

The function of p27^{KIP1} during tumor development

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Abbreviations: CDKs, cycle dependent kinases; CKIs, cyclin dependent kinase inhibitors; CRM1, chromosome region maintenance 1 protein; Pin1, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; RSK1, ribosomal S6 kinase 1; SCF, skp1-cullin1-F-box protein; Skp2, S-phase kinase-associated protein 2

Abstract

Timely cell cycle regulation is conducted by sequential activation of a family of serine-threonine kinases called cycle dependent kinases (CDKs). Tight CDK regulation involves cyclin dependent kinase inhibitors (CKIs) which ensure the correct timing of CDK activation in different phases of the cell cycle. One CKI of importance is p27^{KIP1}. The regulation and cellular localization of p27^{KIP1} can result in biologically contradicting roles when found in the nucleus or cytoplasm of both normal and tumor cells. The p27^{KIP1} protein is mainly regulated by proteasomal degradation and its downregulation is often correlated with poor prognosis in several types of human cancers. The protein can also be functionally inactivated by cytoplasmic localization or by phosphorylation. The p27^{KIP1} protein is an unconventional tumor suppressor because mutation of its gene is extremely rare in tumors, implying the normal function of the protein is deranged during tumor development. While the tumor suppressor function is mediated by p27^{KIP1}'s inhibitory interactions with the cyclin/CDK complexes, its oncogenic function is cyclin/CDK independent, and in many cases corre-

lates with cytoplasmic localization. Here we review the basic features and novel aspects of the p27^{KIP1} protein, which displays genetically separable tumor suppressing and oncogenic functions.

Keywords: cell cycle; cyclin-dependent kinase inhibitor p27; cyclin-dependent kinases; tumor suppressor proteins

Introduction

Extracellular environments initiate cell cycle division or arrest by activating or deactivating cycle dependent kinase (CDK) complexes. Since timely regulation of CDK complexes is critical for proper cell cycle regulation, multiple signals can integrate to control the activity of cyclin/CDK complexes. Activity of cyclin/CDK complexes is regulated by the accumulation of cyclins and by phosphorylation/dephosphorylation of specific complex components (Norbury and Nurse, 1992; Sherr *et al.*, 1993; Malumbres and Barbacid, 2005; De Clercq and Inzé, 2006). Another important regulation of G1 cyclin/CDK complexes lies in their association with cyclin dependent kinase inhibitors (CKIs), such as p27^{KIP1}. Cyclin dependent kinase inhibitors are often associated with diseases when mutated or deregulated. The p27^{KIP1} protein binds to various cyclin/CDK complexes throughout the cell cycle, and is one exemplary CDK inhibitor whose misregulation, and not genetic mutation, is found in diverse cancer types (Tsihlias *et al.*, 1999; Besson *et al.*, 2008; Chu *et al.*, 2008).

The p27^{KIP1} protein was first identified as an inhibitor of cyclin E/CDK2 complexes during TGFβ-induced G₁ arrest (Sheaff *et al.*, 1997). The p27^{KIP1} protein inhibits cyclin/CDK activity by binding cyclin/CDK complexes through its N-terminal, blocking ATP binding, and physically occluding the catalytic cleft of the CDK (Russo *et al.*, 1996; Hong *et al.*, 2009; Zhang *et al.*, 2009). A major regulatory mechanism of controlling the p27^{KIP1} inhibitory function is to regulate p27^{KIP1} protein levels through transcriptional, translational, and post-translational mechanisms (Hengst and Reed, 1996; Carrano *et al.*, 1999; Boehm *et al.*, 2002; Bagui *et al.*, 2009; Shin *et al.*, 2009; Trabosh *et al.*, 2009). Recent study revealed that a novel regulatory mechanism during postphosphorylation and polyubiquitination of proteins could be another regulatory mechanism for p27^{KIP1}. Pin1, pepti-

dyl-prolyl isomerase, recognizes and stabilizes p27^{KIP1} when phosphorylated on Thr187 by inducing its conformational change (Zhou *et al.*, 2009). Misregulation that results in increased degradation of p27^{KIP1} can be related to cancer development (Hershko, 2008; Mishra *et al.*, 2009). In addition to TGF β , other environmental factors such as serum starvation or contact inhibition can increase p27^{KIP1} protein whose role becomes essential during cell cycle arrest and differentiation. For instance, serum deprivation increases the amount of p27 phosphorylation during muscle differentiation or quiescence approaching G₀ state of colon carcinoma cells and mitogen stimulation then causes cells to enter G₁ with the translocation of p27 to the cytoplasm (Rodier *et al.*, 2001; Boehm *et al.*, 2002; Deng *et al.*, 2003; 2004; Jin *et al.*, 2009). Phosphorylation, as such, is the mechanism primarily used for regulating p27^{KIP1} activity. The p27^{KIP1} protein possesses multiple tyrosine, serine, or threonine phosphorylation sites (Figure 1). The inhibitory actions of p27^{KIP1} on cyclin/CDK complexes are weakened by phosphorylations directed by some signal transduction pathways (Larrea *et al.*, 2008; Morishita *et al.*, 2008; Tossidou *et al.*, 2008; Jin *et al.*, 2009). It appears that different signalling pathways direct the fate of the protein through differential phosphorylation patterns as Thr187 phosphorylation leads p27^{KIP1} to a SCFSkp2 ubiquitin ligase complex and promotes the polyubiquitination and degradation while T198 phosphorylation of p27^{KIP1} by ribosomal S6 kinase 1 (RSK1) promotes cell motility (Grimmler *et al.*, 2007; Larrea *et al.*, 2009). This suggests that signal transduction pathway utilizes regulatory mechanisms of p27^{KIP1} by phosphorylation to control the activity of cyclin/CDK complexes. Moreover, recent studies suggest that post-translational modification of p27^{KIP1} by phosphorylation can play diverse roles for p27^{KIP1} in regulations on its half life and subcellular localization. While it is evident that nuclear p27^{KIP1} through inactivating

cyclinE/CDK2 complex acts as a tumor suppressor, tumorigenic properties of p27^{KIP1} have also been proposed in recent years especially when located in cytoplasm. (Blagosklonny, 2002; Sicinski *et al.*, 2007; Hidaka *et al.*, 2009). Pro-tumorigenic activities include enhancing cell mobility and assisting assembly of cyclin D/CDK4 complexes, thereby promoting metastasis and cell cycle progression (Besson *et al.*, 2004; Jeon *et al.*, 2008; Kim *et al.*, 2008; Vervoorts and Lüscher 2008). Since cytoplasmic localization can be associated with cell migration promoting activity of p27^{KIP1}, removal of p27^{KIP1} from nucleus to degradation is less oncogenic than translocation of nuclear p27^{KIP1} to the cytoplasm. The puzzling dual function of p27^{KIP1} is intriguing and worth additional attention because it answers some mysteries surrounding p27^{KIP1}, such as existence of the p27^{KIP1} protein in malignant cancers. While potential pro-oncogenic activity of p27^{KIP1} awaits further investigation, several research groups have independently generated null mutants in order to better understand biological function of p27^{KIP1} and to provide better evidence of tumor suppressor activity of the protein.

The p27^{KIP1} null (-/-) mouse shows an overall increase in cell proliferation, resulting in approximately 30% increase in body size, multiple organ hyperplasia, and disorganization of sensory epithelia in the retina and inner ear. In addition, the null mice also display female sterility due to defective ovarian and uterine function (Fero *et al.*, 1996; Chien *et al.*, 2007; Kim *et al.*, 2007). The tumor suppressive activity of p27^{KIP1} using null mice was first demonstrated by development of adenomas in the intermediate lobe of the pituitary gland, and by their inclination to develop tumors more easily when challenged with chemical carcinogens or irradiation (Nakayama *et al.*, 1996). It is likely that combined loss of p27^{KIP1} with other tumor suppressor genes further enhances tumorigenesis

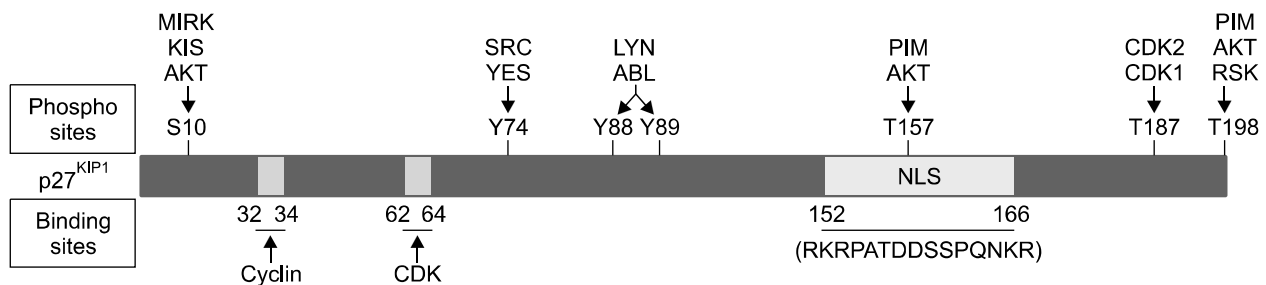


Figure 1. Schematic diagram of p27^{KIP1} phosphorylation and binding sites. Phosphorylation sites (upper) and binding sites (lower) of full length p27^{KIP1} protein are illustrated with the specific site numbers for kinases, or with the binding regions for cyclin/CDK proteins. Nuclear localization sequences (NLS) are also depicted. Note that the threonine subject to AKT phosphorylation, T157, is located within the NLS.

(Di Cristofano *et al.*, 2001; Glover *et al.*, 2009). A 50% reduction in p27^{KIP1} protein level predisposes p27^{KIP1} heterozygous (+/-) mice to tumors in multiple organs, when combined with administration of carcinogens, or when genetically combined with additional oncogenes or tumor suppressors. In humans, p27^{KIP1} deficiency has also been found to be associated with sporadic tumorigenesis. The first human cases reported to have abnormally low amounts of nuclear p27^{KIP1} were associated with increased tumor aggressiveness and a relatively poor clinical outcome for breast and colon cancer (Loda *et al.*, 1997).

Recent findings from knock-out models also show data supporting the pro-oncogenic property of p27^{KIP1} protein. Two groups reported that p27^{KIP1} heterozygous (+/-) mice were more susceptible to mammary and prostate tumors than p27^{KIP1} null (-/-) mice (Muraoka *et al.*, 2002; Gao *et al.*, 2004). Analysis of knock-in mice with CDK mutant p27^{KIP1}, p27^{KIP1} (CK-), that lacks CDK inhibitory function revealed that p27^{KIP1} (CK-/ CK-) mice not only displayed the tumor development phenotype of p27^{KIP1} null (-/-) mice, but also developed a whole range of hyperplasia and neoplasia, suggesting that the p27^{KIP1} (CK-) protein function as an oncogenic protein (Besson *et al.*, 2007). In human cancer cells, homozygous inactivation of the p27^{KIP1} gene in sporadic tumors is extremely rare, but the correlation of cytoplasmic localization of p27^{KIP1} protein with high tumor grade and poor prognosis was discovered (Slingerland and Pagano, 2000). The p27^{KIP1} (CK-) protein was also found in cytoplasm, which correlated with the hypothesis of the p27^{KIP1} inhibitory action of RhoA pathway.

Benefited from many clinical reports, we now understand that the p27^{KIP1} protein can be a prognostic indicator for breast, colon, prostate, lung, esophageal, and gastric cancers. Tumorigenic activity due to abated p27^{KIP1} is dose-dependent, meaning lower doses of the protein are associated with increased malignancy. Also, it is now understood that deregulation of p27^{KIP1} protein, not loss of its gene, is the cause of reduced p27^{KIP1} protein levels. Orderly regulation of p27^{KIP1} includes proper activation of the oncogenic signal proteins, deregulation of which in turn is commonly found in human cancers. Therefore, better understanding of the regulatory mechanisms of p27^{KIP1} function may provide good clinical value with prognostic and therapeutic applications. This fascinating protein has drawn the attention of clinicians as well as scientists, and as a result, understanding the exact cellular functions and mechanisms is currently under active investigation. This report will focus on the regulatory mecha-

nisms and dual roles of p27^{KIP1} in cancer biology.

Interaction of p27^{KIP1} with cyclin/CDK complexes and its inhibitory function

Different from INK4 family proteins' binding to a CDK alone, CDK4 or CDK6, CIP/KIP proteins like p27^{KIP1} interact with the cyclin E/CDK2 complex. The first α -helical loop of a CIP/KIP protein interacts with the cyclin, and the second helix binds to the catalytic cleft of the CDK subunit, thereby blocking ATP loading (Sherr and Roberts, 1999; Yil *et al.*, 2007; Jung *et al.*, 2008). The inhibition of ATP loading blocks activation of the cyclin E/CDK2 complex, and prevents progression through the cell cycle. The p27^{KIP1} protein binds not only to the cyclin E/CDK2 complex, but also to the cyclin D/CDK4,6 complexes. However, the interaction with the cyclin D/CDK complexes is more complicated. It has become consensus that p27^{KIP1} is a required assembly factor for the complex, but whether the binding is inhibitory is still questionable. Moreover, how it might switch between the two modes of inhibitory and non-inhibitory needs to be answered.

Arguing for a two state mechanism, Dr. Blain's group has assiduously sought-after, and shown that p27^{KIP1} can be both a CDK4 bound inhibitor, and a bound non-inhibitor, depending on the growth state of the cell (Ray *et al.*, 2009). They also have discovered that p27^{KIP1} associates with cyclin D/CDK4 constitutively, and that a specific tyrosine phosphorylation converts p27^{KIP1} from a bound inhibitor to a bound non-inhibitor under different growth conditions. To further support this, they showed that *in vitro* tyrosine phosphorylation, by the tyrosine kinase Abl, converts the bound inhibitor to a bound non-inhibitor. Larrea *et al.* reported a similar finding, demonstrating that phosphorylations at Thr157 and Thr198 are required for binding to cyclin D1 and CDK4, but are not sufficient to activate the cyclin D/CDK4 complex (Larrea *et al.*, 2008). In addition, Larrea *et al.* showed that tyrosine phosphorylation by SRC activates the p27^{KIP1} bound cyclin D1/CDK4 complex, but tyrosine phosphorylated p27^{KIP1} does not affect assembly of the complex.

The p27^{KIP1} protein can also bind to the nuclear pore-associated protein (mNAP60), and interact with the nuclear export protein chromosome region maintenance 1 protein (CRM1) (Shin *et al.*, 2005). Since CRM1 mediates nuclear export, CRM1 interaction with p27^{KIP1} causes trans-localization into the cytoplasm and out of nucleus. Furthermore, interaction of p27^{KIP1} with CRM1 also causes displacement of cyclin D1 from CRM1, leading to

increased cyclin D1 levels in the nucleus, and progression through the cell cycle.

p27^{KIP1} is phosphorylated at multiple sites by various signaling pathways

An ability of p27^{KIP1}, together with other cell cycle molecules, is to respond to diverse extracellular demands. In doing so it helps cells adjust to the new environment through proper cell cycle regulation, which is pivotal to maintaining normal cellular homeostasis. The ability to properly respond to different signaling pathways comes from accordingly regulating the p27^{KIP1} protein by intricate, but unmistakable phosphorylations. Misregulation or functional inactivation of p27^{KIP1} protein is caused when oncogenic kinases such as PKB and SRC are over-activated, leading to the development of malignancies. If these oncogenic signaling pathways can be inhibited, it is possible to restore the tumor suppressive functions of misregulated p27^{KIP1} protein. Hence, understanding mechanisms of p27^{KIP1} phosphorylation will provide additional perspectives for finding potential targets for cancer prevention and therapy.

The p27^{KIP1} protein is short-lived, and activity of this protein largely depends on protein levels that are regulated mainly through proteasome-dependent degradation and/or transcriptional control. Recent studies show that potentially oncogenic serine/threonine kinases, PIM kinases, promote

cell cycle progression by phosphorylating Thr157 and Thr198 of p27^{KIP1} leading to its nuclear export and proteasome-dependent degradation. However, while the mechanisms involved are under active investigation, p27^{KIP1} possesses seemingly contradictory actions in facilitating cell motility by interacting with proteins involved in functions aside from cell cycle regulations while located in the cytoplasm. The role of p27^{KIP1} in facilitating cell motility through inhibition of RhoA activation in the cytosol, implies that its cytosolic localization does more than free cyclin/CDK complexes from inhibition. It also does more than subject this inhibitor to proteasomal degradation (Figure 2). The significance of multiple biological functions for p27^{KIP1} protein is generally acknowledged. The onset of environmental cues delivers a new role to this cell cycle inhibitor as a result of post-translational modifications like phosphorylation or ubiquitination. Extensive studies have informed us that p27^{KIP1} protein remodels itself predominantly through phosphorylation and consequently alters its interacting protein partners, changes its cellular function, localization, as well as protein expression levels.

Important roles for p27^{KIP1} in guarding cells against breast cancer have advanced our understanding of the relationship between p27^{KIP1} phosphorylation and its role in CDK inhibition. Phosphorylation of serine or threonine on p27^{KIP1} by ERK1 targets the protein for ubiquitination, and phosphorylation on more than one threonine site by diffe-

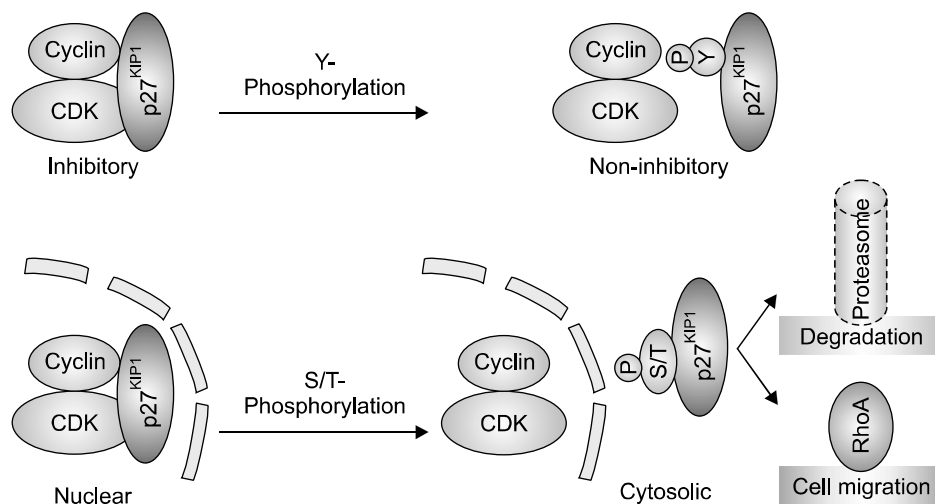


Figure 2. The p27^{KIP1} protein is regulated by phosphorylation on multiple sites. Hypo-phosphorylated or unphosphorylated p27^{KIP1} acts as a cyclin/CDK inhibitor. Multiple phosphorylated forms of the protein, through activation of various mitogenic signals, are present in cells. Phospho-p27^{KIP1} diverges into discrete fates according to the location of phosphate groups. Respective functions of multifarious combinations of individual phospho sites are beyond comprehension at present. However, tyrosine phosphorylation in general renders phospho-p27^{KIP1} non-inhibitory and serine/threonine phosphorylation results in cytosolic localization. Some phospho forms of the cytosolic p27^{KIP1} are subject to proteasomal degradation, while others inhibit RhoA activation and facilitate cell migration.

rent kinases is involved in cytoplasmic localization. Cyclin E/CDK2 phosphorylates p27^{KIP1} on Thr187 and leads to ubiquitin-dependent degradation. The p27^{KIP1} protein is phosphorylated by AKT at Thr157 and Thr198 to become a better assembler of cyclin D/CDK4 complexes, and the binding of p27^{KIP1} to cyclin D/CDK4 facilitates activation of cyclin E/CDK2 through sequestration of the inhibitory protein. Therefore, the differential binding of p27^{KIP1} to distinct CDKs during G₁ cell cycle can also be attributed to the phosphorylation status of p27^{KIP1}. As p27^{KIP1} phosphorylations are cell cycle dependent, the cyclin E/CDK2 inhibitory activity of p27^{KIP1} is maximal during G₀, and falls as cells move through G₁ into S phase. At the same time, the cyclin D/CDK4 bound p27^{KIP1} is maximal in early G₁. All of these findings suggest that anti-mitogenic signaling, which can alter phosphorylation status of p27^{KIP1}, can switch p27^{KIP1} binding from CDK4/6 complexes to cyclin E/CDK2 complexes, promoting restoration of cell cycle control.

p27^{KIP1} exerts anti- and pro-tumorigenic activities

Decreases in p27^{KIP1} expression levels have been implicated in the genesis and progression of many human malignancies. Just as a mouse knock-out system was used to determine anti-tumorigenic activities of p27^{KIP1}, a mouse knock-in model was used to insert one allele of p27^{KIP1} that cannot bind cyclins and CDKs. This knock-in model has been generated to address the question of the tumor-prone phenotype with CDK inhibitory function. The knock-in mouse model shows an increase in spontaneous tumorigenesis in many tissues when compared with either wild-type or p27^{KIP1} null animals (Kim *et al.*, 2005; Susaki *et al.*, 2009). However, many studies indicate that the function of p27^{KIP1} cannot be solely attributed to its CDK inhibitory action. The critical function of p27^{KIP1} in tumorigenesis therefore may extend beyond its ability to regulate CDKs. Indeed, the fact that p27^{KIP1} levels in tumors do not always correlate with proliferative index and that the subcellular localization of p27^{KIP1} is a negative prognostic factor in some tumors, indicates that p27^{KIP1} may not function as a CDK inhibitor in these cases. Another study shows that the haploinsufficient p27^{KIP1} heterozygous mouse is more susceptible to tumor formation than the p27^{KIP1} null animals in mammary and prostate tumor models, suggesting an active contribution of the remaining p27^{KIP1} allele to tumor development (Gao *et al.*, 2004). The group interpreted the enhanced susceptibility to be a result of partial down-regulation of the cyclin/CDK inhibitory activity but maintenance of other pro-tu-

morigenic roles. Mice carrying the p27^{KIP1} S10A allele, which renders p27^{KIP1} to nuclear localization, show a partial resistance to urethane-induced tumorigenesis, despite reduced overall abundance of the p27^{KIP1} protein (Besson *et al.*, 2007). This study further supports the correlation between cytosolic localization and tumorigenic activity of p27^{KIP1}.

The current model generally accepted is that p27^{KIP1} suppresses tumorigenesis by inhibiting cyclin/CDK activity in the nucleus, but exerts other functions in the cytoplasm that are potentially oncogenic. While both roles may be important for homeostasis, inactivation of the nuclear function and/or exaggeration of the cytoplasmic functions may promote tumor progression. The cytosolic functions include the regulation of the actin cytoskeleton and cell migration through modulation of RhoA activity. Cytosolic activities are generated by the C-terminal portion of the p27^{KIP1} protein (Besson *et al.*, 2004; Batsi *et al.*, 2009). During mouse embryonic development, this function is critical for the migration of cortical neurons (Nguyen *et al.*, 2006). Interestingly, cyclin/CDK binding is through the N-terminal portion of p27^{KIP1}, and this same region modulates differentiation of neuronal progenitors *in vivo* via stabilization of neurogenin-2, independent of cyclin/CDK interaction (Nguyen *et al.*, 2006).

Conclusion

The p27^{KIP1} cyclin dependent kinase inhibitor displays apparently contradicting roles by acting as a classic tumor suppressor in one instance, and a pro-tumorigenic oncogene in the other. As its primary function as a CKI is to bind and inhibit cyclin/CDK complexes, the p27^{KIP1} protein functions throughout all cell cycle phases by interacting with different cyclin/CDK complexes. However, this cell cycle inhibitor has emerged to play important roles in other cellular functions, such as cell migration. Being a substrate of several important mitogenic signaling kinases, the p27^{KIP1} protein is post-translationally modified by phosphorylation. Mitogen-induced phosphorylation causes alterations in expression levels, intracellular localization, and induction of p27^{KIP1}'s diverse functions. The proper regulation of p27^{KIP1} expression levels, localization, and differential functions, must be important in maintaining homeostasis and preventing cells from forming tumors. Understanding the diverse roles of p27^{KIP1} protein during normal cell cycle progression and tumor development provides new insights into the field of tumor prognosis and

therapeutics.

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