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Award Number: DAMD17-01-1-0725

TITLE: Cyclin Dependent Kinase Inhibitors as Targets in Ovarian Cancer

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REPORT DATE: October 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2002	Annual (29 Sep 01 - 28 Sep 02)

4. TITLE AND SUBTITLE
Cyclin Dependent Kinase Inhibitors as Targets in Ovarian Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0725

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The objective of this proposal is to develop gene therapy strategies that inhibit ovarian cancer cell growth and selectively improve sensitivity of ovarian cancer cells to chemotherapy and radiation. The proposal focuses on *in vitro* and *in vivo* "proof-of-concept" studies of target discovery.

Four highly interactive projects make up this proposal. We have identified key genes that may be effective targets in ovarian cancer therapy. The first three projects seek to identify alterations in these genes which, either alone or in combination with chemotherapy or radiation, will efficiently kill ovarian cancer cells. Project 4 will identify promoters that allow for high expression of our key gene(s) in ovarian cancer cells but minimal expression in normal tissues.

14. SUBJECT TERMS	15. NUMBER OF PAGES
cell cycle control, DNA repair, drug resistance, experimental chemotherapy, gene therapy, cDNA array, tissue specific promoters	49
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

The objective of this proposal is to develop gene therapy strategies that inhibit ovarian cancer cell growth and selectively improve sensitivity of ovarian cancer cells to chemotherapy and radiation. The proposal focuses on *in vitro* and *in vivo* "proof-of-concept" studies of target discovery.

Four highly interactive projects make up this proposal. We have identified key genes that may be effective targets in ovarian cancer therapy. The first three projects seek to identify alterations in these genes which, either alone or in combination with chemotherapy or radiation, will efficiently kill ovarian cancer cells. Project 4 will identify promoters that allow for high expression of our key gene(s) in ovarian cancer cells but minimal expression in normal tissues.

To increase sensitivity of ovarian cell lines to chemotherapy and radiation by genetic modification, Projects 1 and 2 will modify DNA repair pathways, and the most promising gene targets identified will be tested in tumor xenografts. Project 3 will investigate the role of the cyclin dependent kinase inhibitors (CDKI's) in ovarian cancer cell cycle control and test the hypothesis that sustained overexpression of $p27^{kip1}$ and $p57^{kip2}$ will suppress growth and/or cause programmed cell death in ovarian cancer cell lines. The effect of adding chemotherapy will be studied, and the most promising strategies from Project 3 will then be evaluated in xenografts by our animal core. In Project 4, human cDNA microarray technology will be used to identify ovarian cancer specific promoters that will allow effective expression of these altered genes in ovarian tumor cells but limited expression in normal cells.

BODY

Project 1: Therapeutic manipulation of the DNA base excision repair pathway for ovarian tumor sensitization (PI: Mark R. Kelley, Ph.D.)

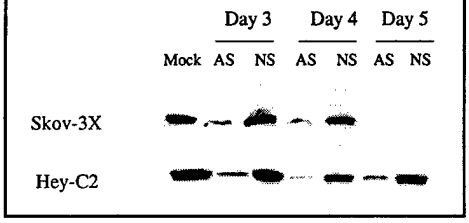
Task 1. Identify altered Ape1 dominant-negative proteins that sensitize ovarian tumor cell lines to chemo-/IR therapy (months 1-24). Mutants of Ape1, which bind substrate DNA with wild-type or better affinity but do not execute repair, will be expressed in ovarian (HeyC2 and SKOV-3X) cell lines using the ecdysone-inducible system (months 1-36). End points such as cell growth, cytotoxicity, DNA damage, and apoptosis will be assessed. Treatments to be used to monitor for improved cytotoxicity include methyl methanesulfonate (MMS), mafosfamide (clinical agent), and ionizing radiation (IR). (months 1-36)

We have identified a number of APE1 mutants that appear to act in a dominant-negative manner *in vitro* (test tube) and are now progressing to *in vivo* (cell lines) studies with these mutants. We have switched from the ecdysone-inducible system to an adenoviral tet-on inducible system due to the leakiness of the ecdysone system and the ease of the adenoviral system. We hope to finish this Task within the next year of the funding period within the time frame as outlined in our original proposal.

Additionally, we have developed the silencing RNA (siRNA) technique for the Ape1 protein and will be using this procedure in the forthcoming year as an additional way to study the role of Ape1 in ovarian tumors and as a potential therapeutic modulator. Some preliminary data is presented below: siRNAs used to decrease AP endonuclease in cells were obtained from Dharmacon Research, Inc., deprotected and hybridized according to directions of the manufacturer [1-4]. Unpurified siRNAs complexed with Oligofectamine in Optimem I (Invitrogen Corp.) according to directions of the manufacturer were applied to cells 30% confluent in Optimem I on six well polylysine coated plates (Biocoat, BD Biosciences) to give final concentrations of 50 or 200 nM. Following a 6 hour incubation at 37°C, 0.5 volume of media was added to each well. 12 hours later the medium was replaced with and incubation was continued until completion of the treatment. As shown in Figure 1, Ape1 levels are dramatically decreased in both SKOV-3X and Hey-C2 cells. Additionally, we have performed time course experiments in a wide variety of cells and have determined that Ape1 protein levels are decreased within 24 hours post siRNA treatment and remain decreased for 3-4 days (data not shown). We are excited about this finding since it now gives us a "window of opportunity" to insert various mutant Ape1 proteins into these cells while decreasing the endogenous wild-type protein.

Figure 1

Knock down of Ape1 using siRNA



Task 2. Determine the effectiveness of MPG and MPG^{mutants} overexpression at killing ovarian tumor cells following alkylating agent chemotherapy. We will make site directed mutations in the active site of MPG which should allow for binding to DNA lesions without removing damaged base. (months 1-12). The MPGs will be overexpressed in ovarian cell lines, and survival will be monitored following exposure to MMS, mafosfamide and cisplatin. (months 1 –36)

We have demonstrated that overexpression of MPG leads to cell killing in breast cancer cells (Cancer Res; manuscript in press) and have confirmed these findings in ovarian cancer cells. We will continue these studies with more detailed studies on the mechanism involved and the use of the more relevant clinical agents mafosfamide and cisplatin.

Task 3. Determine the effects of co-overexpression of Ape1^{mutants}, MPG and MPG^{mutants}.
We will monitor whether combined expression enhances the ovarian tumor cell killing effect of lower doses of chemo-/IR agents administered alone or simultaneously. (months 12-36). Combined expression will be accomplished using either two independent expression plasmids or IRES (internal ribosome entry site) elements and the same expression construct, as well as an adenoviral expression vector. (months 12-36)

Co-expression studies are underway with some of the selected APE1 mutants from Task 1 and the MPG construct from Task 2.

Task 4. Determine *in vivo* chemo- and IR-sensitivity of ovarian cells expressing Ape1^{mutants}, MPG, or MPG^{mutants}. (months 6-36). Ovarian cell lines carrying mutant Ape1, MPG, or MPG^{mutants} genes will be used to produce tumors in the Xenograft Core A. (months 6-36). Dose-response studies will be performed on tumors produced from non-transfected and transfected cells to determine if the addition of the Ape1 mutants or MPG/MPG^{mutant} gene product increases sensitivity to the drugs. (months 6-36). Sensitivity to mafosfamide, cisplatin, and IR will be tested. (months 6-36)

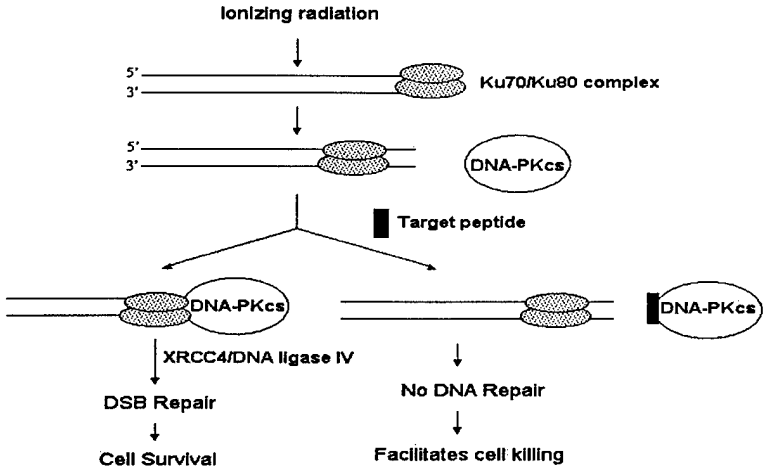
These studies should begin immediately following the completion of Tasks 1 and 2. We have data using a non-invasive imaging system to visualize ovarian cancer cell growth in mice and will be able to generate data in a relatively fast manner once these experiments commence.

Project 2: Targeted inhibition of a key DNA repair enzyme, DNA-dependent protein kinase, in ovarian cancer co-therapy (PI: S-H. Lee, Ph.D.)

Task 1. To develop a peptide that specifically inhibits DNA-PK kinase activity by interfering with the interaction of DNA-PK catalytic subunit (DNA-PKcs) and Ku70/Ku80 (months 1-18)

Screening for a peptide inhibitor of DNA-PK:
Ku70 and Ku80 form a heterodimeric complex that is important for DNA-termini binding; neither Ku70 nor Ku80 alone is active in DNA binding activity (Wu and Lieber, 1996; Gell and Jackson, 1999). The C-terminus of both Ku70 and Ku80 are necessary for heterodimer assembly as well as for DNA-termini binding (Wu and Lieber, 1996; Gell and Jackson, 1999). A recent protein interaction study indicated that DNA-PKcs interacting domain is localized at the extreme C-terminus of Ku80 (amino acids 720-732) (Gell and Jackson, 1999). Since the C-terminus of Ku80 is also likely involved in heterodimer assembly and DNA termini binding, this region (amino acids 720-732 of

Ku80) was selected to synthesize a target peptide that would prevent DNA-PKcs from binding to Ku70/Ku80 regulatory subunits (see Figure 1A). To deliver a peptide to the cancer cells, a cell-permeable peptide import domain and the nuclear localization domain were added to the target peptide to obviate the need for permeabilization or microinjection of individual cells (Lin et al., 1995; Figure 1B).



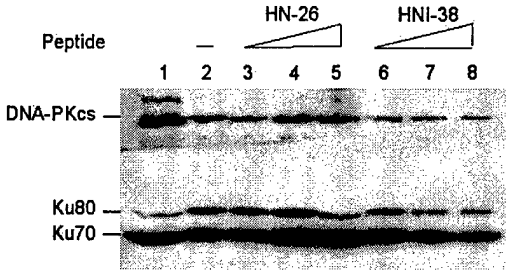
HNI-38: AAVALLPAVLLALLAP**VQRKRQKLM***Y*–▭
HN-26: AAVALLPAVLLALLAP**VQRKRQKLM***Y*
HI-29: AAVALLPAVLLALLAP*Y*–▭
NI-22: **VQRKRQKLM***Y*–▭

Figure 1A (upper) A peptide co-therapy strategy for targeted inhibition of DNA-PK in cancer cell co-therapy. Treatment of cells with ionizing radiation (or chemotherapy drug) induces strand-break DNA damage. To repair DNA damage, DNA-PK heterotrimeric complex (Ku70, Ku80, and DNA-PKcs) needs to be assembled at the ends of DNA. Target peptide representing amino acids 720-732 of Ku80 not only interacts with DNA-PKcs, but may also be involved in Ku heterodimer assembly and DNA termini binding. As a result, cells treated with target peptide will exhibit poor or no DNA repair and become highly sensitive to ionizing radiation or chemotherapy drug. **1B (lower)** The Sequences of synthetic peptide-based inhibitor and control peptides (single-letter amino acid code). Membrane-translocating hydrophobic signal sequence is underlined and the nuclear localization sequence is shown in **bold face**. Twelve residue of peptide inhibitor region is indicated as bar (▭) at the C-terminus. The tyrosine residue (*Y*) is included for ^{125}I-labeling to determine the import efficiency of the peptide into the cells. Peptide-based inhibitor contains the hydrophobic region localization sequence, so-called membrane-translocating carrier, which not only facilitates secretion of proteins, but also is important for importing synthetic peptides into the cell (Lin YZ *et al*, 1995). This localization peptide is capable of carrying a functional domain such as nuclear localization signal (NLS) (Boulikas T, 1994). We therefore synthesized a 38-residue peptide (HNI-38) comprising the signal peptide sequence (AAVALLPAVLLALLAP) and NLS (VQRKRQKLM) followed by a tyrosine (Y) residue and 12-residue of peptide inhibitor sequence (EGGDVDDLLDMI) representing the C-terminus of Ku80 (amino acids #721-732). The tyrosine residue was used for ^{125}I-labeling to determine the import efficiency of synthetic peptides into the cells (and nuclei) (Lin YZ *et al*, 1995).

Effect of target peptide (HNI-38) on the interaction between DNA-PKcs and Ku complex

A target peptide interrupts the interaction between DNA-PKcs and Ku70/Ku80 as well as the binding of Ku complex to DNA. DNA-PKcs and Ku70/Ku80 are abundant proteins approximately 5 x 10^5 molecules per human cells (Lee and Kim, 2002) and most of Ku70/Ku80 heterodimer exists in cell extracts without forming a complex with DNA-PKcs in the absence of DNA (Hammarsten and Chu, 1998). Therefore, target peptide (HNI-38) was analyzed for its effect on interaction between DNA-PKcs and Ku70/Ku80 in the presence of dsDNA. Varying concentrations of either control (HN-26) or target peptide (HNI-38) was incubated with cell extracts containing DNA-PKcs and Ku complex in the presence of dsDNA cellulose, and examined for its effect on binding of Ku complex and DNA-PKcs to DNA following the dsDNA cellulose pulldown assay (Figure 2A). Although it was marginal, the addition of increasing amount of target peptide (HNI-38) not control peptide (HN-26) led to a decrease in DNA-PKcs associated with dsDNA, suggesting that target peptide binds to DNA-PKcs and inhibits its binding to Ku70/Ku80. It is also noted that the addition of target peptide affected the binding of Ku70/Ku80 to the dsDNA cellulose (Figure 2A).

To further examine the effect of HNI-38 on Ku's DNA binding activity, target peptide was incubated with purified Ku70/Ku80 complex in the presence of dsDNA cellulose, and the reaction mixtures were analyzed for the presence of Ku70 and Ku80 following the dsDNA pulldown assay (Figure 2B). In keeping with Figure 2A, target peptide (HNI-38) significantly interfered with binding of Ku complex to dsDNA under the conditions where control peptide (HN-26) showed virtually no effect (Figure 2B). This result suggests that target peptide not only affects the interaction between DNA-PKcs and Ku, but also interferes with Ku's DNA binding activity.



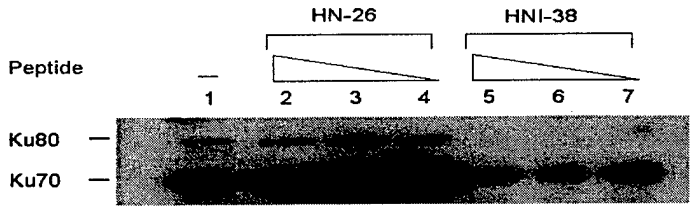


Figure 2. Effect of the target peptide on interaction of Ku70/Ku80 with DNA-PK or with dsDNA. **Panel A (upper).** The target peptide (HNI-38) interferes with association of DNA-PKcs with dsDNA. Partially purified DNA-PK fraction (100 ng) was incubated with 0 nM (lane 2), 10 nM (lanes 3 & 6), 50 nM (lanes 4 & 7), and 100 nM (lanes 5 & 8) of either control peptide or target peptide prior to dsDNA cellulose pull-down assay. Lane 1 contained partially purified DNA-PK without dsDNA pulldown assay. The protein-dsDNA cellulose complex was analyzed by the procedure described in Methods section. **Panel B (lower).** Effect of HNI-38 on DNA binding activity of Ku70/Ku80 complex. Purified Ku70/Ku80 complex (100 ng) was incubated with 10 nM (lanes 4 & 7), 50 nM (lanes 3 & 6), and 100 nM (lanes 2 & 5) of either control peptide or target peptide prior to dsDNA cellulose pull-down assay. Following the dsDNA cellulose pull-down, Ku70 and Ku80 were analyzed by 10% SDS-PAGE and Western blot.

Effect of target peptide (HNI-38) on DNA-PK kinase activity

Interaction of DNA-PKcs with Ku complex are necessary for activation of its kinase activity (Gottlieb and Jackson, 1993; Hartley et al., 1995), therefore, the efficacy of target peptide was analyzed by measuring DNA-PK kinase activity *in vitro* in the presence of either HI-26 or HNI-38. DNA-PK kinase activity was inhibited up to 50% in the presence of HNI-38 under the conditions where a control peptide (HI-26) showed minimal effect (Figure 3), strongly supporting a notion that target peptide specifically binds to DNA-PKcs and interferes with interaction between DNA-PKcs and Ku complex. Inhibitory effect of target peptide on DNA-PK occurred at low peptide concentration (>20 □M) and, in the presence of 20□M or higher, both target and control peptides inhibited DNA-PK activity (data not shown).

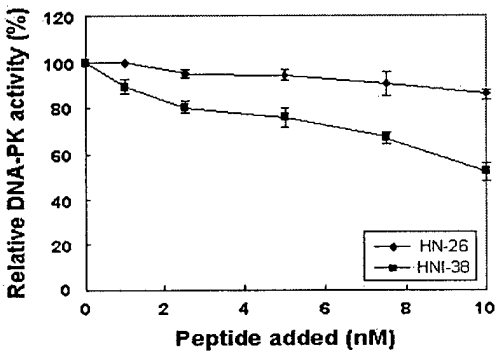


Figure 3. Effect of target peptide on DNA-PK kinase activity *in vitro*. Partially purified DNA-PK fraction was incubated with various concentrations of either control peptide or target peptide prior to the addition of substrate peptide and other components for DNA-

PK kinase assay. DNA-PK activity was measured as the relative amounts of ^{32}P transferred to the substrate peptide.

Task 2. To determine whether DNA-PK plays a role in DNA repair and/or the chemotherapy drug resistance among ovarian cancers (months 1-36)

Target peptide interferes with repair of double-stranded DNA breaks induced by IR

IR-induced double-stranded DNA breaks are efficiently repaired by non-homologous end-joining (NHEJ) process. Genetic and biochemical studies strongly indicated that DNA-PK plays an essential role in NHEJ (Jeggo, 1998; Jin et al., 1997; Blunt et al., 1995). Hence, an alternative way to determine the efficacy of peptide inhibitor is to measure the repair of double-stranded DNA breaks following IR. Cells grown in the presence of [^{14}C]-thymidine (DiBiase et al, 2000) were treated with either a control or target peptide for 24 hrs. Following irradiation (40 Gy), cells were harvested at various time points and intact chromosomal DNA and DSBs were separated by pulsed field gel electrophoresis (0.5% agarose). Treatment of NCI cells with IR (40 Gy) induced substantial amounts of dsDNA breaks, most of which were repaired within 4 hrs. Cells treated with target or control peptide did not show any difference in generating DSBs following IR (Figure 4A; lane 2 vs. lanes 8 & 14). On the other hand, cells treated with target peptide (Figure 4A; lanes14-18) compared with those treated with control peptide (Figure 4A; lanes 8-12) showed a noticeable decrease in DSB repair activity. This result suggests that target peptide interfered with dsb repair *in vivo* through the targeted inhibition of DNA-PK activity.

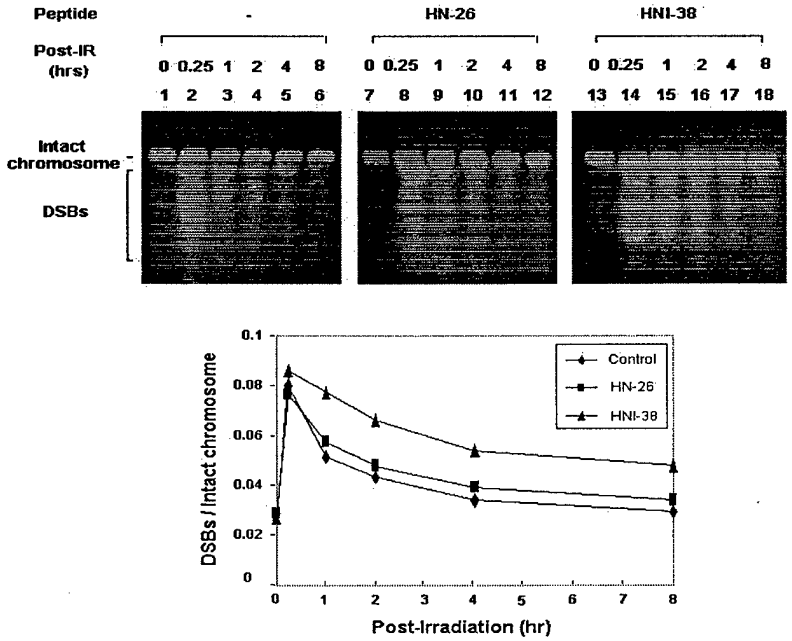
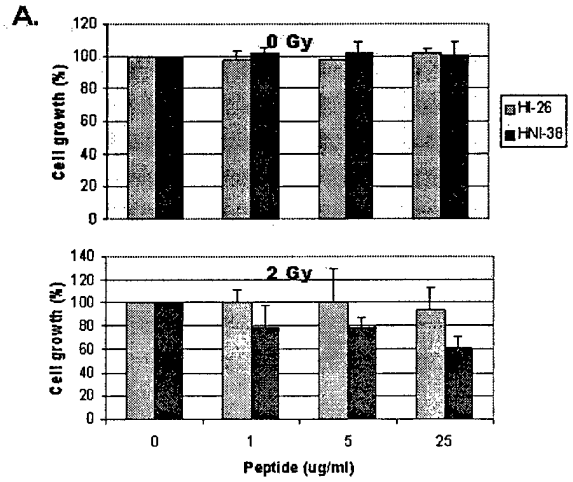


Figure 4. *Effect of target peptide on double-stranded DNA breaks (dsb) repair. Cells grown in ^{14}C-containing media were treated with ionizing radiation (40 Gy) in the presence of no peptide (lanes 1-6), 50 nM of control peptide (lanes 7-12), or 50 nM of target peptide (lanes 13-18). After harvesting the cells at various time points, intact chromosomes and double-stranded DNA breaks (DSBs) were separated by gel electrophoresis (**upper panel:** fluorography) and were quantified by liquid scintillation counter (**lower panel**).*

Target peptide inhibits ovarian cancer cell growth only in the presence of DNA damage

Cells lacking DNA-PK catalytic subunit showed increased sensitivity to DNA damaging drugs or ionizing radiation (IR) (Lees-Miller et al., 1995; Kirchgessner et al., 1995), suggesting that DNA-PK activity is essential for DNA repair and cell survival upon DNA damage. We therefore tested whether a targeted inhibition of DNA-PK by a peptide HNI-38 would sensitize ovarian cancer cells upon treatment of ionizing radiation or chemotherapeutic drug (cisplatin). Two ovarian cancer cells (Hey and Hey-C2) were treated with either control (HI-26) or target peptide (HNI-38) and tested for the efficacy of DNA-PK inhibitory peptide on lowering resistance of cells in response to ionizing radiation using standard colony count cell survival assay. Both control and target peptides did not show any effect on cell growth in the absence of ionizing radiation. However, cells treated with IR showed significant cell growth inhibition in the presence of target peptide but not with control peptide (Figure 5A), suggesting that cell growth inhibition by target peptide occurs through targeting DNA-PK activity. Cells treated with cisplatin, although not as effective as those treated with IR, also showed inhibitory effect on cell growth in the presence of HNI-38 (Fig 5B).



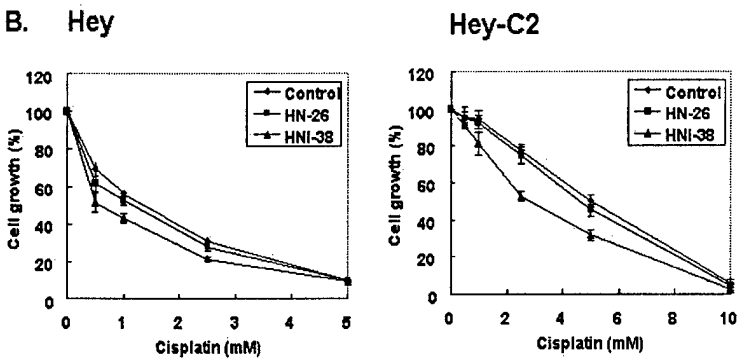


Figure 5. *Effect of control (HN-26) and target (HNI-38) peptides on the growth of ovarian cancer cells (Hey) treated with ionizing radiation (**upper panel**) or cisplatin (**lower panel**). Values expressed are means (+ S.E.) of the three replications (*, p < 0.01; ** p < 0.01). The clonogenic assay was used for the cells treated with ionizing radiation and the cell survival assay (MTT) was employed for those treated with cisplatin.*

In summation, DNA-PK activity is essential for DNA repair as well as cell cycle arrest in response to DNA damage, which contributes to cell survival by protecting cells from apoptosis. Cells treated with target peptide not control peptide showed a noticeable decrease in DSB repair following high dose (40 Gy) of IR, suggesting that HNI-38 specifically targets DNA-PK *in vivo* and interferes with dsb repair activity through inhibition of DNA-PK activity. Targeted inhibition of DNA-PK by HNI-38 also caused cell growth inhibition only when cells were treated with IR, suggesting that HNI-38 targeted DNA-PK and lowered resistance of cells in response to ionizing radiation, which eventually causes growth inhibition of both ovarian cancer cells. Treatment of cells with HNI-38 also showed additive effect on cell growth inhibition in response to cisplatin treatment. This observation is in keeping with previous findings that DNA-PK is directly involved in NER action in mammals (Muller et al., 1998). It also supports a notion that a targeted inhibition of DNA-PK would sensitize cancer cells upon treatment of chemotherapeutic drugs such as cisplatin. Together, our study described here not only validates DNA-PK as a useful molecular target for the treatment of drug-resistant cancer cells, but also supports physiologic role for DNA-PK in IR or chemotherapy drug resistance of cancer cells.

Project 3: DNA repair and cell cycle therapeutic targets for ovarian cancer (PI: Maureen Harrington Ph.D.)

Task 1: Mutations will be generated in the *KIP2* gene at amino acid residues in common with $p27^{Kip1}$ protein that are known to control $p27^{Kip1}$ protein stability. Using the $p57^{Kip2}$ mutants, we will determine if

the mutations affect $p57^{Kip2}$ protein stability, subcellular localization and/or function in normal and in tumor derived ovarian epithelial cell lines (months 1-12).

•Site-directed mutagenesis of the $p57^{Kip2}$ gene, subcloning of the cDNAs encoding wild-type and mutant *KIP2* into mammalian expression vectors. (months 0-6).

Methodology: Site-directed mutagenesis was performed to mutate the threonine at position 342 of the $p57^{Kip2}$ protein, a site shared in common with the $p27^{Kip1}$ protein (see Fig. 1) using the Altered Sites Mutagenesis System II (Promega, Madison, WI). The cDNA encoding the $p57^{Kip2}$ mutation, as well as the wild-type $p57^{Kip2}$ cDNA, was subcloned into the mammalian expression vector pcDNA3.1(-)/Myc-His A (Invitrogen Corp., San Diego, CA).

```
p27   N R T E E N V S D G S P N A G T V E  185
p57   G C P - - - S P N V A P G V G A V E  340
p21   R L V F C K R K P                    159

          *
p27   Q T P K K P G L R R Q T              197
p57   Q T P R K R L R                      348
```

Fig 1: SEQUENCE COMPARISON OF Cip/Kip FAMILY OF CDKIs - CARBOXY TERMINUS

Cloning into pcDNA3.1 generated a plasmid encoding $p57^{Kip2}$ containing a myc-epitope, which will allow us to follow expression of the exogenous wild-type or mutant $p57^{Kip2}$ by Western analysis using antisera to the myc-epitope (Roche, Indpls, IN.;
Fig. 2).

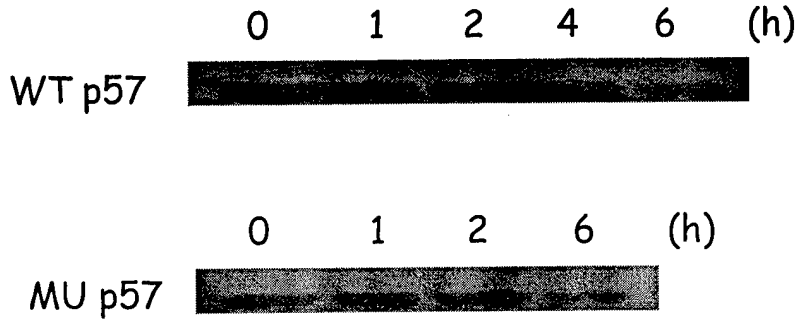


Fig. 2: T342A mutation does not stabilize p57 protein.

•Introduction of mammalian expression vectors into non-tumorgenic immortalized ovarian surface epithelial cell line (IOSE) and into epithelial ovarian cancer cell lines (HeyC2/SKVO3X). (months 6-12).
•Comparison of wild-type and mutant $p57^{Kip2}$ protein activity, stability and subcellular localization by Western blotting and CDK2 kinase assays. (months 6-12).

Methodology: Mammalian expression vectors encoding wild-type, and mutated $p57^{Kip2}$ were transfected into the SKOV3 epithelial ovarian cancer cell line using Fugene 6 (Roche, Indpls, IN) according to manufacturers' specifications. 24h later cultures transfected with the same construct were pooled, replated and 24h later cultures were treated with cycloheximide for the indicated period of time. Cultures were harvested, lysed, Western blots were generated and probed with myc-antisera (Fig. 2). Results of these studies revealed that $p57^{Kip2T342A}$ and wild-type $p57^{Kip2}$ have similar half-lives.

Subcellular localization of the wild-type and mutated $p57^{Kip2}$ protein was determined in the transfected cultures using confocal microscopy (Fig. 3). Results of these studies reveal that wild-type and mutant $p57^{Kip2}$ are both nuclear proteins.

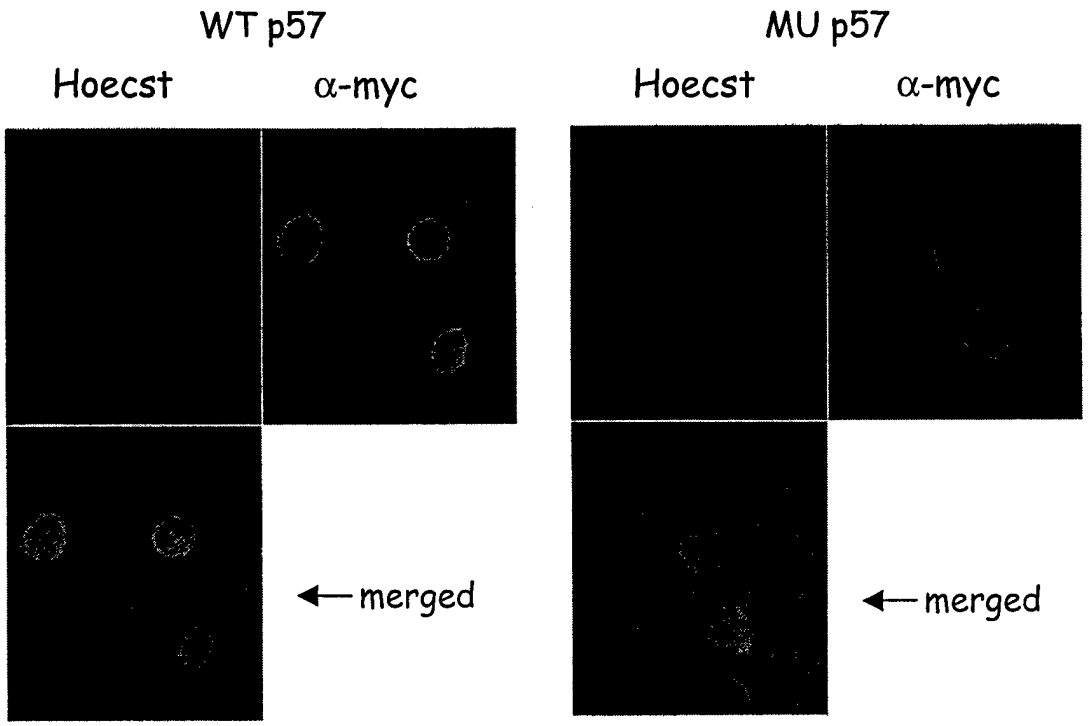


Fig. 3: Subcellular localization of wild-type and mutated p57.

Coordinate with these studies we proposed to determine whether the wild type and mutated KIP2 proteins mechanistically function as CDKIs. These

studies are underway and we are going to first: confirm by Western analysis that we can immunocomplex p57 and cdk2 (antisera from Santa Cruz Biotechnology, Santa Cruz, CA); once we can successfully document this interaction we will measure the ability of KIP2 protein (wild type and mutant) to inhibit the activity of CDK2 complexes.

Certain of the studies outlined under Task 2 were initiated this year as well.

Task 2: To determine if transduction of stabilized forms of $p27^{Kip1}$ and/or mutated $p57^{Kip2}$ inhibit the growth of epithelial ovarian cancer cell lines grown *in vitro* and as xenografts in nude mice, will be pursued in the ensuing year.

•Subcloning of cDNAs encoding wild-type $p27^{Kip1}$ and $p27^{Kip1/T187A}$ (encodes the stabilized $p27^{Kip1}$ protein) into inducible mammalian expression vectors. Generation of stable epithelial ovarian cancer cell lines (HeyC2/SKVO3X) containing these cDNA under the control of an ecdysone-inducible promoter. (months 0-12).

Methodology: cDNAs encoding wild-type $p27^{Kip1}$ and a myc-tagged-$p27^{Kip1/T187A}$ mutant (stabilized $p27^{Kip1}$ protein) were subcloned into the plasmid vector, pIND (Invitrogen) placing the cDNAs under the control of a minimal heat shock promoter flanked by 5 hybrid ecdysone/glucocorticoid response elements (5XE/GRE-HSP). A second vector, pVgRXR, encodes a heterodimeric ecdysone receptor. Exogenously added ponasterone A binds the heterodimeric ecdysone receptor, VgRXR, promoting its binding to the hybrid ecdysone/glucocorticoid response element thereby promoting expression of the $p27^{Kip1}$ and $p27^{Kip1/T187A}$ cDNAs. As described in Projects 1 and 2, the HeyC2 epithelial ovarian cancer cell line has been stably transfected with pVgRXR, generating ECR-HeyC2. For studies outlined in this task we first needed to generate stable cell lines expressing either, $p27^{Kip1}$ or $p27^{Kip1/T187A}$ (pIND-KIP1WT and pIND-KIP1M, respectively) in ECR-HeyC2 to generate ECR-HEYC2-KIP1WT and ECR-HEYC2-KIP1M, respectively. We were unable to generate stable cell subclones expressing either the wild-type or the mutated proteins. We next tried a tetracycline inducible system (Clontech). We also were unable to generate stable cell lines expressing either the wild-type or mutated proteins. Further study has revealed that the promoter elements used in the ecdysone and the tetracycline inducible systems are "leaky" in the ovarian epithelial cell lines. Thus we will not be able to use this strategy for regulating the expression of wild-type or mutated proteins in cells.

The remaining studies outlined (in Task 2) will be addressed in the ensuing grant year. Our strategy for changing the expression system is outlined in the Conclusion section of the proposal. The studies outlined in Task 3 will be initiated in the last year of funding.

Project 4: Identification of Ovarian Tumor-Specific Promoters (PI: Ken Nephew, Ph.D.)

The first task of Project 4 was to identify highly expressed cDNAs in ovarian cancer. In order to accomplish this objective, we categorized genes that were known to be over-expressed in ovarian tumors. We performed extensive data mining of the of several studies (1-3). Based on our analysis of several large data bases, we concluded that the HE4 gene is over-expressed approximately ten-fold in early and late stage ovarian cancer. It is a novel promoter, and no papers as of yet have been published on the HE4 promoter. In fact, there are only two papers published specifically on HE4 (4, 5). The HE4 gene met all the criteria outlined in the project and is thus our number one choice at this time as a promoter to drive ovarian specific trans-gene expression. In addition to HE4, we selected two other gene promoters, OSP-1 (6, 7) and hTERT (7-9), and we are currently assessing their activity in ovarian cancer cells. The OSP-1 promoter is active specifically in ovarian cells and the most heavily researched ovarian specific promoter to date. A recent paper from Dr. Hamilton's lab (6) showed that OSP-1 can selectively direct suicide gene therapy in ovarian cancer cells and in vivo efficacy was improved using a new lipid GL67 delivery system, raising the possibility of using OSP-1 to drive TSG expression in our PPG. The hTERT promoter is considered a cancer specific promoter but has some non-specific expression in immune cells (10).

As outlined in the Statement of the Work for Project 4, *Task 2 was to obtain the promoter region of candidate genes. First, we assessed promoter availability.* The hTERT and OSP-1 promoters had been described in the literature (8, 11) and we obtained the constructs from the corresponding laboratories. By searching available data bases, we realized that the HE4 promoter had not yet been described in the literature or data bases. Thus, it fell into our previously defined category of being an unknown or previously uncharacterized promoter, and we proceeded with a strategy to isolate the HE4 promoter. Our initial multiple PCR attempts made on genomic DNA from ovarian tumors and cancer cell lines yielded poor results; therefore, we ordered a PAC clone *Children Hospital Oakland Research Institute, Accession #* AL031663 and used it to obtain HE4 promoter by PCR amplification. The PAC clone was digested with *Dra*1 and a 5 kb fragment was obtained. The ligation of this piece of DNA was problematic due to its blunt ends and no success was achieved trying to clone it into a useful vector. Because *Dra*I digests did not work, PCR using new primers minus restriction sites was performed and a 2071bp product was successfully obtained Restriction digestion suggested it is the DNA of interest *Acc*I /*Sph*I or *Pvu*II digests, and ligation into pGEM3Z was performed followed by transformation of competent bacteria. A 1300 bp *Acc*I /*Sph*I fragment containing both putatitve HE4 promoters was obtained. The 1300 bp *Acc*/*Sph* fragment was then cloned into pGEM3z (a plasmid that has the cloning sites of interest), and a blunt end ligation into pGL2-Basic was performed as well. The orientation of

insertion of the fragment was checked using restriction digestion (Figure 1, below).

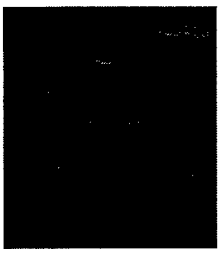


Figure 1.
Lane 1-Ladder
Lane 2-2071 bp PCR product
Lane 3-2071 cut with AccI/SphI
Lane 4-HE4p1308 pGEM3z
Lane 5-HE4p1308 pGEM3z cut with AccI/SphI
Lane 6-HE4p1308 pGL2, HE4p1308 pGL2 cut with KpnI/PvuII.

Task 3 of Project 4 is to identify the most specific and active promoter. We performed additional sequential deletions on the HE4 promoter and assembled the constructs into pGL2-Basic luciferase reporter vector. The multiple HE4 promoter constructs we now have are shown in Figure 2.

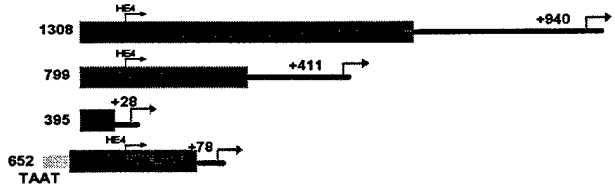
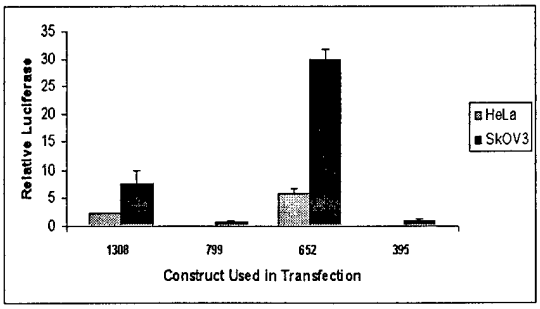


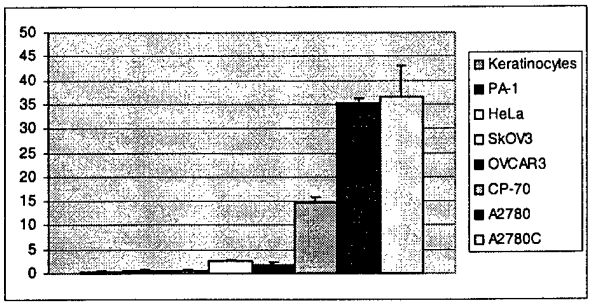
Figure 2 (left). HE4 promoter constructs.
Green arrow indicates start site for luciferase.
HE4p1.3 940 bp from luciferase;
HE4p799 411 bp from luciferase;
HE4p395 28 bp from luciferase;
HE4p652 78 bp from luciferase. The Chip2 promoter analysis program was used to determine HE4p652.
MATInspector was used to determine the other 3 HE4 promoter constructs. DNA sequencing was performed on all HE4 promoter constructs HE4p1.3, p799, p652, and p395. The results confirmed the identity of the constructs Fig 2.



The constructs have been transiently transfected into various ovarian cancer cell lines, and reporter gene activity has been assayed using the dual luciferase reporter assay system. The results are shown in the figure to the left. Transient transfection assays were performed with HE4 constructs. HE4p395 and p799 showed little if any activity in the cell lines tested Hela, SKOV,

Figure 3 (above). Comparison of promoter constructs in HeLa and SkOV3 cell lines.

OVCAR3, CP70. HE4p652 showed greater activity in SKOV and OvCar compared to Hela cells. HE4p1.3 showed some activity in SkOv cells but at higher concentrations of DNA 300ng; no activity in Hela cells.



The next step was to optimize transient transfection assays for HE4p652 for all ovarian cancer cell lines. Using various amount of DNA (-1000 ng) the HE4p652 promoter activity was optimized in the following ovarian cancer cell lines: SkOV, OvCar, CP70, A2780C, PA1. Activity of the promoter was

Figure 4. Comparison of HE4p652 activity in various cell lines.

high in CP70 and A2780, moderate in SkOv and OvCar, and low/no in PA1 and low/no in two non ovarian cell lines, keratinocytes and Hela cells (Figure 4, above).

We will continue the optimization experiments using three additional ovarian cancer cell lines (Hey, HeyA8 and Hey C2). We will also continue to analyze the hTERT-luciferase, SV40- luciferase and TATA- luciferase constructs along with HE4 promoters in the transient transfection assays. The hTERT promoter constructs 1kb and 444 bp both in pGL2 vector are active in ovarian cancer cell lines, Hela cervical cancer cells and MCF7 breast cancer cells (data not shown) and we are actively pursing the possibility of using these promoters in our studies. In agreement with previous reports on the OSP-1 promoter by Hamilton and co-workers (6), our results also show that the OSP-1 promoter is active in ovarian and other cancer cell lines (data not shown), and we will continue to investigate the usefulness of this promoter.

Core A: Animal Models Core (PI-Robert Bigsby, Ph.D.)

The goal of the Animal Models Core is to test the effectiveness of mutated genes developed in Projects 1-3 for their ability to enhance therapeutic index in an in vivo setting. Cells harboring the mutant genes will be grown as tumors in athymic mice and the mice will be treated with a chemotherapeutic agent or with X-irradiation. Dose-response studies will determine the sensitivity of the altered tumors and this will be compared to the sensitivity of tumors from parent cell lines.

The original SOW indicated two tasks for year one.

Task 1: Establish parameters for in vivo induction of gene expression. Although it was originally proposed to use an ecdysone-inducible promoter to

drive expression of the mutant target genes, it was found that another system, the tetracycline controlled promoter worked well in culture. Since the tet-on system would be much more economical, it was decided that this would be the used throughout. Three attempts to grow cells expressing an inducible form of mutated APE have failed. Cells stably transfected with empty vector did grow. Apparently, control of expression of the mutant APE did not allow for null expression in the absence of an exogenous stimulator. We are awaiting construction of further cell lines to be tested in the animal system.

Task 2: Establish tumor lines from engineered HeyC2 and Skov 3X tumor cell sublines supplied from Projects 1-4. As these sublines have not been established in the other projects, we have not been able to begin to make tumor lines.

In lieu of engineered sublines to use in generation of tumor lines, we have focused on establishing procedures required by the Animal Models core and developing the necessary baseline data on therapeutic responses of parent cell lines. This baseline data will be necessary for comparison in experiments using the engineered sublines.

The protocol for growing cells as required by the Animal Core was established. Typically, a xenograft experiment requires 500 million cells. The cells need to be grown in T-500 flasks and the flasks need to handed over to the Animal Core for final processing. Preparing cell suspensions in the other labs and transporting these preparations to the Animal Core lab proved unworkable; the quality of the cell suspension deteriorated quickly and the injections were variable when the cells were processed away from the core.

Baseline X-irradiation experiments using grafted SKOV3x cells were performed (Fig. 1). A dose of 9 Gy produced a dramatic decrease in growth of the tumors, increasing tumor volume doubling time from 6 to 14 days in one experiment and from 6 to 21 days in another; a dose of 6 Gy produced an intermediate growth rate. Similar experiments are underway with HeyC2 cells. These preliminary experiments allowed us to develop the system for X-irradiation and to define the dose range for experimental tests in engineered cells.

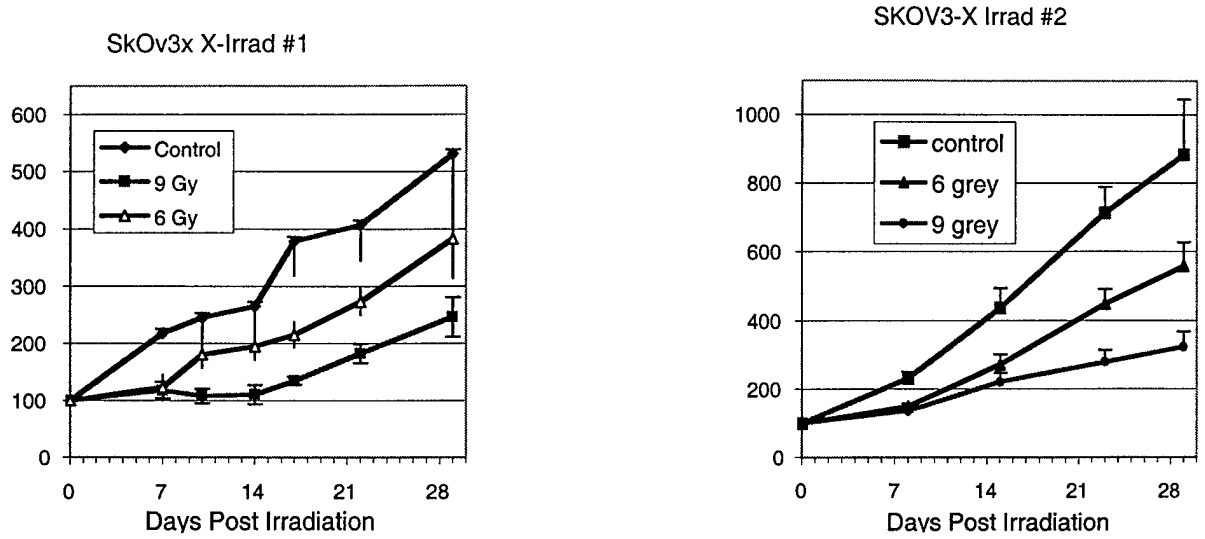


Figure 1. Two experiments were performed with Skov3X cells grown as subcutatneous xenografts. After tumors had reached approximately 300-500 mm^3, they were irradiated with the indicated dose of X-rays. Tumor growth was monitored and the change in size described as relative to the volume of each tumor at the time of irradiation.

Development of cell line expressing the mutant p27 (Project 3) has been problematic. The mutant gene apparently arrests the cells, even during a transient transfection. An experiment was performed to determine the fate of the transiently transfected cells in vivo. Transfection of 293 cells with empty vector, reduced their ability to form tumors slightly compared to non-transfected cells. Transfection with the mutant p27 produced cells that did not make tumors (Table 1). An inducible gene expression system is required for further study of this mutant.

Effect of Mutant p27 on Tumor Growth

vector	cell inocul	Tumor incidence
$pcDNA_3$	**$0.5X10^6$**	**0/4**
	$1X10^6$	**0/4**
	$2X10^6$	**3/4**
	$4X10^6$	**2/4**
p27	**$0.5X10^6$**	**0/4**
	$1X10^6$	**0/4**
	$2X10^6$	**0/4**
	$4X10^6$	**0/4**

In addition, we are attempting to develop a system to follow intraperitoneal tumor growth. One method is to analyze the urine of animals bearing tumors

that secrete ß-hCG Animals were inoculated with BT melanoma cells stably transfected to express ß-hCG and the amount of hCG in the host animal's urine was measured. This system is easy to use and there is a good correlation between urine hCG and tumor growth. The hCG gene has been swapped to a new vector that expresses bleomycin resistance, rather than neomycin resistance, and this is being used to make stable transfectants in SKOV3x and HeyC2 cells. As an aside, we have found that the parent cell lines already express ß-hCG, without transfection, but in small amounts; it will be advantageous to develop lines expressing large quantities of ß-hCG in order to accurately monitor in vivo growth.

KEY RESEARCH ACCOMPLISHMENTS

Project 1:

--Mutants of APE1 act in a dominant-negative manner
--Constructs have been made to express these mutants in cell lines and mouse model

--New inducible cell lines have been made using the Adeno-X system (Tet-On inducible).

--Ape1 siRNA has been optimized and will be used in ovarian cancer cell lines to study the role of Ape1 in cell growth, apoptosis, etc.

--Adenoviral Ape1 constructs have been made and are being tittered and evaluated in ovarian cancer cell lines.

--Overexpression of MPG leads to increased ovarian cancer cell killing with alkylating agents.

--Animal model using non-invasive imaging technologies is underway; will be used with the APE1 mutants or MPG adenoviral constructs.

Project 2:

--Demonstration of the relationship between DNA-PK activity and drug resistance of ovarian cancer cells

--Validation of a peptide-based inhibitor of DNA-PK on lowering the growth of ovarian cancer cells following radiation treatment

Project 3:

--Generated the mutated version of p57.

--The subcellular location of wild-type and mutated p57 was determined to be nuclear.

--The mutation introduced into p57(T342A) does not stabilize the p57 protein.

--The ecdysone and tetracycline inducible systems are "leaky" and thus cannot be used to generate cells lines with either wild-type or mutated versions of the Kip1 or Kip2 genes in their genomes.

Project 4:

--Cloned multiple HE4 promoter constructs HE4p1308, p799, p652, and p395 and tested their activity using cell-based assays.

--Optimized the activity of HE4p652 in ovarian cancer cell lines.

--Determined that HE4p652 displayed greater activity in ovarian cancer cell lines compared to a non-ovarian cancer cell line.

--Obtained hTERT and OSP1 promoters.

--Tested activity of hTERT and OSP1 promoters in ovarian and other cancer cell lines.

Core A:

--Established protocols for transferring cell lines into athymic mouse hosts

--Determined baseline responses of Skov3x cells to X-irradiation

--Determined the effect of transient transfection of mutant p27 tumor take

--Established a non-invasive method for following intraperitoneal tumor growth

REPORTABLE OUTCOMES

Manuscripts

Kreklau, E. L., Limp-Foster, M., Liu, N., Xu, Y., **Kelley**, M. R. and Erickson, L. C. (2001) A novel fluorometric oligonucleotide assay to measure O^6-methylguanine DNA methyltransferase, methylpurine DNA glycosylase, 8-oxoguanine DNA glycosylase, and abasic endonuclease activities: DNA repair status in human breast carcinoma cells overexpression methylpurine DNA glycosylase. *Nucleic Acids Res.* 29(12):2558-66.

Hsieh, M.M., Hegde, V., **Kelley**, M.R. and Deutsch, W.A. (2001) Activation of APE/Ref-1 redox activity is mediated by reactive oxygen species and PKC phosphorylation. *Nucleic Acids Res.* 29(14):3116-3122.

Kelley, M. R. and Parsons, S. H. (2001) Reduction-Oxidation (Redox) regulation of the DNA repair function of the human AP endonuclease Ape1/ref-1. *Antioxid Redox Signal.* 2001 Aug;3(4):671-83

Fishel, M., Seo, Y.R., Smith, M.L. and **Kelley**, M.R. Imbalancing the DNA base excision repair pathway in the mitochondria; Targeting and overexpressing n-methylpurine DNA glycosylase in mitochondria leads to enhanced cell killing. *Cancer Research*, in press.

Lee, S-H. and Kim, C-H. (2002) DNA-Dependent Protein Kinase Complex: a Multifunctional Protein in DNA Repair and Damage Checkpoint. *Molecules and Cells* **13,** 159-166.

Abstracts

Kelley, M.R., Rinne, M., Fishel, M., Caldwell, D. and Xu, Y. Imbalancing the DNA base excision repair (BER) pathway using nuclear and mitochondrial-targeted human N-methylpurine DNA glycosylase (MPG/AAG): Sensitization of breast and ovarian cancer cells to chemotherapy. AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics, Miami, FL, Oct. 29-Nov. 2, 2001.

Michael, H., Moore, D.H. and **Kelley**, M.R. Diagnostic expression of the DNA repair/redox enzyme Ape1/ref-1 in epithelial ovarian cancers. U.S. and Canadian Academy of Pathology Annual Meeting, Chicago, IL, Feb 23-March 2, 2002.

Luo, M. and **Kelley**, M.R. Inhibition of the DNA repair activity of human apurinic/apyrimidinic endonuclease (Ape1/ref-1) by lucanthone: Enhancement

of alkylating agent cell killing in breast and ovarian cancer cells. AACR Annual Meeting, San Francisco, CA, April 6-10, 2002. (platform talk)

Hurteau, J.A., Brutkiewicz, S.A., Wang, Q., Allison, B.M., Goebl, M.G. and Harrington, M.A. (2002) Overexpression of a stabilized mutant form of the cyclin-dependent kinase inhibitor p27(Kip1) inhibits cell growth. Gynecol Oncol. 86(1):19-23.

Hurteau, J.A., Allison, B.M., Brutkiewicz, S.A., Goebl, M.G., Heilman, D.K., Bigsby, R.M. and Harrington, M.A. (2001) Expression and subcellular localization of the cyclin-dependent kinase inhibitor p27(Kip1) in epithelial ovarian cancer. Gynecol Oncol. 83(2):292-8.

CONCLUSIONS:

Project 2:

The overall goal of this proposal is to explore the role of DNA-PK in the development and progression of ovarian cancer. Since DNA-PK is a key DNA repair factor as well as involved in damage signaling pathway, levels of DNA-PK activity among breast cancer would contribute to their drug resistance and also provide the basis for selection of patients for treatment with chemotherapy drugs.

From the first two years of study, we concluded that a peptide-based inhibitor preventing DNA-PKcs from forming a complex with Ku70/Ku80 significantly lowered DNA-PK activity. Furthermore, treatment of these breast cancer cells with target peptide significantly lowered the cell growth only in the presence of ionizing radiation, indicating that the peptide-based inhibitor exhibited a positive effect of on lowering drug resistance by specifically targeting DNA-PK *in vivo*. This result also validates a physiologic role for DNA-PK in chemotherapy drug resistance of ovarian cancers.

Project 3:

An intriguing result has arisen from these studies. We were quite surprised to learn that the $p57^{Kip2T342A}$ mutant was not stablized. This result is surprising given the functional conservation between p27 and p57. These data argue that p27 and p57 phosphorylation events have distinct functions. These data suggest we need to focus our research efforts on p27 if we plan to pursue using a CDKI to gene therapeutic. Our inability to generate stable cell lines, in which either p27 or p57 can be induced suggests we will need to change our gene delivery system. We are currently pursuing the use of folate targeted liposomes.

Project 4:

The information derived has allowed us to determine that the HE4p652 candidate promoter has the potential to give the highest levels of reporter gene activity specifically in ovarian cancer cells. This kind of information can be applied to the development of our ovarian specific transgenic cassette. Ultimately, the smallest HE4 construct capable of driving high levels of ovarian cancer cell-specific expression will be used to develop our gene therapy vector. In addition, recent reports on two other gene promoters, survivin and osteopontin, suggest that these two promoters may be candidates for future investigation by our group.

For the "so what section", which evaluates the knowledge as a scientific or medical product to also be included in the conclusion of this report, we would like to point out that currently there is no animal model for epithelial ovarian cancer, which is a severe limitation both for understanding the basic biology of this devastating disease and testing novel therapeutics for epithelial ovarian cancer. It is clear that the lack of promoters to drive oncogene expression specifically in the ovarian epithelium is a key limitation, if not the major barrier, to developing a transgenic mouse model of the disease. Providing that the novel promoters identified in Project 4 are expressed in normal ovarian epithelium, it seems likely they could be used to target oncogenes specifically to the mouse ovarian epithelium and thus be useful in the future for developing a transgenic model for epithelial ovarian cancer.

Core A:

--Better control of gene expression is required before in vivo studies can be conducted with cells transfected with the mutant genes.

--Baselines are established for X-ray effects on Skov3x tumors.

--The secreted protein, ß-hCG appears to provide a feasible means of tracking intraperitoneal tumor growth.

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APPENDICES:

Lee, S-H. and Kim, C-H. (2002) DNA-Dependent Protein Kinase Complex: a Multifunctional Protein in DNA Repair and Damage Checkpoint. *Molecules and Cells* **13,** 159-166.

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Mol. Cells, Vol. 13, No. 2, pp. 159-166

Minireview

Molecules and Cells

DNA-dependent Protein Kinase Complex: a Multifunctional Protein in DNA Repair and Damage Checkpoint

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(Received February 26, 2002; Accepted March 5, 2002)

DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase that is activated upon DNA damage generated by ionizing radiation or UV-irradiation. It is a three-protein complex consisting of a 470-kDa catalytic subunit (DNA-PKcs) and the regulatory DNA binding subunits, Ku heterodimer (Ku70 and Ku80). Mouse and human cells deficient in DNA-PKcs are hypersensitive to ionizing radiation and defective in V(D)J recombination, suggesting a role for the kinase in double-strand break repair and recombination. The Ku heterodimer binds to double-strand DNA breaks produced by either DNA damage or recombination, protects DNA ends from degradation, orients DNA ends for re-ligation, and recruits its catalytic subunit and additional factors necessary for successful end-joining. DNA-PK is also involved in an early stage of damage-induced cell cycle arrest, however, it remains unclear how the enzyme senses DNA damage and transmits signals to downstream gene(s) and proteins.

Keywords: Damage Checkpoint; DNA Damage; DNA-PK; DSB Repair; Ku Complex; Nucleotide Excision Repair.

Introduction

DNA damage is constantly generated by radiation and chemotherapy drugs, which must be repaired to prevent genomic alterations that could otherwise contribute to cancer progression and/or generation of cancer. For this reason, cells invoke various mechanisms to repair DNA damage while operating damage checkpoint pathways that are responsible for sensing DNA damage and causing arrest of cell cycle progression until the damage is repaired. It is not clear, however, which genes are involved in this pathway or how DNA damage induces cell cycle arrest while permitting DNA repair. The DNA-dependent protein kinase (DNA-PK) is a member of the phosphatidylinositol-3 kinase (PI-3 kinase) family and shares amino acid sequence homology in its carboxy-terminal kinase domain with other family members, including ataxia telangiectasia mutated gene (ATM), ATM-related (ATR), and p110 PI-3 kinase (Hartley *et al.*, 1995; Poltoratsky *et al.*, 1995). All members of the PI 3-kinase family are activated by stress; PI-3 kinase is regulated by heat shock and DNA-damage, and ATM, ATR, and DNA-PK are activated by DNA damage (Gottlieb and Jackson, 1993; Lin *et al.*, 1997; Rotman and Shiloh, 1997; Yuan *et al.*, 1997). The DNA-PK is a unique damage-response element not only because it functions as a DNA damage sensor, but also it is a key player in various DNA repair pathways. In this review, we focus on recent advances on the DNA-PK in DNA repair and damage checkpoint pathways.

The DNA-PK complex and its regulation

Association of the DNA end-binding Ku70/Ku80 heterodimer with the 470 kDa serine/threonine kinase catalytic subunit forms the DNA-PK holoenzyme that is essential for double-strand break (DSB) repair by non-homologous recombination in mammalian cells. The DNA-PK catalytic subunit (DNA-PKcs) is a very abun-

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Abbreviations: ATM, ataxia telangiectasia mutated; DNA-PK, DNA-dependent protein kinase; DSB, double-strand break; JNK, c-Jun N-ternus protein kinase; NHEJ, non-homologous end joining; PCNA, proliferating cell nuclear antigen; PI-3 kinase, phosphatidylinositol-3 kinase; RPA, replication protein A.

dant nuclear protein in human cells and a very large polypeptide of 4127 amino acids giving a mass of 470 kDa (Gottlieb and Jackson, 1993). Its imaging structure indicates that DNA-PKcs has an open, pseudo 2-fold symmetric structure with a gap separating a crown-shaped top from a rounded base (Chiu *et al.*, 1998). With the exception of the 400 amino acids of the C-terminus, the amino acid sequence of DNA-PKcs shows little amino acid similarity to any protein in the databases. The C-terminus of DNA-PKcs are similar to proteins of the phosphatidylinositol (PI) 3-kinase superfamily (Hartley *et al.*, 1995; Poltoratsky *et al.*, 1995), both of which possess the DXXXXN and the DXG amino acid motifs that are found in protein kinase and are required for catalysis (Fig. 1). Despite the amino acid similarity with lipid kinases, DNA-PKcs acts as protein kinase, and no lipid kinase activity has been detected with purified DNA-PKcs or DNA-PK complex *in vitro* (Hartley *et al.*, 1995).

Ku complex, consisting of Ku70 and Ku80 subunits, serves as regulatory subunits for DNA-PK kinase activity essential for dsb rejoining (Kurimasa *et al.*, 1999). It is a very abundant nuclear protein, estimated at 4×10^5 molecules per cell and is present in vertebrates, insects, yeast, and worms (Mimori *et al.*, 1986). The Ku complex binds to DNA ends, nicks, gaps, and regions of transition between single and double-stranded structure (Blier *et al.*, 1993; Falzon *et al.*, 1993). *In vitro* photocross-linking of Ku protein bound to single DNA-strand ends showed that the preferred orientation is when Ku70 contacts the major groove and Ku80 contacts the minor groove (Yoo *et al.*, 1999). The crystal structure of the human Ku heterodimer revealed that Ku70 and Ku80 share a common topology and form a dyad-symmetrical molecule with a preformed ring that encircles duplex DNA (Walker *et al.*, 2001). The binding site can cradle two full turns of DNA while encircling only the central 3–4 base pairs. Surprisingly, Ku makes minimal contacts with DNA bases and with the sugar-phosphate backbone, however its ring structure nicely fits into major and minor groove contours in order to support its interaction with broken DNA ends and to recruit DNA-PKcs to the DNA (Walker *et al.*, 2001).

Regulation of DNA-PK activity While the activation of DNA-PK kinase requires DNA ends, its activity can be regulated by several mechanisms. Heterodimerization is not only essential for Ku-dependent DNA repair *in vivo*, but may be also a way to regulate DNA-PK activity. A central region within Ku80 is required for heterodimerization with Ku70. Evidences suggest that heterodimerization is required for the stabilization of Ku70 and Ku80. Each Ku subunit can translocate to the nucleus not only through its own nuclear localization signal but also through heterodimerization with each other, suggesting that the heterodimerization of these Ku subunits is important for their nuclear entry (Gell and Jackson, 1999; Koike *et al.*, 1999; 2001; Osipovich *et al.*, 1999). *In vitro* work showed that DNA-PK kinase activity involving DNA-PKcs controls Ku entry into DNA which may regulate DNA transactions including transcription in the vicinity of double-strand breaks (Frit *et al.*, 2000).

Phosphorylation of both DNA-PKcs and Ku subunits (Ku70 and Ku80) was observed *in vivo* and *in vitro*. Phosphorylation of either DNA-PKcs or Ku subunit resulted in inactivation of the serine/threonine protein kinase activity, suggesting that protein phosphorylation is a way to regulate DNA-PK protein kinase activity (Chan and Lees-Miller, 1996; Douglas *et al.*, 2001) and that the protein phosphatase responsible for reactivation *in vivo* is a PP2A-like enzyme (Douglas *et al.*, 2001). Post-translational regulation of DNA-PK kinase activity may also occur through modulation of Ku70/80 DNA-binding activity observed in human neoplastic tissues, suggesting a possible role for heterodimer activity in tumor development (Pucci *et al.*, 2001). Also, irradiation induces the expression of Ku70, but not Ku80, in both p53 and ATM-dependent manner, providing cells with means of assuring proper DNA repair and/or cellular response to DNA damage (Brown *et al.*, 2000).

Role in DSB repair

DSBs are deleterious and potentially lethal form of DNA damage because they physically disrupt the continuity of the genome. Accordingly, eukaryotic cells have evolved two distinct DSB repair pathways. One pathway is homologous recombination repair (HRR) that predominantly occurred in lower eukaryotes such as *S. cerevisiae* and is mediated by Rad51 and Rad52 family proteins. The other pathway is non-homologous end joining (NHEJ) that is a major DSB repair pathway in mammals, at least in G0 or G1-phase of the cell cycle (Lee *et al.*, 1997). The NHEJ machinery consists of the DNA-PK complex and a complex of XRCC4 and DNA ligase IV. Although both HRR and NHEJ require the Rad50/Mre11/NBS1 complex in yeast and presumably also in mammalian cells (Critchlow and Jackson, 1998), it is unclear how DSB repair proteins are coordinated at the damage site. The current model of DNA-PK complex activation by dsDNA break is based on the tenet that without DNA, DNA-PKcs is inactive and incapable of binding Ku complex (Suwa *et al.*, 1994). When a DSB is introduced, Ku binds to the DNA because of its high affinity for DNA ends. The binding of Ku elicits conformational changes that allow it to bind DNA-PKcs. Thus the presumed roles of Ku complex are to first bind to DNA and then recruit DNA-PKcs to the DNA. Ku may also serve as an alignment factor that not only increases NHEJ efficiency but also the accuracy. Furthermore, a secondary NHEJ activity is present in the absence of Ku, which is error-prone and possibly assisted by base

pairing interaction (Feldmann *et al.*, 2000). Upon the assembly of DNA-PK holoenzyme on DNA breaks, this DNA repair complex activates its serine/threonine protein kinase activity and phosphorylates target substrates that colocalize with it on the ends of broken DNA.

The assembly and activation of the DNA-PK complex at a DNA strand break is central to NHEJ, although the *in vivo* study suggests that Ku complex but not DNA-PKcs may be essential for DSB repair (Gu *et al.*, 2000). The Ku complex and DNA-PKcs bind to DNA ends and are capable of physically tethering two ends of DNA molecules (Cary *et al.*, 1997; Yaneva *et al.*, 1998). Low resolution structures of DNA-PKcs revealed that DNA-PKcs has a cage-like structure with channels and cavities within the interior of the structure (Chiu *et al.*, 1998; Leuther *et al.*, 1999). Nonetheless, it is still unclear how the Ku/DNA complex activates the kinase activity of DNA-PKcs during DSB repair. One hypothesis is that DNA-PKcs undergoes a conformational change upon association with the Ku/DNA complex and this conformational change accounts for the activation of kinase activity. The kinase activity associated with DNA-PK is needed for DSB repair *in vivo*, since expression of a kinase-dead form of DNA-PKcs fails to complement the radiosensitive phenotype of a mammalian cell line that lacks the DNA-PKcs protein (Kurimasa *et al.*, 1999). However, the physiological targets of DNA-PK *in vivo* are unknown. The DNA-PK complex can physically tether two ends of a DSB in close proximity *in vitro* suggesting the hypothesis that the DNA-PK complex acts as a scaffold to assemble the NHEJ pathway proteins at a DSB (Cary *et al.*, 1997). In support of this, Ku recruits XRCC4/LigaseIV to DNA termini (McElhinny *et al.*, 2000), and Ku binds to MRE11/Rad50/NBS complex (Goedecke *et al.*, 1999). In *S. cerevisiae*, NHEJ of plasmid DSBs requires Ku, Xrcc4, and DNA ligase IV, as well as Mre11, Rad50, Xrs2, and DNA damage checkpoint proteins. In *S. pombe*, however, DSB repair is dependent on Ku complex and DNA ligase IV, but does not require Rad32, Rad50 (the *S. pombe* homologues of Mre11 and Rad50, respectively) and checkpoint proteins for NHEJ, suggesting that NHEJ pathway in eukaryotes may occur in more than one mechanism (Manolis *et al.*, 2001).

Other repair pathways DNA-PKcs-deficient cells exhibited sensitivity not only to ionizing irradiation but also in response to chemotherapy drugs and were associated with lower nucleotide excision repair activity, suggesting that DNA-PK may be involved in the repair of various types of DNA damage (Britten *et al.*, 1999; Frit *et al.*, 1999). Also, studies with drug-resistant or drug-sensitive cancer cells suggested that higher levels of DNA-PK expression lead to a drug-resistant cells, whereas the low DNA-PK activity was associated with cells with drug-sensitive phenotype (Shen *et al.*, 1997) and was linked to cell death via the accumulation of damaged DNA. More than 20-fold overexpression of the catalytic subunit of DNA-PK (DNA-PKcs) was observed with a drug-resistant cancer cells (Muller and Salles, 1997; Shen *et al.*, 1997). The Ku complex also interacts with a key base excision repair enzyme, AP-endonuclease (Chung *et al.*, 1996), suggesting a possible involvement of DNA-PK complex in various DNA repair pathways. It is not clear however whether the Ku or DNA-PK complex has a direct role in these repair pathways.

Role in damage checkpoint

Upon DNA damage, DNA-PK phosphorylates a number of proteins, including p53, RNA-polymerase II, RPA, topoisomerases, hsp90, SV-40 large T antigen, and many transcription factors such as c-Jun, c-Fos, oct-1, sp-1, c-Myc, TFIID, and many more. Among them, p53 is one of the legitimate targets for damage-induced cell cycle arrest. The p53 deficiency prolongs the survival of DNA-PKcs-deficient cells harboring DNA damage by allowing the accumulation of aneuploid cells, suggesting that the DNA-PKcs mutation links to a p53-mediated DNA damage checkpoint (Guidos *et al.*, 1996). The *in vivo* role of DNA-PK in the transduction of the DNA damage signal to p53 remains unclear (Jimenez *et al.*, 1999; Woo *et al.*, 1998), however, DNA-PK along with ATM and ATR collectively detect DSBs and transmit this signal to p53 by phosphorylation. This phosphorylation dissociates p53 from its negative regulator, mdm2, which allows p53 undergo further modification and activate transcription of the genes responsible for cell cycle arrest such as p21waf1/cip1. Both control and DNA-PKcs-null murine embryonic fibroblast cells showed phosphorylation and accumulation of p53 in response to irradiation. However, the rise in p21cip1/waf1 and mdm2 was found to be delayed and attenuated in DNA-PK-deficient cells, which correlated in time with delayed onset of G1/S arrest by flow cytometric analysis, suggesting that loss of DNA-PK activity appears to attenuate the kinetics of p53 to activate downstream genes, implying that DNA-PK plays a role in post-translational modification of p53, without affecting the increase in levels of p53 in response to DNA damage (Kachnic *et al.*, 1999).

Another potentially important role for DNA-PK in damage signaling stems from a functional interaction with c-Abl in response to DNA damage (Kharbanda *et al.*, 1997; Kumaravel *et al.*, 1998). Ionizing radiation stimulates the binding of c-Abl to DNA-PK and induces the association of c-Abl with Ku antigen (Jin *et al.*, 1997a; Kharbanda *et al.*, 1997). DNA-PK phosphorylates c-Abl *in vitro* and activates its tyrosine kinase activity. Moreover, via a potential feedback mechanism, c-Abl phosphorylates DNA-PK, which can then no longer form a complex with DNA

(Kharbanda *et al.*, 1997). Furthermore Ku associates with c-Abl and p21cip1/waf1 after irradiation (Kumaravel *et al.*, 1998), and interestingly, the p21cip1/waf1 only transiently associates with Ku complex at the low dose of irradiation, suggesting a possible role for DNA-PK in the damage-checkpoint pathway to control downstream DNA metabolism. The DNA-PKcs and Ku70/Ku80 heterodimer also can associate with another stress-response protein, the c-Jun N-terminus protein kinase (JNK), and the interaction was significantly stimulated following DNA damage. DNA-PKcs-proficient cells compared to those lacking DNA-PKcs exhibited a tight regulation of JNK activation in response to genotoxic stress. Based on the observation that the DNA-PK phosphorylates JNK *in vitro*, it is possible that DNA-PK is involved in regulation of JNK signaling pathway in response to stress/DNA damage (Park *et al.*, 2001).

G1/S-phase arrest Mounting evidences point to the role of DNA-PK in damage-induced S-phase arrest. Both *in vivo* and *in vitro* studies suggested that DNA-PK kinase activity is necessary for damage-induced replication arrest and its reversal (Park *et al.*, 1999; Wang *et al.*, 1999; 2001). Damage-induced S-phase arrest was reversed *in vitro* by the addition of a DNA-PK inhibitor or by immunodepletion of DNA-PKcs, suggesting that DNA-PKcs may be directly involved in damage-induced S-phase arrest through modulation of replication protein(s) (Park *et al.*, 1999; Wang *et al.*, 1999). Replication protein A (RPA) is a trimeric, multifunctional protein complex involved in DNA replication, DNA repair, and recombination and is a likely target for DNA-PK in damage-induced S-phase arrest because phosphorylation of the RPA2 subunit is observed after exposure of cells to ionizing radiation. DNA-PKcs interacted directly with RPA, and causes RPA2 phosphorylation in response to DNA damage. Phosphorylated RPA has a higher affinity for nuclear structures than unphosphorylated RPA suggesting the modified protein may be involved in the regulation of DNA replication after DNA damage or in DNA repair. RPA phosphorylation is delayed in cells deficient in ATM or DNA-PK, suggesting that DNA-PK and ATM may cooperate to phosphorylate RPA after DNA damage to redirect the functions of the protein from DNA replication to DNA repair (Wang *et al.*, 2001). DNA-PK-mediated RPA phosphorylation requires ongoing DNA replication but is prevented by the cell-cycle checkpoint abrogator, suggesting that the replication fork-associated RPA, when encounters DNA double-strand end-associated DNA-PK, leads to RPA phosphorylation which may signal the presence of DNA damage to an S-phase checkpoint mechanism (Shao *et al.*, 1999). Phosphorylation of RPA in yeast is also dependent on the central checkpoint regulator Mec1p. Because RPA's ssDNA binding activity of the RPA heterotrimer and is required for DNA replication, repair and recombination, it is possible that phosphorylation of this subunit is directly involved in modulating RPA activity during the checkpoint response (Brush and Kelly, 2000).

DNA damage induces the change in other repair/replication proteins that also affects DNA repair and/or G1/S-phase cell cycle arrest. The proliferating cell nuclear antigen (PCNA) forms specific foci in response to DNA damage and an increased interaction of PCNA with the Ku heterodimer after DNA damage, suggesting a role for PCNA in the NHEJ repair pathway of DNA strand breaks (Balajee and Geard, 2001). Similarly, Ku80-deficient cells showed a significant decrease in the amount of PCNA associated with chromatin following ionizing radiation, suggesting that the Ku-complex by binding at the ends of damaged DNA may also protect the key replication factors from dissociating from chromatin (Park *et al.*, submitted). The Ku (or DNA-PK complex) also associates with ors8 and ors12 and this association was significantly enhanced in cells synchronized at the G1/S border. In addition, *in vitro* DNA replication activity with the use of extracts from Ku80-deficient cells was significantly lower compared to that with wild-type cell extracts, suggesting a novel function for Ku that may act at the initiation step of DNA replication and dissociates after origin firing (Novac *et al.*, 2001).

G2 arrest In addition to its role in damage-induced G1/S-phase arrest, DNA-PK may also have a distinct role in G2 checkpoint traversal in response to DNA damage since its activity is required for exit from a DNA damage-induced G2 checkpoint arrest (Lee *et al.*, 1997). This finding is supported by the observation that the activity of the DNA-PK complex is regulated in a cell cycle-dependent manner peaking its activity at the G1/ S boundary and at G2, which is suggestive of the involvement of DNA-PK at multiple checkpoints (Lee *et al.*, 1997). Ku80-deficient cells compared to wild-type cells were highly cytotoxic to various stress agents, which led to their long-term accumulation in the G2 phase. This differential response was not due to differences in DNA repair, since DNA damage was repaired with comparable efficiency in both wild-type and Ku80-deficient cells, but was associated with differences in the expression of important cell cycle regulatory genes, supporting the notion that Ku80-mediated cytoprotection and G2-progression are not only dependent on DNA repair but may also reflect its influence on other cellular processes (Arrington *et al.*, 2000).

Other DNA metabolism

Besides their role in DNA repair, Ku complex also binds to chromosome ends (telomeres) protecting them from

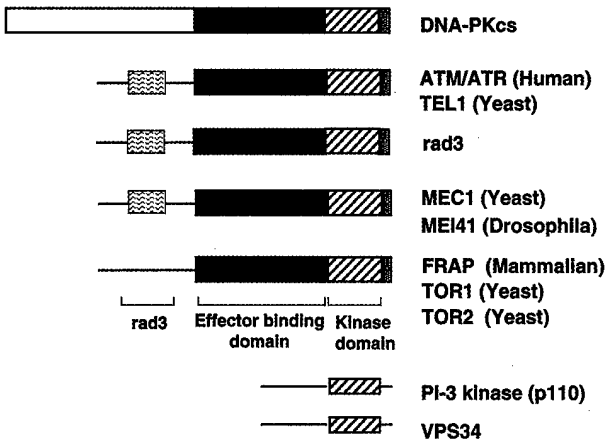

Fig. 1. DNA-PKcs and the related PI-3 kinase family among eukaryotes.

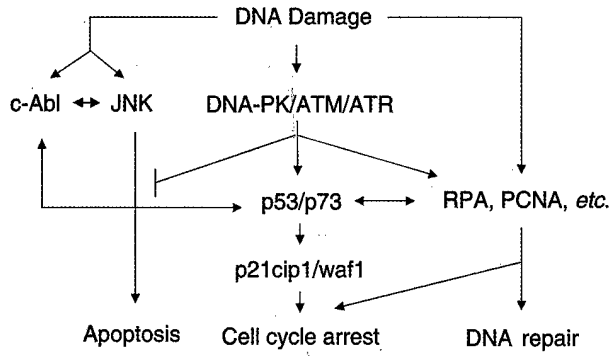

Fig. 2. Involvement of DNA-PK in damage-induced cell cycle arrest and DNA repair.

telomeric shorting and end-to-end fusions. Inactivation of Ku70 or Ku80 in mouse yields telomeric shortening in various primary cell types at different developmental stages and showed failure to proliferate in culture and show signs of premature senescence, suggesting that chromosomal instability of Ku-deficient cells results from a combination of compromised telomere stability as well as defective NHEJ (d'Adda *et al.*, 2001; Featherstone and Jackson, 1999). Localization of Ku to the telomere in yeast does not depend on the DNA-dependent protein kinase catalytic component, indicating that Ku complex not DNA-PK kinase activity is involved in telomere maintenance (Hsu *et al.*, 1999). In mammals, although not a significant change in telomere length or in deregulation of the G-strand overhang at the telomeres, DNA-PKcs cells display an increased frequency of spontaneous telomeric fusions and anaphase bridges, suggesting that DNA-PKcs or DNA-PK complex may have a role in telomeric end-capping (Bailey *et al.*, 1999; Goytisolo *et al.*, 2001; Song *et al.*, 2000). DNA-PK associates with the RNA polymerase I and II transcription complexes and likely negatively regulates them (Anderson, 1993; Bryntesson *et al.*, 2001; Chibazakura *et al.*, 1997; Kuhn *et al.*, 1995; Labhart, 1995; Peterson *et al.*, 1995). Ku recruits Werner syndrome protein (WRN) to DNA. Moreover, Ku complex stimulates and alters WRN exonuclease activity, suggesting that Ku-mediated activation of WRN exonuclease activity may play an important role in a cellular pathway that requires processing of DNA ends (Li and Comai, 2001). Ku also interacts with clusterin, a protein related to apoptosis signaling. Overexpression of nuclear clusterin in nonirradiated cells dramatically reduced cell growth with increased G1 checkpoint arrest and increased cell death, implicating a role for DNA-PK in controlling apoptosis (Jackson, 1996; Jin *et al.*, 1997b; McConnell and Dynan, 1996; Wang *et al.*, 2000; Yang *et al.*, 2000).

Concluding Remarks

DNA-PK is a unique protein kinase that is not only essential for DSB repair but also involved in damage checkpoint pathway in eukaryotes. For that reason, DNA-PK has long been a suspected factor involved in sensing and transmitting DNA damage signals to the downstream targets such as p53 and RPA, which eventually contribute to damage-induced cell cycle arrest and DNA repair (Anderson, 1993; Jackson, 1996). On the other hand, the observation that DNA damage induces the interaction of Ku complex with PCNA (Balajee and Geard, 2001) may indicate a potential role for DNA-PK in mediating DNA repair and replication in response to DNA damage (Fig. 2). The Ku complex seems to play a role in keeping PCNA and other replication proteins associated with the chromatin following DNA damage, which may be essential for subsequent DNA replication following the repair of damaged DNA and cell survival (Fig. 2; Park *et al.*, submitted).

JNK activation and its signaling pathway are linked to the induction of cell cycle arrest and DNA repair. Prolonged activation of JNK, however, can lead to apoptosis (Fig. 2; Auer *et al.*, 1998; Johnson *et al.*, 1996; Sanchez-perez *et al.*, 1998; Widmann *et al.*, 1997), suggesting that the JNK pathway may mediate either cell protection or programmed cell death following DNA damage. These lines of evidences implicate a role for DNA-PK in protecting cells from apoptosis (Canman *et al.*, 1994; Lees-Miller *et al.*, 1995; Park *et al.*, 1999). In this regard, it is obvious why DNA-PK is the primary target for the CPP-32 proteosome (Song *et al.*, 1996).

Acknowledgments This work was supported by grants from the National Institute of Health (CA92111) and the U.S. Army (DAMD17-00-1-0295 and DOD-OC000113).

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Gynecologic Oncology 83, 292–298 (2001)
doi:10.1006/gyno.2001.6376, available online at http://www.idealibrary.com on IDEAL®

Expression and Subcellular Localization of the Cyclin-Dependent Kinase Inhibitor $p27^{Kip1}$ in Epithelial Ovarian Cancer[1,2]

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Received December 6, 2000

Objective. To determine if $p27^{Kip1}$ expression was altered in epithelial ovarian cancers as compared to normal ovarian surface epithelial (NOSE) cells and to determine if subcellular localization of $p27^{Kip1}$ was an important feature.

Methods. Thirteen tumor samples (1 Stage IC [early] and 12 Stage III/IV [advanced]) from patients with epithelial ovarian cancer and five NOSE samples were evaluated. Samples were surgically dissected to obtain an enriched population (90%) of cancer cells. The level of $p27^{Kip1}$ protein expression was determined by Western blot analysis. Actin was used as a loading control, and results were quantified by scanning densitometry using the ratio of the $p27^{Kip1}$ signal to the actin signal for comparison. To evaluate the subcellular localization of $p27^{Kip1}$, immunocytochemical staining was performed. Clinical pathological parameters were correlated to nuclear $p27^{Kip1}$ staining to establish if any association existed.

Results. When comparing the expression of $p27^{Kip1}$ between NOSE and ovarian cancer samples, only 2 of 13 ovarian cancer samples had altered $p27^{Kip1}$ expression. No correlation was found between the expression level of $p27^{Kip1}$ on Western blot and clinical pathological correlates. While no correlation between expression level of $p27^{Kip1}$ and subcellular localization was found, decreased nuclear staining (1+) was associated with shorter survivals using the log-rank test ($P < 0.001$). More importantly, in all tumor samples examined under the microscope, no nuclear $p27^{Kip1}$ staining was noted in cells that were undergoing mitosis.

Conclusions. $p27^{Kip1}$ protein degradation may not be modified in ovarian cancer cells undergoing mitosis. Altered expression of $p27^{Kip1}$ is not an overwhelming feature in certain epithelial ovarian cancers. Decreased nuclear staining of $p27^{Kip1}$ is associated with poor survival in some epithelial ovarian cancers.

Key Words: ovarian cancer; $p27^{Kip1}$; cyclin-dependent kinase inhibitor.

INTRODUCTION

Cell cycle progression is regulated by proteins called cyclin-dependent kinases (CDKs) that are activated by binding to subunits known as cyclins and inhibited by proteins called CDK inhibitors (CDKIs) [1–3].

CDK activity, and therefore cell cycle progression, is controlled by two distinct families of CDKI proteins [1, 4]. The first proteins to be defined as CDKIs were the Ink4 family (also known as MTS1 or p16), which includes p15, p18, and p19. The second family of CDKI proteins are structurally related and referred to as the Kip/Cip family; they include $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ [5, 6].

$p27^{Kip1}$ is a CDKI checkpoint protein that inhibits cell growth and is encoded by the *KIP1* gene [6–9]. $p27^{Kip1}$ functions at the nuclear level by binding to and inhibiting cyclin/CDK complexes. Although it appears that $p27^{Kip1}$'s major target is the cyclin E/CDK2 complex, it has also been shown to inhibit cyclin A and cyclin D containing complexes [6–9].

$p27^{Kip1}$ protein levels are regulated by translation controls, degradation of the $p27^{Kip1}$ protein by the ubiquitin/proteasome pathway, and noncovalent sequestration [6–10]. Recently, a protein encoded by the *Jab 1* gene was found to interact with $p27^{Kip1}$ and translocate the $p27^{Kip1}$ protein from the nucleus to the cytoplasm, thereby decreasing the amount of $p27^{Kip1}$ in the cell by accelerating its degradation [11]. Thus it is likely that much of the control of $p27^{Kip1}$ protein levels is at the point of protein degradation [10].

Reduced expression of $p27^{Kip1}$ has been shown to predict poor survival of patients with breast, colorectal, gastric, and prostate carcinomas [12–14]. In epithelial ovarian cancer, five studies on $p27^{Kip1}$ have been published and two further studies have been presented in abstract form [15–21]. Five of these seven studies have shown that reduced expression of $p27^{Kip1}$ is associated with a poorer prognosis. Thus, loss of $p27^{Kip1}$ may be a contributory factor to oncogenesis and tumor progression. Furthermore, $p27^{Kip1}$ has been implicated as a regulator of drug resistance in solid tumors and it is suggested that targeting

[1] This study was supported by the Eva Metzger Gynecologic Cancer Foundation and Indiana University Showalter Foundation Awards.

[2] Presented in part at the 90th annual meeting of the American Association for Cancer Research, Philadelphia, Pennsylvania, April 1999, and the annual meeting of the Society of Gynecologic Investigators, Chicago, Illinois, March 2000.

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0090-8258/01 $35.00

TABLE 1
Immunocytochemical Staining, Densitometry Readings from Western Blots, and Clinical Parameters of 13 Patients with Ovarian Cancer[a]

Clinical parameters								p27		
#	Age (years)	Stage	Histology	Grade	Residual disease	Survival (months)	Current status	Sn	Sc	p27/actin ratio W
OC1	75	IIIC	Pap. serous	3	Suboptimal	12	DOD	2+	3+	0.65
OC2	57	IV	Pap. serous	3	Suboptimal	1	DOD	1+	3+	0.87
OC3	80	IIIC	Pap. serous	3	Suboptimal	17	DOD	3+	3+	0.76
OC4	51	IV	Pap. serous	3	Suboptimal	27	DOD	2+	3+	3.64
OC5	75	IIIC	Pap. serous	3	Optimal	26	DOD	3+	3+	0.20
OC6	51	IIIC	Pap. serous	2	Optimal	10	DOD	2+	3+	5.74
OC7	54	IV	Pap. serous	2	Suboptimal	14	DOD	1+	3+	1.27
OC8	62	IIIC	Pap. serous	2	Suboptimal	29	NED	2+	3+	0.91
OC9	43	III	Undifferentiated	3	Suboptimal	6	DOD	1+	3+	0.30
OC10	52	IV	Pap. serous	3	Suboptimal	26	DOD	2+	3+	0.98
OC11	45	IIIC	Clear cell	3	Suboptimal	20	NED	3+	3+	
OC12	69	IIIC	Pap. serous	2	Suboptimal	16	AWD	2+	2+	1.13
OC13	72	IC	Clear cell	3	Optimal	32	NED	3+	3+	1.87
NOSE										1.30

[a] Pap. serous, papillary serous; optimal, residual disease less than 1 cm; suboptimal, residual disease more than 1 cm; Sn, nuclear staining; Sc, cytoplasmic staining; W, Western densitometry reading and p27/actin ratio; NOSE, normal ovarian surface epithelium; OC, ovarian cancer; DOD, dead of disease.

p27^{Kip1} may be useful as a chemosensitizer in conjunction with conventional cytotoxic chemotherapy [22].

In these preliminary studies, surgically dissected enriched epithelial ovarian cancer cells were analyzed to determine the level of p27^{Kip1} expression as compared to the expression level of p27^{Kip1} in normal ovarian surface epithelium (NOSE). This is the first study to quantify the level of p27^{Kip1} expression in NOSE and compare this to the level of expression of p27^{Kip1} in enriched epithelial ovarian cancer samples. We also wanted to determine if subcellular localization of p27^{Kip1} was lost or altered in the same tissue samples. Since the site of p27^{Kip1} action is thought to be nuclear, we were interested in learning if, when p27^{Kip1} levels were elevated, this would be manifested in an alteration in p27^{Kip1} subcellular localization. We hypothesized that since p27^{Kip1} is an inhibitory cell cycle checkpoint protein, p27^{Kip1} levels would not be altered in epithelial ovarian cancer cells compared to NOSE cells.

MATERIALS AND METHODS

Tissue sample isolation. Thirteen tumor samples (1 Stage IC [early] and 12 Stage III/IV [advanced]) from patients with epithelial ovarian cancer who underwent surgery at Indiana University Hospital from 1995 to 1998 were evaluated (Table 1). To enrich the tumor samples, thereby decreasing the amount of contaminating inflammatory cells that could interfere with the expression levels of p27^{Kip1}, unfixed tumors obtained directly from the operating room were immediately surgically dissected by a pathologist. One cubic centimeter, or smaller, fragments of tumor were directly quick-frozen in liquid nitrogen. Unstained 10-μm-thick sections were cut onto charged slides from this frozen material for use in these experiments. A second portion of the tumor specimen was formalin-fixed and paraffin-embedded for routine and immunocytochemical staining. All samples were prepared and stored in the tumor bank at Indiana University Medical Center in accordance with institutional review board policy. All tumor samples were confirmed to be epithelial ovarian cancer by hematoxylin and eosin staining (H+E). Only samples with more than 90% of cells confirmed to be epithelial ovarian cancer in origin, by H+E staining, were used for study. Samples in which fewer than 90% of cells were found to be epithelial ovarian cancer cells underwent further surgical dissection to obtain an enriched population (90%) of cancer cells. If this ratio was not achieved, the sample was not considered for study. This accounts for the low number of samples used in this preliminary study from about 60 screened.

Five NOSE samples were obtained from patient ovaries that had undergone surgery for benign conditions in accordance with institutional review board protocols. Briefly, the surface epithelium was gently scraped from the ovarian stroma and grown in culture. Cells were transferred aseptically and suspended in culture media (1:1 mixture of MCDB 105 and M 199; Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum. The cells were allowed to grow in short-term monolayer cultures until confluent for 24 h to mimic the senescent nature of NOSE cells. An aliquot was grown on slides for staining purposes and the

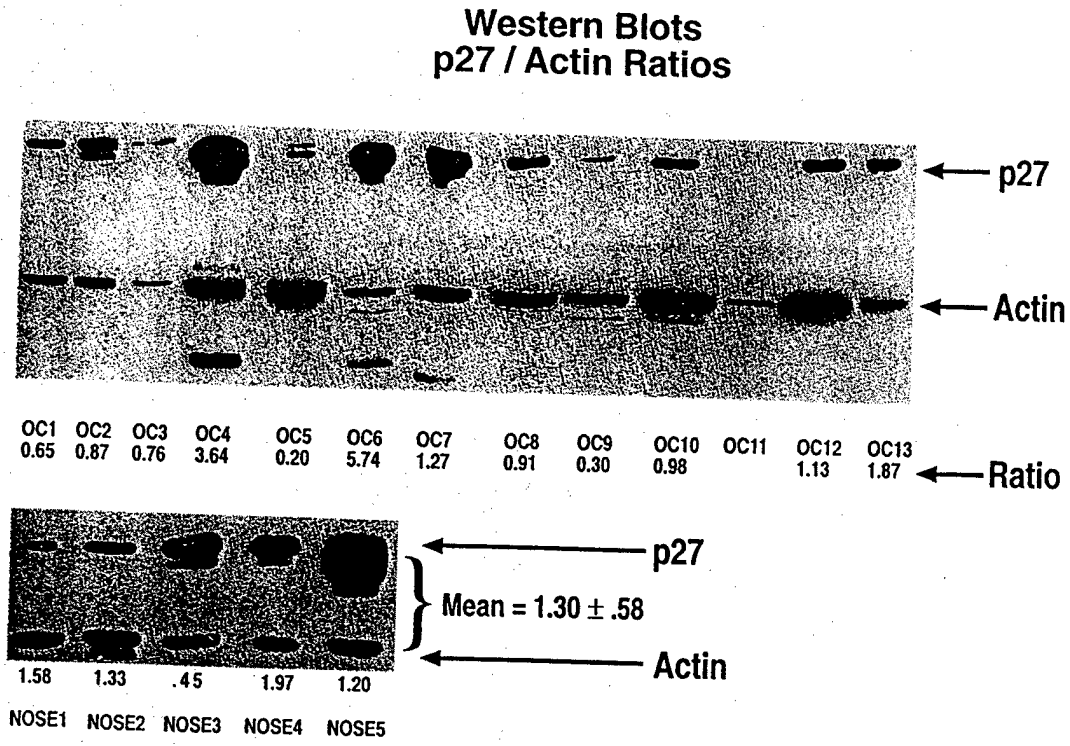

FIG. 1. $p27^{Kip1}$ Western blot analysis of 13 epithelial ovarian cancer samples (OC 1–13) and 5 normal ovarian surface epithelial samples (NOSE). $p27^{Kip1}$ densitometry readings were normalized to actin loading controls to obtain the $p27^{Kip1}$/actin ratio. NOSE mean with SD is indicated.

growth media was removed; slides were washed, air-dried, and stored at −80°C.

Validation of control tissue samples. Cells were immunocytochemically stained with anticytokeratin AE1/AE3 antibodies (500 μg/0.5 ml diluted 1:200, mouse monoclonal) (Boehringer Mannheim Biochemicals, Indianapolis, IN) to confirm their epithelial origin. Purified mouse immunoglobulin (Ig) specific for nonhuman tissue (Coulter Immunology, Hialeah, FL) was used as a negative control. Immunocytochemical staining was performed with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) as described previously [23]. The epithelial ovarian cancer cell line SKVO3 was used as positive control for confirmation of epithelial origin. Vimentin antibodies (1:100 dilution mouse monoclonal) (Novocastra/Vector Laboratories) were used to assess possible stromal contamination of the epithelial cultures. The slides were developed for 4 min with enzyme substrate diaminobenzidine (0.5% diaminobenzidine in 0.05% Tris buffer and 0.6% hydrogen peroxide). Slides were rinsed in water, dehydrated, and mounted.

Western blot analysis. Sodium dodecyl sulfate–polyacrylamide electrophoresis was performed using a discontinuous system consisting of 12% separating gels with 4.8% stacking gel according to the method of Laemmli [24]. Lanes were loaded with 25 μg of protein from the total cell lysate of each tissue extract. Hela cell extract (15 μg) was used as a positive control as well as a molecular weight standard. For Western blotting, the gels were transferred to nitrocellulose membranes at 4°C (25 V) overnight, using a Tris-glycine buffer with 20% methanol. At the completion of transfer, blots were air-dried and stored at 4°C prior to immunoblotting.

Prior to immunoblotting, membranes were stained with Ponceau S stain to locate molecular weight standards. The membranes were blocked with 3% nonfat dried milk in TBS-T (10 mM Tris, pH 7.4, 50 mM NaCl, 0.1% Tween 20) for 1 h at room temperature (RT), rinsed, and incubated with primary antibodies for 1 h at RT. Membranes were rinsed with PBS-T (phosphate-buffered saline [PBS] and 0.1% Tween 20) and incubated with horseradish peroxidase-labeled sheep anti-mouse IgG in blocking solution at a dilution of 1:5000 for 1 h at RT. $p27^{Kip1}$ antibody (mouse monoclonal) (Transduction Laboratories, Lexington, KY) was added to a concentration of 0.1 μg/ml. Actin was used as the loading control, and results were quantified by scanning densitometry using the ratio of the $p27^{Kip1}$ signal to the actin signal for comparison within experimental groups. Scanning densitometry for quantitation of protein bands was performed using enhanced chemiluminescence (ECL) with the automated digitizing system UN-SCAN-IT gel (Silk Scientific, Orem, UT). For NOSE, the five $p27^{Kip1}$/actin ratio values were averaged with SD and used as a measure of

basal expression (Fig. 1). Samples with ECL readings 3.0 times above or below the averaged NOSE value were considered to have altered expression of p27^{Kip1} (Fig. 1, Table 1). The cutoff level of 3.0 was based on SDs of samples used in the analysis and determined to be significant by our biostatistician (DKH).

Immunocytochemical staining. Immunocytochemical detection for subcellular localization of p27^{Kip1} was performed using the DAB detection kit (Ventana Medical Systems, Tucson, AZ). Immunocytochemical staining for the p27^{Kip1} proteins was performed on samples in paraffin-embedded sections. Briefly, tumor sections were deparaffinized and blocked with 3% hydrogen peroxide in methanol, followed by incubation with normal horse serum (1:20 dilution) in 0.1 M PBS at pH 6.0 and incubated overnight with monoclonal p27^{Kip1} antibody (Transduction Laboratories) diluted 1:1000 (0.25 μg/ml) in PBS. Slides were incubated with biotin-labeled anti-mouse IgG and preformed avidin-biotin-peroxidase complex (Vector Laboratories). Sections were counterstained with hematoxylin, dehydrated, and mounted. To assess subcellular localization, a grading system based on the percentage of cells that exhibit any staining was used: 1+ = 0–10%; 2+ = 10–50%; and 3+ = >50% when examined in 10 high-powered fields. Staining for each compartment (nucleus vs cytoplasmic) was independent and not additive.

Statistical analysis. Patient charts were reviewed for clinical parameters that included age, tumor grade, stage, residual disease, and median survival (Table 1). Cox's proportional hazard regression analysis was used to identify the best predictors for survival and disease-free survival. Kaplan-Meier survival and disease-free survival curves were also generated. Log-rank and Wilcoxon statistics were used to assess significance. The association between discrete variables was tested using χ^2 analysis or Fisher's exact test.

RESULTS

When comparing the expression of p27^{Kip1} between NOSE (mean, 1.30 ± 0.58) and ovarian cancer samples, only 2 of 13 ovarian cancer samples (OC4 and OC6) were considered to have altered expression of p27^{Kip1} (Table 1, Fig. 1). No correlation was found between the expression levels of p27^{Kip1} on Western blot and any of the examined clinical pathological correlates (Table 1).

Survival analysis revealed that follow-up data were available on all 13 patients (mean follow-up, 18 months; range, 1–32 months). Decreased nuclear staining (1+) was associated with lower survivals using the log-rank test ($P < 0.001$) (Fig. 2). Patient samples with 1+ nuclear staining showed significantly lower survivals when compared to patient samples with 2+ or 3+ nuclear staining ($P < 0.001$). All patients with 1+ nuclear staining were dead of disease prior to reaching the mean follow-up of 18 months. Patient OC2's death at 1 month was due to disease progression and not a surgical mortality.

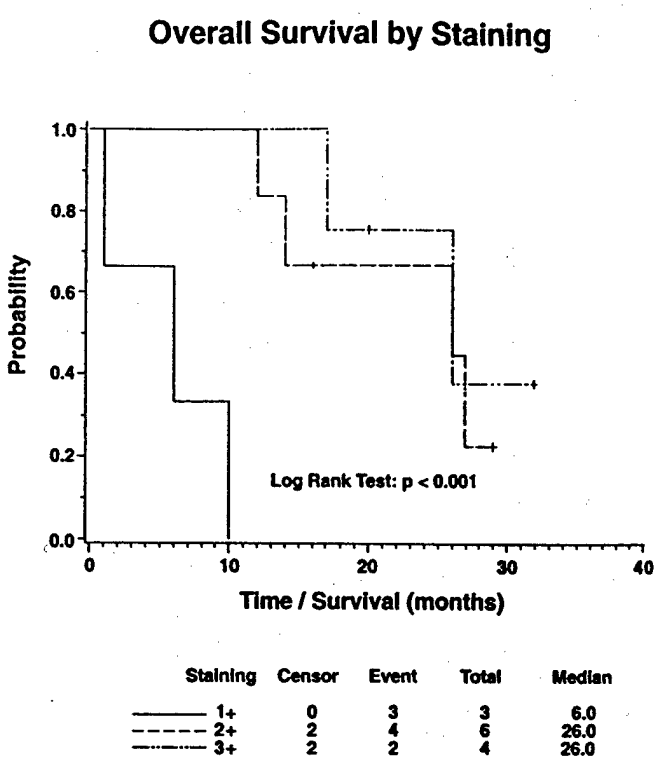

FIG. 2. Log-rank test of survival based on nuclear p27^{Kip1} immunocytochemical staining. Patient samples with 1+ nuclear staining showed significantly lower survivals when compared to patient samples with 2+ and 3+ nuclear staining ($P < 0.001$).

In evaluating subcellular localization of p27^{Kip1}, all tumor samples expressed cytoplasmic p27^{Kip1} staining at roughly the same level, indicating a fairly stable p27^{Kip1} protein pool within the cytoplasm (Table 1, Fig. 3). Most important, however, was that in all tumor samples examined under the microscope, no nuclear p27^{Kip1} staining was noted in cells that were undergoing mitosis (Fig. 3). There was no correlation between expression level of p27^{Kip1} and subcellular localization.

CONCLUSIONS

The present series is the largest thus far that has assessed p27^{Kip1} expression levels in epithelial ovarian cancer by two different and complementary techniques: immunocytochemical staining and Western blot analysis. We are aware of only seven series in the current literature that address the issue of p27^{Kip1} in epithelial ovarian cancer [15–21]. Of these studies, six used immunocytochemical staining and one used a combination of immunocytochemical staining and Western blot analysis [18].

To our knowledge, this is the only p27^{Kip1} study that has attempted to enrich ovarian tumor samples by surgical dissection to obtain a population of epithelial ovarian cancer cells

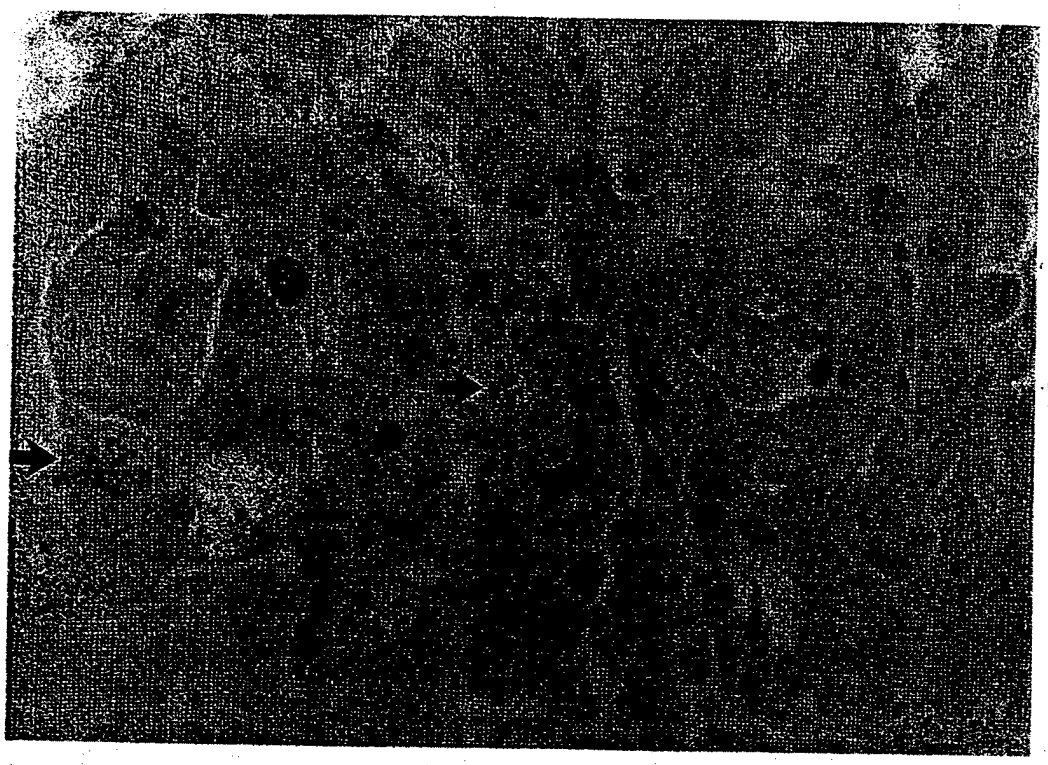

FIG. 3. Immunocytochemical staining of epithelial ovarian cancer samples for $p27^{Kip1}$. Both nuclear and cytoplasmic staining are noted. Dark arrows indicate cells in mitosis where no nuclear staining for $p27^{Kip1}$ was noted.

above 90%. This is also the first study that has quantitated $p27^{Kip1}$ levels in NOSE cells and compared these to enriched ovarian cancer samples.

Despite these technical efforts, the series herein has a small number of patients from which to draw definitive conclusions and therefore must be viewed with some caution. However, these results are important as they show that altered expression of $p27^{Kip1}$ is not an overwhelming feature of some epithelial ovarian cancers. It is probable that a selection bias was introduced because tumor samples were discarded if fewer than 90% of cells were not of epithelial ovarian cancer in origin. This may account for the fact that 83% (10 of 12) of our advanced cases were suboptimal cytoreductions. Although a recent study revealed that residual disease after initial cytoreductive surgery was not associated with $p27^{Kip1}$ expression, our samples may be indicative of a very select subset of epithelial ovarian cancers [19]. The alternative approach of no microdissection would have produced samples with an overabundance of stromal or inflammatory cells that could have altered the levels of $p27^{Kip1}$ artifactually and thereby made results suspect.

This study confirms the findings of previous investigators that decreased $p27^{Kip1}$ nuclear staining as measured by immunocytochemical means is associated with a poor survival [18–21]. With the potential for a selection bias, these results should also be interpreted with caution. We found no association between $p27^{Kip1}$ expression level on Western blot and subcellular localization. However, the small sample size may not have allowed the association to be detected even if it existed (type II error).

The observation that 2 of 13 epithelial ovarian cancer samples had altered expression of the $p27^{Kip1}$ protein was an interesting result in a population of rapidly proliferating cells. Only the nuclear component of the $p27^{Kip1}$ protein binds to and inhibits active cyclin/CDK complexes [25]. It is thought that cytosol sequestration inactivates $p27^{Kip1}$ function [25]. Therefore, activity of the $p27^{Kip1}$ protein is thought to be influenced by its subcellular localization [25]. In order to evaluate the clinical significance of subcellular localization of the $p27^{Kip1}$ protein, the 13 tumor samples also underwent immunocytochemical staining. Our goal was to determine if the increased expression of $p27^{Kip1}$ could be correlated with a difference in subcellular localization between the nucleus vs the cytoplasm.

The immunocytochemical staining of the two ovarian cancer samples that showed altered expression of $p27^{Kip1}$ on Western blot (OC4 and OC6) revealed that $p27^{Kip1}$ staining was more prominent in the cytoplasm when compared to the nucleus (Table 1). This result suggests that increased expression of $p27^{Kip1}$ may be due to a relatively stable but inactive pool of $p27^{Kip1}$ protein within the cytoplasmic cellular compartment or, alternatively, a reflection of the more rapid $p27^{Kip1}$ protein turnover within the nucleus.

The observation of altered expression of the p27^{Kip1} protein in a population of rapidly proliferating cells is not unique to this paper. Recently, high levels of p27^{Kip1} have been reported in highly proliferative human breast cancers [26]. The increased p27^{Kip1} levels may possibly indicate the existence of a yet-unknown mechanism through which malignant cells may resist the inhibitory effect of p27^{Kip1} [26]. It has been speculated that highly proliferative cells respond to a continued proliferative state by increasing the levels of the inhibitory protein p27^{Kip1} [26]. The overexpression of p27^{Kip1} is thought to be overcome by the coordinate overexpression of cyclin D/CDK4 or cyclin E. Resistance to the growth-inhibitory effects of p27^{Kip1} may also be due to a mutation in the p27^{Kip1} gene. However, to date, most studies have shown that the p27^{Kip1} gene is rarely mutated in human cancers [27]. Furthermore, recent data have demonstrated that overexpression of p27^{Kip1} can trigger apoptosis in several different human cancer cell lines [28]. The relationship between the mechanism(s) controlling p27^{Kip1} protein levels and malignant cell transformation remains to be elucidated.

The novel finding of this study was that in all tumor samples examined under the microscope, no nuclear p27^{Kip1} staining was noted in cells undergoing mitosis (Fig. 2). Although these observations are very intriguing and will require confirmation with larger sample sizes, they do suggest that high levels of p27^{Kip1} can be found in rapidly dividing cells as long as the protein is eliminated, either by degradation or cytoplasmic sequestration, prior to mitosis.

In summary, altered expression of p27^{Kip1} is not an overwhelming feature in some epithelial ovarian cancers. Decreased nuclear staining of p27^{Kip1} is associated with poor survival, and no association between expression level of p27^{Kip1} and subcellular localization was noted in this subset of epithelial ovarian cancers. The novel observation that p27^{Kip1} protein is not detected in ovarian cancer cells undergoing mitosis suggests that cells can cope with high levels of p27^{Kip1} expression as long as it can be eliminated or inactivated.

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Gynecologic Oncology 86, 19–23 (2002)
doi:10.1006/gyno.2002.6657

Overexpression of a Stabilized Mutant Form of the Cyclin-Dependent Kinase Inhibitor p27^{Kip1} Inhibits Cell Growth[1]

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Received October 19, 2001

Objective. The purpose of this study was to test the hypothesis that the expression of the mutant p27^{Kip1} protein enhances cell growth inhibition and is more stable than that of the wild-type p27^{Kip1}.

Methods. Site-directed mutagenesis was used to mutate threonine 187 to an alanine residue, generating the mutant p27^{Kip1}. To study the effects of the p27^{Kip1} mutant on cell growth, luciferase assays were performed. Cells were transiently transfected with the *Renilla* luciferase reporter construct and empty vector, wild-type p27^{Kip1}, or mutant p27^{Kip1} using Fugene 6. The transfected cells were lysed and assayed for luciferase activity 24 h later with a dual-luciferase reporter assay system. To further assess the effects of the p27^{Kip1} mutant on cell growth, colony count assays were performed. The experiments were repeated in duplicate and a standard two-tailed Student *t* test was use to analyze the data.

Results. Wild-type p27^{Kip1} protein has a half-life of approximately 2 h while the p27^{Kip1} mutant has a half-life of greater than 12 h. Furthermore, the p27^{Kip1} mutant retained the ability to inhibit CDK2-associated H1 kinase activity. Cells expressing the p27^{Kip1} mutant had an 88% reduction in luciferase activity compared to cells expressing the wild-type p27^{Kip1} ($P = 0.001$). Colony assays revealed that cells expressing the p27^{Kip1} mutant had fewer colonies compared to cells expressing the wild-type p27^{Kip1} ($P = 0.04$).

Conclusions. These data are consistent with the hypothesis that the mutated form of p27^{Kip1} is more effective in cell growth inhibition than the wild-type p27^{Kip1} protein.

INTRODUCTION

Cell cycle progression is regulated by proteins called cyclin-dependent kinases (CDKs) that are activated by binding to subunits known as cyclins and inhibited by proteins called cyclin-dependent kinase inhibitors (CDKIs) [1–3].

Cyclin-dependent kinase activity and therefore cell cycle progression is controlled by two distinct families of CDKIs [1–5]. One CDKI family is defined by the Ink4 protein and includes p15, p16, p18, and p19. Ink4 family members inhibit cell cycle progression by competing with a cyclin for binding to the cyclin-dependent kinase. A second CDKI family, initially defined by the Cip1 or p21 protein, includes Kip1 (p27^{Kip1}) and Kip2 (p57^{Kip2}) [6]. In contrast to the Ink4 family, Kip/Cip family members bind to cyclin/CDK complexes and inhibit cyclin-dependent kinase activity. The Kip/Cip CDKIs inhibit a broader range of cyclin/CDK complexes compared to the Ink4 family.

The p27^{Kip1} protein is a CDKI encoded by the *KIP1* gene [7–10]. The major targets of p27^{Kip1} are the CDK2-containing complexes; however, CDK4/6-containing complexes are also inhibited. These data suggest that p27^{Kip1}'s inhibitory effects are more global during the cell cycle compared to the other CDKIs [7–11]. Therefore, compared to other CDKIs, p27^{Kip1} is a more attractive target for molecular manipulation [12]. The p27^{Kip1} protein is degraded, for the most part, through the ubiquitin/proteasomal pathway. To be degraded, the p27^{Kip1} protein must be targeted to the proteasome through the process of phosphorylation. We hypothesized that by mutating the p27^{Kip1} protein at a critical site, thus preventing its phosphorylation and its targeted degradation, we would be able to increase its half-life and therefore its inhibitory activity.

It is not clear that overexpression of wild-type p27^{Kip1} will achieve an antiproliferative effect in ovarian epithelial cancer cells. However, support for our strategy is found in studies by Zhang *et al.* [13]. Treatment of the ovarian carcinoma cell line CA-OV3 with retinoic acid inhibited cell growth concomitant with an increase in the level of wild-type p27 protein, an effect which was mediated at the posttranscriptional level. Further support is found in the studies by Sgambato *et al.* [14] in which wild-type p27^{Kip1} was overexpressed and the growth of both normal and transformed human mammary epithelial cells was inhibited. A concern with the overexpression of the wild-type p27^{Kip1} approach as a therapeutic tool is that growth inhibition

Presented at the 32nd annual meeting of the Society of Gynecologic Oncologists, March 5, 2001, Nashville, Tennessee.

[1] This study was supported by the Eva Metzger Gynecologic Cancer Foundation and the Indiana University Showalter Foundation Awards.

[2] Supported by a grant from the National Institutes of Health, National Service Award (1 T32 HI07910).

0090-8258/02 $35.00

may be limited by the eventual decay of the cellular $p27^{Kip1}$ pool. Specifically, wild-type $p27^{Kip1}$ protein is known to have a short half-life (~2 h [11, 12]). Loss of $p27^{Kip1}$ protein would enable transduced cells to reinitiate a proliferative response. Therefore, in the proposed studies, we examined the feasibility of using the approach outlined by Sgambato *et al.* [14], direct overexpression of Kip1 to inhibit epithelial ovarian cancer cell growth. However, we modified their strategy and determined if a biologically active, stabilized form of $p27^{Kip1}$ that has an increased half-life would achieve a more prolonged period of growth inhibition. We therefore hypothesized that expression of the $p27^{Kip1}$ mutants is significantly more stable than that of their wild-type counterparts. We also hypothesized that the $p27^{Kip1}$ mutants would be significantly better at inhibition of cell growth *in vitro* and may have a greater inhibitory effect upon tumor growth in a xenograft animal model.

MATERIALS AND METHODS

DNA Manipulations and Plasmid Constructions

Site-directed mutagenesis was used to substitute threonine at position 187 to an alanine residue, as previously described, generating the mutant $p27^{Kip1}$ (Altered Sites Mutagenesis System II; Promega) [15, 16] (Fig. 1). The cDNA encoding the $p27^{Kip1}$ mutation, as well as the wild-type $p27^{Kip1}$ cDNA, was subcloned into the mammalian expression vector pcDNA3.1(−)/Myc-His A (Invitrogen Corp., San Diego, CA). Subcloning into pcDNA3.1 generated a plasmid encoding the $p27^{Kip1}$ protein containing a myc epitope, which allowed us to follow expression of the exogenous wild-type or mutant $p27^{Kip1}$.

Half-Life Determination and Western Blot Analysis

The half-lives of the wild-type and the mutated proteins were determined by Western blotting as previously described [16]. Briefly, to determine the $p27^{Kip1}$ half-life, HeyC2 cell cultures were incubated for 16 h following transient transfection and harvested by trypsinization. Equal numbers of cells were replated into 60-mm dishes. Twenty-four hours after replating, one set of cultures was harvested and nuclear extracts were prepared. The remaining cultures were treated with cycloheximide (35.5 μM final concentration). At the indicated time points, one set of cultures was harvested and nuclear extracts were prepared. Equal volumes of extracts from each sample were separated by 10% SDS–PAGE and analyzed by Western blotting with affinity-purified anti-$p27^{Kip1}$ (Transduction Laboratories, Lexington, KY). The relative amounts of $p27^{Kip1}$ were quantitated with a Bio-Rad GS 250 molecular imager. The half-lives were calculated as described previously [16]. To control for transfection efficiency, cells were cotransfected with a mammalian expression vector encoding the *lac Z* gene and Western blots containing cell lysates were probed with anti-β-galactosidase antibodies. Where indicated, blots were reprobed with anti-Cdc2 antibodies following the manufacturer's instructions (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). SDS–PAGE and Western blotting were performed as described previously [16].

Immunoprecipitations and Histone H1 Kinase Assays

To determine whether the wild-type and mutated $p27^{Kip1}$ protein are mechanistically functioning as inhibitor proteins we measured the ability of the $p27^{Kip1}$ protein (wild type and mutant) to inhibit the activity of CDK2 complexes. Lysates were prepared from *HeyC2* cells transiently transfected with pcDNA3.1 constructs encoding wild-type or mutant $p27^{Kip1}$. CDK2 antisera (Santa Cruz Biotechnology) was used to isolate CDK2-containing complexes. The ability of immunocomplexed proteins to phosphorylate histone H1 in *in vitro* kinase assays was measured.

Luciferase Assay

293 human embryonic epithelial kidney cell lines purchased from ATCC were transiently transfected with the *Renilla* luciferase reporter construct (Promega, Madison, WI) and pcDNA3.1 (empty vector), wild-type $p27^{Kip1}$ or mutant $p27^{Kip1/T187A}$ using Fugene 6 (Roche, Indianapolis, IN). The $p27^{Kip1}$ cDNAs were under control of a CMV promoter (pcDNA3.1). The transfected cells were lysed and assayed for luciferase activity 24 h later with a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's specifications.

Colony Assays

293 human embryonic epithelial kidney cell lines were transfected with pcDNA3.1 (empty vector), wild-type $p27^{Kip1}$, or mutant $p27^{Kip1/T187A}$ using Fugene 6 (Roche). Twenty-four hours after transfection, the cells were removed from the culture plate and counted and 200 cells were replated into 60-mm tissue culture dishes. After 12 days, the cell colonies were washed once with PBS and allowed to dry and fixation was performed by the addition of methanol to the dishes for 5 min. Cells were air-dried, incubated with Wright Giemsa stain for 10 min, and rinsed with water. Images of the stained colonies were obtained by using a Hitachi KP-M2U CCD camera and a Dell Optiplex GX110 computer.

RESULTS

$p27^{Kip1}$ Mutations

The cellular levels of $p27^{Kip1}$ are controlled through a variety of regulatory mechanisms, including the rate of initiation of $p27^{Kip1}$ gene transcription and translation and the rate of $p27^{Kip1}$ protein degradation [17–19]. $p27^{Kip1}$ is a substrate of the CDK2/cyclin E complex and $p27^{Kip1}$ protein phosphorylation is thought to play a role in $p27^{Kip1}$ degradation [19]. $p27^{Kip1}$ can

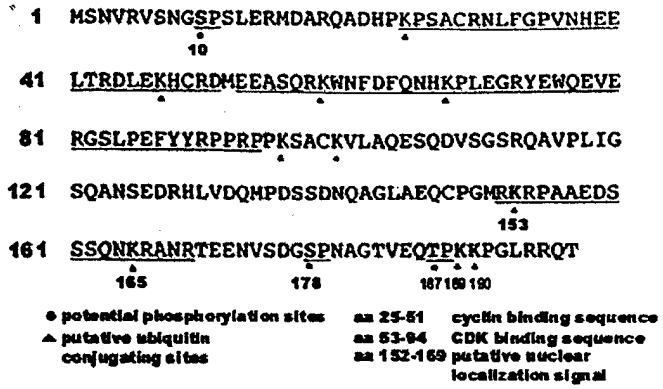

FIG. 1. The structure of the p27^{Kip1} protein. Threonine 187 lies within a CDK2/cyclin E phosphorylation site.

associate with the CDK2/cyclin E complexes to exert its inhibitory effects. Phosphorylation of p27^{Kip1} by CDK2/cyclin E-containing complexes on threonine 187 results in loss of p27^{Kip1} protein from the CDK2/cyclin E-containing complexes. Threonine 187 lies within a CDK2/cyclin E phosphorylation site [11] (Fig. 1). Therefore, the site of phosphorylation essential for the short half-life of nuclear p27^{Kip1} in ovarian cancer cells is predicted to be the threonine at position 187. Site-directed mutagenesis was used to substitute threonine 187 to alanine, generating p27$^{Kip1/T187A}$. The half-lives of the wild-type and mutated p27^{Kip1} proteins were subsequently determined (Fig. 2). While wild-type p27^{Kip1} protein has a half-life of about 2 h, the p27$^{Kip1/T187A}$ mutant has a half-life of greater than 12 h.

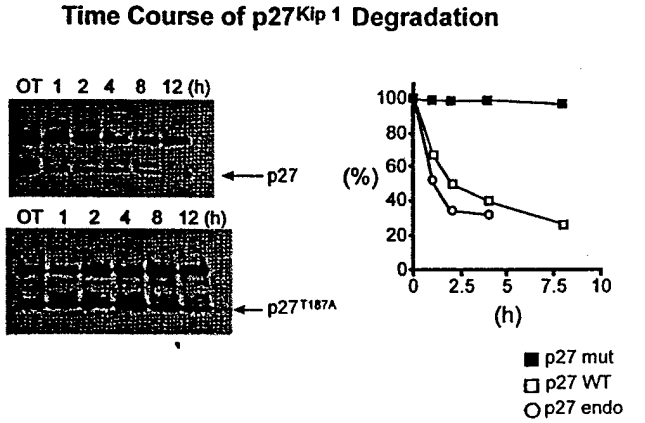

FIG. 2. The T187A mutation stabilizes nuclear p27^{Kip1} and increases its half-life. HeyC2 human ovarian carcinoma cell lines were transfected with wild-type p27 (p27 WT) and mutant p27 (p27 mut). A time course was performed with equal amounts of nuclear extract subjected to SDS–PAGE followed by Western blot analysis probed with anti-p27 antibody. To control for transfection efficiency, cells were cotransfected with the *lac Z* gene and Western blots were probed with anti-β-galactosidase antibodies. While wild-type and endogenous p27^{Kip1} (p27 endo) protein has a half-life of about 2–4 h, the p27^{Kip1} mutant was found to have a half-life of greater than 12 h.

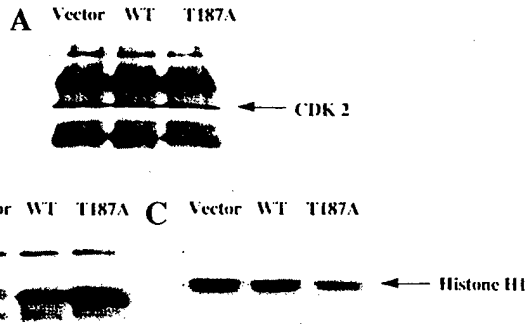

FIG. 3. The p27$^{Kip1/T187A}$ functions as a CDK inhibitor. HeyC2 human ovarian carcinoma cells were transfected with vector alone or vector containing wild-type p27^{Kip1} or p27$^{Kip1/T187A}$. Forty-eight hours later, cell lysates were prepared and subjected to immunoprecipitations using anti-Cdk2 antibody. Immunoprecipitates were used to perform *in vitro* phosphorylation assays with histone H1 as substrate (C). Immunoprecipitates were subjected to SDS–PAGE. Western blots were prepared and probed with either anti-p27 (B) or anti-Cdk2 (A).

Biological Activity of p27$^{Kip1/T187A}$ Mutant

To verify that the p27$^{Kip1/T187A}$ mutant retained its inhibitory function, we measured CDK2-associated histone H1 kinase activity in HeyC2 cells. As shown in Fig. 3, CDK2-associated histone H1 kinase activity is maintained with the p27$^{Kip1/T187A}$ mutant. We infer from these results that the p27$^{Kip1/T187A}$ mutant is a nuclear protein that has the potential to cause cell growth inhibition in epithelial ovarian cancer cells.

Luciferase Inhibitory Assay

To further address the effects of the p27$^{Kip1/T187A}$ mutant on cell function, we examined whether the p27$^{Kip1/T187A}$ mutant compared to wild-type p27^{Kip1} would compromise the efficiency of the cellular transcription/translation machinery. A decrease in *Renilla* luciferase activity could reflect a decrease in transcription and/or translation of the reporter construct. Our hypothesis was that a decrease in reporter activity would reflect a loss of cell viability. Results of these studies revealed that the cells expressing the p27$^{Kip1/T187A}$ mutant had an 88% reduction in luciferase activity, whereas in cells expressing wild-type p27^{Kip1}, the amount of luciferase activity was equivalent to that detected in cells expressing the control vector (pcDNA3.1 alone) (Fig. 4).

Colony Counts

To characterize the effects of the p27$^{Kip1/T187A}$ mutant on cell growth, cells were transfected with the control vector (pcDNA3.1) or the plasmids encoding the wild-type p27^{Kip1} or the mutant p27^{Kip1}. Twenty four hours after transfection, transfectants were harvested and replated at a density of 200 cells per 60 mm-dish. Two weeks later, the number of colonies per plate were determined. Colony assays revealed a significant decrease ($P = 0.04$) in the number of colonies detected for

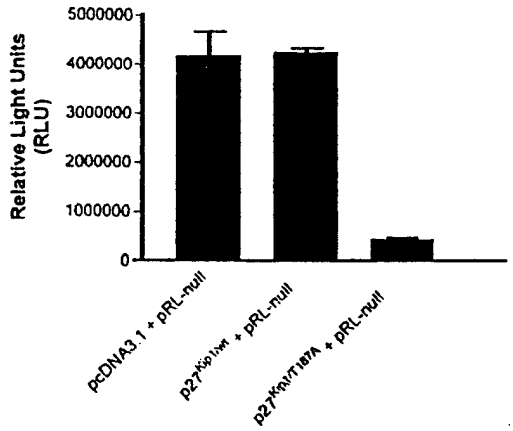

FIG. 4. Cells were transiently transfected with the *Renilla* luciferase reporter construct and: pcDNA3.1 (empty vector), wild type $p27^{Kip1}$, or mutant $p27^{Kip1/T187A}$ using Fugene 6. Luciferase inhibitory assay revealed that the cells transfected with the $p27^{Kip1/T187A}$ mutant plasmid had an 88% reduction in luciferase activity compared to cells transfected with $p27^{Kip1/wt}$ ($P = 0.001$) or the control vector (pcDNA3.1 alone) ($P = 0.017$).

cells expressing the $p27^{Kip1}$ mutant (21 ± 1.4) compared to cells expressing the wild-type $p27^{Kip1}$ (36 ± 4.2). When cells were transfected with the control vector (pcDNA3.1), 60 ± 9.9 colonies were detected (Fig. 5).

CONCLUSIONS

Site-directed mutagenesis of the $p27^{Kip1}$ gene increased the half-life of the $p27^{Kip1}$ protein by presumably preventing its degradation. We noted that the half-life was increased to over 12 h. As mentioned previously, the ubiquitin/proteasomal pathway is the major pathway through which $p27^{Kip1}$ degradation is initiated. It is, however, not the only pathway as other minor pathways may work together to cause decay of the $p27^{Kip1}$ protein, thus accounting for its increased but not infinite half-life.

To confirm that $p27^{Kip1}$ still retained its biological activity we performed a histone H1 kinase assay. Histone H1 is a CDK2 substrate and CDK2/cyclin complexes are $p27^{Kip1}$ protein targets. Binding of either wild-type or the mutated $p27^{Kip1}$ protein to the CDK2/cyclin complex induced H1 phosphorylation. These data indicate that the affinity of the mutated $p27^{Kip1}$ protein for the CDK2/cyclin complex and its inhibitory function was not altered by the site-directed mutagenesis (Fig. 3). We did infer from these results that the mutated $p27^{Kip1}$ protein had the potential to inhibit the growth of human ovarian cancer cells.

To investigate the influence of the mutated $p27^{Kip1}$ protein on general cellular function, luciferase assays were performed. A statistically significant inhibitory response was noted in cells expressing the mutated $p27^{Kip1}$ compared to either cells expressing the wild-type $p27^{Kip1}$ or cells transfected with the vector alone. We were surprised that wild-type $p27^{Kip1}$ protein did not induce a stronger inhibitory response and we attributed this result to the short half-life of the wild-type $p27^{Kip1}$ protein and to the mechanistic limitations of the luciferase assay. We believe that in this assay, a net increase in the amount of the transfected mutated $p27^{Kip1}$ protein, caused by its increased half-life, was necessary to induce an inhibitory response of the cellular transcriptional apparatus as manifested in the luciferase assay.

To determine if the more pronounced inhibitory effect upon cell function detected for the mutated $p27^{Kip1}$ protein would also result in a more pronounced effect upon cell growth, colony assays were performed. Results of the colony assays confirmed that the mutant form of $p27^{Kip1}$ was more proficient at inhibiting cell growth compared to the wild-type $p27^{Kip1}$ ($P = 0.04$). We also noted that the wild-type $p27^{Kip1}$ was an effective inhibitor of cell growth and the results were compa-

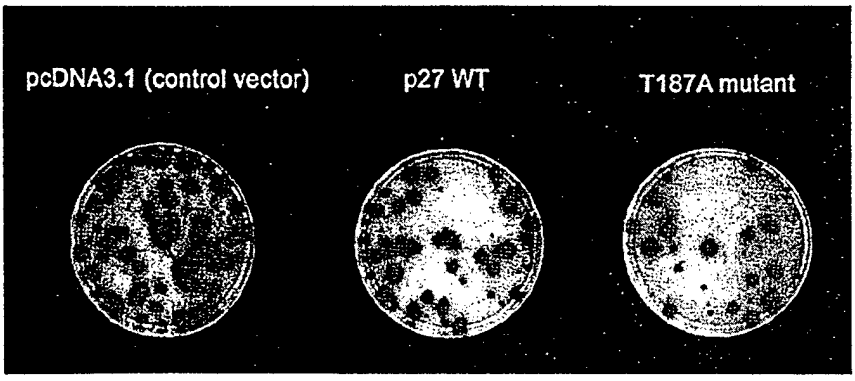

FIG. 5. Cells were transfected with pcDNA3.1 (empty vector), wild-type $p27^{Kip1}$, or mutant $p27^{Kip1/T187A}$ using Fugene 6 (Roche). Twenty-four hours after transfection, the cells were removed from the cell culture plate. The cells were counted and 200 cells were replated into a 60-mm tissue culture dish. After 12 days the cell colonies that had grown on the tissue culture plates were fixed, stained, and counted. The cells transfected with the $p27^{Kip1}$ mutant plasmid had a mean number of colonies of 21 (SD ±1.4) compared to cells transfected with wild-type $p27^{Kip1}$, which had a mean number of 36 (SD ±4.2) ($P = 0.04$). The control vector (pcDNA3.1) had a mean number of colonies of 60 (SD ±9.9).

rable to those reported by Sgambato *et al.* [14]. We believe that the wild-type $p27^{Kipl}$ is not as proficient at growth inhibition as the mutant $p27^{Kipl}$ form due to its shorter half-life and thus eventual decay.

The data presented herein are consistent with the hypothesis that the mutated form of $p27^{Kipl}$ compared to the wild-type $p27^{Kipl}$ is a more effective inhibitor of cell growth. The data are also consistent with the hypothesis that overexpression of the wild-type $p27^{Kipl}$ is not sufficient to alter cell growth as the ubiquitin/proteasomal pathway is still intact. These data support our hypothesis that introduction of stabilizing mutations into CDKIs such as $p27^{Kipl}$ represents a viable strategy that merits further study for use as a therapeutic modality.

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