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TITLE: CDK2 Phosphorylation on Threonine39 by AKT and Its Implication on Cyclin Binding, Cellular Localization, and Cell Cycle Progression

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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, G1 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 G1 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>
<thead>
<tr>
<th>a. REPORT</th>
<th>b. ABSTRACT</th>
<th>c. THIS PAGE</th>
</tr>
</thead>
<tbody>
<tr>
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<td>U</td>
<td>U</td>
</tr>
</tbody>
</table>

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19b. TELEPHONE NUMBER (include area code)
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Body</td>
<td>3</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>4</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>5</td>
</tr>
<tr>
<td>Conclusion</td>
<td>6</td>
</tr>
<tr>
<td>References</td>
<td>6</td>
</tr>
<tr>
<td>Appendices</td>
<td>8</td>
</tr>
</tbody>
</table>
INTRODUCTION

CDK2 is one of the regulators of G1 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 phosphorylated, AKT early in G1. This bump in activity may be required to properly prime CDK2 through phosphorylation on T39. This phosphorylation in turn may allow the phosphorylation of monomeric CDK2 at T160. This phosphorylation is then stabilized by cyclin binding.
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 is 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 from 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 transfected 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<sup>CDK2/-</sup> cells with HA
tagged CDK2 (figure 3). 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 the CDK2R36A 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<sup>flox</sup> cells obtained from Dr Barbacid. In our hands, these cells did
not behave optimally 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<sup>15</sup>. Berthet et. al.
showed that the CDK2<sup>flox/flox</sup> cells entered S phase 14 hours after release and that
CDK2<sup>flox/flox</sup> 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 (Figure 6).

**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\textsuperscript{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\textsuperscript{+/-}HACDK2wt Cell line
- Constructed MEF CDK2\textsuperscript{+/-}HACDK2T39A Cell line
- Constructed MEF CDK2\textsuperscript{+/-}HACDK2T39E Cell line
- Constructed MEF CDK2\textsuperscript{+/-}HACDK2R36A Cell line
- Immunoprecipitated HACDK2 from transformed MEF cells.
- I was able to completely knock out CDK2\textsuperscript{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 \textit{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.

REFERENCES


APPENDICES

SUPPORTING DATA

Figure 1: 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) Thrombin Cleaved Cdk2. 1) Thioredoxin tagged Cdk2. 2) Thrombin cleaved Cdk2. 3) Thioredoxin.
Figure 3: Transduction of Cdk2−/− MEF cells with pBabe Cdk2 MEF cells were transduced with pBabe Cdk2 wt, T39A, T39E and R36A. Lysates were immunoblotted 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\textsuperscript{-/-} MEF cells were serum starved and transduced with 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\textsuperscript{-/-}). A) western blot of Cdk2\textsuperscript{-/-} cells 72 hours after Ad Cre transduction. B) Percent of cells in S phase 15 and 16 hours after release from quiescence.

Figure 6. Cdk2 sequence homology among eukaryotes. Notice that the AKT consensus motif\textsuperscript{RXRXXS/T} is conserved.