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14. ABSTRACT Cyclin Dependent Kinase 6 (CDK6) binds to and is activated by cyclin D1, and thereby enhances the transition of cells through the G1 phase of the cell cycle. We discovered that in human prostate cancer cells CDK6 binds to the androgen receptor (AR) and markedly stimulates its transcriptional activity in the presence of dihydrotestosterone (DHT). This effect of CDK6 does not require its kinase activity and is inhibited by both cyclin D1 and p16 ^{INK4a} . An AR mutated at codon 877, which is found in 24% of advanced cases of prostate cancer displays exaggerated stimulation of transcriptional activity by CDK6. LNCaP prostate cancer cells engineered to overexpress CDK6 have increased expression of PSA and enhanced growth in the presence of DHT. CDK6 is present in the chromatin structure of these cells in association with the AR and the PSA gene. Furthermore, we found that primary human prostate cancers frequently displayed increased expression of CDK6. Thus, CDK6 may play an important role in the development and progression of prostate cancer.					
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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. It is estimated that in 2005 there will be about 232,000 new cases diagnosed and about 30,000 men will die from this disease (1). 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 (2). 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 (2). 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) (Fig. 1). 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 (Fig. 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. The overall goal of this project was to establish the fact that CDK6 can bind to and activate the transcriptional activity of the AR, to elucidate the mechanism of this effect, and to study the possible relevance of these findings to human prostate cancer.

Body:

Research accomplished on specific tasks during years 1-3 (Final Report)

Statement of Work

Task 1. Analyze in detail the molecular mechanisms by which CDK6 markedly stimulates the activity of the androgen receptor (AR) in prostate cancer cells

- a. **Determine whether CDK6 can physically associate with the AR, independent of cyclin D1, and the effects of increased expression of cyclin D1 and p16^{INK4a}**

HA-tagged CDK6 and FLAG-tagged-AR cDNA were co-transfected and transiently overexpressed in 293T cells, using standard protocols. 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 were 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 also in 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 (3). 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 as Task 1b, in which we would examine *in vitro* interactions between recombinant CDK6 and AR proteins produced in bacteria.

b. Determine the specific sequences in CDK6 that are responsible for its stimulatory effects of the AR.

The full length CDK6 gene comprises 885 base pairs. We 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 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 (see Figs. 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 (Δ R31C), the ATP binding site (Δ D146N) or the cyclin D1 binding site (Δ RE60,61AA) 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 (Δ RE60, 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 a stimulatory effect on the transcriptional activity of the PSA promoter equal to 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.).

Taken together the above results indicate that the binding sites for p16^{INK4a}, the ATP binding site, and the cyclin D1 binding site in CDK6 are not required for the ability of CDK6 to stimulate the transcriptional activity of the AR. These findings also indicate that CDK6 does not stimulate the transcriptional activity of the AR by phosphorylating the AR. Therefore, Task 2 in our original proposal, that addressed this question, was completed.

c. Effects of decreasing the cellular level of CDK6.

To extend our studies on CDK6, we examined the effects of the compound indole-3-carbinol (I3C), a known inhibitor of CDK6 expression (5). PC3 human prostate cancer cells that lack expression of the AR were-co-transfected with an androgen-responsive probasin luciferase reporter construct together with an AR expression plasmid and a plasmid that encodes CDK6. The cells were then grown in the presence of 20 nM DHT and treated with 200 or 500 μ M I3C for 24 or 48 hours. We found that I3C caused a significant decrease in cellular levels of the endogenous CDK6 protein (Fig. 5; left panel), and that this was associated with a marked decrease in DHT stimulation of PSA luciferase activity (Fig. 5; right panel). These results also suggest that

the basal PSA luciferase reporter activity observed in PC3 cells not transfected with exogenous CDK6 is due, in part, to endogenous expression of the CDK6 protein. Our results also suggest that it might be possible to develop therapeutic agents, like I3C, that could be used to decrease CDK6 expression in patients with prostate cancer.

d. The roles of specific functional domains of the AR in mediating the stimulatory Effects of CDK6.

CDK activation of the AR pathway requires the full length AR. We found that deletions in the transactivation (AR Δ 37-494) or the DNA binding (AR Δ 557-653) domains of the AR markedly impaired the ability of CDK6 to stimulate PSA-luciferase reporter activity (Fig. 6). A mutant in the ligand binding domain (AR Δ 653-910) which displays constitutive activity in the absence of DHT was still active but not as active as the wildtype AR activity with respect to CDK6 stimulation of reporter activity (Fig. 6). Thus it appears that the ability of CDK6 to stimulate the activity of the AR requires multiple domains in the AR.

Increased length of trinucleotide repeats in the AR decreases CDK6 activation of the AR pathway. The transcriptional activation domain in exon 1 of the AR gene contains a polymorphic CAG repeat sequence. African American (AA) men with prostate cancer are more likely to have shorter CAG repeats in the AR than Caucasian men with prostate cancer. It has been suggested that this may explain, in part, the earlier age of onset of the disease and poorer prognosis in AA men (6,7). Therefore, it was of interest to determine whether CDK6 activation of the AR is dependent on CAG repeat length. Hence, we tested the effects of CDK6 activation on three AR constructs that vary in CAG repeat lengths, using the above described PSA- luciferase reporter assays. In the presence of DHT but absence of CDK6, AR proteins containing 48 (AR75), 20 (AR) or no (AR70) CAG repeats displayed approximately equal activities, although the protein with 48 repeats had somewhat lower activity. In the presence of DHT and CDK6, the AR protein with 48 repeats displayed negligible stimulation by CDK6, but the AR protein with no repeats gave an even higher stimulation by CDK6 than that obtained with the wildtype AR (20 CAG repeats; Fig 7). These results indicate that stimulation of the AR by CDK6 is enhanced with ARs that contain shorter CAG repeats. Therefore, in AA men whose AR has short CAG repeats overexpression of CDK6 might exert an exaggerated stimulation of the AR and, thereby, markedly enhance the development of prostate cancer.

CDK6 activation of the AR pathway is markedly enhanced with the T877A mutant of the AR. The ligand binding domain of the AR is frequently mutated in human prostate cancer (8). Mutations that convert leucine to histidine in codon 701 (AR L701H) and threonine to alanine in codon 877 (AR T877A) of the AR are of particular interest since these mutant ARs bind and are activated by steroids other than androgens. Thus, they may lead to the development of an androgen-independent state (8). Therefore, we examined the ability of CDK6 to enhance the transcriptional activities of these two mutant ARs in the above-described PSA promoter-luciferase reporter assays (Fig. 8). In the presence of DHT, the activity of the mutant AR L701H was stimulated by CDK6 to about the same extent as that obtained with the wildtype AR (results not shown). However, in the presence of DHT, CDK6 produced a dramatic stimulation of the activity of the AR T877A mutant. Indeed, this activity was about 9-fold higher than the comparable activity obtained with the wildtype AR (Fig.8). We then examined the effects of various steroid nuclear receptor ligands in similar PSA promoter reporter assays using cortisol, DHT, β -estradiol, flutamide and progesterone (Fig. 8). In the presence of AR T877A and absence of CDK6, β -

estradiol and progesterone stimulated this reporter activity to an even greater extent than DHT. When wildtype AR was tested in the absence of CDK6, the other ligands did not stimulate reporter activity. When AR T877A was tested in the presence of CDK6, β -estradiol and progesterone caused a dramatic stimulation, which was equal to that obtained with DHT, and significant stimulation was also seen with cortisol and flutamide (Fig. 8). These results suggest that in the subset of prostate tumors that express the T877A mutant form of the AR, CDK6 expression may play a critical role in enhancing the transition from an androgen-dependent prostate tumor to an androgen-independent tumor.

Task 3. Effects of stable overexpression of CDK6 in LNCaP prostate cancer cells

To demonstrate that the ability of CDK6 to stimulate the transcriptional activity of the AR is not confined to transient transfection reporter assays, we engineered LNCaP cells to stably overexpress CDK6 (as described in Methods). Western blot analysis of both a pool of these derivatives (Fig. 9a, lane 2) and especially two clonal derivatives (Fig. 9a, lanes 4 and 5) of LNCaP cells indicated that they stably overexpressed higher levels of CDK6 than parallel vector control cells. It is of interest that these derivatives also showed increased expression of the PSA protein (Fig. 9a) when compared to the vector control cells. The increased expression of PSA was especially striking in the two clonal derivatives, which is consistent with the fact that they express higher levels of CDK6 than the pooled cells (Compare lanes 4 and 5 to lane 2, Fig. 9a). Since expression of the AR gene is itself stimulated by the AR it is also of interest that the two clonal derivatives that expressed high levels of CDK6 also expressed increased levels of the AR (Fig. 9a, lanes 4, 5). However, this was not seen with the pool of CDK6 cells (Fig. 9a, lane 2), perhaps because a higher level of CDK6 is required to stimulate transcription of the AR gene than the PSA gene. RT-PCR analysis indicated that these derivatives also expressed increased levels of PSA mRNA when compared to the vector control cells (Fig. 9a). Therefore, increased expression of CDK6 can markedly increase the expression of the endogenous androgen receptor responsive gene PSA in human prostate cancer cells.

The level of PSA in serum is often used as a biomarker in patients with prostate cancer (9). In order to mimic PSA secretion *in vivo*, we also studied the secretion of PSA into the culture medium of these cells. Immunoblot analyses of the media indicated when incubated in a medium containing charcoal-stripped FBS to reduce basal levels of androgens, the LNCaP cells that overexpress CDK6 secreted more PSA into the medium in response to the addition of 0.1 nM DHT than the vector control cells (Fig. 9c).

We also assessed the effects of stable overexpression of CDK6 on the growth of LNCaP cells by determining their ability to form colonies when plated at low density in medium containing charcoal-stripped FBS. Without the addition of DHT, the vector control HA clone 1.1 cells and the CDK6 overexpressor HA CDK6 clone 2.23 cells yielded 72 ± 4 and 74 ± 4 colonies/plate. When grown in the presence of 0.1 nM DHT, the respective values were 107 ± 4 and 159 ± 9 colonies per plate. Thus, overexpression of CDK6 in LNCaP cells enhances the growth stimulatory effects of DHT in LNCaP cells ($P < 0.02$). We also examined the effects of stable overexpression of CDK6 on the growth of monolayer cultures of LNCaP cells in medium containing 10% charcoal-stripped serum, plus 0.1 nM DHT. Under these conditions the exponential doubling times of the vector control clone HA 1.1 and the CDK6 overexpressor clone HAK62.23 were 32 and 17h, respectively. These results provide further evidence that overexpression of CDK6 stimulates the growth of LNCaP cells.

We then utilized the Chromatin Immunoprecipitation (ChIP) assay to determine if CDK6 can physically associate with a transcriptional complex that contains the AR and the promoter sequence of the endogenous PSA gene (Fig. 9d). As expected, immunoprecipitation with the AR antibody pulled down the -149 to -48 DNA region of the promoter sequence of the PSA gene (Fig. 9d, lane 5). Immunoprecipitation of CDK6 in the chromatin isolated from LNCaP cells that overexpress CDK6 also pulled down the same promoter sequence (Fig. 9d, lane 6). Control studies confirmed the specificity of these results (Fig. 9d, lanes 4, 7-9). These findings provide strong evidence that CDK6 is part of the AR transcriptional machinery that binds to the PSA promoter sequence in cellular DNA.

Task. 4 Expression of CDK6 in human prostate cancer

(Note that this task replaced the original the original Task 4 since it is more relevant to human prostate cancer then the original proposal to develop a mouse model).

To address whether CDK6 overexpression is biologically relevant to human prostate cancer, we randomly selected 79 cases of primary prostate cancer from the Herbert Irving Comprehensive Cancer Center Tumor Bank. Using immunohistochemistry (IHC), we assessed the level of CDK6 expression in tissue sections of these tumors. IHC staining of the normal prostatic epithelium showed nuclear staining of the basal cells (Fig. 9e, panel A, single arrow) but no detectable staining of the glandular cells (Fig. 9e, panel A, double arrow). The normal basal cells are proliferative and AR independent, while the normal glandular secretory cells are androgen dependent, express PSA and have a low rate of proliferation. In contrast to the normal prostate tissue, the prostate tumor sections often displayed increased expression of CDK6. A representative example is shown in Fig. 9e, panels B and C. Thus, 62 percent of prostate tumors (49/79) displayed at least a 1+ increase in staining for CDK6, and 20 per cent (16/79) demonstrated a strong increase, i.e. 3+ staining, for CDK6. Since the Gleason Score is often used as a marker of prognosis in prostate cancer, we also examined possible correlations between this histologic parameter and CDK expression. For this purpose the set of cases were divided into 3 groups of Gleason Scores: 3-6, 7, and 8-10, and the data analyzed only with respect to cases that were 3+ positive for CDK6. No major differences between these 3 groups were seen with respect to the percent of cases that were 3+ positive (Table 1). We also sought a possible correlation between tumor grade and high expression (3+ staining) of CDK6 by comparing T1 plus T2 tumors to T3 plus T4 tumors. In the former group only 7% (7/45) of the tumors expressed high levels of CDK6 but in the latter group 33% (9/27) of the tumors expressed a high level of CDK6 (Table 1). The serum levels of PSA were available on 56 of these cases and covered a wide range from 1 to 476. We did not find any correlation between the serum levels of PSA and overexpression of CDK6 (data not shown). This is not surprising since numerous factors can influence serum PSA levels in patients with prostate cancer.

Thus, it is clear that CDK6 is frequently overexpressed in human prostate cancers and our current data suggest that this occurs more frequently in high grade tumors. However, a larger series of cases must be examined to establish the statistical significance of the latter finding, and to further examine possible correlations between overexpression of CDK6 and other clinical and pathologic parameters. It will be of particular interest to determine whether overexpression of CDK6 plays a role in hormone refractory cases of prostate cancer. Recent studies have shown that decreased expression of p16^{INK4a} in high-grade prostate cancers can predict early relapses in

these patients and that increased expression of p16^{INK4a} in prostate cancer cells leads to senescence (43). In view of our evidence that p16^{INK4a} can inhibit CDK6 stimulation of the transcriptional activity of the AR (Fig. 1c), it will also be of interest to examine the relationship between overexpression of CDK6 and the expression of p16^{INK4a} in these cases of prostate cancer. Hopefully, these clinical studies and further mechanistic studies will indicate whether CDK6 might provide a useful prognostic marker or a novel target for the therapy of prostate cancer.

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.
6. The ability of CDK6 to stimulate the transcriptional activity of the AR is markedly affected by known naturally occurring variations in the amino acid sequence of the AR. African-American men who are at higher risk for prostate cancer than Caucasian or Asian men more frequently display shorter CAG repeats in exon 1 of the AR. We found that a decrease in CAG repeats in the AR enhances stimulation of its transcriptional activity by CDK6. This suggests that overexpression of CDK6 could be especially effective in stimulating the activity of the AR in African-American men, thereby enhancing the development of prostate cancer in these men.
7. A point mutated AR originally identified in human prostate cancers, designated AR T877A, displayed dramatic stimulation of transcriptional activity by CDK6 in the presence of DHT, β -estradiol or progesterone. These results suggest that in some cases of “androgen-independent” prostate cancers, AR mediated pathways of gene expression that enhance growth are maintained, even when there are low levels of DHT or other steroids, by the stimulatory effects of CDK6 on mutant forms of the AR.
8. Our evidence that CDK6 can stimulate the transcriptional activity of the AR is not confined to transient transfection assays. Thus, we engineered LNCaP human prostate cancer cells to stably express increased levels of CDK6 and found that these cells display increased expression and secretion of PSA, increased cellular levels of the AR enhanced growth stimulation by DHT.
9. Chromatin Immunoprecipitation (CHIP) assays indicated that in the above derivatives of LNCaP cells CDK6 is part of the transcriptional machinery that binds to the PSA promoter sequence in cellular DNA.

10. Finally, we obtained evidence that these laboratory findings may be directly relevant to human prostate cancer, since we found that CDK6 is frequently overexpressed in primary human prostate cancers, especially in high grade (poor prognosis) tumors.

Reportable Outcomes:

During the past year a student in Dr. Weinstein's laboratory, J. Terry Lim, received his Ph.D. degree from Columbia University. This DOD Award played a critical role in supporting his thesis research. The results in this Report constituted a major part of his Ph.D. thesis. Abstracts and Posters describing these studies were presented at the 2003 and 2004 annual meetings of the American Association for Cancer Research (AACR). A related manuscript describing the major findings in this final DOD Report is now in press in the *Proceedings of the National Academy of Sciences, USA*. This DOD award and the results obtained made it possible for us to obtain funding from the T.J. Martell Foundation for further prostate cancer research.

Abstracts, Presentation, Publications

1. Lim, J.T.E. and Weinstein, I.B. A novel role of CDK6 in androgen receptor signaling pathways. *Proc. Am. Assoc. Cancer Res.* 44, 818, 2003.
2. Lim, J.T.E and Weinstein, I.B. Overexpression of CDK6 in LNCaP prostate cancer cells increases cell growth, colony formation and prostate specific antigen (PSA) expression and secretin. *Proc. Am. Assoc. Cancer Res.* 45, 445, 2004.
3. Lim, J.T.E., Mansukhani, M. and Weinstein, I.B. Cyclin-dependent kinase 6 associates with the androgen receptor and enhances its transcriptional activity. *Proc. Natl. Acad. Sci., USA*, in press, 2005.

Personnel Supported

1. J.T.E. Lim, Postdoctoral Research Scientist
2. Steven Xing, Staff Associate

Conclusions:

This award led to the novel discovery that the cell cycle control protein CDK6 can bind to the androgen receptor (AR) and stimulate its transcriptional activity in human prostate cancer cells. Furthermore, we found that this effect of CDK6 is markedly affected by naturally occurring variations in the amino acid sequence of the AR. These results may have important clinical implications with respect to why African American men with prostate cancer have a poorer prognosis. They may also help explain the mechanism by which prostate cancers often progress from an "androgen-dependent" to an "androgen-independent" stage. Finally, our results suggest that CDK6 may provide a prognostic marker in individual cases of prostate cancer and also a novel target for chemoprevention and therapy.

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Fig. 1 CDK6 activates the AR signaling pathway. The effect of various CDKs on activation of the probasin promoter. AR negative PC3 cells were co-transfected with plasmids that encode the probasin luciferase reporter, the CMV- β -gal reporter, the AR and various CDKs as indicated. Relative luciferase reporter activity was measured after cells were then incubated in the absence (■) or presence (■) of 20nM DHT for 20 hours.

Fig. 2 CDK6 associates with the AR in intact cells. 239T cells were transfected with a plasmid encoding HA-tagged wildtype (WT) CDK6, with or without a plasmid encoding Flag (Fl)-tagged WT AR. Whole cell extracts were immunoprecipitated with either the HA or the AR antibody and immunoblotted with either the HA or AR antibody, as indicated.

Fig. 3 Dose-dependent inhibition of CDK6-mediated activation of the AR by cyclin D1 and p16^{INK4a}. NIH3T3 cells deficient in the cyclin D1 gene were co-transfected with the indicated amounts (μ g) of p16^{INK4a}, 2 μ g of cyclin D1 and 3 μ g of CDK6. The cells were then incubated in the absence (■) or presence (■) of 10 nM DHT for 20 hours and extracts assayed for relative luciferase reporter activity. Error bars designate standard deviations for triplicate assays. *, $P < 0.05$.

Fig. 4 Effects of mutations in CDK6 and the AR on the association between the two proteins and activation of the PSA promoter. **a.** Structures of HA-tagged CDK6 constructs. **b.** The effect of CDK6 point mutations on activation of the PSA promoter. PC3 cells were transfected with the PSA luciferase reporter and CDK6 WT or the CDK6 mutant constructs. As indicated, the cells were also transfected with the AR expression plasmid (■, ■). Relative luciferase reporter activity was measured after the cells were grown in the absence (□, ■) or presence (■, ■) of DHT for 20 hours. **c.** The effects of CDK6 truncation mutants on activation of the PSA promoter. PC3 cells were transfected with the PSA luciferase reporter and either full-length CDK6 or the indicated CDK6 truncation constructs. The AR expression plasmid was also co-transfected as indicated

(■, ■). Relative luciferase activity was measured after the cells were grown in the absence (□, ■) or presence (■, ■) of DHT for 20 hours.

Fig. 5 Dose-dependent inactivation of the effects of CDK6 by 13C. PC3 cells were transfected with the PSA luciferase reporter and the AR. The cells were then incubated with increasing concentrations of 13C for 24 or 48 hours, in the absence (■) or presence (■) of 10 nM DHT and relative luciferase activity was determined. Extracts of these cells were also immunoblotted for the AR and CDK6 as shown in the left panel.

Fig. 6 The effects of AR deletion mutants on CDK6 activation of the PSA promoter. PC3 cells were transfected with the PSA luciferase and β -gal plasmids. As indicated, the cells were also co-transfected with one of the AR deletion constructs (AR Δ 37-494, AR Δ 653-910, AR Δ 557-653 or the full length AR) and the full-length CDK6 expression plasmid (■, ■). Relative luciferase activity was then measured after the cells were grown in the absence (□, ■) or presence (■, ■) of 10nM DHT for 20 hours. The structures of the deletion mutants are shown schematically on the left.

Fig. 7 The effects of CAG polymorphism in the AR on CDK6 activation of the PSA promoter. PC3 cells were transfected with the PSA luciferase reporter and β -gal plasmids. As indicated, the cells were also co-transfected with an AR plasmid encoding 48 (AR75), 20 (AR) or no (AR 70) CAG repeats and CDK6.

Fig. 8 The effects of an AR mutant found in human prostate cancer on CDK6 activation of the PSA promoter in the presence of various steroids. PC3 cells were transfected with the PSA luciferase reporter and β -gal plasmids. As indicated, the cells were also co-transfected with wildtype AR or the AR mutant AR T877A and CDK6 and grown in the absence or presence of cortisol, DHT, β -estradiol, flutamide or progesterone. *, $P < 0.05$.

Fig. 9 Overexpression of CDK6 in LNCaP cells and in prostate tumors. **a**, Stable overexpression of CDK6 in LNCaP cells increases expression of the endogenous PSA protein. Levels of expression of CDK6, the AR and PSA were determined using western blot analysis, with the respective antibodies, of extracts of the following derivatives of LNCaP cells: a pool of vector control cells, lane 1; a pool of CDK6 overexpressing cells, lane 2; a clone (HA 1.1) of vector control cells, lane 3; a clone (HAK6 2.23) of CDK6 overexpressing cells, lane 4; and a second clone (HAK6 2.24) of CDK6 overexpressing cells, lane 5. **b**, CDK6 overexpression upregulates expression of PSA mRNA. RT-PCR analysis of total cellular RNA. Lane 1, no RNA; lane 2, pooled vector control cells; lane 3, pooled CDK6 overexpressing cells; lane 4, no RNA; lane 5, clone (HA 1.1) cells; lane 6, clone (HAK6 2.23) cells. **c**, CDK6 overexpression confers increased sensitivity to induction of secreted PSA by DHT. The indicated derivatives of LNCaP cells were incubated in the presence or absence of 0.1 nM DHT in charcoal-stripped 10% FBS medium for 6 (lanes 1, 7), 20 (lanes 2, 8), 24 (lanes 3, 9), 30 (lanes 4, 10), 48 (lanes 5, 11) or 54 (lanes 6, 12) hours. The media were collected and assayed for secreted PSA by western blot analysis. Insulin growth factor binding protein-2 (IGFBP-2) was used as a loading control for secreted PSA(18). **d**, CDK6 associates with the AR transcription complex. ChIP assays were done on extracts of the vector control clone (HA 1.1), top panel, or the CDK6 overexpressing

clone (HA K6-2.23), bottom panel. Lane 1, no DNA; lane 2, molecular weight marker; lane 3, total DNA; lane 4, no antibody; lane 5, AR IP; lane 6, CDK6 IP; lane 7, normal IgG_{2a} IP; lane 8, no crosslink; lane 9, CDK6 IP. PSA primers were used for lanes 1 and 3-8 and GAPDH primers for lane 9. Bottom schematic depicts the PSA primers used and the expected PCR fragments. **e**, Overexpression of CDK6 in primary human prostate tumors. Immunohistochemical staining for CDK6 expression in normal prostate tissue (top panel) and prostate tumor tissue (middle panel, low magnification; bottom panel, high magnification). The single arrow depicts basal cell staining and the double arrow depicts glandular cell staining for CDK6 in normal prostatic tissue.

Table 1

Increased Expression of CDK6 in Primary Human Prostate Tumors

<u>Gleason Score</u>	<u>CDK6 3+</u>	
	<u>Number</u>	<u>Percent</u>
3-6	3/16	18
7	6/34	17
8-10	7/29	24

Tumor Grade

T1 and T2	3/45	7
T3 and T4	9/27	33

Paraffin-embedded histologic sections were immunostained with a CK6 antibody