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13. ABSTRACT ***(Maximum 200 Words)***

PI3K signaling is involved in many aspects of cell cycle progression and is often deregulated in malignant cells. Akt, a downstream kinase effector of PI3K, stabilizes cyclin D1 protein and phosphorylates the cdk inhibitor p21 preventing its anti-proliferative effects in breast cancer cells. In this report we attempt to identify PI3K-dependent molecules necessary to rescue cyclin A expression. We examined if loss of p27 together with forced expression of cyclin D1 was sufficient to rescue cyclin A expression in p27-null MEFs and demonstrated that S phase entry could not be restored by forced cyclin D1 expression. As it has been reported that the cyclin A promoter contains a cyclic AMP responsive element and that Akt phosphorylates Ser-133 of CREB in response to mitogens, we examined the phosphorylation state of CREB in the presence or absence of LY. No significant differences in CREB activation were observed suggesting additional transcription factors are required for PI3K effects on cyclin A transcription. Lastly, examination of the activation of cyclin A promoter in MEFs with constitutively active Akt or p70/S6 kinase suggests that constitutively active p70 is sufficient to rescue cyclin A promoter activity after inhibition of PI3K.

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INTRODUCTION

Cell proliferation occurs through transition from G1 to S phase in response to stimulation of mitogens and extracellular matrix. This event requires the coordinated activities of two cyclin-cdk complexes, cyclin D-cdk4/6 and cyclin E-cdk2. Their activation leads to phosphorylation of multiple residues of the pocket proteins pRb and p107 with consequent disruption of E2F-pocket protein complexes (1). Release of E2F allows the beginning of transcription of Rb-regulated genes, such as cyclin A, whose protein expression is required for S phase entry (2, 3, 4). Moreover the cdk inhibitors, p21 and p27, in early G1 phase are sequestered from cyclin E-cdk2 complexes to contribute to the assembly and the stability of cyclin D-cdk4/6 complexes, while their downregulation occurs only in mid-late G1 phase (5).

Tumorigenesis arises as a result of abnormal cell proliferation which is due to either loss of function of tumor suppressor genes or to the overexpression and/or mutation oncogenes. Cyclin D1 protein levels has been reported to be overexpressed in over 50% of human mammary carcinoma (6, 7). In particular cytogenetic studies of breast cancer cell lines and biopsies indicated that overexpression of cyclin D1 plays an important role in breast tumor oncogenesis although other genetic lesions might be required to confer full malignant phenotype (8).

The phoshatidylinositol 3- kinase (PI3K) signaling pathway is involved in many aspects of cell cycle progression. In particular the serine-threonine kinase Akt, one of the downstream effector of PI3K, has been reported to stabilize cyclin D1 protein level through phosphorylation of glycogen synthase kinase 3β(GSK)(9, 10). Moreover, recent reports show that in breast cancer cells overexpressing HER2-neu, Akt phosphorylates the cdk inhibitor p21 preventing its nuclear localization and anti-proliferative effects (11). Similarly, p27 phosphorylation by Akt results in its retention in the cytoplasm by binding with the scaffold protein 14.3.3.(12). In addition pharmacological inhibition of p70/S6 kinase, a serine-threonine kinase downstream in PI3K signaling involved in the translation of oligopyramidine-rich mRNAs, results in reduction of neoplastic proliferation and tumor size in animal models(13, 14). Lastly, the lipid phosphatase PTEN, a negative regulator of PI3K, has been reported to be mutated in high percentage of tumor (15, 16, 17). Linkage with PI3K signaling molecule has been shown in tumors derived from PTEN-null mice where Akt is consitutively active and restoration of PTEN expression restores Akt regulation (18, 19).

These results prompted us to investigate the role of PI3K signaling in cell cycle progression given its relevance in cyclin D1 expression and cdk inhibitors p21 and p27. We were specifically interested in identifying cell cycle molecules whose expression is regulated by PI3K and that are essential for cell cycle progression. We concluded our previous report showing that LY294002 (LY), a PI3K inhibitor, reduces cyclin D1 protein levels and completely blocks cyclin A expression in mouse embryo fibroblasts (MEFs). However, forced expression of cyclin D1 is not sufficient to rescue cyclin A expression in cells treated with LY. Moreover, we have shown that inhibition of PI3K leads to p27 accumulation and inhibition of cyclinE/cdk2 in a p27-dependent manner consistent with data reported in literature. Lastly, we reported that, while cyclin E-cdk2 activity persisted in p27-null MEFs treated with LY, cyclin A was not induced. This

result suggested that another PI3K-dependent pathway is require for cell cycle progression that is independent from the level of the cdk inhibitor p27.

In this annual summary report we decided to pursue two lines of investigation in order to identify molecules necessary to rescue cyclin A expression and consequent S phase entry. We examined if forced expression of cyclin D1 was sufficient to rescue cyclin A expression in p27-null MEFs. However we still were unable to restore S phase entry. Given the multiplicity of roles played by Akt in cell cycle progression we decided to transiently overexpress myristoylated Akt (the active form of Akt) in MEFs in the presence of LY. Although we were able to detect an effect of Akt on p27 in LY treated cells, still we did not rescue cyclin A expression. It has been reported that cyclin A promoter contains a cyclic AMP responsive element (CRE) and, in addition to that, Akt phosphorylates Ser-133 of CREB (20, 21) in response to mitogenic stimulation. Therefore we want to look at the phosphorylation state of CREB in the presence or absence of LY. Our study shows no significant differences in CREB activation. In addition to examining the rescue of cyclin A at a protein level we also focus on the activation of its promoter in MEFs transfected with either constitutively active Akt or p70/S6 kinase. Our preliminary data suggest that a constitutively active form of p70 might be sufficient to rescue cyclin A promoter activity in the presence of LY.

From this study we can conclude that PI3K signaling is controlling the transition of cell cycle entry from G1 to S phase, primarily by modulating p27 levels and stability of cyclin D1. In addition our preliminary data show a novel function of p70/S6 kinase whose activity seems to be play a major role in cell cycle progression and enter in S phase likely through mTOR pathway.

BODY OF WORK

Experimental procedures

Reagents.
Wild type and p27-null mouse embryo fibroblasts (MEFs) overexpressing cyclin D1 under a Tetracycline(Tet)-regulated promoter were generated in our laboratory. p27-null MEFs were the gift of Jim Roberts. Rabbit polyclonal antibody against cyclin A was prepared in this laboratory . All other antibodies were purchased: anti-cdk4 (sc-260), and anti-cyclin D1 (sc-8396) from Santa Cruz Biotechnology, anti-cdk2 (06-505) and anti-phospho-CREB (#06-519) from UBI, anti-CREB (#9192) from NEB, anti-p27 (K25020) from Transduction Laboratory, and anti-actin from Chemicon. The p322/cyclin A promoter-firefly luciferase construct was a gift of Christian Bréchot, plasmids containing Myristoylated Akt or constitutively active p70/S6 kinase constructs were a kind gift of Margaret Chou. LY294002 (LY) was purchased form Calbiochem.

Cell Culture and Transfection.
Wild type or p27-null MEFs were stably transfected with the mouse cyclin D1 cDNA, subcloned into a Tet-operator vector, together with a plasmid containing the transactivator molecule to the Tet-promoter. Stable transfectants were selected in the presence of geneticin and hygromycin. Tet was added to growth media at 2 μg/ml to avoid ectopic expression of cyclin D1 during culturing. MEFs and all derivative cell lines were grown in DMEM + 10% FCS. For G0-synchronization, cells were brought to confluence in 150-mm dishes, washed once with serum-free DMEM, and then cultured in 20 ml serum-free DMEM + 1 mg/ml BSA for 48 hours. To stimulate entry into the cell cycle, G0-synchronized cells were trypsinized, and reseeded in tissue culture dishes in the presence of 10% FCS. In the case of MEFs overexpressing cyclin D1, cells were starved and then stimulated in the presence or absence of Tetracycline. In experiments with LY, the inhibitor was added at the time of serum-stimulation at a final concentration of 25 μM. For all experiments, cells were washed 2-3 times with PBS, collected by scraping and extracted for immunoblotting, or immunoprecipitation and kinase assay.

Extractions for immunoblotting, immunoprecipitation, and kinase assay.
Cells were collected and lysed in 50 ul TNE (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 2 mM EDTA, 1% NP-40, 10 ug/ml leupeptin, 10 ug/ml aprotinin, 1 mM PMSF, 50 mM sodium fluoride and 10 mM sodium orthovanadate) and analyzed by immunoblotting using enhanced chemiluminescence. Protein concentrations were determined by Coomassie binding (Bio-Rad Protein Assay). Equal amounts of protein from each cell lysate were fractionated on reducing SDS-gels containing 12% acrylamide and then analyzed by immunoblotting. For cdk4 and cdk2 immunoprecipitations, cell lysates (500 ug) were brought to 200 μl with TNE and incubated (1 h at 4°C with rocking) with ~5 μg of antibody. 25 μl of the immunocomplexes were then processed for kinase assay

while the rest were used for western blot analysis. The volume of all samples were brought to 200 µl with TNE and the immunocomplexes were collected (1 h, 4°C with rocking) with 50 µl anti-mouse IgG-agarose (Sigma). Collected immunoprecipitates were washed twice with cold TNE and suspended in 100 µl SDS-sample buffer, fractionated on reducing SDS-gels containing 12% acrylamide and analyzed by immunoblotting with cdk2, cdk4 and p27 antisera. For kinase assay, samples were further washed and resuspended in 30 µl of the kinase buffer (50 mM Tris-HCl pH 7.5 and 10mM $MgCl_2$). The kinase reaction was performed at 30^0 C for 30' using histone H1 as a substrate (1 µg), ^{32}P-γATP (10 µCi) and cold ATP (25 µM). SDS-sample buffer was added to stop the reaction and samples were boiled and resolved into a 12% acrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membrane and then exposed to X-ray film.

Transcriptional Reporter assay.

The cyclin A promoter-firefly luciferase (called luciferase) construct p322/cycA-luc (1.3-kb promoter) contains bases 1 to 1,293 of the human cyclin A promoter upstream of the luciferase reporter (22). Transient transfections of MEFs cells with promoter luciferase vectors were performed in 35-mm dishes by using 1 µg of cyclin A promoter-luciferase plasmid, 1 µg of Myristoylated Akt or 1 µg of activated p70 plasmid, and 0.1 µg of a *Renilla* luciferase expression plasmid (pRL-SV40 Promega) to normalize for transfection efficiency. After an overnight recovery, the cells were synchronized in G_0 by incubation in serum-free DMEM for 48h. The G_0-synchronized transfectants were trypsinized, reseeded at subconfluence in 35-mm dishes (~10^5 cells in 2 ml), and then stimulated with 10% FCS in the absence or presence of 25 µM LY. Cells were washed with phosphate-buffered saline, collected, lysed, and analyzed for luciferase and *Renilla* luciferase activity by using the Dual-Luciferase reporter assay system (Promega). Cyclin A promoter-driven luciferase activity was then normalized to a constant activity of *Renilla* luciferase.

Results

In our previous reports we showed that inhibition of PI3K signaling leads to blockage of cyclin A expression in serum-stimulated MEFs. This effect is a result of the lack of phosphorylation of cdk2 and consequent inactivation of cyclinE/cdk2 complex. However, in our cell based model it was not clear if the inactivation of cdk2 was due to an increase of p27 levels in the protein complex. Therefore, we inhibited PI3K with LY in p27-null MEFs and showed that activation of cdk2 by phosphorylation and its kinase activity was partially rescued. This data suggested that accumulation of p27 may block the access of cyclin activating kinase (CAK) in the complex to phosphorylate and thus activate cdk2.

In this current grant year we determined if forced expression of cyclin D1, using a Tet-regulated model in p27-null MEFs, was sufficient to rescue cell cycle entry. We synchronized p27-null MEFs, stably transfected with cyclin D1 under a tetracycline (Tet) regulated promoter, by 48 hours starvation in the presence or absence of Tet. Cells were then reseeded and stimulated with 10% FCS for the time indicated below in the presence or absence or of LY. The experiment turned out technically difficult because LY inhibited the level of expression of the exogenous cyclin D1. However, we were able to almost completely rescue cdk2 phosphorylation, as shown by the presence of phosphorylated cdk2 (P-cdk2), even in the LY treated samples but we did not rescue cyclin A expression (Fig. 1).

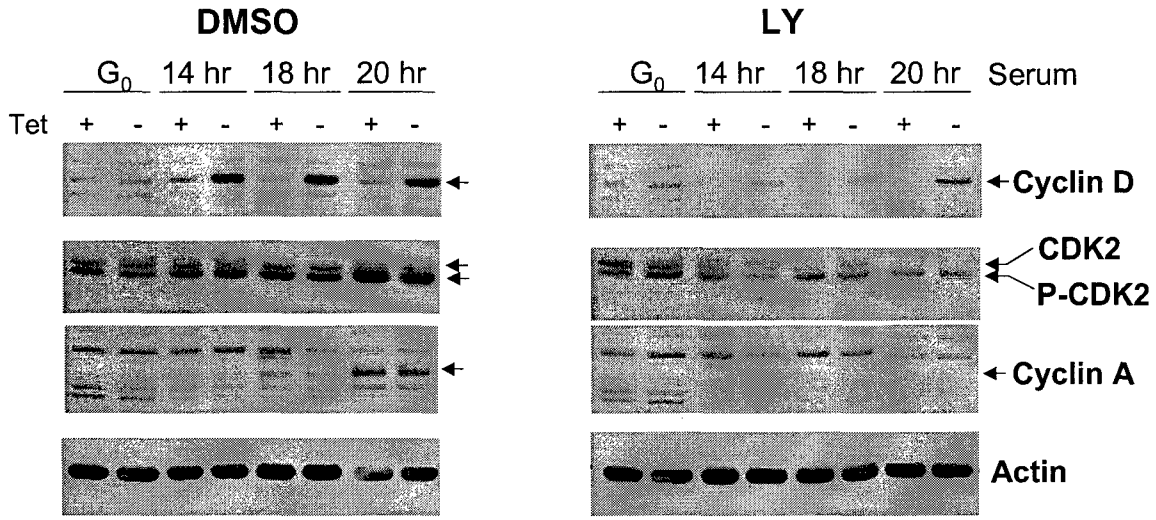

Figure 1. ***Cyclin A induction is not rescued in p27-null Tet D1 MEFs.*** *Western blot analysis of serum-stimulated p27-null Tet D1 MEFs in the presence or absence of Tet and LY. Protein samples were collected after serum-stimulation at the time indicated and fractioned on a 12% acrylamide reducing SDS-gel. Western blot analysis was performed to examine the level of CyclinA, D1 and cdk2. Note the phosphorylated form of cdks (P-cdk2) has a greater mobility. Actin was used as a loading control.*

In order to investigate when Cyclin D1 expression is independent of PI3K, we repeated a similar experiment adding LY at different times after serum stimulation. Consistent with literature observations (23), we were able to rescue expression of exogenous cyclin D1 between 6 and 8 hours following addition of serum to the same level of the control cells (Fig. 2 panel A). However, lack of p27 and rescue of cyclin D1 were not sufficient to allow cyclin A protein expression (Fig. 2 panel B) suggesting that another PI3K dependent pathway is required for cell cycle progression.

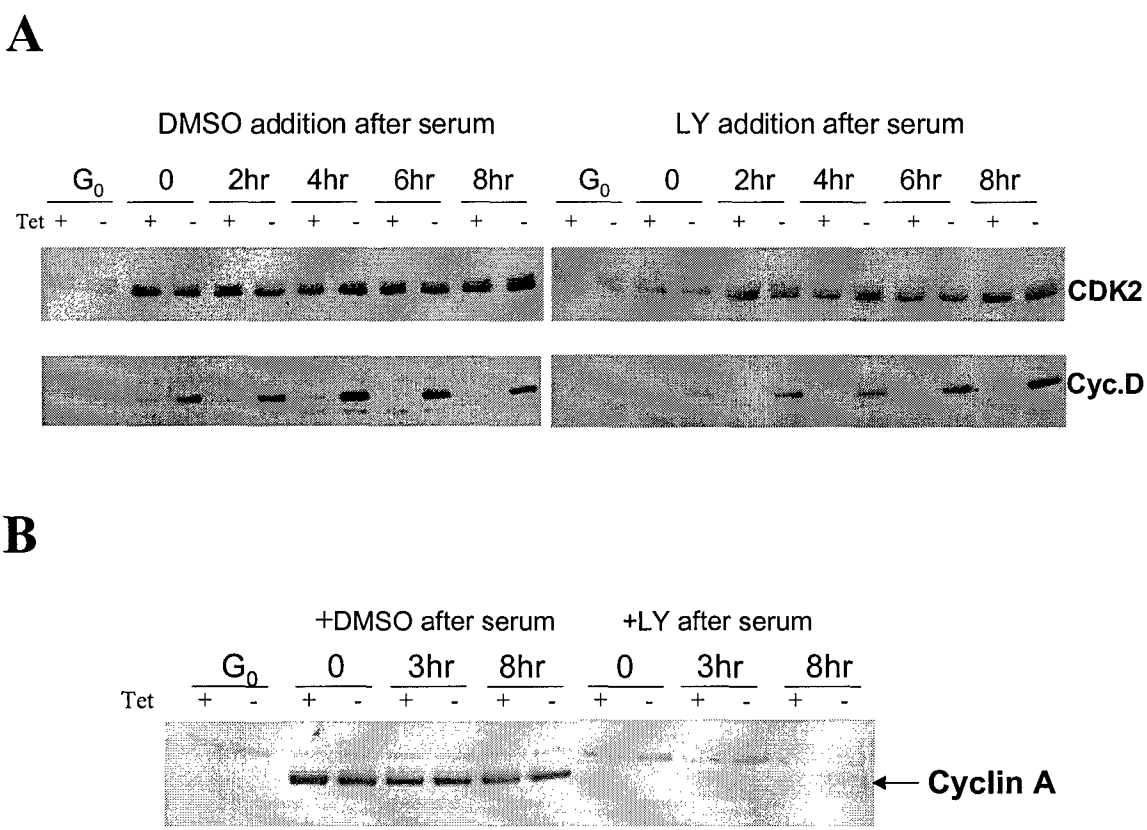

Figure 2. ***Rescue of exogenous cyclin D1 in p27-null Tet D1 MEFs by addition of LY at diferent time after serum stimulation.*** *MEFs were processed as described above Fig.1 with the only difference that LY inhibitor was added at different time after release from starvation at a final concentration of 25 μM. The time indicate the interval between serum stimulation and LY addition. Cell samples were all collected and lysed after 18 hours from addition of serum and processed for Western Blot analysis.*

We were also interested in analyzing the activation state of cyclin A promoter and particularly how PI3K signaling was regulating transcription of cyclin A gene. It has been reported that cyclin A promoter contains a cyclic AMP responsive element (CRE) and that phosphorylation of CREB by Akt is required in order to activate gene transcription.

Therefore, we next examined the phosphorylation state of CREB in the presence or absence of LY in MEFs and p27-null MEFs. As shown in Fig. 3, we were unable to detect significant differences in CREB activation not only between LY–tretaed cells and control cells but also between the wild type MEFs and p27-null MEFs. These observations suggest that PI3K induced transcription of the cyclin A promoter requires additional transcriptional responses.

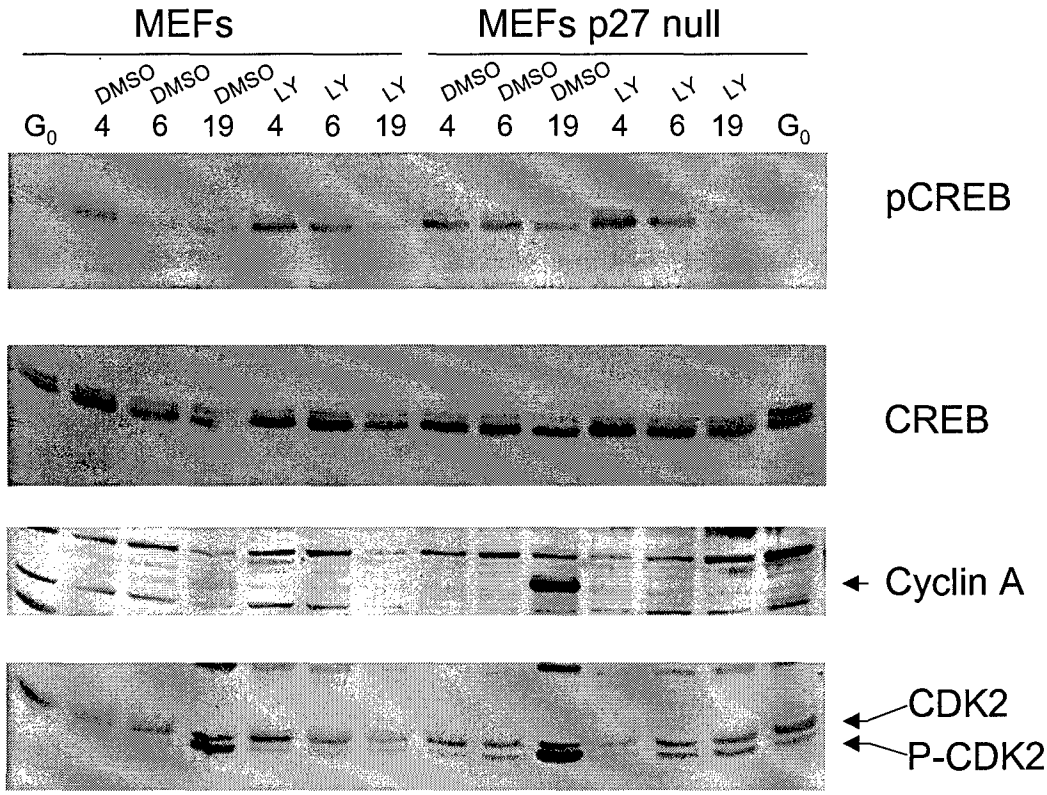

Figure 3. ***Effect of LY on the phosphorylation of CREB in MEFs and p27-null MEFs.*** *Western Blot analysis of cells samples collected 19 hours of serum stimulation after 48 hours of starvation. Lysates were resolved on 10% reducing acylamide gel. Note the peak of phosphorylation of CREB is between 4 and 6 hours and is independent by p27 and treatment with 25 μM LY.*

Lastly we want to use a more sensitive assay to investigate the activation of cyclin A promoter. Our question was whether downstream effector molecules of PI3K signaling involved in cell cycle regulation and proliferation such as Akt or p70/S6 kinase would rescue transcription of cyclin A gene by overriding the blockage of S phase entry due to LY-treatment. We transiently transfected MEFs with the cyclin A-luciferase reporter construct together with either an activated form of Akt or p70/S6 kinase. After recovery from the transfection, cells were serum-starved for 48 hours and reseeded with serum in

the absence or presence of LY. Cells were collected and lysed after 18 hours serum-stimulation and processed for luciferase counts. Luciferase activity was normalized to *Renilla* luciferase which was used as an internal control (Fig.4).

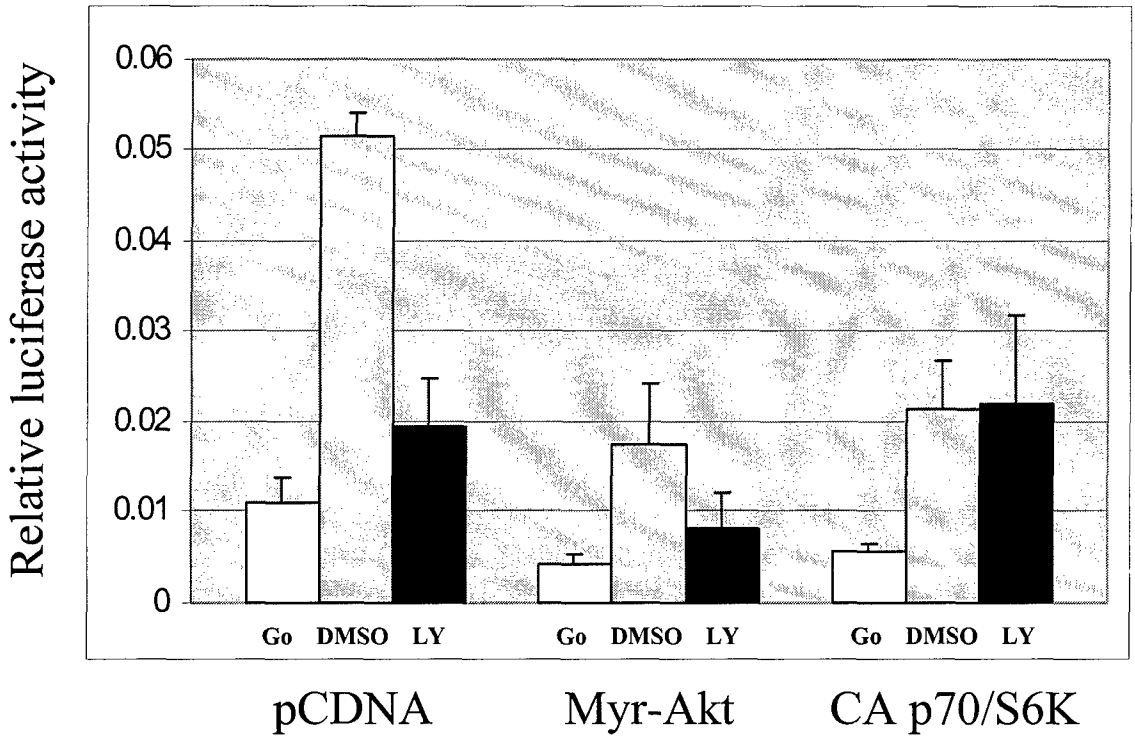

Figure 4. ***Reporter assay to see the effects of either activated Akt or p70 on cyclin A gene transcription in the absence or presence of LY.*** *MEFs were transiently transfected with 1 μg of the cyclin A promoter-luciferase construct, 100 ng of Renilla luciferase and 1 μg of pCDNA(as control), Myr- Akt or constitutively active(CA) p70/S6 kinase. After recover from transfection cells were G_0-synchronized and serum-stimulated for 18 hours. Samples were collected and processed for luciferase measurement. Luciferase activity was plotted as normalized value to a constant activity of Renilla luciferase. Results show mean ± SD of triplicates.*

Our preliminary data indicate that an activated form of p70 might override the blockage of LY and with consequent activation of cyclin A gene transcription required for the transition from G1 to S phase. Although the mechanism is not clear, recent reports underscore the relevance of p70 as an important mediator of cell proliferation through the mTOR pathway. Particularly it has been shown that constitutively form of p70 contribute to tumor formation suggesting that this molecule acts as an important mediator of cell cycle progression.

Conclusions

The current annual summary report focuses on defining the signaling pathways affecting S phase entry with particular emphasis on the role of PI3K and its downstream targets, whose aberrant signaling are implicated in tumorigenesis. The specific conclusions of our work are three-fold. Firstly, cyclin D1 expression together with the loss of the cdk inhibitor, p27, is not sufficient to induce cyclin A expression in MEFs. It will be important in this regard to investigate the activity of cyclin D1/CDK4 complex in this system to fully support the hypothesis that the observed cyclin D1 levels reflect the enzymatic activity of the complex. Secondly, CREB phosphorylation state was unaltered by blockage of PI3K signaling by LY. Thirdly, p70, a kinase involved in regulation of mRNA translation, appears to override LY effects on S phase entry in a reporter assay. This latter conclusion is consistent with recent literature reports suggesting that p70 signals downstream of the mTOR pathway, both signaling molecules implicated in cell proliferation as well as tumor progression (14).

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APPENDIX

- Cyclin A induction is not rescued in p27-null Tet D1 MEFs.
- Rescue of exogenous cyclin D1 but not cyclin A in p27-null Tet D1 MEFs.
- No difference in CREB phosphorylation upon LY treatment in either p27-null or parental MEFs.
- Rescue of cyclin A gene transcription by activated p70 in the presence of LY.

Reported outcomes:

a) manuscripts: none

b) abstracts/presentations: none

c) patents and licenses applied for: none

d) degrees obtained: none

e) development of cell lines: none

f) funding applied for: none

g) employment opportunities applied for: none

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