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14. ABSTRACT

One of the hallmarks of cancer is the deregulation of the cell proliferation. This deregulation promotes genetic errors that contribute to genomic instability. Our hypothesis is that Cdk2 exists in two freely exchangeable conformations: that seen in the active, cyclin—bound crystal and that of the inactive monomeric Cdk2, with the latter predominating in the absence of cyclin. We propose that phosphorylation of Cdk2T39 shifts the equilibrium in the direction of the active conformation that best fits cyclin and therefore facilitating cyclin binding, G_1 progression and initiation of DNA synthesis. We will test this hypothesis by treating recombinant cdk2 with AKT and sending it for mass spectroscopy so we can determine if Cdk2 is indeed phosphorylated by AKT. We will also determine the effect of AKT phosphorylation on Cdk2 by constructing a phosphomimetic mutant of Cdk2 and determining if this has an effect on cyclin binding and G_1 progression. Ultimately, this research may elucidate a novel method of cell cycle control through which mitogenic signals may influence the cell cycle.

15. SUBJECT TERMS
Cell Cycle, Cdk2, AKT, Phosphorylation, Cyclin

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Table of Contents

Page

INTODUCTION

CDK2 is one of the regulators of G_1 to S phase in mammals. Recent work has shown that $CDK2^{-/-}$ animals are viable[1], however it has also been shown that a dominant negative form of CDK2 is capable of halting cell cycle progression[2]. There is abundant evidence for aberrant activation of CDK2 in human carcinogenesis or progression[3-5]. CDK2 gene amplification and CDK2 overexpression and activation is observed in primary colorectal[6,7], lung[8] and ovarian carcinoma[9]. However, there are no known malignancies caused by CDK2 mutations. It has also been shown that overexpression of Cdc25 occurs often in human cancers[10] and recent studies indicate that restricting CDC25A can limit tumorigenesis induced by the HER2/neu-RAS oncogenic pathway without compromising normal cell division or viability[11]. Thus, the faithful maintenance of CDK2 activity is of importance for proper cell cycle progression. Two of the upstream signaling pathways controlling proper cell cycle progression – the Ras/RAF/MEK/MAPK and the PI3K/PKB pathways - are often activated in cancers [12]. Our preliminary data supports a scenario where AKT may phosphorylate CDK2 and influence its conformational structure and therefore its association with cyclin and the timing of S phase entry. Larochelle et al have been working on the kinetics of CDK2 /cyclin binding. They have shown that the mammalian CAK (CDK7) can phosphorylate CDK2 as a monomer whereas CAK requires a CDK1 heterodimer in order to act on it[13]. These recent observations may add another level of complexity to cell cycle regulation but we believe that they dovetail nicely with previously published data and our preliminary data. The difference in kinetics of CAK for CDK2 and CDK1 may be due in part to AKT activity since there is a peak in fully active, S473 phsphorylated, AKT early in G1. This bump in activity may be required to properly prime CDK2 through phosphorylation on T39. This phosphorylation in term may allow the phosphorylation of monomeric CDK2 at T160. This phosphorylation is then stabilized by cyclin binding.

BODY

The past year has been dedicated to obtaining all the required tools with which to complete all the aims outlined in the proposal. We have cloned CDK2 in a thioredoxin-tagged vector so as to increase its solubility. We have had some problems with the bacterial expression of CDK2 as this protein in rather insoluble when expressed in a prokaryotic system. This has also been the case in the past and techniques for its purification have been previously described[14] (see appendix). We reasoned it may be better to use a new system in order to determine if we could achieve greater solubility than previously recorded. At the very least, we were able to achieve the same yield using a thioredoxin tagged CDK2 (cloned into a Pet 102 vector) than previously described using a GST-tag expression and purification system. We have also established the best possible condition for thrombin cleavage. Here too, we have run into difficulties as the purchased thrombin (Calbiochem) seems to have proteolytic activity to CDK2 despite the lack of a cleavage site within the protein sequence. Figure 1 shows that we obtain the best possible cleavage of CDK2 without evident endoproteolytic activity by using 3.2 Units of thrombin per milligram of purified protein.

Previous assays using uncleaved GST-tagged CDK2 (data not shown) were not successful as were kinase assays with uncleaved thioredoxin (figure 2) since the former tag inhibits the kinase activity and the latter is a substrate for AKT (despite the lack of any clear AKT substrate motifs). Therefore, we were left without any preliminary data to report before we pass our cleaved CDK2 through a size exclusion column and separate it form the tag and the thrombin. We are just now ready to perform this task. I expect to be done with my part and send the appropriate amount of radiolabeled protein to Dr Busby at the Scripps Institute for mass spectroscopy by the end of November, 2007.

For our aim II we proposed to test the effect of pT39-CDK2 on CDK2 activity in MCF-7 cells. However in order to avoid direct competition for cyclin partners between both the endogenous CDK2 and the ectopically expressed CDK2, we decided to transfect HA tagged CDK2 vectors into CDK2 null MEF's. It has been shown that interphase cyclins can bind to S phase CDKs [15-17] (see appendix). Our hope, however, is that this interaction

only occurs in the absence of interphase cyclins and that re-expression of CDK2 in a CDK2 null background will give a greater number of HA-tagged CDK/cyclin complexes we can immunoprecipitate. We have successfully transformed MEF$^{CDK2-/-}$ cells with HA tagged CDK2 (figure3). Our initial experiments have been very favorable. They indicate, as we have proposed, that there is an increase in cyclin association with the phosphomimetic CDK2T39E when compared to CDK2wt and CDK2T39A. We have yet to test the effect of theCDK2R36A mutation and the effect of LY treatment on cyclin association to CDK2 wt vs. CDK2T39E.

In our aim III, we proposed to test the effects of CDK2T39 phosphorylation on cell cycle by using a MEFCDK2^{flox} cells obtained from Dr Barbacid. In our hands, these cells did not behave optimmaly and we were unable to serum starve these cells without at least 90% cell death. Beyond this, the surviving cells did not appear to be well synchronized and we were not able to observe any difference in cell cycle progression between the control cells and the Cre infected (and CDK2 knockout) cells. Previous publications have indicated, using primary MEF cells, that this experiment is feasible[15]. Berthet et. al. showed that the CDK2$^{flox/flox}$ cells entered S phase 14 hours after release and that CDK2$^{flox/flox}$ cell transduced with adenovirus encoding cre recombinase had a 4 hour lag in S phase entry (see appendix). However, we were unable to achieve similar results using immortalized MEF cells (Figure 5). Therefore, we are planning on using a different model organism to accomplish this aim. We will try to observe any difference in S-phase entry using budding yeast as the model organism. The Yeast CDK2 homologue – cdc28, does contain the same AKT substrate motif and since there are no other interphase CDK's this organism would be ideal to study the effect of mutations of this site on cell cycle progression (Figure6).

KEY RESEARCH ACCOMPLISHMENTS

- Determined that thioredoxin-tagged CDK2 cannot be used as a substrate in an AKT kinase assay.

- Showed differential *in-vivo* association of Cyclin A for CDK2wt, CDK2T39A and CDK2T39E
- Determined that immortalized CDK2$^{flox/flox}$ cell line is not a viable model system with which to study cell cycle progression in the presence of episomally expressed wt or mutant CDK2.

REPORTABLE OUTCOMES

- Constructed BL21(DE3) pET102CDK2wt cell for recombinant protein expression
- Constructed BL21(DE3) pET102CDK2T39A cell for recombinant protein expression
- Constructed BL21(DE3) pET102CDK2T39E cell for recombinant protein expression
- Constructed BL21(DE3) pET102CDK2R36A cell for recombinant protein expression
- Expressed recombinant CDK2
- Determined optimal thrombin concentration for thioredoxin tag cleavage
- Constructed MEF CDK2$^{-/-}$HACDK2wt Cell line
- Constructed MEF CDK2$^{-/-}$HACDK2T39A Cell line
- Constructed MEF CDK2$^{-/-}$HACDK2T39E Cell line
- Constructed MEF CDK2$^{-/-}$HACDK2R36A Cell line
- Immunoprecipitated HACDK2 from transformed MEF cells.
- I was able to completely knock out CDK2$^{flox/flox}$ using adenovirus encoding Cre recombinase

CONCLUSION

We have been able to show, as proposed by the use of a phosphomimetic mutant, that phosphorylation of CDK2T39 has an effect on cyclin association. We have yet to definitively prove that it is CDK2T39 that is phosphorylated by AKT and that it is indeed AKT that phosphorylates this site *in-vivo*. We also have determined that we will need another model system to study the effect of phosphorylation of CDK2T39 on the cell cycle. We think that the budding yeast may prove useful in this task. The data that we have to date does support our hypothesis and it may indicate a novel method through which cyclin dependent kinases may be regulated by mitogenic signaling. This is important, especially in the context of cancer, because these signaling pathways are commonly over activated in transformed cells.

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APPENDICES

SUPPORTING DATA

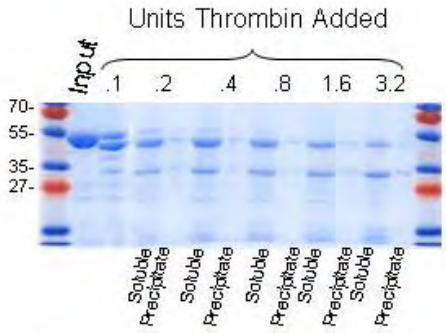

Figure1: purification and cleavage of cdk2. Purified Cdk2 was cleaved using different amounts of Thrombin to determine optimal thrombin concentration required for cleavage. Amount of thrombin added are stated in units of thrombin per mg of recombinant protein

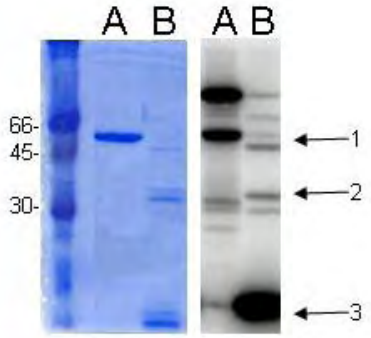

Figure 2: Kinase assay of recombinant Cdk2. Thioredoxin-tagged Cdk2 was used in a kinase assay Using 100ng recombinant AKT. The thioredoxin tag seems to act as a substrate of AKT (1 and 3). A) Un-cleaved recombinant Cdk2. B) Trombin Celaved Cdk2. 1) Thioredoxin tagged cdk2. 2) Thrombin cleaved Cdk2. 3) Thioredoxin

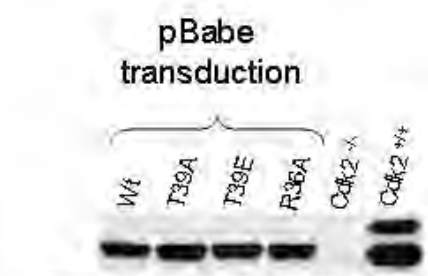

Figure 3: Transduction of Cdk2$^{-/-}$ MEF cells with pBabe Cdk2 MEF cells were transdced with pBabe Cdk2 wt, T39A, T39E and R36A. Lysates were immunoprobed for Cdk2. Lysates from Cdk2$^{-/-}$ and Cdk2$^{+/+}$ cells were used as controls

Figure 4: IP blot of pBabe Cdk2 transduced Cdk2$^{-/-}$ MEF's expressing Cdk2wt, Cdk2T39A and Cdk2T39E. 800ug of lysate were used per lane. Cyclin A is better coimmunoprecipitated with T39E than with T39A or wt Cdk2

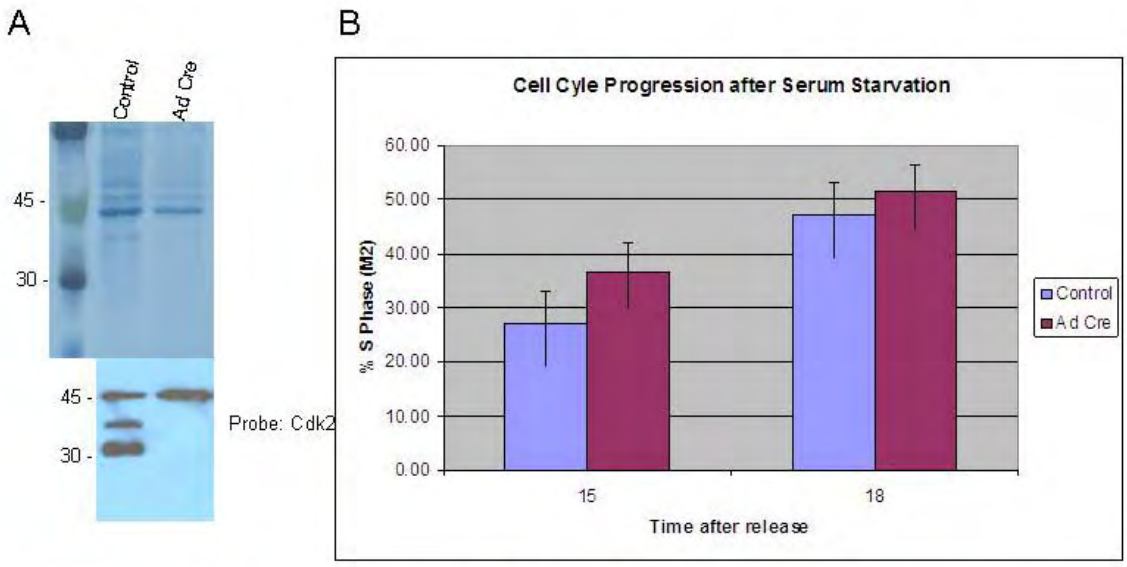

Figure 5: Cell cycle synchrony of Cdk2 knockout MEF. $Cdk2^{flox/flox}$ MEF cells were serum starved and transduced wiith adenovirus containing Cre recombinase. After 72 Hrs, cells were released by addition of serum rich media. There was no noticeable difference in cell cycle progression between control cells and Cre transduced cells (should be $Cdk2^{-/-}$) A) western blot of $Cdk2^{flox/flox}$ cells 72 hours after Ad Cre transduction. B) Percent of cells in S phase 15 and 18 hours after release from quiescence.

Human	Cdk2	21	ARNKLTGE-VVALKKIRLDTETEGVPSTAIRE	51
Mouse	Cdk2	21	AKNKLTGE-VVALKKIRLDTETEGVPSTAIRE	51
Rat	Cdk2	21	AKNKLTGE-VVALKKIRLDTETEGVPSTAIRE	51
Sea Urchin	Cdk2	21	AKNKKSGK-TVALKKIRLDTESEGVPSTAIRE	51
Human	Cdk1	21	GRHKTTGQ-VVAMKKIRLESEEEGVPSTAIRE	51
Yeast (sc)	Cdk1	27	ALDLRPGQGVVALKKIRLESEDEGVPSTAIRE	58
Yeast (sp)	Cdk1	21	ARHK-LSQRIVAMKKIRLEDESEGVPSTAIRE	51

Figure 6: Cdk2 sequence homolgy among eukaryotes. Notce that the AKT consensus motif K/RXRXXS/T is conserved