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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

This project is based on our discovery that cyclin dependent kinase 6 (CDK6) can markedly stimulate the transcriptional activity of the androgen receptor (AR) in human prostate cancer cells. The research performed during the past year has confirmed and extended these findings and provided insights into the underlying mechanisms. Thus, we found that CDK6 can physically associate with the AR *in vivo*. Using a series of truncated and mutant forms of CDK6 that we constructed we obtained evidence that the cyclin D1 binding domain and the ATP binding site in CDK6 are not essential for the stimulatory effect of CDK6 on the AR. These results, taken together with additional findings, indicate that CDK6 can bind to and directly stimulate the activity of the AR via a cyclin D1 and kinase independent mechanism. Therefore, in addition to its role in cell cycle control, CDK6 may play an independent role in modulating the activity of the AR. Our findings may have clinical significance since they suggest that variations in the level of expression of CDK in human prostate cancers might influence their growth properties and response to anti-androgen therapy.

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Introduction :

Prostate cancer is currently the most frequently diagnosed cancer and the second leading cause of cancer deaths in men in the United States. In 1999 about 179,300 new cases were diagnosed and 37,000 men died from this disease (*1*). Furthermore, prostate cancer incidence is expected to double by the year 2030 (*2*). Despite the magnitude of this disease, the precise exogenous causative factors and the pathogenesis of this disease at the cellular and molecular levels are poorly understood. The Androgen Receptor (AR) plays a critical role in the development of prostate cancer, and abnormalities in the AR and/or in AR-mediated signaling pathways play an important role in the progression of this disease, and in responses to therapy. We have focused on proteins that regulate cell cycle progression because it is becoming increasingly apparent that abnormalities in the expression of these proteins often occurs during the multistage carcinogenic process (*3*). It is known that in vivo cyclin D1 binds to CDK4 or CDK6, thus activating their kinase activities, to enhance the G1 to S progression of the cell cycle progression (*3*).

During the course of our studies on possible effects of these cell cycle control proteins on AR mediated functions we discovered an unexpected stimulatory effect of CDK6 itself. Our assay system utilized the AR negative PC3 human prostate cancer cell line for transient transfection reporter assays, employing either probasin or prostate specific antigen (PSA) promoter-luciferase reporter constructs (*4*), in which these promoters contain AR responsive elements (ARE). We found that co-transfection with a plasmid encoding a full-length CDK6 plus a plasmid encoding the AR markedly stimulated transcription from either of these reporters when the cells were also treated with dihydrotestosterone (20 nM DHT). However, similar co-transfection assays with CDKs 1, 2 or 4 did not stimulate this activity. The stimulatory effect required transfection with the AR, since PC3 cells do not express the AR, and the addition of DHT to activate the AR. These results are displayed in Figure 1. Using PSA promoter reporter assays, we found that CDK6 also markedly stimulated the transcriptional activity of the

endogenous AR present in the LNCaP human prostate cancer cell line (data not shown). Taken together, these results provided the basis for this project.

Body :

Research accomplished on specific tasks during year one :

Task 1a. Determine whether *in vivo* CDK6 can physically associate with the androgen receptor, independent of cyclin D1.

HA-tagged CDK6 and FLAG-tagged-AR cDNA were co-transfected and transiently overexpressed in 293T cells, using standard protocols (Gibco). These cells were chosen since they have a low concentration of cyclin D1 and therefore would have higher levels of unbound CDK6. After 36 hours, the cells were lysed in 300 µl of E1A Lysis Buffer [250 mM NaCl, 50 mM Hepes pH 7.0, 5 mM EDTA, 0.1% NP-40]. Two micrograms of anti-CDK6 (Neomarker) or anti-HA (Pharmacia) antibodies were added to 500 µg of the cell lysate and immunoprecipitates allowed to form for 60 min at 4°C. Twenty microliters of swollen protein A-Sepharose beads (Pharmacia) was added for an additional 30 min, the beads were washed four times with 1 ml of E1A lysis buffer, and the bound proteins separated on a denaturing 10% polyacrylamide gel by electrophoresis (PAGE). The blots was assessed with an anti-Flag antibody to detect coimmunoprecipitated FLAG-AR protein. Likewise, two micrograms of anti-AR (Pharmingen) antibody were used in co-immunoprecipitation studies to determine whether the AR protein co-immunoprecipitates with the HA-CDK6 protein. The results are shown in Fig. 2 and indicate that the immunoprecipitate obtained with the anti-HA antibody which binds to the HA-tagged CDK6 protein contain the Flag-tagged AR protein. Likewise, immunoprecipitates obtained with the anti-AR antibody contain the HA-tagged CDK6 protein. Therefore CDK6 binds to the AR *in vivo*.

The fact that this occurs in 293T cells that have a very low level of cyclin D1 suggests that the formation of the CDK6/AR complex does not require the presence of cyclin D1. To further examine this question, we did probasin reporter assays in a derivative of NIH3T3 cells that totally lacks expression of

cyclin D1(*5*) and found that when CDK6 was coexpressed with the AR in these cells, CDK6 also markedly stimulated the transcriptional activity of the probasin reporter (Fig. 3). Furthermore, we found that overexpression of cyclin D1 actually suppressed CDK6-mediated activation of probasin reporter activity in this cell system (Fig. 3) and PC3 cells (data not shown).

Among the cyclin dependent kinase inhibitors (CDIs), $p16^{INK4a}$ is unique in that it binds directly to a specific site on CDK4 and CDK6 (*6*). It is of interest, therefore, that increasing concentrations of $p16^{INK4a}$ also inhibited probasin reporter activity (Fig. 3). Therefore, binding of $p16^{INK4a}$ to CDK6 inhibits the ability of CDK6 to stimulate the transcriptional activity of CDK6.

Since the above studies provide strong evidence that CDK6 binds to the AR, and that the ability of CDK6 to stimulate the activity of the AR does not require that CDK6 exist as a complex with cyclin D1, we decided to omit the study originally proposed in Task 1b, in which we would examine *in vitro* interactions between recombinant CDK6 and AR proteins produced in bacteria.

Task 1b. Determine the specific sequences in the CDK6 gene that are responsible for its stimulatory effects on the AR.

The full length CDK6 gene comprises 885 base pairs. We have constructed a series of truncated and point mutated HA-tagged CDK6 cDNA sequences incorporating BamHI restriction sites on the 5' and 3' ends using the Polymerase Chain Reaction (PCR). Each of the CDK6 cDNA fragments was ligated into the pCRUZ-HA expression vector, at 15°C overnight. The ligation mixtures were transformed into Epicurian coli XP-10 Gold cells (Stratagene), plated on ampicillin resistant plates and grown. The integrity of these constructs was then determined by restriction enzyme analysis and dideoxy DNA sequencing using an Applied Biosystems 373 automated sequenator.

The structures of the full length wild type (WT) HA-tagged CDK6 construct and the four progressively truncated constructs are shown schematically in Fig. 4a. Also shown in this figure are the 4 constructs with specific point mutations. This series of truncated or mutant constructs of CDK6 were then assessed for their ability to stimulate the transcriptional activity of the probasin-luciferase reporter,

or a PSA promoter-luciferase reporter (since the PSA promoter also contains androgen responsive elements) when cotransfected into DHT treated PC3 cells together with the AR. Similar results were obtained with both reporters and so only the results obtained with the PSA-luciferase reporter are shown in Figures 4b and 4c. These results indicate that a C-terminus truncation (up to amino acid 121) did not interfere with the ability of CDK6 to stimulate the DHT and AR-dependent transcriptional activity of the PSA promoter. Residues 261-287 appear to contain an inhibitory site, since when this region of CDK6 was deleted there was further stimulation of this reporter. Site-specific mutations in the $p16^{INK4a}$ binding site (ΔR31C), the ATP binding site (ΔD146N) or the cyclin D1 binding site (ΔRE60,61AA) of CDK6 did not interfere with the stimulatory effect of CDK6 on ARE reporter assays.

The fact that the cyclin D1 binding site can be deleted provides further evidence that cyclin D1 does not play a role in the ability of CDK6 to stimulate the activity of the AR. The relevance of these findings related to the ATP hydrolysis site are discussed below under Task 2.

Task. 2. Determine whether the ability of CDK6 to stimulate the activity of the AR depends on the kinase activity of CDK6 and whether CDK6 can directly phosphorylate the AR.

It is known that *in vivo*, the AR is phosphorylated but the specific kinases involved and the functional effects of this phosphorylation are poorly understood. (7) Our initial findings that CDK6 binds to and stimulates the activity of the AR raised the possibility that CDK6 might directly phosphorylate the AR, either because the CDK6 bound to the AR exists as a complex with cyclin D1 and CDK6 and is thereby activated, or that when CDK6 is bound to the AR the kinase activity of CDK6 is activated by another, unknown, mechanism. However, the above studies indicating that CDK6 exerts its stimulatory effect in cyclin D1 deficient cells (Fig. 3), that increased expression of cyclin D1 actually exerts an inhibitory effect (Fig. 3) and that a CDK6 construct (ΔRE60, 61 AA) that lacks the cyclin D1 binding site is still active (Fig. 4c), provides strong evidence against a role for cyclin D1 in this process. Furthermore, it appears that the potential kinase activity of CDK6 is not important, since the kinase inactive CDK6 mutant CDK6-D146N exerted as strong stimulatory effect on the transcriptional activity

of the PSA promoter equal that of the wildtype CDK6 (Fig. 4c). In addition, we found that CDK6 treatment of PC3 cells with 3mM 3-ATA, a selective CDK4/6-cyclin D1 kinase inhibitor (*8*), did not inhibit CDK6 activation of the PSA promoter (data not shown.).

Key Research Accomplishments :

1. We have confirmed and extended our discovery that CDK6 markedly stimulates the transcriptional activity of the AR in the presence of DHT.
2. We have demonstrated that CDK6 physically associates with the AR and obtained evidence that this does not require the presence of cyclin D1.
3. We constructed a series of expression vectors of CDK6 that encode truncated or mutant forms of CDK6.
4. Co-transfection studies with these constructs indicate that the cyclin D1 binding site and the ATP kinase domain in CDK6 are not required for the ability of CDK6 to stimulate the activity of the AR.
5. The above results coupled with studies using an inhibitor of CDK6 kinase activity indicate that CDK6 does not stimulate the activity of the AR receptor by phosphorylating this receptor. Studies in progress are pursing alternative mechanisms, and the *in vivo* relevance of this effect of CDK6 with respect to the control of gene expression and proliferation in human prostate cancer cells.

Reportable Outcomes :

A manuscript describing the above results is now being prepared for publication. J. Terry Lim, a pre-doctorate student, has carried out the above studies and they will be an important component of his PhD Thesis. The vectors that encode truncated and point mutant forms of CDK6 constructed as part of this research may be valuable to other investigators.

Conclusions :

The research that we have carried out during the first year of this project indicates that CDK6 can bind to and directly stimulate the activity of the AR via a cyclin D1 and kinase independent mechanism. Therefore, in addition to its role in cell cycle control, CDK6 may play an independent role in modulating the activity of the AR. Our findings may have clinical significance since they suggest that variations in the level of expression of CDK6 in human prostate cancers might influence their growth properties and response to anti-androgen therapy.

Future Studies :

During years 2 and 3 we will verify and extend the above-described results and then concentrate on Task 3, which is described below:

Task 3. We will develop derivatives of LNCaP cells that stably overexpress CDK6 and examine their phenotypic properties.

Task 3a We will develop the CDK6 overexpressing derivatives

Task 3b. Determine if overexpression of CDK6 in LNCaP cells induces transcription of the endogenous AR-responsive genes PSA and Kallikrein 2.

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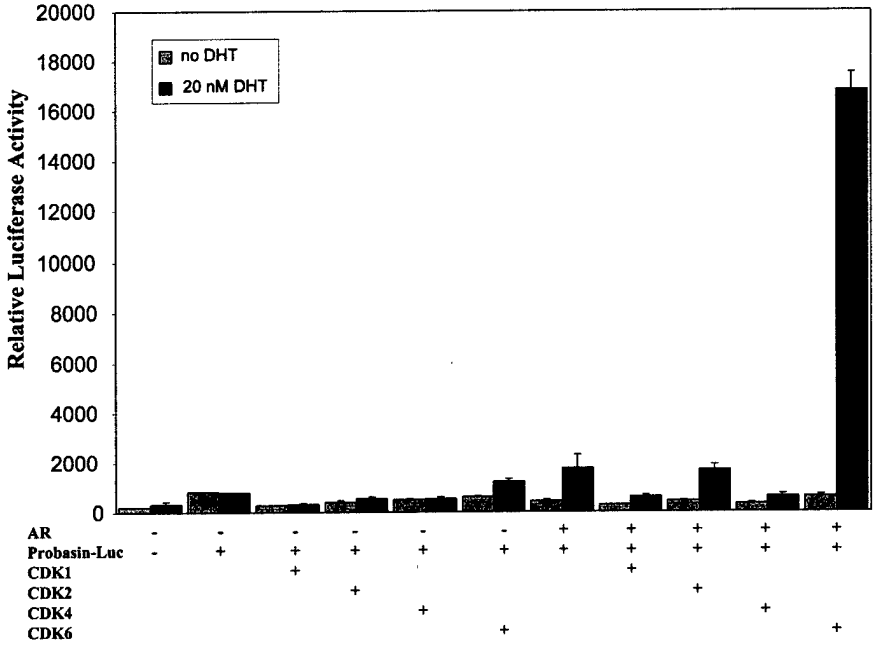
Figure 1

CDK6 Activates Probasin Reporter in PC3 Cells



Increased Exogenous Expression of CDK6 Increases Binding to AR

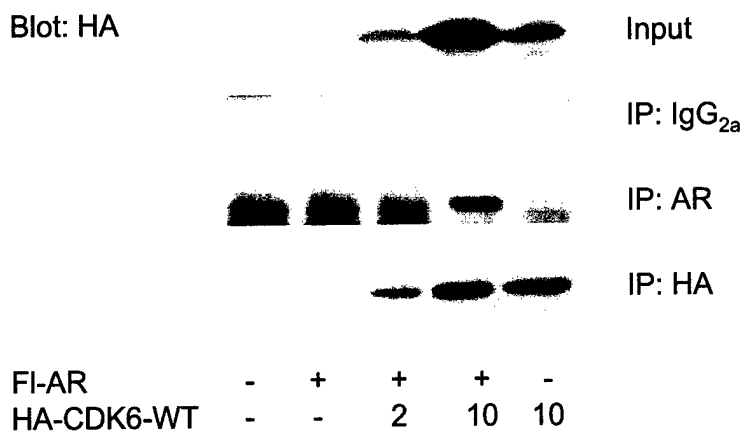
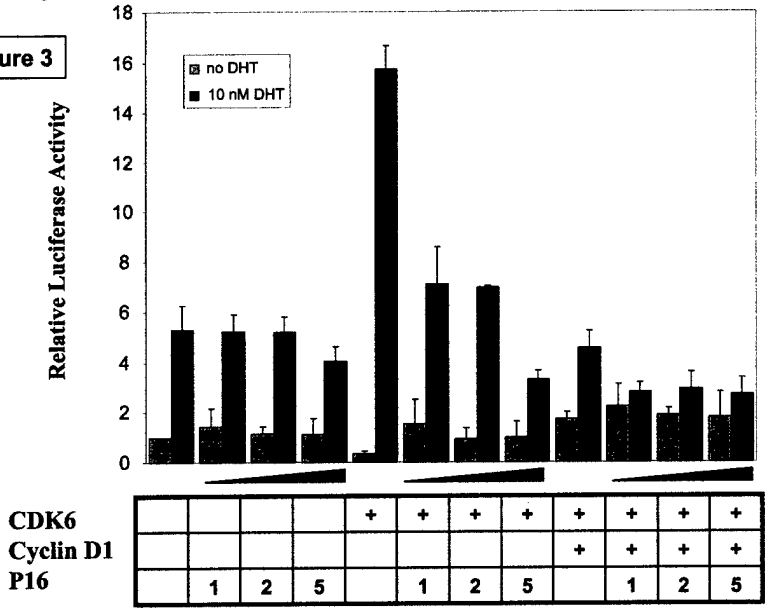


Figure 2

Exogenous P16 and/or Cyclin D1 expression suppress CDK6-AR Activation of PSA Promoter Reporter in NIH 3T3 Cells deficient of Cyclin D1

Figure 3



HA-Tagged CDK6 Constructs

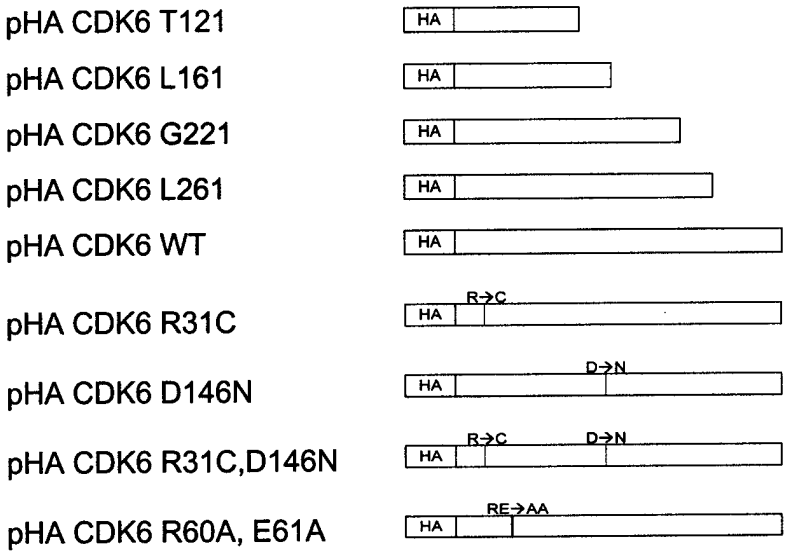
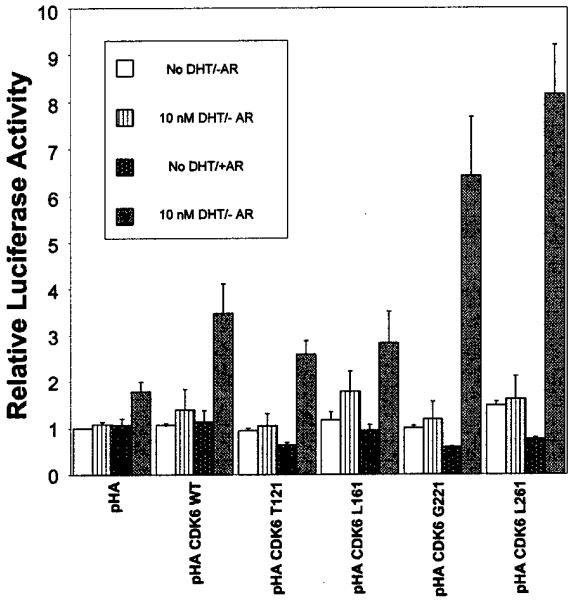


Figure 4a

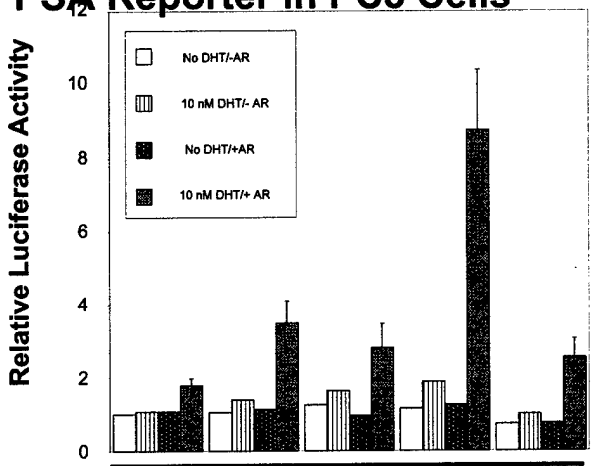
The Effect of CDK6 Truncations on the Activation of PSA Reporter in PC3 Cells

Figure 4b



The Effect of CDK6 Point Mutants on the Activation of PSA Reporter in PC3 Cells

Figure 4c



pHA	+	-	-	-	-
pHA CDK6 WT	-	+	-	-	-
pHA CDK6 D146N	-	-	+	-	-
pHA CDK6 R31C	-	-	-	+	-
pHA CDK6 R60A, E61A	-	-	-	-	+