P27^{Kip1}: Regulation and Function of a Cell Cycle Key Regulator

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REVISIÓN

ABSTRACT

The p27^{Kip1} protein has a dual role in the G1 phase of the cell cycle: it inhibits CDK2 complexes in resting cells and during the G1 phase of the cell cycle, and promotes the assembly and nuclear import of cyclin D–CDK4 complexes by binding to them, thus making the cyclin D–CDK4-bound p27 unavailable for cyclin E-CDK2 binding. The unbound cyclin E-CDK2 can become activated and drive the cells through the G1/S phase. P27 is the only cyclin-dependent kinase inhibitor known to date that has a unique role between cell cycle and mitotic stimuli. P27 functions as a major gatekeeper of the quiescent state in mammalian cells. The P27 protein is abundant in quiescent cells, in contact-inhibited cells, and in cells treated with antimitotic stimuli. Conversely, p27 expression is low in certain aggressively growing tumors and after mitotic stimulation. Several cellular and viral oncogenes are able to downregulate p27 protein. Recently, new functions for p27 have been described, including its role in apoptosis, and drug sensitivity, reinforcing the need to extend the analysis of this important protein. The purpose of this paper is to provide a comprehensive review of current knowledge on p27 functions as a positive and negative regulator of G1 phase progression that could make it an attractive candidate as a therapeutical target.

Keywords: apoptosis, cell cycle, cyclin-dependent kinase inhibitor, p27^{Kip1}, progression in G1

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RESUMEN

P27^{Kip1}: Regulación y función de un regulador clave del ciclo celular. La proteína p27^{Kip1} tiene un papel dual en la fase G1 del ciclo celular: inhibe los complejos CDK2 en células arrestadas y durante la fase G1 del ciclo y además, mediante su unión a los complejos ciclina D-CDK4 promueve el ensamblaje y la entrada al núcleo de los mismos, de manera tal que la p27 unida a dichos complejos es incapaz de unirse al complejo ciclina E-CDK2. El complejo ciclina E-CDK2 libre, es entonces activado y guía a las células a través de la fase G1/S. La p27 es hasta la fecha, el único inhibidor conocido de quinasas dependientes de ciclinas que tiene un papel fundamental entre el ciclo celular y el estímulo mitótico. P27 es abundante y funciona como guardián del estado de quiescencia en las células de mamíferos inhibidas por contacto y en células tratadas con estímulos antimitóticos. Varios oncogenes celulares y virales pueden regular negativamente esta proteína. Recientemente, se describieron nuevas funciones para p27 en la apoptosis y en la respuesta a drogas farmacéuticas, haciendo necesario profundizar en el análisis de esta importante proteína. El objetivo del presente artículo es proporcionar al lector una revisión abarcadora acerca de las funciones conocidas de p27, tanto como regulador negativo como positivo de la progresión de la fase G1, las que podrían hacer a esta proteína un blanco terapéutico atractivo.

Palabras claves: apoptosis, ciclo celular, inhibidor de quinasa dependiente de ciclina, p27^{Kip1}, progresión en G1

Introduction

Progression through or the exit from the eukaryotic cell division cycle is regulated by a series of stringent control mechanisms. The most commonly depicted cell cycle (Figure) consists of two major phases, one where replication of genome occurs (S phase) and another responsible for the segregation of the duplicated genome into daughter cells (mitosis or M phase). These two phases are separated by gaps; G1 between M and S and G2, between S and M. Cells in G0 or G1 can be stimulated by mitogens (growth factors) to progress through G1 towards the S phase. This transition is mitogen-dependent until the cells reach the "restriction point" (R). After this point, cells are irreversibly committed to DNA synthesis regardless the presence of the mitogenic signal. Thus, the restriction point represents a critical checkpoint in the cell cycle. Here, the integration of an array of endogenous and exogenous signals leads either to cell cycle arrest or continuation through the cell cycle to

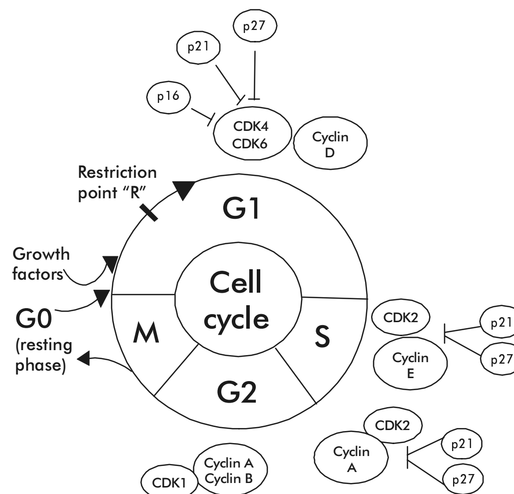

Figure. Schematic diagram of the cell cycle. It is marked by two primary events, DNA synthesis (S phase) and mitosis (M). These phases are separated by gaps, G1 and G2. The cell may also leave the cell cycle (G0) in the absence of mitogenic stimuli to enter a quiescent state or when terminally differentiated. Rate limits to cell cycle progression is the orderly appearance of the cyclins. The D-type cyclins, which associate primarily with the cyclin dependent kinases, CDK4 and CDK6, are expressed first after growth factor stimulation. These are followed by cyclin E, which binds CDK2 exclusively, at the G1-S boundary. Subsequently, cyclins A and B increase in levels as cells approach M. Cyclin-CDK complexes are inhibited by small proteins named CDK inhibitors, the loss of which may contribute to uncontrolled progression through the cell cycle.

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mitosis. Due to its irreversible nature, the restriction point is tightly regulated. Passage through this checkpoint is governed by both positive and negative cell cycle regulatory factors. Two families of G1 cyclins and several classes of cyclin counterparts, the cyclin-dependent kinases (CDKs) cooperatively regulate mitogen-dependent progression through G1 and the initiation of S: D-type cyclins (cyclin D1, D2, and D3) and cyclin E (cyclin E1 and cyclin E2) [1]. The D and E types of cyclins have specificity for different CDK subunits. The D-type cyclins bind and activate CDK4 and CDK6 exclusively, while cyclin E form kinase complexes predominantly with CDK2 [1]. Cyclin E/CDK2 is believed to function downstream of cyclin D [2]. The principal cellular targets of the G1 cyclin-dependent CDKs are the retinoblastoma protein (pRB) family of "pocket proteins", consisting of pRB, p107 and p130 [3]. In their hypophosphorylated forms, these pRB-related pocket proteins associate with members of the E2F family of transcription factors, thereby negatively regulating transcription activity of E2F-regulated genes, which are important for their entry into the S phase of the cell cycle [3, 4]. The activities of CDKs are in turn constrained by CDK inhibitors (CKIs). CKIs that govern these events have been assigned to one of two families based on their structures and CDK targets (Table). The first class includes the INK4 proteins (inhibitors of CDK4), so named for their ability to specifically inhibit the catalytic subunits of CDK4 and CDK6. Four such proteins: $p16^{INK4a}$ [10], $p15^{INK4b}$ [11], $p18^{INK4c}$ [12], and $p19^{INK4d}$ [13] are composed of multiple ankyrin repeats and bind only to CDK4 and CDK6 but not to other CDKs. The other group includes more broadly acting inhibitors, the Kip/Cip family whose actions affect the activities of cyclin D-, E- and A-dependent kinases. The Cip/Kip family comprises $p21^{Cip1/Waf1}$, the first product of a p53-regulated gene and the first mammalian CKI to be discovered [14–16] and two other CKIs, $p27^{Kip1}$ [17] and $p57^{Kip2}$ [18]. More recent work has altered the view of Cip/Kip proteins as CKIs and revealed that these proteins could act as positive regulators of cyclin D-dependent kinases [1]. Several excellent reviews focused on the functions of $p27^{Kip1}$ and its abnormalities found in human tumors have been recently published [8, 19, 20].

Here we focus on structural and functional characterization of $p27^{Kip1}$ as both a negative and positive regulator of G1/phase progression. Moreover, we will discuss how a variety of physiological and viral proteins impact p27 function by multiple and distinct mechanisms.

Table. Cyclin-dependent kinase inhibitors.

CKI	Synonym	Chromosome	Target cyclin-CDK	Ref.
INK4 family				
P15	INK4a, MTS2	9p21	D-CDK4,6	[5]
P16	INK4b, MTS1	9p21	D-CDK4,6	[6]
P18	INK4c	1p32	D-CDK4,6	[7]
P19	INK4d	19p13.2	D-CDK4,6	[7]
CIP/KIP family				
P21	CIP, WAF1, SDI1	6p21.1	D-CDK4,6; E-CDK2 A-CDK2	[4]
P27	Kip1	12p12-13	D-CDK4,6; E-CDK2 A-CDK2	[8]
P57	Kip2	11p15.5	D-CDK4,6; E-CDK2 A-CDK2	[9]

MTS, multiple tumor supressor.

P27 Gene and Protein Structure

The human $p27^{Kip1}$ (hereafter p27) gene resides in a region of chromosome 12p12-13 that is frequently rearranged or deleted in hematologic malignancies [21–23]. It contains two coding and one noncoding exons. The mouse p27 gene is similar to the human p27 gene and its cDNA sequence is more than 90% homologous to the human p27 cDNA. The cDNA for p27 was cloned independently by using protein sequence information from TGFβ-induced growth arrested cells [17] and by the two-hybrid system using the cyclin D/cdk4 complex as bait [24]. The p27 cDNA encodes a nuclear phosphoprotein of 198 amino acids in humans and 197 amino acids in mice. Like other members of the Cip/Kip family, p27 has a CDK-binding domain in the N-terminus, which binds to and inhibits cyclin D-, E-, A-, and B-dependent kinases [25]. This inhibitor shares 47% of the amino acid identity with p21. In contrast to p21, p27 does not bind proliferating cell nuclear antigen (PCNA), it is not regulated by p53 and its protein levels are high in most quiescent cells.

Many, if not all, of the nuclear proteins contain a nuclear localization signal (NLS), which promote the active transport into the nucleus [26]. Classical NLSs are short sequences containing several essential basic amino acids. There are two major types of NLS: a monopartite NLS composed of a single cluster of basic amino acids separated by a spacer region composed of non-basic amino acids. It has been suggested that p27 contains a putative bipartite NLS at the C-terminal region, which consists of an N-terminal cluster of 3 basic residues and a C-terminal cluster of two basic residues [27]. However, the function of this region 152-166 as a NLS has not yet been proven, and the minimal requirement of amino acid residues for the nuclear localization of p27 has not yet been determined. Based on the phenotype of progressive C-terminal deletion mutants of p27, its nuclear import was thought to depend exclusively on the integrity of a bipartite nuclear import signal [24]. However, a triple point mutant that disrupts the NLS only partially abolishes nuclear import of p27 *in vivo.* In a direct comparison, this phenotype differs strikingly from a C-terminal deletion mutant that removes amino acids 91–197. Therefore, deletion of the conserved C-terminus removes additional elements that directly or indirectly influence the apparent nuclear localization of p27 [29].

The crystal structure of the p27 – cyclin A – Cdk2 complex reveals that p27 invades the catalytic subunit to dismantle its ATP binding site and that p27 has separate binding sites on the cyclin and CDK subunits, this explains how the Kip/Cip inhibitors can bind the isolated subunits. The binding to the cyclin – CDK complex is significantly tighter, consistent with cooperative binding to the two subunits [30].

Transcriptional Regulation

While p27 levels are generally constant during the cell cycle, there are certain circumstances when p27 is

regulated at the transcriptional level. Yang *et al.* presented evidence that demonstrated that the p27 gene is a target of transcriptional repression by c-Myc in WEHI 231 immature B cells. Inhibition of the TATA-less p27 promoter by c-Myc was also observed by the same group in Jurkat T cells, vascular smooth muscle, and Hs578T breast cancer cells, extending the observation beyond immune cells [31]. Regulation at the mRNA level also occurs after androgen deprivation of mammary carcinoma cells [32], IFNα2b treatment of human lung carcinoma cells [33], by hypoxia and independent of HIF-1 in murine fibroblasts [34], and in androgen-independent, prostate-specific antigen-positive prostate cancer cells (LNAI cells) established from xenograft tumors of the androgen-dependent LNCaP cell line [35].

The human p27 promoter is TATA-less, and the sequence is highly homologous to the murine p27 promoter sequence [36]. It contains binding sites for several transcription factors including Sp1, CRE, Myb, NFκB, IRF-1, and AFX. AFX is a member of the forkhead family of transcription factors that integrates the PI3K/AKT and Ras signaling pathways to regulate transcription of p27. In prostate cancer, inactivation of the tumor suppressor gene pTEN, which acts as a phosphatase for phosphatidylinositol-3,4,5 triphosphate (PIP_3) leads to the constitutive activation of AKT via the PI3K pathway [37]. AKT phosphorylates and inactivates the forkhead transcription factor AFX and suppresses p27 transcription [35]. IL-3 was found to repress the expression of p27 through activation of PI3K, and this occurs at the level of transcription. This transcriptional regulation occurs through modulation of the forkhead transcription factor FKHR-L1, and IL-3 inhibited FKHR-L1 activity in a PI3K-dependent manner, providing a novel mechanism of regulating cytokine-mediated survival and proliferation [38]. During the early stages of the myelomonocytic U937 cell line, it was shown that vitamin D_3 stimulates the transcription of the p27 gene by a novel mechanism involving Sp1 and NF-Y, but not the vitamin D receptor [39]. The transcriptional activity of the human p27 promoter driving the luciferase reporter gene was activated by IFNα2b in human lung carcinoma cells transiently transfected with this construct most probably by a mechanism involving the IRF-1 transcription factor [40] in correspondence with previous results where an increase of the p27 mRNA upon IFNα2b addition was observed [33].

Although some experiments have shown enhanced degradation by the ubiquitin proteasome pathway as the principal mechanism that regulates levels of p27 (see below), it is possible that epigenetic mechanisms such as DNA methylation or genomic imprinting could also be involved in the inactivation of the p27 gene. Few detailed studies of p27 methylation status in normal and transformed human tissue have been published. In rodent pituitary cell lines and melanocytes, p27 abundance was reduced compared to their normal counterparts. In rat GH3 and mouse GHRH-CL1 pituitary cells, methylated cytosines were observed in exon 1 whereas dense methylation of a CpG island in the promoter region of p27 was detected in 4 of 45 metastatic melanoma (9%) and 3 of 35 cell lines (9%) derived from malignant melanoma [41]. Genomic imprinting is an epigenetic modification in the germline leading to parental allele-specific gene expression in somatic cells. It has been found that imprinted genes can be abnormally expressed or silenced in tumors and that the CKI p57[Kip2] is normally imprinted with preferential expression of the maternal allele [42]. Analysis of the imprinting status of p27 in fetal and adult tissues revealed biallelic expression in all tissues examined [43]. Thus, no evidence for genomic imprinting was found.

Another possibility is that p27 is inactivated at a post-transcriptional level. Comparison of immunohistochemical staining and *in situ* hybridization reveals discordance between p27 mRNA and protein levels in human breast, colon, and pituitary tumors [44–46].

Recently, an element within the 5'-untranslated region (UTR) of the p27 mRNA has been described that is required for the efficient translation of p27 in proliferating and quiescent cells [47]. The proteins that bind *in vitro* to this p27 5' UTR element, HuR and hnRNPC1, are governed in a cell cycle dependent manner. The mutation status of genes whose proteins bind to the p27 5' UTR and posttranslational modifications of such proteins will require further investigation in human cancers.

Nearly all eukaryotic mRNAs are translated through a mechanism involving the recognition of the 5' cap by the eukaryotic initiation factor 4E (eIF4E). In quiescent cells eIF4E activity is repressed, leading to a global decline in translation rates. In contrast, p27 translation is highest during quiescence, suggesting that it escapes the general repression of translational initiation [25]. Miskimins *et al.* demonstrated that the 5' UTR of the p27 mRNA mediates cap-independent translation [48]. This activity is unaffected by conditions in which eIF4E is inhibited. In D6P2T cells, elevated cyclic AMP levels cause a rapid withdrawal from the cell cycle that is correlated with a striking increase in p27. Under these conditions, cap-independent translation from the p27 5' UTR is enhanced. These results indicate that the regulation of the internal initiation of translation is an important determinant of p27 protein levels.

Regulation by Physiological Signals

Various mechanisms for inducing growth arrest in normal cultured cells increase p27 levels including TGFβ [49], lack of IL-2 [50], low serum [51], N-cadherin-mediated contact inhibition [52], and growth in suspension [53]. In TGFβ-arrested epithelial cells, p27 is competing with p15[INK4b] in binding to cyclin D1/CDK4 [54]. Upregulation and binding of p15 to CDK4 serves to destabilize the association of p27 with Cyclin D1/CDK4 and promotes p27 binding to cyclin E/CDK2. In TGFβ-treated epithelial cells, upregulation of p15 protein and increased binding of p15 to Cyclin D1/CDK4 occurs concomitant with the reduction of CDK4-associated p27 and the stabilization of the association of p27 with Cyclin E/CDK2 complexes [54]. P27 mediates responses to several other growth inhibitory signals, including cAMP in macrophages [55], rapamycin in T-lymphocytes [50], IFNβ in human gastric cells [56], IFNγ in mammary epithelial cells [57], and IL-6 in melanoma cells [58]. Signaling through the receptors for oncostatin M and IL-6 leads to the

accumulation of p27 and G1 arrest, and seems to involve the extracellular regulated kinase (ERK) signal and STAT 5 pathways [59]. Although cyclin E/CDK2 and other CDK2-containing cyclins seem to be the principal targets in p27-mediated growth arrest, vitamin D-treated promyelocytic leukemia cells show a specific increase in CDK6-associated p27 protein [60]. P27 has also been demonstrated to mediate G1 arrest by a number of drugs, including lovastatin [61].

The over-expression of the tumor suppressor gene PTEN leads to the suppression of cell growth through the blockade of cell cycle progression, an increase in the abundance of p27, a decrease in the protein levels of cyclin D1 and the inhibition of Akt phosphorylation [62].

Several growth factors trigger a rapid decrease in p27 expression. In breast cancer cells estrogens stimulate cell cycle progression through loss of p27 and p21 [63, 64]. IL-2 causes a decrease of p27 in T-cells, allowing CDK2 activation and entry into the S phase [65]. Stimulation with mitogens such as platelet-derived growth factor (PDGF) or serum also leads to p27 downregulation and growth arrest in 3T3 fibroblasts [66] and epithelial cells [67]. Mitogen-induced Ras signaling promotes the induction of cyclin D1 and downregulation of p27 [68–70]. Expression of oncogenic Ras in epithelial tumor cells is linked to the loss of TGFβ anti-proliferative activity through the disruption of TGFβ-mediated growth inhibition due to a lack of CDK2 inhibition, caused by the sequestration of p27 and CDK2 in different subcellular compartments and by the loss of TGFβ-induced partner switching of p27 from CDK6 to CDK2 [71].

Another oncogen, BCR/ABL suppresses p27 protein levels through PI3K/AKT in hematopoietic cells, leading to its accelerated entry into the S phase [72]. Upon downregulation of p27, cells progress through the G1 phase and often become independent of mitogens. Cancer cells have various means of deregulating p27 expression and to render it unable to interfere with cyclin E/Cdk2 induction, for instance, they are known to display up-regulation of ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC), the key enzymes in the biosynthesis of polyamines that are essential for cellular proliferation. The expression of ODC or AdoMetDC affect cell cycle regulation through the constitutive downregulation of p27 and its loss from the cyclin E/Cdk2 complexes [73].

Degradation of p27

The elimination of p27 during the late G1 phase is required for G1 cyclin/CDK complex activation and cell cycle progression from the G1 to the S phase in various cells lines [50, 51, 74]. Two mechanisms are responsible for p27 breakdown: 1) ubiquitin-mediated and 2) proteolytic-processing [75]. The ubiquitin-mediated and proteasome-dependent degradation of p27 can be triggered by phosphorylation by at least three kinases, CDK2/cyclin E [76], Jab1 [77] and cyclin K/CDK 6 [78]. P27 binds to cyclin E–CDK2 with high and low affinities. Initially, p27 binds with low affinity acting as a substrate, and then the binding slowly shifts to high affinity and p27 becomes an inhibitor [79]. *In vivo,* degradation of p27 requires active CDK2 kinase and the mutation of Thr187 inhibits degradation; *in vitro,* phosphorylation of Thr187 is required for ubiquitination and subsequent degradation of p27 by the proteasome complex. Therefore, the ubiquitin-proteasome pathway was suggested to be involved in the p27 degradation in mammals [75, 80, 81]. The ubiquitin-proteasome pathway is emerging as a major and universal mechanism that regulates the selective and time-controlled elimination of short-lived key regulatory proteins, e.g. p53 [82], IκB [83], β-catenin [84] and others. This pathway requires adenosine triphosphate (ATP) and the covalent conjugation of target proteins with multiple ubiquitin molecules [85]. This multistep process involves ubiquitin activation by a ubiquitin-activating enzyme (E1), followed by the transfer of ubiquitin to a ubiquitin-conjugating enzyme (E2), and the third step is the transfer of ubiquitin to an ubiquitin-ligase (E3), which catalyzes the formation of isopeptide bonds between the C-terminal glycine of ubiquitin and the ε-amino groups of lysine residues on the target proteins. During subsequent cycles, additional ubiquitin molecules are added to the substrate. Then, multi-ubiquitinated proteins are recognized by the 26 S (1500 kD) proteasome complex and rapidly degraded into short peptides. The proteasomes 26 S are multicatalytic protease complexes containing chymotrypsin-like, trypsin-like, and postglutamyl activities together with ATP. Skp2 has been identified as a candidate component of the E3 ligase complex that ubiquitinates p27 [86]. This is an F-box protein that associates with Skp1, Cul1, and Roc1/Rbx1 to form the SCF (Skp2) ubiquitin ligase complex [87]. The ectopic expression of skp2 in quiescent cells stimulates the entry into the S phase of the cell cycle and promotes degradation of p27 [86]. A mutant p27 protein (T187A), which cannot be phosphorylated at Thr187, is resistant to Cdk2, Skp2, and proteasome-mediated degradation [88], thus providing evidence that the amount of both p27 and skp2 are rate limiting for p27 degradation and the transition from quiescence into a proliferative state. In human glioblastoma cells, ectopic pTEN expression led to p27 accumulation, which is accompanied by a reduction of SKP2 through downregulation of the PI3K pathway [89].

Recently, it was demonstrated that p27 ubiquitination activity in cell extracts depends on the presence of the ubiquitin-like protein Nedd8 and enzymes that catalyze Nedd8 conjugation to proteins [90]. Consistent with a role in cell-cycle progression, Nedd8 is expressed in proliferating cells and is itself downregulated upon cellular differentiation. These results suggest that the Nedd8 conjugation pathway may regulate the turnover of p27, independently of p27 phosphorylation, and further establishes the identity of protein components involved in p27 ubiquitination.

In order to exert its function during G1 and to be degraded at the end of G1, p27 has to be imported into the nucleus [77]. Phosphorylation of p27 by CDK2 is restricted to the cell nucleus due to the nuclear localization of CAK [91], which may account for the requirement for nuclear import in degradation. The Jab1 protein, a component of the 450K COP9/signalosome complex, which phosphorylates IκB, the NF-κB precursor and c-Jun and is structurally similar to the proteasome regulatory complex

has been found to interact with p27 and promote its degradation [92]. The Jab1 protein promotes the p27 export from the nucleus, suggesting that degradation of phosphorylated p27 occurs in the cytosol [77]. The signals mediating the nuclear export of p27 are unknown; however, recent findings indicate that the nuclear-pore-associated mNPAP60 is required for intracellular transport and, hence, the cyclin E-mediated elimination of p27 [29]. Whereas binding of Jab1 to p27 occurs independent of phosphorylation; a phosphorylation step is needed for proteasome-dependent degradation. Whether Jab1 and mNPAP60 act sequentially or in parallel, pathways directing the export of p27 from the nucleus will be an interesting point of further investigation. Recently, it was shown that Grb2 and Grb3-3, the molecules functioning as an adaptor in several signal transduction pathways, specifically and directly bind to p27 in the cytoplasm and participate in the regulation of p27. The interaction requires the C-terminal SH3-domain of Grb2/3-3 and the proline-rich sequence contained in p27 immediately downstream of the CDK binding domain. In living cells, enforcement of the cytoplasmic localization of p27, either by artificial manipulation of the nuclear/cytoplasmic transport signal sequence or by the coexpression of ectopic Jab1, markedly enhances the stable interaction between p27 and Grb2. An over-expression of Grb2 accelerates Jab1-mediated degradation of p27, while Grb3-3 expression suppresses it. A p27 mutant unable to bind to Grb2 is transported into the cytoplasm in cells ectopically expressing Jab1 but is refractory to the subsequent degradation [93]. These findings indicate that Grb2 participates in the negative regulation of p27 and may directly link the signal transduction pathway with the cell cycle regulatory machinery.

In parallel with its ubiquitin-dependent degradation, p27 can be processed rapidly at its N-terminus, reducing its molecular mass from 27 to 22 kD, by an ubiquitination-independent but ATP-dependent mechanism with a greater activity during the S than in the G0/G1 phase [75]. This 22 kD intermediary has no cyclin-binding domain at its N-terminus and virtually no CDK2 kinase inhibitory activity.

Although the phosphorylation of p27 on Thr 187 has been shown to be essential in controlling the stability of p27 by ubiquitin-mediated degradation, it was recently discovered that the extent of the phosphorylation of p27 on Thr 187 represents only ~1% of the total extent of phosphorylation of this protein *in vivo.* In contrast, phosphorylation of Ser 10 accounts for 70% of the total extent of phosphorylation of p27 and it is regulated in a cell cycle-dependent manner [94]. Furthermore, the extent of phosphorylation at this site is increased in resting cells, and Ser 10 phosphorylation affects both protein stability and was apparent in various types of cells from several species. These data suggest that phosphorylation of Ser 10 may represent another important mechanism by which the stability of p27 is regulated. However, both the kinase and the phosphatase responsible for phosphorylation and dephosphorylation of Ser 10, respectively as well as the mechanism by which phosphorylation of Ser 10 stabilizes p27, remain unidentified. It will be interesting to see if the regulation of p27 stability by phosphorylation of Ser 10 is linked to external mitogenic signals.

Alternative Inactivation of p27

Unlike classic tumor suppressor genes such as p53 and Rb, the p27 gene contains only rare somatic mutations and shows a low frequency of allelic loss. The loss of heterozygosis (LOH) within the p27 region as defined by the markers D12S133 proximally and D12S142 distally is a feature of certain human cancers, including acute myeloid leukemia, acute lymphoblastic leukemia, ovarian cancer, and prostate cancer [22, 95–97]. Of the more than 500 tumors analyzed, only a handful of somatic mutations in p27 have been detected [98]. Although the majority of bone marrow samples from acute myeloid and acute lymphoblastic leukemia patients show hemizygous deletion of p27 by fluorescent *in situ* hybridization (FISH), no inactivating mutations in the remaining p27 allele have been observed [22]. Analysis of 66 breast tumors revealed only one inactivating mutation of p27, a point mutation leading to the creation of a nonsense codon at position 104 and premature protein truncation [99, 100]. This somatic mutation was accompanied by LOH of markers near p27 on chromosome 12p13, indicating that the loss of p27 played an important role in the genesis of the invasive ductal carcinoma. Additionally, homozygous deletion of the p27 gene was detected by Southern hybridization in one B/immunoblastic lymphoma and in one acute adult T-cell leukemia/lymphoma, indicating that the p27 genetic loss plays a role in the development of at least a small percentage of hematologic malignancies [101].

Consequences of the p27 Gene Inactivation

In 1996 three independent groups created p27 knockout mice using homologous recombination and reported similar phenotypes [102–104]. Targeted disruption of the murine p27 caused a gene dose-dependent increase in animal size, with thymus, pituitary, and adrenal glands and gonadal organs exhibiting striking enlargement. Growth was attributed to an increase in cell number, due to increased cell proliferation. However, increased growth occurs without an increase in the amounts of either growth hormone of IGF-I [102]. In addition, the development of ovarian follicles was impaired, resulting in female mice infertility. Luteal cell differentiation was impaired and a disordered estrus was detected reflecting a disturbance of the hypothalamic-pituitary-ovarian axis. Similar to mice with the Rb mutation, $p27^{-/-}$ mice often develop pituitary tumors spontaneously. The retinas of the mutant mice showed a disturbed organization of the normal cellular layer pattern. Unexpectedly, the cell cycle arrest mediated by TGFβ, rapamycin, or contact inhibition remained intact in $p27^{-/-}$ cells, suggesting that p27 is not absolutely required in these pathways.

An advantage of targeting p27 has been found in transplantation. One group demonstrated that the loss of p27 enhanced the transplantation efficiency of hepatocytes transferred into diseased livers [105]. They found that compared to control hepatocytes, p27 knockout hepatocytes proliferate better in diseased livers to reverse liver failure. The potential

benefit of inactivating p27 is not restricted to hepatocytes as demonstrated by others, who have shown that p27 knockout hematopoietic stem cells had a repopulation advantage after bone marrow transplantation [106].

Interaction of p27 with Oncoproteins

DNA tumor viruses have evolved a number of mechanisms by which they deregulate normal cellular growth. The high-risk types of human papillomavirus (HPV) are agents in the etiology of cervical carcinoma. The products of two early genes, E6 and E7, appear to be the main transforming proteins [107]. Studies of various monolayer cell culture systems have shown that the E7 oncoprotein of human papillomavirus type 16 is able to neutralize or bypass the inhibitory effect of the cell cycle-dependent kinase inhibitors and induces the S phase entry of mammalian cells in the presence of antiproliferative signals [108]. E7 can bypass G0/G1 arrest in response to both serum withdrawal and loss of cell adhesion, two experimental conditions in which elevated levels of the CDK inhibitor p27 accompany cell cycle progression. Besides, it has been shown that E7 can antagonize the ability of p27 to block cyclin E-associated kinase *in vitro* and to inhibit transcription from the cyclin A gene in transfection experiments. E7 associates with p27 both in a reconstituted *in vitro* system and in extracts of mammalian cells, and this association requires the C-terminal part of E7. This interaction between p27 and E7 can also be demonstrated in a yeast two-hybrid system [109]. Therefore, the data suggest that the ability of E7 to override certain forms of G0/G1 arrest is mediated, in part, by binding to and subsequent inactivation of p27.

The adenovirus oncoprotein E1A can not only bind directly to pRB to overcome TGFβ-induced cell cycle arrest, but also directly affects p27 by binding to it and blocking its inhibitory activity in mink lung epithelial cells [110]. This view has been challenged by a report showing that free p27 levels in E1A-expressing Rat1 cells are increased, and yet the cells are proliferating [111]. The polyoma virus small T antigen (PysT) triggers phosphorylation and degradation of p27 protein indirectly by binding to protein phosphatase 2A (PP2A) [112]. The interaction between PP2A and PysT has been shown to induce the MAPK pathway, which could be one mechanism for the phosphorylation of p27.

Recently, a novel mechanism has been described by which viral proteins can subvert normal growth control: a cyclin encoded by human herpesvirus 8 (also known as Kaposi's sarcoma-associated herpesvirus), cyclin K, can confer resistance to its associated catalytic CDK subunit against the CDK inhibitor proteins [78]. K cyclin forms an active complex with CDK6, which is resistant to inhibition by $p16^{INK4A}$, $p21^{CIP1}$ and $p27^{KIP1}$. The mechanism by which cyclin K overcomes a p27-imposed arrest is that this complex can phosphorylate p27 on Thr 187, thus triggering its degradation by the proteasome [113].

The Positive Role of p27 in Proliferation

Cyclin D–CDK can sequester Cip/Kip proteins without being subject to inhibition, whereas the catalytic activities of complexes containing CDK2 are efficiently extinguished by these same CKIs. Not only are cyclin D–CDK complexes resistant to Cip/Kip inhibition but also, their activation is actually facilitated by their interactions with these CKIs. This was first demonstrated by LaBaer *et al.,* who, on recognizing that Cip/Kip proteins bind both to cyclin and CDK subunits, directly tested the possibility that the CKIs actively promoted cyclin D–CDK assembly. Their studies demonstrated that both p21 and p27 promoted interactions between D-type cyclins and their CDK partners *in vitro,* primarily by stabilizing the complexes [114]. Results obtained by Parry *et al.* supported this observation [115]. This group showed that Cyclin D–CDK subunit arrangement is dependent on the availability of competing INK4 and p21 class inhibitors. In a later complementary study, Cheng *et al.* observed that the assembly of cyclin D1/D2-CDK4 complexes was impaired in primary mouse embryo fibroblast (MEF) strains taken from animals lacking the p21 gene, the p27 gene, or both [116]. Moreover, it was also observed that CDK4 disruption in Cdk4 (-/-) mice increased binding of p27 to cyclin E/CDK2 and diminished activation of CDK2 accompanied by impaired pRB phosphorylation, which suggests that at least part of the CDK4 participation in the rate-limiting mechanism for the G0-S transition consists of controlling p27 activity [117]. Based on all these data, the idea was suggested that Cip/Kip proteins act as positive regulators of cyclin D–CDK complexes [1]. Observations made with cells that lack particular G1 phase regulators have demonstrated the major role of the D-type cyclins as growth factor sensors, converting environmental signals into fuel for the cell cycle engine. In normal cells, pRB phosphorylation and Cip/Kip sequestration activate cyclin E/CDK2, thereby propelling cells into the S phase and enabling the completion of the cycle in the absence of further mitogenic cues. In the absence of Cip/Kip proteins, however, the loss of D-type cyclin functions may make it more difficult for cells to enter and exit the cycle in response to environmental signals.

The Role of p27 in Apoptosis

Apoptosis in tumors has become a subject of considerable interest in recent years. A balance between proliferation, growth arrest, and apoptosis regulates cell number. Growing evidence suggests that apoptosis frequently occurs in cells in the G1 phase of the cell cycle and arrest in late G1 or S phase can accelerate or potentiate apoptosis [118]. The first suggestions that p27 may play a role in apoptosis came from results obtained with the over-expression of p27 using an adenoviral system in cancer cell lines [119]. Recombinant adenovirus-overexpressing p27 induces not only apoptosis but also cell cycle arrest and the loss of cyclin-CDK activity in human breast cancer cells [120]. *In vivo,* spontaneous apoptosis in some human cancers with high p27 level expression is significantly higher than in tumors with low p27 levels and has been shown to correlate with BAX expression in oral and oropharyngeal carcinomas [121]. In contrast to the studies discussed above, others have reported antiapoptotic effects of p27. In carcinoma cells and in leukemic cell lines p27 has been shown to prevent

drug-induced apoptosis [122–125]. Levkau *et al.* showed that p27 and p21 undergo specific cleavage by CPP32 and/or CPP32-like caspases upon serum deprivation of endothelial cells [126]. Cleavage of these two proteins results in reduction of their association with CDK2 and a drastic activation of cyclin A/CDK2 suggesting that this is a necessary step leading to apoptosis in this epithelial cell line. In transient transfection experiments using etoposide-treated U937 human leukemic cells as a model system, the p23 and p15 N-terminal peptides generated by p27 proteolysis demonstrated an anti-apoptotic effect similar to that induced by the wild type protein, whereas cleavage resistant mutants have lost their protective effect. Stable transfection of a cleavage resistant mutant of p27 sensitizes U937 to etoposide-induced cell death [127]. Ectopic over-expression of p27 in HT29 human colon cancer cells decreases their tumorigenesis *in vivo* in nude mice [128]. This decreased tumor growth was associated with increased p27 protein expression in tumor extracts. Interestingly, the over-expressing-p27 tumors were significantly more resistant to doxorubicin-induced apoptosis than the control tumors. Moreover, it has been also shown that integrin β1 mediated adhesion of myeloma cells to fibronectin may transduce signals that increase p27 protein levels in these cells resulting in the inhibition of drug-induced apoptosis [129]. Similar to these findings, Sethi *et al.* reported that binding small cell lung cancer cell lines to fibronectin via β1 integrins conferred resistance to DNA damaging drugs [130].

These results indicate that p27, which delays tumor growth could also increase tumor resistance to cytotoxic drugs *in vivo.*

Final Remarks

A great deal of knowledge on the role of p27 in apoptosis and cell cycle regulation has accumulated during the last few years. Whether p27 promotes or inhibits apoptosis depends in part on the cleavage status of p27, cell type, and the status of the cell, i.e., transformed versus nontransformed. Cancer cell lines, given their nature, are aberrant in cell cycle regulation, and it is therefore not surprising that associations discovered there are different from findings in normal diploid cells and tissues. It may be possible that certain tumors cells have a mechanism whereby they induce cleavage of p27, which could lead to decreased apoptosis. The reduced expression of p27 has been correlated with poor prognosis in individuals with breast, colorectal and stomach carcinoma and these tumors exhibited high rates of p27 degradation. The mechanisms that control the stability of p27 thus appear important in cancer development. Characterization of these mechanisms should shed light on how the disturbance of cell cycle regulation results in carcinogenesis and it may lead to the development of anti-cancer drugs with new modes of action.

It is still uncertain how p27 levels correlate with the initial response to chemotherapy. The activation of p27 by chemo- and radio-therapeutic agents was once considered to be a marker of biological efficacy and an indication that treatment had restored G1 checkpoint activity, which is impaired in many tumor cells. However, the activation and accumulation of p27 during this therapy might actually contribute to drug resistance. Therefore, it has been suggested that a transient downregulation of p27 prior to the treatment with cytotoxic agents may have a therapeutic benefit whereas a chronic downregulation of p27 would adversely affect patient survival.

The complex interaction of molecules regulating nuclear import and export and availability and degradation of p27 facilitates the fine tuning of the abundance of p27 molecules that are free to interact with and inhibit cyclin E/CDK2 and block cell cycle progression. Any of the molecules impacting p27 levels in the nucleus might be a target for alterations in cancer.

Finally, a thorough understanding of the central role of p27 in cellular growth and its misregulation in cancer should stimulate therapeutic intervention, which would improve the patient's quality of life and life expectancy.

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Biotecnologías aplicadas a la producción de fármacos y vacunas

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En este libro, Albert Sasson ofrece una valiosa y bien documentada información que abarca desde los antecedentes científicos hasta las tendencias actuales y futuras de la comercialización de los fármacos y vacunas obtenidos por métodos biotecnológicos.

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