., Califano, D., Vinci, F., Chiappetta, G., Tsichlis, P., Bellacosa, A., Fusco, A., and Santoro, M. (2002) *Nat.Med.* 8, 1136-1144.

15. Shin I, Yakes FM, Rojo F, Shin NY, Bakin AV, Baselga J, and Arteaga CL (2002) *Nature Med.* 8, 1145-1152.

16. LaBaer J, Garrett MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A, Harlow E. New functional activities for the p21 family of CDK inhibitors. Genes Dev. 1997. 1; 11(7): 847-862.

APPENDICES

1. Manuscript: Jiyong Liang, Shan Shao, Kathy Han, Joyce M. Slingerland. Regulation of $p27^{Kip1}$-mediated cyclin D1/cdk4 assembly by multisite phosphorylation
2. Reprint: Liang J, Slingerland JM. Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. Cell Cycle. 2003; 2(4): 339-45.
3. Abstract: Jiyong Liang, Shan H. Shao, Kathy Han, Joyce M. Slingerland. Regulation of p27Kip1-mediated cyclin D1-cdk4 assembly by PKB through multisite phosphorylation. 2004 Miami Nature Biotechnology Symposium. February 2004, Miami, FL, US.

Regulation of $p27^{Kip1}$-mediated cyclin D1/cdk4 assembly by multisite phosphorylation

(4941 words; Intro: 883; Results: 2381; Discussion: 1677)

Jiyong Liang[1], Shan H. Shao[1], Kathy Han[1], Joyce M. Slingerland[1,2*]

1. Molecular and Cellular Biology, Sunnybrook & Women's Health Sciences centre, Toronto, ON M4N 3M5, Canada;
2. Braman Breast Cancer Institute, Sylvester Comprehensive Cancer Center, Division of Hematology Oncology, University of Miami, 1475 N. W. 12th Ave (D8-4), Miami, FL 33136, USA

* Corresponding Author. Mailing address: UM Sylvester Comprehensive Cancer Center, Division of Hematology Oncology, 1475 N. W. 12th Ave (D8-4), Miami, FL 33136,USA. Phone: 305-243-6788. Fax: 305-243-9161.

email: jslingerland@med.miami.edu

ABSTRACT

The mammalian Cdk inhibitor $p27^{Kip1}$ is also a key mediator for D type cyclin-cdk assembly and nuclear translocation. p27 also plays a role in stabilization of cyclin D1. Acquisition of assembly function of p27 is known to be dependent on mitogenic signals. We observed that p27 from TGF-β resistant human mammary epithelial cells (HMEC) showed altered phosphorylation, cytoplasmic mislocalization and increased cyclin D-cdk assembly function. These cells exhibit increased PKB activity compared to normal HMEC. Here we present data supporting a role for PKB-dependent p27 phosphorylation in cyclin D-cdk assembly function. Following mitogen stimulation, cyclin D1/cdk4/$p27^{Kip1}$ assembly in early G1 is preceded by PKB activation. Inhibition of the PI3K/PKB pathway resulted in loss of cyclin D1 from p27 complexes and a shift of p27 to cyclin E/Cdk2. Transfection of constitutively active PKB increased the abundance of cellular cylin D1/cdk4/p27 complexes. Moreover, PKB-dependent phosphorylation of p27 *in vitro* increased the ability of p27 to mediate the assembly of recombinant cyclin D1 and cdk4. Two-dimensional phospho peptide mapping revealed that p27 from cyclin D1 immunoprecipitates differed significantly from that in cdk2 immuncomplexes. p27 bound to cyclin D1 is discretely phosphorylated at multiple sites that involve both PKB-dependent and independent mechanisms. Our data suggested that the assembly function of p27 toward cyclin D1 and cdk4 is activated through sequential changes in p27 phosphorylation involving multiples sites. Constitutive PKB activation and consequent changes in p27 phosphorylation would increase cyclin D1-cdk4

assembly and contribute to loss of the cyclin E/Cdk2 inhibitory function of p27 and resistance to TGF-β.

Introduction

Progression through G1 phase of cell division cycle is a rate-limiting step in mammalian cell proliferation. This process is driven by sequential activation of D-type and E-type cyclin dependent kinases (cdks) and requires the continuous presence of mitogenic signals (Sherr, 2000). Following assembly with cyclin D and cyclin E respectively, cdk4 (or cdk6) and cdk2 are activated by CAK (cdk-activating kinase)-mediated phosphorylation in the T-loops of cdks (Solomon MJ and Kaldis P, 1998). G1 cdks are inhibited by Wee-1-dependent T14/Y15 phosphorylation and by INK4 (inhibitor of cdk4) and Kip (kinase inhibitor protein) family proteins (Sherr and Roberts, 1999). The Cdc25A phosphotase dephosphorylates inhibitory sites and plays a role in cdk2 activation (Hoffmann, EMBO, 1994; Draetta G and Eckstein J, 1997).

The accumulation of cyclin D1 in early G1 is up-regulated through mitogen-dependent increases in transcription and translation and by post-translational modification leading to its stabilization (Rosenwald IB, 1995 JBC; Diehl, 1998; Muise-Helmericks, 1998; Sherr, 2000). Cyclin D1 is unstable in G0 cells (Perry and G Peters, EMBO) and does not assemble into cyclin D1/cdk complexes (Cirallo, 2002). The assembly of cyclin D1 and cdk4 is also thought to be dependent on mitogen signaling since exogenously over-expressed cyclin D1 did not form complexes with cdk4 in serum-starved fibroblasts (Matsushime H, 1994). Assembled kinase active cyclin D-cdk4 complexes were found to contain

$p21^{Cip1}$ and $p27^{Kip1}$ (Sherr and Roberts, 1999). Although p21 and p27 were initially identified as members of the Kip family cdk inhibitors (Koff A, 1993; Polyak K, 1994; Slingerland JM, 1994), further studies showed that p21 and p27 also function to mediate cyclin D1 and cdk4 or cdk6 assembly (LaBaer, 1996). Cyclin D1 assembly into Kip-cdk complexes is associated with cyclin D1 stabilization. In mouse embryonic fibroblasts (MEFs) lacking both *p21* and *p27* genes (Cheng M, et al, 1999), D-type cyclin levels were reduced and cyclin D dependent kinase activity was undetectable. In p21/p27 null MEFs, reduced cyclin D1 levels were partly due to accelerated proteolysis. This was reversed by reintroduction of p27 into these cells. In theory, the assembly of cyclin D-cdk4 by Kips may increase cyclin D1 stability via multiple mechanisms. Kip binding may inhibit the GSK-3β-dependent phosphorylation of cyclin D1 on T286 that targets this cyclin for proteolytic degradation (Diehl, 1998). Association with cdk4 may also prevent cyclin D degradation through a phosphorylation-independent mechanism that specifically targets free cyclin D1 (Germain D, 2000).

Except for the assembly of cyclin D and cdk4, the association of p27 with cyclin D-dependent kinase complexes plays at least two additional roles that are important for G1-to-S progression. First, cyclin D and cdk4 or cdk6 lack nuclear localization signals (NLS), p27 binding facilitates the nuclear translocation of the assembled kinase complexes (LaBaer, 1996). Second, shifting of p27 equilibrium toward cyclin D-cdks leads to activation of cyclin E-dependent kinase in G1 and enables cyclin E/cdk2 to mediate p27 phosphorylation and degradation, pRb phosphorylation and E2F release, and phosphorylation of other substrates

(Polyak, G&D, 1994). These late G1 events characterize the restriction point when cell cycle progression escapes the dependence on exogenous mitogenic signals and becomes resistant to G1 arrest by growth inhibitory cytokine TGF-β or by growth factor and nutrient depletion (Blagosklonny MV, Pardee AB., Cell cycle, 2002).

The assembly function of p27 may be implicated in resistance to TGF-β-mediated G1 arrest in human mammary epithelial cells (HMEC). TGF-β induces expression and accumulation of $p15^{INK4B}$, which binds to cdk4, leading to dissociation of p27 and cyclin D1 from cdk4 and an increase in association of p27 with cyclin E and cdk2 (Reynistotir, 1995; Hannon and Beach, 1994; Sandhu, 1997). p27-cyclin D1-cdk4 complexes from TGF-resistant HMEC were shown to be resistant to dissociation by p15 (Sandhu, 1997). Further study showed that p27 phosphorylation was altered in these cells (Ciarallo, 2002). These data suggest that changes in p27 phosphporylation may increase its association with cyclin D1-cdk4, preventing accumulation of p27 in cyclin E-cdk2 in response to TGF-β.

Our recent data showed that in TGF-β resistant HMEC and advanced melanoma cells, the PI3K/PKB pathway is constitutively active and inhibition of PI3K restored sensitivity to TGF-β induced G1 arrest. This suggests over-activation of PI3K may be causally linked to TGF-β resistance (Liang, 2002). A key PI3K downstream effector PKB (Akt), phosphorylates T157 of p27 and impairs nuclear import of p27 (Viglietto, 2002; Shin, 2002; Liang, 2002). This leads to cytoplasmic mislocalization and sequestration of p27.

In the present study we investigated whether PKB-dependent phosphorylation affects the ability of p27 to mediate cyclin D1 and cdk4 assembly. Our data suggest that the assembly function of p27 toward cyclin D1 and cdk4 is activated by both PKB-dependent and independent multisite p27 phopshorylation.

Materials and Methods

Cell Culture

Finite lifespan human mammary epithelial (HMEC) strain 184^{S} was cultured as described previously (refs) and arrested by EGF depletion for 48 hours (Sandhu, 1997). MCF-7 cells were grown in improved Eagle's medium (IMEM) and WM239 cells derived from late stage melanoma were cultured in RPMI1640 medium supplemented with L-glutamine and 5% fetal bovine serum (FBS) as described (Herlyn, 1990; Cariou, 2000).

Plasmids, Site-directed Mutagenesis and Transfections

FLAG-tagged wild-type p27 (F-p27WT) was constructed by inserting human $p27^{Kip1}$ cDNA into an N-terminal pFLAG-CMV-2 vector (Sigma, Oakville, ON). Respective serine or threonine residues were replaced with alanine (A) or aspartic acid (D) by site-directed mutagenesis to generate F-p27S10A, F-p27S10D, F-p27S12A, F-p27S12D, F-p27T157A, F-p27T157D, and F-p27T198A using a QuickChange site-directed mutagenesis kit (Stratagene, Loyola, CA). The YFPp27WT and YFPp27T157A were described previously (Liang, 2002). Five microgram of each vector was transfected into asynchronously growing

MCF-7 cells using lipofectamine PLUS (GIBCO, Grand Island, NY) according to manufacturer's protocol.

Flow Cytometry

Cells were pulse labeled with 10 μM of bromodeoxyuridine (BrdU) for two hours and processed for flow cytometry as described (Sandhu, 1997).

Antibodies

The anti-EGF receptor monoclonal antibody mAb225 was provided by Steve Wiley (University of Utah Medical Center, Salt Lake City, Utah). Antibodies to phospho Ser473 specific PKB and total PKB were obtained from New England Biolab (Beverly, MA); to cdk4 (C-22), p27 (C-19), and cdk2 (M-2) from Santa Cruz Biotechnology; to p27 from Transduction Laboratories (Lexington, KY) to cyclin D1 (DCS-6 and Ab-3) and cdk4 (Ab-4) from Neomarkers (Fremont, CA). The polyclonal anti-p27T157 phospho specific antibody was generated and provided by G. Viglietto (Institute?, Naples, Italy).

Immunoprecipitation and Western Blotting

Cell lysis, immunoprecipitation and immunoblotting were performed as described earlier (Sandhu, 1997). Two hundred and 50 μg of protein lysates were used for immunoprecipitation and immunoblotting respectively unless otherwise indicated. Antibody alone controls were run along side all immunoprecipitates and equal protein loading of all Western blots was verified by blotting for β-tubulin.

Recombinant Proteins and Assembly of cyclin D1, cdk4 and p27

The expression and purification of His-p27 were described earlier (Liang, 2002). To assay cyclin D1 and cdk4 assembly, 200nM of baculoviral flag-tagged human

cyclin D1 and cdk4 (obtained from Alan Diehl, Cancer Biology, University of Pennsylvania Health System) were incubated in an assembly buffer (20 mM HEPES [pH7.4], 10 mM MgCl2, 10 mM MnCl2, 1 mM Dithiothreitol) at 25 °C for 20 min in the presence or absence of p27. The reactions were then diluted with 500 μl of ice-cold Nonidet P-40 lysis buffer (0.5% Nondet P-40, 50mM Tris [pH7.5], 150 mM NaCl, 1 mM Phenylmethylsulfonyl fluoride, and 0.02 mg each of aprotinin, leupeptin, and pepstatin per ml) (Sandhu, 1997) for immunoprecipitation with a cyclin D1 antibody (Ab-3) followed by immunolblotting for cyclin D1 and cdk4.

To compare the activity of cellular p27 from different cyclin-cdk complexes to assemble cyclin D1 and cdk4, immunoprecipitation was performed using appropriate antibodies and the heat stable components were released from the immunoprecipitates by boiling in assembly buffer for 5 min and the supernatants were assayed.

To assay the effects of PKB on the assembly function of p27, His-p27 or cellular p27 released from immunocomplexes by boiling was pretreated with PKB immunoprecipitated from 1 mg of WM239 cell lysates for 30 min as in PKB kinase assay (Liang 2002). The supernatants were recovered by centrifugation and assayed for assembly activity.

Two Dimensional Isoelectric Focusing (2DIEF)

p27 immunoprecipitated from 1 mg protein lysates was used for 2DIEF performed as described by Ciarallo *et al* (2002) using nonlinear (pH3-10) IEF strips.

Two Dimensional Tryptic Phosphopeptide Mapping

Asynchronously proliferating cells were incubated in phosphate free medium containing 5% dialyzed FBS for 4 hours prior to metabolic labeling with 1 mCi of [^{32}P] orthophosphate (Amersham Pharmacia Biotech) per p100 dish for 3 hours at 37°C. Cells were lysed in an ice-cold Nondet P40 lysis buffer. Cell lysates were then precleared with normal rabbit IgG prior to immunoprecipitation of either cyclin D1, cdk2, or p27. The immunoprecipitates were resolved by 12% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a polyvinyl difluoride membrane and subjected to autradiography. p27 bands dissected from the membrane were blocked with 0.5% polyvinyl difluride in 100 mM Tris (pH 7.6) at 37°C for 30 min and digested overnight with 10 μg modified trypsin (Roche Diagnostics, Mannheim, Germany). The samples were then lyophilized and treated with ice-cold performic acid (45 μl of formic acid and 5 μl of H_2O_2 (37%)) unless otherwise indicated. Lyophilized samples were re-suspended in 5ml of pH1.9 buffer (formic acid, 50 ml; glacial acetic acid, 156 ml; and H_2O, 1794 ml) and loaded onto cellulose thin layer plates. Electrophoresis was performed with pH1.9 buffer at 1000V for 1 hour using Hunter thin-layer electrophoresis system (HTLE-7000; CBS Scientific, Del Mar, CA) system. Ascending chromatography was performed using a phosphochromatography buffer (*n*-butanol, 750 ml; pyridine, 500 ml; glacial acetic acid, 150 ml; and H_2O, 600 ml) for 24 hours. The plates were then air-dried and subjected to autoradiography.

Results

PKB activation and the assembly of cyclin D1/cdk4/p27 are early G1 events. Our recent data suggested that the shift of p27 between active cyclin D1 and inactive cyclin E1 complexes may be attributable to changes in p27 phosphorylation (Ciarallo S *et al*, 2002). TGF-β resistant cells showed increased p27-cyclin D1-cdk complexes and cyclin D1-cdk4 activity (Sandhu, 1997). TGF-β resistance is also associated with increased activation of PKB, which phosphorylates p27 at T157. These observations lead us to investigate whether PKB may play a role in regulating the assembly activity of p27 for cyclin D1-cdk4.

Human mammary epithelial cells (HMEC) 184 were first arrested in G0 by EGF depletion for 48 hours. The quiescent cells were then released into the cell cycle by addition of medium containing EGF. Within 18 hours after the addition of EGF, a majority of cells had entered S phase. PKB activation was assayed across the cell cycle by measuring PKB phosphorylation on Ser 473 (P-PKB) (Fig. 1A). Results were confirmed by immunoblotting for phosphorylated GSK3-∀ as a read out of endogenous PKB activity (data not shown). In quiescent 184 cells, phosphorylated PKB was minimal. PKB was maximally activated within 3 hours of release from G0. Phosphorylated PKB remained elevated during the G1/S transition and declined gradually as cells progressed through S into G2/M (at 18 to 24 hours). Total PKB levels did not change across the cell cycle. Cyclin D1 protein levels increased by 6 hours after the peak activation of PKB. Since cyclin D1 protein stability is known to be increased by PKB dependent

inactivation of GSK3-Ǝ (Diehl et al. 1998; Diehl et al. 1997), PKB activation in early G1 may contribute to the ensuing increase in cyclin D1 levels.

Cyclin D1 stabilization has also been shown to be dependent on the assembly of cyclin D1 with its cdk partners (Bates et al. 1994; Parry et al. 1995). To examine cyclin D1-cdk4-KIP complexes, equal amounts of cdk4 were immunoprecipitated from 184 HMEC lysed at intervals from G0 to S phase (Fig. 1B). As the overall cyclin D1 level increased, cdk4-bound cyclin D1 was also increased, however the fold increase of cdk4-bound cyclin D1 was greater than that of cyclin D1 itself. The recruitment of p27 into cyclin D1-cdk4 complexes increased between 3 and 6 hours, despite the reduction of p27 protein during this period in G1. Thus, the assembly of p27 into cyclin D1-cdk4 complexes appears to be actively regulated and does not correlate precisely with either cyclin D1 or p27 availability during G1. The assembly of p21 into cyclin D1-cdk4 complexes followed similar kinetics.

PI3K/PKB inhibition induces dissociation of cyclin D1/cdk4 and formation of p27, cyclin E1 and Cdk2 complexes. Sustained inhibition of the PI3K/PKB pathway with resultant G0 arrest is associated with reduced expression of cyclin D1 and stabilization of p27 protein (Yakes FM, Cancer Res, 2002; JL and JMS, 2003). However, a brief inhibition of PI3K in of MCF-7 cells treated with the PI3K inhibitor LY294002 for 6 hours did not affect the protein levels of cyclin D1, cyclin E, or p27. The effects of PI3K inhibition on p27-cyclin-cdk complexes were analyzed. Similar amounts of p27 were immunoprecipitated from proliferating MCF-7 cells before and at intervals during 6 hours of LY294002 treatment.

Although p27 protein levels were constant, p27-bound cyclin D1 was rapidly reduced in response to LY294002 (Fig. 2A). p27-associated cdk4 were not affected by LY294002 during this 6 hour interval. Thus, PI3K/PKB inhibition induced dissociation of cyclin D1 from cdk4 and p27 complexes. These data suggested that despite its ability to bind to cdk4, the cyclin D1-cdk4 assembly function of p27 is lost soon after inactivation of PI3K/PKB. Concurrent with dissociation of cyclin D1 and p27, p27-bound cyclin E1 increased with 2 hours of treatment with LY294002.

In contrast to the effect of PI3K inhibition, p27-cyclin D1-cdk4 dissociation was not observed following MEK inhibition by UO126 (Fig. 2 B). Inhibition of PKB and MAPK activation is shown by diminished P-PKB and P-MAPK respectively (Fig. 2B top panel). An increase of cyclin E and Cdk2 and loss of cyclin D1 from p27 immunocomplexes is seen in cells treated with LY294002, but not in cells treated with UO126 (Fig. 2B bottom panel).

LY294022 treatment induces a progressive G1 arrest with an increase in the proportion of cells in G1 phase and a concomitant decrease in the proportion of cells in S and G2/M phases (Fig. 2C). To demonstrate that the dissociation of p27-cyclin D1-cdk4 complexes resulting from LY29004-mediated PI3K inhibition was not merely a reflection of G1 arrest, G1 arrest of MCF-7 was induced by transfection with p27 and then the effects of PI3K inhibition were examined. Transfection of p27 induced G1-arrest (Decreased %S shown in Fig. 2D) without affecting PKB phosphorylation, whereas LY294002 treatment for 6 hours reduced both the %S phase cells and markedly reduced P-PKB (Fig. 2D left

panel). Rather than inhibiting the association of cyclin D1/cdk4, p27 transfection reproducibly increased cdk4-associated cyclin D1 by approximately 2-fold (Fig. 2D right panel). Treatment with LY294002 resulted in loss of cyclin D1 from cdk4 immunocomplexes regardless of p27 transfection.

The effects T157 phosphorylation on p27/cyclin D1 association. PKB has been shown to phopshorylate T157 of p27, leading to cytoplasmic mislocalization of p27. In *in vitro* assays, PKB has also been shown to phosphorylate p27 at T198 (Fujita *et al,* 2002). The effects of PKB-dependent p27 phosphorylation on p27-cyclin-cdk complexes were investigated. As shown earlier (Liang, 2002), PKB activity is at least 5-fold higher in the late stage melanoma line WM239 than in an early stage melanoma derived WM35 line. The $WM35^{PKBDD}$ stably expresses constitutively active PKBDD and $WM35^{E}$ was transfected with an empty vector. To investigate the effect of PKB on the assembly function of p27, cdk4 inmmunocomplexes from lysates of WM239, WM35, $WM35^{E}$ and $WM35^{PKBDD}$ were analyzed. In WM239 cells and in $WM35^{PKBDD}$ there was a 2-3-fold increase in cyclin D1 and p27 bound to cdk4 compared to that in non-transfected WM35 and $WM35^{E}$ cells (Fig. 3A). Thus, the assembly of cellular p27/cyclin D1/cdk4 is increased in PKB activated cells.

To test how the potential for T157 phosphorylation would affect association of p27 with cyclin and cdks, equal amounts of YFPp27WT and YFPp27T157A were transfected into MCF-7 cells and immunoprecipitation was performed using antibodies against cyclin D1, and cdk2 respectively. Less YFPp27T157A bound to cellular cyclin D1 or cdk4 than YFPp27WT (Fig. 3B and

C), whereas the amounts of Cdk2-associated YFPp27WT and YFPp27T157A were similar (Fig. 3D). These data suggest that loss of T157 phosphorylation of p27 reduces the association of p27 with cyclin D1 and cdk4 while binding to cdk2 is unaffected. In agreement with this result, cellular p27 bound to cdk4 showed strong reactivity to a phosphoT157 specific antibody (Fig. 3E).

PKB activates the activity of p27 to mediate cyclin D1/cdk4 assembly. PKB-dependent T157 phosphorylation of p27 leads to cytoplasmic mislocalization of p27. This may differentially affect the proximity of p27 to cyclin D1/cdk4 and cyclin E1/cdk2 complexes. Therefore, reduced binding to cyclin D1/cdk4 of the YFPp27T157A following transfection might be due to altered subcellular compartmentalization of this p27. We next examined whether PKB-dependent phosphorylation would modulate cyclin D1-cdk4 assembly by p27 *in vitro* by using recombinant proteins. Recombinant baculoviral produced cyclin D1 and cdk4 were mixed and their assembly was detected by immunoprecipitating cyclin D1 followed by immunoblotting cyclin D1 and cdk4. While purified cyclin D1 and cdk4 did not form complexes after incubation (Fig. 4 lane 1), the addition of purified recombinant p27 (Rec-p27) was able to mediate the assembly of cyclin D1 and cdk4 (Fig. 4 lane 2), consistent with previous studies (LaBaer, 1997). Treatment of recombinant p27 with PKB kinase for 30 min prior to the assembly reaction markedly increased the ability of p27 to mediate the assembly of cyclin D1 and cdk4 (Fig. 4 lane 3). The assembly activity of cellular p27 from discrete cyclin-cdk complexes was similarly assayed. Immunoprecipition of cdk2 and cyclin D1 were performed. The complexes were boiled and the heat-stable

cellular p27 from these immunocomplexes was recovered. As observed with recombinant p27, cdk2-associated cellular p27 was able to mediate the assembly of cyclin D1 and cdk4, but it was less effective than equivalent amounts of p27 isolated from cellular cyclin D1 complexes (see Fig. 4 lane 4 *vs* 6). The assembly activity of p27 recovered from cdk2 precipitation was also increased following pretreatment with PKB kinase (Fig. 4 lane 4 and 5). These data suggest that PKB-dependent phosphorylation of p27 positively regulates p27 assembly function for cyclin D1 and cdk4 in a manner independent of the subcellular localization of p27.

Cycin D1 associated p27 is hyper-phosphorylated. The spectrum of p27 phosphorylation in cyclin D1/cdk4 complexes differs from that of p27 bound to cyclin E/cdk2 (Ciarallo S *et al*, 2002). p27 phosphorylation was further characterized by phosphate labeling and two-dimensional peptide mapping of p27 from cdk2 and cyclin D1 immunoprecipitates. As shown in Fig. 5A, phospho peptides x, y, 2, 3, and 6 were present in both cyclin D1-bound and cdk2-bound p27 maps, indicating that consistent phosphorylation patterns were obtained in p27 isolated from cyclin D1 and Cdk2 complexes respectively. However, in cyclin D1 associated p27, peptide 2, 3, and 6 were strongly phosphorylated whereas these peptides were only minimally phosphorylated in cdk2-bound p27. In addition, phosphorylation of peptides 4 and 7 were observed in p27 that associated with cyclin D1- but not in Cdk2-bound p27. Thus, cyclin D1 associated p27 is phosphorylated to a greater extent and at more sites than is cdk2 associated p27.

p27 has been shown to be phosphorylated at T187 (Sheaff, 1997; Vlach, 1997) S10 (Ishida, 2000; Rodier, 2002; Boehm, 2002), S178 (Ishida, 2000), T157 (Viglietto, 2002; Shin, 2002; Liang, 2002), and T198 (Fujita, 2002). To identify the sites that are specifically phosphorylated in cyclin D1-bound p27, FLAG-tagged p27S7A, S10A, S12D, T157A, T198A were generated by converting the respective serine (S) or threonine (T) residues to either alanine (A) or aspartic acid (D) and two-dimensional phosphopeptide mapping of phosphate labeled FLAG p27 precipitates was performed following transfection of these different alleles into MCF-7 cells.

Sites 1 to 7 were detected in wild-type FLAG-p27 (F-p27WT) (Fig. 5B). Sites 1, 2, and 3 were barely detectable in p27S10A (Fig. 5C), suggesting these sites are the peptides containing phosphorylated S10. The detection of multiple S10-related spots suggests that site 1 could contain S10 single phosphorylation whereas sites 2 and 3 might represent the S10 peptide phosphorylated at multiple sites. S7A mutation did not affect the numbered sites on tryptic maps or the mobility of p27 on SDS PAGE (Fig. 5D). Since this peptide contains also S12, its mutant allele was mapped. Given that S10A mutation abolished these sites altogether, the phosphorylation of these sites might be interdependent. For this reason S12D that mimics S12 phosphorylation instead of S12A was used for mapping. S12D mutation abolished site 1 (Fig. 5E), thus site 1 likely contains single phosphorylation whereas sites 2 and 3 in F-p27WT are the same peptide phosphorylated at both S10 and S12. The detection of two different sites could

be partly due to an oxidation side effect of performic acid treatment (Ishida *et al, 2000*).

In F-p27T157A site 5 is absent (Fig. 5F), hence site 5 likely contains phospho T157. Due to limited abundance of site 5, we could not perform Edman degradation on this peptide. The intensity of sites 4, 6, and 7 is also decreased in F-p27T157A (Fig. 5F), suggesting that stable detection of phosphorylation at sites 4, 6, and 7 is at least partly dependent on T157 phosphorylation. Both sites 6 and 7 were absent in p27T198A (Fig. 5G) and phospho amino acid analysis showed that both sites contain phospho threonine exclusively (Data not shown). Therefore, sites 6 and 7 possibly contain phospho T198 and the presence of two distinctive sites is likely due to partial tryptic digestion. Incomplete tryptic digestion could result from the present of multiple arginine residues within the fragment of p27 containing T198 (194RRRQT198). Site 4 is not mapped, but this peptide migrates in the position shown to contain phospho S178 and T187 by Ishida et al (Ishida, 2000). Sites x and y in cyclin D1- as well as Cdk2-bound p27 are not present in transfected p27 alleles and their identity is unknown.

Based on present data and published phosphopeptide maps of p27, p27 assembled with cyclin D1 appears to be discretely phosphorylated at S10/S12, S178/T187, and T198 and these sites are either not or only weakly phosphorylated in Cdk2-associated p27.

PKB and p27 cooperate to support cyclin D1 accumulation. Cyclin D1 appears to be stabilized in vivo following assembly into cdk4 complexes than its monomeric form (Parry, EMBO J, 1995; Diehl, 1999). Cyclin D1 is undetectable

in p21/p27 double null MEFs. In the presence of mitogen signals, expression of either p27 or p21 can help establish nearly normal cyclin D1 levels in mouse fibroblasts lacking both p27 and p21 genes (Cheng, EMBO). We postulated that PKB-dependent activation of p27's assembly function might contribute to the accumulation of cyclin D1. To investigate this possibility, F-p27WT and F-p27S10DT157D, the mutant that mimics S10 and T157 phosphorylation, were transfected into MCF-7 cells. Following transfection, cells were treated with or without LY294002 for 12 hours. At 12 hours after PI3K inhibition, endogenous cyclin D1 levels fall notably (Data not shown). Inhibition of PKB activation by LY294002 is shown by a reduction in the levels of P-PKB but not total PKB (Fig. 6A). Transfection of F-p27WT led to an increase in cyclin D1 levels and this effect was slightly more pronounced following F-p27S10DT157D transfection. Treatment with LY294002 induced a 4-fold down-regulation of cyclin D1 in cells transfected with an empty vector and F-p27WT. The reduction in cyclin D1 levels observed after PI3K/PKB inhibition was diminished in F-p27S10DT157D compared to that in vector or F-p27WT transfected cells (Fig. 6A). Cyclin D1 detected in F-p27S10DT157D immunoprecipitates was two-fold higher than F-p27WT and this ratio was not affected by LY294002 (Fig. 6B). These data are in agreement with increased p27 assembly activity conferred by the phosphomimetic S10DT157D mutations. Thus, cyclin D1 loss following inhibition of the PI3K pathway is in part attributable to inhibition of the assembly activity of p27. The ability to support cyclin D1 accumulation in the presence of LY294002

is seen only with F-p27S10DT157D but not F-p27S10D or F-p27T157D (data not shown).

Discussions

Initially identified as cdk inhibitors, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ also function as assembly factors to promote the association of D type cyclins and cdks (Sherr and Roberts, 1999). Several lines of evidence suggest that this process require mitogenic signals, but the mechanisms of its regulation are unclear. Two of the major questions remaining to be addressed are whether cyclin-cdks themselves or the assembly function of Kips is regulated by mitogen-dependent mechanisms (Cheng et al, 1999) and what is the signaling pathway(s) responsible for this regulation.

We recently showed that p27 phosphorylation is altered in TGF-β resistant HMEC cells. The changes in p27 phosphorylation appear to favor the binding of p27 to cyclin D1-cdk4 and prevent its accumulation in cyclin E1-cdk2 in response to TGF-ß (Ciarallo, 2002). In the same cells, p27was shown to be phosphorylated by PKB (Viglietto et al, 2002) and inhibition of PI3K/PKB restored TGF-β-induced accumulation of p27 in cyclin E-cdk2 complexes (Liang et al, 2002). These data suggest that PKB-dependent phosphorylation might influence p27 association with cyclin-cdks via regulating its assembly function towards cyclin D-cdks.

In consistent with this hypothesis, we now show that during cell cycle progression induced by EGF, PKB is activated early in G1, preceding cyclin D1-cdk4 assembly with Kips. Although PKB is not the only kinase that is activated in

early G1 and required for G1-to-S progression (Jones et al, 2000), our data show that inhibition of PI3K/PKB but not MEK/MAPK led to disassociation of cyclin D1 from cdk4 and p27. Further, the association of cyclin D1-cdk4-p27 is markedly increased in WM35PKBDD as well as WM239 cells, which express constitutively active PKB and lack tumor suppressor PTEN, respectively.

p27 was previously shown to be phosphorylated by PKB on T157 (Viglietto et al, 2002; Shin et al, 2002; Liang et al, 2002). YFP-p27T157A show reduced association with cyclin D1-cdk4 but not cdk2. Furthermore, pretreatment of recombinant and cellular p27 with PKB increased the activity of p27 to mediate cyclin D1 with cdk4 assembly *in vitro*. These data collectively support for a role of PKB in facilitating p27-mediated cyclin D1-cdk4 assembly via phosphorylation of p27 on T157.

It is unclear however, whether phosphorylation on sites other than T157 is also involved in the activation of p27 assembly function. To address this question, phosphorylation of p27 in cyclin D1 and cdk2 complexes was analyzed by phopshopeptide mapping. Our data show that p27 in cyclin D1 immunocomplexes is phosphorylated at multiple sites that are either not or only weakly phosphorylated in cdk2-associated p27. The preferentially phosphorylated sites in cyclin D1-bound p27 are likely the peptides containing S10/S12, S178/T187, and T198 respectively, according to two-dimensional phosphopeptide mapping of mutant p27 alleles. At least some, if not all, of these differences in phosphorylation may affect p27 function since cyclin D1-associated

p27 also show increased activity to assemble cyclin D1-cdk4 *in vitro* than cdk2-associated p27.

Interestingly, p27T157A mutation attenuated S178/T187 and T198 phosphorylation. Thus, T157 phosphorylation appears to facilitate phosphorylation on the remaining C-terminal sites. However, site 5 that likely contains phospho-T157 is not visible on two-dimensional phosphopeptide map of cyclin D1-associated p27, which may be due to insufficient sensitivity of this experiment. Indeed, site 5 can only be weakly detected in F-p27WT. Nevertheless, T157 phosphorylation is readily detectable using phospho-T157 specific antibody in cdk4 immunocomplexes. Thus, an alternative explanation is that once assembled with cyclin D1, dephosphorylation of T157 occurs rapidly to allow nuclear import of the complex. This model is consistent with previous data showing that T157 phosphorylation presents only in the cytoplasm (Viglietto, 2002). Of note is that T157 phosphorylation is mediated by PKB, which was also shown to phosphorylate T198 *in vitro* (Fujita et al, 2002). Our data now suggest that these sites may be phosphorylated in a processive manner. Although it is unclear whether PKB is responsible for T198 phosphorylation *in vivo*, PKB may play a crucial role in initiating this sequential C-terminal phosphorylation by phosphorylating T157. The possibility that other AGC family kinases such as p70S6K and SGK may act cooperatively with PKB in this process cannot be excluded.

Unlike T157A mutation, T157 pseudophosphorylation (T157D mutation) showed no effect on p27 association with cyclin–cdks (data not shown).

Therefore, despite its unique role, T157 phosphorylation may be insufficient for full activation of p27 assembly function.

S10 phosphorylation has been described by several groups (Ishida, 2000; Boehm, 2002; Rodier, 2002). Our two-dimensional phosphopeptide mapping data further show that S12 in the same peptide is also phosphorylated. More importantly, S10/S12 are preferentially phosphorylated in cyclin D1-associated p27. Although neither S10A nor S10D mutation alone affects p27 association with cyclin-cdks, F-p27S10D/T157D double mutant show a 2-fold increase in association with cyclin D1. This effect is retained even when PI3K/PKB pathway is inhibited. In parallel, F-p27S10D/T157D but not F-p27WT supports the accumulation of cyclin D1 in the presence of LY294002. The levels of cyclin D1 are controlled by complex mechanisms (Gille H and Downward J, 1999; Diehl A, et al, 1999; Cheng M, et al 1999; Treinies I, 1999), in which PI3K/PKB pathway plays a central role (Liang and Slingerland, 2003). Our data suggest that up-regulation of the assembly function of p27 that favors the association of p27 with cyclin D1 may also lead to cyclin D1 stabilization, unless F-p27S10D/T157D but not F-p27WT may have unexpected activities that regulate cyclin D1 at transcriptional or translational level.

Although recombinant p21 and p27 were effective in mediating the association of cyclin D1-cdk4 (LaBaer, 1997), our data are most consistent with that the assembly function of p27 may be actively regulated as previously suggested (Matsumishi et al, 1994; Cheng M, et al, 1999). Interestingly, this process appears to involve multisite phosphorylation on p27 centered at its N-

terminal and C-terminal regions respectively. PKB plays an important role in the C-terminal phosphorylation whereas the mechanism(s) of N-terminal phosphorylation is less clear.

In NIH 3T3 cells as well as Rat1 cells, S10 was identified as a major phosphorylation site in p27 by several groups (Ishida, 2000; Boehm, 2002; Rodier, 2002), although inconsistency exists regarding the role and timing of S10 phosphorylation in cell cycle. Ishida *et al* (2000) showed S10 phosphorylation was enriched in the cells synchronized by contact inhibition and serum starvation and S10 phosphorylation appeared to increase p27's stability. Data from Rodier *et al* (Rodier, 2002) suggest that the timing of S10 phosphorylation may be cell type dependent. In NIH 3T3 cells and mouse T lymphocytes, S10 phosphorylation occurred in G0 and early G1, whereas maximal p27 phosphorylation was detected between 6 and 12 hours following serum stimulation in Rat1 cells, in which S10 phosphorylation is necessary for nuclear to cytoplasmic translocation of p27 upon cell cycle re-entry. Boehm, et al (Boehm, et al, 2002) showed that S10 phosphorylation is mediated by a kinase known as hKis, whose expression is induced in response to growth factors. In agreement with the notion that S10 phosphorylation is mitogen-dependent and not restricted to arrested cells, we detected strong S10/S12 phosphorylation in actively proliferating human cancer cells. Further, strong S10/S12 phosphorylation is detected in p27 associated with cyclin D1 but not cdk2.

S10 phosphorylation facilitates nuclear to cytoplasmic translocation (Rodier, 2002; Connor, 2003) and T157 phosphorylation impairs nuclear import

of p27 (Liang, 2002), mechanistically both causing cytoplasmic retention of p27. It is conceivable that cytoplasmic p27 that is phosphorylated at S10 and T157 binds to D type cyclins and cdk4 or cdk6 and mediate the assembly of these kinase complexes in the cytoplasm. The evidence to support such a view is the observation that cyclin D3 complexes that contain p27 were cytoplasmic (Baldassarre G, JCI, 1999). One of the possibilities is that the cyclin D1-cdk4-p27 complexes assembled in the cytoplasm could be redirected to nucleus, which may require T157 dephosphorylation. Alternatively, since hKis is a nuclear kinase (Boehm, 2002) and activated PKB also translocates to the nucleus (Coffer, 1997), it is plausible that p27 could be phosphorylated and conferred preference for association with cyclin D1 in nucleus as well. Nevertheless, regardless of the subcellular distribution of the cyclin D-cdk-p27 complexes, the assembly of p27 with cyclin D-cdk would theoretically embargo p27 from inhibiting cyclin E-cdk2, a mechanism that is a prerequisite to enable cyclin E-cdk2 to further phosphorylate and down-regulate p27 by proteolysis.

Phosphorylation is a common mechanism whereby protein interactions are facilitated or optimized, as exemplified by many signaling and non-signaling molecules (Pawson and Nash, 2003). However, given that neither of these residues is located in cyclin/Cdk binding domains, why S10 and T157 phosphorylation are needed for the activation of the assembly function of p27? As suggested by LaBaer *et al* (1997), p27-mediated cyclin D1/cdk4 assembly, involving complex formation and stabilization, which as suggested by our data is likely a two-step process. First, PKB-dependent phosphorylation of p27 at the C-

terminal tail might be sufficient for the assembly activity of p27, since *in vitro* phosphorylation of p27 by PKB can mediate cyclin D1/cdk4 assembly effectively. Second, S10/S12 phosphorylation at the N-terminal region may create additional phosphorylation-dependent interaction between phosphorylated S10 cluster and cyclin D, further stabilizing assembled cyclin D/cdk/p27 complexes. Both of these two steps appear to be essential for the formation of stable cyclin D dependent kinase complexes, since neither p27S10D nor p27T157D but the p27S10DT157D double mutant allele is capable of supporting the accumulation of cyclin D1 when the activation of PKB is inhibited.

Our data is consistent with a model (Figure 7) in which the activity of p27 to mediate the assembly of D type cyclin and cdks is activated by phosphorylation at multiple sites through mitogen-dependent mechanisms, which contributes to and is likely accompanied by the loss of cyclin E-cdk2 inhibitory function of p27. This process, representing one of the critical mechanisms that are essential for G1/S transition and S phase progression, may be misregulated in TGF-β resistant HMECs and human cancer lines. Since at least over-activation of PKB, one of the kinases that participate in the activation of this function, is frequently seen in human cancers, deregulation of this mechanism might likewise be relevant to tumor development and loss of sensitivity to growth inhibitory mechanisms including that induced by TGF-β.

Figure legends

Figure 1. PKB activation and cylin D1-cdk4-Kip assembly in G1. HMEC strain 184 at 60% confluence were maintained in quiescence (G0) by EGF depletion

and treatment with a monoclonal EGF receptor antibody for 48 hours and released by addition of EGF (0.1μg) at time 0. At intervals thereafter, cells were assayed for cell cycle entry by flow cytometric analysis and cell lysates for protein analysis. **A**. Total PKB, phospho S473 PKB isoform (PKB-P), cyclin D1 and p27 were detected by Western blotting with respective antibodies. Cell cycle entry is indicated by %S. **B**. Cyclin D-dependent kinase complexes were analyzed by immunoprecipitation using a cdk4 antibody and immunoblotting for cdk4 and the associated proteins.

Figure 2. Disassociation of cyclin D1/Cdk4 and formation of p27, cyclin E1 and cdk2 complexes upon PI3K inhibition. **A.** Lysates were prepared from asynchronously proliferating MCF-7 cells and MCF-7 cells treated with 10μM LY294002 (LY) for indicated times. Left panel, the total cellular levels of the relevant proteins were detected by Western blot; right panel, p27 immunocomplexes were analyzed by immunoprecipitation with a p27 antibody (c-19) and p27 and the associated proteins were detected by immunoblotting with respective antibodies. **B.** MCF-7 cells were treated without (-) or with (+) an MEK inhibitor, UO126 (5 μM), or LY294002 (LY) for 24 hours and the cell lysates were probed for phospho S473 PKB (P-PKB), total PKB (PKB), phospho MAPK (P-MAPK) and total MAPK by Western blots (top panel) and p27 immunoprecipitation followed by immunoblotting for p27 immunocomplexes (bottom panel). **C.** Asynchronously proliferating MCF-7 cells and MCF-7 cells treated with LY294002 (10μM) for indicated times were pulse-labeled with 10μM BrdU for two hours and the cell cycle profiles were analyzed by flow cytometry.

Data represent the averages of three independent experiments. **D.** MCF-7 cells were transfected with an empty vector (-) or YFP-tagged p27 and 24 hours post transfection the cells were treated with or without LY294002 (LY). Cell lysates (left panel) and cdk4 inmmunoprecipitates (right panel) were detected for S473 phospho PKB (P-PKB), total PKB (PKB), cdk4, cyclin D1, FLAG p27 (F-p27), and endogenous p27 (Endo-p27) by Western blots. Numbers indicate %S phase measured by flow cytometry.

Figure 3. T157 phosphorylation of p27 affects association with cyclin D1-cdk4. **A.** Cdk4 immunoprecipitation was performed with lysates of WM239 cells, WM35 cells and WM35 stably expressing an empty vector ($WM35^{E}$) or PKBDD ($WM35^{PKBDD}$). cdk4 and the associated cyclin D1 and p27 were probed with respective antibodies. **B, C** and **D.** Cell lysates from MCF-7 cells 24 hours post transfection with YFP-tagged wild type (WT) or T157A mutant p27 were used for immunoprecipitation (IP) using indicated antibodies. The immunoblots were probed with indicated antibodies and a p27 antibody for YFP-p27. **E.** Cdk4 was immunoprecipitated from lysates of synchronously growing MCF-7 cells. The associated p27 were first probed with a p27T157 phopsho-specific antibody (p27-T157-P) and the blot was then stripped and re-probed with a regular p27 antibody (p27).

Figure 4. PKB-dependent phosphorylation increases the activity of p27 to assemble cyclin D1-cdk4. Recombinant human cyclin D1 and cdk4 were incubated for 20 min at 25 °C without (No p27) or with recombinant (Rec-p27) or cellular p27 released by boiling from cdk2 (K2-p27) and cyclin D1 (D1-p27)

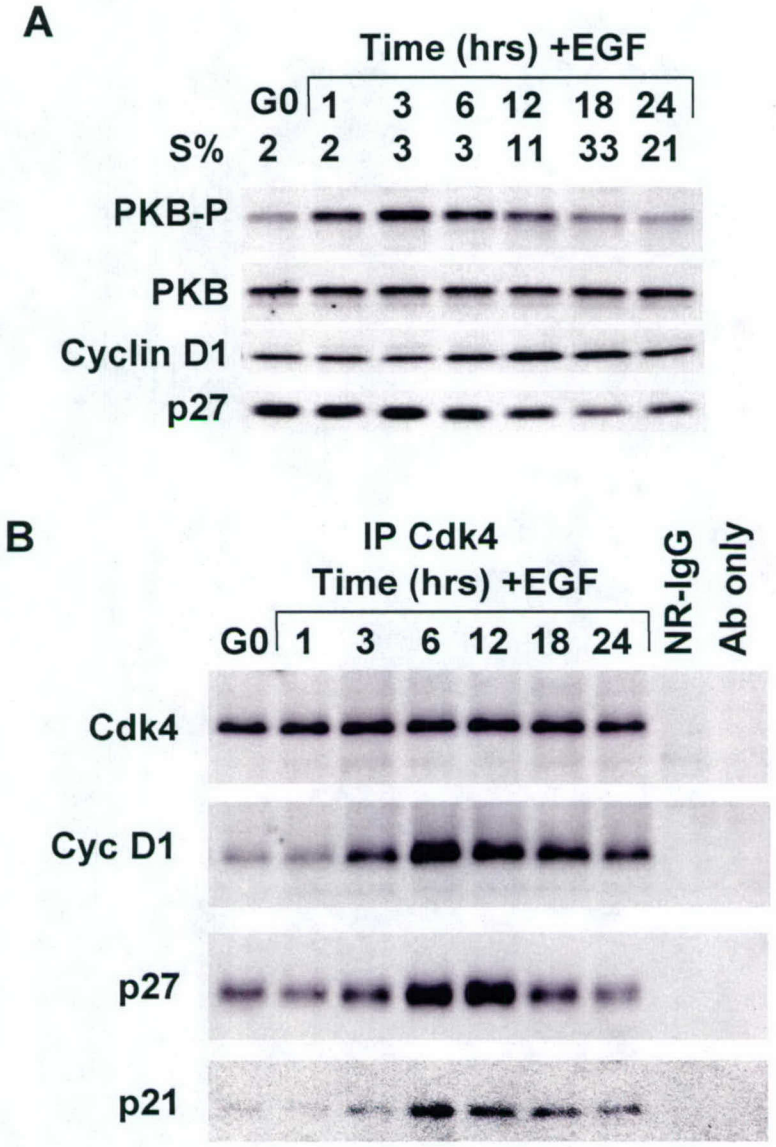
immunocomplexes from MCF-7 cells treated without (-) or with (+) PKB immunoprecipitates from WM239 cells. Cyclin D1 was then immunoprecipitated and cyclin D1 and the associated cdk4 were detected by Western blot. Bottom panel shows Western blots of the input proteins.

Figure 5. Cyclin D1-associated p27 is phosphorylated at more sites than cdk2-associated p27. **A.** MCF-7 cells were metabolic labeled with ^{32}p-orthophosphate. Cyclin D1 and cdk2 immunoprecipitations were performed using respective antibodies (Ab-3 for cyclin D1 and M-2 for cdk2) and resolved by SDS PAGE. p27 from immunoprecipitates of cyclin D1 (D1-p27) and cdk2 (K2-p27) was isolated and two-dimensional tryptic phosphopeptide mapping was performed. **B-G.** FLAG-tagged wild-type and the indicated mutant p27 alleles were transfected into MCF-7 cells and 24 hours post-transfection the cells were labeled with ^{32}p-orthophosphate. Immunoprecipitation was performed using a p27 antibody (c-19). The immunoprecipitates were boiled in the presence of 1% SDS for 5 min and re-suspended and immunoprecipitation was re-performed using p27 antibody. The final immunoprecipitates were then resolved on SDS PAGE and F-p27s were isolated for two-dimensional tryptic mapping. All detected phosphopeptides are numbered or lebaled with letters and indicated by arrows. The directions of chromatography (TLC) and electrophoresis (TLE) separation are indicated by arrows and the origins of the maps are marked as asterisks.

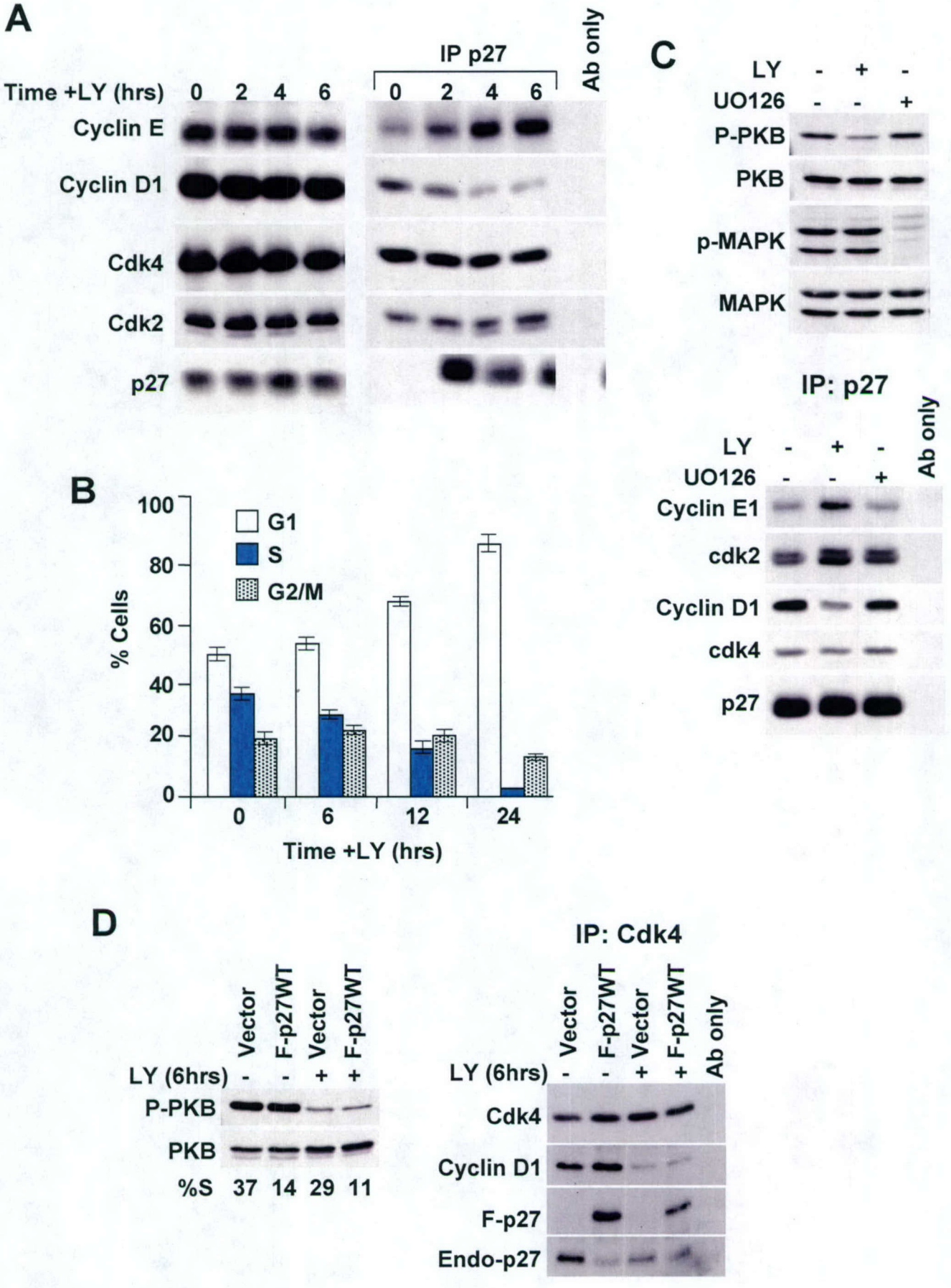
Figure 6. F-p27S10DT157D constitutively associates with cyclin D1 and increase scyclin D1 levels. An empty FLAG vector (Vector), FLAG-tagged wild-type p27 (F-p27WT) and its mutant allele (F-p27S10DT157D) were transfected into MCF-7

cells. 24 hours post transfection, cells were treated with (+) or without (-) 10 μM LY294002 (LY) for 12 hours. **A.** 25 mg protein per lane was used for Western blotting for cyclin D1, FLAG-p27 (F-p27), phospho S473 PKB (P-PKB) and total PKB (PKB) with respective antibodies. **B.** 200μg of cell lysates were used for immunoprecipitation with a FLAG antibody and Western blotting with cyclin D1 and F-p27.

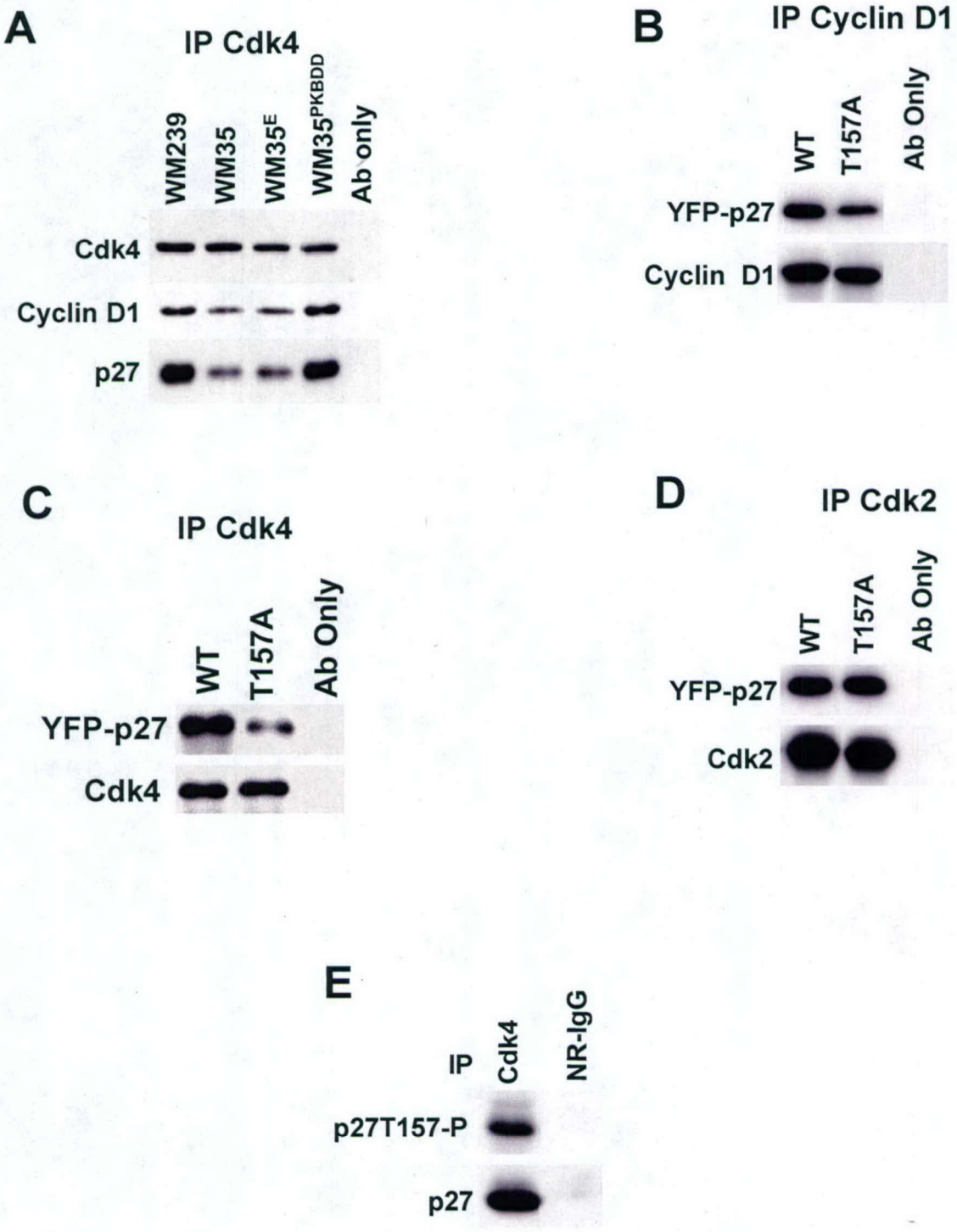
Figure 7. Two-step assembly of cyclin D1/Cdk4/p27. Depending on whether p27 is first phosphorylated on S10 or T157, the assembly function of p27 could be activated via two possible processes. a) S10 phosphorylation may create additional phopshorylation-dependent interaction between phosphorylated S10 cluster and cyclin D. Further phosphorylation of T157 by PKB induces conformational changes that allow for optimized contact of p27 with cdk4. b) PKB-dependent phosphorylation of p27 at the C-terminal tail is sufficient for p27 to mediate cyclin D and cdk4 assembly and subsequent p27 phosphorylation on S10 cluster further stabilizes the assembled tripartite complexes by creating extra contact between p27 and cyclin D.



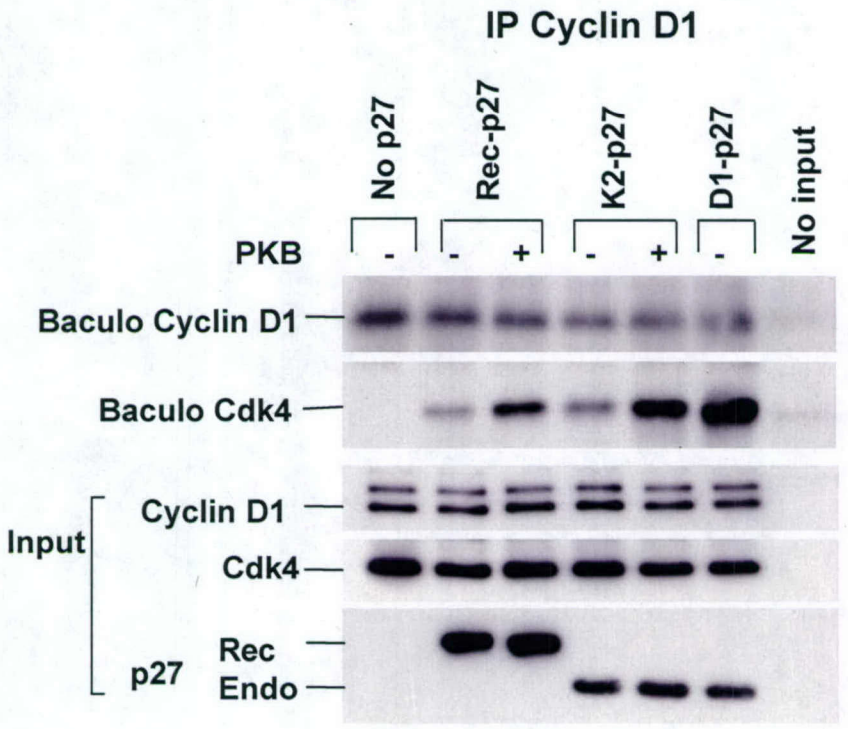
J Liang *et al*
Figure 1



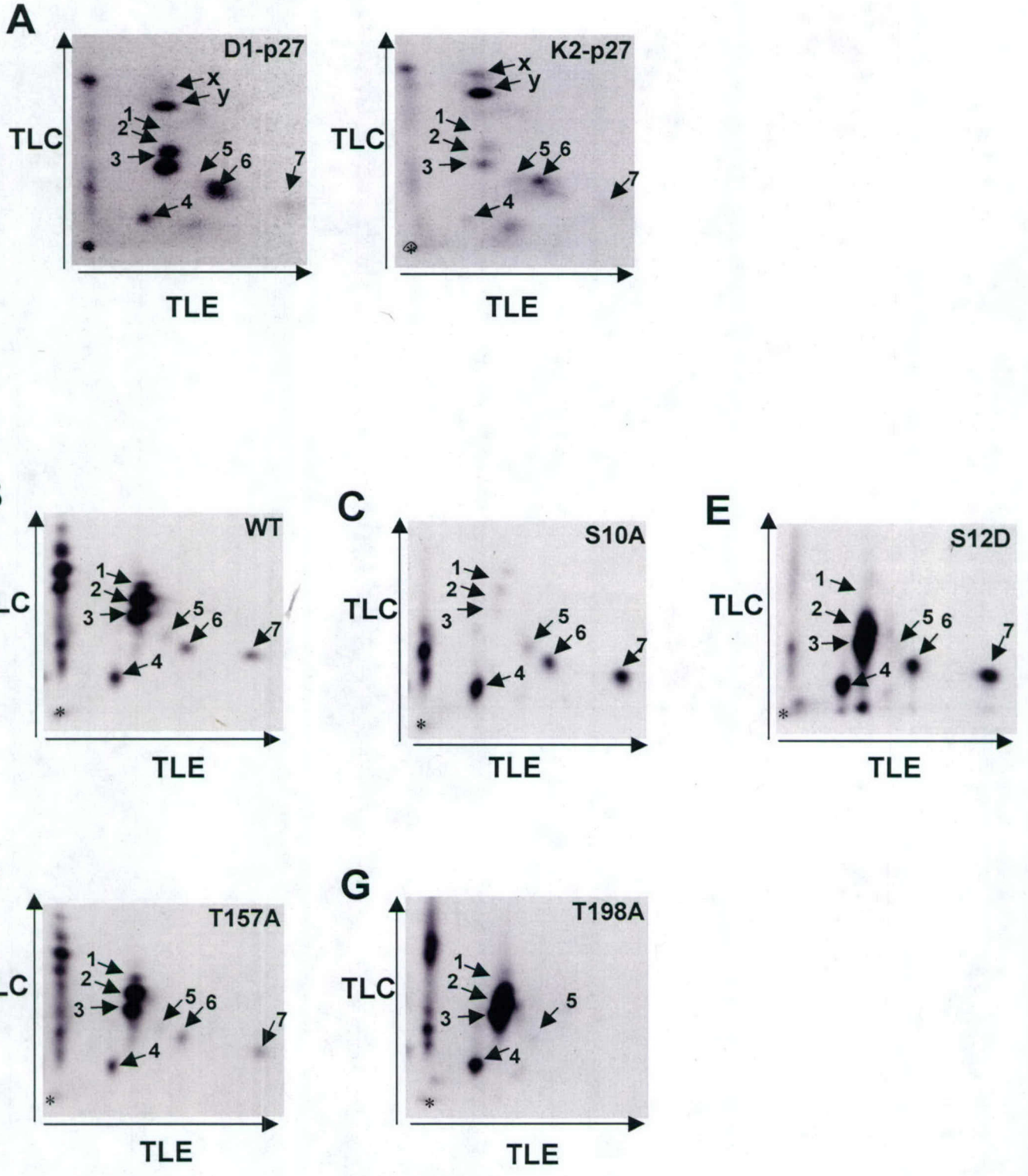
J Liang *et al,*
Figure2



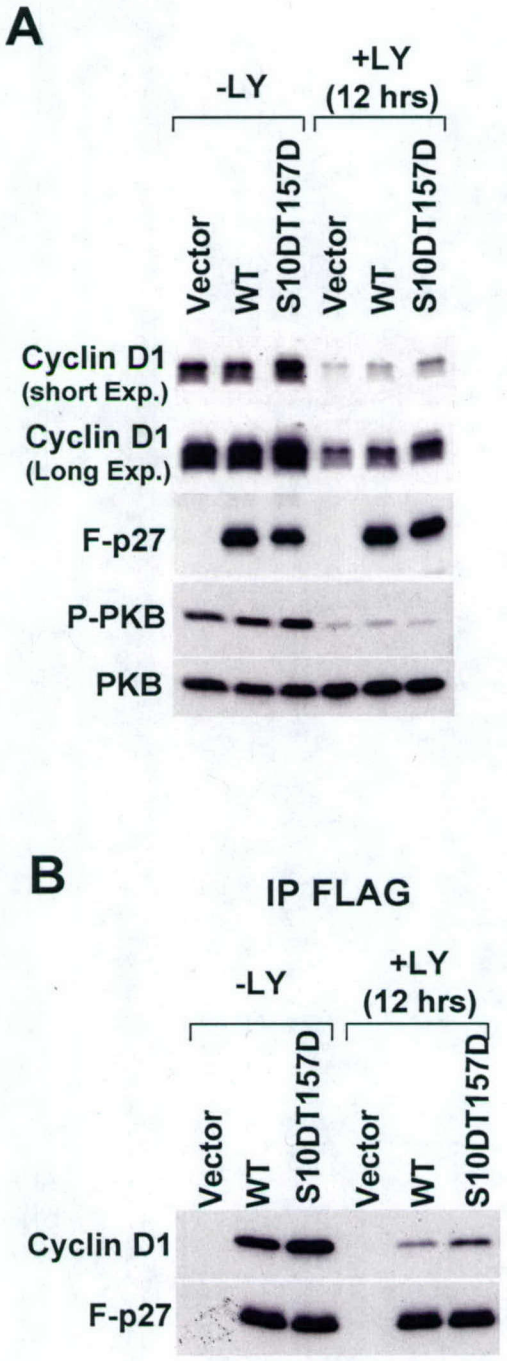
J Liang *et al*
Figure3



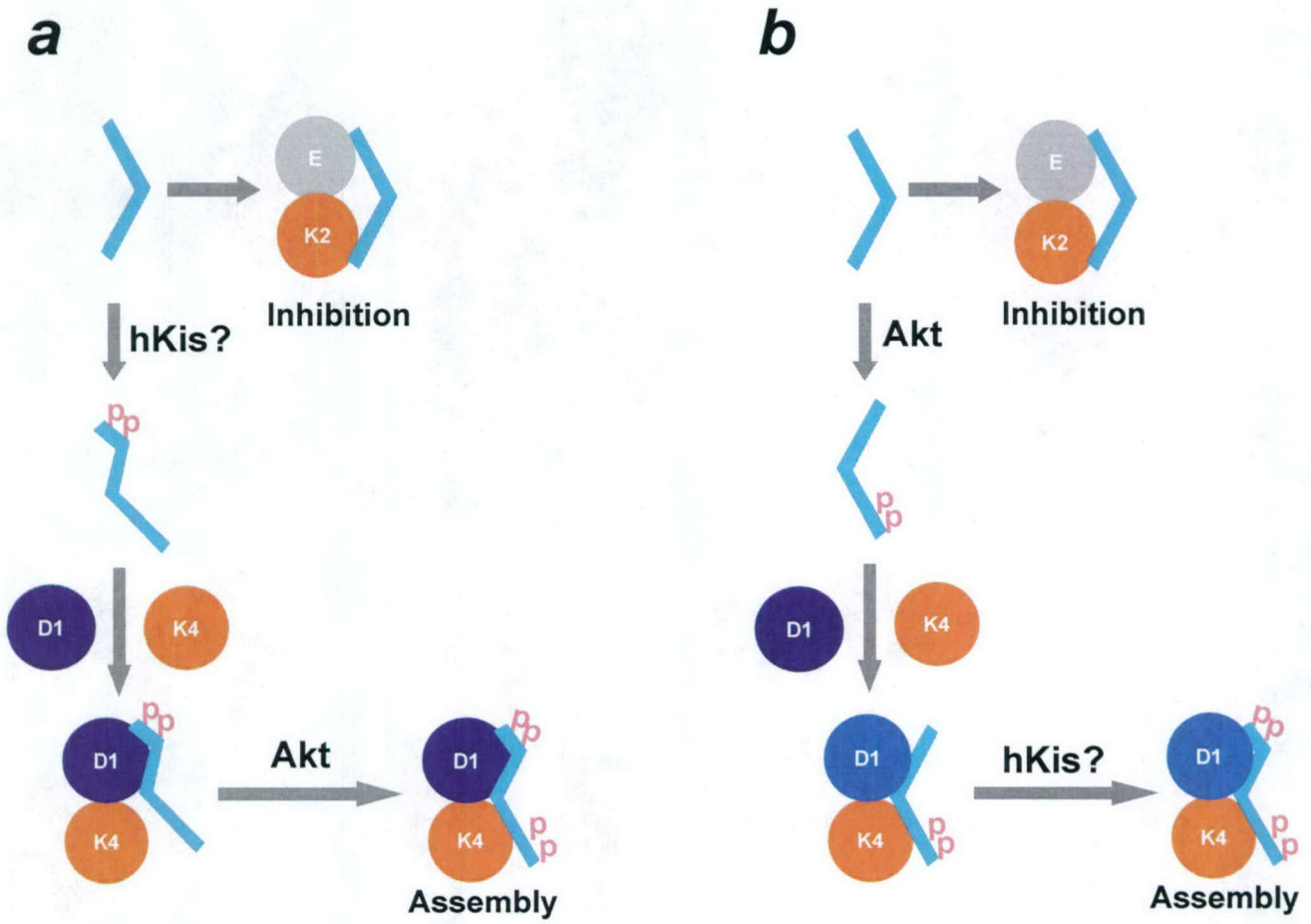
J Liang *et al*
Figure 4



Liang et al, Figure5



J. Liang, Figure 6



Liang et al, Figure 7

[Cell Cycle 2:4, 339-345, July/August 2003]; ©2003 Landes Bioscience

Review

Multiple Roles of the PI3K/PKB (Akt) Pathway in Cell Cycle Progression

Jiyong Liang[1]
Joyce M. Slingerland[2,*]

[1]Molecular and Cell Biology; Sunnybrook and Women's Health Sciences Centre; University of Toronto; Toronto, Ontario, Canada

[2]Braman Breast Cancer Institute; Sylvester Comprehensive Cancer Center; University of Miami School of Medicine; Miami, Florida USA

*Correspondence to: Joyce M. Slingerland; Braman Breast Cancer Institute; Sylvester Comprehensive Cancer Center; University of Miami School of Medicine; 1475 N.W. 12th Avenue (D8-4); Miami, Florida 33136 USA; Email: jslingerland@med.miami.edu

Received 05/16/03; Accepted 06/01/03

Previously published online as a *Cell Cycle* Paper in Press at:
http://www.landesbioscience.com/journals/cc/toc.php?volume=2&issue=4

KEY WORDS

PI3K, PKB/Akt, cell cycle, $p27^{Kip1}$, phosphorylation

ABSTRACT

As its role in tumor progression emerges, the PI3K/PKB (Akt) pathway presents an appealing cancer therapeutic target. Recent studies have investigated the mechanisms underlying the tumor-promoting effects of this pathway. PKB triggers a network that positively regulates G_1/S cell cycle progression through inactivation of GSK3-beta, leading to increased cyclin D1, and inhibition of Forkhead family transcription factors and the tumor suppressor tuberin (TSC2), leading to reduction of $p27^{Kip1}$. The identification of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ as novel substrates of PKB provided new insights into mechanisms whereby hyperactivation of this lipid signaling pathway may lead to cell cycle deregulation in human cancers. The PI3K pathway may also play a key role in the G_2/M transition and its constitutive activation may lead to defects in DNA damage checkpoint control.

INTRODUCTION

Differentiated mammalian cells require the presence of mitogenic signals to initiate cell cycle re-entry and exit quiescence (G_0) and to progress through the restriction (R) point in late G_1.[1-3] Serum and combinations of growth factors are known to induce the activation of cyclin-dependent kinases (Cdks) that drive cell cycle progression. Proliferative signals can also be transduced by proto-oncogenes even in the face of reduced or absent growth factors and constitutive oncogene activation plays a key role in carcinogenesis and tumor progression.

The class 1A family of phosphoinositide 3-kinases (PI3K) is activated by insulin, by attachment to extra-cellular matrix, by oncogene products Ras, Her2/neu, cKit, and cAbl, and by growth factors via their receptor tyrosine kinases (RTKs).[4-6,7] Once activated, PI3K phosphorylates its lipid substrate phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), converting PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3, an important intra-cellular lipid second messenger. This process is opposed by the tumor suppressor PTEN (**p**hosphatase and **t**ensin **h**omolog), also known as MMAC1 (**m**utated in **m**ultiple **a**dvanced **c**ancers) and TEP1 (TGF-β-regulated and epithelial cell-enriched phosphatase). PTEN is located on chromosome 10 and is frequently deleted or mutated in human cancers.[8] PTEN dephosphorylates the lipid product of PI3K, PtdIns(3,4,5)P_3.[9,10] Genetic loss of PTEN results in accumulation of high levels of PtdIns(3,4,5)P_3 and constitutive activation of this lipid pathway. An important down-stream effector of the PI3K pathway is protein kinase B (PKB), also known as Akt. Through its pleckstrin homology (PH) domain, PKB interacts with PtdIns(3,4,5)P_3 and undergoes a conformational change that allows phosphorylation of PKB at threonine (T) 308 by the 3-phosphoinositide-dependent protein kinase-1 (PDK1). Maximum activation of PKB requires both T308 phosphorylation and phosphorylation at a second site serine (S) 473 by PDK2, a kinase whose identity remains unclear. PKB phosphorylates its substrates at serine/threonine residues residing in the consensus motif RXRXXS/T and is involved in many of the biological effects of the PI3K pathway including cell proliferation, survival, and cellular responses to insulin and nutrients.[11,12] Overactivation of PI3K and/or its downstream effectors is frequently observed in a wide range of human tumor types (see Vivanco, 2002 for a comprehensive review, ref. 8). This review will focus on recent studies that shed light on the role of the PI3K/PKB (Akt) pathway in cell cycle progression.

THEROLE OF PKB IN G_1/S PROGRESSION

An accumulation of evidence supports a key role for the PI3K pathway in cell cycle progression. The PI3K pathway is activated during the G_1/S transition.[13] Its activity in G_1 is required for subsequent initiation of DNA synthesis induced by PDGF, EGF, and following MEK activation in mouse fibroblasts.[14-16] P13K activity is also required for G_1/S phase progression in lymphocytes and in human mammary epithelial cells (HMEC).[17,18] Studies[19,20] with mutant PDGF receptors, pharmacological inhibitors, and synthetic PI3K lipid products suggest that PI3K activity is dispensable for exiting G_0 in early G_1 (termed G_{1E} by the authors), even though it is activated at this time in response to mitogens. Whereas PI3K activation in the rest of G_1 phase is specifically required for the commitment of mouse fibroblasts to G_1 progression.[19,20] Thus P13K activation is linked to restriction point progression and coincides with G_1 events that culminate in cyclin E/Cdk2 activation.

Inhibition of Cyclin D1 and c-Myc Proteolysis. Cyclin D1 and c-Myc play distinct roles in cell cycle progression through G_1 phase. Cyclin D-dependent kinases initiate the phosphorylation of pRb, which facilitates subsequent pRb phosphorylation by cyclin E/Cdk2.[21-24] c-myc induces the expression of D-type cyclins and a heat labile protein(s) that binds and inhibits p27.[25,26] c-Myc can also suppress the gene expression of multiple negative cell cycle regulators including p15, p21, and p27 (reviewed in ref. 27). c-Myc also facilitates G_1/S progression through mechanisms independent of p27 inactivation.[28] Cyclin D1 and c-Myc expression are induced early in G_1 upon growth factor stimulation through the MAPK pathway.[24,29-32] However, it appears that MAPK pathway is not the only signaling pathway required for the accumulation of cyclin D1 and c-Myc in G_1.

Cyclin D1 and c-Myc are short-lived proteins whose levels are regulated by phosphorylation-dependent proteolytic degradation. Proteolysis of both c-Myc and cyclin D is regulated by the P13K pathway. Induced expression of a constitutively active MEK failed to achieve an increase in cyclin D1 levels in the presence of a PI3K inhibitor LY294002.[16] Diehl et al showed that glycogen synthase kinase 3 beta (GSK-3β) phosphorylates cyclin D1 at T286.[33-35] T286 phosphorylation promoted association of cyclin D1 with its nuclear exporter CRM1 and triggered nuclear-cytoplasmic translocation of cyclin D1, leading to ubiquitin-mediated proteolytic degradation of cyclin D1 in the cytoplasm. T286 to alanine (T286A) mutation stabilized cyclin D1 and resulted in cyclin D1 accumulation in the nucleus. The activity of GSK-3β is inhibited by PKB-dependent phosphorylation[36] and thus PKB mediated GSK-3β inhibition stabilizes cyclin D1. Overexpression of constitutively active PKB (MyrAkt) was shown to extend the half-life of cyclin D1 protein whereas treatment with the PI3K inhibitor Wortmannin accelerated cyclin D1 degradation.[33]

The stability of c-Myc is controlled by phosphorylation at serine 62 (S62) and T58 in a hierarchical fashion.[37] S62 phosphorylation by MAPK mediates Ras-dependent stabilization of c-Myc. Interestingly, S62 phosphorylation also creates a priming site that is necessary for subsequent phosphorylation by GSK-3β at T58, which appears to activate the ubiquitin dependent degradation of c-Myc. Thus, growth factor mediated PKB activation would stabilize c-myc through inhibition of GSK-3β. This processive phosphorylation is typical for GSK-3 substrates in that phosphorylation of specific sites allows interaction of the substrate with the phosphate-docking motif of GSK-3. Thus, Ras and growth factor signaling mediators, MAPK and PKB, act cooperatively to ensure a rapid increase in c-Myc levels in response to mitogen. Since PKB activation is periodic in early G_1, the relative inhibition of c-Myc proteolysis would be released when mitogenic stimuli are removed and when PKB activity falls in late G_1 and S phases. It seems paradoxical that MAPK-dependent phosphorylation that stabilizes c-Myc would facilitate the subsequent destruction of c-Myc by creating a priming site for GSK-3β mediated phosphorylation and activation of c-Myc destruction. However, this may represent a safeguard against sustained over-expression of c-Myc following growth factor withdrawal. Thus, activation of GSK-3β would lower c-myc levels and prevent c-Myc induced apoptotic cell death when growth factor signaling and PKB dependent survival signals are reduced.[38]

PKB also promotes transcriptional activation of *cyclin D* levels and c-Myc genes.[39,40] Moreover, translation of cyclin D1 mRNA has been shown to be dependent on the activation of PI3K/PKB/eIF4E pathway.

Regulation of p27 Degradation. High levels of $p27^{Kip1}$ are required to maintain many cell types in quiescence. p27 translation and its protein stability decrease in response to mitogenic signals.[41-44] Downregulation of p27 is critical for cell cycle re-entry in Ras induced transformation.[45] p27 levels are importantly regulated by ubiquitin-dependent proteolysis mediated through at least two distinct mecahnisms. One of these pathways requires cyclin E/Cdk2-dependent T187 phosphorylation of p27, which triggers SCF^{SKP2}-mediated ubiquitination and degradation of p27.[43,46-48] p27 proteolysis is also activated in early G_1 in a mitogen dependent and T187 phosphorylation independent manner.[49-51]

Recent studies suggest that the PI3K pathway regulates p27 protein stability. Activation of the PI3K pathway reduces p27 levels and inhibition of PI3K either by a chemical inhibitor, LY294002, kinase dead PKB/Akt, or through overexpression of the tumor suppressor PTEN can lead to accumulation of p27 in a cell-type-dependent manner.[18,52-54] PI3K activation increased p27 proteolysis in hematopoietic cells expressing BCR/Abl, in prostate cancer cells, and in PTEN null ES cells.[55-57]

p27 degradation may be regulated by the PTEN/PI3K/PKB pathway through an upregulation of SKP2. SKP2 is a key component of the SCF^{SKP2} ubiquitin ligase complex that mediates cyclin E-Cdk2 dependent ubiquitination and degradation of p27. PTEN is a lipid phosphatase that opposes P13K activation. Mamillapalli et al. observed that p27 levels were decreased and SKP2 was increased in PTEN-deficient mouse embryonic stem (ES) cells.[58] Retroviral-mediated introduction of the PTEN gene into a PTEN-deficient cell line led to a reduction of SKP2 mRNA and an accumulation of p27. Inhibition of the PI3K by LY294002 also increased SKP2 gene expression. Treatment with LY294002 was shown to down-regulate SKP2 protein in multiple myeloma cell lines.[59] In human prostate cancer, SKP2 protein levels were found to be inversely correlated with p27 and the PTEN tumor suppressor protein.[60] While PTEN activity may cause repression of SKP2 gene expression, the loss of SKP2 following PTEN overexpression or LY294002 treatment could also result from the destabilization of SKP2 protein associated with G_1 arrest. SKP2 stability is cell cycle dependent and its rate of degradation is maximal in quiescent cells.[61]

SKP2 overexpression may play an oncogenic role in a variety of human malignancies. In a subset of human oral squamous cell carcinoma, lymphoma, breast carcinomas, and gastric carcinoma, SKP2 overexpression is associated with reduced p27 levels.[62-64] Since PTEN transfection and LY294002 treatment can both reduce Skp2 mRNA expression, PI3K activation may enhance SKP2

transcription. Future studies investigating whether SKP2 gene expression is regulated by the Forkhead family transcription factors in a PKB-dependent manner may provide insights into the underlying mechanisms.

The stability of p27 may also be linked to the P13K/PKB pathway via PKB dependent phosphorylation of the TSC2 protein, tuberin. The tumor suppressor hamartin (TSC1) and tuberin (TSC2) are encoded by *TSC1* and *TSC2*, which are mutated in tuberous sclerosis (TS), an autosomal dominant disorder affecting 1 in 6,000 individuals. TS family members develop benign hyperproliferative tumors with high frequency. *TSC2* mutations are also seen in up to 30% of high-grade astrogliomas and occur spontaneously in human lung cancers (reviewed in refs. 65 and 66). Several studies showed that loss of functional tuberin protein encoded by *TSC2* is associated with cytoplasmic p27 mislocalization, reduced p27 levels due to increased p27 proteolysis, and increased cyclin E-Cdk2 activity.[67-69] The accelerated p27 proteolysis resulting from mutational inactivation of TSC2 appears to be independent of T187 phosphorylation of p27.[67] In cells genetically lacking TSC2, the half life of p27 is reduced and p27 stability can be restored by reintroduction of TSC2.[67,70]

Recent studies have shown that PKB phosphorylates and inactivatesTSC2 (see recent reviews, refs. 65 and 66). PKB mediated TSC2 phosphorylation causes dissociation of TSC1 and TSC2 and loss of their inhibitory action on mTOR. Inhibition of mTOR by Rapamycin has been shown to stabilize p27.[71,72] Thus, the increased stability of p27 following P13K inhibition by PTEN or LY294002 may result in part from PKB inhibition and the consequent activation of *TSC2*. It will be of interest to determine how downstream effectors of mTOR regulate p27 proteolysis.

Since mutational inactivation or deletion of TSC loci (9q34 for *TSC1* and 16p13.3 for *TSC2*) has been observed in human cancers,[73-75] the effects of the PI3K pathway on p27 stability may differ depending on whether TSC1-2 is functional. While PI3K activation may modulate SKP2 transcription and may also inactivate TSC2 through PKB mediated phosphorylation, the effects of this pathway on p27 stability are clearly cell type dependent.[18] Activation of PKB alone may not suffice to trigger p27 degradation in some tumors. In primary breast cancers, high PKB activity was not always associated with reduced p27 protein levels.[18]

Transcriptional Regulation of $p27^{Kip1}$, p130, and D Type Cyclins through PKB-Mediated Inhibition of Forkhead Family Transcription Factors. p27 transcription was recently shown to be regulated by PKB. The Forkhead family transcription factors are the mammalian orthologues of *Caenorhabditis Elegans* DAF-16. Three Forkhead transcription factors FoxO1a (FKHR), FoxO3a (FKHR-L1), and FoxO3 are substrates of PKB (Akt). PKB dependent phosphorylation of these transcription factors leads to their nuclear exclusion and thus inhibition of FoxO factor-mediated gene expression.[76,77] The $p27^{kip1}$ gene promoter contains multiple putative FoxO4 (initially named AFX) binding sites. Medema et al reported that FoxO4-like Forkhead family transcription factors can upregulate p27 gene expression.[78] In mouse fibroblasts, overexpression of FoxO4 resulted in increased p27 mRNA levels, a marked increase in p27 protein, inhibition of cyclin E/Cdk2 activity, and cell cycle arrest in G_1 phase. Since p27 mRNA levels are relatively constant across the cell cycle[41] and p27 protein levels are largely regulated by translation and proteolysis,[41] the significance of PKB mediated transcriptional regulation of p27 is unclear. There is no evidence at present that the reduction of p27 protein in human cancers has a transcriptional basis.[44]

The increased expression of p27 was likely only partially responsible for Cdk2 inhibition and G_1 arrest induced by FoxO4, since immortalized p27-deficient cells are still arrested by FoxO4 overexpression.[78] Downregulation of cyclins D1 and D2 may also contribute to cell cycle inhibition by forced expression of FoxO4.[79] It is paradoxical that p27 stability was unaltered in FoxO4-arrested cells, despite the inhibition of Cdk2 activity.[78] Most forms of G_1 arrest are associated with increased p27 stability and Cdk2 inhibition would block Cdk2-dependent T187 phosphorylation of p27 and SCF^{SKP2} mediated p27 proteolysis. FoxO4 mediated effects on p27 stability and transcription warrant further investigation in different cell culture models and in different physiologically relevant cell cycle arrest states.

In addition to regulating p27 gene expression, Forkhead transcription factors may also transactivate the gene encoding p130.[80] p130 plays a role in cyclin-Cdk inhibition in G_0 and early G_1.[81] Thus PI3K/PKB (Akt) activation may contribute to G_1 cell cycle progression through inhibition of forkhead mediated p130 transcription.

Inactivation of p21 by PKB-Dependent Phosphorylation. Recent reports indicate that PKB can directly phosphorylate the cyclin-dependent kinase inhibitors $p21^{cip1/Waf1}$,[82-84] and $p27^{kip1}$.[18,85,86] In cells over-expressing HER-2/neu, PKB was shown to phosphorylate p21 at T145, within its nuclear localization sequence (NLS).[84] As is the case for PKB mediated phosphorylation of Forkhead transcription factors, these authors suggested that PKB-dependent phosphorylation of p21 caused cytoplasmic accumulation of p21, preventing its access to nuclear cyclin-Cdk targets. Intriguingly, T145 of p21 is also located in the binding site for proliferating cell nuclear antigen (PCNA) and Rossig et al. (2001) found that T145 phosphorylation inhibited p21 binding to PCNA without affecting its sub-cellular distribution in human umbilical vein endothelial cells.[82] Li et al (2002) showed that, in addition to T145, S146 of p21 was also phopshorylated by PKB.[83] Phosphorylation at S146 was shown to stabilize p21. Since PKB-mediated p21 phosphorylation was also correlated with increased p21-cyclin D-Cdk4 complexes, it was postulated to increase cyclin D-Cdk4 assembly. In addition to phosphorylation by PKB, both T145 and S146 of p21 can be phosphorylated in vitro by protein kinase C (PKC) and by the cAMP-dependent protein kinase A (PKA), although the biological significance of this is not yet clear.[87]

Inactivation of p27 by PKB-dependent Phosphorylation. Unlike the classic tumor suppressor p53 and pRb, p27 gene mutations are rare in human cancers. However, reduced levels of p27 protein have been observed in up to 60% of human carcinomas due to enhanced proteolysis and this is associated with aggressive tumor behavior and poor patient outcome.[44,88] In breast and other cancers, reduced p27 levels constitute an independent poor prognostic indicator.[44] As noted earlier, in certain cancers, the accelerated proteolysis of p27 may reflect increased PI3K activity and while PKB is not likely the mediator of this effect, the relative roles of other PI3K effector pathways in the loss of p27 protein in human cancers remain to be elucidated.

In addition to reduction in p27 levels resulting from accelerated proteolysis, the antiproliferative effects of p27 may be functionally impaired in many human cancers by sequestration in cyclin D-Cdk4/6 complexes, and through mislocalization in the cytoplasm away from nuclear targets. The accumulation of p27 in cyclin D3 complexes has been observed in human lymphomas.[89] This is associated with reduced ability of p27 to inhibit cyclin E-Cdk2.[23,90]

While p27 is exclusively nuclear in quiescent normal epithelial

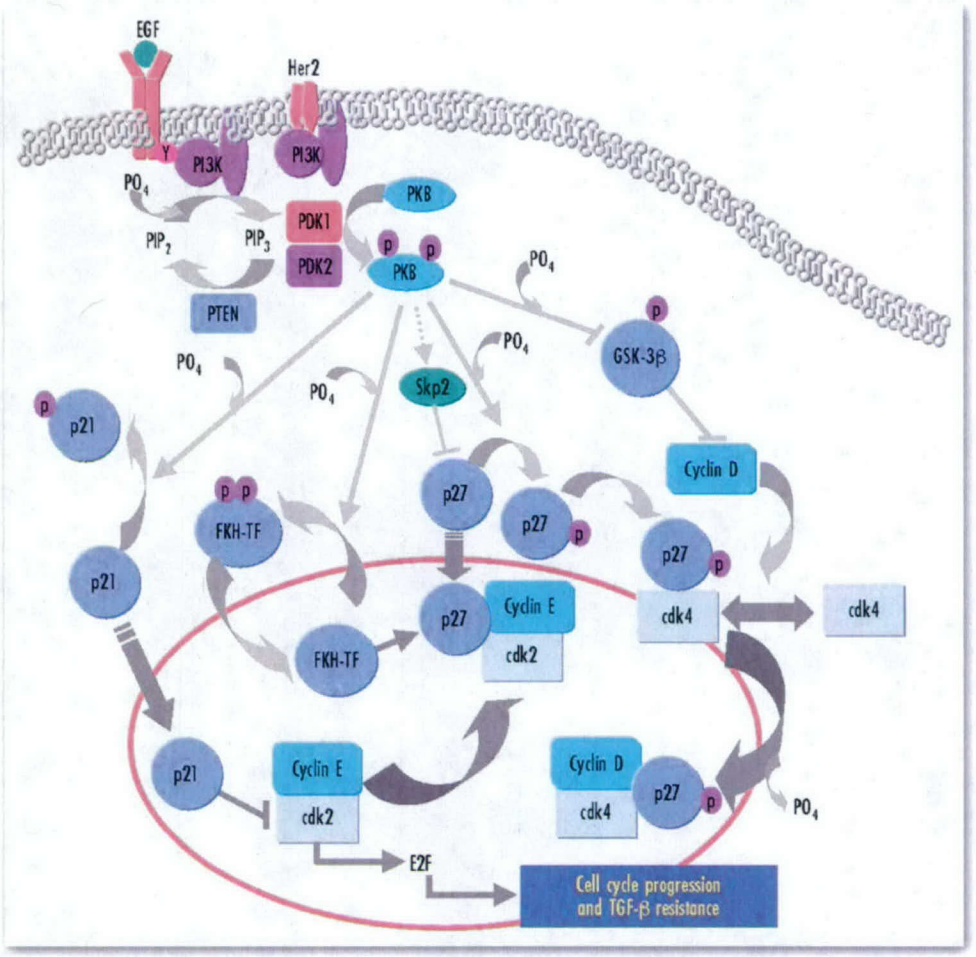


Figure 1. Mechanisms of G_1/S progression by the PI3K/PKB pathway. PI3K is activated by growth factors and oncogenes such as *Her2/neu* and catalyzes the production of the lipid PIP3, which recruits PKB, PDK1 and PDK2. PDK1 and PDK2 phosphorylate and activate PKB, which phosphorylates a number of substrate proteins that promote Cdk2 activation and G_1/S transition. PKB-mediated phosphorylation of the NLS domains of p21 and p27 causes cytoplasmic mislocalization of these Cdk inhibitors. Phosphorylation of p27 by PKB impairs nuclear import of p27 and PKB phosphorylation also facilitates the assembly of cyclin D-cdk4-p27, which prevents the inhibition of cdk4 by p15, specifically antagonizing the TGF-β-p15 pathway.

cells, p27 expression has been detected in the cytoplasm of tumor cell lines and cancer tissues.[89,91-93] Cytoplasmic sequestration would prevent p27 from binding and inhibiting nuclear cyclin-Cdk targets.[94] The nuclear import of p27 depends on a bipartite NLS in its carboxyl terminal region. Three recent reports provide new insights into mechanisms regulating the subcellular localization of p27.[18,85,86] These studies showed that PKB phosphorylates p27 at T157 in its NLS. Liang et al showed that p27 phosphorylation by PKB impaired the nuclear import of p27 in vitro.[18] Cells with genetic loss of PTEN or amplification of *HER2/neu* showed cytoplasmic mislocalization of p27 that was reversed by the PI3K inhibitor, LY294002. Cells with constitutive PKB activation were also resistant to G_1-arrest induced by proliferation inhibitory cytokines such as TGF-β and IL-6.[18] Partial inhibition of PKB activation by either LY294002, or PTEN transfection restored both the nuclear localization of p27 and its ability to mediate G_1-arrest in response to TGF-β and IL-6.[18] The biological relevance of these findings was underscored by the observation that up to 40% of human breast cancers showed cytoplasmic p27.[18,85,86] Cytoplasmic p27 was correlated with tumor de-differentiation (increased tumor grade), and poor prognosis.[18] All three studies showed a consistent association between PKB activation and cytoplasmic p27 staining in these breast cancers. Moreover, cytoplasmic p27 but not nuclear p27 isolated from primary cancers reacted with a T157 phosphorylation specific p27 antibody.[85] Thus, excessive activation of the PI3K/PKB pathway and PKB-dependent phosphorylation of p27 is likely one of the mechanisms underlying cytoplasmic mislocalization of p27 in human cancers.

While PKB-dependent T157 phosphorylation affected the sub-cellular localization of p27, PKB activity did not directly affect p27 stability.[18] PKB activation and mislocalization of p27 were not statistically associated with reduced p27 levels in primary tumors. Moreover, transfection of activated PKB did not activate p27 proteolysis. Thus, the increased p27 proteolysis observed following PI3K activation in many cell types may involve PI3K dependent effectors other than PKB.

In cells transfected with both *PKB* and *p27*, p27 has been shown to be phosphorylated at T198. T198 does not lie within a classical PKB consensus motif. T198 phosphorylation of p27 increased its

association with overexpressed 14-3-3.[95] Since these studies were based on over-expression of transfected p27, *PKB* and a specific *14-3-3* isoform, further investigation will be required to clarify whether endogenous cellular p27 is phosphorylated at T198, whether this site is a physiologic target of PKB, and the biological consequences of this phosphorylation.

Some aspects of these mechanisms are only beginning to be understood. PKB-dependent phosphorylation and the subsequent cytoplasmic sequestration of cdk inhibitors may be oversimplified as a mechanism of inactivation. Cytoplasmic sequestration of p27 and p21 may not be equivalent to loss of function of these cdk inhibitors. Cytoplasmic p27 does not likely exist as a monomer. If it remains bound to cyclin D-dependent kinases such binding may not lead to kinase inhibition. Sequestration of cdk inhibitors is thought to be a non-catalytic function of cyclin D-cdk complexes. It is tempting to speculate that cytoplasmic p27 or p21 may have functions other than Cdk inhibition. The phosphorylation event(s) that regulate the subcellular localization of p21 and p27 may also lead to altered KIP function.

A ROLE FOR PKB/AKT IN G_2/M PROGRESSION

PI3K pathway activation is required for G_1/S progression and PI3K inhibition leads to G_1 arrest in many cell types.[14,17,59,96,97] Although mitogens are no longer essential for S phase entrance once cells have progressed past the R point in late G_1, the PI3K/PKB pathway may also regulate the efficiency of G_2/M phase progression. Inhibition of PI3K by LY294002 was shown to delay G_2/M progression in Rat-1 cells released from an aphidicolin-mediated S phase blockade.[98] When MDCK cells synchronized by double thymidine block were released from S phase, addition of a PI3K inhibitor led to G_2 arrest and apoptosis.[98] Kandel et al found that LY294002 treatment of HEK293 cells mediated a G_2 arrest rather than the G_1 arrest seen in most other cell lines.[99] Thus, PI3K activity may be required for G_2/M phase progression.

Recent studies have shown that constitutive activation of PI3K pathway components can override DNA damage-induced cell cycle arrest. Kandel et al showed that constitutive PKB activation could overcome a G_2/M cell cycle checkpoint induced by DNA damage.[99] Transfection of constitutively active MyrAkt abrogated Cdc2 inhibition and the G_2 arrest induced by exposure to the guanine analog 6-thioguanine, a DNA damaging agent that induces DNA mismatch repair, or following gamma irradiation in Rat1a cells. Interestingly, the effects of MyrAkt were independent of p53 and were not merely due to enhanced proliferation by MyrAkt. Thus, MyrAkt functions that are independent of its antiapoptotic and pro-mitogenic effects are implicated in its ability to overcome this DNA damage mediated Cdc2 inhibition and G_2 checkpoint arrest.

Although the pathway whereby MyrAkt prevented DNA damage induced Cdc2 inhibition and G_2/M arrest were not elucidated by Kandel et al., a number of potential mechanisms are emerging. G_2/M progression is regulated by activation of cyclin B-Cdc2. Gadd45a (Growth arrest and DNA damage 45a) is activated following DNA damage and opposes cyclin B-Cdc2 activation.[100] Tran et al recently demonstrated that the Gadd45a gene is transactivated by FOXO3a.[101] Thus, activation of PI3K and PKB would lead to FOXO3a phosphorylation and its sequestration in the cytoplasm, thereby reducing Gadd45a expression. The effective induction of Gadd45a following DNA damage may require inactivation of PI3K. Thus MyrAkt transfection and constitutive PKB/Akt activity would attenuate the induction of Gadd45a and oppose Gadd45a mediated Cdc2 inhibition. However, while the PI3K inhibitor LY294002 increased FOXO3a induced Gadd45a expression, PI3K inhibition still delayed G_2/M progression in Gadd45a$^{-/-}$ cells, suggesting that PI3K regulated mechanisms independent of Gadd45a may oppose DNA damage induced G_2 arrest.

Tyrosine 15 (Y15) phosphorylation of Cdc2 by Wee1-like kinases inhibits Cdc2 activity.[102,103] Cdc25 phosphatases dephosphorylate this inhibitory site, thus positively regulating Cdc2 activity.[104,105] Following DNA damage, Chk1 has been shown to phosphorylate Wee-1 and Cdc25C, leading to inhibition of Cdc2 (reviewed by Walworth, 2001, ref. 106). It was recently shown that PKB can phosphorylate human Chk1 kinase at S280 and inactivate it in vitro. Inhibition of cellular PI3K led to a substantial increase in Chk1 kinase activity.[98] Thus, inactivation of Chk1 by constitutively active MyrAkt would impair Chk1 mediated Cdc25C phosphorylation following genotypic injury and prevent the inhibition of cyclin B1-Cdc2 leading to resistance to DNA damage mediated G_2/M arrest. While available data supports this model, in vivo phosphorylation of Chk1 by PKB remains to be demonstrated.

Okumura et al (2002)[107] showed that microinjection of either a PKB blocking peptide or a PKB antibody into immature *Asterina* (starfish) oocytes can inhibit G_2/M transition. Moreover, microinjection of mRNA encoding a constitutively active PKB was sufficient to induce meiotic re-initiation in the absence of maturation-inducing hormone. In this system, PKB was shown to bind and phosphorylate Myt1 at S75 and impair the activity of Myt1-mediated Y15 phosphorylation of Cdc2. Thus, PKB may also facilitate the G_2/M transition by inactivating Wee1-like kinases. It is not clear whether similar mechanisms are at work in mammalian or somatic cells.

While PI3K/PKB activation may be required for G_2/M progression, the subsequent inactivation of PI3K pathway may also be required for mitotic exit. Alvarez et al. (2001)[108] demonstrated that sustained PI3K activation following transfection of p110CAAX in NIH3T3 cells increased the proportion of cells in G_2/M, delayed the M to G_1 phase transition and led to defective cytokinesis. Failure to downregulate PI3K activity during G_2 impaired FOXO3a-mediated transactivation of both cyclin B and polo-like kinase (Plk) genes. Thus, constitutive activation of the PI3K or PKB led to reduced cyclin B synthesis and interfered with the timely degradation of cyclin B1, leading to defective G_2/M progression and impaired mitotic exit respectively.

CONCLUDING REMARKS AND PERSPECTIVES

Tumor cells are distinguished from their normal counterparts by aberrant cell cycle progression resistant to growth inhibitory signals and insensitive to DNA damage checkpoints. As evident from the present review, constitutive or increased activity of the PI3K dependent signaling cascade presents a major means whereby tumor cells achieve uncontrolled proliferation. A key effector of this pathway is PKB. Consitutive PKB activation can lead to cytoplasmic mislocalization of p21 and p27, elevation of c-Myc and cyclin D and suppression of p130. Activation of the PI3K pathway may also modulate p27 stability through effects on SKP2 gene expression and indirectly by shifting p27 localization to the cytoplasm. Consitutive PKB activation may also lead to loss of normal G_2/M checkpoint responses through changes in the transcriptional regulation of Gadd45a and modulation of Chk1 phosphorylation and activity. Although many studies have revealed mechanisms whereby PKB

regulates the cell cycle, a complete understanding of the involvement of PI3K pathway in normal and malignant cell cycle progression will require additional investigation. Further studies are needed to define the roles of other components or targets of the PI3K pathway that may link cell size and cell cycle regulation including mTOR, $p70^{S6K}$ and other PKB/Akt-like kinases such as SGK (serum and glucocorticoid-inducible kinase) in normal cell cycle regulation and in human cancers.

References

1. Sherr CJ. The Pezcoller lecture: cancer cell cycles revisited. Cancer Res 2000; 60:3689-95.
2. Burdon T, Smith A, Savatier P. Signalling, cell cycle and pluripotency in embryonic stem cells. Trends Cell Biol 2002; 12:432-8.
3. Gille H, Downward J. Multiple ras effector pathways contribute to G(1) cell cycle progression. J Biol Chem 1999; 274:22033-40.
4. Sattler M, Mohi MG, Pride YB, Quinnan LR, Malouf NA, Podar K, Gesbert F, Iwasaki H, Li S, Van Etten RA, Gu H, Griffin JD, Neel BG. Critical role for Gab2 in transformation by BCR/ABL. Cancer Cell 2002; 1:479-92.
5. Cantley LC. The phosphoinositide 3-kinase pathway. Science 2002; 296:1655-7.
6. Roymans D, Slegers H. Phosphatidylinositol 3-kinases in tumor progression. Eur J Biochem 2001; 268:487-98.
7. Linnekin D. Early signaling pathways activated by c-Kit in hematopoietic cells. Int J Biochem Cell Biol 1999; 31:1053-74.
8. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2002; 2:489-501.
9. Cantley LC, Neel BG. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci U S A 1999; 96:4240-5.
10. Di Cristofano A, Pandolfi PP. The multiple roles of PTEN in tumor suppression. Cell 2000; 100:387-90.
11. Coffer PJ, Jin J, Woodgett JR. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochem J 1998; 335 (Pt 1):1-13.
12. Lawlor MA, Alessi DR. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? J Cell Sci 2001; 114:2903-10.
13. Auger KR, Serunian LA, Soltoff SP, Libby P, Cantley LC. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell 1989; 57:167-75.
14. Roche S, Koegl M, Courtneidge SA. The phosphatidylinositol 3-kinase alpha is required for DNA synthesis induced by some, but not all, growth factors. Proc Natl Acad Sci U S A 1994; 91:9185-9.
15. Takuwa N, Takuwa Y. Ras activity late in G1 phase required for p27kip1 downregulation, passage through the restriction point, and entry into S phase in growth factor-stimulated NIH 3T3 fibroblasts. Mol Cell Biol 1997; 17:5348-58.
16. Treinies I, Paterson HF, Hooper S, Wilson R, Marshall CJ. Activated MEK stimulates expression of AP-1 components independently of phosphatidylinositol 3-kinase (PI3-kinase) but requires a PI3-kinase signal To stimulate DNA synthesis. Mol Cell Biol 1999; 19:321-9.
17. Brennan P, Babbage JW, Burgering BM, Groner B, Reif K, Cantrell DA. Phosphatidylinositol3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. Immunity 1997; 7:679-89.
18. Liang J, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catzavelos C, Beniston R, Franssen E, Slingerland JM. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. Nature Med 2002; 8:1153-60.
19. Jones SM, Klinghoffer R, Prestwich GD, Toker A, Kazlauskas A. PDGF induces an early and a late wave of PI 3-kinase activity, and only the late wave is required for progression through G1. Curr Biol 1999; 9:512-21.
20. Jones SM, Kazlauskas A. Growth-factor-dependent mitogenesis requires two distinct phases of signalling. Nat Cell Biol 2001; 3:165-72.
21. Hatakeyama M, Brill JA, Fink GR, Weinberg RA. Collaboration of G1 cyclins in the functional inactivation of the retinoblastoma protein. Genes Dev 1994; 8:1759-71.
22. Resnitzky D, Gossen M, Bujard H, Reed S. Differential acceleration of the G1/S transition by conditional overexpression of cyclins D1 and E. Mol Cell Biol 1994; 14:1669-79.
23. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 1999; 13:1501-12.
24. Blagosklonny MV, Pardee AB. The restriction point of the cell cycle. Cell cycle 2002; 1:103-10.
25. Vlach J, Hennecke S, Alevizopoulos K, Conti D, Amati B. Growth arrest by the cyclin-dependent kinase inhibitor p27Kip1 is abrogated by c-Myc. EMBO J 1996; 15:6595-604.
26. Perez-Roger I, Solomon DL, Sewing A, Land H. Myc activation of cyclin E/Cdk2 kinase involves induction of cyclin E gene transcription and inhibition of p27(Kip1) binding to newly formed complexes. Oncogene 1997; 14:2373-81.
27. Gartel AL, Shchors K. Mechanisms of c-myc-mediated transcriptional repression of growth arrest genes. Exp Cell Res 2003; 283:17-21.
28. Beier R, Burgin A, Kiermaier A, Fero M, Karsunky H, Saffrich R, Moroy T, Ansorge W, Roberts J, Eilers M. Induction of cyclin E-cdk2 kinase activity, E2F-dependent transcription and cell growth by Myc are genetically separable events. EMBO J 2000; 19:5813-23.
29. Weber JD, Hu W, Jefcoat SCJr, Raben DM, Baldassare JJ. Ras-stimulated extracellular signal-related kinase 1 and RhoA activities coordinate platelet-derived growth factor-induced G1 progression through the independent regulation of cyclin D1 and p27 Kip1. J Biol Chem 1997; 272:32966-71.
30. Sears RC, Nevins JR. Signaling networks that link cell proliferation and cell fate. Biol Chem 2002; 277:11617-20.
31. Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J Biol Chem 1996; 271:20608-16.
32. Balmanno K, Cook SJ. Sustained MAP kinase activation is required for the expression of cyclin D1, p21Cip1 and a subset of AP-1 proteins in CCL39 cells. Oncogene 1999; 18:3085-97.
33. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev 1998; 12:3499-511.
34. Diehl JA, Sherr CJ. Identification of a protein kinase that regulates cyclin D1 turnover and subcellular localization. [The Cell Cycle], xviii. 1998. Cold Spring Harbor, New York, Cold Spring Harbor Laboratories. 5-20-1998.
35. Alt JR, Cleveland JL, Hannink M, Diehl JA. Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. Genes & Dev 2001; 14:3102-14.
36. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 1995; 378:785-9.
37. Sears R, Nuckolls F, Haura E, Taya Y, Tamai K, Nevins JR. Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. Genes Dev 2000; 14:2501-14.
38. Pelengaris S, Khan M, Evan G. c-MYC: more than just a matter of life and death. Nat Rev Cancer 2002; 2:764-6.
39. Ahmed NN, Grimes HL, Bellacosa A, Chan TO, Tsichlis PN. Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. P N A S USA 1997; 94:3627-32.
40. Chen C, Sytkowski AJ. Erythropoietin activates two distinct signaling pathways required for the initiation and the elongation of c-myc. J Biol Chem 2001; 276:38518-26.
41. Hengst L, Reed SI. Translational control of p27Kip1 accumulation during the cell cycle. Science 1996; 271:1861-4.
42. Millard SS, Yan JS, Nguyen H, Pagano M, Kiyokawa H, Koff A. Enhanced ribosomal association of p27(Kip1) mRNA is a mechanism contributing to accumulation during growth arrest. J Biol Chem 1997; 272:7093-8.
43. Pagano M, Tam SW, Theodoras AM, Beer-Romero P, Del Sal G, Chau V, Yew PR, Draetta GF, Rolfe M. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science 1995; 269:682-5.
44. Slingerland J, Pagano M. Regulation of the cdk inhibitor p27 and its deregulation in cancer. J Cell Physiol 2000; 183:10-7.
45. Leone G, DeGregori J, Sears R, Jakoi L, Nevins JR. Myc and Ras collaborate in inducing accumulation of active cyclin E/Cdk2 and E2F [published erratum appears in Nature 1997 Jun 26; 387(6636):932]. Nature 1997; 387:422-6.
46. Sheaff RJ, Groudine M, Gordon M, Roberts JM, Clurman BE. Cyclin E-CDK2 is a regulator of p27Kip1. Genes & Dev 1997; 11:1464-78.
47. Vlach J, Hennecke S, Amati B. Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. EMBO J 1997; 16:5334-44.
48. Montagnoli A, Fiore F, Eytan E, Carrano AC, Draetta GF, Hershko A, Pagano M. Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes & Dev 1999; 13:1181-9.
49. Malek NP, Sundberg H, McGrew S, Nakayama K, Kyriakidis TR, Roberts JM. A mouse knock-in model exposes sequential proteolytic pathways that regulate p27Kip1 in G1 and S phase. Nature 2001; 413:323-7.
50. Hara T, Kamura T, Nakayama K, Oshikawa K, Hatakeyama S, Nakayama KI. Degradation of p27Kip1 at the G0-G1 transition mediated by a Skp2-independent ubiquitination pathway. J Biol Chem 2001; 276:48937-43.
51. Connor MK, Kotchetkov R, Cariou S, Resch A, Lupetti R, Beniston RG, Melchior F, Hengst L, Slingerland JM. CRM1/Ran-Mediated Nuclear Export of p27(Kip1) Involves a Nuclear Export Signal and Links p27 Export and Proteolysis. Mol Biol Cell 2003; 14:201-13.
52. Narita Y, Nagane M, Mishima K, Huang HJ, Furnari FB, Cavenee WK. Mutant Epidermal Growth Factor Receptor Signaling Down-Regulates p27 through Activation of the Phosphatidylinositol 3-Kinase/Akt Pathway in Glioblastomas. Cancer Res 2002; 62:6764-9.
53. Weng LP, Brown JL, Eng C. PTEN coordinates G(1) arrest by down-regulating cyclin D1 via its protein phosphatase activity and up-regulating p27 via its lipid phosphatase activity in a breast cancer model. Hum Mol Genet 2001; 10:599-604.
54. Yakes FM, Chinratanalab W, Ritter CA, King W, Seelig S, Arteaga CL. Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt Is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. Cancer Res 2002; 62:4132-41.
55. Forti FL, Schwindt TT, Moraes MS, Eichler CB, Armelin HA. ACTH Promotion of p27(Kip1) Induction in Mouse Y1 AdrenocorticalTumor Cells is Dependent on Both PKA Activation and Akt/PKB Inactivation. Biochemistry 2002; 41:10133-40.

56. Murillo H, Huang H, Schmidt LJ, Smith DI, Tindall DJ. Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state. Endocrinology 2001; 142:4795-805.
57. Gesbert F, Sellers WR, Signoretti S, Loda M, Griffin JD. BCR/ABL regulates expression of the cyclin-dependent kinase inhibitor p27Kip1 through the phosphatidylinositol 3-Kinase/AKT pathway. J Biol Chem 2000; 275:39223-30.
58. Mamillapalli R, Gavrilova N, Mihaylova VT, Tsvetkov LM, Wu H, Zhang H, Sun H. PTEN regulates the ubiquitin-dependent degradation of the CDK inhibitor p27(KIP1) through the ubiquitin E3 ligase SCF(SKP2). Curr Biol 2001; 11:263-7.
59. Pene F, Claessens YE, Muller O, Viguie F, Mayeux P, Dreyfus F, Lacombe C, Bouscary D. Role of the phosphatidylinositol 3-kinase/Akt and mTOR/P70S6-kinase pathways in the proliferation and apoptosis in multiple myeloma. Oncogene 2002; 21:6587-97.
60. Yang G, Ayala G, Marzo AD, Tian W, Frolov A, Wheeler TM, Thompson TC, Harper JW. Elevated Skp2 protein expression in human prostate cancer: association with loss of the cyclin-dependent kinase inhibitor p27 and PTEN and with reduced recurrence-free survival. Clin Cancer Res 2002; 8:3419-26.
61. Wirbelauer C, Sutterluty H, Blondel M, Gstaiger M, Peter M, Reymond F, Krek W. The F-box protein Skp2 is a ubiquitylation target of a Cul1-based core ubiquitin ligase complex: evidence for a role of Cul1 in the suppression of Skp2 expression in quiescent fibroblasts. EMBO J 2000; 19:5362-75.
62. Gstaiger M, Jordan R, Lim M, Catzavelos C, Mestan J, Slingerland J, Krek W. Skp2 is oncogenic and overexpressed in human cancers. P N A S USA 2001; 98:5043-8.
63. Signoretti S, Di Marcotullio L, Richardson A, Ramaswamy S, Isaac B, Rue M, Monti F, Loda M, Pagano M. Oncogenic role of the ubiquitin ligase subunit Skp2 in human breast cancer. J Clin Invest 2002; 110:633-41.
64. Masuda TA, Inoue H, Sonoda H, Mine S, Yoshikawa Y, Nakayama K, Nakayama K, Mori M. Clinical and biological significance of S-phase kinase-associated protein 2 (Skp2) gene expression in gastric carcinoma: modulation of malignant phenotype by Skp2 overexpression, possibly via p27 proteolysis. Cancer Res 2002; 62:3819-25.
65. McManus EJ, Alessi DR. TSC1-TSC2: a complex tale of PKB-mediated S6K regulation. Nat Cell Biol 2002; 4:E214-E216.
66. Marygold SJ, Leevers SJ. Growth Signaling: TSC Takes Its Place. Curr Biol 2002; 12:R785-R787.
67. Soucek T, Yeung RS, Hengstschlager M. Inactivation of the cyclin-dependent kinase inhibitor p27 upon loss of the tuberous sclerosis complex gene-2. Proc Natl Acad Sci USA 1998; 95:15653-8.
68. Howe SR, Everitt JI, Goldsworthy TL, Wolf DC, Walker C. Rodent model of reproductive tract leiomyomata. Establishment and characterization of tumor-derived cell lines. Am J Pathol 1995; 146:1568-79.
69. Pasumarthi KB, Nakajima H, Nakajima HO, Jing S, Field LJ. Enhanced cardiomyocyte DNA synthesis during myocardial hypertrophy in mice expressing a modified TSC2 transgene. Circ Res 2000; 86:1069-77.
70. Uhlmann EJ, Apicelli AJ, Baldwin RL, Burke SP, Bajenaru ML, Onda H, Kwiatkowski D, Gutmann DH. Heterozygosity for the tuberous sclerosis complex (TSC) gene products results in increased astrocyte numbers and decreased p27-Kip1 expression in TSC2+/- cells. Oncogene 2002; 21:4050-9.
71. Nourse J, Firpo E, Flanagan WM, Coats S, Polyak K, Lee MH, Massague J, Crabtree GR, Roberts JM. Interleukin-2-mediated elimination of the p27kip1 cyclin- dependent kinase inhibitor prevented by rapamycin. Nature 1994; 372:570-3.
72. Firpo EJ, Koff A, Solomon MJ, Roberts JM. Inactivation of a Cdk2 inhibitor during interleukin 2-induced proliferation of human T lymphocytes. Mol Cell Biol 1994; 14:4889-901.
73. Lininger RA, Park WS, Man YG, Pham T, MacGrogan G, Zhuang Z, Tavassoli FA. LOH at 16p13 is a novel chromosomal alteration detected in benign and malignant microdissected papillary neoplasms of the breast. Hum Pathol 1998; 29:1113-8.
74. Lininger RA, Zhuang Z, Man Y, Park WS, Emmert-Buck M, Tavassoli FA. Loss of heterozygosity is detected at chromosomes 1p35-36 (NB), 3p25 (VHL), 16p13 (TSC2/PKD1), and 17p13 (TP53) in microdissected apocrine carcinomas of the breast. Mod Pathol 1999; 12:1083-9.
75. Marygold SJ, Leevers SJ. Growth Signaling: TSC Takes Its Place. Curr Biol 2002; 12:R7785-R787.
76. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 1999; 96:857-68.
77. Brownawell AM, Kops GJ, Macara IG, Burgering BM. Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX. Mol Cell Biol 2001; 21:3534-46.
78. Medema RH, Kops GJ, Bos JL, Burgering BM. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature 2000; 404:782-7.
79. Schmidt M, de Mattos SF, van der Horst A, Klompmaker R, Kops GJ, Lam EW, Burgering BM, Medema RH. Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. Mol Cell Biol 2002; 22:7842-52.
80. Kops GJ, Medema RH, Glassford J, Essers MA, Dijkers PF, Coffer PJ, Lam EW, Burgering BM. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol Cell Biol 2002; 22:2025-36.
81. Coats S, White P, Fero ML, Lacy S, Chung G, Randel E, Firpo E, Roberts JM. A new pathway for mitogen-dependent Cdk2 regulation uncovered in p27Kip1-deficient cells. Current Biology 1999; 9:163-73.
82. Rossig L, Jadidi AS, Urbich C, Badorff C, Zeiher AM, Dimmeler S. Akt-Dependent Phosphorylation of p21(Cip1) Regulates PCNA Binding and Proliferation of Endothelial Cells. Mol Cell Biol 2001; 21:5644-57.
83. Li Y, Dowbenko D, Lasky LA. AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival. J Biol Chem 2002; 277:11352-61.
84. Zhou BP, Liao Y, Xia W, Spohn B, Lee MH, Hung MC. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat Cell Biol 2001; 3:245-52.
85. Viglietto G, Motti ML, Bruni P, Melillo RM, D'Alessio A, Califano D, Vinci F, Chiappetta G, Tsichlis P, Bellacosa A, Fusco A, Santoro M. Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. Nature Med 2002; 8:1136-44.
86. Shin I, Yakes FM, Rojo F, Shin NY, Bakin AV, Baselga J, Arteaga CL. PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. Nature Med 2002; 8:1145-52.
87. Scott MT, Morrice N, Ball KL. Reversible phosphorylation at the C-terminal regulatory domain of p21(Waf1/Cip1) modulates proliferating cell nuclear antigen binding. J Biol Chem 2000; 275:11529-37.
88. Viglietto G, Fusco A. Understanding p27(kip1) deregulation in cancer: down-regulation or mislocalization? Cell cycle 2002; 1:394-400.
89. Sanchez-Beato M, Camacho FI, Martinez-Montero JC, Saez AI, Villuendas R, Sanchez-Verde L, Garcia JF, Piris MA. Anomalous high p27/KIP1 expression in a subset of aggressive B-cell lymphomas is associated with cyclin D3 overexpression. p27/KIP1-cyclin D3 colocalization in tumor cells. Blood 1999; 94:765-72.
90. Ciarallo S, Subramaniam V, Hung W, Lee JH, Kotchetkov R, Sandhu C, Milic A, Slingerland JM. Altered p27(Kip1) phosphorylation, localization, and function in human epithelial cells resistant to transforming growth factor beta-mediated G(1) arrest. Mol Cell Biol 2002; 22:2993-3002.
91. Singh SP, Lipman J, Goldman H, Ellis FH, Aizenman L, Cangi MG, Signoretti S, Chiaur DS, Pagano M, Loda M. Loss or altered subcellular localization of p27 in Barrett's associated adenocarcinoma. Cancer Research 1998; 58:1730-5.
92. Mizumatsu S, Tamiya T, Ono Y, Abe T, Matsumoto K, Furuta T, Ohmoto T. Expression of cell cycle regulator p27Kip1 is correlated with survival of patients with astrocytoma. Clin Cancer Res 1999; 5:551-7.
93. Ciaparrone M, Yamamoto H, Yao Y, Sgambato A, Cattoretti G, Tomita N, Monden T, Rotterdam H, Weinstein IB. Localization and expression of p27KIP1 in multistage colorectal carcinogenesis. Cancer Res 1998; 58:114-22.
94. Blain SW, Massague J. Breast cancer banishes p27 from nucleus. Nat Med 2002; 8:1076-8.
95. Fujita N, Sato S, Katayama K, Tsuruo T. Akt-dependent phosphorylation of p27Kip1 promotes binding to 14-3-3 and cytoplasmic localization. J Biol Chem 2002; 277:28706-13.
96. Li DM, Sun H. PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. Proc Natl Acad Sci U S A 1998; 95:15406-11.
97. Collado M, Medema RH, Garcia-Cao I, Dubuisson ML, Barradas M, Glassford J, Rivas C, Burgering BM, Serrano M, Lam EW. Inhibition of the phosphoinositide 3-kinase pathway induces a senescence-like arrest mediated by p27Kip1. J Biol Chem 2000; 275:21960-8.
98. Shtivelman E, Sussman J. A role for PI 3-kinase and PKB activity in the G2/M phase of the cell cycle. Curr Biol 2002; 12:919-24.
99. Kandel ES, Skeen J, Majewski N, Di Cristofano A, Pandolfi PP, Feliciano CS, Gartel A, Hay N. Activation of Akt/protein kinase B overcomes a G(2)/m cell cycle checkpoint induced by DNA damage. Mol Cell Biol 2002; 22:7831-41.
100. Zhan Q, Antinore MJ, Wang XW, Carrier F, Smith ML, Harris CC, Fornace AJ Jr. Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene 1999; 18:2892-900.
101. Tran H, Brunet A, Grenier JM, Datta SR, Fornace AJ Jr, DiStefano PS, Chiang LW, Greenberg ME. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science 2002; 296:530-4.
102. O'Connell MJ, Raleigh JM, Verkade HM, Nurse P. Chk1 is a wee1 kinase in the G2 DNA damage checkpoint inhibiting cdc2 by Y15 phosphorylation. EMBO J 1997; 16:545-54.
103. McGowan CH, Russell P. Cell cycle regulation of human WEE1. EMBO J 1995; 14:2166-75.
104. Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H, Elledge SJ. Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. Science 1997; 277:1497-501.
105. Raleigh JM, O'Connell MJ. The G(2) DNA damage checkpoint targets both Wee1 and Cdc25. J Cell Sci 2000; 113:1727-36.
106. Walworth NC. DNA damage: Chk1 and Cdc25, more than meets the eye. Curr Opin Genet Dev 2001; 11:78-82.
107. Okumura E, Fukuhara T, Yoshida H, Hanada Si S, Kozutsumi R, Mori M, Tachibana K, KT. Akt inhibits Myt1 in the signalling pathway that leads to meiotic G2/M-phase transition. Nat Cell Biol 2002; 4:111-7.
108. Alvarez B, Martinez-A C, Burgering BM, Carrera AC. Forkhead transcription factors contribute to execution of the mitotic programme in mammals. Nature 2001; 413:744-7.

REGULATION OF P27^{KIP1}-MEDIATED CYCLIN D1-CDK4 ASSEMBLY BY PKB THROUGH MULTISITE PHOSPHORYLATION

Jiyong Liang[1], Shan H. Shao[1], Kathy Han[1], Joyce M. Slingerland[1,2*]
[1]Department of Medical Biophysics, University of Toronto, Molecular and Cellular Biology, Sunnybrook & Women's Health Sciences Centre, Toronto, ON M4N 3M5, Canada; [2]Braman Breast Cancer Institute, University of Miami Sylvester Comprehensive Cancer Center, 1475 N. W. 12th Ave (D8-4), Miami, FL 33136, USA

Progression through the G1 phase of the cell division cycle is a rate-limiting step in mammalian cell proliferation. This is driven by sequential activation of D-type and E-type cyclin dependent kinases (cdks) and requires the presence of mitogenic signals until the restriction point is passed [1] The accumulation of cyclin D1 in early G1 is up-regulated through mitogen-dependent increases in its transcription and translation and by post-translational modification leading to its stabilization [2,3] In G0 cells, cyclin D1 is unstable [4] and newly synthesized cellular cyclin D1 does not assemble into cyclin D1-cdk complexes [5]. The assembly of cyclin D1 and cdk4 appears dependent on mitogen signaling since exogenously over-expressed cyclin D1 cannot form complexes with cdk4 in serum-starved fibroblasts [6]. Although p21 and p27 were initially identified as members of the kinase inhibitor protein (KIP) family of cdk inhibitors, further studies showed that p21 and p27 also function to mediate cyclin D1-cdk4 and cdk6 assembly [7]. While acquisition of KIP assembly function is known to be dependent on mitogenic signals, mechanisms regulating cyclin D1-cdk4 assembly had not been fully elucidated.

We have shown that phosphorylation, subcellular localization and the assembly function of p27 are misregulated in TGF-β resistant human mammary epithelial cells [5,8]. We and others showed that PKB phosphorylates T157 of p27 and impairs nuclear import of p27 leading to its mislocalization in the cytoplasm[9].

The present study provides a new link between PKB activation and cell cycle regulation. In response to growth factor stimulation, the assembly of cyclin D1/cdk4/p27^{Kip1} in early G1 is preceded by PKB(Akt) activation. Inhibition of the PI3K/PKB pathway resulted in loss of p27 from cyclin D1 complexes, a shift of p27 to cyclin E-cdk2 both of which precede G1 arrest. Transfection of constitutively active PKB conferred resistance to TGF-βand increased cyclin D1-cdk4 complexes. The non-phosphorylatable mutant p27T157A showed reduced binding to cellular cyclin D1 and cdk4.

Two-dimensional phospho peptide mapping revealed that p27 from cyclin D1 immunoprecipitates differed significantly from that in cdk2 complexes. Cellular p27 from cyclin D1 complexes was more phosphorylated at S10, T157 and T198 and also mediated cyclin D1-cdk4 assembly more effectively *in vitro* than did p27 from cdk2 complexes. PKB-dependent phosphorylation of p27 *in vitro* increased the activity of p27 to mediate the assembly of recombinant cyclin D1 and cdk4. Phosphatase treatment of p27 abolished its assembly function. Taken together, our data suggest that while PKB-dependent phosphorylation leads to cytoplasmic mislocalization of p27, PKB also activates the assembly function of p27 toward cyclin D1 and cdk4 through sequential changes in p27 phosphorylation at multiples sites.

While T157 phosphorylation of p27 in early G1 would impair nuclear import of newly synthesized p27 [9] facilitating access of p27 to newly synthesized cyclin D1-cdk4 in the cytoplasm, cyclin D-cdk assembly occurs at a time when new p27 synthesis is dramatically reduced [10]. Indeed, new synthesis of p27 may be insufficient to support the assembly of rapidly synthesized and stabilized cyclin D1 required for the burst of cyclin D1-cdk4/6 activity that promotes G1 progression. S10 phosphorylation is required for p27 export from the nucleus [11-13]. Mitogen stimulated S10 phosphorylation promoting nuclear export of p27 may be required at the G0-G1 transition and in early G1 to provide sufficient cytoplasmic p27 to assemble and import newly

synthesized cyclin D1-cdk complexes. In cancers, constitutive PKB activation and subsequent changes in p27 phosphorylation may sequester p27 in cyclin D1-cdk complexes contributing to loss of the cyclin E-cdk2 inhibitory function of p27 and resistance to TGF-β.

1. Sherr,C.J. The Pezcoller lecture: cancer cell cycles revisited. *Cancer Res* **60**, 3689-3695 (2000).

2. Diehl,J.A., Zindy,F., & Sherr,C.J. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev.* **11**, 957-972 (1997).

3. Diehl,J.A., Cheng,M., Roussel,M.F., & Sherr,C.J. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.* **12**, 3499-3511 (1998).

4. Parry,D., Bates,S., Mann,D.J., & Peters,G. Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of p16INK4/MTS1 tumour suppressor gene product. *EMBO J.* **14**, 503-511 (1995).

5. Ciarallo,S., Subramaniam,V., Hung,W., Lee,J.H., Kotchetkov,R., Sandhu,C., Milic,A., & Slingerland,J.M. Altered p27(Kip1) phosphorylation, localization, and function in human epithelial cells resistant to transforming growth factor beta-mediated G(1) arrest. *Mol Cell Biol* **22**, 2993-3002 (2002).

6. Matsushime,H., Quelle,D.E., Shurtleff,S.A., Shibuya,M., Sherr,C.J., & Kato,J.Y. D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol* **14**, 2066-2076 (1994).

7. LaBaer,J., Garrett,M.D., Stevenson,L.F., Slingerland,J.M., Sandhu,C., Chou,H.S., Fattaey,A., & Harlow,E. New functional activities for the p21 family of CDK inhibitors. *Genes Dev.* **11**, 847-862 (1997).

8. Sandhu,C., Garbe,J., Daksis,J., Pan,C.H., Bhattacharya,N., Yaswen,P., Koh,J., Slingerland,J., & Stampfer,M.R. Transforming growth factor β stabilizes $p15^{INK4B}$ protein, increases $p15^{INK4B}$ -cdk4 complexes and inhibits cyclin D1/cdk4 association in human mammary epithelial cells. *Mol. Cell Biol.* **17**, 2458-2467 (1997).

9. Liang,J., Zubovitz,J., Petrocelli,T., Kotchetkov,R., Connor,M.K., Han,K., Lee,J.H., Ciarallo,S., Catzavelos,C., Beniston,R., Franssen,E., & Slingerland,J.M. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat. Med.* **8**, 1153-1160 (2002).

10. Hengst,L. & Reed,S.I. Translational control of p27Kip1 accumulation during the cell cycle. *Science* **271**, 1861-1864 (1996).

11. Rodier,G., Montagnoli,A., Di Marcotullio,L., Coulombe,P., Draetta,G.F., Pagano,M., & Meloche,S. p27 cytoplasmic localization is regulated by phosphorylation on Ser10 and is not a prerequisite for its proteolysis. *EMBO J* **20**, 6672-6682 (2001).

12. Ishida,N., Hara,T., Kamura,T., Yoshida,M., Nakayama,K., & Nakayama,K.I. Phosphorylation of $p27^{Kip1}$ on serine 10 is required for its binding to CRM1 and nuclear export. *J. Biol. Chem.* **277**, 14355-14358 (2002).

13. Connor,M.K., Kotchetkov,R., Cariou,S., Resch,A., Lupetti,R., Beniston,R.G., Melchior,F., Hengst,L., & Slingerland,J.M. CRM1/Ran-Mediated Nuclear Export of p27(Kip1) Involves a Nuclear Export Signal and Links p27 Export and Proteolysis. *Mol. Biol. Cell* **14**, 201-213 (2003).