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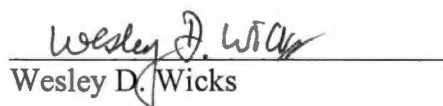


Jay Wimalasena, Major Professor

We have read this dissertation  
And recommend its acceptance:



Albert T. Ichiki

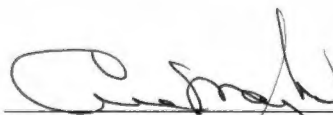


Wesley D. Wicks



Philip N. Bochsler

Accepted for the council:



Interim Vice Provost and  
Dean of the Graduate School

**MOLECULAR MECHANISMS OF ZEARALENONE-  
INDUCED G1/S PHASE TRANSITION IN MCF-7 BREAST  
CARCINOMA CELLS**

**A Dissertation Presented for the Doctor of Philosophy Degree  
The University of Tennessee, Knoxville**

**SHAMILA AHAMED  
December 2000**

## **DEDICATION**

**This dissertation is dedicated to:  
SHRI PARTHASARATHI RAJAGOPALACHARI  
"Chariji"**

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## ABSTRACT

Zearalenone is a naturally occurring estrogenic contaminant of moldy feeds and is present in high concentrations in dairy products and cereals. Zearalenone has been postulated to contribute to the overall estrogen load of women, however, the mechanisms of its action are not known. We demonstrate the ability of zearalenone to stimulate growth of the estrogen-receptor positive human breast carcinoma cell line, MCF-7. Zearalenone has multiple effects in the regulation of the MCF-7 cell cycle. Treatment of these cells with zearalenone induced cell cycle transit following increases in expression of Myc and the cyclins D1, A, and B1, as well as downregulation of p27. G1/G2 phase kinase activity and phosphorylation of the retinoblastoma gene product (Rb) was evident as well. Flow cytometric analysis demonstrated entry of cells into S and G2/M phases of the cell cycle, and phosphorylation of Histone H3 (HH3) occurred by 36 hr of zearalenone treatment.

We further examined how zearalenone treatment induces cyclin E-associated kinase activity within 9-12 hr. This increase in activity could be due to the sequestration of p27 by newly formed cyclin D1/Cdk4 complexes and/or due to zearalenone-induced downregulation of p27. The activity of cyclin E/Cdk2 complexes from zearalenone treated lysates could be inhibited in a dose dependent manner by His-tagged p27. This inhibition was relieved by addition of increasing levels of recombinant cyclin D1/Cdk4 complexes. Boiling released latent inhibitory activity from lysates of cells treated with zearalenone, and could be depleted by anti-p27 and anti-p21 antibodies. Overexpression of the Cdk4/6 specific inhibitor, p16, was associated with increased association of p27 with Cdk2 concomitant with disruption of D cyclin Cdk4 complexes. Nevertheless, in p16-arrested cells downregulation of p27 by zearalenone was present and resulted in cells entering S phase after a lag of 18-24 hr. These data suggest that sequestration of CKI by cyclin D1/Cdk4 complexes as well as downregulation of p27 protein play major roles in the induction of cyclin E-associated kinase activity and in S phase entry in zearalenone-treated MCF-7 cells.

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## LIST OF ABBREVIATIONS

**AF-1:** transcription activation function-1,

**AF-2:** transcription activation function-2,

**BrdU:** bromodeoxyuridine,

**Cdk:** cyclin dependent kinase,

**Cdc2:** cell division cycle 2,

**CAK:** Cdk activating kinase,

**CKI:** Cdk inhibitor,

**CRE:** cAMP response element,

**DBD:** DNA binding domain,

**DES:** diethyl stilbestrol,

**DDT:** bis(4-chlorophenyl)-1,1-dichloroethene

**DDE:**

**DMEM:** Dulbecco's minimal Eagle's  
medium,

**E1:** estrone,

**E2:** 17 $\beta$ -estradiol,

**E3:** estriol,

**ERKO:** ER knockout,

**ER:** estrogen receptor,

**ERE:** estrogen response element,

**Erk:** extra-cellular signal related  
kinases,

**FCS:** fetal calf serum,

**HH1:** Histone H1,

**16 $\alpha$ -OH E1:** 16 $\alpha$ -hydroxy estrone,

**INK4:** inhibitors of Cdk4/6,

**LBD:** ligand binding domain,

**MAPK:** mitogen activated protein  
kinase,

**MPF:** maturation promotion factor,

**MTT:** dimethylthiazoldiphenyl-  
tetrazoliumbromide,

**PCB:** polychlorinated biphenyls

**p27:** p27<sup>Kip-1</sup>,

**Rb:** retinoblastoma gene product,

**rasN17:** dominant-negative p21<sup>ras</sup>,

**SDS:** sodium dodecyl sulphate,

**PART I.**

**Literature Review**

# THE MAMMALIAN CELL CYCLE

## Introduction

The cell cycle describes the ordered process by which the cell executes the main events required to produce a duplicate of itself, chromosomal replication and cell division. The production of genetically identical and viable daughter cells require that these two processes be coordinated with each other, thus cells replicate their DNA before dividing and then divide before re-replicating their DNA (1-3). DNA synthesis (S phase), Mitosis (M phase) and the gaps between them, G1 (before S) and G2 (before M) provide an operational subdivision of the cell cycle into 4 discrete phases. Cells can withdraw from the mitotic cycle and exist in a quiescent state ( $G_0$ ), and the rate of proliferation in a given cell population is determined by the relative proportions of cycling versus quiescent cells (4). Proliferation is not the only options for cells in complex multicellular organisms and often these cells subordinate their replicative capacity for the greater good of the organism (5,6). A typical somatic cell must integrate a complex array of both intra- and extra-cellular signals to ascertain whether proliferation is desirable and feasible as a wrong decision can end in the death of the organism. Therefore, the cell cycle machinery is highly organized to both control G1 progression and link the mitotic program to the extra-cellular environment.

Study of how extra cellular mitogenic signaling impinges on the cell cycle to influence proliferation has identified a temporal location in the cellular control mechanism called the restriction point (R point). Nearly twenty-five years ago

Pardee made the observation that there is a window of time in G1 during which serum factors are absolutely required for cell cycle transit and the point at which the cell loses its dependency upon extra-cellular conditions for cell cycle progression he defined as the R point (7) According to this hypothesis, at the R point in mid G1, the cell evaluates the available information and decides whether or not to continue with the mitotic cycle The cell can advance through the R point and commit to completing the cycle, or it can withdraw from the mitotic cycle into the quiescent G<sub>0</sub> state. It was speculated that the molecular basis for the R point switch was an unstable restriction protein, which is synthesized in G1 and has to accumulate above a pre-determined level for continuous cell cycling (8,9) The R point hypothesis has proven to be a useful framework to explain the complex events involved in G1 progression

## **The Cell Cycle Machinery**

The complex events of cell division, from DNA replication to mitosis are controlled primarily by a simple chemical reaction: the transfer of a  $\gamma$  phosphate from ATP to the hydroxyl group of serines and threonines in regulatory proteins (10). The enzymes that catalyze this reaction and govern cell cycle progression are the cyclin dependent kinases (Cdk's) (11) Discovery of these key enzymes began with the study of frogs and yeast. In amphibian eggs an activity capable of inducing M phase, called maturation promoting factor (MPF), was shown to consist of two protein components, a catalytic subunit and a regulatory subunit (12,13) The catalytic component was identified as cell division control protein 2

(cdc2), which previously had been shown to have a function in promoting M phase in yeast. The regulatory component of cdc2/MPF, which was responsible for periodic activation of the complex, was named cyclin. In contrast to yeast, where the kinase cdc2 (Cdk1) controls both the G1/S and G2/M transition (14), in mammalian cells each phase of the cell cycle is characterized by a unique pattern of Cdk activity (11). Cdk's coordinate the cell cycle by acting as on/off switches. When a Cdk is off, the cell cycle stops when it reaches the stage controlled by that Cdk (11). So far eight Cdk's have been identified in mammalian cells, each with a specific profile of activity (14) (Table 1). They are closely related in size (35-40Kd) and sequence (>40% identity), and are associated with an activating cyclin subunit (14). The Cdk's 2,3,4 and 6 are required for progression through the G1 phase while Cdk1 is the major Cdk involved in mitosis (11). As the fate of the cell hangs on the whims of the Cdk enzymes, these kinases are extensively regulated to ensure that they are responsive to the extra cellular environment. The small Cdk catalytic subunit, which contains the conserved catalytic core found in all protein kinases, is extensively regulated and its activity controlled by four highly conserved biochemical mechanisms (14) (figure 1).

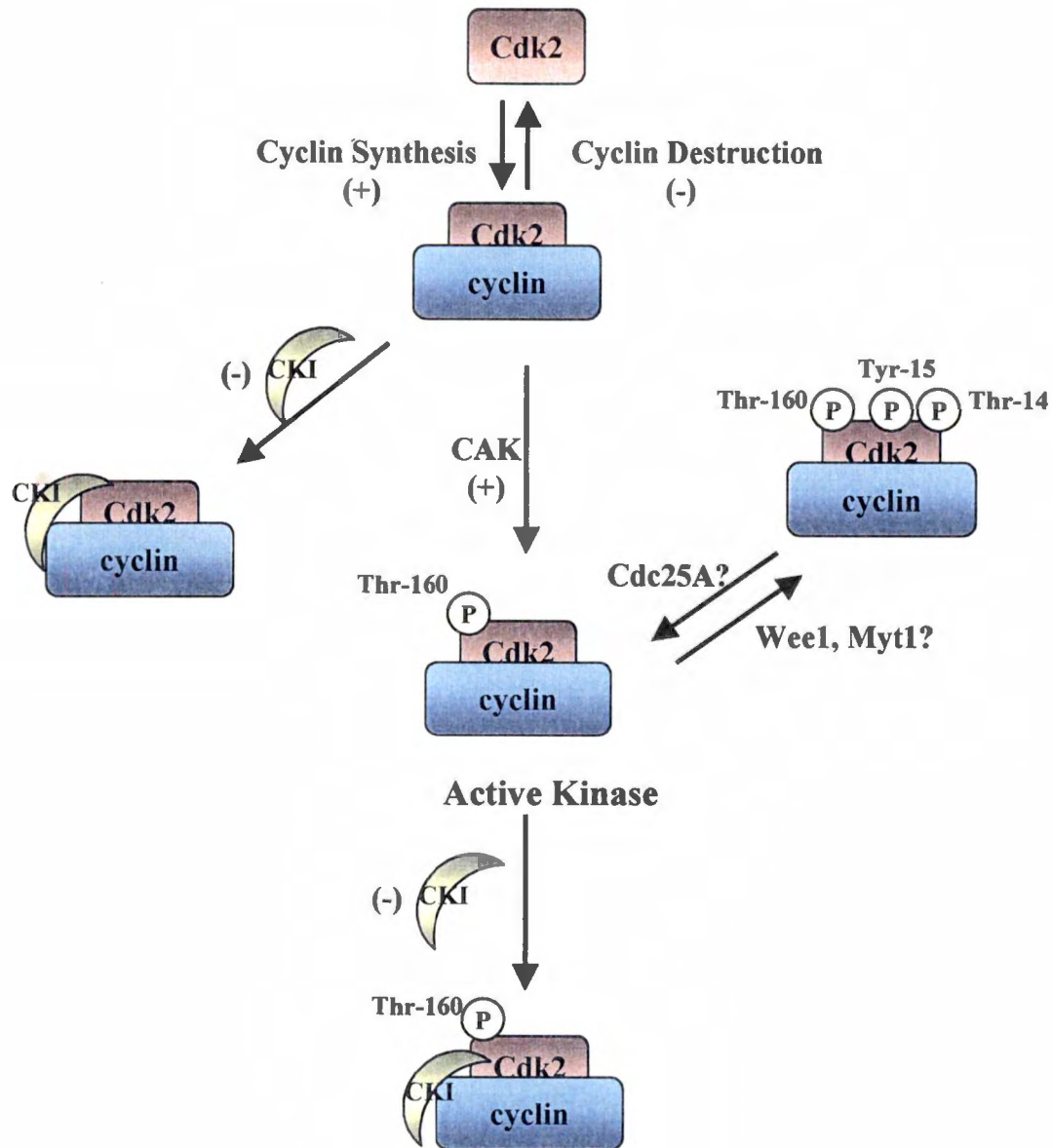
### **Cdk Activation by Cyclin Binding**

The naked Cdk subunit is completely inactive with partial activation occurring upon binding of the positive regulatory subunit, cyclin (10). Originally defined as proteins whose levels oscillate during the cell cycle, cyclins are now more accurately defined as members of a group of structurally related proteins

**Table 1: Major Cdks and cyclins in humans (14)**

| <b>Cdk</b>  | <b>Cyclin Partner</b> | <b>Function</b>        |
|-------------|-----------------------|------------------------|
| Cdc2 (Cdk1) | Cyclin B              | M phase                |
| Cdk2        | Cyclin E<br>Cyclin A  | G1/S<br>S phase and G2 |
| Cdk3        | ?                     | G1?                    |
| Cdk4        | Cyclin D              | G1                     |
| Cdk5        | p35                   | Neural differentiation |
| Cdk6        | Cyclin D              | G1                     |
| Cdk7 (MO15) | Cyclin H              | CAK, transcription     |





**Figure 1. Regulation of Cdk2.** The Cdk monomer can be activated by cyclin binding as well as addition of the activating phosphate group Thr-160. Inhibitory phosphorylations by Wee1/Myt1 as well as binding of CKI blocks the cyclin-bound Cdk activity. Cdc25A by removing the Thr-14/Tyr-15 inhibitory phosphate groups in turn leads to induction of kinase activity.

that bind and activate the Cdk catalytic subunit (14). Homology among cyclins is mostly limited to a relatively conserved domain of about 100 amino acids, the cyclin box, which is responsible for Cdk binding (11). The primary mammalian G1 cyclins are the D cyclins (1,2 & 3), which associate with Cdks 4 and 6; and cyclin E, which associates with Cdk2 and perhaps Cdk3 (15). Cyclin A is expressed at the G1/S boundary and is involved in DNA replication, while B cyclins are considered to be mitotic cyclins (Table 1).

Cyclin function is primarily controlled by changes in cyclin levels by regulation of synthesis and/or degradation (14). By controlling cyclin expression the cell succeeds in having periodicity of Cdk activity in order to phosphorylate different substrates specific for each stage of the cell cycle (14). Although the hypothesis that cyclins impart substrate specificity to Cdk's is still largely unsupported by experimental data, it is possible that cyclins may target the Cdk to specific substrates by interacting with the substrate *in vivo*; cyclins D and E interact with Rb (15,16), or by changing the structure of the Cdk catalytic cleft through allosteric interactions to alter the affinity of the Cdk for a particular substrate (16).

### **Cdk Activation by Phosphorylation**

Complete activation of the cyclin bound Cdk subunit requires phosphorylation of the activation loop of the cyclin-bound Cdk subunit on a single threonine residue (Thr-161 in Cdk1, Thr-160 in Cdk2, Thr-172 in Cdk4 and Thr-177 in Cdk6) (14) (figure 1). This phosphorylation is mediated by the Cdk-

activating kinase (CAK), a multi-subunit enzyme whose catalytic subunit is a highly conserved, Cdk-related protein kinase called MO15 (17-21). In addition to its catalytic subunit, CAK also contains regulatory subunits. Cyclin H, a member of the cyclin C family has a three dimensional structure similar to cyclin A (18,22,23). Addition of purified cyclin H to MO15 *in vitro* reconstitutes CAK activity (22), demonstrating that CAK is a Cdk-cyclin complex, and therefore MO15 has been renamed Cdk7. An additional regulatory subunit termed MAT1 was originally identified by its co-immunoprecipitation with MO15 and cyclin H from human cell extracts and promotes the assembly of MO15 and cyclin H, producing an active trimeric complex (17,24,25). Cyclin H, Cdk7 and MAT1 are also a part of transcription factor IIIH, which phosphorylates the C-terminal domain (CTD) of the large subunit of RNA polymerase II (26,27). A single CAK can activate all of the major Cdk-cyclin substrates involved in vertebrate cell-cycle control and its activity does not change during the cell cycle (14). Instead the phosphorylation of Thr160/161 rises and falls in parallel with cyclin binding and reflects the ability of cyclin binding to stimulate Cdk phosphorylation. Dephosphorylation of Cdk1 on Thr161 occurs following degradation of cyclin B at anaphase and resets the system for the next cycle (28,29), and the phosphatase responsible for dephosphorylating the Thr160/Thr161 site in both Cdk2 and Cdk1 is termed the Cdk-associated phosphatase (KAP) (28).

### **Cdk Inhibition by Phosphorylation**

The activity of the Cdk-cyclin complex can be reduced by phosphorylation of the Cdk subunit on two inhibitory sites at the amino terminus (Thr-14/Tyr-15 in Cdk1 & 2, Thr-17 in Cdk4, Thr-24 in Cdk6) (14) (figure 1). In Cdk2 the side chains of these residues are oriented from the ceiling of the ATP-binding site and when phosphorylated are in a position to affect the kinase activity (30). Surprisingly, phosphorylation does not inhibit ATP binding, suggesting a more subtle effect on the positioning of ATP for catalysis (31). Both residues are buried beneath the T loop and phosphorylation only takes place when the T loop is moved and therefore thought to be cyclin-dependent (11). Phosphorylation of Thr14/Tyr15 is of particular importance in the control of Cdk1 activation at mitosis, and roughly parallels the rise in cyclin B levels that occurs when cells approach mitosis (11). The cyclin B bound Cdk1 is kept in an inactive state until dephosphorylation of Thr-14/Tyr-15 occurs at the end of G2 resulting in the activation of the Cdk1/cyclin B complex. Wee1, a major candidate for Tyr-15 kinase activity was originally identified in *S. pombe* (32), and phosphorylates Cdk1 *in vitro* at Tyr-15 but not at Thr-14 (33). Subsequently during a PCR-based screen for wee1-like genes in *Xenopus* oocytes, a novel Thr-14/Tyr-15 kinase activity was isolated and the encoded protein was termed "Myt1", for membrane-associated, Tyr/Thr specific, Cdk1 inhibitory kinase (34). The human Myt1, which is 46% identical to *Xenopus* Myt1, localizes to the endoplasmic reticulum and preferentially phosphorylates Thr-14 (35) (figure 1).

Thr-14 and Tyr-15 are both dephosphorylated by the dual-specificity phosphatase Cdc25 (14) (figure 1) Three Cdc25 phosphatases have been identified in mammals, and the different isoforms, A, B, and C, share 40-50% amino acid identity (32,36-38). Cdc25C preferentially acts to remove inhibitory phosphates from Cdk1-cyclin B complexes, triggering its activation at the G2/M transition and initiating the onset of mitosis (39). The activity of this phosphatase is increased during mitosis largely due to increased phosphorylation in its N-terminal half (40). Cdk1 is reported to stimulate this phosphorylation leading to a positive-feedback system that could induce the abrupt mitotic dephosphorylation of itself (40) Although Cdc25A and Cdc25B can dephosphorylate cyclin-Cdk2 complexes *in vitro* (41-44), their physiological *in vivo* substrates have not yet been formally assigned (45). However, Cdc25A gene is induced in G1 phase and microinjection of Cdc25A specific antibodies into fibroblasts arrests them in G1 (46), suggesting that this phosphatase is required for entry into S phase. Cdc25B is expressed later in S and G2 phase, and like Cdc25A, is capable of transforming primary mouse fibroblasts when co-expressed with activated forms of ras (46,47). *In vitro* evidence for phosphorylation and activation of Cdc25A by cyclin E-Cdk2 implies that similar to activation of Cdc25C by Cdk1 at G2/M transit, Cdc25A could be controlled by cyclin E/Cdk2 at the G1-S transition (41), suggesting that this amplification loop could be a general phenomenon.

### **Cdk Inhibition by Inhibitory Subunits**

One mechanism whereby physiological signals that prevent growth and proliferation do so is by inducing expression of Cdk inhibitors (CKIs) (figure 1). These CKI bind and inactivate Cdk-cyclin complexes and thus result in cell cycle arrest (14). There are several different ways by which these CKIs might regulate cell cycle progression (48). By determining the duration of G1 phase by setting a threshold above which G1 Cdk activity must accumulate before initiation of S phase, i.e.: the steady state levels of CKIs counteract the temporal accumulation of G1/cyclin Cdk complexes

- (i) By acting as checkpoints inducing cell cycle arrest transiently when genomic fidelity is threatened. Chromosome damage or inappropriate segregation of chromosomes may induce cell cycle arrest and initiate DNA repair mechanisms or apoptotic programs.
- (ii) By inducing withdrawal of cells from the cell cycle and a concomitant change in cell fate, i.e.: cell differentiation, cell senescence etc
- (iii) By functioning as gatekeepers of quiescence, keeping G<sub>0</sub> cells from inappropriately resuming proliferation

Two classes of CKIs have been identified and can be distinguished by their targets. (a) INK4 (Inhibitors of Cdk4/6) proteins (p15<sup>INK4B</sup>, p16<sup>INK4A</sup>, p18<sup>INK4C</sup>, and p19<sup>INK4D</sup>), which specifically target Cdk4/6 kinases, and (b) the Cip/Kip family of inhibitors (p21<sup>CIP1</sup>, p27<sup>KIP1</sup>, and p57<sup>KIP2</sup>), which are thought to be more general Cdk inhibitors (49). The members of the INK4 family of CKIs have a common ankyrin repeat motifs (structural elements involved in binding

interactions with other proteins) flanked by unique sequences and they interfere with Cdk activity by either, binding monomeric Cdk4/6 and preventing association with the D type cyclins or by directly binding and inhibiting cyclin D-Cdk complexes (48). Identification of p16 occurred simultaneously during the hunt for melanoma tumor suppressors (50), and for proteins that associate with Cdk4 (51). These studies indicated that a 16 kD protein, termed p16<sup>INK4A</sup>, was involved in both. Two hybrid screens and sequence homology cloning led to the identification of p15, p18, and p19 (52-56). In contrast to the Cip/Kip family, the members of the INK4 family, especially p16, are considered to be candidates for tumor suppressor genes (57). In a large number of tumor-derived cell lines and clinical tumors, p16 activity is lost due to mutations, deletions, or methylation of the gene (58). The spontaneous tumorigenesis observed in p16 knockout mice also supports this conclusion (57). However, the p16 locus encodes two different transcripts from different reading frames, p16 and p19ARF (59). Both p16 and p19 are growth suppressive *in vitro*, and both are disrupted in the knockout mice, thus, the contribution of p19ARF to the tumorigenicity of p16 remains to be clarified. Progressive accumulation of p16 in primary cells undergoing terminal cell divisions suggests a role for p16 in senescence and fibroblasts obtained from p16 knockout mice fail to undergo senescence *in vitro* (60). These fibroblasts grow more rapidly in culture than wild-type cells, also suggesting a role for p16 in determination of G1 duration (60).

The Cip/Kip class inhibitors, p21, p27, and p57, share a conserved 60 amino acid cyclin/Cdk-binding domain, and p21 and p57 also have a PCNA

binding domain (48). The members of the Kip subclass, p27 and p57, have an FY motif at the carboxyl region while members of the Cip subclass (p21) do not. p21, was identified by several different laboratories; as a protein that physically interacts with cyclin/Cdk complexes (61-63), is involved in DNA damage-induced cell cycle arrest mediated by p53 (64), as well as in the withdrawal from the cell cycle required for terminal differentiation (65). However, p21 levels also rise as cells exit quiescence and enter the cell cycle in apparent contradiction to its role as a CKI (66,67), thus, p21 appears to function as an assembly factor (68). In some cases p21 has been found to associate with active cyclin-Cdk complexes (69,70). Therefore, formation of higher order structures containing multiple p21 molecules may be required for Cdk inhibition. In addition, mice lacking p21 are viable and show little physiological effects, although cell lines established from these animals show a defective response to DNA damage (71,72).

An inhibitory activity present in TGF $\beta$ -treated mink lung epithelial cells, which led to decreased cyclin E/Cdk2 activity, was purified by its ability to bind Cdk2-cyclin E complexes and the protein was named p27 (73,74). Simultaneously p27 was also identified using a modified two-hybrid screen with cyclin D/Cdk4 as the bait (75). Levels of p27 are high in quiescent cells as well as those cells inhibited by cell-to-cell contact (76). The fall in p27 levels when cells reenter the cell cycle correlates with increased insensitivity to the inhibitory effects of TGF $\beta$ , suggesting that upregulating p27 may be part of a generalized mechanism responsible for withdrawal from the cell cycle (74,77). Using antisense message to decrease p27 levels led to decreased serum dependence of cells growing in



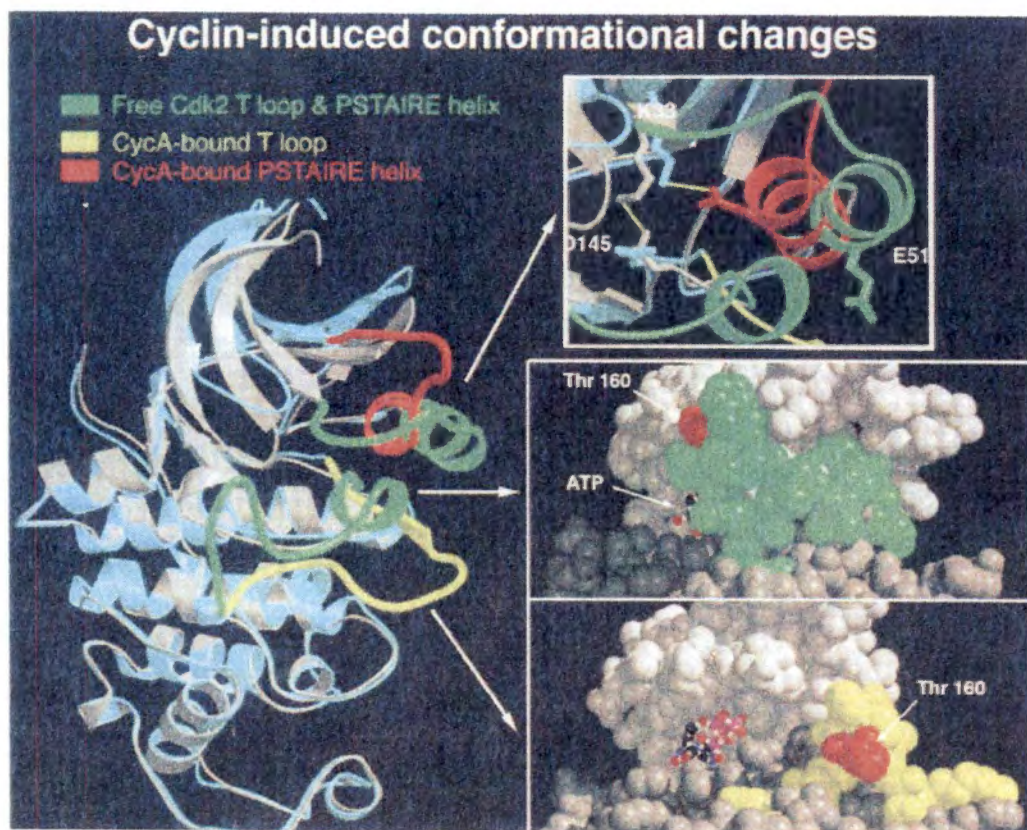
culture (78), and p27 knockout mice grow twice as fast as wild types (79-81). The expression pattern of p27 in most cell lines, as well as primary cells, also suggests that p27 has a more direct role in the cell cycle, such as regulating the rate of S phase entry (48). The p27 deficient mice develop tumors in the intermediary lobe of the pituitary, similar to pRb deficient mice, implying a functional connection between pRb and p27 (82). However, primary cells lacking p27 do not display a significant alteration in kinetics in withdrawing from the cell cycle due to serum starvation (79). This suggests that in primary cells, in contrast to immortalized cells, cell cycle exit may require components in addition to p27 (83), any of which is sufficient to cause cell cycle arrest.

The third member of the Cip/Kip family, p57, was identified as a homologue of p21 (84,85). Targeted disruption of p57 has demonstrated that it is critical for endochondral ossification and development of the palate (86,87). Most p57-deficient mice die within a day after birth, displaying short limbs, cleft palate and an inflated GI tract. In addition, cells of some types of tissue from p57-deficient mice remain in the cycle as with p27, perturbing differentiation (48). Although the members of these two families of CKIs target different Cdks and exhibit different mechanisms of inhibition, their overexpression invariably results in G1 arrest

## Mechanisms of Cdk Regulation

The crystal structures of the Cdk2/cyclin A complex, the phosphorylated Cdk2/cyclin A complex, the phosphorylated complex bound to the p27 inhibitor as well as that of Cdk6 bound to the p16 inhibitor are now known and have helped in the understanding of the regulatory processes mentioned above. The structure of the monomeric Cdk2 consists of an N-terminal lobe rich in  $\beta$ -sheet (N lobe), a larger C-terminal lobe rich in  $\alpha$ -helix (C lobe), and a deep cleft at the junction of the two lobes that is the site of ATP binding and catalysis (88). Overall the Cdk2 monomer appears to have a structure similar to other eukaryotic protein kinases, first seen in the cAMP-dependent protein kinase (PKA). Two regions of the monomeric Cdk2 differ from the canonical kinase structure, an  $\alpha$ -helix having the unique sequence PSTAIRE (one letter amino acid code) and a T loop that has the activating phosphorylation site. Both regions have been predicted to function as regulatory elements (88). The crystal structure of the human Cdk2 shows that it is held in an inactive state by at least two major structural restraints: the substrate-binding site is blocked by the T loop and the side chains in the ATP-binding site are poorly positioned for efficient phospho-transfer (10).

The crystal structure of the cyclin bound Cdk2 enzyme shows that the cyclin A makes contact with the PSTAIRE helix and helps in forming a continuous protein interface (89) (figure 2). Other key contacts are also made with the T loop, and parts of the N and C lobes. Binding of the cyclin to the PSTAIRE moves the helix into the catalytic cleft and rotates it by 90°. The PSTAIRE helix carries on it a catalytic site residue, Glu51, conserved among eukaryotic protein

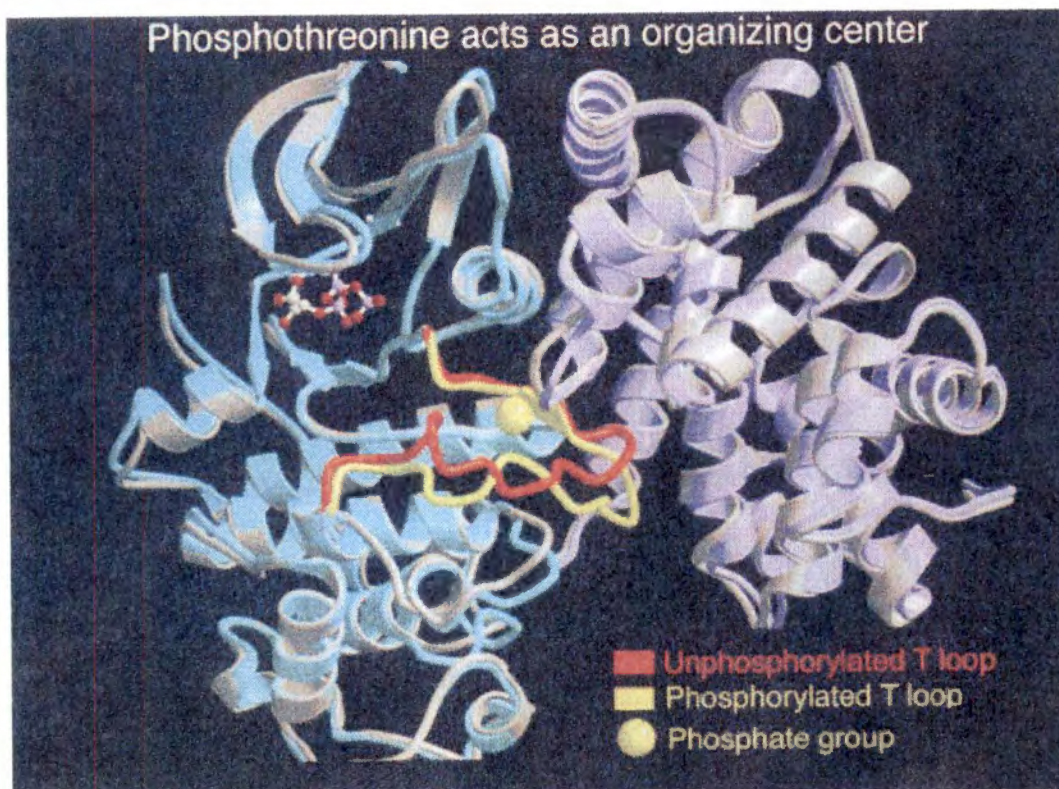


**Figure 2.** Cyclin binding partially activates the Cdk by causing conformational changes in the PSTAIRE helix and T loop, as shown in the superposition of free Cdk2 (grey) and cyclin A-bound Cdk2 (blue). The PSTAIRE helix and T loops of cyclin A-bound Cdk2 are high-lighted in red and yellow, respectively, while those of free Cdk2 are green. The close-up view of the PSTAIRE helix shows the realignment of Glu51, Lys33, and Asp145; the ATP and magnesium ions are not shown. The close-up views of the T loop, before and after cyclin A binding, are in space filling representation. Thr160, which is phosphorylated by CAK, is colored red (88).

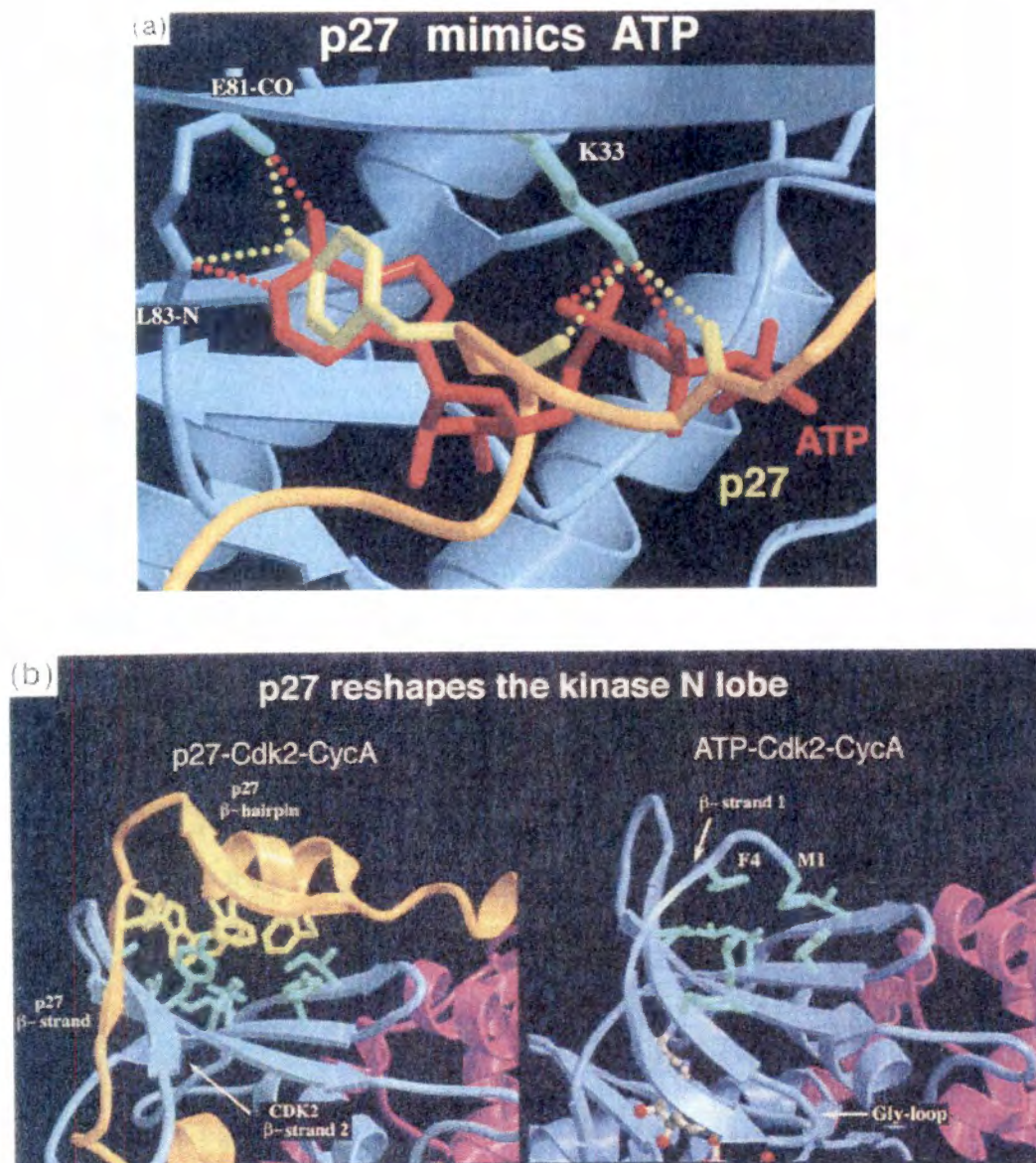
kinases (88) When the cyclin binds, the change in the helix brings the glutamic acid side-chain into the catalytic cleft, where, together with a lysine residue, an aspartic acid residue and a magnesium ion it coordinates the ATP phosphate atoms and correctly orients them for catalysis. Binding of cyclin A also changes the T loop structure and position, moving parts of it by over 20°A and largely relieves the blocking of the catalytic cleft. This exposes the phosphorylation site on the T loop allowing CAK access for the activating phosphorylation at Thr160 (figure 3). This phosphothreonine can act as an organizing center and bind three arginine residues coming from the N lobe, C lobe, and the T loop, thus completing the reorganization of the substrate-binding site (90). After these changes, the Cdk bears a striking resemblance to the canonical Ser/Thr kinase structure of PKA (88).

Binding of a member of the Cip/Kip family can completely shut down the fully active Cdk2 enzyme by reshaping the N lobe and mimicking ATP binding (88) The glycine-rich loop of the kinase  $\beta$ -sheet which are needed to interact with the ATP phosphate groups are not free in the Cdk2-cyclin A-p27 complex because of the changes in the shape of the catalytic cleft from p27 binding. When p27 binds it removes the first  $\beta$ -strand of the Cdk2 to gain access to the hydrophobic core, forms an intermolecular  $\beta$ -sandwich with the N-lobe and refolds into a new structure Another way by which p27 can inhibit the kinase is through the insertion of a small helix into the catalytic cleft. This helix mimics the ATP substrate, both its position and in the contacts it makes to the active site groups and thus directly blocks ATP binding (figure 4)





**Figure 3.** Phosphorylation causes additional conformational changes in the T loop, highlighted in the superposition of the unphosphorylated (grey) and phosphorylated (blue and purple) Cdk2-cyclin A complexes. The unphosphorylated T loop is in red, and the phosphorylated loop in yellow. The phosphate group is indicated by a yellow sphere and ATP is shown in ball-and-stick representation (88).



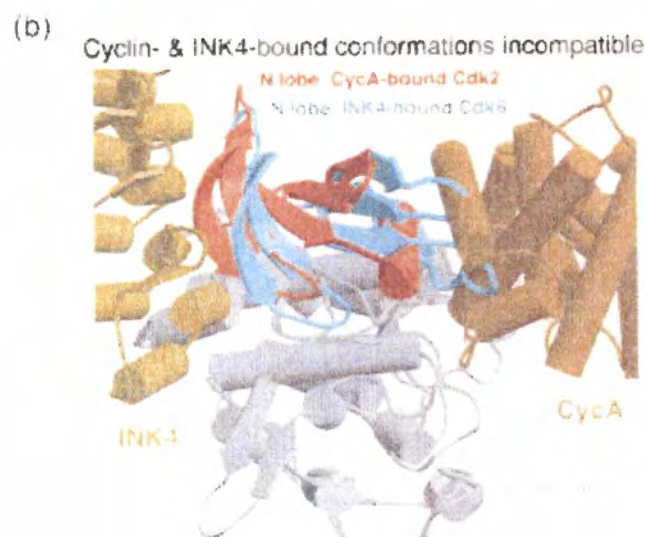
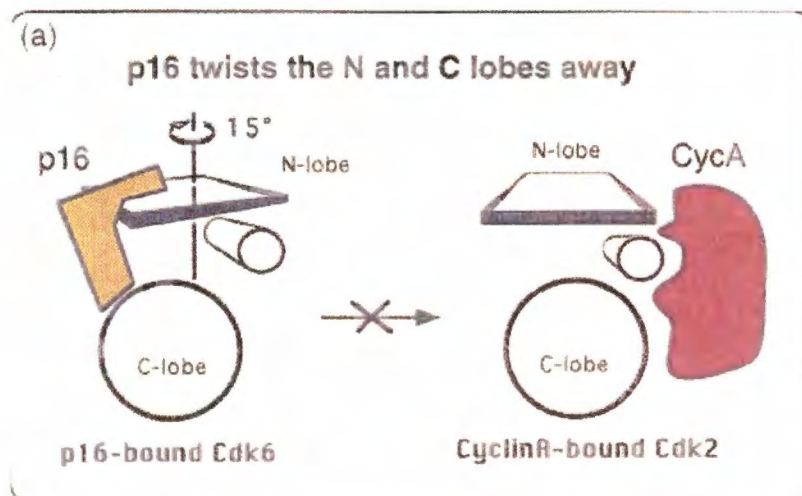
**Figure 4.** p27 inhibits the Cdk-cyclin complex by changing the shape of the catalytic cleft and by inserting a helix inside the ATP binding site. **(A)** Close up view from the superposition of the ATP-cdk2-cyclin A and p27-Cdk2-cyclin A complexes showing the coincidence in the positions and contacts of the Tyr88 side-chain from p27 and the ATP purine group. Hydrogen bonds are indicated by dotted lines. **(B)** Side by side comparison of the p27-bound N lobe. Hydrophobic residues involved in the hydrophobic core of the N lobe in the two complexes are shown (88).



The binding and mechanisms of action of the INK4 family members of Cdk inhibitors are distinct from the Cip/Kip family of Cdk inhibitors (88). The structure of the p16-bound Cdk6 shows that p16 blocks the formation of the active, cyclin bound conformation of the kinase indirectly, by causing allosteric changes that propagate to alter the cyclin binding site (91,92). The interaction of p16 with the N and C lobes of the kinase causes these lobes to twist away from each other (by around 15°), distorting the cyclin binding site and blocks the translocation of the PSTAIRE helix into the catalytic cleft (figure 5). The binding of the p16 also leads to the distortion of the ATP binding site, which significantly reduces ATP affinity (91). In cross-linking studies with an ATP analogue, p16 has been reported to decrease the apparent  $K_i$  of the analogue for Cdk6 by 20 fold. p16 can also reduce the ATP binding of Cdk6-cyclin D complexes to a similar extent by affecting the catalytic cleft structure. The intrinsic structural flexibility of Cdk's, especially around the catalytic cleft, appears to be utilized by the CKI in inhibiting the enzymatic activity of these kinases: the Cip/Kip family members by binding to the N lobe and refolding it into a different structure, and the INK4 inhibitors by twisting the structure and distorting the ATP binding site, succeed in this task (88).

### **The retinoblastoma protein (Rb)**

The rare childhood cancer retinoblastoma is caused in part by inactivating mutations in the retinoblastoma gene. The product of the retinoblastoma gene, Rb,



**Figure 5.** p16 would block the formation of the active, cyclin-bound conformation of the kinase indirectly, by causing allosteric changes that propagate to the cyclin binding site. **(A)** Schematic illustration showing that the binding of p16 is associated with a rotation of the N and C lobes by about 15°, relative to the other Cdk structures. **(B)** Superposition of the p16-bound Cdk6 and the cyclin A-bound Cdk2 structures, performed by aligning the C lobes (88).



is an 110kD protein and a member of the family of pocket proteins, whose other members include p130 and p107 (93). An important function of Rb is to control progression through the G1 phase of the cell cycle and entry into S phase (94,95). Rb appears to connect the cell cycle clock with the transcriptional machinery necessary for initiating events downstream of R point. Active Rb can hold the cell cycle in check by turning off this transcriptional program, and at a time close to the R point Rb appears to become phosphorylated and inactivated. Throughout the rest of the cell cycle Rb, is in a state of hyperphosphorylation and only loses its multiple phosphate groups upon emergence from M phase (96). A number of kinases and phosphatases operating upstream of Rb determine its functional state and Rb is one of the most important Cdk substrates identified to date (97-99). Cyclins and/or cyclin/Cdk complexes have been shown to interact with Rb/p107/p130 and ternary complexes between Rb, D-type cyclins and Cdk4/6 have been observed in mammalian cells (100-102). p107 and p130, and to a significantly lesser extent Rb, can form stable complexes with both cyclin E and cyclin A-Cdk2 (102-104). In the hypophosphorylated state Rb is thought to inhibit cell proliferation by sequestering the transcription factor E2F, which inhibits E2F's ability to drive the transcription of genes important for growth control and cell cycle progression (94,95). Observations such as ectopic expression of E2F inducing quiescent cells to enter S phase or overcoming an Rb-imposed cell cycle arrest imply that release of this transcription factor from Rb is rate determining for cell cycle progression through G1 (105). In addition to its regulatory role in the G1/S transition, Rb also plays a role in S phase progression since

unphosphorylated Rb has been demonstrated to inhibit DNA synthesis that is independent of the inhibition of the known G1/S Cdk-cyclin activities (106). The negative effects of Rb on S phase progression can be overridden by high levels of E2F1 expression implying that E2F-activated genes may be needed continuously throughout S phase for efficient replication of genomic DNA (106).

The Rb protein is compromised of several distinct protein binding domains (107). The A/B pocket was the first domain to be identified and was shown to complex with oncoproteins encoded by simian virus 40 (SV40), adenovirus and human papillomavirus (HPV), namely T antigen, E1A, and E7, respectively. This binding region is composed of a linear sequence: A domain-insert domain (ID)-B domain, which interacts with both upstream (D type cyclins) and downstream (E2F) factors in the Rb pathway. The carboxy terminal region of Rb contains another protein binding domain, the C pocket, which interacts with the c-Abl tyrosine kinase (108). Phosphorylation appears to inhibit the protein binding activity of Rb. 16 Ser/Thr-Pro motifs in Rb are demonstrated to be potential Cdk targets and 11 of these sites are phosphorylated *in vivo* (109-111). Specific phosphorylation sites appear to regulate distinct protein binding functions: phosphorylation of Thr821 and Thr826 disrupts binding of proteins with the LXCXE motifs while the C pocket is inhibited by phosphorylation of two serines (Ser807 and Ser811) (112).

Mutations in or loss of Rb is one of the most common abnormalities associated with human cancers and Rb is the best-characterized example of a tumor suppressor (113,114). Re-introduction of functional Rb into retinoblastoma

or osteosarcoma cells that have inactivated endogenous Rb genes results in reduced tumorigenicity in nude mice. As inappropriate inactivation of Rb can lead to uncontrolled proliferation and ultimately cancer, the normal cell has linked loss of Rb to apoptosis; which requires the activity of the p53 tumor suppressor gene (115). Many of the most aggressive cancers display loss of both Rb and p53 (116). Rb knockout mice are only viable until day 12-13 of gestation and, although mice heterozygous for Rb survive longer, they eventually succumb to cancer after losing the 2nd Rb allele (117,118). In contrast, knocking out the other two members of the family of pocket proteins, p130 and p107, is not fatal and these mice show no obvious abnormalities (119). Fibroblasts from Rb knockout mice appear to have lost the R point criterion and display a tendency to escape G1 arrest associated with mitogen deprivation, although eventually most of these cells arrest in G<sub>0</sub> in response to low serum (120,121). This implies the presence of Rb-independent factors contributing to R point control, especially the CKIs. These cells also show increased expression levels of transcription factor E2F-responsive genes leading to a shorter G1 phase and smaller cells, which grow faster than the wild type cells (122).

The E2F family is composed of six related but distinct family members, which show strong preference for specific Rb family members: E2F 1-3 associate almost exclusively with Rb; E2F5 binds to p130; E2F4 binds with high affinity to p107 and p130 but also associates with Rb in some cell types (123-126). In G<sub>0</sub> cells p130/E2F4 complexes predominate and repress transcription of certain genes, i.e., E2F1 and Cdk1, and thereby contribute to the maintenance of the

quiescent state (127-129) E2Fs associate with members of the DP family (DP1 and 2) to form DP/E2F complexes and target to the consensus binding sequence TTCGCGC in the promoter regions of responsive genes (130). Expression of dominant-negative DP1 leads to cell cycle arrest (131), and DP1 is also reported to be regulated by phosphorylation in a cell cycle-dependent manner (132). The cyclin A mediated-phosphorylation of DP1 in the E2F/DP complex inactivates this complex (133).

The region of E2F1 responsible for binding to Rb is embedded within its transactivation domain. Therefore, complex formation between Rb and E2F results in inhibition of E2F-mediated transactivation (134,135). Recently it has come to light that the E2F-mediated targeting of the Rb-E2F and p107-E2F complexes to an E2F site within a promoter can also actively represses transcription (136-138). This is highlighted by the observation that inactivation of the E2F gene in mice leads to hyperplasia in certain tissues (139,140). Therefore, transcriptional repression by Rb-E2F complexes could be a mechanism whereby Rb acts as a tumor suppressor and this could also explain the dual behavior of E2F as both oncogene and tumor suppressor.

About 50 different polypeptides have been reported to physically interact with Rb, of which only a few are components of E2F (E2F 1,2,3,4 and DP 1&2). Despite the fact that E2F and Rb have antagonistic activities, it is still not clear what fraction of Rb's functions, as a growth suppressor/tumor suppressor properties, is attributable to its regulation of E2F. The C pocket of hypophosphorylated Rb also binds the active catalytic domain of c-abl, a nuclear

tyrosine kinase, blocking its function (141,142). By binding multiple effectors such as E2Fs and Abl, Rb may be able to modulate simultaneously the activity of a number of downstream growth controlling pathways.

## **Regulation of G1 progression**

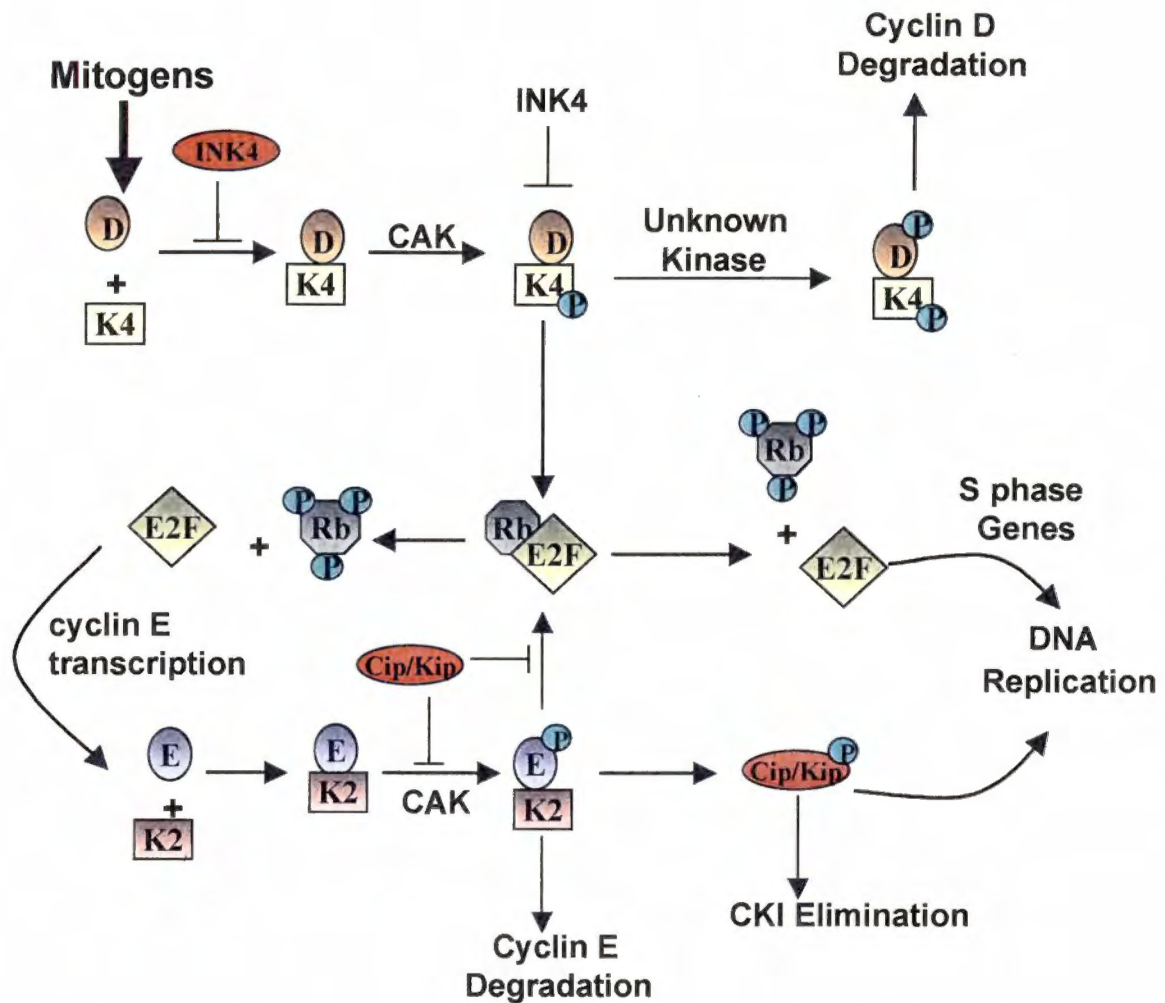
As quiescent cells are stimulated to reenter the cell cycle by exposure to mitogens, cyclin D expression is transcriptionally upregulated in early G1 (1). Cyclin D1 expression is upregulated by ras via the MAP kinase cascade in response to growth factor signaling (143) and its levels appear to decline after mitogen removal implying a linear connection between cyclin D expression and extracellular mitogenic signals (144). As the half-life of cyclin D is only ~15 min, the cell cycle can be kept tightly under mitogen control by manipulation of cyclin D levels (145). Its turnover is mediated by phosphorylation on Thr-286 leading to ubiquitination and proteolytic degradation (146). The assembly and activation of cyclin D-Cdk complexes are also regulated by mitogenic signals (1,147). Injection of monoclonal antibodies directed against cyclin D arrests cells in early G1 but not after the cell has progressed into late G1 (148-150). This suggests that the function of cyclin D is completed in early G1 and once its role in early G1 is completed, the level of cyclin D declines and does not fluctuate dramatically with the cell cycle (1). Therefore, it is possible that cyclin D1 may play a specialized role in cells, reentering the cell cycle from a quiescent state.

The diverse processes negatively regulated by Rb are linked to the cell cycle machinery by the Cdks, which are responsible for phosphorylating and inactivating Rb (107,151). Cyclin D was implicated in phosphorylation of Rb when anti-mitogenic signals, which interfere with cyclin D-associated kinase activity, were seen to prevent Rb phosphorylation *in vivo* (152,153). Inhibition of ras in cycling cells also caused the cells to arrest in G1 accompanied by downregulation of cyclin D levels and accumulation of hypophosphorylated Rb (149,154). However, blocking ras signaling in Rb negative cells did not result in cell cycle arrest (154). This led to the conclusion that the growth factor activated ras-dependent signal transduction pathway induced cell proliferation by inactivating Rb. Also, inactivation of cyclin D by microinjection with anti-cyclin D antibodies or overexpression of p16 resulted in G1 arrest only in cells that have Rb (152,153). Furthermore, the Rb negative cells proceed uninterruptedly through the cell cycle in the absence of cyclin D associated kinase activity (155,156). These observations, together with the fact that overexpression of cyclin D1 accelerated progression through G1 only if the cells are Rb positive (157), convinced some researchers that phosphorylation of Rb was the only function of cyclin D in cell cycle progression (155).

As with cyclin D, antibodies injected against cyclin E arrest cells in G1, while its over-expression shortens G1 (158). The expression patterns of RNA, protein and kinase activity suggest that cyclin E functions after cyclin D in the cell cycle (159,160). Cyclin E mRNA and protein are reported to rise during mid G1 and to decline with degradation taking place late in G1 (158). Cyclin E is

autophosphorylated when associated with Cdk2, which may result in complex disassembly and subsequent degradation of the free cyclin by the ubiquitin-proteasome system (161,162). It is possible that the cell cycle machinery has ensured periodicity of the kinase activity by linking cyclin E/Cdk2 activity to destruction of cyclin E and restrained the cyclin E/Cdk2 kinase activity to a specific point in G1.

Purified cyclin E/Cdk2 complexes phosphorylate Rb *in vitro* and this effect binding of Rb to E2F (133). To determine whether cyclin E normally phosphorylates Rb *in vivo*, a cyclin E mutant was constructed recently. This mutant is deficient in phosphorylating Rb although it can form catalytically active complexes with Cdk2 and phosphorylating histone H1 (HH1), a ubiquitous Cdk substrate (163). This led to the discovery of a conserved Rb binding domain in cyclin E (the VxCxE motif) and mutations of this motif, similar to mutations in the LxCxE motif in cyclin D, decreased the ability of cyclin E to bind Rb both in cells and *in vitro* (163). It has also been suggested that the effects of cyclin D and cyclin E overexpression on G1 acceleration are additive (164). A positive feedback model has been proposed where phosphorylation of Rb by cyclin E releases E2F, which in turn upregulates cyclin E expression with subsequent activation of cyclin E-associated kinase activity (165) (figure 6). This model could explain how Rb phosphorylation becomes independent of cyclin D and mitogenic stimulation as well as explain the observations from the cyclin D1 knockout/cyclin E knockin mice. These transgenic mice, which express cyclin E from the cyclin D1 promoter, have a normal cell cycle and completely reverse all



**Figure 6. Model of cell cycle events mediating progression through G1.** The activation of cyclin D-CDK4 by mitogenic stimulation results in phosphorylation of Rb and release of E2F. E2F activates cyclin E transcription, as well as downstream components needed for initiation of DNA replication. Cyclin E has the potential to form self-regulating circuitry which is responsible for the periodicity of cyclin E-CDK2 kinase activity. The conversion from a cyclin D-activating cycle to a cyclin E self-sustaining cycle may represent the R point switch from mitogen dependence to mitogen independence. Cyclin E may control the timing of DNA replication in two ways: by activating components required for initiation and/or by inactivating the negative regulator p27 (49).



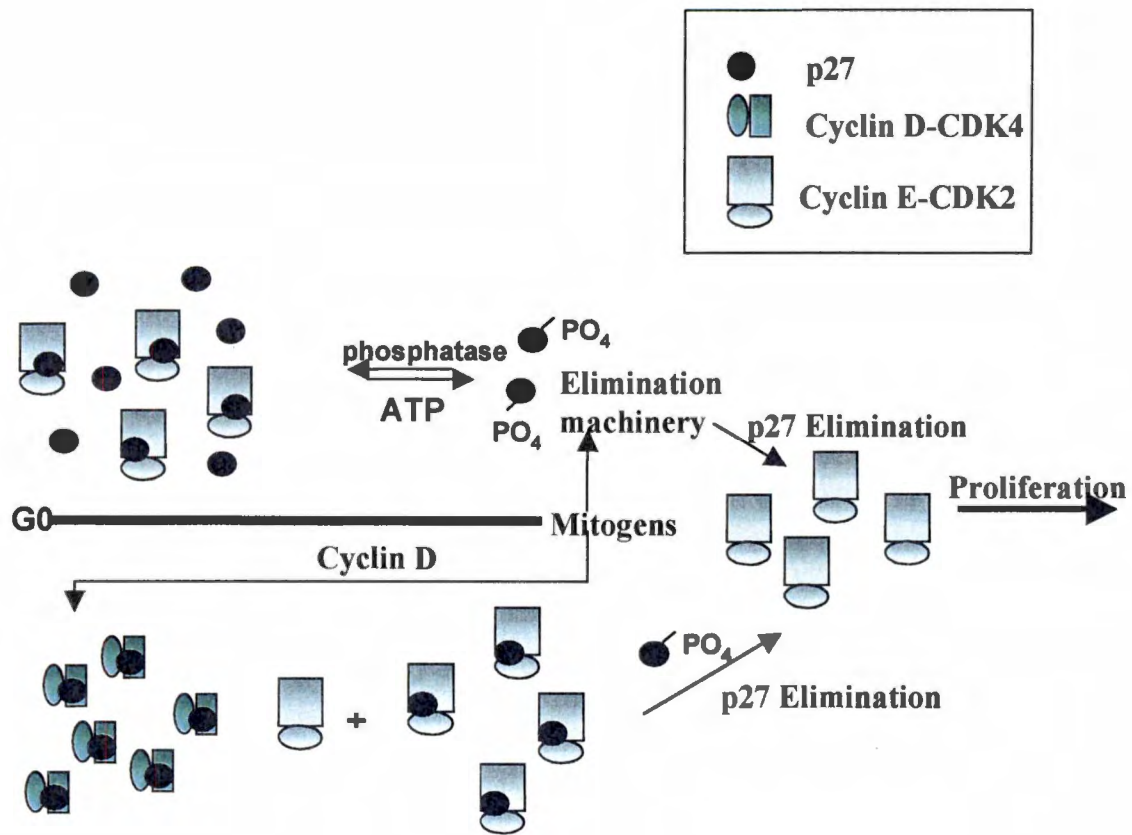
the known defects of the cyclin D1 knockout mice (166). The early expression of cyclin E appears to have short-circuited the necessity for cyclin D1 for cell cycle transit and this poses the question of the redundancy of cyclin D in normally cycling cells (see also below)

It was not well understood how cyclin E/Cdk2 catalyzes the G1 to S phase transition, although it has been demonstrated that cyclin E can function independent of Rb (158,164). Microinjection of antibodies directed against cyclin E, arrests both Rb-positive and Rb-negative cell lines in G1 (158). The "classic" Rb/E2F pathway may not be the only physiological way to commit mammalian cells to DNA replication, as there is evidence that cyclin E is under the control of a parallel G1/S-regulatory pathway that pivots around Myc (167). It was recently demonstrated that Myc, like cyclin E, could rapidly induce S phase entry and persistent DNA replication independent of E2F activity (167). Myc appears to perform this G1/S-promoting function by inducing cyclin E-associated kinase and Cdc25A phosphatase activities. Blocking either the classical Rb pathway or the parallel Myc pathway significantly delayed and decreased, but did not prevent DNA replication, while blocking both pathways abolished DNA synthesis. This implies both Myc and E2F activities are necessary to allow for timely and proper initiation of DNA synthesis in mammalian cells, however, it is possible that alternative mechanisms of cyclin E control both dependent and independent of E2F exist.

One non-Rb substrate of cyclin E is p27 and it has been reported that phosphorylation of p27 on Thr-187 by cyclin E-bound Cdk2 initiates the

elimination of this CKI from the cell (168). Kinetic analysis of cyclin E/Cdk2/p27 complexes demonstrates that p27 initially engages cyclin E/Cdk2 complexes in a transient loosely bound state (168). This can later convert to the catalytically inactive form and the equilibrium of these two states is modulated by the ambient ATP concentration. In the loosely bound state, p27 can be phosphorylated by Cdk2 and released from the complex, thus becoming the substrate instead of inhibitor of the cyclin E/Cdk2 complex. Growth factors or other mitogenic signals can tip the balance towards proliferation by causing a small increase in the amounts of active cyclin E/Cdk2 complexes, which could trigger a feedback loop in which cyclin E/Cdk2 downregulates p27 and activates more of the cyclin E-associated kinase (figure 7)

All mammalian G1 cyclin/Cdk complexes may not be inhibited by p27 as cyclin D-Cdk4/6 complexes are active early in G1 when p27 levels are high and these complexes bound to p27 have demonstrable Rb kinase activity (68,169,170). Similarly, it was found that p27 immunoprecipitates contained a kinase activity with strong preference for Rb but not for HH1 (169). *In vitro*, cyclin D-Cdk4-p27/p21 complexes with a stoichiometry of 1:1:1 failed to be inhibited significantly (68,170,171), implying that D cyclin-Cdk complexes bound to Cip/Kip inhibitors are relatively resistant to functional disruption. These data lead to the proposal of a model where kinase complexes associated with only one inhibitor molecule remain active and, for inhibition of enzymatic activity, binding of more than one CKI molecule is necessary. However, Hengst et al.,



**Figure 7. Control of proliferation by p27 and its Cdk catalyzed elimination.** In the upper panel cyclin E/Cdk2 actively phosphorylates p27, but a pool of free p27 is maintained by the counter balancing effect of phosphatases. Mitogens tip the balance in favor of cell cycle progression by activating elimination machinery so that phosphorylated p27 is eliminated from the cell. The lower panel depicts an alternative model in which cyclin E/Cdk2 complexes are inhibited in G0 by p27. Mitogenic activation of cyclin D/Cdk4 results in the redistribution of p27 to these complexes, and the subsequent activation of low levels of active cyclin E/Cdk2. These in turn catalyze p27 elimination by phosphorylation, thereby activating more cyclin E/Cdk2 and driving G1 progression.

using analytical ultracentrifugation of purified p21, cyclin A and Cdk2 complexes as well as several other techniques demonstrated that cyclin A/Cdk2 complexes bound to a single p21 molecule were in a completely inhibited state (172). This lead them to conclude that the level of Cdk inactivation by p21 is determined by the fraction of kinase complexed with the inhibitor and not by the stoichiometry of inhibitor bound to the kinase or the phosphorylation state of the inhibitor, at least in the case of p21 and cyclin A bound Cdk2.

Studies with U2OS cells also demonstrated an adaptor function for the Cip/Kip family of CKI (68). In these cells the association of Cdk4 with D-type cyclins were facilitated by the presence of p21, p27 and p57, both *in vivo* and *in vitro*. All three Cip/Kip inhibitors were also demonstrated to target Cdk4 and cyclin D1 to the nucleus. The p27 and/or p21 knockout mice further demonstrated that binding of these CKIs actually facilitate assembly of cyclin D1/D2-Cdk4 and activation of these enzymes (173). Therefore, early induction of cyclin D by mitogens and formation of complexes with Cdk4/6 could lead to redistribution of p27 from cyclin E/Cdk2 to D cyclin-Cdk4/6 complexes and pave the way for the formation of a small p27-free active cyclin E/Cdk2 pool. This could tip the balance towards p27 elimination and G1 transit.

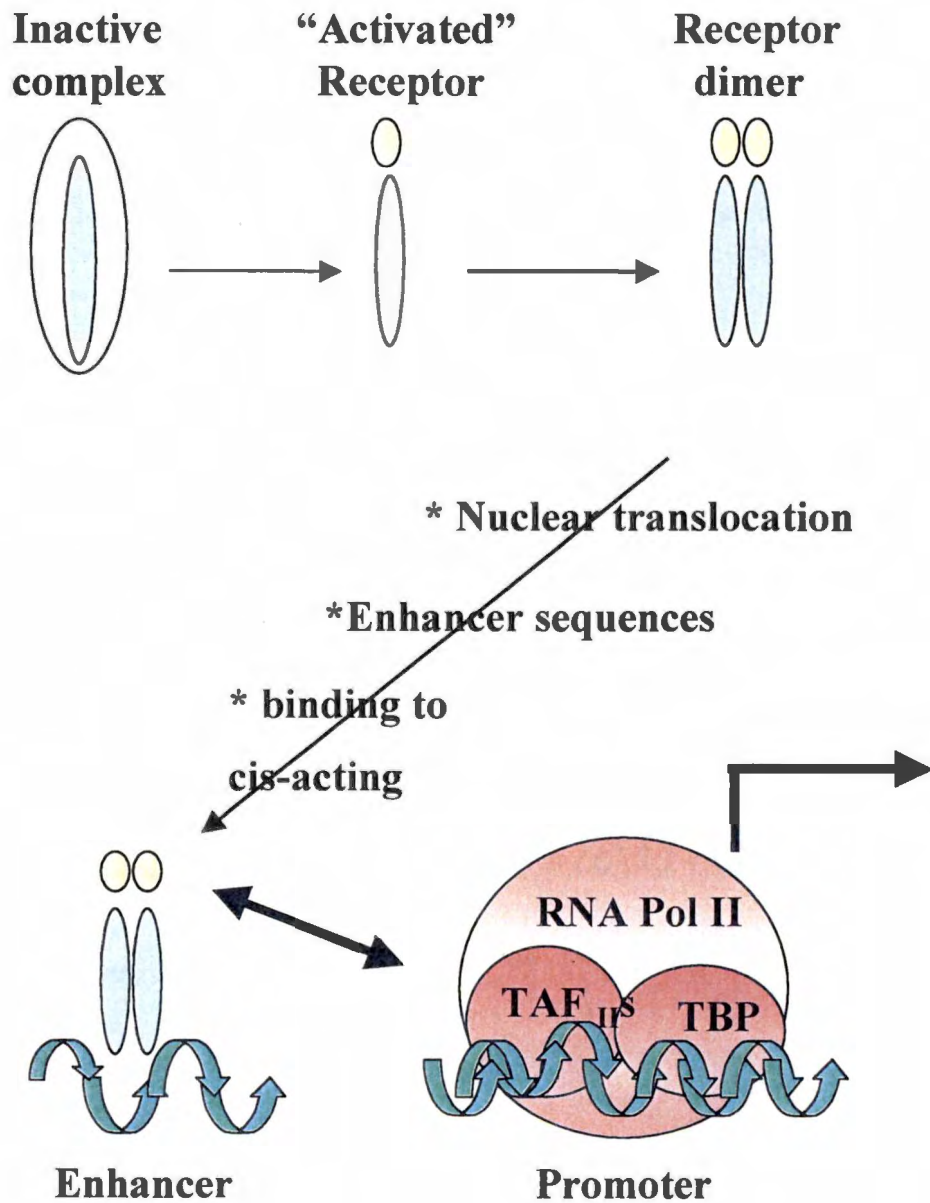
The above observations indicate that when Cip/Kip family members are neutralized, cyclin D-associated kinase activity may not be required for cell cycle progression. This is further confirmed by the mouse embryo fibroblasts (MEFs), which lack p27 and p21 but exhibit normal cell cycles, although, cyclin D-dependent kinase activity was reduced below detection limits (173). In the

absence of CKI, cyclin E and A-associated Cdk2 can phosphorylate Rb unopposed in these MEFs, rendering both the catalytic and CKI-titration function of the D cyclins superfluous. The replacement of cyclin D1 with cyclin E leading to rescue of the developmental defects observed in D1 deficient mice also implies that cyclin E can function downstream of cyclin D1 (166).

## **ESTROGENS AND XENOESTROGENS**

### **17 $\beta$ -estradiol**

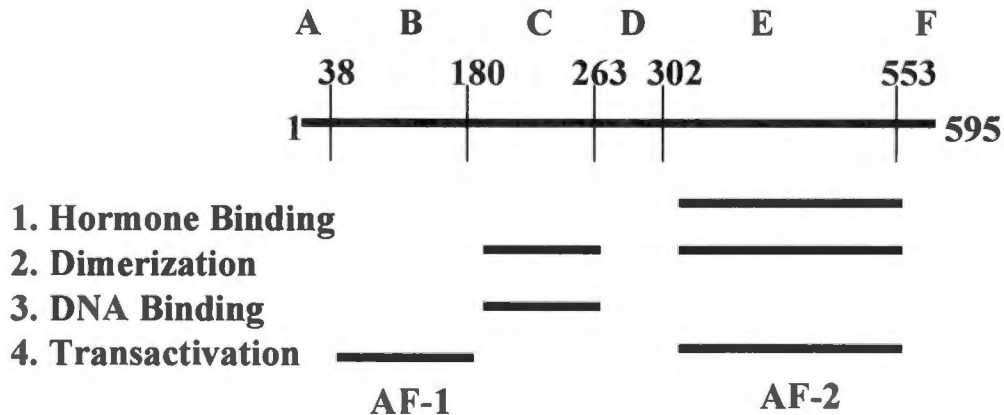
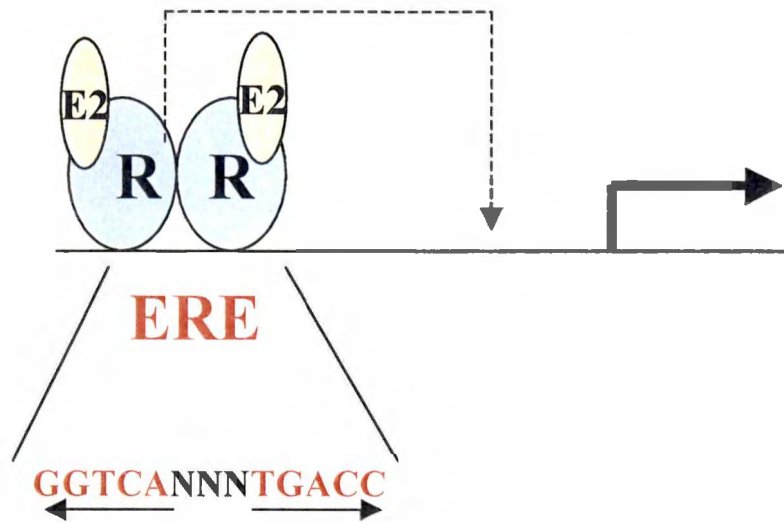
Classical steroid hormones such as estrogen, progesterone, androgens, glucocorticoids, and mineralocorticoids are synthesized and secreted by endocrine cells. These hormones travel via the blood stream, bound to serum hormone binding globulins (SHBG) and diffuse passively into target cells. Steroids are preferentially retained in target cells as stable complexes with specific intracellular receptor proteins. These intracellular receptors are transcription factors that exist in inactive apoprotein forms either in the cytoplasm or nucleus (174). A model for the general mechanism of action of steroid hormones was originally proposed by Jensen et al (175) and Gorski et al in 1968 (176). They suggested that upon binding the respective hormonal ligand, these receptors undergo transformation or "activation" which allows them to bind effectively to a DNA element (the hormone response element, HRE). The DNA bound steroid-



**Figure 8. Model for steroid receptor transactivation.** The ligand bound receptor dimerizes and enters the nucleus, prior to binding enhancer elements on the promoters.

receptor complex then acts as a transcription factor modulating the transcription of hormone-responsive genes (figure 8). The naturally occurring estrogens are 18-carbon steroids, which have an aromatic A ring with a phenolic hydroxyl group. The nonpregnant female ovaries are the major sites of production of  $E_2$ , while in the pregnant female the ovaries produce  $E_2$  until the demise of the corpus luteum. Estrinol ( $E_3$ ) is produced by the fetal-placental-unit by conversion of fetal androgens, primarily dehydroepiandrosterone sulfate. Androgens produced by the adrenal glands also serve as substrates for  $E_2$  production in a variety of tissues, including fat, brain, and breast. In males, the testes under some circumstances can produce  $E_2$ , and in both males and females, the adrenal cortex can generate small quantities of estrones. The actions of estrogenic hormones are mediated through the estrogen receptor (ER), a member of a large superfamily of nuclear receptors that function as ligand-activated transcription factors. The presence of the estrogen receptor in a tissue is presumptive evidence for the biological actions of estrogen at that location. In 1986, Greene et al. reported that the human estrogen receptor present in MCF-7 human breast cancer cells is comprised of a single polypeptide chain of 595 amino acids, with a molecular weight of 66 kDa (177) and later this receptor was called  $ER\alpha$ . Amino acid sequence analysis and mutational dissection of this intracellular receptor indicates that it can be divided into several domains that are responsible for ligand binding, DNA binding, and transcriptional activation (178,179) (figure 9).

The central DNA binding domain (DBD) of the ER, demarcated as the C region, contains two highly conserved zinc fingers and is responsible for DNA



**Figure 9.** (A) Model of estrogen receptor action. Receptor dimer occupied by E2 binding to the ERE to activate gene transcription. (B) Domain Structure of the human estrogen receptor (595 aa in length) and the Localization of receptor functions (numbered 1-4) to specific regions (A-F) of the receptor (184).



recognition, dimerization, and nuclear localization. The carboxy-terminal E/F domain encompasses the ligand-binding region (LBD), which has the hormone recognition property and is relatively large and functionally complex. The E/F domain also contains the hormone-dependent transactivation functions (AF-2) of the receptor (180,181). Downstream of the C domain is a variable hinge region, the D region, which allows the protein to bend or alter conformation after ligand binding. The amino-terminal A/B region contains a transactivation function (AF-1), which is independent of E<sub>2</sub> binding (180). The inactive receptor is thought to exist in a complex with heat-shock proteins (hsp90, hsp70, and hsp56) and it has been proposed that these proteins interfere with its transactivation function (182). The ligand-bound ER is reported to undergo conformational changes, which disperse the heat-shock-proteins, cause dimerization, and subsequent binding to estrogen response elements (ERE) located in the 5' flanking region of estrogen responsive genes (182,183)

The ERE comprises of an inverted palindrome separated by three nucleotides (GGTCAnnnTGACC). Three amino acids in the "P box" (EGckA), situated at the base of the first zinc finger in the DBD of ER, is essential for ERE recognition, and mutations in this region from EgckA to GSckV changes ERE recognition to glucocorticoid response element recognition (174). These DNA sequences function as enhancers conferring estrogen inducibility on the genes (184). The E<sub>2</sub>-bound receptor then interacts with basal transcription factors, coregulator proteins, and other transcription factors to modulate gene transcription (185) The phenomenon of squelching, or transcriptional interference between

receptors, in transient receptor/reporter co-transfection assays indicate that factors other than general transcription factors interact with activated receptors (186). The titration of a cellular pool of factors for which the activated receptors compete for limits the overall reporter activity of the receptors and indicated that common cofactors might be an important functional link between the receptor and basal transcriptional machinery (187). It is now recognized that a host of co-activator and co-repressor proteins are involved in the transcriptional modulation of the ligand-bound ER. Binding of  $E_2$  allows the formation of a hydrophobic cleft on the surface of the ER that serves as a docking site for co-activators (188). Antiestrogens such as tamoxifen, competitively bind ER and block its hormone-dependent AF-2 function, by displacing part of the receptor and occluding co-activator access (188). The finding that antiestrogen-bound ER can have different estrogenicity in different target tissues (eg, breast vs. bone and uterus) also raised the possibility of differential tissue distribution of co-activators and co-repressors (189-191). It is also reported that the estrogen can regulate genes, which do not have a "classical" ERE, via different mechanisms.

Several genes which have a half palindromic ERE also can be regulated by  $E_2$  in conjunction with other transcription factors such as SP-1 (192). In the case of the insulin-like growth factor-1 gene, the ligand-bound ER is demonstrated to interact with the Fos/Jun complex and enhances Fos/Jun binding to the AP-1 motif (193). It is suggested that this is possible because ER binds coactivators, CBP and GRIP1, which have been recruited by Jun/Fos and, through this contact, trigger these co-activators into full activity independent of the DBD of ER (188).

In this circumstance, ER appears to be part of the co-activator complex for Jun/Fos. On the other hand, the agonist activity of tamoxifen in tissues such as the uterus is believed to be due to the DBD-dependent transcriptional activity at AP-1 sites (194). The amino terminal activation function of the ER, AF-1, appears to be essential in this case and may involve synergism between ER activation domains and Jun. The estrogen-like effects of Tamoxifen such as increased cervical hyperplasia, increased risk of uterine cancer, and unpredictable stimulation of breast tumor growth during tumor progression, are reported to be due to "agonism" at AP-1 sites.

A separate subtype of ER, termed ER $\beta$ , was identified from cDNA libraries of human testes and rat prostate, tissues not generally considered to be major estrogen target tissues (195,196). ER $\beta$  is not an isoform of ER $\alpha$ , but a distinct protein encoded by a separate gene on a different chromosome. The initial studies indicated that the rodent ER $\beta$  cDNA encodes a protein of 485 amino acid residues with a calculated mol wt of 54 kDa. However, unlike the ER $\alpha$  gene, Northern blot analysis of ovarian RNA from both rat and mouse appear to indicate the presence of multiple ER $\beta$  transcripts (197-199). ER $\alpha$  mRNA appears to be predominantly present in the uterus, mammary gland, testis, pituitary, liver, kidney, heart, and skeletal muscle, whereas ER $\beta$  transcripts are significantly expressed in the ovary and prostate (198,200-202). While relatively equal levels of mRNA for the two receptors appear to be present in the epididymis, thyroid, adrenals, bone, and various regions of the brain, there appear to be differences among species in tissue distribution (200,202,203).

The rat ER $\beta$  is highly homologous to the rat  $\alpha$  subtype at the DBD, with >90% amino acid identity, suggesting that ER  $\alpha$  and  $\beta$  interact with similar DNA response elements (200,204). However, the overall homology of the LBD region is only 55% while the A/B domains and AF-1 regions are quite different. The ligand binding affinities of ER  $\alpha$  and  $\beta$  appear to be quite similar for most physiological ligands and so far a unique physiological ligand for ER $\beta$  has not been identified (200). However, ER  $\alpha$  and  $\beta$  appear to respond differently to certain ligands at an AP-1 site, implying different regulatory functions for the two ER subtypes. Transfection of Hella cells with either the ER  $\alpha$  or  $\beta$  expression plasmids, along with an ERE-luciferase reporter gene, demonstrate same transactivation profiles with E<sub>2</sub> (205). However, ER $\beta$ -transfected cells showed no transcriptional activation at an AP-1 site with E<sub>2</sub> or diethylstilbestrol (DES), a potent synthetic estrogen. In contrast, antagonists such as raloxifen, tamoxifen, and ICI 164,384, which block the stimulatory activity of the agonists when bound to an ER $\alpha$ /AP-1 complex, acted as potent agonists when bound to an ER $\beta$ /AP-1 complex. Such differences in activity between the two subtypes of the ER together with the different distribution in the body may explain estrogen's broad spectrum of activity, especially in tissues where no ER $\alpha$  is found.

The precise role of estrogens in humans is not fully understood. There have been reports of E<sub>2</sub> synthesis in fetal ovaries of several species suggesting the involvement of estrogen signaling system in ovarian development (206). The generation of knockout animals for both receptors, as well as the double knockout

has furthered knowledge of the precise role of each ER, and provided invaluable data for possible cooperative roles of both receptors within the same tissue. It has been demonstrated that both the  $\alpha$ ERKO and  $\beta$ ERKO mice were viable and both male and female mice exhibited a life span comparable to wild type (207,208). Both sexes of the double knockout,  $\alpha\beta$ ERKO mice, survived to adulthood and exhibited normal reproductive tract development but were infertile (209). This challenged the prevailing view that disruption of ER would be lethal during development as a result of the defective implantation of the embryo (210). However, the dependency on  $E_2$  for postnatal growth was illustrated by the severe hyperplasia in the uteri of female  $\alpha\beta$ ERKO mice, and observations of postnatal sex reversal in the ovaries. The ovaries of the  $\alpha\beta$ ERKO mutants exhibited follicle transdifferentiation to structures resembling seminiferous tubules of the testis, indicating that both receptors are required for ovarian function and oocyte survival in the adult.

The classical ER signaling pathway explains the slower genomic signaling, which takes hours or more to achieve its final downstream effects. However, estrogens also exert a variety of short-term effects seen in seconds to minutes on their target organs.  $E_2$  rapidly affects cation transport, glucose uptake, and water imbibition in the rat uterus (211). In neurons and GH3 pituitary tumor cells, estrogens elicit changes in electrical activity within minutes (212,213). Estrogens have also been demonstrated to induce extremely rapid increases in the concentrations of the intracellular second messengers, calcium and cAMP (214-216). The time course of these events is similar to those elicited by growth

factors, and peptide hormones, implying that they do not involve the classical genomic action of estrogen through its receptor. Cell surface forms of ER that are coupled to cytosolic signal transduction proteins have been proposed to mediate the rapid estrogenic effects seen in the vasculature, breast, bone and neuronal tissue (217,218). Razandi et al transfected CHO cells with ER  $\alpha$  and  $\beta$  and demonstrated that the membrane and nuclear form of the ER originate from a single transcript, and that the membrane forms of  $\alpha$  and  $\beta$  receptors can activate the mitogen-activated protein kinase (MAPK) pathway leading to cell proliferation (219). In MCF-7 human breast carcinoma cells the estrogen-induced activation of MAPK pathway requires intracellular calcium mobilization (220).

### **E<sub>2</sub> and Carcinogenesis**

The dominant actions of estrogens occur in the female reproductive tract, although there are also significant biological actions mediated in the liver, central nervous system, bones, cardio vascular system, as well as other visceral organs. E<sub>2</sub>, together with progesterone, profoundly influences mammary epithelial cell proliferation and morphogenesis (221,222). In the normal breast, E<sub>2</sub> stimulates the growth of the ductal systems whereas lobular development depends on progesterone secretion. The cumulative exposure of breast tissue to E<sub>2</sub> appears to predispose for carcinogenesis (223). E<sub>2</sub> affects the rate of cell division and proliferating cells are susceptible to genetic errors during DNA replication and, if

uncorrected, can ultimately lead to a malignant phenotype. E<sub>2</sub> has long been known to exert growth-promoting effects on a significant proportion of breast cancers *in vivo* (224,225), as well as on breast cancer cell lines *in vitro* (226,227).

Estrogens and antiestrogens regulate cell cycle progression and cyclin expression in ER-positive breast cancer cell lines (228-230). The inhibition of growth of estrogen-responsive MCF-7 breast cancer cells by antiestrogens has also been linked to downregulation of cyclin D1 gene expression leading to accumulation of cells in G1 (231). The mechanisms by which E<sub>2</sub> regulates G1 progression in ER-positive cells were further delineated by Foster et al. when they demonstrated that E<sub>2</sub>, in the absence of additional mitogenic stimulation, regulates G1 progression in MCF-7 cells by direct activation of G1 Cdks, Rb phosphorylation, and elimination of Cdk inhibitors (232). They also demonstrated that, as with other mitogens, E<sub>2</sub> was able to induce synthesis of cyclin D1, although, a classical ERE has not been identified in the cyclin D1 promoter.

Within a short time Altucci et al. demonstrated that in MCF-7 cells treatment with E<sub>2</sub> resulted in increase in cyclin D1 mRNA and protein due to increases in transcription of the gene (233). These experiments were done in the presence of Symvastatin, a Hydroxy-methylglutaryl-CoA reductase inhibitor, which is reported to make these cells unresponsive to serum-derived growth factors by inhibiting key signal transduction pathways (234). They succeeded in mapping an estrogen-responsive regulatory region upstream of the transcriptional start site of the cyclin D1 promoter that was different from the classical ERE, and suggested that an AP-1 site (-935) might be involved in the transcriptional

regulation of cyclin D1 by E<sub>2</sub>. Recently, it was proposed that a cAMP response element (CRE) is also involved in E<sub>2</sub> transactivation of the cyclin D1 gene. Sabbah et al. in 1999 demonstrated that the activation of cyclin D1 promoter by the E<sub>2</sub> bound receptor is mediated by ATF-2/c-jun heterodimers binding to the CRE in the region between -96 and -29 upstream of the start site (235). Destruction of this CRE motif caused complete loss of estrogen responsiveness and the DBD and both AF-1/AF-2 transactivation functions of the ER were required for the optimal activation of D1 promoter by E<sub>2</sub>.

The recent publication by Swarbrick et al. further elucidates the mechanisms by which the steroid hormone progesterone mediates cell cycle arrest in T-47D breast cancer cells (236). They propose that the decrease in G1 Cdk activity and the consequent G1 arrest resulting from progesterone treatment is due to the concerted inhibition from the Cip/Kip inhibitor, p27, and INK4 inhibitor, p18. They demonstrated that the progesterone-induced p18 leads to redistribution of p27 among the G1 Cdk complexes and results in cell cycle arrest.

In addition to the promotional effects of estrogens on cellular proliferation, estrogens may directly damage DNA. The synthetic estrogen DES is oxidized by a peroxidase-mediated reaction to produce short-lived epoxide- and semiquinone intermediates that are carcinogenic (237). Several metabolites of E<sub>2</sub> can directly, or indirectly, cause oxidative DNA damage (238-241). The hydroxylation of E<sub>2</sub> at positions C2 and C16 produces reactive metabolites that can attack both DNA and proteins (242,243). In the body, circulating E<sub>2</sub> is rapidly converted in the liver to estrone (E<sub>1</sub>) and can be further metabolized to 16 $\alpha$ -



hydroxestrone ( $16\alpha$ -OH  $E_1$ ) or to 2-hydroxyestrone. The remaining  $E_1$  is conjugated to form  $E_1$  sulfate.  $16\alpha$ -OH  $E_1$  is converted to  $E_3$ , which in turn is metabolized to estriol-3-sulphate-16-glucuronide before excretion by the kidney. Investigators have found that the levels of  $16\alpha$ -OH  $E_1$  is significantly elevated in women with clinically identifiable breast cancer and in women at increased risk for developing breast cancer (244,245). It is suggested that, since the  $16\alpha$ -OH  $E_1$  pathway is selectively elevated well before the appearance of overt cancer, this can provide an endocrine biomarker for cancer risk identification (246). Therefore it appears that estrogens act by both inducing genomic damage (initiating) and increasing cellular proliferation (promotion), i.e. estrogens function as both carcinogens and tumor promoters.

### **Xenoestrogenic compounds**

Natural, environmental, or manufactured substances, which exhibit estrogenic activity; i.e. substances that affect the estrogen-dependent development and function of reproductive systems in humans and animals, are considered xenoestrogenic compounds. The ability of a specific compound to induce cell proliferation of estrogen-dependent cells or organs via binding to the ER, either *in vivo* or *in vitro*, can be used as a definition of estrogenicity (247). According to this definition, a number of synthetic chemicals and naturally occurring plant compounds can be defined as Xenoestrogens. However, a xenoestrogen can induce its estrogenic effect in multiple ways: it may act directly through the ER,

or it may disturb estrogen metabolism, thus increasing the levels of endogenously produced ligand. These estrogenic compounds can be categorized broadly as natural or synthetic (Table 2). The natural estrogens can be manufactured by the body (endogenous estrogens), or by plants (phytoestrogens), or fungi (mycoestrogens). The synthetic estrogens can be pharmaceutical (drugs) or industrial xenoestrogens (pesticides, plasticizers etc.).

### **Industrial Chemicals**

The industrial xenoestrogens include the organochlorine pesticides (dichlorodiphenylethanes, cyclodienes, hexachlorobenzene, and hexachlorocyclohexanes) (248), polychlorinated biphenyls (used as heat transfer and hydraulic fluids, adhesives, flame retardants, dielectric fluids for capacitors and transformers, waxes etc.) (249), and alkylphenol polyethoxylates (industrial surfactants) (250). The best known and most extensively studied xenoestrogen is the organochlorine pesticide bis(4-chlorophenyl)-1,1-dichloroethene (DDT) (251). Extensive use of DDT as an agricultural pesticide and malaria control agent began in late 1930s when its potential as an insecticide was recognized by the Swiss chemist Paul Muller, who was awarded the 1948 Nobel Prize for Medicine and Physiology for his contribution to public health. Substantial evidence for the hormone-like effects of these chemicals in fish, wildlife, and humans surfaced over the next few decades and led to the banning of DDT and other polychlorinated biphenyls (PCBs) (252-255). Although the organochlorine compounds are less potent than endogenous estrogens, they cause concern due to

**Table 2. Classification of natural and synthetic estrogens.**

| Subtype                        | Source                                                                    | Examples                                                                       |
|--------------------------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| <b>Natural Estrogens</b>       |                                                                           |                                                                                |
| <b>Endogenous Estrogens</b>    | Ovarian, adrenal, adipose tissue                                          | Estradiol, estrone, estriol                                                    |
| <b>Exogenous Estrogens</b>     |                                                                           |                                                                                |
| Replacement Therapy            | Pharmaceutical                                                            | Estradiol, estrone, estriol, equinilin                                         |
| Phytoestrogens                 | Foods, herbs, spices                                                      | Genistein, coumestrol, equol, enterolactone                                    |
| Mycoestrogens                  | Fungi                                                                     | Zearalenol, zearalenone                                                        |
| <b>Synthetic Estrogens</b>     |                                                                           |                                                                                |
| Replacement Therapy            | Pharmaceutical                                                            | Ethinyl estradiol, diethylstilbestrol                                          |
| Xenoestrogens                  | Industrial<br>Pesticides<br><br>Preservatives<br>Solvents<br>Plasticizers | DDT, methoxychlor, dieldrin, kepone<br>BHA, BHT<br>Hydrocarbons<br>Bisphenol-A |
| <b>Synthetic Antiestrogens</b> |                                                                           |                                                                                |
| Cancer Therapy                 | Pharmaceutical                                                            | Tamoxifen                                                                      |
| Xenoantiestrogens              | Industrial                                                                | TCDD                                                                           |

Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene  
TCDD, 2,3,7,8,-tetrachlorodebenzo-p-dioxin

their persistence in the environment, resistance to degradation, and sequestration and storage in fatty tissue of humans and animals (256). It has been reported that the levels of organochlorines in fat are 200-300 times greater those measured in serum (257).

Numerous reports link industrial chemicals to adverse reproductive, developmental and behavioral effects on fish and wildlife Fry et al. In 1987 (253) noted poor breeding success, a skewed sex ratio favoring females, female-female pairing, and supernormal cultures in western gulls on the Channel Islands offshore southern California This area was reported to receive approximately 2 million kg of DDT via sewerage discharge from the Los Angeles area and Central Valley of California from 1950-1970. The feminizing effects of o,p'-DDT was confirmed in egg injection studies and gull eggs collected from the contaminated area contained 6-30 ppm total DDT, an amount sufficient to cause abnormal development (253). On Lake Apopka in Florida, a dramatic decline in adult alligator populations was noted in 1980 and which persisted (255). The juvenile male alligators in the lake exhibited microphallus, altered plasma E<sub>2</sub> to testosterone ratios and abnormal testicular development, while the females exhibited abnormal ovarian development, polyovular follicles, and multinucleated oocytes. A tributary of the lake was contaminated by an industrial spill of DDT, dicofol, and sulfuric acid the year before the decline in reproduction was observed which could have lead to the feminization of the male alligator and the hyper-feminization of the females. The above studies, as well as other reports such as the wildlife studies in the Great Lakes (258,259), observation of hermaphroditism

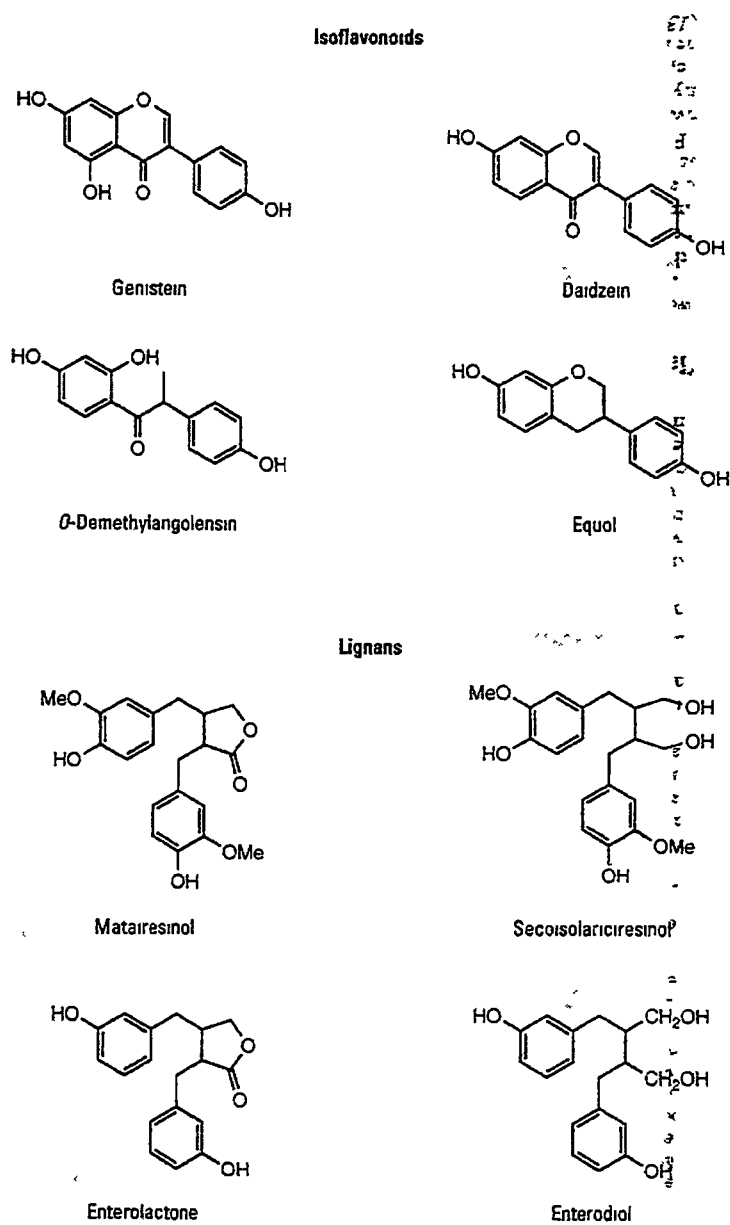
in fish in sewage treatment water lagoons in England and Wales (260,261), the decline in the population of Florida panthers (*Felis concolor coryi*) (262,263), demonstrate that industrial xenoestrogens such as DDT and its analogues or other endocrine-disruptive contaminants have caused significant adverse reproductive effects in exposed birds, fish, reptiles and mammals.

These organochlorine compounds have been shown to promote tumorigenesis in rodents (264,265), however, their role in human cancers appears to be controversial. It has been hypothesized that environmental estrogens may contribute to the increased incidence of breast cancer in women (266), as well as falling sperm concentrations and disorders of the male reproductive tract (267). Although some studies showed that PCB or DDE levels, a metabolite of DDT, were elevated in breast cancer patients (268-270) these increases were not consistently observed in all studies (271-273). Some researchers are of the opinion that the linkage between estrogenic organochlorine compounds and breast cancer is highly unlikely since DDE, which is the dominant metabolite of DDT that persists in the environment, is primarily the p,p'-DDE isomer, which is not estrogenic (274,275). However there are also reports to the contrary that demonstrate estrogenic actions of p,p'-DDE on the rat uterus (276) and in MCF-7 cells (277), as well as potent anti-androgenic effects (278). Carlsen et al. performed a metaanalysis of sperm concentrations from over 60 data sets reported between 1938-1990 and found that the sperm concentration had declined >40% from 1940 ( $113 \times 10^6/\text{ml}$ ) to 1990 ( $66 \times 10^6/\text{ml}$ ) in otherwise normal men (279). During the same time period, the incidence of testicular cancer has markedly

increased in many countries. It was suggested that xenoestrogenic compounds might be involved in the global fall in sperm counts and increased disorders of the male reproductive tract (267). Reanalysis of these data, however, indicated that in the second half of the study period from 1960-1990, the decrease in sperm count was not significant (280). It was also pointed out that during the early part of this time period, organochlorine use was rampant and therefore the environmental residues might have been very high and could have affected sperm concentrations before the 1960s (275). Therefore, the role of the industrial estrogens on breast cancer in women and reproductive disorders in males appears to be highly complex and requires further investigation

### **Phytoestrogens**

Structurally, phytoestrogens can be divided into 3 classes. isoflavonoids (biochanin A and genistein), coumestans (coumesterol) and lignans (enterolactone and enterodiol). Isoflavonoids and coumestans are formed in numerous edible plants, especially in soy and leguminous plants. The lignans are derived from unrefined grain as precursors, which are activated by intestinal bacteria (281) (figure 10). Large amounts of isoflavones are ingested daily by humans, especially in a vegetarian diet. In countries such as Japan and China, traditionally isoflavone-containing soy is a staple (daily intake around 20-50 mg/d) (282), while in Western women the daily intake is typically negligible (<1 mg/d) (283). The phytoestrogens appear to possess mixed estrogen agonist-antagonist properties that are organ-specific *in vivo* (281). The compounds genistein,



**Figure 10.** Structure of the most important isoflavonoids and lignans identified in human biological samples

coumestrol, and zearalenone (a fungal estrogen) are reported to have high binding affinity for ER (284), and It has been suggested that the phytoestrogens act as antiestrogens through competition for the binding sites of ER with the hormonally more potent endogenous estrogens (275). However, other studies have demonstrated that genistein, coumestrol and zearalenone act through ER-mediated mechanisms and stimulate growth of different breast cancer cell lines (285-287)

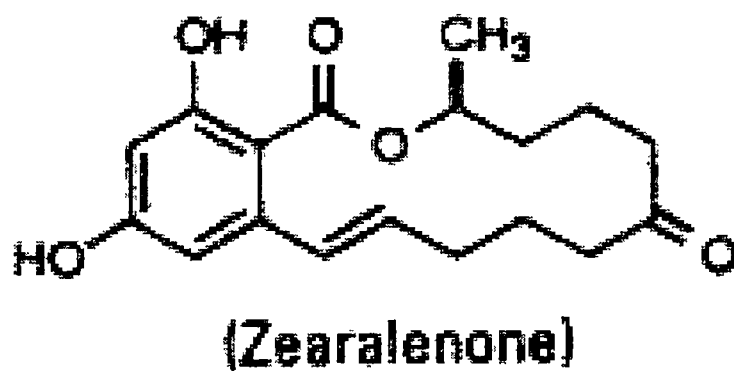
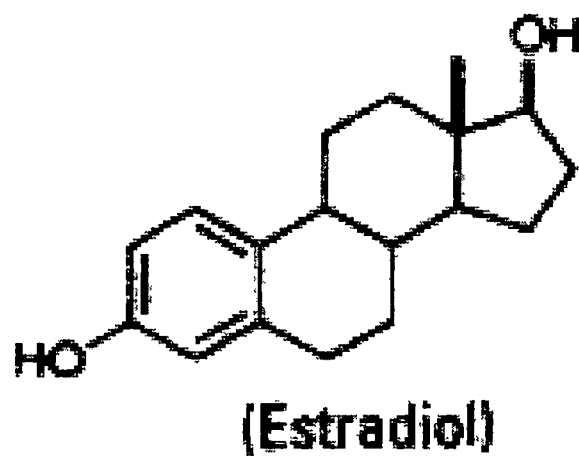
On the other hand isoflavonoids are reported to increase the hydroxylation of  $E_2$  to 2-OH metabolites (288), increasing the formation of catechol estrogens that act as antiestrogens (289). The antiestrogenic actions of isoflavones were also demonstrated in women who consumed a soy protein-enriched diet containing 45 mg isoflavones daily for one month, probably due to the phytoestrogen-induced production of serum hormone binding hormones (290). The resultant decrease in bioavailability of endogenous estrogens could lead to the observed menstrual cycle changes, including prolongation of the follicular phase and suppression of mid cycle surge of LH and FSH hormones. Menstrual cycle length is one of the risk factors for breast cancer (291) The mean cycle length in Western countries, where breast cancer risk is high, is 28-29 days, whereas in Japanese women, who have 4-5 times lower breast cancer risk, it is around 32 days (292). Also, plasma circulating estrogen concentrations in Asian women are 20-30% lower than in their Western counterparts (293-296), and the combined effect of longer menstrual cycles with lower estrogen concentrations appear to result in overall lower lifetime exposure to estrogens (283). Tamoxifen, a commonly used



chemotherapeutic antiestrogen, also has similar effects on the endocrine regulation of the menstrual cycle and is used in preventing breast cancer in women at high risk for this disease (297-299).

### Mycoestrogens

The mycotoxin zearalenone (figure 11) is a resorcylic acid lactone mainly produced by the mould *Fusarium graminearum* present naturally in a variety of host plants and debris from soil around the world (300). Chemical reduction of zearalenone yields two epimers, the  $\alpha$ -zearalenone is 4.8 times more estrogenic than zearalenone and exhibits anabolic properties (301), while the  $\beta$ -epimer is a less potent estrogen. Zearalenone has been implicated in numerous incidences of hyperestrogenism in farm animals, especially pigs (302-304). Zearalenone and some of its derivatives have been shown to competitively bind to ER in a number of *in vitro* systems (284,305,306). It stimulates the growth of MCF-7 breast cancer cell lines *in vitro* (281,284,287), and has been reported to enlarge mammary glands and induce spontaneous mammary tumors in mice (307), reflecting ER agonist properties. Zearalenone is also reported to have been used as a contraceptive (308), as well as estrogen therapy in postmenopausal women (309). A synthetic derivative of zearalenone, zearanol, is used in cattle and lambs in the United States and Canada as an anabolic agent to enhance growth (310). Zearalenone is also reported to be present in barley, wheat, corn, corn flakes, and rice at concentrations varying from 35-115  $\mu\text{g/kg}$  (300,311-313). The estimated



**Figure 11.** Chemical structures of estradiol and zearalenone.

daily intake of zearalenone for young children in Canada, the highest consumption group on a body weight basis, is reported to be around 50-100 ng/kg bw/day (the mean and 90th percentile of eaters respectively) (301). In 1999, based on studies in ovariectomized female cynomolgus monkeys, the joint WHO/FAO Expert Committee on Food Additives (JECFA) set an acceptable daily intake for zearanol at 0.5 µg/kg bw (30 µg/day for a person weighing 60 kg) (301).

### **The Role of Xenoestrogens in Breast Cancer**

Breast cancer is second most frequent cause of cancer death among American women, accounting for 15% of all cancer deaths (314). Based on data from the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program, 182,800 new breast cancer cases are estimated for the year 2000 while the lifetime risk among US women of being diagnosed with breast cancer is 12.2%, or 1 in 8 (314). Risk for breast cancer is strongly age-related with a progressive rise in incidence with increasing age (315). Breast cancer is generally recognized as a disease that occurs often among women of upper social classes (as measured by higher educational status, family income etc ) reflecting the effects of life-style factors such as later ages at first birth (316)

Links between breast cancer and reproductive functions are well established. Compared with controls women with breast cancer are more likely to be unmarried, to have married later in life, to have become pregnant for the first time at an older age, and to have fewer children. Single women over 40 years

have been found to have higher risk of breast cancer than married women, while nuns have a higher than average risk (317,318). A study in which women with a first birth after age 36 years were shown to have approximately twice the risk of those with a first birth before age 18 years, demonstrated the importance of the age at first birth, as a determinant of breast cancer risk (319). The importance of early first birth suggests that early maturation of mammary epithelium makes it less susceptible to carcinogens. Numerous studies have also shown that women with early onset of menarche are at an increased risk of breast cancer, and that women who begin menstruating before 12 years of age have approximately a 50% higher risk than those with menarche at 15 years of age or later (320). Similarly women at late ages at menopause have been shown to be at an increased risk of breast cancer, the relative risk being almost two fold for natural menopause after age 55 year, compared with menopause before age of 45 years (321). Early menopause resulting from removal of the ovary is similarly associated with a reduction in risk, compared with a natural menopause around 50 years. Bilateral oophorectomy before the age of 40 years has been shown to reduce the incidence of breast cancer by 75% in both parous and nulliparous women (322). These factors appear to increase risk by increasing the length of time the breasts are exposed to high (menstrual cycle levels) of E<sub>2</sub> and progesterone during a woman's reproductive lifetime. Additionally, removal of the ovaries from premenopausal women with metastatic breast cancer could, in some cases, cause regression of the disease and improve the prognosis of the patient (323). These observations have contributed to the theory that exposure of the mammary gland

to ovarian estrogens and progestins are critical to onset and malignant progression of breast cancer.

Observation that estrogen enhances the growth of murine mammary tumors (324), and that chemical/carcinogen induced rat mammary tumors regress upon ovariectomy or antiestrogen administration (325), suggest that estrogens in general or E<sub>2</sub> in particular may be potent mammary tumor promoters. Factors that operate during early life can play an important role in affecting subsequent risk of developing breast cancer due to the fact that critical events of mammary gland development occur *in utero* (326,327). Female rats exposed to 20ng E<sub>2</sub> *in utero*, through maternal injections, have been demonstrated to exhibit significantly elevated incidence of carcinogen-induced mammary tumors (328). It is suggested that early E<sub>2</sub> exposure may increase mammary tumorigenesis by preventing mammary cell differentiation (308). Many of the effects of E<sub>2</sub> and its metabolites, could also be induced by phytoestrogenic and mycoestrogenic compounds, which could influence breast cancer risk by mimicking or inhibiting these effects.

These xenoestrogenic compounds, which have the potential to disrupt the endocrine system of animals, including man, may have different effects on the embryo, fetus, or perinatal organisms than on the adult (252) These effects are most often manifested in offspring, rather than the exposed parent. The timing of the exposure is also important, because even though the critical exposure occurred during embryonic development, obvious manifestations may not occur until maturity. The synthetic estrogen diethylstilbestrol (DES), which was administered to pregnant women to prevent spontaneous abortion, caused disruption of the

development of the reproductive system of both male and female offspring (329). The detrimental effects on the reproductive system from exposure to DES during embryonic life were not observed until puberty in either men or women. There is further evidence that neonatal exposure to environmentally derived hormones, such as zearalenone, affects tumorigenesis. In female mice, treatment with zearalenone during the neonatal period causes ovarian dysfunction and preneoplastic and/or neoplastic changes in the cervicovaginal epithelium (330). Treatment with zearalenone during postnatal days 7 and 14 has been seen to induce an increased incidence of tumors in Wistar rats (313). The proliferation and differentiation of mammary epithelial structures obtained from female Sprague-Dawley rats exposed *in utero* to zearalenone, also closely reflected those obtained in female rats exposed to E (308). These data imply that zearalenone appears to act as an estrogen in influencing the morphology of the developing mammary gland.

Several animal studies with zearalenone have demonstrated hyperestrogenic signs such as endometrial hyperplasia, vaginal hyperkeratosis, vulvular swelling, enlarged mammary glands and nipples, in females (301). In male mice zearalenone given in the diet for 13 weeks caused atrophy of seminal vesicles and testes, and squamous metaplasia of the prostate gland was seen (301). This mycotoxin is also thought to affect at the pituitary and hypothalamic levels. Zearalenone has been reported to competitively bind to the hypothalamic ER in immature rats (301), and in ovariectomized monkeys serum gonadotropin concentrations were altered by zearalenone in a manner similar to the endogenous

E<sub>2</sub> (331). Additionally, zearalenone caused non-estrogenic signs such as pituitary hyperplasia and osteopetrosis in rats and mice (301). Zearalenone also has been demonstrated to have anabolic effects in the animals studied (332). Of the species tested pigs, especially prepubertal gilts were most sensitive to the estrogenic effects of zearalenone, while poultry, especially chickens were quite resistant (303,333).

The relative potency of the resorcyclin acid lactones (zearalenone and its metabolites) in general have been consistently reported to be 1000 times less active than E<sub>2</sub> in a number of uterotrophic and vaginal cornification assays (301). However, in the neonates, effects similar to those of E<sub>2</sub>, was seen with 10-25 fold doses of zearalenone (334). Similarly, in reproduction studies with rats, effects similar to those of DES were seen with 30-fold doses of zearalanol (335,336). However, metabolic disposition of the resorcylic acid lactones in man appears to differ from that in the rat or monkey, the two species in which estrogenic potency has most often been measured. In man and pig greater amounts of  $\alpha$ -zearalenol, the more estrogenic metabolite is formed compared to rodents (301). Also due to differences in excretion methods the peak plasma concentration of zearalenone and its metabolites achieved in man and rabbit is reported to be many times higher than in other species studied, including the monkey (337). Zearalenone has a very low affinity to SHBG (5% vs. 100% for E<sub>2</sub>) and consequently it may exhibit elevated free (unbound) serum concentrations, increasing its ability to activate estrogenic pathways that lead to malignant growth (284). Taken together this implies that although the levels of ingested zearalenone appears to be lower than

the acceptable daily intakes (see Introduction), the risk from increase in the estrogenic burden from zearalenone in prepubertal and postmenopausal females, may be significant. An outbreak of precocious puberty in Puerto Rico in the early 1980's, where thousands of young children were affected with premature telarche, premature pubarche and prepubertal gynecomastia, was linked to zearalenone or zearalanol (338). These compounds were detected in the blood of some of the patients and it was suspected that a plant or a cereal source of zearalenone could have lead to the outbreak.

Some researchers have suggested that the potential role of environmental estrogens as etiologic agents in female breast cancer should be evaluated by determining the human exposures to natural estrogens such as isoflavonoids and lignans (275). Asian women who consume traditionally low-fat-high-soy diet have a four to six fold lower risk of developing breast cancer than women living in the industrialized Western world (293,339,340) Migration studies also show that the incidence of breast cancer in Asian women approaches that of American women after several generations of residence in the United States and adoption to the high-fat American diet (341). Lower incidence of prostate and breast cancer in Japanese and other oriental populations is suggested to be directly related to their consumption of high levels of phytoestrogens and to correlate with higher plasma and urinary levels of these compounds (342) It is proposed that the less estrogenic phytoestrogens from the diet might compete with the endogenous estrogens for the ER in the body, and thereby prevent these women from the risk of breast cancer (275) However, the isoflavonoids such as genistein elicit many



other biological responses, including inducing levels of SHBG and thus reducing the bioavailability of estrogens, endogenous or from the diet in premenopausal women (290). In postmenopausal women dietary soy is reported to induce a tendency for higher percentage of superficial cells in the vaginal epithelium, indicative of estrogenicity (343,344).

## RATIONALE & OBJECTIVE

### Rationale

1. Breast cancer is a serious health concern in the US and many other countries. There has been an inexorable rise in incidence of breast cancer over the past 50 years and for women in the US the life time odds of getting breast cancer now stands at 1 in 8, double the risk of 1940. Most of the identified risk factors reiterate the significance of cumulative exposure of the breast to estrogen and perhaps progesterone in getting breast cancer.
2. Data showing breast cancer incidence rates can vary across time periods and geographic areas have given an impetus for researchers to look for environmental causes of breast cancer. Recent reports have linked xenoestrogenic compounds in the environment to reproductive disorders in wild life as well as mammary carcinomas in humans.
3. Zearalenone, a phenolic resorcylic acid lactone produced by fungi of the fusarium species, is known to cause hyperestrogenism in farm animals and spontaneous mammary tumors in mice and rats exposed to zearalenone *in utero*. Trace amounts of zearalenone have been found in Barley, maize, corn, wheat, rice, oats etc. Additionally, the use of a synthetic analogue of zearalenone as a growth-promoting agent in cattle and lamb has resulted in residues of this compound present in milk and meat.
4. The possibility of zearalenone as a potential promoter of breast carcinoma makes it essential to study the molecular mechanisms of this compound in inducing breast cell proliferation.

## Objectives

- 1 Does zearalenone induce proliferation of the estrogen receptor positive human breast carcinoma cell line, MCF-7?
- 2 If zearalenone mimics E2 in causing MCF-7 cells to proliferate, I would like to identify the key molecular targets regulated by zearalenone in inducing G1 transit
3. Anti-apoptotic effects of estrogens could also significantly contribute to tumor growth I would like to study the role of zearalenone in preventing programmed cell death in MCF-7 mammary carcinoma cells.
4. Zearalenone may regulate key targets of G1 transit by binding to the ER and transactivating E2-responsive genes. It may also be possible that zearalenone can regulate key molecules in cytoplasmic signal transduction pathways. I would like to study the potential non-genomic signaling pathways utilized by zearalenone in implicating its growth-promoting effects.

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## **PART II**

### **Signal Transduction via the Ras/Erk Pathway Essential for Zearalenone-Induced Cell Cycle Progression in MCF-7 Cells**

## ABSTRACT

Zearalenone is a naturally occurring estrogenic contaminant of moldy feeds and is present in high concentrations in dairy products and cereals. Zearalenone has been postulated to contribute to the overall estrogen load of women, however, the mechanisms of its action are not known. Here we demonstrate the ability of zearalenone to stimulate growth of the estrogen-receptor positive human breast carcinoma cell line, MCF-7. In addition, zearalenone functions as an anti-apoptotic agent by increasing the survival of MCF-7 cell cultures undergoing apoptosis due to serum withdrawal. Treatment of these cells with zearalenone induced cell cycle transit following increases in expression of Myc and D1, A, and B1 cyclins, as well as downregulation of p27<sup>Kip-1</sup>. G1/G2 phase kinase activity and phosphorylation of Rb was evident as well. Flow cytometric analysis demonstrated entry of cells into S and G2/M phases of the cell cycle, and phosphorylation of Histone H3 (HH3) occurred by 36 hr of zearalenone treatment. Ectopic expression of a dominant-negative p21<sup>ras</sup> (rasN17) completely abolished the zearalenone-induced DNA synthesis in these cells and the specific inhibitor (PD98059) for mitogen/extracellular-regulated protein kinase kinase arrested S phase entry induced by zearalenone. These data suggest that the mitogen activated protein kinase-signaling cascade may be required for zearalenone's effects on cell cycle progression in MCF-7 cells. Given the presence of this mycotoxin in cereals, milk and meat, the possibility that zearalenone may be a potential promoter of breast cancer tumorigenesis should be further evaluated.

## INTRODUCTION

There is a great deal of evidence that  $E_2$  plays a critical role in breast cancer tumorigenesis (1,2). Epidemiological evidence in women indicates that the length of exposure of the mammary glands to ovarian-derived estrogenic stimuli is directly proportional to breast cancer risk (3). Early menarche, late menopause, and late age at first full-term pregnancy can all increase the total exposure of the breast to estrogenic stimuli from endogenous estrogens and estrogen exposure is the most well defined cause of sporadic breast carcinoma in women (4-7). Estrogens affect the rate of cell division and therefore may manifest their effect on risk of breast cancer by causing proliferation of breast epithelial cells.

In recent years environmental contaminants that disrupt estrogenic pathways, known as xenoestrogens, have been proposed to be a significant factor contributing to the higher incidence rates of breast cancer (8-10). Nonsteroidal dietary estrogens have been historically known to cause reproductive problems in farm and wild animals (11-13). Though their relative contribution to the genesis of breast cancer in humans appears to be controversial (8,14-20), xenoestrogenic compounds could add to the estrogenic burden. Therefore, they could be a potential source of estrogenic exposure in women, especially in pre-pubertal and post-menopausal years. However, the antiestrogenic actions of some plant estrogens such as isoflavones too, have been demonstrated in women who consumed a soy protein-enriched diet (21). This could be probably due to increased production of serum hormone binding globulins. The resultant decrease in bioavailability of endogenous estrogens is thought to result in overall lower

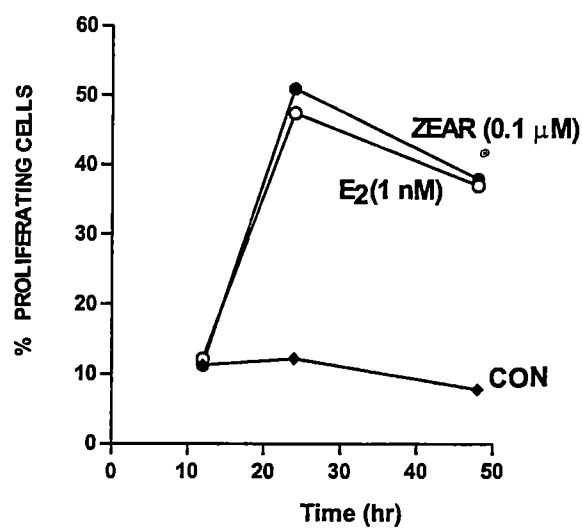
lifetime exposure to estrogens in Asian women who consume diets high in soy proteins (22).

The fungal metabolite zearalenone, a resorcylic acid lactone, is produced by numerous species of *Fusarium* (23). Zearalenone is a nonsteroidal estrogenic mycotoxin present in grains and grain-based foods at concentrations varying from 35-115 mg/kg (24-28). Zearalenone and its major metabolites,  $\alpha$  and  $\beta$  zearalenols, also occur in meat and milk (23). Consumption of corn-based breakfast cereals and cow's milk are the major routes of zearalenone intake in humans (29-31). The estrogenic and anabolic properties of zearalenone have long been known in animals (23,32-36). Zearalenone has been used as a contraceptive (37), as estrogen therapy in post-menopausal women (38) and zearanol, a closely related compound is used as an anabolic agent to enhance growth in cattle and lambs (39). Zearalenone stimulates the growth of MCF-7 breast cancer cell lines in vitro (40-42), and has been reported to enlarge mammary glands and induce spontaneous mammary tumors in mice (43), reflecting its estrogen receptor-agonist properties. Zearalenone also has a very low affinity to serum hormone binding globulins (5% vs. 100% for  $E_2$ ) and consequently it may exhibit elevated free (unbound) serum concentrations, increasing its ability to activate estrogenic pathways (41). Thus, although the levels of ingested zearalenone are low (suggested to be around 1-5  $\mu\text{g/day}$  for Canadians) (23), the risk from increased estrogenic burden from zearalenone in pre-pubertal and post-menopausal women, who have low endogenous estrogens, has to be further evaluated.

Previously we have demonstrated that zearalenone induces increases in cyclin D1 protein levels, Cdk2 activation, and hyperphosphorylation of pRb in the estrogen receptor-positive MCF-7 breast carcinoma cells (42). In this report, we have attempted to further elucidate the molecular mechanisms of zearalenone-induced cell proliferation. We demonstrate that the activation of G1 and G2 phase kinases by zearalenone may result from induction of cyclin D1 protein via signal transduction pathway(s) involving ras and extra-cellular signal related kinases (Erks) and a reduction in the cyclin dependent kinase (Cdk) inhibitor p27. These actions in turn lead to an increased rate of G1 to S phase transition and result in mitosis. Furthermore, we demonstrate that prevention of apoptosis as well as enhanced proliferation can contribute to the effects zearalenone may have as a potential promoting agent in breast cell carcinogenesis

## RESULTS

We used MCF-7 breast cancer cells to demonstrate the mitogenic effects of zearalenone in a cell culture system. Growth-arrested MCF-7 cells were treated with 0.1  $\mu$ M zearalenone, harvested at the indicated times and cell cycle status was determined by flow cytometric analysis. Approximately 50% of zearalenone-treated cells entered S phase by 24 hr of treatment as compared to 10% of control cells (Figure 1). Our data demonstrate that zearalenone-induced MCF-7 cells enter the cycle to the same extent as E<sub>2</sub>



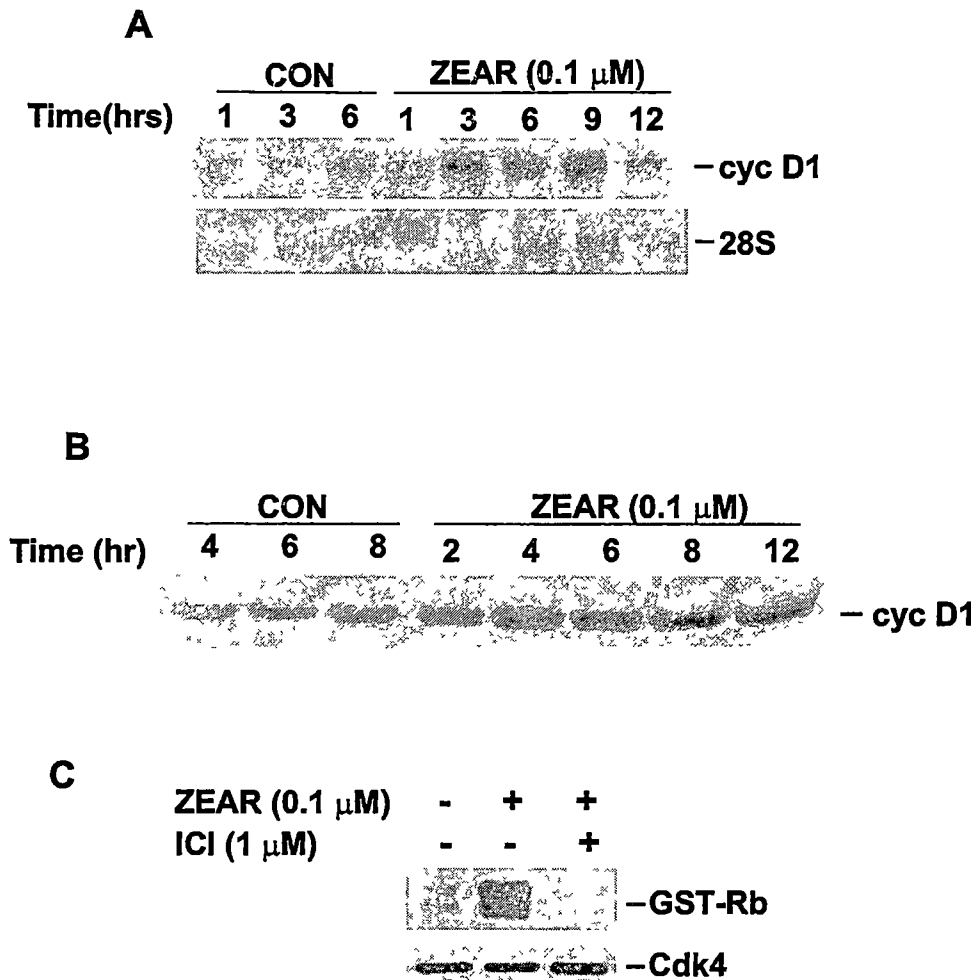
**Figure 1. Zearalenone induces proliferation of MCF-7 breast carcinoma cells.** MCF-7 cells growth arrested as described in Materials and Methods, were treated with zearalenone (ZEAR) or E<sub>2</sub> in the presence of 1 nM ICI 182,780. The control cells (CON) were treated with 1 nM ICI 182,780 only. At indicated times cells were trypsinized and fixed with 70% ice-cold ethanol. The cell cycle status was analyzed by flowcytometry



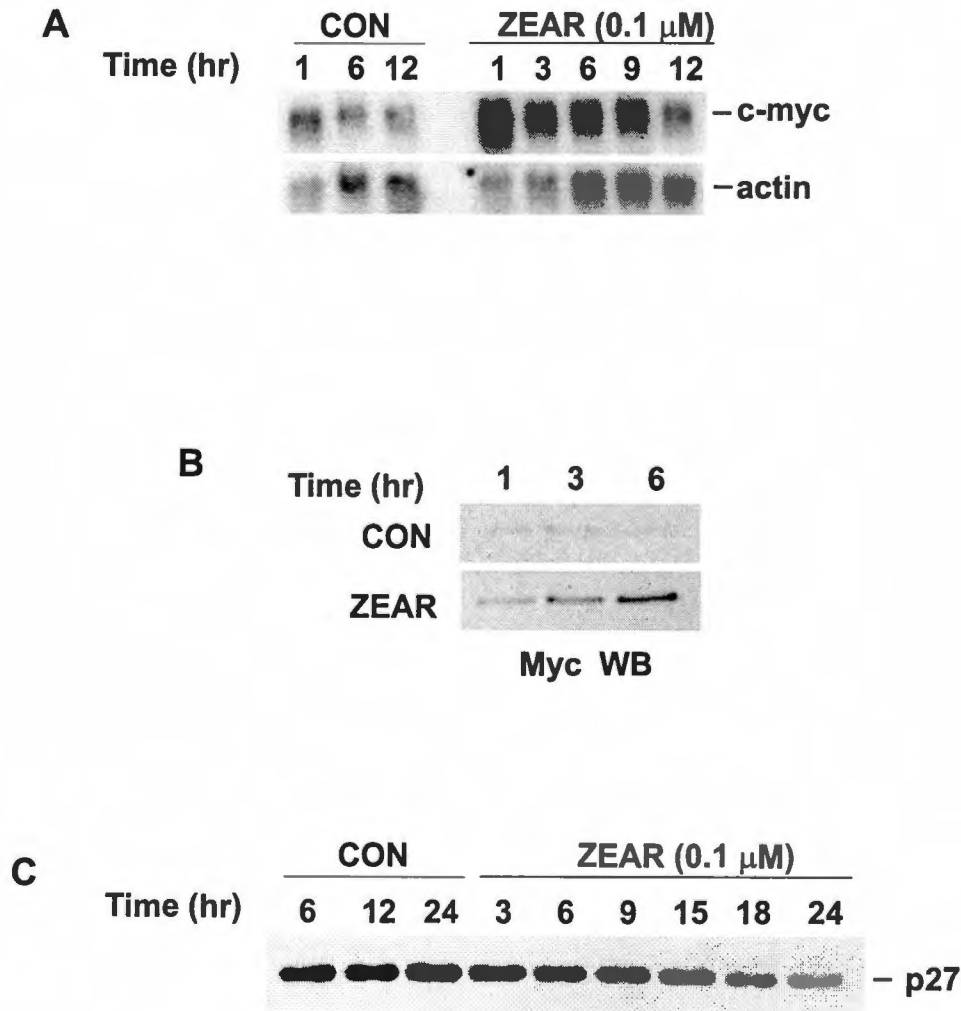
To delineate the molecular mechanisms leading to zearalenone's mitogenic effects in MCF-7 cells, we examined expression of cyclin D1, a sensor of growth-stimulating conditions in most cell types (44). Zearalenone induced an increase in cyclin D1 mRNA expression as early as 3 hr after treatment, which was sustained for up to 9 hr before returning to control levels by 12 hr (Figure 2A). The increase in cyclin D1 mRNA resulted in elevated protein levels by 4-6 hr, which was maintained at high levels up to 12 hr (Figure 2B). Finally, to confirm that this increase in cyclin D1 protein is functionally significant, we measured the cyclin D1-associated kinase activity. Zearalenone induced cyclin D1/Cdk4 activity approximately 3-4 fold by 6 hr (Figure 2C) when the cyclin D1 protein level was elevated. The activation of cyclin D1/Cdk4 was completely abolished by addition of ICI 182,780, a specific inhibitor of estrogen receptor action (45).

We next examined if zearalenone regulates c-myc, a proto-oncogene that is over expressed or activated in a variety of tumors (46) and is an early response gene following E<sub>2</sub> stimulation (47). Zearalenone transiently induced c-myc mRNA expression within one hour and the mRNA levels gradually declined to control levels by 12 hr (Figure 3A). This increase in mRNA expression was followed by a corresponding increase in Myc protein levels by 3-6 hr (Figure 3B) suggesting that zearalenone may regulate the MCF-7 cell cycle by inducing immediate early genes such as c-myc.

Myc is reported to be an upstream activator of Cdk2 kinase activity in G1 at least in part through regulation of p27 levels bound to the cyclin E/Cdk2



**Figure 2. Zearalenone induces cyclin D1 expression with concomitant increase in Cdk4 kinase activity.** (A) MCF-7 cells growth arrested for 48 hr were treated with 0.1  $\mu$ M zearalenone (ZEAR) or left untreated (CON) for the indicated times. Total RNA was extracted and analyzed by Northern blotting for cyclin D1 message. Methylene blue stained 28S rRNA is shown as a control for RNA loading. (B) Similarly treated cells were lysed and cyclin D1 protein levels analyzed by western blotting with the anti-cyclin D1 antibody. (C) Growth-arrested MCF-7 cells similarly treated with zearalenone +/- ICI 182,780 (ICI) for 6 hr and lysates were prepared and immunoprecipitated with anti-cyclin D1 antibody to assay for cyclin D1-associated kinase activity with GST-Rb as substrate. Cdk4 protein levels in these lysates were analyzed by Western blotting staining with the anti-Cdk4 antibody.



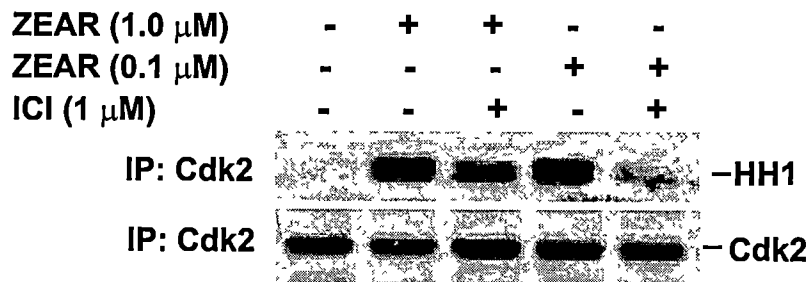
**Figure 3. Zearalenone induces c-myc and causes downregulation of the p27 inhibitor in MCF-7 cells.** (A) Zearalenone-treated cells (ZEAR) or untreated cells (CON) were lysed at the indicated times and total RNA was extracted to analyze for c-myc mRNA by Northern blotting. Actin is shown as a control for RNA loading. (B) Myc protein levels were measured by immuno-blotting from similarly treated extracts. (C) Western blotting for total p27 protein levels in zearalenone +/- cell extracts.

complex (48). Thus, we hypothesized that zearalenone may lead to the induction of Cdk2 activity and S phase entry through Myc-mediated downregulation of cyclin dependent kinase inhibitors. Therefore, we examined the p27 levels in MCF-7 cells after zearalenone treatment. A steady decline in p27 protein levels was observed by 18-24 hr after zearalenone treatment (Figure 3C), while the untreated cultures maintained high levels of p27 even at 24 hr. The zearalenone-induced downregulation of p27 by mid to late G1 phase was also observed in immunohistochemical staining of MCF-7 cells with peroxidase-conjugated anti-p27 antibody (data not shown).

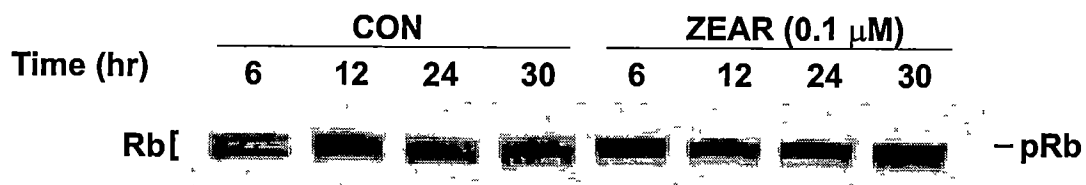
The ability of zearalenone to induce cyclin D1 protein expression and downregulate p27 levels could result in increased Cdk2 activity. This was confirmed by experiments showing increased Cdk2 kinase activity 18 hr after zearalenone treatment (Figure 4A). Both 0.1  $\mu$ M and 1  $\mu$ M zearalenone induced significant levels of Cdk2 activity and this increase was completely abolished by a ten-fold excess of ICI 182,780. Both G1 kinases, Cdk2 and Cdk4, are reported to phosphorylate and thereby inactivate Rb, resulting in transcriptional activation of S phase genes (13). Therefore, we examined the phosphorylation status of Rb in zearalenone treated cells over a period of 30 hr by Western blotting. The slower migrating upper band that corresponds to phosphorylated Rb is detectable as early as 6 hr, and Rb phosphorylation was sustained up to 30 hr, after zearalenone treatment (Figure 4B).

Transition from G2 to M phase is triggered by the cyclin B1-associated kinase activity (49). Therefore, we measured G2 phase kinase activity in

**A**



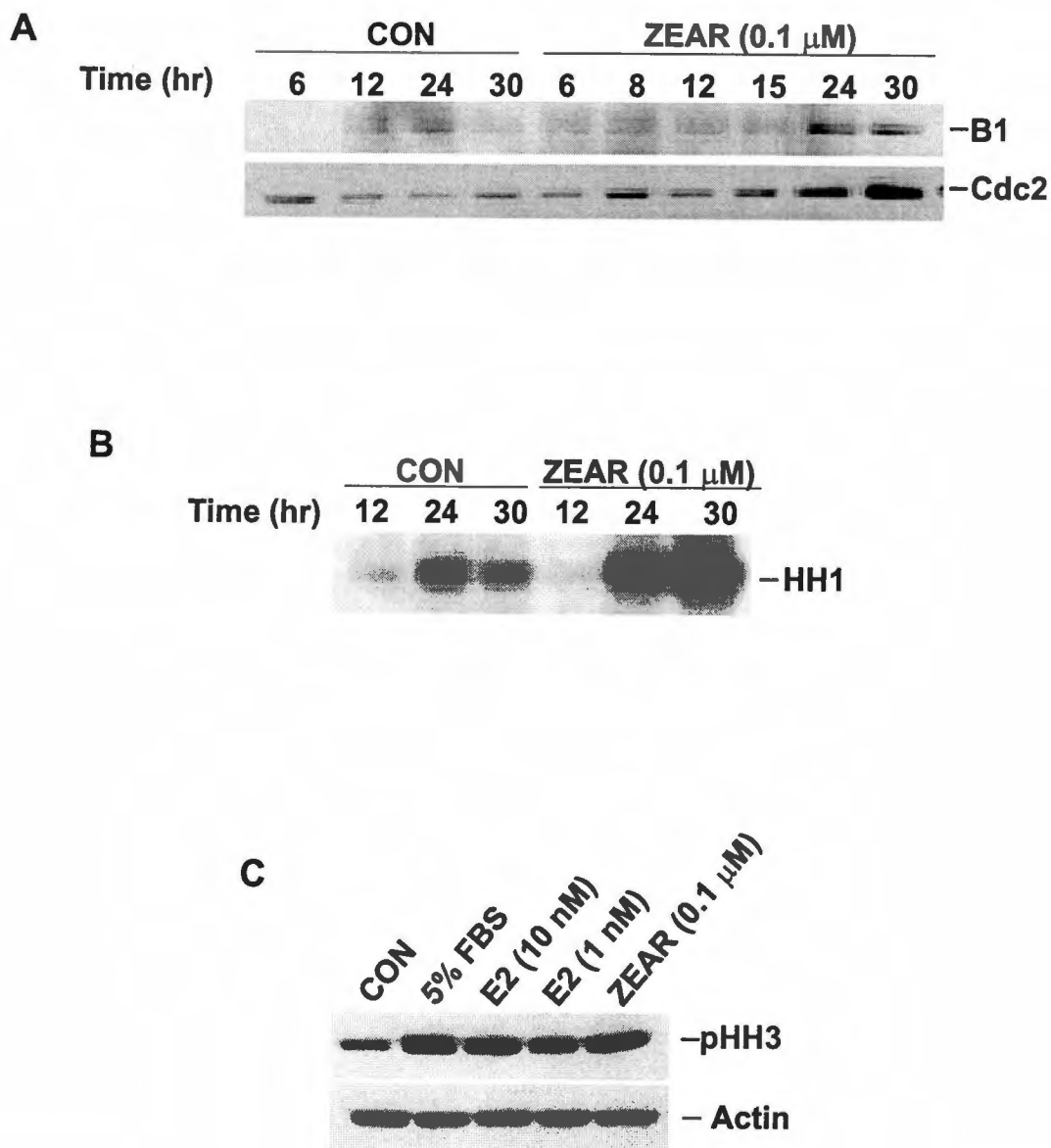
**B**



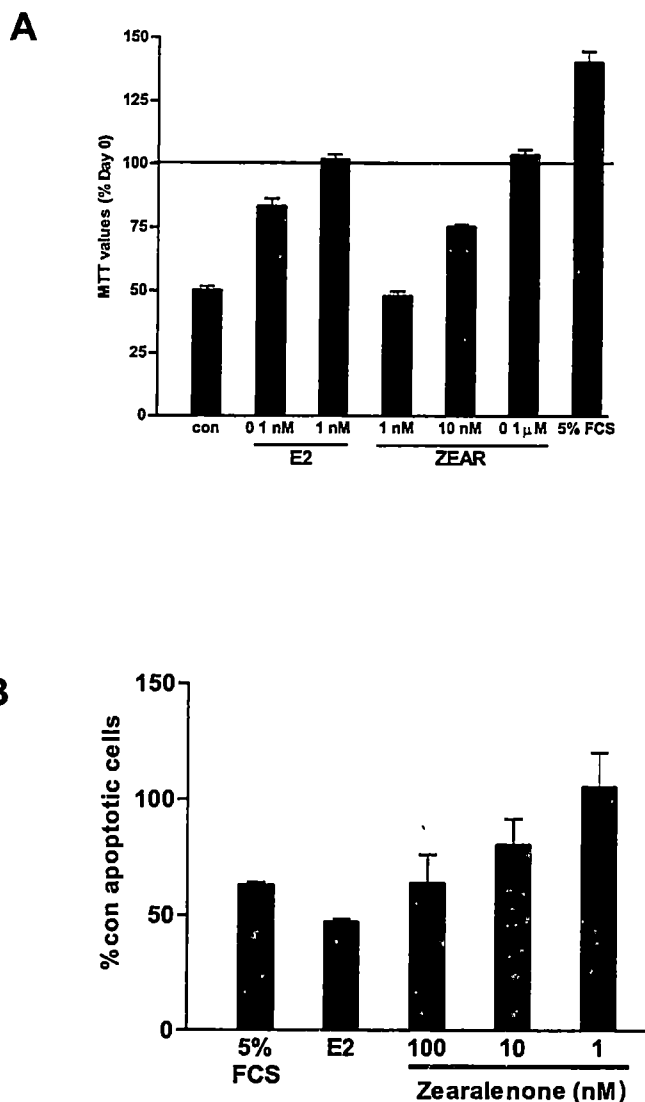
**Figure 4. Increased Cdk2 activity and Rb hyperphosphorylation in zearalenone-treated cells. (A)** MCF-7 cells were treated with indicated doses of zearalenone (ZEAR) in the presence of an excess of ICI 182,780 (ICI). Lysates were prepared at 18 hr after treatment and immuno-precipitated with anti-Cdk2 antibody to assay for associated kinase activity with Histone H1 (HH1) as substrate. Total Cdk2 protein levels demonstrated by Western blotting with the anti-Cdk2 antibody. **(B)** Similar treated cell lysates were analyzed for the phosphorylation status of the Rb protein by western blotting with an anti-Rb antibody. The slower migrating band indicated as pRb, is the hyperphosphorylated and inactive Rb.

zearalenone treated MCF-7 cells. MCF-7 cells treated with 0.1  $\mu$ M zearalenone were analyzed for cyclin B1 and Cdc2 protein levels by western blotting analysis of whole cell lysates (Figure 5A). Zearalenone treatment induced expression of cyclin B1 and Cdc2 by 24-30 hr and this was accompanied by an increase in cyclin B1-associated kinase activity, which was approximately 8 fold above control levels by 30 hr (Figure 5B). Treatment of MCF-7 cells with zearalenone for 36 hr also induced phosphorylation of HH3, a marker of mitotic activity (50-52), (Figure 5C). This confirms that zearalenone can induce MCF-7 cells to undergo mitosis. The MTT values of similarly growth-arrested and treated MCF-7 cells measured 40 hr after zearalenone treatment demonstrated about 30-40% increase in cell numbers (data not shown).

MCF-7 cells grown for several days in the presence of zearalenone and 0.2% FCS, demonstrated an increase in the number of viable cells compared to control cultures that were grown in the absence of zearalenone (data not shown). Therefore, to determine if zearalenone functions as a survival factor as well as inducing proliferation, MCF-7 cells were maintained in serum-free conditions in the presence or absence of increasing doses of zearalenone, or  $E_2$  (Figure 6A). In the absence of serum, zearalenone and  $E_2$  protected MCF-7 cells from undergoing cell death in a dose-dependent manner. To determine whether this represents an antiapoptotic effect of zearalenone, MCF-7 cells that were undergoing serum starvation in the presence or absence of increasing doses of zearalenone were stained with Annexin V. Flow cytometric analyses of the stained cells demonstrated that zearalenone-treated cells, similar to those treated with  $E_2$  or 5%



**Figure 5. Zearalenone induces cyclin B and Cdc2 levels with concomitant increase in cyclin B1-associated kinase activity.** (A) Growth-arrested MCF-7 cells treated with zearalenone (ZEAR) or left untreated (CON) were harvested at the indicated times and analyzed for cyclin B1 and Cdc2 protein levels by western blotting. (B) Similarly treated lysates were assayed for cyclin B1-associated kinase activity with HH1 as substrate. (C) Growth-arrested MCF-7 cells were treated as indicated for 36 hr and the histones were acid extracted as described in Materials and Methods. The phosphorylation of the Ser-10 residue of the HH3 mitotic marker was analyzed by Western blotting with the anti-phospho-HH3 antibody.

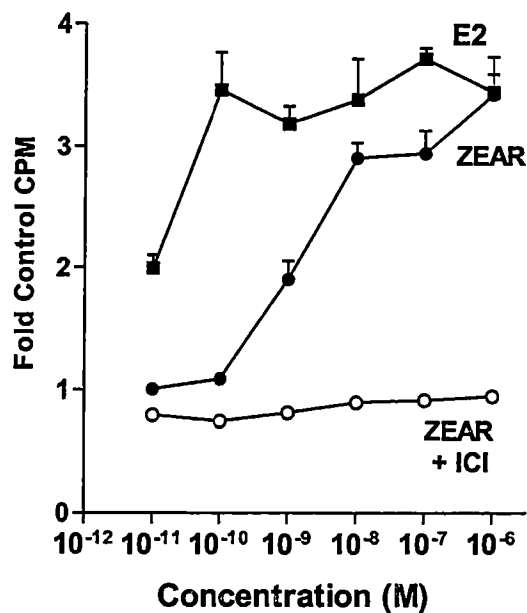


**Figure 6. Increased cell survival and reduced serum withdrawal-induced apoptosis in the presence of estrogens.** (A) Growing MCF-7 cultures were serum starved in the presence of indicated concentrations of zearalenone and E<sub>2</sub>, while control cells received no treatment. Cells maintained in 5% FCS is depicted as positive control (FCS). Cell viability determined 4 days later by MTT uptake is plotted as a percentage of day 0 values (100%). Results are given as mean  $\pm$  S.D. of four replicate cultures. (B) Cycling cells were placed in serum-free phenol red-free medium and treated as indicated for 72 hr. Cells were harvested and stained with Annexin V and propidium iodide for analysis with flow cytometry as described in Materials and Methods. The percentage of Annexin V stained cells is given relative to that of control cultures (30% of the 20,000 gated events were stained with Annexin V in the control cultures indicating the fraction of apoptotic cells).

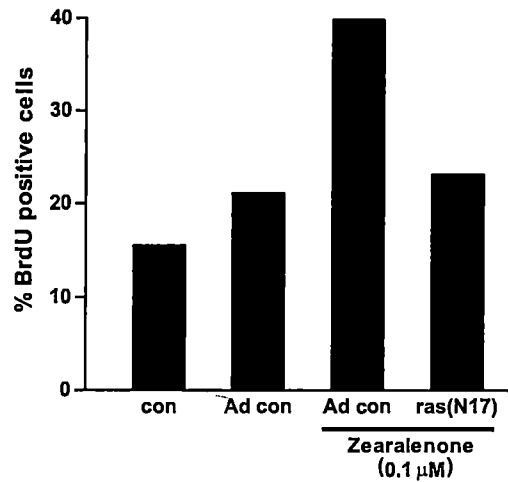
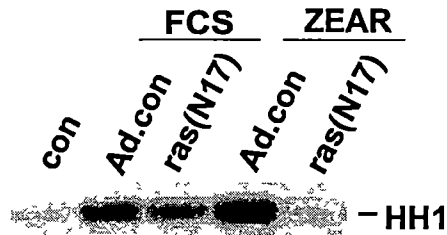


FCS, exhibited decrease in Annexin V staining (Figure 6B). This suggests that estrogens may play a role in preventing programmed cell death in ER-positive breast cancer cells

The classical mode of estrogenic action is thought to be via binding to the intracellular estrogen receptor and direct transcriptional activation of genes that are responsive to estradiol. In our system we observed that zearalenone induced transcriptional activation of an estrogen response element/Luciferase reporter construct. The dose dependent activation of this construct by zearalenone was completely abolished by addition of an excess of ICI 182,780 (Figure 7) indicating that zearalenone binds intracellular ER and cause transactivation of estrogen-responsive genes. However, it is also possible that zearalenone may regulate cytosolic signal transduction pathways To examine the involvement of non-nuclear pathways in zearalenone-induced proliferation of MCF-7 cells, we utilized an adenovirus encoding the dominant negative form of rasN17. Ectopic expression of rasN17 in MCF-7 cells completely arrested zearalenone-induced DNA synthesis as measured by analyzing the BrdU incorporation into newly synthesized DNA (Figure 8A) Expression of rasN17 also caused a decrease in the G1 phase Cdk2 kinase activity induced by zearalenone whereas the control virus had no effect Similarly, rasN17 reduced serum-induced Cdk2 activity (Figure 8B) This suggests that the ras/Erk pathway may be involved in zearalenone-mediated mitogenic activity. Therefore, we pretreated MCF-7 cells with PD98059 an inhibitor of mitogen/extra-cellular-regulated protein kinase kinase, prior to zearalenone treatment. We observed that this inhibitor prevented zearalenone-



**Figure 7. Zearalenone stimulates transcriptional activation at estrogen response elements (ERE) in MVLN cells.** MVLN cells were grown in 5% charcoal stripped calf serum (CSCS) and treated with the indicated doses of E2 and zearalenone (ZEAR) +/- ICI 182,780 (ICI). Cells were lysed and luciferase assay performed after 24 hr as indicated in experimental procedures. The values for E2 and zearalenone treated cultures are given as fold induction of untreated cultures (the mean control value for untreated MVLN cells was 912,838 counts per minute)

**A****B**

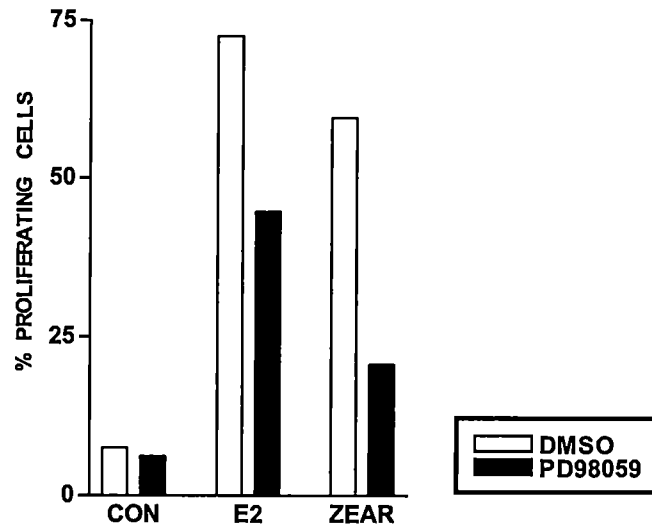
**Figure 8. Zearalenone-induced DNA synthesis and Cdk2 activation is blocked by a dominant negative ras.** (A) Cells infected with either the adenoviral control or adenoviral rasN17 were growth arrested and treated with zearalenone. Six hours prior to harvesting, cells were incubated with BrdU and at 24 hr of zearalenone treatment cells were harvested and analyzed for BrdU staining by flowcytometry as indicated in Materials and Methods. (B) Similarly infected and growth arrested cells treated with zearalenone and serum for 20 hr were assayed for Cdk2 activity.

induced cyclin D1 induction (Figure 9B) and S phase entry (Figure 9A). A dominant-negative Erk construct (DN-Erk2) also in a similar manner was effective in preventing E<sub>2</sub>- and zearalenone-induced cell proliferation (Appendix A.1). These data imply that the mitogen activated protein kinase-signaling cascade may play a significant role in the zearalenone-induced breast cell proliferation

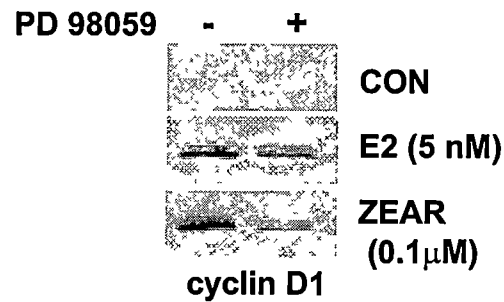
## DISCUSSION

In this report we provide evidence indicating that growth-arrested MCF-7 cells treated with zearalenone are induced to enter the cell cycle by activating early G1 phase events. An increase in c-myc and cyclin D1 expression together with downregulation of the Cdk inhibitor, p27, resulted in enhanced Cdk4 and Cdk2 activity, phosphorylation of Rb, and culminated in S phase entry. Increased levels of cyclin B and induction of cyclin B1-associated kinase activity was followed by G2/M transit and cell division. Previous studies on estrogen-induced cell proliferation have demonstrated that E<sub>2</sub>-induces G1 transit in estrogen receptor positive cell lines by transcriptional activation of the cyclin D1 gene (53), and by increased expression of G1-regulatory proteins Myc and cyclin D1, which results in the induction of G1 phase kinase activity (54). Additionally the E<sub>2</sub>-induced downregulation of Cdk-inhibitory activity also is correlated with increased Cdk2 activation and prolonged Rb phosphorylation resulting in S phase

**A**



**B**



**Figure 9. Inhibition of the MAPK pathway reduces zearalenone-induced cyclin D1 expression and prevents G1/S transition in MCF-7 cells. (A)** Cells pre-incubated with 50  $\mu$ M PD98059 for 30min prior to treatment with 0.1  $\mu$ M zearalenone (ZEAR) or 5 nM E<sub>2</sub>, were analyzed by flowcytometry by 18 hr of treatment. **(B)** Cells treated similarly were harvested by 6 hr to analyze for cyclin D1 levels by Western blotting.

entry (55,56). Data from several laboratories using different cell types together with data presented in this paper suggests that estrogens regulate multiple events leading to G1/S transit (54-56). Our study demonstrates that the molecular mechanisms whereby zearalenone regulates MCF-7 cell cycle are similar to that of E<sub>2</sub>, and that these actions of zearalenone are exerted through the ER.

In addition to inducing cell cycle progression in MCF-7 cells, zearalenone also exhibited a protective effect on these cells by preventing the programmed cell death caused by serum withdrawal. There have been several studies in variety of cell types, which suggest that E<sub>2</sub> is a potent inhibitor of apoptosis (57-59). MCF-7 cells growing as tumors in oophorectomized nude mice given estradiol implants showed both decreased proliferation and increased apoptosis of the cells in the xenografts following withdrawal of the implants (58). An anti-apoptotic effect of estrogens *in vivo* would be important to tumor growth especially in the clonal expansion of tumor cells carrying mutations. Our results demonstrate that zearalenone and E<sub>2</sub> have similar roles as survival factors as well as mitogens.

The mitogen activated protein kinase pathway is activated within 2-5 min by E<sub>2</sub> in MCF-7 cells via the activation of c-src/ras/raf/Erk pathway (60). This non-transcriptional activation of cytoplasmic signal transduction pathways is reported to play a major role in cell cycle progression (61). Several studies indicate that that membrane-bound receptors may be involved in regulating E<sub>2</sub>-activated signal transduction pathways (62,63). Our observation of the ability of rasN17 to block the zearalenone-induced MCF-7 cell cycle progression implies that ras function is involved in the induction of G1/S transition in MCF-7 cells by

estrogens We have also observed that prolonged activation of Erks is involved in estrogen-induced cell cycle progression in MCF-7 cells (Fernando et al. in preparation). Inhibiting the mitogen activated protein kinase pathway with PD98059 resulted in decreased cyclin D1 protein, and blocking ras function resulted in reduced Cdk2 activity. Thus, the ras/Erk pathway appears significant for zearalenone-induced cyclin D1 expression Cyclin D-Cdk4 complex formation is suggested to be required for Cdk2 activation via sequestration of Cdk inhibitors from E cyclin-Cdk2 complexes (64). Therefore, blockade of cyclin D1 expression may lead to the observed decrease in Cdk2 activity and cell cycle arrest. Our data suggest that these non-genomic signaling pathways are important for growth promoting activities of zearalenone (as for E<sub>2</sub>), and may play an important role in the effects of this mycoestrogen in breast epithelium.

## **MATERIALS AND METHODS**

### **Reagents and antibodies**

Cell culture media, dialyzed fetal calf serum (FCS), zearalenone, E<sub>2</sub>, histones, RNase A, propidium iodide, and other chemicals unless otherwise mentioned were from Sigma Chemicals Co. (St. Louis, MO). PD08059 was purchased from Calbiochem (La Jolla, CA) and dissolved in dimethyl sulfoxide ICI 182,780 was kindly supplied Dr Alan Wakeling at Zeneca Pharmaceuticals (Alderley Park, Cheshire, UK ). Mouse monoclonal antibody to human Rb protein

was from Pharmingen (San Diego, CA). Recombinant GST-Rb, protein A/G beads, affinity-purified antibodies to human Cdk2 (M2), p27 (C19), cyclin D1 (H295), as well as monoclonal antibodies to cyclin E (HE111 and HE12) and c-myc (C-33), were from Santa Cruz Biotechnology (Santa Cruz, CA). Cyclin D1 monoclonal antibodies DCS-6 and DCS-11 were from Neomarkers (Freemont, CA) and monoclonal antibody to human actin was from Boehringer Mannheim (Indianapolis, IN). Horseradish peroxidase-conjugated protein A/G and secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

## Cell Culture

MCF-7 cells were maintained in Dulbecco's minimal Eagle's medium (DMEM) supplemented with antibiotics and 5% FCS. For assays requiring growth arrest, MCF-7 cells were grown in DMEM with 5% FCS for 1-2 days, at which time the medium was changed to phenol red-free DMEM with 0.2% FCS and 10 nM ICI 182,780 for an additional 48 hr. Stock solutions of zearalenone and E<sub>2</sub> were prepared in ethanol and added as indicated in the text, in the presence of 1 nM ICI 182,780. The MVLN cells, as previously described (65), are MCF-7 cells that have been stably transfected with a firefly-luciferase reporter gene that is under the control of an estrogen response element of the *Xenopus vitellogenin* A2 gene and a thymidine kinase promoter of the Herpes simplex virus.



## **Flow cytometric analysis and Bromodeoxyuridine (BrdU) labeling**

MCF-7 cells treated as indicated were trypsinized and fixed overnight in ice-cold 70% ethanol. Prior to flow cytometric analysis, cells were washed with phosphate buffered saline to remove the ethanol and treated with 100 µg/ml RNase A and 50 µg/ml propidium iodide for 30 min. The stained cells were analyzed with a Becton-Dickinson FACScan (Becton-Dickinson Pharmingen, San Jose, CA) using a Becton-Dickinson cell FIT software to determine percentages of cells in the G1, S, and G2+M phases of the cell cycle as previously described (55).

For analysis of BrdU incorporation 10 µM BrdU was added to the cell cultures by 18 hr of zearalenone treatment. The cells were harvested at 24 hr and fixed as described for flow cytometry. The fixed cells were stained with Fluorescein Isothiocyanate-conjugated anti-BrdU antibody and analyzed by flow cytometry according to the manufacturer's protocol (Becton-Dickinson Pharmingen, San Diego, CA)

## **Cell Lysate Preparation and Western Blotting**

MCF-7 cells to be harvested were washed with ice-cold phosphate buffered saline and lysed by addition of ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% nonidet P-40, 5 mM EDTA, 10 mM EGTA) containing 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation at 11,000 X g for 20 min at 4°C and protein concentrations was determined by the Bradford

assay (66). For assays of cyclin D1-dependent Rb kinase activity, cells were lysed in Rb kinase buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Tween-20, 10% glycerol, 10 mM  $\beta$ -glycerophosphate) containing 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride (67).

For Western blotting, equal quantities of protein resolved on sodium dodecyl sulphate (SDS)-poly acrylamide gels were transferred to nitrocellulose membranes and blocked with 0.5% casein and 0.1% Tween 20 for 30 min. The primary antibody was used at a concentration of 0.5  $\mu$ g/ml in the blocking buffer for 2-4 hr at room temperature or overnight at 4°C. Bound antibodies were visualized with horseradish peroxidase-conjugated anti-mouse antibody and chemiluminescent substrate according to the manufacturer's protocol (Amersham Life Science Inc., Arlington Heights, IL). For Myc western blots, cells were lysed in 10 mM Tris (pH 7.5), 50 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 0.5% SDS, 1mM EDTA supplemented with 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride (68). The lysates were sonicated briefly and clarified by centrifugation prior to continuing as mentioned above. Acid extraction of histones was performed as specified by manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY). MCF-7 cells were incubated in the lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM  $MgCl_2$ , 10 mM KCl, 0.5mM dithiothreitol, 1.5mM phenylmethylsulfonyl fluoride) containing 0.2M sulfuric acid, and placed on ice for 30 min. The supernatant fraction containing the acid soluble proteins were dialyzed twice against 0.1M acetic acid, 1-2 hr each and

three times against distilled water for 1 hr, 3 hr, and overnight respectively. The proteins were quantified by the Bradford assay and stored at  $-80^{\circ}\text{C}$ .

## **Kinase Assays**

Equal amounts of protein ( $\sim 150\text{ }\mu\text{g}$ ) of cell lysates were immunoprecipitated with 200 ng of affinity purified rabbit antibody to human Cdk2, cyclin E, B1 or monoclonal antibodies to cyclin D1 (HD-11), and protein A/G agarose beads. The immune precipitates were washed three times with the lysis buffer and twice with the kinase buffer (50 mM Tris-HEPES, pH 7.5, 10 mM  $\text{MgCl}_2$ ). For cyclin E-, Cdk2- or cyclin B1-associated kinase assays, the immune precipitates were resuspended in 20  $\mu\text{l}$  of kinase buffer supplemented with 400  $\mu\text{g/ml}$  histones (Sigma type III-SS), 10  $\mu\text{M}$  ATP, 0.5 mM dithiothreitol, 0.5 mM EGTA, and 5  $\mu\text{Ci}$  [ $\gamma^{32}\text{P}$ ]ATP (7000 Ci/mmol) for 30 min at room temperature. For Rb kinase assays, 0.2  $\mu\text{g}$  of GST-RB was added to the kinase reaction mix and incubated for 1 hr at room temperature. Assays were stopped with 20  $\mu\text{l}$  of 2x SDS-PAGE sample buffer. Reaction products were separated on SDS-poly acrylamide gel electrophoresis followed by autoradiography using Kodak XAR film.

## **Northern Blot Analysis**

Total RNA was extracted from cells using the guanidine thiocyanate/acid phenol procedure. RNA was resolved on 1.2% agarose/2 M formaldehyde gels, transferred by capillary blotting to nylon membranes, and immobilized by UV

crosslinking. Transcripts were detected using  $^{32}\text{P}$ -labeled cDNA probes (DECAprime II DNA labeling kit, Ambion, Austin, TX) hybridized overnight at 42°C in 50% formamide, 0.1% SDS, 5X SSC, 5X Denhardt's with 100 µg/µl sonicated, denatured salmon sperm DNA. Probed membranes were rinsed with 2X SSC, 0.1% SDS twice at room temperature then washed once more with 0.2X SSC, 0.1% SDS. Membranes were exposed to Kodak X-OMAT film  $\beta$ -actin or methylene blue staining of 28S rRNA was used as loading control.

### **MTT and Luciferase Assays**

MCF-7 cells were grown until cells are around 40% confluent, and the monolayers were washed with phosphate buffered saline and medium replaced with DMEM and treated as indicated. At indicated times cells were treated with a solution of dimethylthiazoldiphenyl-tetrazoliumbromide (MTT; Sigma Chemicals Co., St. Louis, MO) and left to incubate for 2 hr. The medium was aspirated and plates dried prior to reading the optical density at the wavelength of 462 nm.

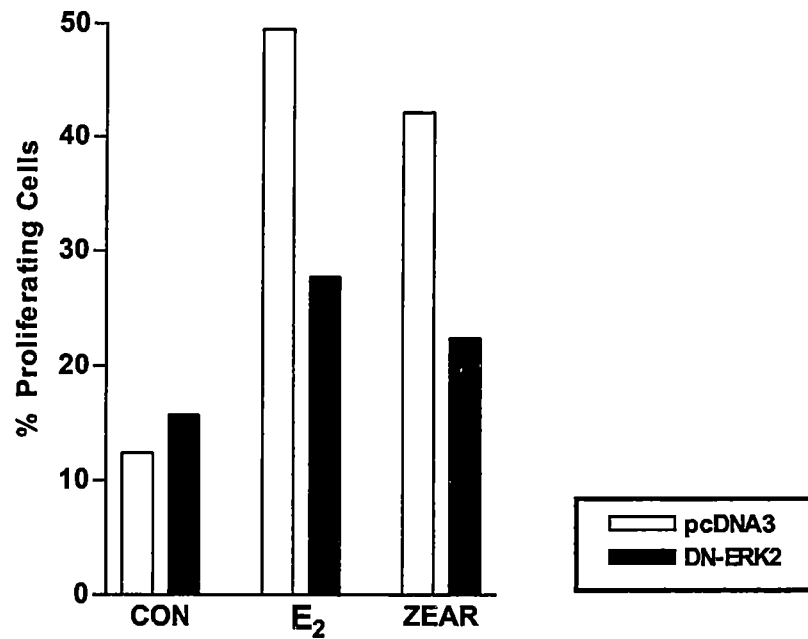
The ability of  $\text{E}_2$  and zearalenone to stimulate the expression of luciferase production under the control of an estrogen response element was measured by arresting MVLN cells with 10 nM ICI 182,780 for 48 hr and treating with  $\text{E}_2$  or zearalenone in the presence or absence of ICI 182,780. After 24 hr of treatment, the cells were lysed and luciferase activity determined according to the manufacturer's protocol (Promega Corp., Madison, WI) with a  $\beta$ -scintillation counter.

## **Annexin V / Propidium Iodide Staining**

About 50% confluent MCF-7 cells were placed in serum-free DMEM to induce apoptosis and indicated treatments were added. Seventy-two hours later, attached cells were trypsinized and pooled with floating cells. The harvested cells ( $0.1 \times 10^6$ ) were suspended in 500  $\mu$ l of binding buffer (10 mM Hepes pH 7.4, 140 mM NaCl, 2.5 mM  $\text{CaCl}_2$ ) containing 5  $\mu$ l of Fluorescein-conjugated Annexin V and 10  $\mu$ l of 50  $\mu$ g/ml propidium iodide. After 15 min of incubation the cells were analyzed by flow cytometry with FACStar Plus (Becton Dickinson, Pharmingen, San Diego, CA)

## **Adenoviral Infections**

The adenoviral vector for rasN17 was kindly provided by Dr. J.R. Nevins (69). The control adenovirus was constructed by homologous recombination in 293 human embryonic kidney cells (ATCC CRL1573) as previously described (70). The adenoviral backbone (Ad type 5, 9.24-100mu) was co-transfected by calcium phosphate precipitation without an insert for the control virus. The viral vectors were propagated in 293 cells and titered by a standard plaque assay. Growth-arrested MCF-7 cells were infected for 1-2 hr with the adenoviral vector at an m.o.i. of 40 in DMEM/0.1% FCS (phenol red-free). The infection medium was replaced with phenol red-free DMEM/0.1% FCS and the cells were growth-arrested for further 48 hr before treatment and harvest.



**Appendix A.1. Arrest of estrogen-induced MCF-7 cell proliferation by dominant-negative Erk2.** Cells were co-transfected with pEGFP and DN-Erk2 or an empty vector (pcDNA3) and treated as previously with 0.1  $\mu$ M zearalenone (ZEAR) or 5 nM E<sub>2</sub>. By 18-24 hr cells were harvested and analyzed by flowcytometry. The fraction of S+G<sub>2</sub>/M cells is demonstrated graphically.

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### **PART III**

**Sequestration of CKI by Cyclin D1/Cdk4 Complexes Essential in**

**Induction of Cyclin E-Associated Kinase Activity in**

**Zearalenone Treated MCF-7 Cells**

## ABSTRACT

Zearalenone, a fungal estrogen, has multiple effects in the regulation of the MCF-7 cell cycle. Zearalenone treatment induced cyclin E-associated kinase activity within 9-12 hr. This increase in cyclin E/Cdk2 activity could be due to the sequestration of the CKI, p27, by newly formed cyclin D1/Cdk4 complexes and/or due to zearalenone-induced downregulation of p27. The activity of cyclin E/Cdk2 complexes from zearalenone treated lysates could be inhibited in a dose dependent manner by His-tagged p27. This inhibition was relieved by addition of increasing levels of recombinant cyclin D1/Cdk4 complexes. Thus sequestration of p27 by cyclin D1/Cdk4 resulted in activation of the enzyme, *in vitro*. Boiling released latent inhibitory activity from lysates of cells treated with zearalenone, and this activity was depleted by anti-p27 and anti-p21 antibodies. Overexpression of the Cdk4/6 specific inhibitor, p16, was associated with increased association of p27 with Cdk2 concomitant with disruption of D cyclin Cdk4 complexes. Nevertheless, in p16-arrested cells downregulation of p27 by zearalenone was present and resulted in cells entering S phase after a lag of 18-24 hr. These data suggest that sequestration of CKI by cyclin D1/Cdk4 complexes as well as downregulation of p27 protein play major roles in the induction of cyclin E-associated kinase activity and in S phase entry in zearalenone-treated MCF-7 cells.

## INTRODUCTION

The mammalian cell cycle is positively regulated by Cdks, which, via sequential phosphorylations, control DNA replication and mitosis (1). Cdk activity is subjected to multiple levels of regulation with cyclin binding being the major determinant of its activity (2-4). Complete activation of the cyclin bound Cdk subunit requires phosphorylation by CAK on a single threonine residue (Thr-161 in Cdk1 and Thr-160 in Cdk2) (1). However, even in the presence of cyclin binding and activating phosphorylation by CAK, phosphorylation of certain residues within the catalytic cleft (Thr-14/Tyr-15 in Cdk1/2) can affect Cdk activity (2). The Cdc25 phosphatases are reported to activate Cdk's by dephosphorylating these inhibitory residues (2). The catalytic activity of Cdk's is further regulated by two families of structurally divergent Cdk Inhibitors (CKIs), the Cip/Kip family and the INK4 family (5). Members of the Cip/Kip family (p21, p27, and p57) inhibit a broad range of Cdks (6-8). They have characteristic motifs in their amino terminal domains, which allow them to bind cyclin/Cdk complexes (9, 10). The INK4 family members (p15, p16, p18, and p19) contain 4-5 ankyrin repeats, which are required for binding with Cdk4 and Cdk6 (11-14). Association of INK proteins with Cdk4/6 prevents cyclin D binding and thereby eliminates cyclin D-associated kinase activity (14).

The Retinoblastoma protein (Rb), a member of the pocket protein family, is the most important Cdk substrate identified to date (15, 16). As cells progress through G1, Rb is hyperphosphorylated and inactivated releasing the transcription factor E2F for transactivation of genes important for initiation of DNA replication



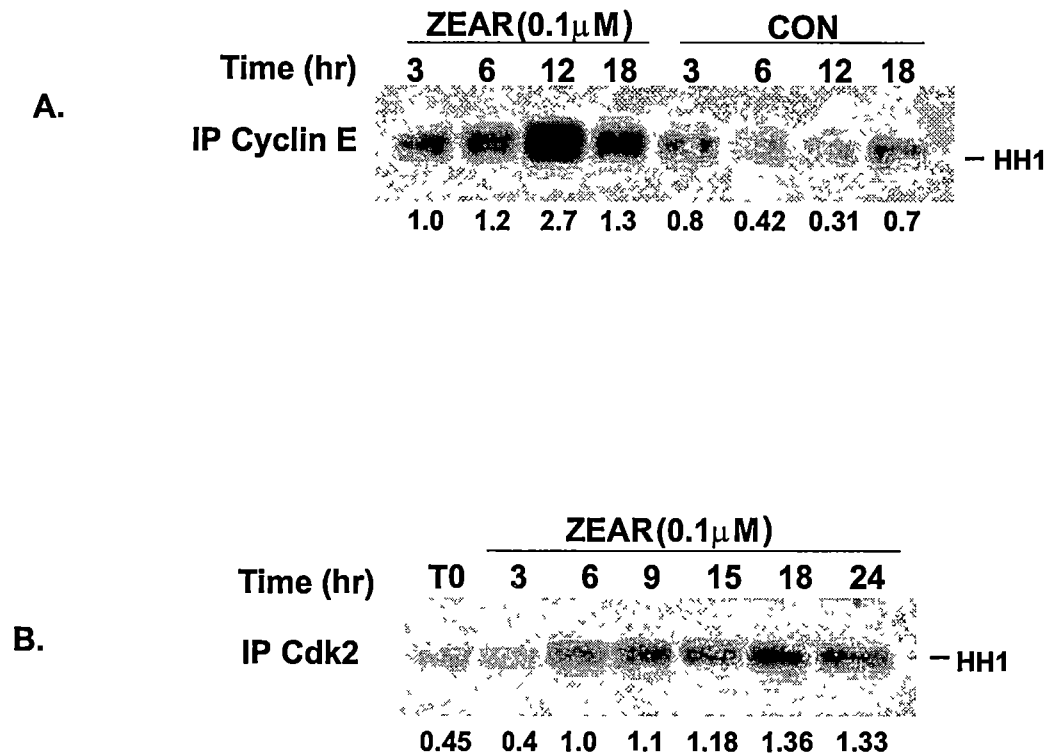
and cell cycle progression (17). Expression of D-type cyclins is induced as cells enter the cell cycle from G<sub>0</sub> and these cyclins form complexes with Cdk4 or Cdk6 kinases (18). These kinases play a critical role in G1 transit, both by phosphorylating Rb and sequestering p27, leading to the activation of cyclin E/Cdk2 complexes (5, 19). Most p27 in proliferating cells is associated with cyclin D dependent kinases (8, 20). Overexpression of cyclin D1 and Cdk4 abrogates the cell cycle arrest imposed by p27 overexpression, without a decline in overall p27 levels (21). Cyclin E/Cdk2 regulates p27 expression through phosphorylation leading to nuclear export and ubiquitin-targeted degradation of the CKI (21). In this model any stimulus, which elicits even minimal accumulation of active cyclin E/Cdk2, may trigger a positive feedback loop whereby p27 is phosphorylated and eliminated, thus, paving the way for G1/S transit.

The mycotoxin zearalenone is present in a wide variety of grains, grain derived foods (22), and milk and meat (23) and is reported to have a variety of estrogen-like effects in mammals (24-26). Since breast cancer in women is related to estrogen exposure it is important to delineate the mechanisms by which xenoestrogens like zearalenone may influence the cell cycle in estrogen-responsive tissue. Previously we have demonstrated that zearalenone has multiple actions in the G1 phase of the cell cycle (Ahamed et al. submitted) We have observed that zearalenone induces expression of cyclin D1 and Myc, as well as the degradation of p27, resulting in increased cyclin D1/Cdk4, cyclin E/Cdk2 activities, Rb phosphorylation and S phase entry by 18-24 hr.

The data presented in this paper demonstrates that cyclin D1/Cdk4 complex formation plays a key role in G1/S phase transit induced by zearalenone treatment in MCF-7 cells. The sequestration of CKI by cyclin D1/Cdk4 complexes is critical for activation of cyclin E-associated kinase in mid G1 phase. Our data also demonstrate that in addition to sequestration, the downregulation of p27 occurring late in G1 may contribute significantly to activation of the Cdk2 enzyme and G1/S transition.

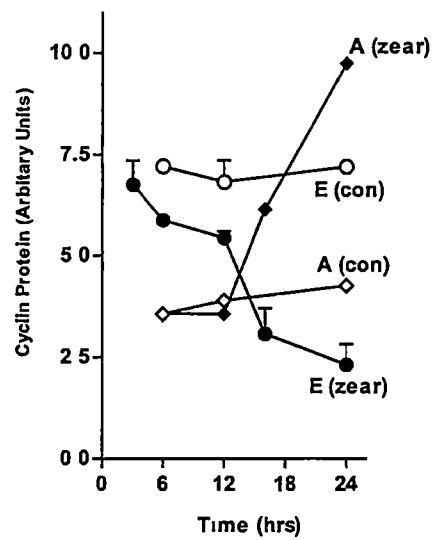
## RESULTS

Zearalenone treatment of MCF-7 cells induced cyclin E-associated kinase activity 2.7 fold by 9-12 hr unlike in quiescent cells where very little activity was present (Figure 1A and data not shown). The increase in cyclin E/Cdk2 activity is not due to increased cyclin E levels as there is no observable increase in total cyclin E levels with zearalenone treatment (Figure 1C). Instead, a decrease in cyclin E levels could be observed over time with less than 50% of the protein observed by 18-24 hr. Cyclin E is associated with Cdk2 in zearalenone-treated as well as G1-arrested cells (Figure 2A) indicating that in the arrested cells catalytically inactive cyclin E/Cdk2 complexes are present. Zearalenone-induced activation of cyclin E/Cdk2 complexes correlated with reduced binding of negative regulators; as the p27 bound to cyclin E/Cdk2 complexes in zearalenone treated cells decreased with time with minimal binding being present by 12 hr

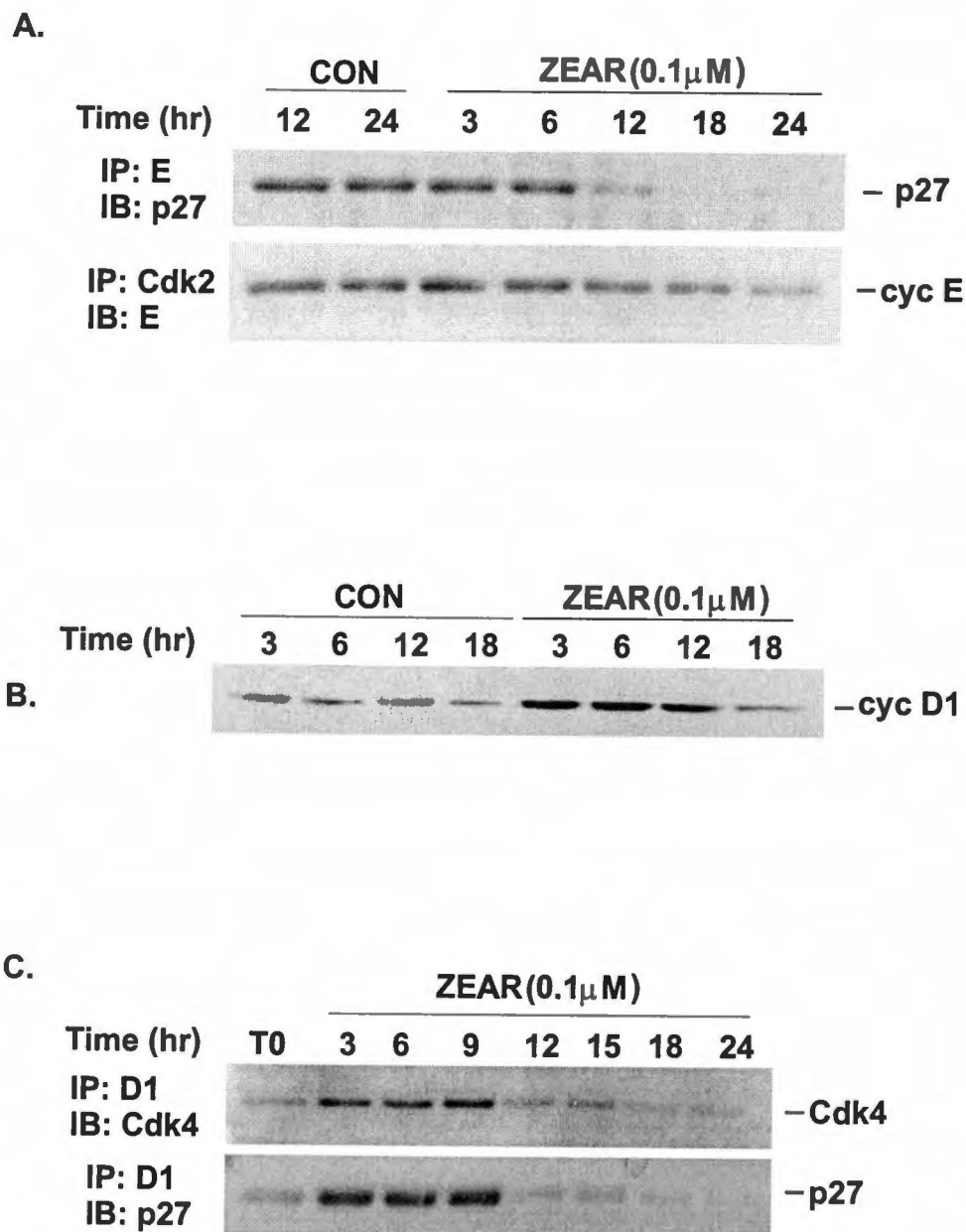


**Figure 1. Cyclin E associated kinase activity is induced in zearalenone treated MCF-7 cells.** G1 arrested MCF-7 cells were treated with 0.1  $\mu$ M zearalenone and at the indicated times treated and untreated cells were harvested and lysates prepared as indicated in materials and methods. Equal concentrations of proteins were immunoprecipitated with anti bodies to (A) cyclin E, or (B) Cdk2, and associated kinase activity was measured using HH1 as substrate. Representative results are shown.

C.



**Figure 1. Cyclin E associated kinase activity is induced in zearalenone treated MCF-7 cells. (C)** Similarly growth-arrested and zearalenone treated cells were analyzed for cyclins E (circles), and A (diamonds) protein levels by Western blotting.

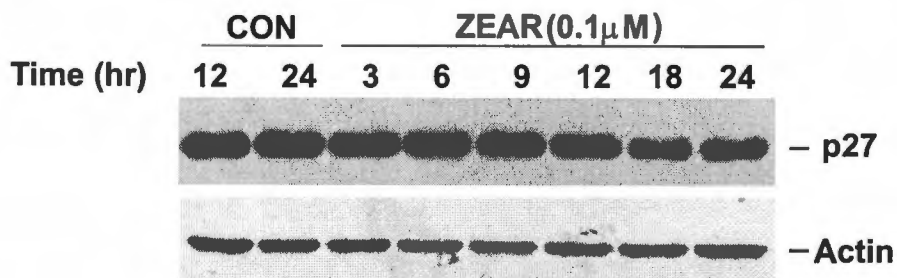


**Figure 2. Regulation of Cdk bound p27 levels and induction of cyclin D1 protein expression by zearalenone.** (A) Lysates treated similar to figure 1 were immunoprecipitated with anti-cyclin E antibody to measure the levels of associated p27 and with anti-Cdk2 antibody to measure the associated cyclin E. (B) Similarly treated lysates Western blotted with anti-cyclin D1 antibody to measure the changes in total cyclin D1 levels following zearalenone treatment. (C) Above lysates were immunoprecipitated with cyclin D1 antibody to demonstrate bound Cdk4 and p27 over time.

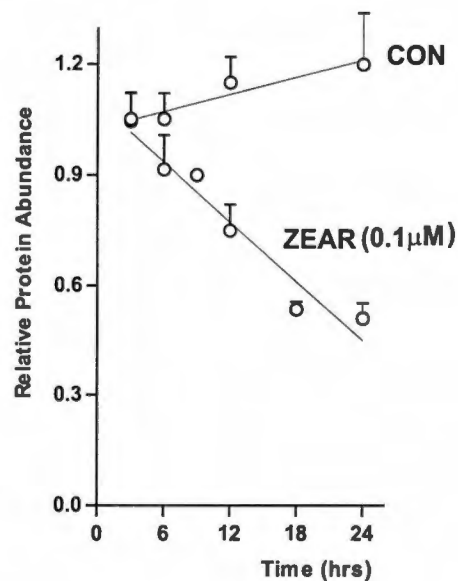
(Figure 2A). Therefore zearalenone-induced activation of cyclin E/Cdk2 complexes by 9-12 hr likely relates, at least in part, to the reduced p27 bound to these complexes, which could lead to formation of a p27-free, active cyclin E/Cdk2 pool. The fall in cyclin E levels observed by 15-18 hr was concomitant with an increase in cyclin A levels at this time (Figure 1C) and could give rise to the increasing Cdk2 activity (Figure 1B). In contrast to cyclin E-associated kinase activity the total Cdk2 activity remains elevated up to 24 hr, when peak S phase entry is observed with zearalenone treatment (Part II).

Recent studies demonstrate that loss of Cip/Kip inhibitors from cyclin E/Cdk2 complexes could arise due to sequestration by cyclin D-Cdk4/6 complexes (27-29). We therefore, investigated what occurs in the latter complexes during zearalenone-treatment. Previously we have observed that zearalenone treatment of MCF-7 cells leads to induction of cyclin D1 protein expression and down regulation of Cip/Kip inhibitors, which results in G1 kinase activation (Part II). The induction of cyclin D1 by 3 hr (Figure 2B) was followed by association with its catalytic subunit, Cdk4, as early as 3 hr after zearalenone treatment, and persisted till 12 hr (Figure 2C). The amount of p27 bound to cyclin D1/Cdk4 complexes also was seen to change with an almost similar time course (Figure 2C). Total p27 levels decreased about 30% by 12 hr of zearalenone treatment (Figure 3A) coinciding with the peak induction of cyclin E-associated kinase activity (Figure 1A). These data imply that formation of cyclin D1/Cdk4/p27 complexes as well as the down regulation of total p27 induced by zearalenone result in less available p27 levels for association with cyclin E/Cdk2 complexes.

A.



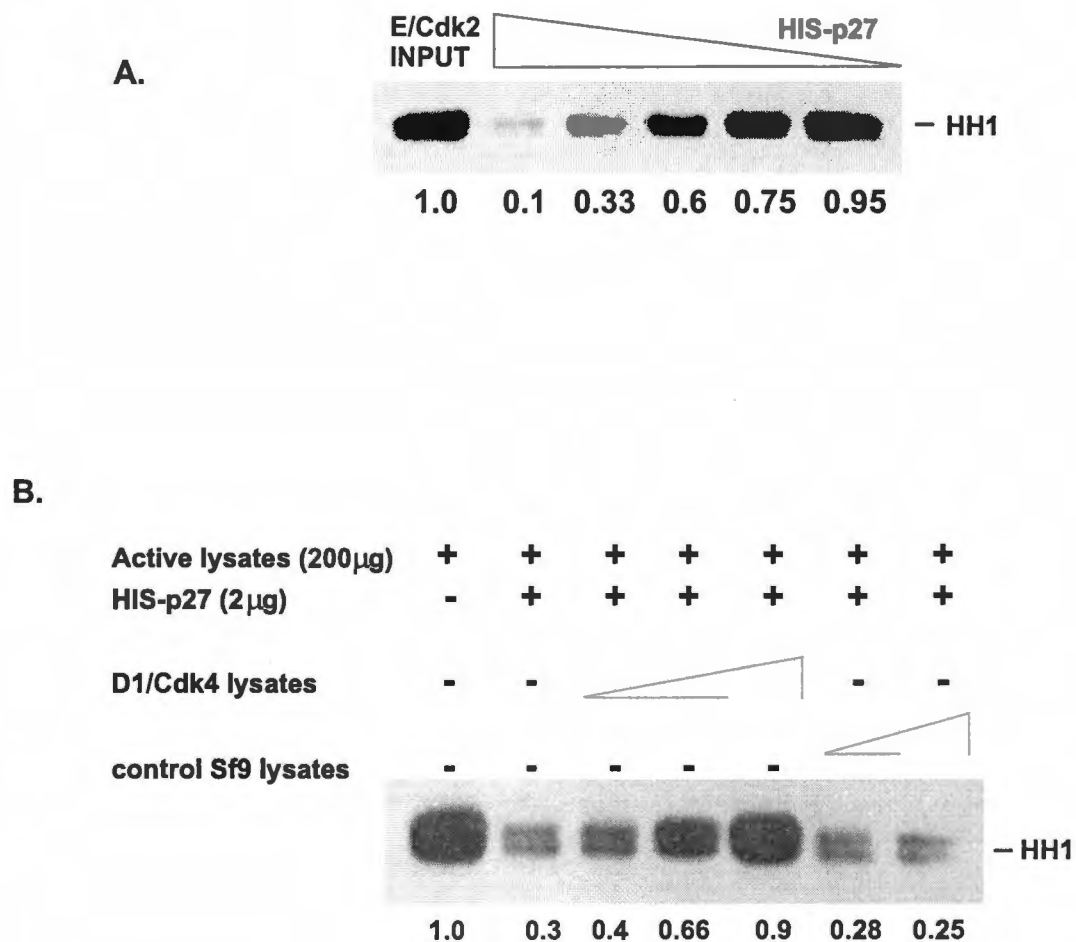
B.



**Figure 3. Downregulation of total p27 in zearalenone treated MCF-7 cells.** (A) Zearalenone-treated cell lysates were analyzed by Western blotting with anti-p27 antibody. Actin is shown for loading control. (B) Data representing mean values from two independent experiments are shown graphically.

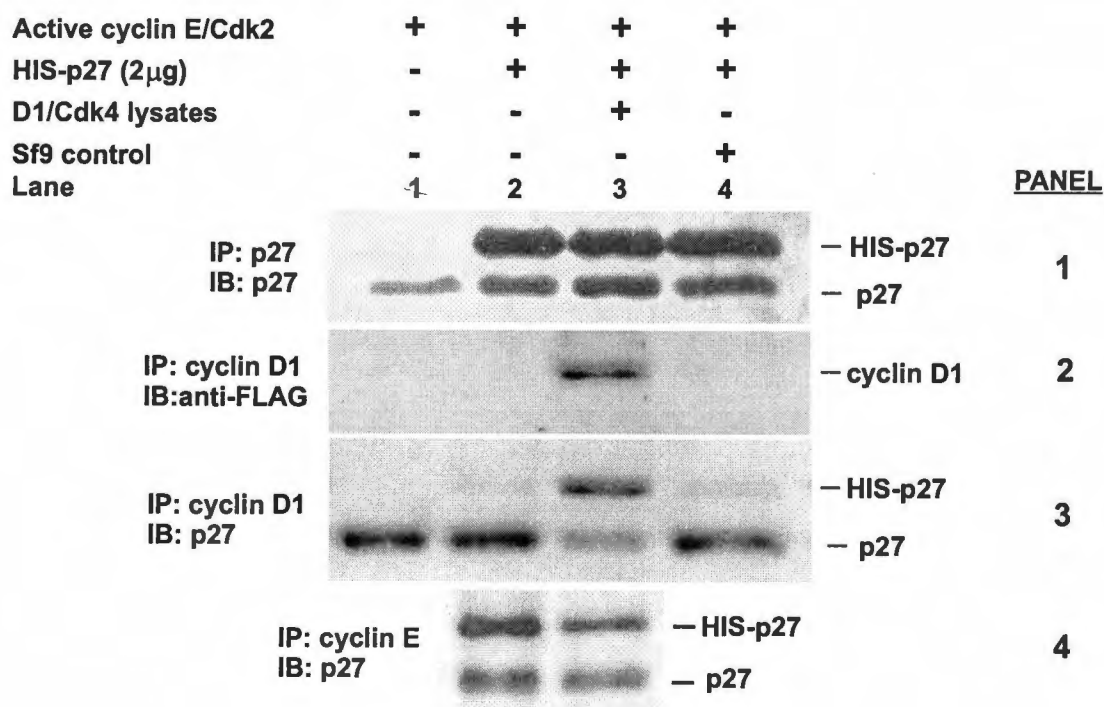
To demonstrate that association of p27 to cyclin E/Cdk2 complexes could regulate cyclin E-associated kinase activity, increasing concentrations of recombinant His-tagged p27 protein (His-p27) was incubated with active cyclin E/Cdk2 complexes from estrogen-treated MCF-7 cell lysates. The recombinant p27 inhibited the cyclin E-associated kinase activity in a concentration dependent manner with almost 90% decrease in activity seen with 2  $\mu$ g of His-p27 (Figure 4A). Presuming that sequestration of p27 by cyclin D/Cdk4 can lead to the activation of cyclin E/Cdk2, the p27 bound cyclin E/Cdk2 complexes were incubated with recombinant flag tagged-cyclin D1 and His tagged-Cdk4 complexes. These proteins were produced by infection of Sf9 insect cells with appropriate baculoviruses (30, 31). Addition of increasing concentrations of active cyclin D1/Cdk4 complexes succeeded in reversing the inhibition caused by the excess p27 (Figure 4B). However, control Sf9 lysates that do not express the cyclin D1/Cdk4 recombinant proteins had no effect. To demonstrate the changes in associated p27, similarly treated lysates were immunoprecipitated with antibodies specific for cyclins D and E and blotted for p27 (Figure 4C). The added recombinant His-p27 and Flag tagged cyclin D1 are visible in panel 1 (lanes 2,3 & 4) and panel 2 (lane 3) respectively. Increased binding of His-p27 to cyclin D1 (panel 3, lane 3) is concurrent with reduced binding of p27 to cyclin E (panel 4 lane 3), which resulted in increased cyclin E-associated kinase activity (Figure 4B). These data suggests that the recombinant D1/Cdk4 complexes can overcome the inhibitory effect of the CKI on cyclin E-associated kinase activity. These results further support the notion that the newly formed cyclin D1/Cdk4





**Figure 4. Cyclin D1/Cdk4 complexes titrate p27 and relieve inhibition of Cdk2-cyclin E complexes.** (A) MCF-7 lysates treated with zearalenone for 12 hr were incubated with different concentrations of recombinant p27 (His-p27), and the cyclin E-associated kinase activity was measured with HH1 as substrate. (B) Similarly treated MCF-7 lysates were incubated with His-p27 (2 μg) and increasing concentrations of Sf9 lysates that have been infected with baculoviruses encoding for flag-cyclin D1 and His-Cdk4. Non-infected Sf9 lysates are used as control. Cyclin E-associated kinase activity is measured as above.

C.



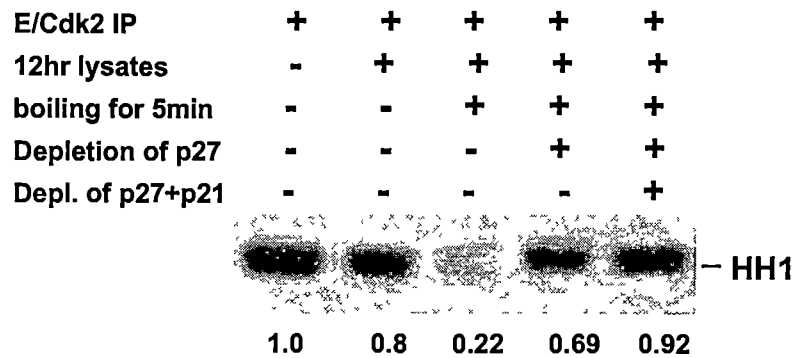
**Figure 4. Cyclin D1/Cdk4 complexes titrate p27 and relieve inhibition of Cdk2-cyclin E complexes. (C)** Lysates from "B" were immunoprecipitated with indicated antibodies and blotted for p27 (panels 1,3, and 4) and flag-tagged cyclin D1 (panel 2). The faster moving band in panels 1,3, and 4 is the endogenous p27. (The decrease in endogenous p27 in panel 3/lane 3, is due to limiting cyclin D1 antibody).

complexes in zearalenone-treated MCF-7 cells are capable of sequestering p27 bound to cyclin E/Cdk2, thus leading to induction of cyclin E-associated kinase activity.

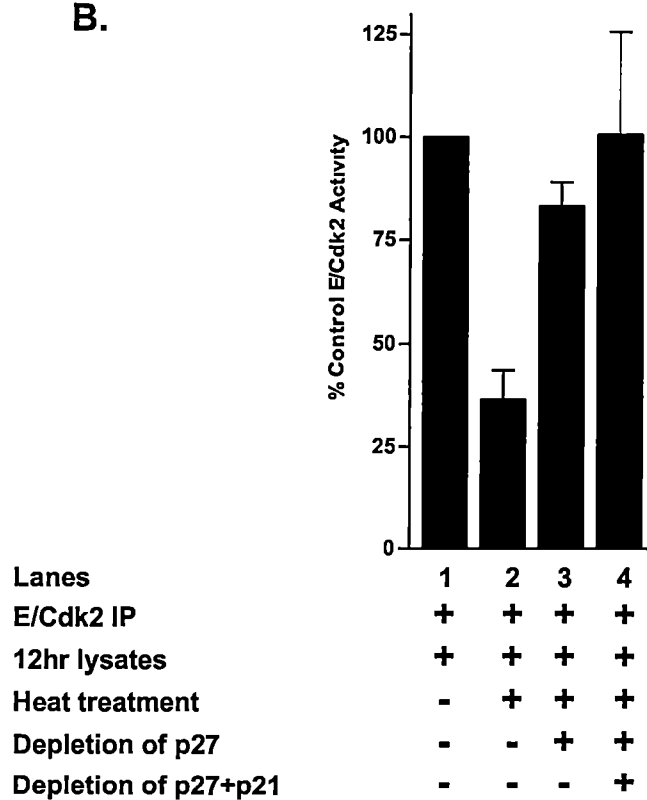
However, zearalenone treatment also results in downregulation of p27 protein in MCF-7 cells (Figure 3), therefore the reduced binding of p27 to cyclin E/Cdk2 complexes may partly be due to the decrease in total p27 levels. Since p27 is heat stable (32) and can be released from bound complexes by boiling, active cyclin E immunoprecipitates isolated from cells treated with estrogens for 12 hr were incubated with similarly treated lysates that has been boiled (Figure 5). The boiled lysates exhibit significantly more inhibitory activity than the non-boiled lysates (Figure 5B lane 2 vs. lane 1) and this inhibitory activity could be largely abrogated by depletion of p27 and p21 from the boiled lysates (lane 4). This suggests that the heat stable inhibitory activity is due to the p27/p21 bound to cyclin/Cdk complexes. Since maximal cyclin E/Cdk2 activity is seen 12 hr after estrogen treatment, it is likely that these CKI are mostly released from cyclin D1/Cdk4 complexes. Therefore, sequestration of p27 by cyclin D1/Cdk4 complexes appears to be essential for cyclin E/Cdk2 activation by mid G1.

To further investigate if the importance of sequestration by D cyclin-Cdk4 complexes for cyclin E/Cdk2 activation, the CKI p16 was overexpressed in G1-arrested MCF-7 cells. The tumor suppressor p16, a specific inhibitor of Cdk4 and Cdk6 kinases (11), is commonly deleted, mutated, or transcriptionally silenced in tumors that retain wild type Rb (33-36). It specifically binds to, and competes with cyclin D1 for a binding site on Cdk4 (37, 38) and therefore should prevent

**A.**



**B.**

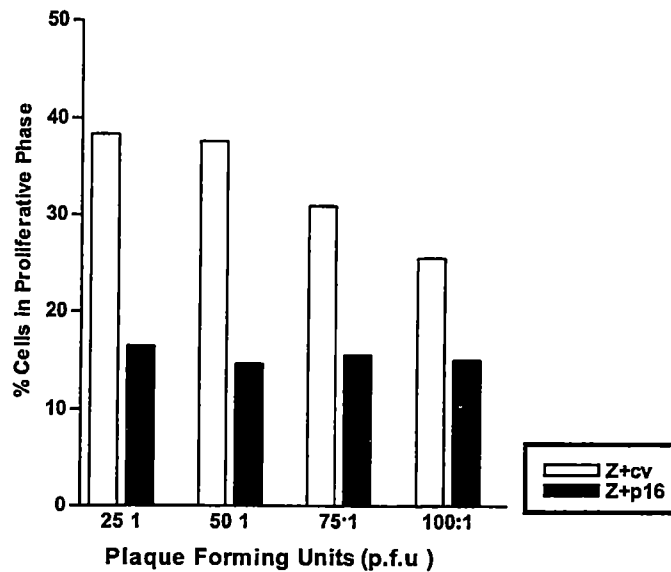


**Figure 5. Disassociation of cyclin/Cdk complexes by heat treatment increases the inhibitory activity in zearalenone treated lysates. (A)** Cyclin E immunoprecipitated from MCF-7 lysates that have been treated with 0.1  $\mu$ M zearalenone for 12 hr (E/Cdk2 IP), were incubated with similarly treated total lysates (12 hr lysates) that have been boiled (heat treatment) or depleted of p27 and or p21 after boiling. The cyclin E-associated kinase activity was measured after incubation as described in materials and methods **(B)** Means of two independent experiments are shown graphically.

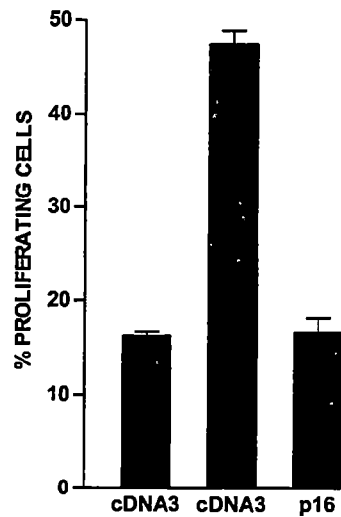
the sequestration of p27 by the cyclin D/Cdk4 complexes. Also, MCF-7 cells are reported to lack endogenous p16 protein due to a deletion in the p16 locus (39, 40). Overexpression of the p16 inhibitor by transfection of a p16 expression plasmid successfully arrested zearalenone-induced S phase entry measured by Flow cytometric analysis by 18 hr of treatment (Figure 6B). Similarly infection with an adenoviral vector overexpressing p16 (Ad.p16) also succeeded in arresting the estrogen-induced S phase entry (Figure 6A). The G1 arrest-induced by p16 was accompanied by reduced cyclin D1 and cyclin E associated kinase activities (Figure 6C & E respectively) and overall Rb phosphorylation (Figure 6D). The overexpressed p16 and the lack of endogenous p16 in MCF-7 cells are depicted in Figure 6F.

We have previously observed that with zearalenone treatment c-myc expression is induced as early as 1 hr in MCF-7 cells (Part II), and could lead to the induction of cyclin E-associated kinase activity. To see whether the observed abrogation of cyclin E/Cdk2 activity is due to reduced c-myc expression, p16-arrested cells were analyzed by Northern blotting for c-myc mRNA levels (Appendix B Figure 1). However, the zearalenone-induced c-myc message by 1 hr was present in the Ad.p16 infected cells, similar to the Ad Con infected cells. On the other hand, overexpression of cyclin E was successful in overriding the p16-induced block of cyclin E/Cdk2 activity (Appendix B Figure 2). This suggests that the overexpressed cyclin E can override the CKI bound to cyclin E/Cdk2 complexes, and thereby overcome the p16-induced abrogation of cyclin

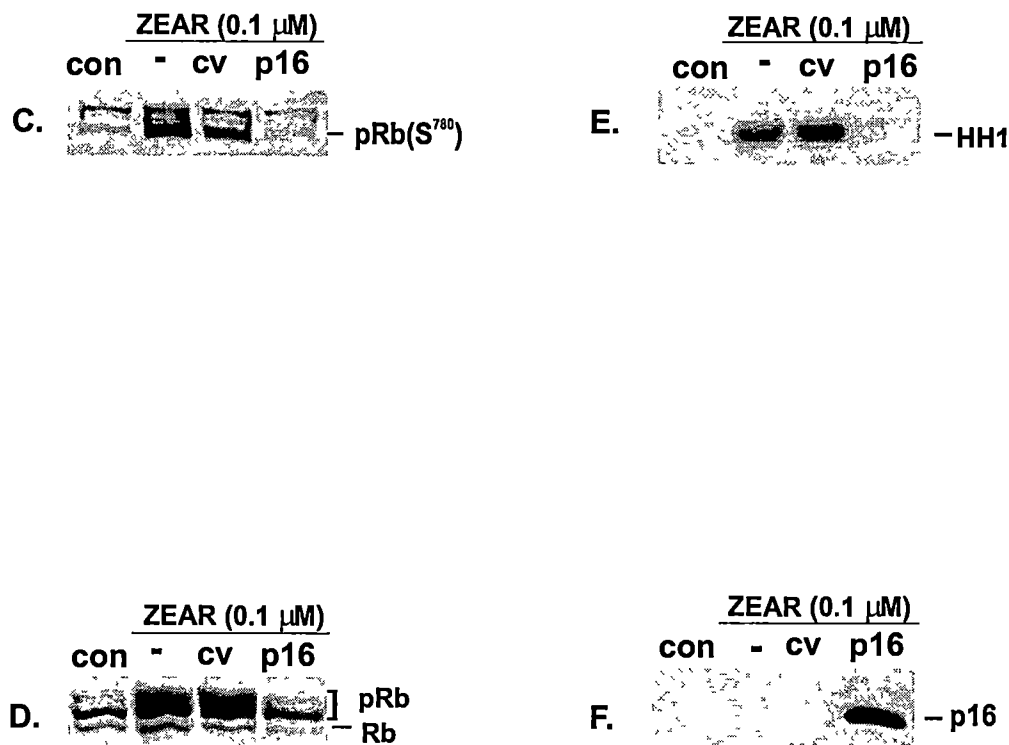
**A.**



**B.**



**Figure 6. The CKI, p16, arrests cells in G1 phase by blocking G1 kinase activity.** (A) MCF-7 cells infected with increasing p.f.u.'s of an adenovirus encoding for p16 (Ad.p16) or a control adenoviral vector (Ad.Con) were growth arrested and treated with 0.1  $\mu$ M zearealenone for 18 hr and percentage (%) proliferating cells were analyzed by flow cytometry. (B) Cells were co-transfected with pEGFP and an expression vector for p16 or a control vector (pcDNA3). Cells were growth arrested and treated with 0.1  $\mu$ M zearealenone for 18 hr and analyzed by flowcytometry. The DNA was measured in green transfected populations and the mean of 3 independent experiments is shown graphically



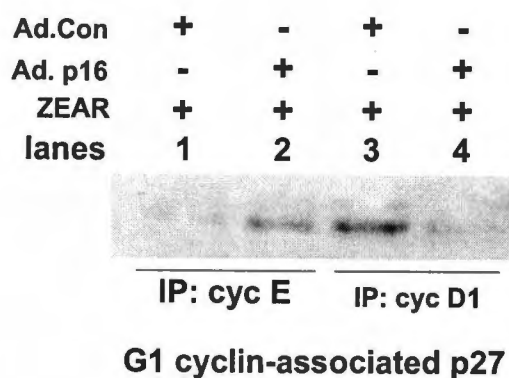
**Figure 6. The CKI, p16, arrests cells in G1 phase by blocking G1 kinase activity.** MCF-7 cells infected with 40 p.f.u. of Ad.p16 or Ad.Con viruses were treated with 0.1 μM zearalenone for 12 hr. Cell lysates were prepared and phosphorylation of the retinoblastoma protein was analyzed by Western blotting with the cyclin D/Cdk4 specific anti-phospho-Rb (S<sup>780</sup>) antibody (C) or with an anti-human phospho Rb antibody (D). Lysates were also assayed for cyclin E associated kinase activity with HH1 as substrate (E). The presence of p16 was verified by performing western blots with the anti-p16 antibody (F).

E-associated activity Therefore we examined cyclin E/Cdk2 and cyclin D/Cdk4 complexes in cells ectopically expressing p16. As expected from reduced cyclin D1-Cdk4 association due to the presence of p16, reduced levels of p27 were bound to cyclin D1 in p16-overexpressing cells by 6 hr (Figure 7A, lane 4). There was also corresponding increase in p27 bound cyclin E in these cells (Figure 7A lane 2 vs. lane 1) and this was not due to a failure in total p27 downregulation as zearalenone-induced decrease in p27 levels appears to be unaffected by overexpression of p16 (Figure 7B). Therefore it appears that p16, by blocking the sequestration of p27 by cyclin D1/Cdk4, can inhibit cyclin E/Cdk2 kinase activity independent of p27 downregulation. However, the decrease in total p27 levels by late G1 in the Ad.p16-arrested cells, appear to facilitate a delayed entry into the cell cycle (Figure 7C). By 36 hr after zearalenone treatment about 25-30% of Ad.p16-infected MCF-7 cells were proliferating, as compared to 40% of Ad.Con infected cells by 18-24 hr. The cyclin E-associated activity in these cells also was induced by 24 hr (Figure 7D), in agreement with the increase in proliferation. The increase in cyclin E-associated activity was not due to a fall in p16 levels, as the p16 protein level in these cells does not change (Figure 7D). These results indicate that the degradation of p27 induced by zearalenone can activate S phase entry in cells where p27 sequestration by cyclin D1/Cdk4 is blocked by overexpression of p16.

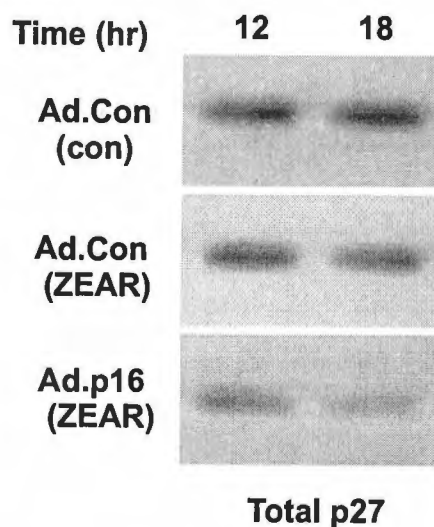
If cyclin D1-Cdk4 complex formation is essential for zearalenone-induced G1 transit, catalytically inactive cyclin D/Cdk4 complexes should not have a



**A.**

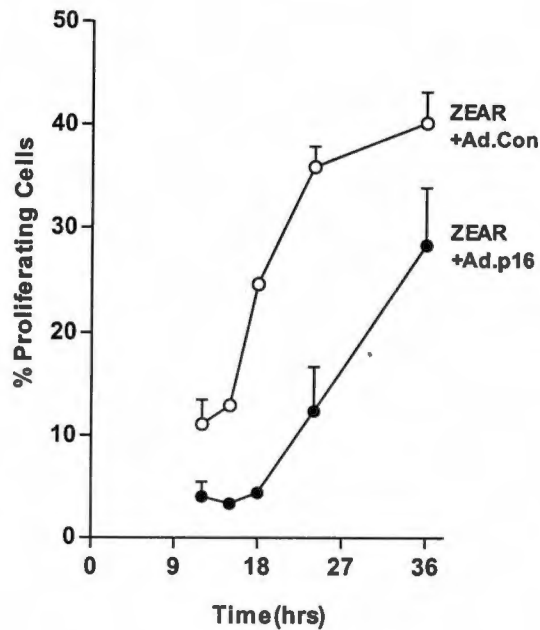


**B.**

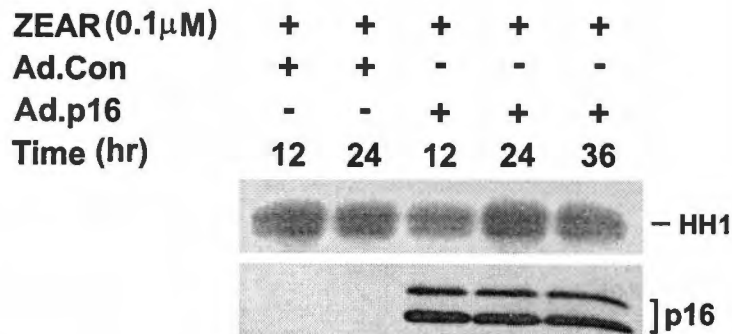


**Figure 7. Adenoviral p16 causes redistribution of the CKI, p27 from cyclin D1 to cyclin E independent of degradation of p27. (A)** Lysates prepared similar to figure 6C were immune precipitated with cyclin E or cyclin D1 antibodies as indicated and analyzed for bound p27 by western blotting. **(B)** Lysates from similarly infected and growth arrested cells that have been untreated (CON) or treated with 0.1  $\mu$ M zearalenone (ZEAR) were blotted for p27 levels.

C.



D.

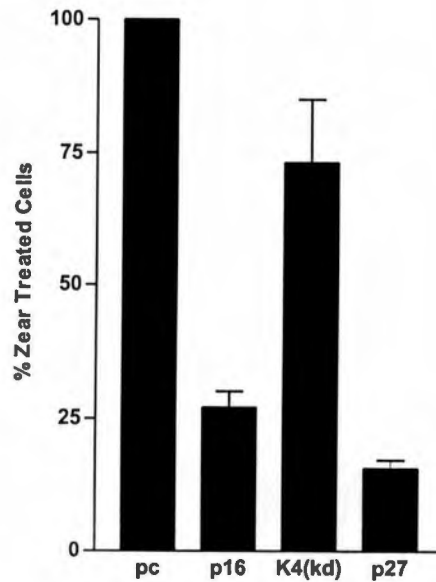


**Figure 7. (C) The S phase blockade imposed by p16 overexpression is reversible.** MCF-7 cells infected with Ad.Con or Ad.p16 and treated as in figure 6 were growth arrested and at the indicated times was analyzed by flowcytometry to measure the percentage of proliferating cells. Mean of two independent experiments is shown graphically. **(D)** Similarly infected and treated cells were lysed and cyclin E associated-kinase activity was measured with HH1 as substrate. The presence of the ectopically expressed Ad.p16 is shown as a control.

significant effect on G1/S transit. Therefore, we transfected a plasmid expressing an ATP-binding mutant Cdk4, which forms catalytically inactive complexes with D cyclins (29). This mutant Cdk4 protein was unable to block S phase entry significantly (Figure 8A) suggesting that zearalenone-induced S phase entry in MCF-7 cells is largely independent of Cdk4 activity. In contrast, zearalenone-induced G1/S transit in these cells appears to be completely dependent on Cdk2 activity as overexpression of p27 completely abrogated S phase entry. We also used a mutant Cdk4, that is not phosphorylated by CAK but forms catalytically inactive complexes with cyclin D1 (41), to reverse a p27-induced arrest of cyclin E/Cdk2 complexes in an *in-vitro* kinase assay (Figure 8B). This mutant Cdk4 complexed to cyclin D1 was able to overcome the inhibitory activity of excess recombinant p27 on cyclin E/Cdk2 complexes as effectively as the wild type Cdk4-cyclin D1 complexes.

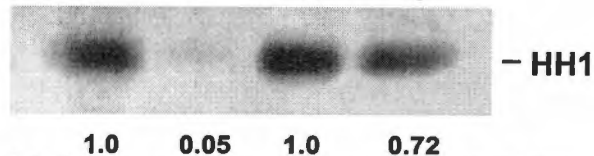
In the p16-arrested cells zearalenone induction of cyclin D1 expression was present, however, the cyclin D1 protein levels appear to be degraded at a faster rate (Appendix B, Figure 3A). To ensure that the cyclin D1 protein levels are not limiting for activation of the cyclin E/Cdk2 complexes, we co-transfected a stable mutant of cyclin D1 (42), with p16, and analyzed the cell cycle profile of these cells (Appendix B, Figure 3B). Our data demonstrate that overexpression of the mutant cyclin D1 failed to overcome the cell cycle arrest caused by the presence of p16. Thus, cyclin D1 is not limiting in these cells and the high levels of p16 compete with cyclin D1 for Cdk4 and prevent the complex formation between D cyclins, Cdk4 and p27. Collectively these data demonstrate that

**A.**



**B.**

|                        |   |   |   |   |
|------------------------|---|---|---|---|
| Active lysates (200μg) | + | + | + | + |
| HIS-p27 (2μg)          | - | + | + | + |
| D1/Cdk4 lysates        | - | - | + | - |
| D1/Cdk4 (m)            | - | - | - | + |
| Lane                   | 1 | 2 | 3 | 4 |



**Figure 8. A mutant Cdk4, which forms catalytically inactive complexes with cyclin D1, fails to block the zearalenone-induced cycling.** (A) Cells co-transfected with pEGFP and/or expression vectors for a control vector (cv), p16 (p16), a kinase-dead Cdk4 (Cdk4[kd]), p27 (p27) were growth arrested and treated with zearalenone to induce cycling. DNA levels of the green population of cells were analyzed by flowcytometry and the percentage of proliferating cells was measured. The mean of 3 independent experiments is demonstrated graphically. (B) The experiment as in figure 3B was performed with a mutant Cdk4/cyclin D1 complex [D1/Cdk4 (m)] and the cyclin E associated kinase activity was measured with HH1 as substrate.

sequestration of CKI is a major role of cyclin D1/Cdk4 complexes that lead to cyclin E-associated kinase activity and cell cycle transit in estrogen-treated MCF-7 cells.

## DISCUSSION

Data presented in this communication demonstrates that both redistribution and degradation of p27 are essential for activation of Cdk2 and the resultant G1/S phase transition induced by estrogens in MCF-7 cells. The induction of cyclin E associated kinase activity in zearalenone treated MCF-7 cells by mid G1 (9-12 hr) is primarily a result of redistribution of the CKIs from E cyclin-Cdk2 to D cyclin-Cdk4 complexes. The decrease in p27 observed in mid to late G1 (after 12 hr) is probably more important in upregulating total Cdk2 activity, which peaks around 18-20 hr following treatment with zearalenone. We observed abundant levels of functionally inactive cyclin E bound-Cdk2 in growth arrested MCF-7 cells (Figure 2A) indicating that cyclin E protein levels in growth-arrested MCF-7 cells are not limiting (Figure 1C). Thus the increase in cyclin E/Cdk2 activity in MCF-7 cells is probably not a result of increased cyclin expression. Activation of cyclin E/Cdk2 complexes could result from both changes in the phosphorylation state of the cyclin bound Cdk2 as well as through formation of a pool of cyclin E/Cdk2 complexes free of CKI. CAK activity responsible for activation phosphorylation of Cdk2 is not limiting during cell cycle transit and Thr160 phosphorylation parallels cyclin levels (2), therefore, it is unlikely that CAK activity is limiting in

these cells. Cdc25A phosphatase activity is required for removal of the inhibitory Thr14/Tyr15 phosphorylations within the catalytic cleft of Cdk2 (43, 44) and regulation of Cdc25A activity is important for G1 transit (45-47). Cdc25A expression is stimulated by Myc (48), an early estrogen target (49, 50). Cdc25A message and protein are induced in estrogen-treated MCF-7 cells, and this induction is abrogated by c-myc antisense oligonucleotides (51), (Foster et al submitted). Therefore the increase in cyclin E-associated kinase activity after mitogenic stimulation may also relate to removal of the inhibitory phosphate groups in the Cdk2 catalytic subunit by the Cdc25A phosphatase.

Previous work has demonstrated that cyclin E degradation is mediated by autophosphorylation and inhibition of Cdk2 activity *in vivo* stabilizes cyclin E in mammalian cells (52, 53). Thus, coupling cyclin E/Cdk2 activation to cyclin E degradation is apparently important for effecting a decisive cell cycle transition. Our data indicates that levels of cyclin E decreased approximately 60-70% late in G1, 15-18 hr after treatment with zearalenone (Figure 1C). This decrease in cyclin E levels corresponding with a reduction in cyclin E-associated kinase activity (Figure 1A). Unlike cyclin E-associated activity, total Cdk2 activity in zearalenone treated MCF-7 cells remains elevated as cells enter S phase (figure 1B). The zearalenone-induced increase in cyclin A levels observed by 15-18 hr after treatment (figure 1C) may account for this prolonged increase in Cdk2 activity. Phosphorylation of the E2F/DP-1 transcription factor by cyclin A/CDK2 may also abolish its DNA binding activity (54-56). Inactivation of E2F/DP1

transcription factors may prevent the induction of cyclin E expression in late G1 contributing to the observed decrease in cyclin E levels.

The decrease in levels of p27 associated with cyclin E/Cdk2 complexes (figure 2A) could be brought about by downregulation of the total CKI (Figure 3), or through sequestration of the CKI by newly formed cyclin D1/Cdk4 complexes (Figure 2C). Increased levels of cyclin D1 protein associated with Cdk4 and p27 appear within 3-4 hr of estrogen treatment and persist until 12 hr or more (Figure 2C). Formation of these ternary complexes coincides with cyclin D1-associated kinase activity (Ahamed et al submitted). The cyclin D1/Cdk Rb kinase activity in proliferating cells is found in complexes containing Cip/Kip proteins (8), and *in vitro*, p27 is a more effective inhibitor of cyclin E/Cdk2 than of cyclin D/Cdk4 complexes (8, 20). The reported absence of cyclin D1/Cdk4 complex formation in p21 (-/-) and/or p27 (-/-) mouse embryo fibroblasts (MEFs) has demonstrated that CKI are essential for cyclin D1/Cdk4 complex formation (57). In this context, the relative absence of cyclin D1/Cdk4 complexes later in G1 phase (Figure 2C), even in the presence of substantial levels of cyclin D1 (Figure 2B) may be due to reduced p27 availability for binding to these complexes (Figure 3). The modest decline in p27 levels at 12 hr appears to be sufficient for a large decrease in cyclin D1/Cdk4 complexes and this is concurrent with a reduction in cyclin D1 levels between 12-18 hr (Figure 2B).

Formation of ternary complexes composed of cyclin D1-Cdk4-p27/p21 facilitates the induction of cyclin E-associated kinase activity in U2OS cells (27-29). Our *in vitro* results demonstrate that cyclin E/Cdk2 complexes rendered

inactive by recombinant p27 could be reactivated by addition of cyclin D/Cdk4 (figure 4B). This activation of cyclin E/Cdk2 is accompanied by sequestration of p27 into the exogenous cyclin D/Cdk4 complexes (Figure 4C). Furthermore boiling of MCF-7 lysates exhibiting maximal cyclin E-associated kinase activity released p27 and p21 probably from cyclin D/Cdk4 complexes resulting in inhibition of Cdk2 activity (Figure 5). These studies confirm the importance of Cip/Kip inhibitor sequestration by cyclin D1-Cdk4 complexes, and suggest that expression of cyclin D1 and its association with Cdk4 is essential for subsequent cyclin E-associated kinase activation in response to zearalenone. Ectopic expression of p16 in Rb positive cells prevents association of Cdk4/6 with D cyclins resulting in growth arrest (5, 37, 58). Expression of p16 in MCF-7 cells, which have no endogenous p16, prevented zearalenone-induced S phase entry concomitant with decrease in G1 kinase activities and accumulation of hypophosphorylated Rb (Figure 6). Several studies have indicated that G1 arrest mediated by p16 is due to abrogation of complex formation between cyclin D1 and Cdk4 and the resulting inhibition in cyclin E-dependent kinase activity (27-29). In the p16-arrested MCF-7 cells we observed a shift in p27 from cyclin D1 to cyclin E (Figure 7A), which in turn could have resulted in blockade of cyclin E-associated kinase activity and G1 arrest (Figure 6).

Whereas catalytically inactive Cdk2 that binds cyclins E and A resulted in G1 arrest, mutant Cdk4 that form catalytically inactive complexes with D cyclins failed to do so (59). Subsequently it was found that inhibition of cyclin D-dependent kinase activity is insufficient for G1 arrest in actively proliferating



U2OS cells (29). In contrast, cyclin D-dependent kinase activity is required for cell cycle progression in mouse fibroblasts as expression of a dominant negative D1 mutant results in growth arrest (60). We observed that a kinase-dead mutant of Cdk4 is a relatively poor inhibitor of G1 transit in estrogen-treated MCF-7 cells compared to p16 (Figure 8A). Furthermore, a CAK-mutant Cdk4 that forms catalytically inactive complexes with cyclin D1 substantially activates p27-inhibited cyclin E/Cdk2 complexes, in vitro (Figure 8B). Taken together our data agrees with the hypothesis that the ability of cyclin D1/Cdk4 complexes to sequester p27 is essential for regulating G1 transit. Rb hyperphosphorylation is maintained at a high level in zearalenone-stimulated MCF-7 cells even at 30 hr post treatment (Ahamed et al. submitted), long after Cdk4-bound cyclin D1 complexes have disappeared (Figure 2C). Thus it is likely that a substantial part of Rb kinase activity in these cells is due to Cdk2. As the only established enzymatic activity of cyclin D/Cdk4 appears to be phosphorylation of Rb (61), the role of cyclin D1/Cdk4 catalytic activity in G1 transit appears to be unclear. Previous observations in cyclin D1 (-/-) mice where abnormalities are highly restricted (62, 63) were taken to suggest either that cyclin D1 is not essential development of most tissues, or that cyclins D2/D3 are functionally redundant with cyclin D1. Cyclin E expressed from the cyclin D1 promoter rescues most of the morphologic defects in cyclin D1 (-/-) mice (64), implying that expression of cyclin E renders the upstream role of cyclin D in G1 transit redundant. These observations together with the fact that cyclin E can drive Rb-negative cells into S phase (65) has lead to the hypothesis that D cyclins function primarily as upstream sensors for

regulating cyclin E/Cdk2 activity. Our data agrees with this hypothesis in that the function of cyclin D1 in MCF-7 cells appears largely to facilitate cyclin E/Cdk2 activation via the sequestration of p27/p21

Cdc25A phosphatase activity may also be limiting in p16-arrested cells as addition of recombinant GST-Cdc25A to p16-arrested cell lysates *in vitro* partially restores cyclin E-associated kinase activity (data not shown). Cdc25A is reported to interact with cyclin E/Cdk2 complexes (46, 66) and the catalytic activity of Cdc25A is increased *in vitro* (46) and *in vivo* (Foster et al. submitted) upon phosphorylation by cyclinE/Cdk2. Thus in p16-arrested cells the feed forward loop between cyclin E/Cdk2 and Cdc25A may be dysfunctional. The decrease in p27 levels by late G1 in p16-arrested cells could lead to the formation of p27-free active cyclin E/Cdk2 complexes, which in turn activates the feed forward loop between Cdc25A and cyclin E/Cdk2 complexes. Our observation of increased cyclin E-associated kinase activity by 18-24 hr (Figure 7D) and the delayed G1/S transit (by 36 hr) induced by zearalenone in Ad.p16 infected MCF-7 cells (Figure 7C) lends support to this hypothesis. The fact that the p16-induced blockade of G1/S transition is reversible concurrent with the substantial decrease in p27 between 18-24 hr demonstrates the importance of p27 downregulation in Cdk2 activation and S phase entry. Therefore in MCF-7 cells both, sequestration of p27 as well as its downregulation, appear to play important roles in activation of the central regulator of G1/S transition, Cdk2.

Our data demonstrates that in zearalenone-treated MCF-7 cells cyclin D1 couples the mitogenic signal to the cell cycle machinery by forming stable ternary

complexes with Cdk4 and p27/p21, which leads to reduced CKI levels in cyclin E/Cdk2 complexes and results in a pool of active cyclin E/Cdk2. Increased E2F transcriptional activity consequent to Rb phosphorylation together with an increase in Cdc25A activity may further increase cyclin E-associated kinase activity. The rapid increase in cyclin E associated kinase activity in mid G1 probably results in a feedback reduction in cyclin E and consequent reduction in cyclin E/Cdk2 activity. However, because cyclin A levels are induced and p27 is rapidly degraded in mid-late G1 total Cdk2 activity peaks at 18-20 hr associated with entry of cells into S phase

## **MATERIALS AND METHODS**

### **Cells, Culture conditions and Transfections**

MCF-7 cells were provided by Dr Robert P.C Shiu (67) and were maintained in Dulbecco's minimal Eagle's medium (DMEM) supplemented with antibiotics and 5% fetal bovine serum (FBS). *Spodoptera frugiperda* Sf9 insect cells were grown in Grace's media supplemented with 10% heat-inactivated FBS as previously explained (41) MCF-7 cells were transfected with expression vectors for pBPSTR-1-p16, provided by Dr. Gordon Peters, pCG-N-BL-HA-p27, provided by Dr Jun-Ya Kato (68), pCMV-Cdk4-dn, provided by Dr. Ed Harlow (59), or pcDNA3 control vector (Invitrogen, Carlsbad, CA) using Superfect Reagent (Qiagen, Hilden, Germany). A CMV promoter-driven enhanced green fluorescent

protein construct, pEGFP (CLONTECH, Palo Alto, CA), was co-transfected as a selectable marker for transfected cells

## **Generation and Use of Recombinant Proteins**

His-p27 was expressed using a construct encoding Histidine-tagged p27 was generously provided by Dr. Joan Massague. Protein was expressed in *E.coli* and purified by sonication and nickel-NTA chromatography. Purified protein was dialyzed against PBS at 4 °C/overnight, the protein concentration determined by Bradford protein assay (69) and stored at -80 °C. The recombinant Flag-tagged cyclin D1, wild-type CDK4, and CDK4(T172A) were produced by infection of insect Sf9 cells with the appropriate baculoviruses (30, 31, 41). The infected cells were harvested 48 hr post-infection and lysed in Tween 20 lysis buffer (50 mM HEPES, pH 7.5, 10 mM MgCl<sub>2</sub>, 2.5 mM EGTA, 1 mM EDTA, 1 mM PMSF, 20 U/ml of aprotinin, 5 µg/ml leupeptin, 0.4 mM NaVO<sub>4</sub> and 0.4 mM NaF). Whole cell lysates were cleared by centrifugation for 10 min at 4 °C and stored at -80 °C. For inhibitory assays MCF-7 cell lysates (200 µg) treated for 9-12 hr with zearalenone was incubated (30min at 30°C) with increasing concentrations His-p27. Cyclin E-associated kinase activity, of the above lysates was measured with HH1 as substrate. For reversal of inhibitory activity 200 µg of total lysates (9-12 hr zearalenone treated) was incubated with 10 µl (0.2 µg/µl) of recombinant His-p27 +/- baculovirus infected Sf9 lysates (10, 20 or 40 µl) or non-infected control

Sf9 lysates (20 or 40  $\mu$ l) for 30 min at 30 °C. The treated lysates were immunoprecipitated and followed by kinase assay or western blotting.

## **Growth Arrest and Cell Lysis**

For assays requiring growth arrest, MCF-7 cells were grown in DMEM with 5% FBS for 1-2 days, at which time the medium was changed to phenol red-free DMEM with 0.2% FBS and 10 nM ICI 182,780 (Dr. Alan Wakeling, Zeneca Pharmaceuticals, Alderley Park, Cheshire, UK) for an additional 48 hr. Treatment with zearalenone was done in the presence of 1 nM ICI 182,780. This protocol results in 85%-90% of the cells in G0/G1 phase (Ahamed et al. submitted). MCF-7 cells to be harvested were washed with ice-cold PBS and lysed by addition of ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% nonidet P-40, 5 mM EDTA, 10 mM EGTA) containing 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation at 11,000 X g for 20 min at 4°C and protein concentrations was determined by Bradford protein assay (69).

## **Antibodies**

The antibodies used in this study are Cdk2 (M2), Cdk4 (C22), p27 (C19), p16 (H-156), cyclin D1 (H295), cyclin A (BF683) as well as monoclonal antibodies to cyclin E (HE111 and HE12) and are from Santa Cruz Biotechnology (Santa Cruz, CA) Mouse monoclonal antibody to human Rb protein was from Pharmingen

(San Diego, CA) and the antibody for phospho Rb(Ser780) was from Medical & Biological Laboratories Co. Ltd. (Nagoya, Japan). The anti-flag antibody (M2) was from Sigma Chemical Co (St. Louis, MO).

### **Western Blot Analysis and Immunoprecipitation**

For immunoprecipitations, 200-500 µg of cell lysates pre-cleared with protein A/G beads (15 µl/sample for 1 hr at 4 °C) were immunoprecipitated with antibodies for cyclin E, Cdk2, Cyclin D1, or p27 for 2 h at 4 °C and washed with lysis buffer and resuspended in sample buffer (63 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol). Total protein lysates or the immunoprecipitated samples were boiled at 100 °C for 3-5 min, resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes. The membranes were incubated in blocking buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20, 0.5% casein) for 10-15 min prior to incubating with the primary antibody (0.5 µg/ml blocking buffer) for 2-4 hr at room temperature or overnight at 4 °C. Bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies and chemiluminescent substrate according to the manufacturer's protocol (ECL, Amersham, Irvine, CA). Equal protein loading was verified by reprobing for actin expression.

## **Cyclin Dependent Kinase Assays**

Histone kinase assays were performed as previously described (70). Briefly, equal amounts of protein (~200 µg) of cell lysates were immunoprecipitated with 200 ng of monoclonal antibodies to cyclin E and protein A/G agarose beads. The immune precipitates were washed three times in the lysis buffer and twice with the kinase buffer (50 mM Tris-HEPES, pH 7.5, 10 mM MgCl) and resuspended in 20 µl of kinase buffer supplemented with 400 µg/ml histones (Sigma type III-SS), 10 µM ATP, 0.5 mM DTT, 0.5 mM EGTA, and 5 µCi [ $\gamma$ 32P]ATP (7000 Ci/mmol) for 30 min at room temperature. The assay was stopped with 20 µl of sample buffer, and the reaction products separated on SDS-PAGE gels followed by autoradiography using Kodak XAR film.

## **Inhibition Analysis**

The inhibition experiments (modified from (71, 72)) were performed by incubating active cyclin E/Cdk2 complexes with boiled MCF-7 cell lysates. Cyclin E protein immunoprecipitated from 200 µg of mid-G1 (9-12 hr, zearalenone treated) MCF-7 lysates were immobilized on protein G-sepharose beads. 200 µg of similarly treated, total lysates were subjected to boiling at 100 °C for 5 min and clarified by centrifugation. For serial depletion of the inhibitor activity, boiled and clarified lysates were incubated with 5 µl (200 µg/ml) of p27 and/or p21 antibodies and 10 µl of protein A/G beads for 2 hr at 4 °C. This procedure was repeated three times. The cyclin E immunoprecipitates (Ips)-bound

to protein G beads was incubated with PBS, 9-12 hr MCF-7 lysates, boiled lysates, or boiled/CKI depleted lysates (for 30 min at 30 °C). The beads were washed several times with the lysis buffer and kinase assay was performed with HH1 as substrate. The Cdk2 activity from cyclin E Ips treated with PBS was approximately 10-20% more than that in the Ips mixed with lysates as shown in lane 1 of figure 5. This decrease is probably due to the presence of free CKI in the 9-12 hr treated lysates, which associate with the bead-bound cyclin E/Cdk2.

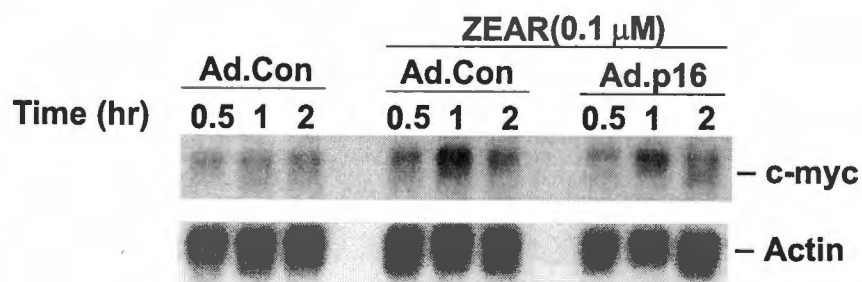
### **Adenoviral Infections**

The Replication-defective adenoviral vector for expression of p16 (Ad p16) was kindly given by P. Seth (73). The control adenovirus was derived by homologous recombination in human embryonic kidney 293 cells (ATCC CRL1573). The adenoviral backbone (Ad type 5, 9.24-100mu) was co-transfected by calcium phosphate precipitation with the shuttle vector pCC2 (73). The viral vectors were propagated in 293 cells and tittered by a standard plaque assay. For experiments 60-70% confluent MCF-7 cells were infected for 1-2 hr with the adenoviral vector at an M.O.I. of 60 p.f.u./cell, in DMEM/0.1% FBS (phenol red-free). The infection medium was replaced with phenol red-free DMEM/0.2% FBS and 10 nM ICI 182,780 and the cells were growth arrested for further 48 hr before treatment and harvest.

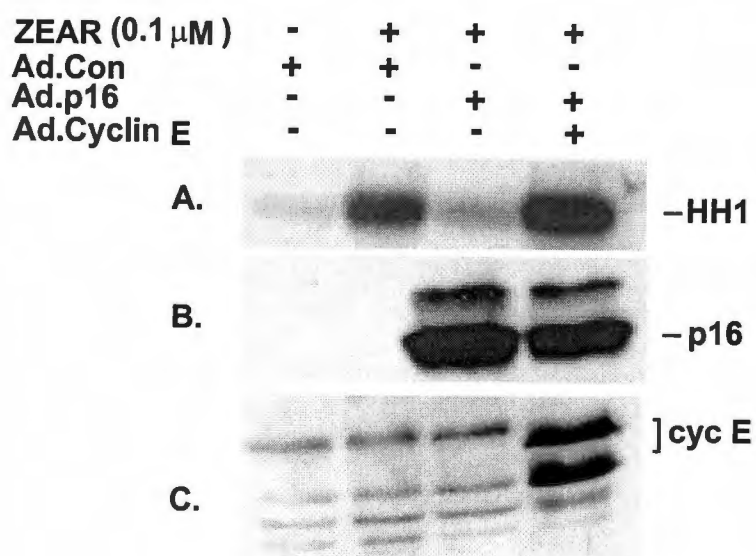


## **Cell Cycle analysis**

MCF-7 cells co-transfected with pEGFP, control vector or the expression vectors for p16, HA-p27, or Cdk4 (dn) were trypsinized at the indicated times and fixed overnight in ice-cold 70% ethanol. Prior to flow cytometric analysis cells were washed with PBS to remove the ethanol and treated with 100 µg/ml RNase A and 50 µg/ml propidium iodide for 30 min. The stained cells were analyzed with a Becton-Dickinson FACScan (Becton-Dickinson, San Jose, CA) with a minimum of 15000 events collected for analysis using a Becton-Dickinson cell Quest software.

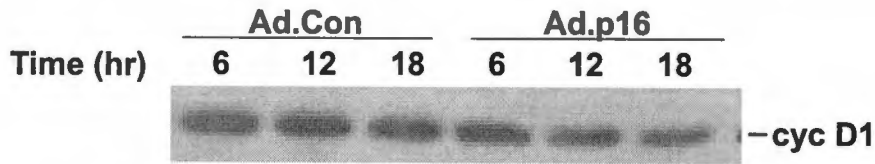


**Appendix B Figure 1. p16 does not affect the c-myc expression.** Cells infected with Ad.Con or Ad.p16 were growth arrested and treated with zearalenone (ZEAR). At the indicated times c-myc mRNA levels were analyzed by Northern blotting. The Actin mRNA is demonstrated for loading control.

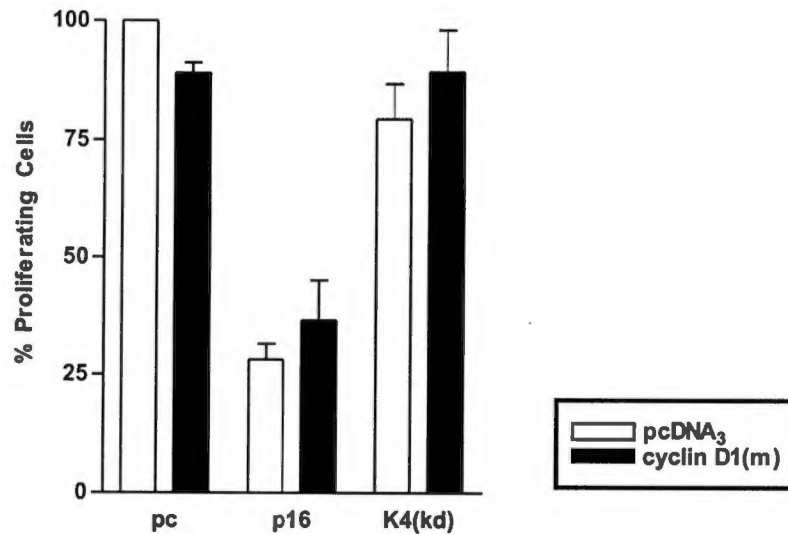


**Appendix B Figure 2. Overexpressed cyclin E prevents the p16-induced abrogation of the cyclin E-associated kinase activity.** MCF-7 cells infected with p16 (Ad.p16), cyclin E (Ad.Cyclin E) or an empty vector (Ad.Con) were growth-arrested and treated as previously. The cells were harvested by 12 hr and the cyclin E-associated kinase activity measured with HH1 as a substrate. The levels of p16 and cyclin E were analyzed by Western blotting and demonstrated as controls.

**A.**



**B.**



**Appendix B Figure 3. Overexpression of cyclin D1 fails to rescue cells from p16-induced arrest.** (A) MCF-7 cells infected with Ad.Con or Ad.p16 were treated with 0.1  $\mu$ M zearalenone and at the indicated times the levels of cyclin D1 protein was measured. (B) Cells were co-transfected with pEGFP and/or expression vectors for a control vector (pcDNA3), p16 (p16), a kinase-dead Cdk4 [Cdk4(kd)] in the presence or absence of a stable mutant of cyclin D1 [cyclin D1(m)]. Growth arrested and treated cells were analyzed by flow cytometry for DNA levels of the green population and the percentage of proliferating cells was measured. The mean of 3 independent experiments is demonstrated graphically.

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**PART IV**  
**GENERAL DISCUSSION**

Globally breast cancer is a very important disease of women. In terms of number of new cases, breast cancer is the third most frequent cancer in the world and the most common cancer, affecting women. It is also the leading cause of cancer mortality in women. The steady increase in breast cancer incidence over the past few years has been attributed to exposure of xenoestrogenic compounds in the environment. The fungal mycoestrogen, zearalenone, has wide variety of hyperestrogenic effects in mammals, including induction of spontaneous mammary tumors. Previous observations indicate that zearalenone is a potent natural xenoestrogen and can induce growth of ER positive breast carcinoma cell lines. In an attempt elucidate the potential role of zearalenone in the promotion of breast carcinomas, I studied the effects of this mycoestrogen on the growth of MCF-7 cells and examined the molecular mechanism of zearalenone-induced G1 progression.

## **THE ROLE OF ZEARALENONE IN PROLIFERATION OF MCF-7 CELLS**

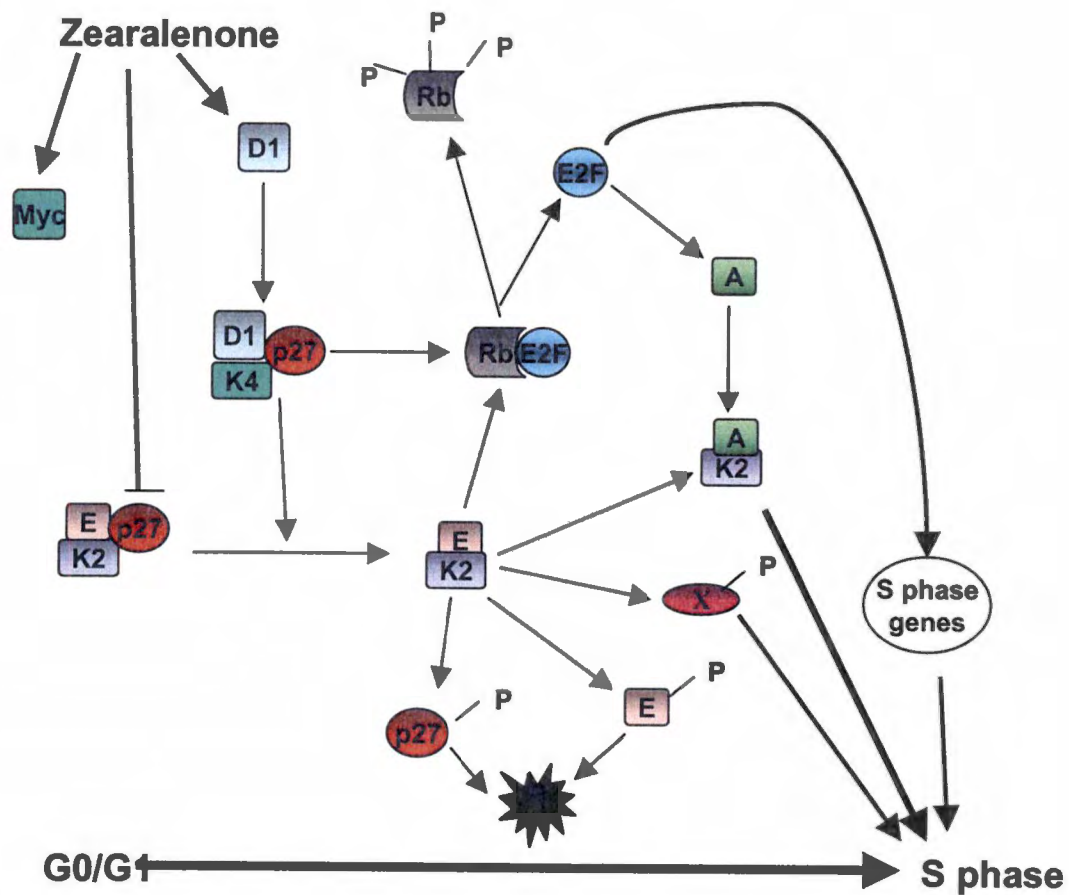
Data from part II demonstrate that treatment of MCF-7 cells with zearalenone results in an increase in cell numbers, in the absence of other mitogens. These observations also suggest that zearalenone may promote breast cell carcinogenesis by inducing breast cell growth and reducing programmed cell death. Zearalenone appears to induce cell cycle transit in MCF-7 cells by inducing the expression of c-myc and cyclin D1. This results in formation of ternary complexes between D cyclins, Cdk4 and CKI, and in activation of cyclin E/Cdk2

complexes. Cyclin E/Cdk2 activity may contribute to the downregulation of p27, and phosphorylation of Rb, both of which appear to be essential for S phase entry and DNA synthesis (Figure 1).

## **DIRECTIONS FOR FURTHER RESEARCH**

### **The Role of Myc in zearalenone-induced G1 transit**

Results of my study have raised several questions, especially regarding the role of Myc in zearalenone-induced G1 transit. Zearalenone induces c-myc expression within an hour of treatment. Myc is reported to be a key regulator of cell proliferation and apoptosis (1, 2). Ectopic expression of Myc induces S phase entry in quiescent cells (3). Activation of cyclin E/Cdk2 is regarded as an essential event in Myc induced G/S progression and number of observations suggests that Myc directly controls the activity of the cyclin E/Cdk2 kinase in mammalian cells. In resting RAT1 fibroblasts induction of Myc resulted in rapid activation of the cyclin E-associated kinase activity, by triggering a change in the molecular weight of cyclin E/Cdk2 complexes (4). Other studies have reported that Myc induces loss of p27 from cyclin E/Cdk2 complexes with a concomitant activation of the kinase activity (5) This loss of p27 from Cdk2 complexes was facilitated by phosphorylation of p27 by the Cdk2 kinase, and resulted in degradation of p27 in a process, which was sensitive to inhibitors of proteasome function



**Figure 1. Molecular mechanism of G1/S transit induced by zearalenone in MCF-7 cells.** Treatment of growth-arrested MCF-7 cells with zearalenone is followed by increased expression of Myc, cyclin D1, and downregulation of p27. The resultant activation of the cyclin E/Cdk2 complexes together with phosphorylation and inactivation of Rb, leads to S phase entry and cell proliferation.

One way by which Myc induces cyclin E/Cdk2 complexes is by causing the inactivation of the CKI p27 through the induction of a p27-sequestering protein(s) (4, 6). Vlach et al. demonstrated that constitutive Myc expression abrogates the growth-suppressive function of p27, and this effect does not require ubiquitin-mediated degradation of p27 (6). Other reports suggest that Myc induces cyclins D1 and/or D2 protein synthesis, which results in the inhibition of CKI binding to cyclin E/Cdk2 complexes in RAT1 fibroblasts (7). Consistent with this observation, primary cells from cyclin D1  $-/-$  and cyclin D2  $-/-$  mouse embryos, unlike wild type controls, do not respond to Myc with increased proliferation (8). In these cells Myc sensitivity could be restored by overexpression of D cyclins, suggesting the sequestration function of D cyclins is essential for Myc-induced cell cycle progression. Also in the c-myc  $-/-$  cells a 12 fold reduction in the activity of D cyclin-Cdk4/6 have been reported as well as delayed induction of cyclins E and A-associated kinase activity.

To examine the role of Myc in zearalenone-induced cell cycle transit several experimental techniques could be used. The first step would be to demonstrate that blocking transactivation function of the Myc protein arrests zearalenone-induced G1/S transit in MCF-7 cells. A MadMyc (MM) chimera is available which behaves as a dominant-negative protein in that this protein binds the E box sequence (CACGTG) and actively represses Myc target genes (9). This MM chimeric protein, the Myc amino acids 1-263, comprising the highly conserved N-terminal domains necessary for Myc-dependent transactivation, transrepression, and biological activities are substituted for the transcriptional



repression domain of Mad. Expression of MM in cycling NIH3T3 cells caused G1 arrest concomitant by reduction in cyclin E/Cdk2 activity (9). Co-expression of the cyclin D1 protein with the MM chimeric protein in zearalenone-treated cells might rescue these cells from G1 arrest. This will confirm that inducing D cyclin expression is a major step in Myc induction of cyclin E/Cdk2 activity. To further confirm this observation MM could be co-transfected with the EGFP protein and sorted for the green fluorescent marked cells. The levels of cyclin D1 protein (Westerns) and message (Ribonuclease Protection Assays) in control vector vs. MM vector transfected cells could be compared. If these observations do confirm that there is a difference in cyclin D1 expression in MM arrested cells this suggests that induction of D cyclins and the resultant CKI titration is one way by which Myc Induces cyclin E/Cdk2 activity in zearalenone-treated cells. This could also explain the observation in p16-overexpressed cells where Myc is induced by zearalenone (Appendix B, Figure 1), but fails to prevent the G1 arrest caused by p16. By preventing complex formation between D cyclins and its catalytic partners p16 blocks the sequestration of CKI. Therefore, the downstream regulatory step of Myc in activating cyclin E/Cdk2 is also blocked by p16.

Another way by which Myc may activate the cyclin E/Cdk2 enzyme is by inducing the expression of the Cdc25A phosphatase. Myc has been reported to transactivate the Cdc25A via an E box in the Cdc25A gene (10). Stimulation of Cdc25A expression may increase cyclin E/Cdk2 activity and indirectly phosphorylate p27, which leads to its degradation. Previous observations indicate that Cdc25A message and protein are induced by E2 in MCF-7 cells (Foster JS

manuscript submitted) Further, antisense Cdc25A oligonucleotides inhibit E2-induced Cdk2 activation and DNA synthesis (Foster JS manuscript submitted). These observations indicate that Cdc25A may have an *in vivo* role in estrogen-induced G1/S transition. Zearalenone induces c-myc expression very early in the cell cycle (part B, figure 3 A&B) and indirectly via Myc could lead to induction of Cdc25A. The MM chimera could be used here too, to demonstrate that in MM arrested cells, the zearalenone-induced Cdc25A message and protein is reduced. However, the analysis of Cdc25a levels in MM arrested cells may be complicated because Cdc25A is also transcriptionally elevated by E2F1 (11). It is reported that E2 can induce E2F1 expression by direct transactivation of the promoter (12). On the other hand, the E2F1 gene also has E2F response elements upstream to the promoter start site, which could be transcriptionally repressed by p130-E2F4 (13). If in the MM-arrested cells most of the E2F-1 is tethered to pocket proteins, the repressive function of the sequestered E2F family proteins, may override the enhancer activity of estrogens at the promoter. This will result in repression of E2F synthesis even in the presence of estrogens. This point could be further clarified by examining the E2F1 levels in p16 overexpressing MCF-7 cells. In presence of p16 Rb is in the hypophosphorylated state (part III, figure 6D) and therefore most of the E2F would be tethered. Similarly, a constitutively active Rb mutant that could not be phosphorylated due to phosphorylation site mutations (14) and will tie up all free E2F could be used instead. If the repression by the pocket protein bound E2F family members takes upper hand the E2F-1 protein

levels in zearalenone-treated control cells will be greater than in the p16-arrested cells or cells transfected with the mutant Rb

Myc by inducing D cyclin levels and thereby sequestration of CKI from functionally inactive cyclin E/Cdk2 complexes, could lead to the formation of a small pool of active cyclin E/Cdk2 complexes. In turn, the active cyclin E/Cdk2 enzyme could phosphorylate and activate the Myc-induced Cdc25A protein, which could tip the balance towards further activation of cyclin E/Cdk2, which can phosphorylate p27, triggering them for destruction and amplifying the initial signal. Inactive cyclin E/Cdk2 complexes in p16-arrested cells could be activated by recombinant GST-Cdc25A, *in vitro*, suggesting that in the p16 overexpressing cells, the Cdc25A activity is limiting (data not shown). However, the Cdc25A message and protein levels are not affected in these cells (Foster JS manuscript submitted). Further the inactive cyclin E/Cdk2 complexes in the p16-arrested cells could be activated *in vivo* in Cdc25A overexpressing cells (Foster JS manuscript submitted). This implies a positive feedback loop between cyclin E/Cdk2 and Cdc25A similar to that between cyclin B/Cdk1 and Cdc25C (15). These observations validate the hypothesis that Myc induction of D cyclin-CKI sequestration function and the positive Cdc25A/Cdk2-cyclin E loop, work in concert in zearalenone treated MCF-7 cells to activate cyclin E-associated kinase activity (figure 2).



## **The Role of Cyclin E in G1/S Transit**

As previously discussed in part I, cyclin E-associated kinase activity appears essential for G1 transition. The mitogen-induced cyclin D1 protein expression as well as complex formation with Cdk4/6 also appears to revolve around CKI sequestration that leads to the activation of cyclin E/Cdk2 complexes. These observations imply that cyclin E-associated kinase activity is the major prerequisite not only for progression through the "R point" and for cells to achieve mitogen independence, but for inducing DNA synthesis also. Our data demonstrates that cyclin E-associated kinase activity peaks by 9-12 hr and falls back to control levels prior to cells entering S phase. However, the total Cdk2 activity, i.e. the cyclin A-associated Cdk2 activity, and the pattern of cyclin A protein expression, suggest that this cyclin plays an important role in cells entering S phase. Further, cyclin A is localized at sites of DNA replication and can promote DNA replication in a cell-free system (16, 17), and overexpression of cyclin A in cells accelerates exit from G1 phase (18, 19). The cyclin E/Cdk2-induced inactivation of Rb results in the transcriptional activation function of E2F/DP family members and gives rise to increased cyclin A expression as well as other proteins essential for DNA synthesis (20, 21). Also, the cyclin E/Cdk2 activity leads to phosphorylation mediated ubiquitination and degradation of p27 (22), which further facilitates the cyclin A-associated kinase activity. Cyclin A/Cdk2 complexes in turn phosphorylate proteins that are essential for firing of the replication origins of DNA, resulting in DNA synthesis (23). The cyclin A/Cdk2 complexes, in a negative regulatory loop, phosphorylate DP-1, which

affects the ability of the E2F/DP-1 heterodimeric transcription factors to bind DNA and cause transactivation of E2F responsive genes (24).

The crystal structures of p27 bound cyclin A/Cdk2 complexes demonstrate that one molecule of p27 can effectively block cyclin A/Cdk2 activity (25), suggesting that downregulation of p27 is extremely important for induction of cyclin A-associated kinase activity. Microinjection of anti-cyclin E antibodies blocks cells in G1 (26), and the two most well studied substrates of the cyclin E/Cdk2 complexes are Rb and p27 (22, 27). Cyclin E is reported to phosphorylate p27 resulting in the ubiquitin-mediated downregulation of this CKI (22). All three pocket protein family members have been demonstrated to form stable complexes with cyclin A/Cdk2 (28-30), implying that the cyclin A bound-Cdk2 enzyme too has the ability to phosphorylate and inactivate Rb. Taken together these facts could suggest that one of the major functions for the active cyclin E/Cdk2 complexes is to facilitate the activation of cyclin A/Cdk2 complexes by late G1. Further, these data could be interpreted as that the activity of cyclin A/Cdk2 complexes and not cyclin E/Cdk2 activity is the major requisite for S phase entry. In cyclin D1 (-/-) mice, the expression of cyclin E from the cyclin D1 promoter rescued most of the morphological defects and rendered the upstream role of cyclin D1 redundant (31). Cyclin E by activating a downstream step in effect completely bypassed the need for cyclin D1. If our hypothesis is valid, overexpression of cyclin A may rescue the G1 arrest induced by anti-cyclin E antibodies, while the reverse may not be true; i.e. overexpression of cyclin E may not be able to override the G1 arrest induced by anti-cyclin A antibodies.

Dominant-negative proteins for cyclins E and A could also be used instead of antibodies for the cyclins. A kinase-dead Cdk2 could be used as a negative control in this instance to demonstrate that overexpression of neither cyclin E nor A could override the G1 arrest induced by this mutant construct. Flow analysis of transfected and growth-arrested cells, treated with estrogens will demonstrate the fraction of cells in different phases of the cycle. These experiments could prove that the major function of cyclin E-associated kinase activity is to induce activation of the cyclin A/Cdk2 complexes, or suggest that other targets of cyclin E/Cdk2 are important for cell proliferation in estrogen-treated MCF-7 cells.

Assuming that the previous experiments suggest that the upstream role of cyclin E/Cdk2 complexes is to induce the activity of cyclin A/Cdk2 in late G1, we could further examine the role of cyclin A in G1/S transit by overexpression of this cyclin in growth-arrested MCF-7 cells. However, cyclin A may not be effective in overriding the high levels of p27 present in these cells. The crystal structure of p27 bound cyclin A/Cdk2 complexes demonstrate that p27 binds a hydrophobic patch on the surface of cyclin A bound to the Cdk monomer (25). This hydrophobic patch on the surface of cyclin A is thought to be essential for the ability of the cyclin A/Cdk2 complex to interact with and phosphorylate proteins with the RXL motif (32). The RXL motif is conserved in both substrates and inhibitors of cyclin-cdks (33, 34). Mutation of the RXL motif in p27 may interfere with the ability of this CKI to bind to and inhibit the enzymatic activity of the cyclin/Cdk complexes. Expression of this mutant p27 with cyclin A should bypass all previous steps and allow the cells to start synthesizing DNA. If the

latter experiment succeeds, it firmly establishes that the sole role of cyclin E in G1 transit is to indirectly, via phosphorylation of Rb, to induce cyclin A expression, which together with the cyclin E-dependent phosphorylation and removal of p27, facilitates the induction of cyclin A/Cdk2 activity.

In mammalian cells one way the periodicity of Cdk activity appears to be controlled is by cyclin expression, which could result in the phosphorylation of different substrates specific for different stages of the cell cycle. It is possible that cyclins affect the Cdk substrate choice by changing the structure of the Cdk substrate-binding site, or by directly binding to the substrate, facilitate phosphorylation by the Cdk. In the case of cyclin E and A, the substrates for the cyclin A/Cdk2 complexes may not be phosphorylated by the cyclin E bound-Cdk2. The cyclin A bound-Cdk2 phosphorylates and inactivates DP-1 while the cyclin E-bound Cdk2 complexes have not been demonstrated to phosphorylate this substrate. Phosphorylation of DP-1 by cyclin E/Cdk2 could shut off the E2F/DP-1 transcriptional unit prematurely, before sufficient cyclin A or other key enzymes of DNA synthesis is expressed. Similarly, firing of the replication origins activated too early in G1 phase by cyclin E/Cdk2 complexes may be detrimental for the cell. Thus it is possible that the mammalian cell has safeguarded against such calamities by ensuring sequential phosphorylation activation/inactivation functions, especially for the Cdk2 enzyme, which appears to be the major kinase essential for progression through both G1 and S phase. By regulating the expression of cyclins in a sequential manner the cell may control the phosphorylation of specific substrates by the Cdk2 enzyme at specific stages



of the cell cycle. This suggests that by inducing cyclin A expression and activity later in G1 phase, the cell may have protected against premature firing of the replication origins, as well a way of shutting off the E2F/DP-1 transcriptional activation unit after its function is done

The reason why the cyclin A bound- and not cyclin E bound-Cdk2 can phosphorylate the E2F/DP complexes could be that the cyclin binding causes conformational changes in the Cdk2 catalytic cleft altering the affinity for a particular substrate. On the other hand it is also possible that cyclin A can form stable interaction with the E2F/DP complex more efficiently than cyclin E, and therefore can act as a better targeting subunit for the cyclin the Cdk2 enzyme. Residues 67-108 of E2F-1 are necessary for binding cyclin A and an E2F-1 mutant which can no longer bind cyclin A/Cdk2 has prolonged DNA binding activity in S phase (24, 35). Mutations of a conserved hydrophobic patch on cyclin A, eliminated the interaction with proteins containing the RXL motif without affecting the binding to Cdk2, and this binding was critical for phosphorylation of these substrates (36). The crystal structure of cyclin A/Cdk2/p27 also revealed that p27 binds this hydrophobic patch and severely impaired the phosphorylation function of cyclin A/Cdk2 (25). These observations suggest that substrate recruitment to Cdk2 by protein-protein interactions, result in high local concentration of substrates, and are critical for efficient phosphorylation by the kinase. The residues that influence substrate binding by cyclin A are also conserved in a number of mammalian cycles, including cyclin E, and may serve as binding sites (36). However, although the hydrophobic patch on

cyclin E can serve as a docking site for E2F-1, its accessibility may be limited by surrounding side chains, or by other unknown sites. The crystal structures of cyclin E/Cdk2 bound to Rb, p27/p21 as well as the structures of cyclin A/Cdk2 bound to various substrates will prove invaluable in determining substrate specificities of cyclins E and A. It is also possible that the crystallographic data will demonstrate that it is not the amount of Cdk2 activity but the targeting cyclin which is essential for S phase entry, i.e. the qualitative model vs. the quantitative model (37).

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## VITA

Shamila Ahamed was born in Kandy, Sri Lanka, on March 19, 1966. She received a Bachelors degree in Veterinary Medicine from the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka, in December, 1993. In August, 1994, she joined the University of Tennessee to pursue the Doctorate of Philosophy in Comparative and Experimental Medicine. She received her Ph D degree in December, 2000.

Shamila is presently working as a Senior Lecturer in Physiology in the Faculty of Medicine, University of Peradeniya, Sri Lanka.