Cell Cycle Control, Checkpoint Mechanisms, and Genotoxic Stress

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The ability of cells to maintain genomic integrity is vital for cell survival and proliferation. Lack of fidelity in DNA replication and maintenance can result in deleterious mutations leading to cell death or, in multicellular organisms, cancer. The purpose of this review is to discuss the known signal transduction pathways that regulate cell cycle progression and the mechanisms cells employ to insure DNA stability in the face of genotoxic stress. In particular, we focus on mammalian cell cycle checkpoint functions, their role in maintaining DNA stability during the cell cycle following exposure to genotoxic agents, and the gene products that act in checkpoint function signal transduction cascades. Key transitions in the cell cycle are regulated by the activities of various protein kinase complexes composed of cyclin and cyclin-dependent kinase (Cdk) molecules. Surveillance control mechanisms that check to ensure proper completion of early events and cellular integrity before initiation of subsequent events in cell cycle progression are referred to as cell cycle checkpoints and can generate a transient delay that provides the cell more time to repair damage before progressing to the next phase of the cycle. A variety of cellular responses are elicited that function in checkpoint signaling to inhibit cyclin/Cdk activities. These responses include the p53-dependent and p53-independent induction of Cdk inhibitors and the p53-independent inhibitory phosphorylation of Cdk molecules themselves. Eliciting proper G1, S, and G2 checkpoint responses to double-strand DNA breaks requires the function of the *Ataxia telangiectasia* mutated gene product. Several human heritable cancer-prone syndromes known to alter DNA stability have been found to have defects in checkpoint surveillance pathways. Exposures to several common sources of genotoxic stress, including oxidative stress, ionizing radiation, UV radiation, and the genotoxic compound benzo[*a*]pyrene, elicit cell cycle checkpoint responses that show both similarities and differences in their molecular signaling. — *Environ Health Perspect* 107(Suppl 1):5–24 (1999). *http://ehpnet1.niehs.nih.gov/docs/1999/Suppl-1/5-24shackelford/abstract.html*

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Biology of the Cell Cycle

The development of microscopy in the seventeenth century allowed early microscopists to examine a large number of protozoa, bacteria, molds, animal cells, and other "animalcules" for the first time (*1,2*). With the development of cell theory, and improvements in microscopy and sample preparation in the nineteenth century, the study of cell division became possible. Early examinations of cell division were limited to the observation that cells increased in size from the completion of one cell division or mitosis (M phase) to the initiation of the next. The period between mitoses was termed interphase (*3*). Later, DNA replication was found to occur at a discrete time during interphase, termed DNA synthesis phase or S phase (*4,5*). The period between mitosis and the subsequent S phase was termed Gap 1 (G_1), while the period between S phase and the following mitosis was termed Gap 2 (G_2). Thus the cell cycle was divided into four major phases (*3,6,7*). Cells in a metabolically active state but not progressing to, or through DNA synthesis or cell division, were said to be quiescent or resting (G_0). In the typical dividing eukaryotic cell, G_1 phase lasts approximately 12 hr, S phase 6 to 8 hr, G_2 phase 3 to 6 hr, and mitosis about 30 min, although the exact length of each phase varies with cell type and growth conditions (Figure 1) (*6,8*).

The description of the cell cycle being divided into four phases led to many questions about the regulatory mechanisms cells employ to ensure an ordered and sequential progression from G_1 to M phase, as well as the mechanisms ensuring DNA stability. Some of these questions were summarized as the "completion" and "alternation" problems (*8,9*). In the completion problem, the question is raised as to how cells ensure that specific events are completed before subsequent events are initiated. For example, cells must ensure that once DNA is condensed for segregation during cytokinesis, it remains condensed throughout M phase and does not prematurely decondense. In the alternation problem, the question is raised as to how cells ensure that once an event is completed, it is not inappropriately repeated. For example, cells must ensure that once DNA replication in S phase is completed, it is followed by DNA condensation and not by another round of replication.

Insight into the completion and alternation problems came from cell fusion experiments carried out by Rao and Johnson (*10,11*). When S phase cells were fused to G_1 or G_2 cells, the G_1 cells began premature DNA replication, but the G_2 cells did not re-replicate their DNA. Also when S phase cells were fused with G_1 cells, the resulting cell fusion did not enter M phase until the G_1 nuclei had completed DNA replication. These results indicated that *a*) S phase cells contain an S phase-promoting factor (SPF) activity that is *trans*-dominant acting on G_1 cells but not on G_2 cells, *b*) G_2 cells contain a block that prevents SPF from initiating DNA replication in G_2 cells, and *c*) S phase cells contain a feedback control factor that prevents the initiation of M phase until DNA replication is complete. In other experiments, fusion of M phase cells with G_1, S, or G_2 cells resulted in interphase nuclear membrane breakdown and in chromosome condensation, demonstrating that M phase cells carried a *trans*-dominant M phase-promoting factor (MPF) activity.

Another important question in understanding cell cycle biology deals with the ability of cells to pause transiently during the cell cycle in response to agents that cause damage, particularly to DNA. Surveillance control mechanisms that check to ensure proper completion of early events

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Abbreviations used: ATM, *Ataxia telangiectasia* mutated; B[*a*]P, benzo[*a*]pyrene; Cdk, cyclin-dependent kinase; IR, ionizing radiation; MMS, methyl methanesulfonate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MPF, mitosis-promoting factor; pRB, retinoblastoma protein; SPF, S phase-promoting factor; UV, ultraviolet.

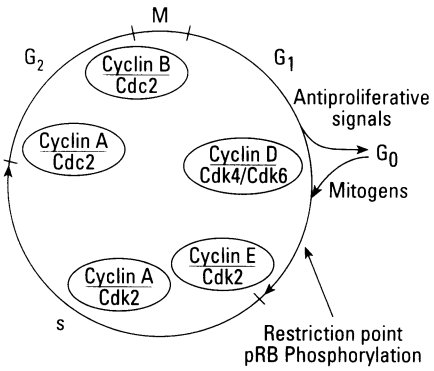


Figure 1. Schematic representation of Cyc/Cdk protein complexes and the cell cycle.

and cellular integrity before initiation of subsequent events in cell cycle progression are referred to as cell cycle checkpoints and can cause a transient delay that has been suggested to allow the cell more time to repair damage before progressing to the next phase of the cycle [for reviews, see (*12,13*)]. Alternatively, if the damage is too severe to be adequately repaired, the cell may undergo apoptosis or enter an irreversible senescencelike state (*13*).

Molecular Biology of the Cell Cycle

The experiments by Rao and Johnson (*10,11*), although important, did not provide molecular information about the nature of SPF, MPF, or cell cycle checkpoint mechanisms. Since those initial observations, studies in budding and fission yeast, and frog and marine invertebrate oocytes and embryos, *Drosophila* embryos, and mammalian cells have led to the molecular characterization of SPF, and MPF, as well as a greater understanding of the molecular events that govern the cell cycle, the alternation/completion problems, and checkpoint function (*8*). SPF and MPF have now been characterized as protein complexes whose key components consist of a regulatory protein subunit, referred to as a cyclin, and a protein kinase, called a cyclin-dependent kinase (Cdk). Different cyclin/Cdk complexes are expressed in different phases of the cell cycle, with each cyclin having a specific time of appearance and kinase activity [for reviews, see (*8,14–16*)]. In this review we discuss the known cyclin/Cdk activities that characterize each phase of the cell cycle, the cellular signal transduction pathways of cell cycle checkpoints, and several genotoxic insults that can initiate checkpoint function.

Cell Cycle Control

The G_1 Restriction Point

In early G_1, a series of molecular events occur that eventually commit the cell to progression through the cell cycle and division. Early events in the commitment to division include the induction of the D-type cyclins in response to growth factors and subsequent retinoblastoma protein (pRb) phosphorylation by G_1 cyclin/Cdk protein kinase complexes. This later event is necessary for progression through G_1 phase, as described below. In early to mid G_1, the withdrawal of external growth factors can result in a rapid lowering of cyclin D levels and exit of proliferation into a G_0 state (*17*). However, as cells proceed through G_1, a point is reached where the withdrawal of growth factors no longer halts cell cycle progression (*6*). This point is called the restriction point and is thought to coincide with pRb phosphorylation (Figure 1) (*18,19*). The G_1 restriction point has been found to be lost in many human tumors (*20*).

G_1 Cyclins

In mammalian cells, cyclins D and E form active protein kinase complexes with Cdk proteins, which are required for progression of cells through G_1 into S phase. There also is evidence suggesting that cyclin A/Cdk2 complexes may have a role in $G_1 \rightarrow S$ progression, although this is less clear. Cyclin D kinase activity is maximal in early to mid-G_1 (*21*). In G_0 cells, cyclin D levels are low but may be induced by mitogenic stimuli, whereas in continually cycling cell populations, cyclin D protein levels do not significantly oscillate throughout the cell cycle, although there is generally more cyclin D protein in late G_1 (*8,17,21–23*). Cyclin D has a relatively short half-life (~20 min) and rapidly disappears with the removal of mitogenic stimuli or the addition of antiproliferative agents (*17,24,25*). The requirement for cyclin D in regulating the $G_1 \rightarrow S$ transition was demonstrated by the microinjection of antibodies to cyclin D1 and by microinjection of cyclin D1 antisense plasmid into G_1 fibroblasts, both of which resulted in a block of progression into S phase. The same procedures failed to block S phase entry in fibroblasts near the G_1/S border (*26,27*). Overexpression/deregulation of cyclin D has been found in a variety of human tumors, implying that cyclin D can function as a positive growth regulator (*28–30*). In fact, overexpression of cyclin D was found to accelerate G_1 phase in rodent fibroblasts and decrease their dependency on mitogens (*27,31*). However, in cells that constitutively express cyclin D/Cdk4, the assembly of the active kinase complex depends on growth factors (*21*). On the basis of these data, cyclin D is thought to move cells from $G_1 \rightarrow S$ and participate in the transduction of external mitogenic/antiproliferative signals to other components of G_1/S transition cell cycle machinery, thus moving G_0 cells into G_1, and early G_1 cells into the G_1/S transition [(*17,21–25*); for reviews, see (*8,32*)].

Three mammalian isoforms of cyclin D occur (types D1, D2, and D3) and each is differently expressed in different cell types (*22,23,28,33,34*). The D cyclins show some functional redundancy, as cyclin D1 nullizygous mice are viable, although they are smaller than heterozygous or wild-type littermates and exhibit problems in retina and mammary gland development (*35*). Cyclin D2 nullizygous mice are also viable (*36*). However, cyclin D2-deficient females are sterile because of abnormalities in ovarian development, whereas cyclin D2-deficient males display hypoplastic testes. Interestingly, this observation led Sicinski et al. (*36*) to examine human testicular and ovarian tumors for abnormal cyclin D2 expression. Unusually high cyclin D2 mRNA expression was found in some of these tumors. Other differences between the three D-type cyclins have been documented. For example, although cyclin D1 is dysregulated in many tumors, there is little evidence implicating similar dysregulation of cyclins D2 and D3 in tumorigenesis [for review, see (*37*)]. Also, most cell types express cyclin D2 and either D1 or D3, suggesting that cyclins D2 and D1/D3 are not functionally equivalent (*32*).

The D-type cyclins normally associate with Cdk4 and Cdk6 (Figure 1) (*23,38,39*). Like the D-type cyclins, Cdk4 and Cdk6 show some degree of tissue-specific expression and have been found to be amplified-/overexpressed in human tumors and tumor cell lines (*38–44*). The cyclin D/Cdk4–Cdk6 complexes appear to function, at least in part, by phosphorylating the pRb protein (*38,39*). Support for this comes from the observations that in pRb-deficient cells, cyclin D activity is dispensable for passage through the cell cycle (*45*). The pRb and the pRb-related proteins act to suppress progression from $G_1 \rightarrow S$ by sequestering and thereby inactivating a number of regulatory factors [for review, see (*46*)]. Of these factors, the E2F-DP1 transcription factor

families are the best characterized [for reviews, see (*32,47*)]. In G_0 and early G_1 cells, E2F is bound to hypophosphorylated pRb and is inactive. With progression into G_1, the cyclin D/Cdk protein kinase complexes phosphorylate pRb, releasing E2F from pRb. E2F proteins can then form complexes with members of the DP-1 family of proteins and these complexes can act as transcriptional activators for several genes required for S phase. Included among these genes are dihydrofolate reductase, thymidine kinase, histone H2A, DNA polymerase α, proliferating cell nuclear antigen, as well as cyclin E, cyclin A, Cdc2, and E2F1 itself (*45,48–61*). The induction and activation of cyclin D is summarized in Figure 2.

Another cyclin/Cdk complex that plays a crucial role in the G_1/S phase transition is cyclin E/Cdk2. The expression and activity of cyclin E follows that of cyclin D, with increases in cyclin E expression occurring in the nucleus in early G_1, peaking at the G_1/S border (where cyclin E-associated protein kinase activity is maximal), and declining thereafter (Figure 1) (*62–64*). Cyclin E associates with a single Cdk, Cdk2 (*63,65*). Unlike the cyclin D/Cdk4 and cyclin D/Cdk6 complexes that show apparent limited substrate specificity for pRb and related proteins, cyclin E/Cdk2 protein complexes show *in vitro* protein kinase activity toward a number of exogenous protein substrates including pRb and histone H1 (*63,65*). As seen with cyclin D, microinjection of anticyclin E antibody blocks progression of G_1 cells into S, but fails to block cells at the border of G_1/S from proceeding into S phase. Cyclin E differs from cyclin D in that it is required for S phase progression in cells that lack pRb function, demonstrating that it has a function different from that of D-type cyclins (*66*). Similarly, Cdk2 expression has been found to be required for S phase entry, although it was not clear whether this was due to its association with cyclin E and/or cyclin A (*67,68*). Cyclin E dysregulation has been found in human cancers, with amplification of the cyclin E gene common in gastric and colorectal cancers [for review, see (*69*)]. Like cyclin D, overexpression of cyclin E shortens the time cells spend in G_1 (*31,66*).

Lundberg and Weinberg (*70*) have recently demonstrated that cyclin D and E act cooperatively. When either Cdk4/6 or Cdk2 was selectively inhibited, cyclin D/Cdk4-6 complexes where unable to phosphorylate pRb completely. Furthermore, the cyclin E/Cdk2 complex was found to be incapable of phosphorylating pRb unless pRb had previously been partially phosphorylated by a cyclin D/Cdk4-6 complex. Together these observations indicate that pRb inactivation and E2F transcriptional activity require the combined action of at least two distinct cyclin/Cdk complexes. Although cyclin A is believed to function mainly in S and G_2 phase, there is evidence that it can influence G_1 progression as well, since ectopic expression of cyclin A in G_1 cells can cause them to advance prematurely into S phase (*71*).

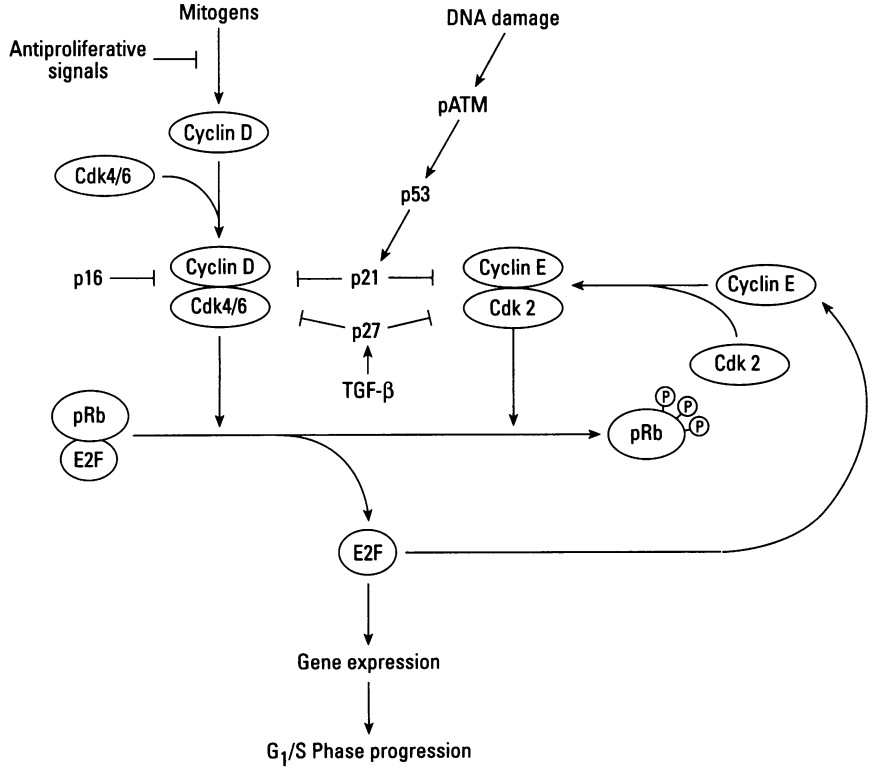


Figure 2. Schematic representation of cyclin D/Cdk and cyclin E/Cdk protein kinase complexes regulation in the G_0/G_1 transition into S phase.

S Phase Cyclins

DNA replication occurs in a discrete portion of the cell cycle referred to as S phase (*3,6*). Expressed at low levels in G_1, cyclin A protein levels steadily increase from S phase through G_2, with degradation occurring during M phase (*72,73*). Cyclin A activity is thought to contribute to the G_1/S transition, S phase progression, and $G_2 \rightarrow M$ transition. Support for this comes from the observations that microinjection of cyclin A antibody resulted in a failure to replicate DNA in fibroblasts, and that cyclin A null *Drosophila* embryos cannot enter mitosis (*74,75*). In extracts of *Xenopus* eggs, ablation of cyclin A mRNA resulted in the dysregulation of S phase progression and M phase entry (*76*).

Cyclin A associates with two Cdks, Cdk2 and Cdc2 (or Cdk1) (*73,77*). It has been hypothesized that cyclin A/Cdk2 activity is required for S phase progression, whereas cyclin A/Cdc2 activity is required for $G_2 \rightarrow M$ progression. Support for this hypothesis comes from the observation that mouse cells with temperature-sensitive Cdc2 mutations arrest only in G_2, whereas in *Xenopus* cell-free extracts Cdk2 is essential for DNA synthesis (*78,79*). Also, although cyclin A/Cdk2 activity is present in both S and G_2 phase, cyclin A/Cdc2 activity is present only in G_2 (*80*). The endogenous targets of these protein kinases are not known. However, *in vitro* protein substrates for cyclin A/Cdk2 include histone H1 and pRb, and for cyclin A/Cdc2 complexes include histone H1 protein (*72,73,81*). Recently, Knudsen and colleagues (*82*) found that a phosphorylation-site-mutated pRb was capable of blocking progression through S phase, suggesting that the continued hyperphosphorylation of pRb may be a necessary part of cell cycle progression. It is interesting to note that pRb represses both cyclin A and Cdc2 expression, putting these gene products

under G_1 cyclin control (*83,84*). It appears that this repression involves binding of pRb–E2F complexes to and actively repressing transcription from E2F promoters, thus in fact inhibiting gene expression [for review, see (*61*)]. Like cyclins D and E, there is evidence that cyclin A is dysregulated in some human cancers (*85*).

G_2/M Cyclins

$G_2 \rightarrow M$ progression and entry into M phase is regulated by MPF, an activity that is due principally to the protein kinase activity of cyclin B/Cdc2 protein complexes (*86–92*). Cyclin B levels oscillate through the cell cycle, with cyclin B first appearing in S phase, increasing through G_2, and being abruptly degraded at anaphase (Figure 1) (*93*). Cyclin B-associated activity peaks at the G_2/M border and remains until cyclin B degradation (*93*). Three major mammalian cyclin B isoforms have been characterized, cyclin B1, B2, and B3. During interphase, cyclins B1 and B2 are cytoplasmic, whereas cyclin B3 appears to be nuclear (*94–97*). At the G_2/M transition, the cytoplasmic B cyclins translocate to the nucleus prior to nuclear envelope breakdown (*94,95,98–100*). This nuclear translocation appears to be necessary for normal cyclin B activity and is regulated at least in part by phosphorylation (*100*). Cyclin B3 is unusual in that it is nuclear throughout interphase, associates *in vivo* with Cdc2 and Cdk2, and has structural features that resemble cyclin A (*97*). *In vitro*, cyclin B/Cdc2 protein complexes have kinase activity toward a variety of exogenous protein substrates including histone H1 (*92,101*).

Mice have been developed that are nullizygous for either cyclin B1 or B2 (*102*). Mice nullizygous for cyclin B2 developed normally. In contrast, no cyclin B1 homozygous null pups were born, demonstrating that cyclin B1 is an essential gene.

Regulation of Cyclin/Cdk Protein Kinase Activity

Regulation of cyclin/Cdk protein kinase activity during cell cycle progression involves not only regulation of the timing of cyclin protein accumulation and degradation, but also the binding of Cdk inhibitory polypeptides, and phosphorylations and dephosphorylations of both the cyclin proteins and the Cdk's (for reviews, see (*15,103–106*)]. The regulatory consequences of cyclin phosphorylation are not totally clear. Phosphorylation of B-type cyclins appears to influence subcellular localization and activation (*100*). More is known about the regulatory consequences of Cdk phosphorylation. Once complexed with their cyclin subunit, Cdk2 and Cdc2 must be phosphorylated on a regulatory threonine residue (Thr-160 and Thr-161 in humans, respectively) to become active. This activating phosphorylation is accomplished by an activity known as the Cdk-activating kinase, or CAK, which is composed of Cdk7, cyclin H, and a RING-finger protein MAT1 (*107,108*). Cdc2 molecules are phosphorylated on threonine 14 and tyrosine 15 amino acid residues in late S phase and G_2, as they associate with cyclin B molecules. These phosphorylations inhibit the activity of cyclin B/Cdc2 complexes (*109–111*). Thus, these inhibitory phosphorylations appear to be one important mechanism employed by cells to prevent premature activation of cyclin B/Cdc2 complexes before entry into mitosis. Phosphorylations of Cdc2 on Thr-14 and Tyr-15 can be accomplished through the actions of several dual-specificity protein kinases, including Wee1, Mik1, and Myt1 (*112–114*). Thr-14 and Tyr-15 are positioned within the Cdc2 ATP-binding cleft and phosphorylations of these residues are thought to inhibit kinase activity by disrupting the orientation of ATP molecules bound in this cleft (*109,115*). Activation of the cyclin B/Cdc2 complex occurs through dephosphorylation of Thr-14 and Tyr-15 on Cdc2 by the duel-specificity phosphatase Cdc25C (*116–118*). The extremely rapid activation of cyclin B/Cdc2 at the G_2/M border is thought to be brought about by an autocatalytic positive feedback loop involving cyclin B/Cdc2 and Cdc25C (*119*). This occurs when Cdc25C binds to cyclin B/Cdc2, dephosphorylating Cdc2 and activating the protein kinase complex. Cyclin B/Cdc2 in turn phosphorylates Cdc25C, which increases its phosphatase activity, resulting in the activation of more cyclin B/Cdc2 complexes, and in turn resulting in a rapid activation of both the Cdc25C phosphatase and cyclin B/Cdc2. Support for this model comes from the observations that hyperphosphorylation of Cdc25C correlates with increased phosphatase activity (*119,120*).

Regulation of Cell Cycle Checkpoint Function

Under normal circumstances the cell cycle proceeds without interruptions. However, when damage occurs, most normal cells have the capacity to arrest proliferation in G_1, S, and G_2, and then resume proliferation after the damage is repaired. Alternatively, cells may undergo apoptosis with or without growth arrest or enter an irreversible G_0-like state. Cells are acutely sensitive to broken DNA. Even a single double-strand DNA break appears to be sufficient to bring about cell cycle arrest in normal human fibroblasts (*121*). Cellular surveillance pathways that monitor successful completion of early cell cycle events and the integrity of the cell and generate delays in cell cycle progression in response to DNA damage and other events have been given the term checkpoints (*12,13, 122*). Cells exposed to a genotoxic agent while in early G_1 may arrest at a point in mid G_1 phase, whereas those in late G_1 or S phase will slow the initiation of DNA synthesis. Similarly, those exposed to a damaging agent in early to mid G_2 may delay in mid G_2, whereas those in late G_2 or early M phase may delay in mitosis. Thus, checkpoints appear to operate in all phases of the cell cycle. Checkpoint function often involves a delay in activation or inactivation of a particular cyclin/Cdk complex (*122,123*).

The G_1 Checkpoint

Cells exposed to genotoxic agents in early to mid G_1 may delay proliferation in G_1 at the G_1 checkpoint (*124*). G_1 cell cycle arrest in response to DNA damage has been found to depend heavily on the action of the p53 gene product (*125*). p53 has been characterized as a tumor suppressor gene product and is known to be mutated in more than 50% of human cancers (*20*). p53 is normally a short-lived protein, but is induced through posttranscriptional stabilization in response to DNA damage (*125,126*). Agents such as ionizing radiation, radiomimetic chemicals, and UV can all induce p53 (*125–128*). The dependence of the G_1 checkpoint function upon p53 function is demonstrated by the observation that cells containing wild-type p53 alleles undergo a dose-dependent G_1 arrest in response to γ-radiation. However, cells lacking functional p53 alleles enter S phase regardless of dose of γ-radiation (*129*). Similarly, cells from individuals with *Ataxia telangiectasia* (AT) induce p53 poorly in response to ionizing radiation. Not surprisingly, they also exhibit a severely attenuated G_1 checkpoint response after exposure to ionizing radiation (*130*).

Once induced, p53 can function as a transcription regulatory factor, binding to

the regulatory sequences and *trans*-activating a number of genes, including p21, Mdm2, and GADD45 (*131–134*). p53 can also act as a transcriptional repressor by interfering with the binding of basal transcription factors to the TATA motif (*135*). This observation may account for some of the ability of p53 to interfere with neoplastic processes (*135*). p21, also known as Cip1 /Waf1, binds directly to cyclin/Cdk complexes and acts as a Cdk inhibitor, or Cki (*136,137*). p21 can inhibit the kinase activity of cyclin E/Cdk2, cyclin D1/Cdk4, cyclin A/Cdk2, and to lesser extent, cyclin B/Cdc2 (*134,138–140*). Overexpression of p21 can result in G_1 arrest, while p21-deficient murine fibroblasts exhibit a defective G_1 arrest following γ-irradiation (*139,141*). It is important to note however, that p21-deficient fibroblasts exhibit an attenuated G_1 checkpoint, not an ablated one, indicating that other events are required in the G_1 checkpoint (*141*). Interestingly, basal p21 expression is not p53 dependent. Furthermore, p21 expression can be induced in a p53-independent manner under certain conditions such as during cellular differentiation and following serum stimulation and exposure to carbon tetrachloride (*142–145*). Also, p21 is normally associated with active cyclin/Cdk complexes (*146*). It appears that two or more p21 molecules are required per cyclin/Cdk complex to inhibit kinase activity (*147*). p21 is also associated with proliferating nuclear antigen and has been suggested to directly inhibit DNA replication (*148*). However, p21 is not required for inhibition of DNA replication in response to DNA damage in normal human fibroblasts (*149*). The N-terminal half of p21 shares homology with the Cdk inhibitor proteins p27 and p57, and these inhibitors also interact with Cdks in response to other signals (*150,151*).

Another important regulator of the G_1/S cyclin/Cdk complexes is the association of members of the INK4 family of proteins, especially p16, although the role, if any, of the INK4 proteins in cell cycle checkpoint function is not clear. p16 is known to inhibit cyclin D/Cdk4-6 complexes and therefore probably acts as an inhibitor of pRb phosphorylation (*152*). Support for this view comes from the observation that p16 overexpression leads to arrest in G_1 in pRb+/+ cells, but not in pRb–/– cells (*153*). p16-deficient mice develop normally, but show an elevated cancer rate in the presence of carcinogens (*154*). Both somatic and germline p16 mutations have been found in human cancers/familial cancers syndromes, as well as inactivating hypermethylation of the p16 gene in human tumors, demonstrating the importance of p16 as a tumor-suppressor gene [(*155–157*); for review, see (*158*)]. The gene locus encoding p16, INK4a, has recently been found to encode another protein, $p19^{ARF}$, which is produced through splicing of an alternative first exon into an alternative reading frame of the shared second exon. Many p16 mutations arise in the second exon and therefore are also shared mutations in $p19^{ARF}$. Although $p19^{ARF}$ loss has not yet been associated with human tumors, $p19^{ARF}$ null/p16 wild-type mice develop spontaneous tumors at a high rate, indicating that $p19^{ARF}$ functions as a tumor suppressor (*159*). $p19^{ARF}$ has been shown to interact with the MDM2 protein, neutralizing MDM2's inhibitory regulation of p53, resulting in an activation of p53 and, following transient $p19^{ARF}$ expression, may induce a p53-mediated cell cycle arrest in rodent fibroblasts (*160,161*).

Another factor in the G_1 checkpoint is the inhibitory phosphorylation of Cdk proteins on threonine and tyrosine residues, as described above. Phosphorylations and dephosphorylations of G_1 Cdk's are normal components of regulation of G_1 cyclins/Cdk complexes. Specifically, Cdk2 is phosphorylated on Thr-14 and Tyr-15 during the cell cycle (*162,163*). Treatment of cyclin E/Cdk2 and cyclin A/Cdk2 immunoprecipitates with a bacterially expressed Cdc25M2 (the murine homolog of huCDC25 phosphatase) increased the histone H1 kinase activity of these complexes 5- to 10-fold (*163*). Similarly, Cdk4 is phosphorylated on Tyr-17 in response to ultraviolet (UV) treatment and transfection of cells with a mutant Cdk4 that could not be phosphorylated on Tyr-17 resulted in a loss of the UV-induced G_1 checkpoint (*164*). Furthermore, treatment of Daudi Burkitt's lymphoma cells with interferon-α resulted in a G_0-like arrest and rapid elimination of the phosphatase (Cdc25A) required for removal of Cdk2 tyrosine phosphorylation (*165*). Inhibition of the Cdc25A phosphatase by antibody microinjection also resulted in G_1 arrest (*166*). Together these results implicate the regulation of Cdk tyrosine phosphorylation as an important component of regulation of G_1 cyclin/Cdk activity in the G_1 checkpoint response to genotoxic agents.

The S Phase Checkpoint

Less is known about the S phase checkpoint function than the G_1 and G_2 checkpoint functions. Upon exposure to DNA-damaging agents, such as ionizing radiation, mammalian cells exhibit a dose-dependent reduction in DNA synthesis within a few minutes (*167–170*). The suppression is biphasic, with a strong initial suppression at low doses of radiation and less additional suppression at higher dosages. The biphasic response has been attributed to a suppression of radiation-sensitive new replicon initiation followed by the suppression of initiated replicons, the latter being less radiation sensitive (*169,171*). The suppression of replicon initiation is mediated by a *trans*-acting factor, as ionizing radiation inhibits both chromosomal replication and the replication of a resident autonomously replicating plasmid, even when the radiation dosage is not sufficient to damage the autonomously replicating plasmid (*172*). S phase cyclin A/Cdk2 activity, which is thought to be necessary for S phase progression (see previous discussion), is suppressed by treating cells with ionizing radiation. Interestingly, neither the inhibition of DNA synthesis nor the inhibition of cyclin A/Cdk2 activity is seen in cells from patients with AT (*173*). Thus, the AT gene product appears to be required for appropriate S phase checkpoint response to DNA damage.

The G_2 Checkpoint

Ionizing radiation and other agents that trigger the G_2 checkpoint response suppress cyclin B/Cdc2 kinase activation at the G_2/M border (*174,175*). Treatment of mammalian cells with genotoxic agents results in accumulation of $p34^{cdc2}$ molecules that are phosphorylated on amino acid residues Thr-14 and Tyr-15, resulting in inhibition of cyclin B/Cdc2 protein kinase activity (*174–177*). When HeLa cells were transfected with a tetracycline-repressible Cdc2 mutant that could not be phosphorylated on Thr-14/Tyr-15, the G_2 checkpoint was partially ablated, indicating that these phosphorylations are an important inhibitory component of the G_2 checkpoint (*178*). As mentioned previously, activation of the cyclin B/Cdc2 complex occurs through Cdc2 dephosphorylation on Thr-14/Tyr-15 by the duel-specificity protein phosphatase Cdc25C (*116–118*). Hyperphosphorylation of Cdc25C correlates with increased Cdc25 protein phosphatase activity (*119,120*), and in DNA-damaged cells, Cdc25C does not reach its hyperphosphorylated state (*179*). In addition, although cyclin B/Cdc2-Cdc25C association normally occurs at the G_2/M border, this interaction

does not occur in cells arrested in G_2 by DNA damage (*179*).

This interaction might be prevented through the action of the Chk1 kinase. This kinase phosphorylates Cdc25C on Ser216, leading to its binding by 14-3-3 proteins and apparent sequestration from its physiologic substrate, the cyclin B/Cdc2 protein complex (*180,181*). When a nonphosphorylatable Cdc25C mutant (Ser216→Ala216) was expressed in HeLa cells, the cells escaped radiation-induced G_2 checkpoint delay [(*180*); for review, see (*182*)]. As with their G_1 and S phase checkpoint function, cells from individuals with AT have defective G_2 checkpoint function (*173,176,183–186*). It has been speculated that the AT gene product may function as an upstream regulator of Chk1 (Figure 3) (*182*).

An additional component that likely contributes to the G_2 checkpoint is regulation of the subcellular localization of cyclin B/Cdc2 protein complexes. Cyclin B/Cdc2 complexes accumulate in the cytoplasm in S/G_2 phase and then as cells progress from G2→M, cyclin B/Cdc2 complexes move into the nucleus (*94,95*). Cyclin B complexes are retained in the cytoplasm in response to ionizing radiation treatment, suggesting that differential localization might also account for some aspects of the G_2 checkpoint function (*187–189*).

Another mechanism of suppression of cyclin B/Cdc2 protein kinase activity may involve the regulation of cyclin B levels. In S phase-irradiated cells, cyclin B mRNA and protein levels have been reported to be inhibited, whereas in G_2-irradiated cells, cyclin B mRNA stability and promoter activity are suppressed (*190–192*). It is important to note, however, that cyclin B downregulation has not been observed in other studies (*176,193–197*), and the importance of this level of regulation remains unclear.

The Cdk inhibitor p21 has been shown to associate with the cyclin B/Cdc2 complex. Cells in which the function of p53 has been disrupted either by expression of SV40 T-antigen or expression of the human papilloma virus type 16 E6 gene product (both of which bind and functionally inactivate p53, and hence prevent p53-dependent induction of p21 expression) have been found to have an accelerated G_2 entry and higher cyclin A/B kinase activity (*140,198–202*). In fact, it has been suggested that p21 plays a role in the G_2/M transition by inhibiting the activation of cyclin A/Cdk2 kinase complexes, thus delaying the activation of cyclin B/Cdc2 complexes in G_2 and that this delay could contribute to G_2 checkpoint function (*140*). However, normal human fibroblasts expressing the E6 protein for only a few population doublings show a normal initial G_2 checkpoint response to ionizing radiation, suggesting that p21 is not required for the immediate G_2 checkpoint in response to ionizing radiation (*203*). Thus the role of p21 appears to be ancillary for the immediate early G_2 checkpoint delay.

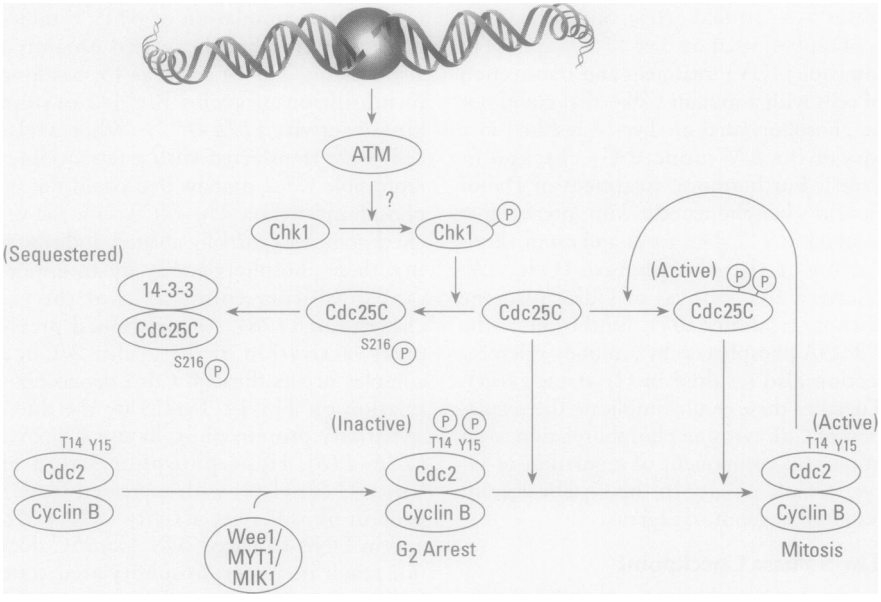


Figure 3. A schematic representation of the known or suggested interactions of proteins in the G_2 checkpoint signal transduction response to double-strand DNA breaks.

The Spindle Checkpoint

Most cells contain a spindle checkpoint that arrests cells in mitosis until all chromosomes are attached properly to the spindle [for reviews, see (*204–206*)]. Much of our understanding of the genes and the gene products that make up the spindle checkpoint pathway comes from studies with budding yeast and frog eggs, in addition to studies with mammalian systems. The critical transition from metaphase to anaphase and the separation of sister chromatids is monitored by the spindle checkpoint gene products that include the Mad (mitotic arrest defective) proteins, Mad1-3p, the Bub (budding uninhibited by benomyl) proteins, Bub1-3p, and Mps1 (*206*). To progress through this transition, cells must proteolytically degrade a number of proteins that are required earlier for entry into mitosis and this is accomplished by the activation of the proteasome, a component of the large multiprotein complex referred to as the anaphase-promoting complex or APC (*207–209*). Ubiquitin conjugation and proteolysis by APC results in the degradation of cyclin B proteins and the inactivation of MPF that is necessary for exit from mitosis (*210,211*) as well as the degradation of proteins involved in sister chromatid cohesion such as Pds1p (*212,213*) and proteins involved in cross-linking spindle microtubules such as Ase1p (*214*). Agents such as nocodazole and colcemid arrest cells in a prometaphase state because of disruption of microtubule reorganization and spindle apparatus formation (*215,216*). Anaphase will not begin until all the kinetochores receive bipolar spindle apparatus attachments (*217*). Li and Nicklas (*218*) showed that an M phase block induced by an unattached chromosome in insect cells was relieved through the application of tension to the unattached chromosome. It was hypothesized that tension resulted in a change in kinetochore chemistry, relieving the M phase arrest. Furthermore DNA-damaging agents, in addition to spindle-damaging agents, can activate the spindle checkpoint surveillance mechanism and this signaling pathway seems to involve Cdc20 proteins that interact with the Mad proteins (*219,220*), Mec1 proteins, which signal through Psd1p (*213*), the Polo-like kinase

(Plk) proteins (*221–224*), and perhaps protein kinase A (PK A), which can regulate the activity of APC (*225*). As with the other checkpoint functions, the spindle checkpoint is disrupted in tumor cells, with both a reduction in the levels of hsMAD2 observed in breast cancer cells (*226*) and mutationally inactive BUB1 found in tumor cells displaying chromosomal instability (*227*).

p53 and pRb were implicated as having roles in the spindle checkpoint response on the basis of the observation that cells lacking either function when cultured in the presence of spindle-damaging agents inappropriately initiate DNA synthesis without undergoing cytokinesis (*228,229*). However, recent evidence indicates that p53 and pRb probably do not function in this checkpoint (*230,231*). In fact,cells that were either wild type or deficient for either p53 or pRb all transiently arrested in M phase in response to nocodazole treatment. After roughly 24 hr all four cell types entered a G_1-like state with an interphase nuclear structure but with a 4N DNA content (a process referred to as adaptation or restitution). However, the p53- and pRb-deficient cells went on to rereplicate their DNA, becoming 8N and higher. These results were interpreted to indicate that cells undergoing the adaptation or restitution process in the continued presence of nocodazole suffered genomic damage that was recognized by the p53-dependent and pRb-dependent G_1 checkpoint surveillance system that monitors genomic integrity and regulates entry into the DNA replicative cycle.

Checkpoint Signaling, Caffeine, and DNA Repair

Checkpoint signaling has been hypothesized to give the cell time to repair broken DNA, or alternatively, to induce a program of either replicative senescence or apoptosis (*8,12,13,121*). On the basis of this hypothesis, suppression of the checkpoint response should result in decreased cell viability. Certain drugs such as the methylxanthines, e.g., caffeine and pentoxifylline, are capable of relieving the G_1, S, and G_2 checkpoint delay periods (*232–238*). When cells are treated simultaneously with these drugs and DNA-damaging agents such as ionizing radiation or alkylating agents, the lethality of the DNA-damaging agent is potentiated (*239–243*). For example, when baby hamster kidney cells synchronized at G_1/S were treated with 0.5 µM nitrogen mustard, 90% survived. However, in the presence of 2 mM caffeine, the same treatment resulted in 5- to 10-fold greater lethality (*244*). The molecular mechanism of caffeine's action remains unclear, but one of the consequences of the abrogation of the induction of the G_1 delay following DNA damage is a failure to induce p53, and hence p21 (*125*). More recently caffeine has been found to inhibit the G_2 checkpoint function by increasing Thr-14/Tyr-15 dephosphorylation on Cdc2 (*197*). The finding that overriding the G_1 and G_2 checkpoints results in lowered cell viability after damage supports the theory that one function of these checkpoints is to allow cells time to stop to repair damage before continuing the cell cycle.

Heritable Human Cancer Syndromes and the Cell Cycle

The molecular defects present in a number of heritable human cancer-prone syndromes have been characterized. Not surprisingly, these defects often compromise the ability of the cell to checkpoint delay in response to DNA damage and/or the ability to repair damaged DNA. Next, we briefly discuss the molecular defects in several heritable human cancer-prone syndromes and their effect on human health.

Ataxia telangiectasia and pATM

Ataxia telangiectasia is an autosomal recessive disease characterized by premature aging, sensitivity to ionizing radiation, sterility, immune dysfunction, acute cancer predisposition, telangiectasias, and progressive ataxia and neuronal degeneration, particularly of the Purkinje cells of the cerebellum (*245–247*). AT heterozygotes are reported to have elevated cancer risk, particularly of developing lymphoproliferative disease and breast cancer (*248–250*). In culture, fibroblasts from patients with AT exhibit premature senescence, increased serum requirements, increased chromosomal instability compared to that of normal human fibroblasts, abnormally rapid telomere shortening, and sensitivity to ionizing radiation and radiomimetic chemicals (*169,251–254*). Recently the gene mutated in AT (AT mutated or *ATM*) was identified (*255,256*). The *ATM* gene product (pATM) has been hypothesized to be a sensor of DNA strand breaks and to be required in the DNA damage response signal transduction pathway that results in the activation of p53 in response to DNA strand breaks (*121,128*). Recently, the protein product of the *ATM* gene was demonstrated to have protein kinase activity that is activated in response to IR but not UV exposure and that is capable of phosphorylating p53 on serine residue 15 (*257,258*). In addition, pATM has been suggested to be a cellular sensor of oxidative stress, making pATM null cells abnormally sensitive to oxidative stress from such sources as ionizing radiation and H_2O_2 [for review, see (*259*)].

Cells in culture from individuals with AT exhibit severely impaired G_1, S, and G_2 checkpoint functions (*170,183,186*). The defect in the G_1 checkpoint in AT cells has been found to be associated with a defect in the induction of p53 protein in response to IR exposures, with an induction that is only slight and occurs with delayed kinetics (*133,260,261*). Interestingly, however, AT cells induce p53 in response to UV exposures (*260,261*). AT cells exposed to IR during S phase show little inhibition of DNA synthesis (i.e., radioresistant DNA synthesis) or inhibition of cyclin A/Cdk2 activity (*170, 172,173,262–264*). AT cells have been found to lack a normal G_2 checkpoint response to IR exposure (*176,183–186*). The exact molecular defect in response to DNA damage in cells from individuals with AT remains to be elucidated. AT cells have shown apparently normal repair of single-strand DNA (ssDNA) breaks and show global double-strand DNA (dsDNA) break repair that appears to have the same kinetics as normal cells (*265–267*). However, evidence supports the interpretation that AT cells are defective in certain types of dsDNA break repair. Initial indication of an inability to repair dsDNA breaks was the observation of increased chromosomal aberrations, in particular both chromatid and total breaks, in AT cells following exposures to DNA-damaging agents and especially exposures in G_2 phase (*252,268–273*). Thus, the molecular defect in AT cells may be an inability to respond correctly to certain types of dsDNA breaks, particularly those arising from reactive oxygen species/oxidative stress [for review, see (*13*); (*274,275*)]. Evidence supporting the involvement of pATM in sensing oxidative stress comes from the observations that pATM null cells resynthesize glutathione unusually slowly after depletion with diethylmaleate and are abnormally sensitive to the damaging effects of hydrogen peroxide, superoxide, and nitric oxide (*267,276–279*). Whatever the exact nature of the defect in pATM function, the inability of AT cells to initiate the checkpoint function in

response to ionizing radiation clearly demonstrates how the ablation of one gene product involved in checkpoint function and maintenance of genomic integrity results in lowered cellular viability and greatly enhanced predisposition to cancer.

Retinoblastoma and pRb

Retinoblastoma (Rb) is a childhood retinal tumor that occurs in approximately 1 in 20,000 births worldwide, which is roughly 3% of all pediatric malignancies. All bilateral and some unilateral Rb cases (approximately 40%) are genetically determined and appear by the age of 15 months. Sporadic Rb (60% of Rb cases) is mainly unilateral, with diagnosis occurring later at 2 to 3 years of age. Analysis by Knudson demonstrated that bilateral (genetic) Rb resulted from a single somatic gene mutation. Analysis of most unilateral Rb cases, however, followed second-order kinetics, indicating that tumor formation required two mutational events. (*280,281*). Individuals with hereditary Rb who survive the Rb tumor are at high risk for later developing a second primary cancer, particularly osteosarcoma (*282*). The *Rb* gene is altered in a variety of human cancers, including breast, lung, and bladder cancers (*283–289*). Relatives of Rb patients often have elevated cancer rates (*290*).

Rb null mice die at day 14 to 16 in embryogenesis, exhibiting neuronal cell death and defective erythropoiesis (*291*). Heterozygous mice with one defective *Rb* allele do not develop retinoblastomas but develop pituitary adenomas in which the wild-type *Rb* gene is lost (*292,293*). The *Rb* gene product appears to play a role in the maintenance of genomic stability (*294,295*). Both White et al. (*294*) and Reznikoff et al. (*296*) introduced human papilloma virus type 16 E6 proteins (which inactivate p53) and E7 proteins (which inactivate pRb) into isogenic human cells and, after extensive passaging, found that although the E6-transformed cells showed significant chromosomal abnormalities, the E7-transformed cells had minimal alterations. However, cells lacking functional pRb were found to amplify the dihydrofolate reductase gene when grown in the presence of methotrexate, indicating that loss of pRb function can contribute in some degree to genetic instability (*294,295*). These data, together with the data on germline *Rb* mutation, demonstrate that the *Rb* gene product plays a significant role in the maintenance of genomic integrity.

Li-Fraumeni Syndrome and p53

Li-Fraumeni syndrome (LFS) is a rare heritable disease characterized by soft tissue sarcomas in children and young adults, early development of breast cancer in close relatives, and high rates of leukemia, brain, and adrenocortical tumors, osteosarcomas, and a number of other neoplasms (*297–300*). LFS shows an autosomal dominant transmission pattern and involves a germline mutation of *p53* (*301,302*). Interestingly, examinations of all 11 exons, the splice junctions, and the promoter regions of the *p53* gene in LFS families has shown that roughly 30% of LFS families do not show *p53* coding region mutations (*303*). The nature of the molecular defect in these families remains unknown.

Studies of cells that lack wild-type p53 function have demonstrated that lack of p53 can result in persistent chromatid damage after exposure to IR, changes in cell cycle checkpoint delay initiated by IR in G_1 phase, dysregulation of apoptosis, increased spontaneous immortalization, and chromosomal instability with long-term growth in culture, even in the absence of DNA-damaging agents (*125,176,201,304–310*). Loss of the wild-type *p53* allele in LFS cells results in abrogation of the G_1 checkpoint. Reintroduction of wild-type *p53* can restore the G_1 checkpoint and genomic stability (*307*). *p53* null mice develop normally, although 75% develop tumors by 6 months of age, usually lymphomas with some sarcomas (*311*). In contrast, mice with a single null *p53* allele had a delayed onset of spontaneous tumors, with osteosarcomas and soft tissue sarcomas predominating (*311*). These mice were also more susceptible to the effects of carcinogens than p53 wild-type mice (*312*). p53 null mice were abnormally sensitive to the effects of IR (*313*). It is interesting to note that some p53 mutations have a *trans*-dominant effect, partially inhibiting the action of the remaining wild-type p53 protein (*305, 314–319*). The tendency toward genomic instability, tumorigenesis, and loss of checkpoint function in LFS cells and p53-deficient transgenic mice, is a good example of how impaired p53 function can have profound effects on cell cycle regulation and cancer development.

Environmental Sources of Genotoxic Stress

Humans come into daily contact with an enormous number of DNA-damaging agents. Therefore, it is not surprising that elaborate molecular regulatory systems exist to maintain cellular genomic integrity. Genotoxic substances may come from both endogenous and exogenous sources. Some of these sources commonly encountered are discussed below.

Ultraviolet Radiation

Exposure to UV light induces a number of cellular changes, including the generation of DNA lesions, the induction of stress proteins (such as p53 and p21), and the initiation of cell cycle checkpoint arrest in cycling cells (*126,127,320–331*). UV radiation is divided into three classes based on wavelengths; UV-A (400–320 nm), UV-B (320–290 nm), and UV-C (290–100 nm). UV-A and UV-B are more biologically relevant, as UV-C is mostly absorbed in the upper atmosphere by ozone (*324*). The main direct UV-induced DNA lesion is the cross-linking of adjacent pyrimidines through formation of a cyclobutane-like four-membered ring structure with saturation of the 5,6 double bonds, referred to as a pyrimidine dimer (*320–322,328–330*). The formation of pyrimidine dimers is a UV-reversible process; however, equilibrium lies far to the right and favors the formation of dimers (*330*):

$$\mathrm{Py} + \mathrm{Py} \Leftrightarrow \mathrm{Py} <> \mathrm{Py}$$

Thymine–thymine dimers are the most common pyrimidine dimers formed following UV exposures, with cytosine–cytosine and cytosine–thymine dimers also occurring (*330*). However, most UV-induced mutations occur at cytosines, suggesting that cells are able to replicate DNA through thymine dimer lesions without error (*332,333*). UV radiation also produces a number of less common DNA lesions such as the mutagenic 6–4 pyrimidine–pyrimidone dimers, thymine glycols, and protein–DNA cross-linking (*330*). UV radiation also generates DNA damage indirectly via through the production of reactive oxygen species (ROS), including superoxide ($O_2^{\cdot -}$), the hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2), all of which rapidly react with each other and surrounding biomolecules. In addition, exposure to UV radiation can cause multimerization, clustering, and activation of cell surface receptor proteins for growth factors and cytokines, with activation of receptor-associated tyrosine kinase activities (*334*). Lastly, UV exposure elicits a number of other events that can lead to DNA damage and the promotion of tumor growth. Among these events are the induction of gene expression and/or activity such

as c-*fos* and protein kinase C (*335,336*), recruitment of inflammatory cells (with the accompanying release of ROS), the production of cytokines, and immunosuppression [for review, see (*337*)].

Exposure to UV radiation is associated with an increased skin cancer risk and premature aging of the skin, particularly among fair-skinned individuals with histories of being sunburned. A strong positive correlation also exists between skin cancer and proximity to the equator, indicating that higher UV doses to human populations result in higher incidences of skin cancer [for reviews, see (*338–342*)]. Enhanced removal of UV-induced pyrimidine dimers lowers skin cancer rates in mice, indicating that unrepaired dimers cause cancer in mammalian skin (*343*). Individuals with the heritable syndrome *Xeroderma pigmentosum* (XP) have impaired ability to remove DNA lesions induced by UV and consequentially are extremely sensitive to UV exposure, which results in an increased risk of developing skin cancers (*344–357*). Generation of mice deficient in *XP* genes have confirmed the important role these gene products play in protecting against UV-induced tumorigenesis (*358–360*). Skin cancer is the most prevalent malignancy in the United States, indicating that the genotoxic effects of UV radiation are a significant health hazard (*342*).

Ionizing Radiation

Ionizing radiation was first demonstrated to be mutagenic by Muller in 1927 (*361*). Since that time, IR has been demonstrated to induce mutations and cause cancer in a dose-dependent manner (*362–368*) [for review, see (*369*)]. IR damages all components of the cell and is known to produce more than 100 distinct DNA adducts (*365*). Data derived from studies on the survivors of the Hiroshima and Nagasaki bombings indicate that exposures to IR resulted in an increased cancer incidence over that in unexposed populations, with increases observed in incidences of leukemia, and breast, stomach, colon, and lung cancers (*370*). These studies also demonstrated that prenatal exposure to IR can also cause mental retardation and microcephaly (*371,372*).

IR damages DNA through direct and indirect mechanisms. Direct damage to DNA occurs as a result of the interaction of radiation energy with DNA. This can result in the generation of a variety of lesions, including the generation of abasic deoxyribose sites in DNA that are produced as a consequence of destabilization of the *N*-glycosidic bond, generation of ssDNA breaks and generation of dsDNA breaks. Indirect DNA damage comes from the interaction of DNA with reactive species formed by IR (*367,373–375*). Water is the predominant cellular constituent and more than 80% of the energy in IR deposited in cells results in the ejection of electrons from water (*376,377*). Subsequent reactions following this event can result in the formation of reactive oxygen species such as superoxide ($O_2^{\cdot-}$), the hydroxyl radical ($^{\cdot}OH$), e^-, $H^{\cdot}$, H_2, and H_2O_2.

Exposure to IR is a potent inducer of cell cycle checkpoint responses, resulting in p53 protein induction and Thr-14/Tyr-15 phosphorylation of Cdks. Environmental sources of IR include natural background radiation, medical procedures such as X-rays, radon, and in some areas such as those effected by the Chernobyl accident, environmental contamination (*378–381*).

Reactive Oxygen Species

Although oxygen is an absolute requirement for the survival of most metazoans, it can damage biologic molecules, including DNA. Normal cellular metabolism, as well as the metabolism of a variety of xenobiotics, produces an array of ROS that are highly reactive and can readily damage DNA. Under conditions of oxidative stress, cycling cells will exhibit cell cycle checkpoint responses (*382–384*). ROS have been implicated as important factors in a large number of biologic events including aging, carcinogenesis, atherosclerosis, strokes, and autoimmune disorders [for reviews, see (*385–390*). Ames and Shigenaga (*388*) have estimated that roughly 2×10^4 lesions occur per day per human genome because of oxidative damage to DNA. The number of different modifications resulting from ROS acting on DNA include both ssDNA and dsDNA breaks, DNA–protein cross-links, and a wide variety of base and sugar modifications (*391*). The number of ROS from both endogenous and exogenous sources that have been proposed to damage DNA is large. Here we focus on several thought to have important affects on biologic processes.

Hydrogen Peroxide/Hydroxyl Radical. H_2O_2 is produced by a wide variety of intracellular events, particularly in normal oxidative electron transport in the mitochondria, and it is normally present in most cells at a concentration of about 10^{-8} M (*392*). H_2O_2 participates in DNA damage through a variety of pathways including the production of $^{\cdot}OH$ through such reactions as the Fenton reaction (*393,394*):

$$Fe^{2+} + H_2O_2 \rightarrow {}^{\cdot}OH + {}^{\cdot}OH + Fe^{3+}$$

$^{\cdot}OH$ is an extremely strong oxidant, with a redox potential of approximately +1.35 V, making it capable of degrading most biologic molecules, including DNA (*367,373,395,396*). The number of different DNA modifications that $^{\cdot}OH$ is capable of producing appears to be over 100 (*365*). $^{\cdot}OH$ has been implicated in the etiology of human cancers such as breast cancer and leukemia (*397,398*). In addition to being produced from endogenous sources, $^{\cdot}OH$ can be generated in the human body after exposures to a variety of exogenous substances including cigarette tars, dietary components high in fat and low in plant fiber, ethyl alcohol, asbestos fibers, and IR (*365,399–402*).

Superoxide. Though less reactive than other ROS such as $^{\cdot}OH$, $O_2^{\cdot-}$ can damage biomolecules, including DNA. Approximately 2% of the oxygen consumed by human cells is converted to $O_2^{\cdot-}$, resulting in a steady concentration of $O_2^{\cdot-}$ within human cells of 1.0×10^{-11} M, this in turn resulting in the generation of an estimated 10,000 DNA lesions per genome per day (*403–405*). Like H_2O_2, $O_2^{\cdot-}$-induced damage is thought to be due mainly to conversion to $^{\cdot}OH$ by such pathways as the Haber-Weiss reaction (*406*):

$$H_2O_2 + O_2^{\cdot-} \rightarrow {}^{\cdot}OH + OH^- + O_2$$

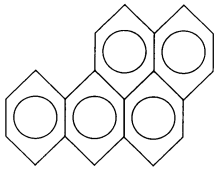
Like H_2O_2-induced damage, much of the $O_2^{\cdot-}$ found within cells is produced from the mitochondrial electron transport chain (*407*). $O_2^{\cdot-}$ is detoxified by conversion to H_2O_2 through the action of superoxide dismutase, which in turn is converted into $H_2O + O_2$ by the action of catalyze (*404*). The toxicity of $O_2^{\cdot-}$ is illustrated by the neurodegeneration seen in Lou Gehrig's disease (in which superoxide dismutase levels are low) and by the recent observation that overexpression of human superoxide dismutase in the motor neurons of *Drosophila* resulted in a 40% increase in lifespan (*404,408*).

Nitric Oxide. $^{\cdot}NO$ is an important proinflammatory mediator produced constitutively by vascular endothelial cells, some neuronal cell types, and activated macrophages (*409*). $^{\cdot}NO$ appears to damage DNA by combining with $O_2^{\cdot-}$ and forming the peroxynitrite radical. The

peroxynitrite radical is a similar to •OH and can readily damage biomolecules (*410*). •NO and cigarette tar synergistically induce DNA breakage, suggesting that •NO might react with many exogenous compounds to produce genotoxic substances (*411*).

Genotoxic Chemicals

Within the environment are an enormous number of both natural and man-made substances that have genotoxic properties. Most of these substances are chemical compounds that have the capacity to covalently modify DNA molecules. Within this category are such compounds as cisplatin and nitrogen mustard, which have been shown to generate strong cell cycle checkpoint responses to DNA damage generated following exposures (*179,412–416*). Also in this class are compounds such as methyl methanesulfonate (MMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) that transfer methyl or ethyl groups to DNA bases. Exposure to methylating agents has been reported to result in cell cycle checkpoint delays, particularly in cells defective in certain aspects of DNA repair (*417–419*). Polycyclic aromatic hydrocarbons (PAHs) comprise a family of compounds that modify DNA with bulky lesions and, because of their prevalence in the environment, pose a significant human health hazard. Here we will focus on one of the better characterized members of this class of compounds, benzo[*a*]pyrene B[*a*]P. B[*a*]P is produced along with other PAHs during the combustion of many organic substances including coal, cigarettes, and gasoline, and for this reason exposures to PAHs in the environment are relatively prevalent (*420*). The carcinogenic effects of PAHs in coal tar was first noticed in 1775 by Percival Pott, who observed a correlation between scrotal cancer and the occupation of chimney sweeping (*421*). B[*a*]P is a relatively unreactive 5-ring polycyclic planer hydrocarbon (Structure 1) (*422*):



Structure 1

However, B[*a*]P, like many other PAHs, is metabolized by components of the NADPH-dependent, cytochrome P450-containing monooxygenase microsomal enzymes through epoxidation to reactive electrophiles that can bind to such cellular nucleophiles as DNA, RNA, and proteins (*423,424*). The ultimate carcinogenic form is thought to be the 7β,8α-diol-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE I) metabolite (*422,425–427*). BPDE I can covalently attach to DNA and form a variety of adducts, with the major adduct formed through linkage between the exocyclic 2-amino group of guanine and the C-10 position of BPDE I (*422,428*). It has also been reported that the process of metabolizing B[*a*]P to its reactive metabolites through the generation of radical cations results in the generation DNA adducts that undergo rapid depurination and contribute significantly to the carcinogenic properties of B[*a*]P (*429,430*). Exposure to B[*a*]P and other PAH carcinogens, which generate bulky DNA adducts and apurinic sites that can be further degraded to DNA strand breaks, has been shown to result in inhibition of DNA synthesis and induction of S phase cell cycle arrest (*431–437*). It is likely that the persistence of BPDE I–DNA adducts and other unrepaired lesions generated after exposure to B[*a*]P during the process of DNA replication can result in generation of base-substitution mutations and chromosomal aberrations (*332,437–441*).

It is important to note that there are many other classes of environmental agents known to modify DNA and to be potent carcinogens. Included among these agents are aflatoxin and the aromatic and heterocyclic amines. Aflatoxin is a potent hepatocarcinogen produced by fungal contamination of foods and readily forms DNA adducts (*442*). The heterocyclic and aromatic amines also readily form DNA adducts and have widespread industrial uses and occur in foodstuffs, cooked meat, and tobacco smoke (*443,444*). Although the effects of these and other important environmental mutagenic toxins upon cell cycle checkpoint function are as yet poorly understood, their ability to induce mutations in critical cell cycle regulatory genes, as has been demonstrated in the case of aflatoxin-induced mutations of p53 (*445*), could seriously compromise checkpoint function.

DNA Repair Ability and Cancer Risk

The importance of DNA repair in maintaining genomic integrity and protecting against development of cancers has been shown in studies involving cancer patients and cancer-prone individuals as well as in studies involving genetically altered mice that exhibit deficiencies in DNA repair. The connection between DNA repair defects and human cancer predisposition was first recognized by Cleaver (*344,345*) in studying cells from individuals with *Xeroderma pigmentosum.* These cells were defective in the nucleotide excision repair pathway required to remove UV-induced DNA lesions. Studies of phytohemagglutinin-stimulated blood lymphocyte cultures from individuals with breast cancer and from individuals from familial breast cancer families showed that these cells were deficient in their DNA repair capacity compared with lymphocytes from control individuals, as measured indirectly by quantifying the generation of chromatid abnormalities following DNA damage (*446,447*). The *BRCA1* and *BRCA2* gene products, which when mutated predispose individuals to development of breast cancer, have been reported to play a role of in DNA repair and cell cycle checkpoint function (*448–457*). Defects in DNA repair, specifically in mismatch repair pathways, are important in the development of a variety of human cancers including cervix–uterine cancer, lung cancer, head and neck cancers, colorectal cancer, and basal cell carcinoma (*458–473*). The postreplication DNA mismatch repair system recognizes and removes inappropriately paired nucleotides that may have been generated by DNA replication errors, errors generated in DNA recombination events, or base damage following exposures to genotoxic agents (*465,474,475*). Mutations in DNA mismatch repair pathways have been reported to affect cell cycle checkpoint function, with the best evidence to date demonstrating an important role of the *MLH1* gene product in a p53-independent G_2 checkpoint response to DNA damage generated by 6-thioguanine, MNNG, and IR exposures (*227,419,476,477*). Furthermore, mice deficient in DNA mismatch repair have increased susceptibility to development of neoplasia (*478–481*).

Summary

Neoplastic progression has been demonstrated to involve increasing genetic instability (*201,470,482–488*). The information gained from studies of the molecular mechanisms governing cell cycle control, DNA repair, and cell cycle checkpoint signaling in normal individuals and in individuals with heritable cancer syndromes, together with the effects of genotoxic substances on these biochemical pathways, demonstrates the importance of these molecular pathways in the maintenance of

genomic integrity. Loss of any aspect of these systems dramatically lessens DNA stability and cell viability and increases cancer susceptibility.

In particular, attenuation or ablation of cell cycle checkpoint signaling pathways results in a dramatic lessening of DNA stability in the face of genomic stress as well as lowered cellular viability and increased cancer susceptibility. These effects are particularly clear in studies involving caffeine-induced "checkpoint function over-ride" after DNA damage [for example (*244*)]. Similarly, the near-complete ablation of the G_1, S, and G_2 phase checkpoint functions in cells from individuals with AT and the loss of the G_1 checkpoint function in p53 mutant cells (accompanied by an attenuation of the G_2 checkpoint function and increased genomic instability) supports the view that cell cycle checkpoint responses function to allow the damaged cell time to repair damage, or alternatively to undergo apoptosis or enter into a permanent G_0-like state.

One important and interesting area for future study is the impact of nongenotoxic chemicals on cell cycle checkpoint function. A number of chemicals found in the environment, compounds such as benzene and 1,4-dioxane, fail to show mutagenic properties as measured in *Salmonella* mutagenesis assays, yet have the ability to induce tumors in rodents (*489*). The mechanism of induction of neoplasia by these environmental chemicals and their effects on cell cycle checkpoint function are not yet clearly understood. However, the study of these agents may give insight into both checkpoint signal transduction pathways and mechanisms of carcinogenesis. It is possible for example, that a nongenotoxic environmental carcinogen may function by ablating some aspects of cell cycle checkpoint function, perhaps leading to genetic instability or heritable alterations of the genome. Interestingly, caffeine, which has been found to have a significant impact on cell cycle checkpoint function (see above), is nonmutagenic in *Salmonella* mutagenesis assays (*490*).

The current model of the cell cycle checkpoint signaling in response to cellular damage and the generation of DNA strand breaks that result in both the G_1 and G_2 checkpoint delays involves activation of the ATM protein, which leads to both p53 and Chk1 activation. p53 initiates p21 transcription and the inhibition of cyclin/Cdk activity. Chk1 activation results presumably in altered CDC25 phosphatase localization, and hence lack of activation of cyclin/Cdk protein kinase complexes. Although less is known about the S phase checkpoint function, signaling through this pathway is known to be ATM-dependent and involves cyclin/Cdk inhibition and the suppression of DNA synthesis.

Together the above data indicate that cell cycle checkpoint responses *a*) are active signaling pathways dependent upon a number of different gene products, *b*) play a vital role in maintaining genomic stability, *c*) generate a transient delay in the progression through the cell cycle, *d*) may be either wholly or partially ablated by the loss/mutation of a single gene such as ATM or p53, and *e*) may be initiated by a wide variety of genotoxic agents that may exert very different effects on the cell. Our increasing understanding of cell cycle checkpoint signaling pathways may help in the design of more efficacious therapeutic strategies for treatment of cancers and other diseases that develop as a consequence of exposures to environmental genotoxins. Furthermore, understanding the role of cell cycle checkpoint responses to environmental exposures promises to aid in the development of more efficacious approaches to disease prevention. Such insight will provide us with a better understanding of the risks associated with exposures for the general population. Moreover, such data may allow more accurate assessment of risk for specific subpopulations of individuals predisposed to development of certain diseases because of genetic susceptibilities. Appropriate measures then can be designed to minimize those exposures associated with significant risks.

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