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Cell Cycle Controls: Potential Targets for Chemical Carcinogens?

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The progression of the cell cycle is controlled by the action of both positive and negative growth regulators. The key players in this activity include a family of cyclins and cyclin-dependent kinases, which are themselves regulated by other kinases and phosphatases. Maintenance of balanced cell cycle controls may be directly linked to genomic stability. Loss of the checkpoints involved in cell cycle control may result in unrepaired DNA damage during DNA synthesis or mitosis leading to genetic mutations and contributing to carcinogenesis.

This overview provides a general review of cell cycle control and describes how chemicals may interfere with these controls, leading to neoplastic development. For us to discuss mechanisms of cell cycle control, the cell cycle must first be defined. The most basic cell cycle, which exists in the cleavage stage of frog embryos, consists of only two phases, DNA synthesis (S phase) and mitosis (M phase) or cell division (*1*). This cell cycle lacks two additional phases or gaps (G_1 and G_2) observed in more complex cycles in adult cells and most embryonic cells. The G_1 and G_2 gaps, intervening between the M- and the S-phase, allow growth control points to regulate cell size and cell number and to monitor the cell's environment for nutrients and growth signals (*2,3*). The existence of G_1 and G_2 phases also allows the cell to insure that certain intracellular events are completed before the cell progresses to the next phase of the cell cycle (*4*). For example, DNA replication and chromosome segregation must be completed before a cell continues through the cell cycle. These controls have been referred to as cell cycle checkpoints (*4*). Several G_1 checkpoints have been proposed for mammalian fibroblasts (*5*). The most studied of these is the R, or restriction, point (*6*). Cells that do not have sufficient nutrients may arrest in late G_1 at this point. It has been proposed that critical proteins must accumulate to a certain level before a cell can pass the R point to enter S-phase and that some cancer cells may stabilize these proteins and therefore override these checkpoints. This may lead to infidelity in replication and provide a partial explanation for the observance of the high level of chromosome aberrations in cancer cells (*4*).

Cell Cycle Control Proteins

Several classes of proteins are important in cell cycle control. Progression through the cell cycle depends on the action of a family of kinases known as cyclin-dependent kinases (cdk) and the interaction of these kinases with another class of proteins called cyclins. The activity of these complexes in turn appears to be regulated by various phosphatases and kinases.

The first member of the cdk family identified was the *S. pombe cdc2* gene (Table 1). Cell division cycle (cdc) mutants in yeast have mutations in specific genes involved in cell cycle progression. Conditional mutations of these genes results in cell arrest at very specific points of the cell cycle when the mutants are placed under restrictive conditions (*7*). Study of these mutants led to the cloning of the *cdc2* gene, which codes for a protein (*8*) that is a key regulator of the cell cycle in eukaryotic cells including yeast and human cells. In fact, the human *cdc2* gene was cloned by functional complementation of the yeast *cdc2* mutant by human cDNA (*9*), indicating that this gene is highly conserved between yeast and humans. In addition, the *cdc2* gene has been cloned in mouse (*10*) and several other species (*11–13*). The conservation of this gene suggests that it plays an important, fundamental role in growth and division. The cdc2 protein ($p34^{cdc2}$) is a serine-

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Table 1. Characteristics of $p34^{cdc2}$.

$p34^{cdc2}$ is required for G_1/S and G_2/M transitions in the cell cycle.
Protein levels of $p34^{cdc2}$ are usually constant during the cell cycle but activity is periodic.
cdc2 protein is the 34 Kd catalytic subunit of a serine-threonine protein kinase complex.
Kinase activity is regulated by protein–protein interactions, particularly with different members of the cyclin family, and also by phosphorylation and dephosphorylation.
Kinase phosphorylates a number of substrates that are possibly involved in regulation of specific events in the cell cycle.
This protein kinase complex is responsible for M-phase-specific histone H-1 kinase activity.
Homologous to important cell control gene, CDC28, of the budding yeast *Saccharomyces cerevisiase*, which was isolated as a mutation that arrested cells at "start" in G_1.
Start defines a central control point in yeast at which the cell decides to continue to grow and divide, to enter into stationary phase, or to mate. This is the first point in the cell cycle under genetic control in *S. cerevisiae*.
cdc2 is a member of a family of genes that are cyclin-dependent kinases (cdk).
$CDC2^{Hs}$ was cloned by complementation of cdc2 ts mutant in fission yeast (*S. pombe*) with human cDNA.

threonine kinase that is constitutively expressed in dividing cells but is down-regulated when cells exit the cell cycle, such as in quiescence, senescence, and differentiation (*14–18*). $p34^{cdc2}$ is required for both S- and M-phase progression (*19–22*). Protein levels are constant during the cell cycle; however, the kinase activity is regulated by interaction of $p34^{cdc2}$ with proteins as well as by phosphorylation (*23–25*). In addition, recent data suggest that there is some regulation at the level of transcription (*26,27*).

Cyclins are proteins that bind cdks and modulate their function (*28*) (Table 2). Cyclins were first identified as proteins whose levels fluctuate during the cell cycle. These proteins share sequence homology in a region known as the cyclin box. Cyclin activity is generally controlled at the level of protein expression since the proteins are synthesized and degraded very rapidly at specific times during the cell cycle (*29,30*). Multiple cyclins are present in the cell and appear to function at different stages of the cell cycle. For example, cyclin B binds $p34^{cdc2}$ at G_2/M and is required for its activation as a mitotic kinase complex (*31*). Cyclin A is expressed earlier than cyclin B in the cell cycle and is probably involved in regulation of S phase (*32*). Other cyclins (cyclins C, D, and E) are involved in the G_1 phase of the cell cycle.

The kinase activation of $p34^{cdc2}$ is subject to negative control by phosphorylation on tyrosine 15 and dephosphorylation of this site is required for activation (*33*). The phosphorylation state of $p34^{cdc2}$ fluctuates through the cell cycle (*34*). Two yeast gene products, the wee1 kinase (*35–37*) and the cdc25 phosphatase (*38,39*), are responsible in part for this regulation. cdc25 is believed to be the factor responsible for initiation of mitosis, which is dependent upon completion of DNA replication. $p80^{cdc25}$ is the tyrosine phosphatase that activates $p34^{cdc2}$ by dephosphorylation of the tyrosine 15 residue of $p34^{cdc2}$ when it is complexed to cyclin B (*39,40–42*). Several cdc25 genes have been identified, suggesting that a family of these proteins exists and association between cyclin B and cdc25 has been observed (*43,44*). Therefore, one function of cyclin B may be to target $p80^{cdc25}$ to $p34^{cdc2}$ for G_2/M activation. The $wee1^+$ gene product negatively regulates entry into mitosis (*36*). The $p107^{wee1+}$ protein is a dual function kinase that phosphorylates serine, threonine, as well as tyrosine residues (*45*). $Wee1^+$ and a related gene product, mik1, are responsible for phosphorylating $p34^{cdc2}$ on tyrosine 15, thereby inactivating it (*35,37*). Analysis of yeast wee1 mutants that have lost cdc25 control have lost mitotic dependency on completion of DNA replication (*46*). The gene was first cloned in fission yeast and a human wee1-like gene has been cloned by complementation of human cDNA into a yeast mutant (*47*).

In addition to $p107^{wee1}$ and $p80^{cdc25}$, activated $p34^{cdc2}$ binds a protein of unknown function, $p13^{suc1}$ (*48*). It is known, however, that binding of $p34^{cdc2}$ to $p13^{suc1}$ is required for $p34^{cdc2}$ activity (*49,50*). It has been proposed that $p13^{suc1}$ may act as a facilitator of the formation or localization of the $p34^{cdc2}$ kinase complex (*49*).

The expression of the $wee1^+$, suc1, cyclins A and B as well as cdc25 homologs in human cells suggest that not only is the structure of cdc2 conserved across species, but also that its regulaton is conserved, further indicating that $p34^{cdc2}$ plays a basic and important role in growth control. Very little is known about the *in vivo* functions of the cdc2/cyclin complexes; however, it has been shown that this kinase is involved in the breakdown of the nuclear envelope during mitosis (*51,52*).

Table 2. Characteristics of cyclin proteins

Identified in marine invertebrates as two proteins (cyclins A and B) whose abundance oscillates in early invertebrate embryonic cell cycles and regulate G_2/M transition.
A family of cyclins exists that regulates progression through the cell cycle.
Cyclin A is required for two points in the cell cycle, S-phase and G_2/M phase.
Different cyclins (G_1 cyclins) regulate the G_1/S transition in yeast (CLN1-3), and at least 5 proteins (cyclins C, D1-3, and E) are identified as candidate G_1 cyclins in mammalian cells.
There are also other cyclins involved in mitosis in yeast (e.g., MCS).
Cyclins combine with $p34^{cdc2}$ (and other cdk proteins) to form an active cdc2 kinase
Cyclins are involved in regulation of phosphorylation/dephosphorylation of $p34^{cdc2}$.
Cyclins are degraded rapidly at specific times in the cell cycle by proteolysis mediated by the ubiquitin pathway.
Cyclins are altered in certain cancer cells.

Studies of cell-free extracts show that $p34^{cdc2}$ may be involved in complex formation at the replication origin prior to intiation of DNA synthesis (*53*). Several proteins have been identified as substrates for the $p34^{cdc2}$ kinase. These include the retinoblastoma protein (*54,56*), nucleolar proteins (*57*), *c*-src (*58*), histone H1 (*59,60*) and other proteins (*61*).

Other cyclin-dependent kinases have been described. Human cdk2 was discovered as a target for binding by the E1A protein of a DNA tumor virus. The $p33^{cdk2}$ protein, like $p34^{cdc2}$, has protein kinase activity and binds cyclin A (*62*). It also binds G_1 cyclins, cyclin E (*63*) and possibly cyclin D (*28*). Its kinase activity peaks in late G_1 or early S phase indicating that it plays an important role at a point earlier in the cell cycle than $p34^{cdc2}$ (*64*). In addition, cdk2 is part of a complex formed with the transcription factor, E2F, indicating its kinase activity may be important in gene regulation (*65,66*). Pines and Hunter have proposed that the functions of cdks are critical for the eukaryotic cell cycle and are required to traverse checkpoints (*28*).

Cyclin A association with $p33^{cdk2}$ has been shown to be required for entry into DNA synthesis in mammalian cells (*67,68*). In addition, several new cyclins that appear to play a role in G_1 have been cloned. Human cyclin D_1 was cloned for its ability to complement a yeast deficient in a G_1 cyclin function and also as a gene induced late in G_1 in growth factor (CSF-1) stimulated mouse macrophages (*69,70*). This gene is the same as the *PRADI* oncogene that is overexpressed in parathyroid tumors (*71*). Cyclins C and E are two other cylin molecules expressed during G_1 (*72,73*). Cyclin E protein is associated with a histone kinase activity that is most likely derived from its interaction with $p33^{cdk2}$ (*74*). Although the exact functions of these different G_1 cyclin/kinase complexes are unknown, the nature of their cycle-dependent expression indicates their importance in the G_1/S transition.

Cell Cycle Checkpoints and Perturbations

While the functions of these cell cycle control proteins are just beginning to be understood, perturbations of these controls are already being observed during abnormal growth states such as transformation. For example, cyclin A and cdk2 are both targets for binding by DNA tumor viral protein E1A (*62*). In addition, the hepatitis B virus is integrated into the cyclin A gene in a hepatocellular carcinoma (*75*). As previously mentioned, cyclin D_1 is overexpressed in parathyroid tumors (*71*). Several cyclins have been shown to be overexpressed in breast cancer (*76*).

However, these control proteins are not the only potential targets for carcinogens or tumor promoters. In addition to cell cycle control proteins involved in normal cell cycle progression, there are other proteins that are important in the regulation of cell cycle checkpoints in response to agents that damage DNA or perturb the cell cycle. For example, the *RAD 9* gene of *S. cerevisiae* is responsible for arresting cells after DNA damage by X-irradiation. The *RAD 9* gene is not required for cell growth, but *RAD 9* mutants fail to arrest after treatment with radiation and therefore have no time to repair DNA damage (*4*). In addition to rad 9, the wee1 kinase has been shown to be required for mitotic delay after irradiation (*77*). Therefore, mutation of this gene not only perturbs normal cell cycle progression but also makes a cell more susceptible to radiation-induced damage. Recently, it has been shown in human cells that p53 protein levels increase in response to radiation damage (*78,79*). This leads to the hypothesis that p53 may be acting similarly to rad 9 as a checkpoint in order to inhibit cell division until repair has occurred. However, p53 is involved in a G_1 checkpoint whereas RAD 9 is a G_2 checkpoint. Loss of this checkpoint may lead to an increase in genetic instability (*80*).

In addition to aberrations in repair after exogenous damage, loss of cell cycle checkpoints can increase the rate of "spontaneous" mutations. For example, RAD 9 mutants in yeast have a 21-fold elevated rate of chromosome loss (*81*) and mutant p53 human and mouse cells have a several hundredfold elevation in the rate of gene amplification (*80,82*). Increased genetic instability may also result from chemical treatments that block the action of proteins involved in checkpoints. For example, caffeine blocks upregulation of p53 protein in irradiated cells and prevents radiation-induced G_1 growth arrest (*83*). Okadaic acid, a tumor promoter, inhibits phosphatases that regulate G_2/M checkpoints and can induce mitotic abnormalities (*84–85*). Chemical carcinogens may also mutate checkpoint genes, and loss of these protein functions might predispose a cell to successive mutational events. This is consistent with a model in which the occurrence of one mutation in a cell increases susceptibility for a second mutation. Neoplastic development is a multistep process requiring at least four to five distinct steps (*86*). Since the probability that a cell will acquire multiple defects is low, the existence of predisposing mutations may explain the ontogeny of many adult cancers.

Role of Cell Proliferation in Carcinogenesis

Cell proliferation can influence carcinogenesis by various mechanisms (Table 3). This has led to the hypothesis that cell proliferation itself may be carcinogenic and carcinogens that increase cell proliferation may be operating exclusively by this mechanism. The failure to detect a measurable mutagenic activity associated with nongenotoxic carcinogens indicates that these chemicals may act by alternative mechanisms of action, increasing cell proliferation being one possibility. This hypothesis is supported by the fact that in some species many types of cancers may arise spontaneously. Normal cell division results in a low level of

Table 3. Mechanisms by which chemicals affecting cell proliferation might influence carcinogenesis.

Increase fixation and expression of premutagenic DNA lesions.
Increase the number of initiated cells occuring spontaneously during cell division.
Increase the number of spontaneous initiated cells by blocking cell death or elimination.
Increase the number of initiated cells by pertubing checkpoints in the cell cycle leading to mutagenic events.
Increase the rate of neoplastic progression by mechanisms 1–4.
Promote clonal expansion of initiated cells.

spontaneous errors during DNA replication, and spontaneous DNA damage can result from cytosine deamination at physiological temperatures, from oxidative damage associated with normal cellular physiology, and from mutagens in food, air, or water (*87*). Thus, mutations occur "spontaneously" from normal cellular processes. There are risk factors for human cancers (e.g., hormones) that also influence the rate of cell proliferation in target tissue (*88*). However, mechanisms in addition to cell proliferation should be considered for these risk factors.

Before cell proliferation can be accepted as the causative mechanism for certain carcinogens, several facts should be considered. First, many toxic and/or hyperplastic stimuli are not carcinogenic (*89–91*). Second, cell division occurs frequently in all organisms; therefore, it is not clear whether cell division is limiting in the carcinogenic process. This, of course, depends on the target tissue. Furthermore, cell division of initiated or intermediate cells may occur at quite different rates than division of normal cells. Finally, the observation that multiple mutations are involved in the development of many neoplasms may suggest that even a weak mutagenic response, which is below the level of detection of current assays, is sufficient to influence the neoplastic process in a specific target tissue. This is a plausible explanation for certain nongenotoxic carcinogens, some of which may act by indirect mutagenic processes.

Conclusion

The cell cycle is controlled by a network of proteins whose activity is intricately regulated. DNA synthesis and cell division are tightly coupled to these controls. The observations presented here support the hypothesis that growth arrest points exist to control genetic fidelity and stability. Disruption of growth arrest checkpoints by mutation or by chemical treatment may lead to increased cell growth and genetic instability (Table 3). Finally, chemicals that induce cell proliferation and genetic instability by interfering in regulatory checkpoints, thus disturbing the cell's process of "checks and balances," are more likely to cause cancer than chemicals that are only mitogenic.

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