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Mini-review

Convergence of mitogenic and DNA damage signaling in the G1 phase of the cell cycle

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Abstract

Research into the molecular basis of cancer has a central tenet. Cancer arises from genetic alterations that disconnect growth and differentiation signaling pathways from the machinery that regulates cellular proliferation. In multi-cellular eukaryotes, proliferation is regulated by external signals, such as the availability of growth factors and nutrients and by internal signals, such as those sensing cellular integrity. Cellular stress created either by lack of mitogens or damage to cellular components, such as DNA, stimulates responses that enforce temporal or permanent withdrawal from the cell cycle. Although these stress responses stem from different sources and activate distinct pathways, they converge on the same components of the cell cycle machinery in the G1 phase of the cell cycle. This review will highlight and compare aspects of the G1 arrest in response to stress generated either by lack of mitogens or damage to DNA.

Keywords: Stress response; Cyclin; DNA damage; Proteasome; Restriction point; Checkpoint; Cyclin-dependent kinases; Mitogen; Cell cycle; G1; G2

1. Introduction

Cellular proliferation depends on the cell's growth and division cycles, which are governed by periodic assembly of the core cell cycle clock, composed of cyclins and cyclin-dependent kinases (CDKs). CDKs are Serine/Threonine protein kinases that are inactive as monomers and need to be activated by binding of a cyclin partner. In eukaryotes, distinct CDKs regulate different cell cycle processes. For instance, CDK4 and CDK6 (CDK4/6) regulate cell cycle progression through mid-G1, CDK2 activation is associated with entry into S-phase, whereas CDK1 primarily regulates mitosis. Distinct cyclins associate and activate different CDKs throughout the cell cycle. D-cyclins (D1, D2, and D3) bind CDK4/6 to wire external signals to the cell cycle and regulate progression through mid-G1. Cyclin E binds CDK2 in late G1 and its activity is rate-limiting for progression from G1 to S phase [1,2]. The function of each of these complexes is quantitatively regulated throughout the cell cycle and qualitatively targets a unique set of substrates [3].

The activity of cyclin/CDK complexes is modulated by both activating and inhibiting phosphorylation of the CDKs, and by binding to cyclin-dependent kinases inhibitors (CKIs). Two families of CKIs exist, the INK4 and the CIP/KIP families. Proteins of the INK4 family interact only with CDK4/6 and inhibit their association with- and activation by the D-type cyclins [4]. In contrast, members of the CIP/KIP

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family, consisting of the proteins p21, p27 and p57, act primarily on CDK2 and CDK4/6 complexes [5]. These CKIs are required for proper cell cycle arrest in response to mitogen deprivation and genotoxic stress. Interestingly, as will be discussed below, different members of this family play distinct and unique roles in these stress responses.

Most of the regulatory events that affect proliferation occur in the G1 phase of the cell cycle. Growth and proliferation of normal cells is regulated by complex interactions between growth factors, cell density and attachment to substrate [6]. Growth factors are necessary to initiate and maintain the transition between G1 and S phase. The time window at which cell cycle progression depends on growth factors is called 'restriction' [7]. Topologically, the restriction period lies in between mid and late G1 phase (Fig. 1A).

In contrast to extracellular cues, the internal integrity of a cell is examined by distinct checkpoints spread throughout the entire cell cycle. Each of these processes tests the successful completion of a previous phase and is defined as 'checkpoint control' [8]. The term 'checkpoint' is mostly used in the context of DNA damage [9] and is in a way misleading, as cells continuously sense damage to the genome rather than checking its integrity only at specific 'checkpoints'. As damaged DNA may propagate during replication and mitosis, the cell cycle arrest occurs mostly in G1 (before S) and in G2 (before mitosis), but can also occur during S phase. Arrest in G1 prevents aberrant replication of damaged DNA and arrest in G2 allows the cells to avoid segregation of defective chromosomes. This review will focus on the arrest in G1 phase of the cell cycle. The G2 arrest was recently reviewed [10].

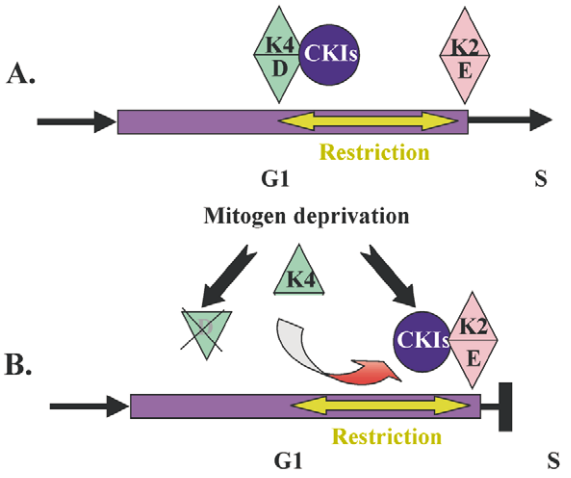

Fig. 1. Model showing the restriction window in the G1 phase of cycling cells. (A) Cyclin D-CDK4 (D/K4) complexes bind CKI proteins and sequester these proteins to prevent them from inhibiting the function of cyclin E-CDK2 (E/K2), active E/K2 complexes allow entry into S phase. (B) Cells deprived of mitogens withdraw from the cell cycle by activating mechanisms that down-regulate D cyclin abundance and increase the amount of CKIs. Both actions culminate in effective inhibition of cyclin E-CDK2 activity.

2. Mechanisms of restricting progression through G1

Growth factors induce cell cycle progression by binding and activating their cognate tyrosine kinase receptors to start a network of pathways that include other tyrosine kinases, PI3K, RAS, MAPKs and other proteins, most of them are proto-oncoproteins (for recent review, see Ref. [11]). This mitogen-activated network culminates in programmed induction of genes whose function is required for promoting G1 progression. Most importantly, mitogenic stimulation requires the core G1-cell cycle progression machinery to exert its function [3].

During progression through the G1 phase of the cell cycle two major types of cyclins are required: D and E. D-cyclins are functional in mid G1 whereas cyclin E functions at the G1/S border. Both the level and activity of cyclin E are regulated by signals that emanate from within the cell, leading to a peak in cyclin E activity in late G1 allowing the cells to enter into S phase. The three D-cyclins serve primarily to connect the cell cycle machinery to external signals. The cyclin D and E/CDK complexes synergize to cause phosphorylation of the retinoblastoma family of tumor suppressor proteins (pRb, p107 and p130) in G1 and abrogate their growth suppressive activity [12].

The gap between expression of D-type cyclins and cyclin E in G1 forms the so-called 'restriction window' (Fig. 1A). The hierarchical relationship between the two types of cyclins was demonstrated genetically by placing the *Cyclin E* coding sequence under the control of the *Cyclin D1* promoter ('*Cyclin E* knockin mice', [13,14]). Mice, ablated for *Cyclin D1*, *D2* or both, exhibit focal developmental anoma-

lies [13,14]. Expression of cyclin E instead of cyclin D1 in the *Cyclin E* knockin mice rescues all the phenotypes of cyclin D1 deficiency, without replacing the specific functions of cyclin D1 [15]. Therefore, ectopic expression of cyclin E bypasses the need of cyclin D1 for cell cycle progression, suggesting that cyclin E is a major downstream target of the D-type cyclins. Most importantly, this function is reflected in mammary tumorigenesis. Whereas mice deficient for *Cyclin D1* are resistant to the development of breast tumors induced by *RAS* and *NEU* (c-*ERB-2*), mice expressing cyclin E instead of D1 are as susceptible as wild type mice to these oncogenes [16].

How do D-type cyclins regulate cyclin E activity? The answer can possibly be found in the function of the CKIs from the CIP/KIP family, especially p21 and p27. These CKIs bind both CDK2 and CDK4/6 complexes, but play a distinct role in each case [17–20]. They inhibit cyclin E-CDK2 activity but are essential for complex formation and activity of cyclin D-CDK4/6 [17,21]. These distinct functions explain at least part of the upstream positive role of cyclin D1 on cyclin E activity. The association of D cyclins with CDK4/6 sequesters p21 and p27 away from cyclin E/CDK2 complexes, thereby releasing them from their inhibitory effect on cyclin E-CDK2. Supporting this idea are the following observations: first, in cycling cells CKIs are bound mostly to CDK4 complexes, which exhibit kinase activity [21]. Second, cells devoid of both p21 and p27 do not have active cyclin D/CDK4 kinase complexes, even though the proteins are expressed [21]. Importantly, these cells are also resistant to p16INK4A mediated growth arrest, indicating that p16INK4A also acts to translocate CIP/KIP inhibitors to cyclin E/CDK2 kinase complexes. Third, the phenotype of *Cyclin D1* deficiency in mice is rescued by ablation of *p27*, restoring nearly normal development to animals [22]. Fourth, ablation of *p27* also rescues the proliferative defects of $CDK4^{-/-}$ cells in culture [23]. Last, whereas over-expression of a kinase dead CDK2 mutant arrests cells in G1, ectopic expression of a kinase dead CDK4 mutant does not affect cell cycle progression [24]. Thus, D cyclin-CDK4 complexes function upstream of cyclin E and CIP/KIP inhibitors, and their major role is to titrate and neutralize CKIs to allow activation of CDK2 at the end of G1.

D-cyclins are sensitive to mitogens as their promoters, protein stability and their assembly into functional complexes is regulated by a variety of mitogenic signals. For instance, activated RAS governs the accumulation of cyclin D1-CDK4 complex activity by transcriptional activation of *Cyclin D1* promoter through a kinase pathway that includes RAF-1, MEK and MAPKs [25–29]. Expression of the c-Myc oncoprotein promotes the accumulation of D1 and D2 cyclins via transcriptional de-repression of their promotes [30,31]. Furthermore, the *Cyclin D1* promoter has been shown to respond to TCF/β-catenin signaling [32,33]. Finally, induction of tumorigenesis and transformation by activated *NEU* requires activation of *Cyclin D1* promoter [16,34]. D-type cyclins are also very unstable proteins, a function that ensures that they will rapidly disappear when cells are deprived of growth factors. Their stability is regulated by GSK3-β that phosphorylates D cyclins at a position analogous to threonine 286 of cyclin D1 and triggers their nuclear export, ubiquitination and proteasomal degradation [21,35,36]. Mitogenic signals activate an inhibitory pathway mediated by activation of PI3K-PKB/Akt pathway that inhibits GSK3-β and stabilizes D-cyclins. As a consequence, the level of D-cyclins in resting cells is low due to the combined effects of reduced promoter activity and decreased protein stability. Cyclin D down-regulation leads to a rapid loss of cyclin D-CDK4/CKI complexes, which in turn results in a release and redistribution of CKIs to bind and inhibit CDK2 kinase activity (Fig. 1B).

In contrast to D cyclins, the level of p27 CKI protein in quiescent cells is high. When cells are deprived of mitogens, accumulation of p27 is required for efficient exit from the cell cycle and entry into a quiescent state [37,38]. Consistent with this view, *p27* knockout mice are one third bigger than normal and are tumor prone [39–41]. During cell cycle progression p27 is a very labile protein and its destruction relies primarily on the E3 ligase complex SCF^{SKP2} [42–45]. Accumulation of p27 by mitogen deprivation depends on inhibition of its proteolysis [46,47], possibly via inhibition of SCF^{SKP2} activity towards p27. Thus, in most cases it appears that mitogen deprivation causes activation of the restriction point and exit from G1 to G0 by combined effects of reduction in cyclin D levels and activity, and elevation of the amount of p27 (Fig. 2).

In many cell types the level of p27 is high and p21 is low during quiescence. Mitogenic stimulation of

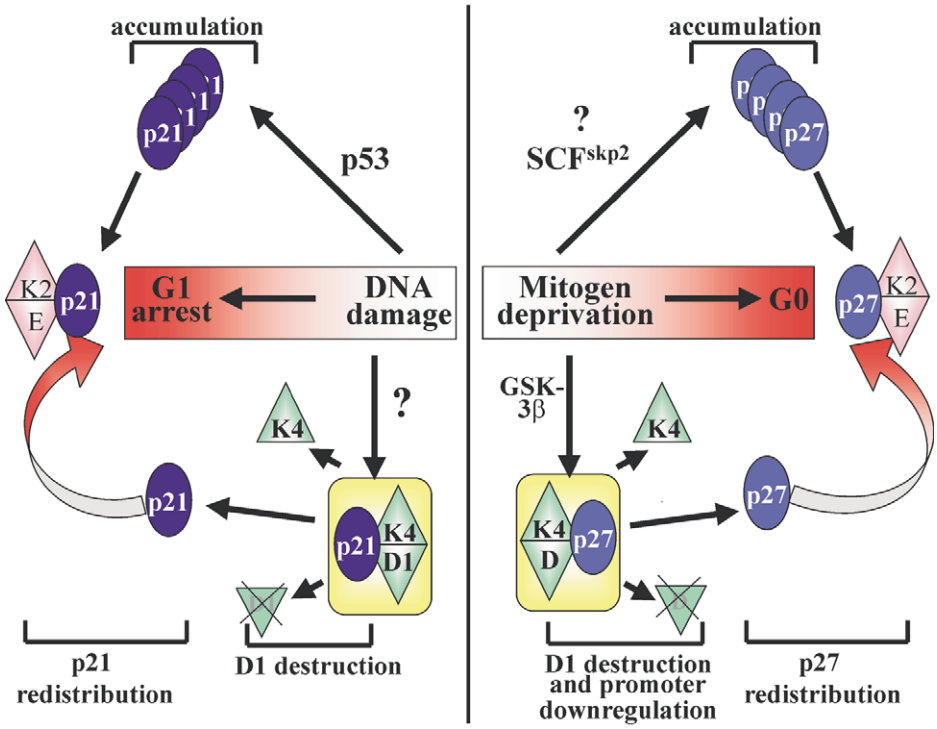

Fig. 2. Mechanisms used for withdrawal from the cell cycle in response to DNA damage and mitogen deprivation. Left: G1 arrest following DNA damage depends on two processes. A rapid release of p21 from CDK4 (K4) complexes caused by destruction of cyclin D1 and accumulation of p21 caused by activation of p53. Right: withdrawal from G1 to G0 in response to mitogen deprivation depends on accumulation of p27 due to combined inhibition of its destruction, maybe via inhibition of SCF^{skp2} activity, and its redistribution from CDK4 to CDK2 complexes caused by reduced level and activity of D cyclins.

quiescent cells leads to a reciprocal reduction in p27 levels and, most intriguingly, to induction of p21 [48–52]. In contrast to p27, p21 is not required for proper G1-exit from the cell cycle in response to mitogen deprivation and mice lacking *p21* develop normally and exhibit no increase in spontaneous tumor development [53]. Whether the phenotype of mice lacking *Cyclin D1* can be rescued by ablation of *p21* remains an open question. Why then do cells express higher amounts of p21 when they are cycling? As will be discussed below, it appears that cyclin D-CDK4 complexes containing p21 differ from those that contain p27 with respect to their ability to respond to acute stress signals, such as produced by DNA damage.

3. The G1 DNA damage checkpoint

Acute damage to DNA, such as double stranded breaks or bulky adducts caused by ionizing radiation or *cis*-platin treatments, activate a checkpoint that arrests the cells in G1. This arrest requires both functional p53 and pRB proteins and is built up through two distinct and independent mechanisms, referred to as ‘initiation’ and ‘maintenance’ [54,55]. The initiation phase of the cell cycle arrest is rapid, and depends uniquely on cyclin D1 destruction. p53 contributes very little to this initial response. Destruction of cyclin D1 releases a wave of p21 molecules from CDK4 complexes to arrest cell cycle progression in G1, at least in part by inhibiting CDK2 activity. Thus, the main feature of this initial arrest is that it does not require protein synthesis, but rather relies on rapid destruction and redistribution of pre-existing cellular components. However, this initial arrest is transient as the pool of p21, held by cyclin D1-CDK4, is rapidly exhausted and newly synthesized CDK2-cyclin E complexes are active to push the cells back into the

cycle in the presence of damaged DNA. In contrast, the 'classical' p53 response does require protein synthesis and is therefore inherently slower. The p53-dependent maintenance phase of cell cycle arrest follows the initiation phase, in which accumulation of p21 by DNA-damage activated p53 arrests the cells in G1 as long as the damage is present (Fig. 2).

The initial response to DNA damage demonstrates an important function of the p21/cyclin D1/CDK4 complex in cycling cells. These complexes constitute intra-nuclear stores loaded with specific inhibitors, allowing a rapid break of cell cycle progression in G1 once these stores are triggered to release their inhibitory content into the nucleus. This may explain why cells in many cases elevate p21 levels when they enter the cell cycle from quiescence. By having significant stores of p21/cyclin D1/CDK4 complexes, cycling cells can respond quickly to acute DNA damage, to arrest and repair the damage in order to survive. Thus, the p21/cyclin D1/CDK4 complex provides cycling cells with a mechanism to quickly respond to acute DNA damage.

Interestingly, similar to mitogen deprivation, a clear functional distinction between the p21 and p27 CKIs is seen in the DNA damage response. Whereas p21 was readily released from CDK4 complexes after DNA damage, p27 was not [55]. Thus, whereas proper cell cycle arrest following mitogen deprivation requires p27 and not p21, the response to DNA damage requires p21 and not p27 (Fig. 2). This difference in the function of these CKIs is intriguing. A likely explanation is that each CKI molecule assembles a distinct cyclin D/CDK4 complex that is functionally devoted to respond to a different set of stress signals. Supporting this hypothesis is the notion that ablation of *p21*, but not of *p27*, prevented destruction of cyclin D1 following DNA damage (R. Agami and R. Bernards, unpublished results). This indicates that p21 is not only a downstream target of cyclin D1 destruction, but is also uniquely required for cyclin D1 destruction, most likely via an ability to recruit specific components of the protein destruction machinery to cyclin D1-CDK4 complexes. Taken together, the G1 arrest following acute DNA damage requires p21 function, but not p27, in three distinct ways. First, p21 is a unique downstream target of cyclin D1 destruction. Second, it is uniquely required for cyclin D1 destruction following DNA-damage. Third, p21 is a direct transcriptional target of p53. It is therefore no surprise that p21-deficient cells are impaired in their ability to arrest in G1 following DNA damage [53].

4. Concluding remarks

We discussed here several aspects of the relationship between the restriction mechanisms (caused by growth factor starvation) and the checking mechanisms (following acute DNA damage) in the G1 phase of the cell cycle. At first glance, these two responses look-a-like as both require the concerted action of reduction in the levels of D-cyclins and an accumulation of CKIs. Both responses inactivate the cyclin E-CDK2 complex at the G1-S transition and induce exit from the cell cycle. However, the mechanisms used by these two types of anti-proliferative signaling are strikingly different. Both the CKIs p21 and p27 originated from a common ancestor (Xic1 in *Xenopus*), and share a highly homologous amino terminus. However, they diverged later in evolution and adopted distinct and highly specialized functions. It appears that p27 is mostly involved in regulation of exit from cell cycle in response to growth factor depletion, whereas p21 is mostly required for both initiation and maintenance of G1 arrest in response to DNA damage (Fig. 2).

In contrast, cyclin D1 can respond to both mitogen and DNA damage signaling through unique and distinct functional domains. Whereas mitogen deprivation activates GSK-3β to destruct cyclin D1 through phosphorylation of threonine 286, this residue is dispensable for cyclin D1 destruction following DNA damage. Instead, genotoxic stress requires a destruction box in the cyclin D1 N-terminus. Intriguingly, threonine 286 of cyclin D1 and flanking sequences are conserved in the other D-type cyclins (D2 and D3), whereas the destruction-box is not. Thus, important differences in their ability to respond to cellular stress signals exist between the three D-type cyclins (Fig. 3). Indeed, the levels of cyclins D2 and D3 are not altered in response to DNA damage, but they are reduced by mitogen starvation [55,56]. In conclusion, with the increased complexity of higher organisms came the need for more sophisticated responses to internal and external signals.

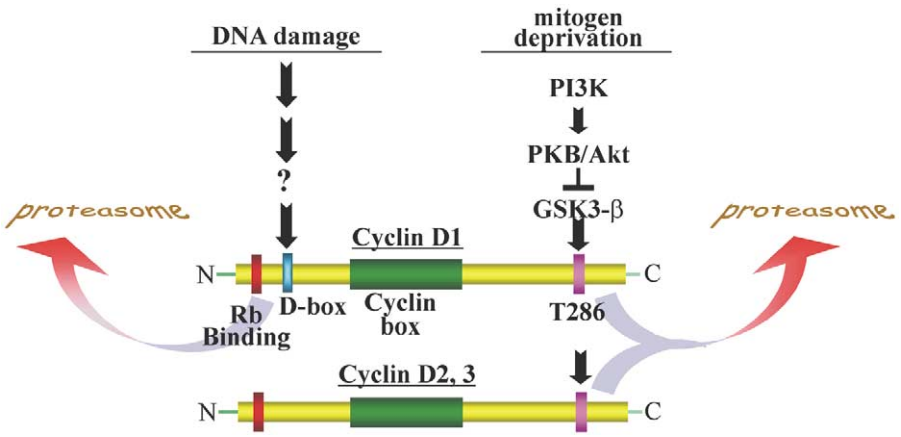

Fig. 3. Different response of the D cyclin family members to mitogen deprivation and DNA damage. Following DNA damage cyclin D1 destruction is accelerated by a process that depends on the proteasome machinery and on a destruction box (D-box) within the N-terminus of cyclin D1. Cyclins D2 and D3 do not contain D-box and also their stability is not altered after DNA damage. Following mitogen deprivation cyclin D1 is destabilized, a process that depends on the proteasome machinery and on its phosphorylation on Threonine 286 (T286) by GSK3-β. T286 residue and surrounding sequences are conserved in both cyclin D2 and D3 and so is their destabilization after mitogen starvation.

During evolution, the CIP/KIP CKIs and the D type cyclin gene family members have evolved to acquire distinct functional motifs that endowed them with the ability to respond to a multitude of signals, allowing a more refined regulation of cellular responses to both internal and external signals.

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