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INDUCTION OF S PHASE BY G1 REGULATORY FACTORS

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1. ABTRACT

The first gap phase (G1) in the mammalian cell cycle plays a pivotal role in determining whether or not cells are to initiate DNA replication. Progression through G1 phase and transition into S phase are positively and negatively regulated by a series of factors, collectively termed G1 regulators. Among them, D-type G1 cyclins and a Cdk inhibitor, $p27^{Kip1}$, function as the target of growth factors to integrate extracellular signals into cell cycle regulators. Another G1 cyclin, cyclin E, and a transcription factor E2F are situated the furthest downstream of known G1 regulators and seem to be directly involved in the initiation of chromosomal DNA replication. Alterations in G1 regulator genes are often present in human tumors, indicating that G1 regulators participate in tumor suppressive mechanisms as well as in cell proliferation.

2. INTRODUCTION

Proliferation of mammalian cells is strictly regulated by extracellular signals, which largely exert their effects on cells during G1 phase of the cell cycle. Mitogenic signals such as growth factor stimulation are sine qua non for target cells to progress through G1 and to commit to replicating chromosomal DNA. However, once cells enter S phase, they can undergo mitosis even if deprived of growth factors during S, G2, and M phase intervals (1). Thus, control of factors that regulate G1 progression (G1 regulators) by extracellular signals is the key event to determining whether or not cells are to initiate DNA replication.

Among cell cycle regulators, only G1 regulators are found mutated or altered in human tumor cells. Thus, regulation of G1 progression plays an important role in tumor suppression as well as in cell proliferation *in vivo* (2). Interestingly, there seems to be an inverse correlation of some G1 regulator gene abnormalities (for example, cyclin D1, $p16^{INK4a}$, and pRb) (3). The rate abnormality of one particular gene is not always dramatic. Yet the rate of mutation that occurs in any of these genes is high, suggesting that disruption of the signaling pathway consisting of these gene products, but not the mutation of one particular gene, contributes to neoplasia. Thus, knowledge of the pathway composed of several G1 regulators and interactions, rather than studying the function of one particular gene product would seem to be an important approach to investigate mechanisms regulating G1 progression. In this review article, I focus on three subjects and present our latest findings on each subject. The functional roles of each G1 regulatory pathway in S phase induction is discussed (figure 1).

3. CELL CYCLE PROGRESSION MEDIATED BY G1 REGULATORS

Among G1 regulators, D-type cyclins serve as targets of growth factors to integrate extracellular signals into the core cell cycle regulators (4). D-type cyclins were identified in three independent approaches; (I) a target gene of chromosomal translocations in a variety of cancers (3, 5), (II) a mammalian cyclin gene that can complement yeast G1 cyclin deficiency (6), and (III) a delayed early growth factor inducible gene (7). D-type cyclins are composed of three different but closely related subfamilies (D1, D2, and D3), all differentially expressed in a wide variety of organs and in a tissue-specific manner (7). D-type cyclins are induced to express in response to a variety of mitogenic signals and function as a regulatory subunit of cyclin-dependent kinases (Cdk) (8). D-type cyclins can interact with 4 different Cdks (Cdk2, 4, 5, and 6), among which Cdk4 and Cdk6 are apparently the major functional catalytic partners in proliferating cells (8, 9). When cells are exposed to growth factor stimulation, the expression of cyclin D is maintained regardless of the point in the cell cycle (7). However, accumulation of active cyclin D/Cdk4 (or Cdk6) complex is rate-limiting and is required for cells to progress through G1 and to commit to entering S phase (10). The overexpression of D-type cyclins shortens the

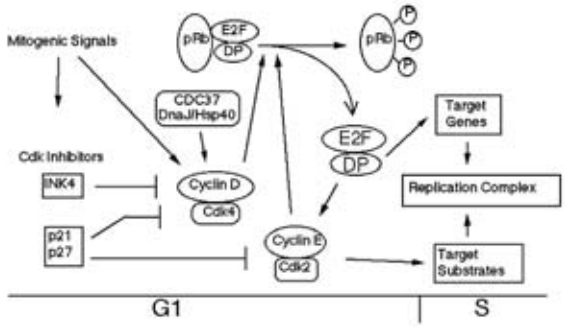

Figure 1. A model of functional interactions among G1 regulatory factors

length of G1 without affecting remainder of the cell cycle (10). This event is clearly different from phenotype of the cells overexpressing another G1 cyclin, cyclin E, in which G1 is shortened but elongation of the S phase compensates this shortening and as a result, doubling time of the cell remains unchanged (11). Thus, the cyclin D/Cdk4 complex largely exerts effects on commitment of cells for the S phase entry during the G1 phase, while functions of cyclin E/Cdk2 kinase are more directly involved in the initiation of chromosomal DNA synthesis.

Much evidence has accumulated to prove that one of the most important functions of cyclin D/Cdk4 complex is to phosphorylate retinoblastoma protein (pRb) in mid to late G1 and to neutralize the anti-proliferative activity of pRb (12). For example, introduction of the anti-cyclin D antibody or Cdk4-specific inhibitory protein (INK4 family of Cdk inhibitors) blocks cell cycle progression of normal cells but not that of cells lacking functional pRb (13). Therefore, function of the cyclin D/Cdk4 complex is required for cells to commit to enter S phase, and pRb is the only known substrate of cyclin D/Cdk4 kinase, at least for G1 progression of cultured cells *in vitro*. Indeed, the timing of pRb phosphorylation in cyclin D-overexpressing cells is accelerated (14). In contrast, although the cyclin E/Cdk2 complex also participates in pRb phosphorylation, the kinetics of pRb-phosphorylation remain the same regardless of whether or not cyclin E is engineered to overexpress (14). Thus, pRb phosphorylation by cyclin E/Cdk2 complex may be a secondary event. Microinjection of anti-cyclin E antibody arrests both Rb-positive and -negative cells in G1 (15), therefore pRb may not be the only substrate for cyclin E/Cdk2 kinase. Other candidate substrates for cyclin E/Cdk2 complex include minichromosome maintenance proteins (MCMs), CDC45-associated proteins (16), and NPAT (17).

Another target of growth factors is a Cdk inhibitor, $p27^{Kip1}$, which is downregulated upon mitogenic stimulation (18, 19). In certain circumstances, even though some growth signals induce the expression of G1 cyclins (cyclins D and E), cells do not progress into S phase until second-growth signals trigger downregulation of $p27^{Kip1}$ (18). Thus, G1 cyclin-production and $p27^{Kip1}$-removal are required for cells to enter S phase. Cellular abundance of $p27^{Kip1}$ is controlled by multiple mechanisms, among which degradation by the ubiquitin/proteasome pathway (20) seems to be the most important. Details of this pathway will be described in chapter 5.

Components that function the furthest downstream of the known G1 regulators are probably directly involved in initiation of chromosomal DNA replication. Such factors include cyclin E and a transcription factor E2F. Cyclin E forms a complex with and activates Cdk2 near the G1/S transition (4). Cyclin E/Cdk2 complex neutralizes the pRb function by phosphorylation (21) and pRb suppresses the activity of E2F by directly binding during G1 (12). These data place E2F downstream from cyclin E. However, the E2F-inducible genes include the cyclin E gene (22, 23). Thus, E2F and cyclin E (cyclin E/Cdk2 complex) create a positive feedback loop, allowing for a rapid rise of both activities as cells approach the G1/S boundary. Recent findings suggest that E2F and cyclin E/Cdk2 kinase have their own pathway to promote S phase entry (24, 25) but details are controversial. In chapter 6, I describe our findings obtained by our unique system used to analyze the initiation of DNA replication.

4. REGULATION OF CYCLIN D/Cdk4 COMPLEXES

Activity of the cyclin D/Cdk4 complex, which plays a central role in regulation of G1 progression, is positively and negatively regulated by multiple mechanisms (13). Activation of Cdk4 requires binding to its regulatory subunit, D-type cyclins (26). Although the half life of Cdk4 is relatively long (>10 hours), D-type cyclins are unstable proteins within the cell (half life = ca 10-20 min) and depend upon extracellular signals for expression (7, 8). Therefore, induction of cyclin D expression is the rate-limiting step for cyclin D/Cdk4 activation. Although analyses of the cyclin D promoter provided little information at to which signal transduction pathway is involved in cyclin D induction, recent studies revealed that the Ras-Raf-MEK pathway positively regulates transcriptional activation of the cyclin D gene (27, 28). The cyclin D protein is located and functions within the nucleus in normally proliferating cells. But upon phosphorylation on Thr286, cyclin D1 proteins are exported from the nucleus to the cytoplasm and are rapidly degraded through ubiquitin/proteasome machinery (29, 30). In mouse fibroblasts, GSK3beta is the only kinase that phosphorylates this residue, which suggests that the Ras-PI3K-Akt-GSK3beta pathway regulates the stability of cyclin D proteins (30). Thus, both induction and degradation of cyclin D are under control of growth factor-mediated signaling pathways.

Within proliferating cells, newly synthesized cyclin D proteins rapidly form a complex with Cdk4 and are transported into the nucleus. However, purified recombinant cyclin D and Cdk4 do not bind with high affinity in a test tube (31). Furthermore, ectopically overexpressed cyclin D3 and Cdk4 do not assemble in serum-deprived NIH3T3 cells (32). These observations imply that, in contrast to other cyclins and Cdks, assembly

of the cyclin D/Cdk4 complex may require other factors (Assembly Factor), the activities of which are regulated by growth factor stimulation. Molecular identity of this "Assembly Factor" remains unknown but there is a recent report that Cdk inhibitors, $p21^{Cip1}$ and $p27^{Kip1}$ are essential activators of the cyclin D/Cdk4 complex in murine fibroblasts (33).

In an attempt to identify regulators of the cyclin D/Cdk4 complex, two types of molecular chaperones were found to be Cdk4 binding proteins (Unpublished observations), one is CDC37 and the other is a novel member of the DnaJ/Hsp40 protein family. Both chaperones are located in the cytoplasm and seem to hold monomeric Cdk4 molecules until newly synthesized cyclin D proteins become available. The function of CDC37 is required for G1 progression of mouse fibroblasts and inactivation of CDC37 downregulates the expression of both cyclin D1 and Cdk4 proteins. Further analyses would be required to identify the most essential targets of CDC37 in G1 progression.

After being transported into the nucleus, Thr172 of the Cdk4 subunit in cyclin D/Cdk4 complex is phosphorylated by Cdk Activating Kinase (CAK) (31, 34). This modification is sine qua non for Cdk4 protein kinase activation (31). CAK is also a cyclin/Cdk complex composed of cyclin H and Cdk7 (35). In cells growth-arrested by TGF-beta treatment or by UV irradiation, the tyrosine residue of the Cdk4 subunit is phosphorylated and the cyclin D/Cdk4 activity is inhibited (36, 37). The kinase and the phosphatase responsible for phosphorylation and dephosphorylation of the tyrosine residue remain to be investigated. However, in analogy to other cyclin/Cdk complexes, the enzymes are most likely to be the Wee1/Myt1 family of kinases and the CDC25 family of phosphatases. Thus, specific phosphorylation and dephosphorylation positively and negatively regulate the activity of cyclin D/Cdk4 complex.

Another negative regulation of cyclin D/Cdk4 kinase is executed by two families of small-molecular-weight inhibitory proteins, termed Cdk inhibitors (38). The Cip/Kip family of Cdk inhibitors ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) inhibits a wide range of cyclin/Cdk activities (cyclin D/Cdk4, cyclin D/Cdk6, cyclin E/Cdk2, and cyclin A/Cdk2) by stoichiometrically binding to the cyclin/Cdk complex. The INK4 family of Cdk inhibitors ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$) specifically targets cyclin D-dependent kinases, Cdk4 and Cdk6. These seven Cdk inhibitors are induced to express or to be activated in response to a variety of anti-proliferative signals, among which $p27^{Kip1}$ seems to play the most essential role in regulating cell proliferation *in vivo* as well as *in vitro* during development and tumor suppression.

5. REGULATION OF Cdk INHIBITOR, $p27^{Kip1}$

$p27^{Kip1}$ is subject to multiple regulatory mechanisms. First, the level of $p27^{Kip1}$ protein expression fluctuates as cells progress through the cell cycle, high in G0/G1 and low after entry into S phase (39, 40). Because the amount of $p27^{Kip1}$ mRNA remains constant throughout the cell cycle (39, 40), $p27^{Kip1}$-regulation is largely post-transcriptional, which is clearly differentiated from that of $p21^{Cip1}$, which is regulated at the transcriptional level. The abundance of $p27^{Kip1}$ within the cell is regulated by at least two factors, the rates of translation (39) and degradation (20). Recent research interest has focused on cell cycle-dependent degradation mechanisms. Pagano *et al.* reported that $p27^{Kip1}$ is degraded through the ubiquitin/proteasome pathway (20). According to these investigators, the human ubiquitin-conjugating enzymes (E2), Ubc2 (Rad6) and Ubc3 (Cdc34), are specifically involved in the ubiquitination of $p27^{Kip1}$. In yeast, the SCF complex containing CDC34 is responsible for ubiquitination of the yeast Cdk inhibitor, Sic1 (41, 42), which suggests that a novel mammalian SCF complex participates in $p27^{Kip1}$ degradation. Sheaff *et al.* (43) and Vlach *et al.* (44) have shown that phosphorylation of Thr187 of $p27^{Kip1}$ by cyclin E/Cdk2 complex is required for $p27^{Kip1}$ degradation. This phenomenon mirrors that of the yeast system, in which phosphorylation of Sic1 by a G1 cyclin-dependent kinase generates the binding site for the SCF ubiquitin-ligase and triggers ubiquitination and subsequent degradation of the yeast Cdk inhibitor (41, 42). Recently, three research groups reported that the mammalian SCF complex containing Skp2 functions as a ubiquitin ligase specific for $p27^{Kip1}$ (45-47).

The breakdown of $p27^{Kip1}$ is regulated not only in time, but also in space. In an attempt to search for novel cellular factors which regulate $p27^{Kip1}$, we isolated Jab1, the specific interactor of $p27^{Kip1}$ (48). Ectopic expression of Jab1 facilitates the degradation of $p27^{Kip1}$, overcomes the cell cycle arrest mediated by this Cdk inhibitor and reduces the serum dependency of the cells. These results clearly indicate that Jab1 is a negative regulator of $p27^{Kip1}$. Interestingly, overexpression of Jab1 induces translocation of $p27^{Kip1}$ from the nucleus to the cytoplasm. Jab1 does not function as the ubiquitin ligase or the specific protease, but rather directly binds to $p27^{Kip1}$ and delivering it to the ubiquitin-conjugation system located in the cytoplasm. Jab1-mediated $p27^{Kip1}$ degradation requires Thr187-phosphorylation of $p27^{Kip1}$. The obvious next questions are (I) what is the exact role of Jab1 in $p27^{Kip1}$ degradation other than shuttling it to the cytoplasm? Does Jab1 accelerate $p27^{Kip1}$ phosphorylation, or does Jab1 directly bind to the ubiquitin ligase or proteasome? and (II) how is Jab1 activity regulated during the cell cycle? Is Jab1 induced to express in response to growth factor stimulation, or is Jab1 subject to post-translational modifications such as phosphorylation or interaction with other cellular factors? Regardless of the exact mechanisms, identification of Jab1 as a negative regulator of $p27^{Kip1}$ should aid explaining how the cell-cycle-dependent proteolytic machinery selects the specific target proteins for degradation.

In proliferating cells, the inhibitory activity of $p27^{Kip1}$, expressed at a low level, is neutralized by other cell cycle regulators. The cyclin D/Cdk4 complex exhibits a higher affinity with $p27^{Kip1}$ than the cyclin E/Cdk2 complex does, therefore, most of the $p27^{Kip1}$ proteins expressed in

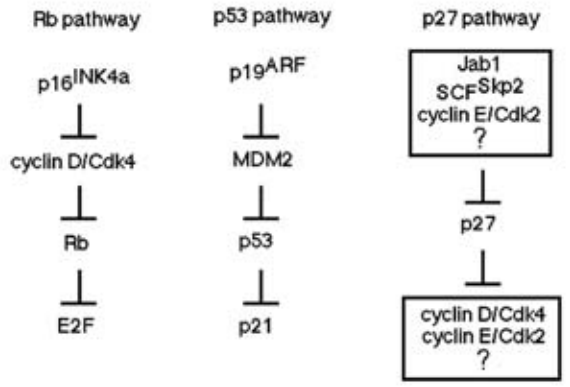

Figure 2. A putative model of three distinct tumor suppressing pathways

proliferating cells are sequestered to a large pool of the cyclin D/Cdk4 complex (49). In another example, proto-oncoproteins, myc and ras, facilitate progression through the G1 phase by downregulating $p27^{Kip1}$. Interestingly,induction of c-myc neutralizes the inhibitory activity of $p27^{Kip1}$ without downregulating expression level of the protein (50). In cells transfected with the c-myc vector, $p27^{Kip1}$ is separated from the cyclin/Cdk complex and moves into an ill-defined large protein complex, suggesting that an unidentified c-myc target gene product sequesters $p27^{Kip1}$. Finally, the E1a protein, an oncoprotein encoded in the adenovirus genome, binds to the C-terminus domain of $p27^{Kip1}$ and neutralizes the inhibitory activity (51).

6. COOPERATIVE ACTION OF E2F AND CYCLIN E/Cdk2 IN S PHASE INDUCTION

The transcription factor E2F and cyclin E/Cdk2 kinase are situated the furthest downstream of the known G1 regulatory factors and maximum activation occurs near the G1/S border (2, 22), suggesting that their functions correlate most with initiation of chromosomal DNA replication instead of progression through G1. The finding that ectopic overexpression induces DNA replication in quiescent cells (24, 25) supports this view.

pRb suppresses E2F activity by direct binding (12, 22). Phosphorylation of pRb by cyclin E-activated Cdk2 kinase neutralizes pRb function and facilitates E2F activation (12, 21). These mean that cyclin E is situated upstream of E2F. On the other hand, the cyclin E gene is one of the most important E2F-inducible genes (22, 23), indicating that cyclin E is located downstream from E2F. Thus, E2F and cyclin E (cyclin E/Cdk2 complex) create a positive feedback loop which allows for a rapid rise of both activities as cells approach the G1/S boundary (2).

The most important question to address is the precise roles of E2F and the cyclin E/Cdk2 complex in S phase induction. It seems likely that both E2F and cyclin E/Cdk2 activities are required to initiate DNA replication. However, ectopic expression of either E2F (24) or cyclin E (25) has been reported to be sufficient to initiate replication without activating the other, revealing the complexity of the E2F/cyclin E pathway. To analyze the functional roles of E2F and cyclin E/Cdk2 complex from a different point of view, we developed a system utilizing *Xenopus* oocytes (52). Although no chromosomal DNA replication actually occurs during *Xenopus* oocyte maturation, the capability develops during the late meiosis I (MI) phase but is suppressed until fertilization. Injection of dominant negative E2F or universal Cdk inhibitors (p21 and p27) blocked induction of the DNA replication ability, while co-injection of E2F-1 and cyclin E initiated DNA replication. Interestingly, neither E2F-1 nor cyclin E alone was sufficient to induce replication, and cyclin E was not regulated at the transcriptional level in this particular system. Thus, differing from D-type cyclins and pRb, E2F-1 and cyclin E are more fundamentally connected with the DNA-replication-initiation machinery. Furthermore, not being restricted to somatic cell cycle regulation, they play an important role in a wide variety of situations.

In a mammalian system, induction of E2F-1 alone is sufficient to initiate DNA replication (53-55). Utilizing the mouse fibroblast system, we found that both Cdk2-dependent and –independent pathways have roles in E2F-mediated S phase induction (Unpublished observations). Our conclusions are as follows: (I) Cyclin E induction and successive Cdk2 activation, but not that of cyclin D and Cdk4, are required for S phase entry. (II) The loading of MCM proteins onto chromatin depends on E2F activity but not on activity of Cdk2. (III) Induction of Cdc45 expression depends solely on E2F but binding to chromatin requires Cdk2 activation. E2F-inducible genes other than cyclin E include a number of genes, the products of which function to regulate cell cycle progression and DNA replication (22), and candidate substrates for the cyclin E/Cdk2 complex contain pRb (12, 21), MCMs, CDC45-associated proteins (16), and NPAT (17). Therefore, molecular mechanisms of S phase induction mediated by E2F and cyclin E are far more complex than heretofore considered to be.

7. PERSPECTIVE

Among regulators of the cell cycle progression, only those specific for G1 phase are often found altered in human tumors (2), indicating that G1 regulation is highly connected with tumor suppression. From this point of view, there are three distinct signaling pathways (figure 2). The first is the Rb pathway composed of $p16^{INK4a}$-Cdk inhibitor, cyclin D/Cdk4 complex, and Rb protein (3). The second is the p53 pathway, which includes $p19^{ARF}$, MDM2, and p53 (56). Interestingly, the furthest upstream components of these two pathways are encoded at a single gene locus, the *INK4a* gene. Therefore, deletions or mutations of one gene locus could disrupt two important tumor suppressive pathways. Consistent with this possibility, the mutation rate within the *INK4a* locus is high among a wide variety of human cancers (3). The third is the p27 pathway (57, 58). This idea stems from findings that the reduced expression of $p27^{Kip1}$ Cdk inhibitor correlates well with short survival time of patients. This correlation was originally found in

breast or colorectal carcinomas (59-61) but recent data indicate that this is the case for most types of cancers. Because malignant tumors expressing low levels of p27 contain wild-type p27 genes, target genes should encode proteins functioning upstream of p27. Although the molecular identity of such gene products remains to be investigated, our finding that Jab1 is a negative regulator of p27 (48) will aid in understanding details of this pathway.

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