Identification of the nuclear localization signal of p21^{cip1} and consequences of its mutation on cell proliferation

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Abstract Overexpression of p21^{cip1} induces cell cycle arrest. Although this ability has been correlated with its nuclear localization, the evidence is not conclusive. The mutants that were used to inhibit its nuclear translocation could no longer bind to several proteins known to interact with the last 25 amino acids of p21^{cip1}. Here we used point mutation analysis and fusion of the proteins to DsRed to identify which amino acids are essential for the nuclear localization of p21^{cip1}. We conclude that amino acids RKR$^{140–142}$ are essential for nuclear translocation of p21^{cip1}. While wild-type DsRed-p21 induces cell cycle arrest in 95% of transfected cells, overexpression of cytoplasmatic p21AAA$^{140–142}$ arrested only 20% of transfected cells. We conclude that cytoplasmatic p21, with no deletion in the C-terminal region, had a much lower capacity to arrest the cell cycle.

Key words: p21^{cip1}; p21; CKI; Cell cycle; Nuclear localization signal; Nuclear transport

1. Introduction

p21^{cip1} (p21) belongs to the CIP/KIP family of cyclin-dependent kinase (cdk) inhibitors. All the members of the family contain conserved cyclin and cdk binding domains near the amino-terminus. These sequences allow them to bind to almost all cyclin/cdk complexes and to inhibit cyclin/cdk2 complexes [1–3]. Furthermore, binding of p21 to cyclin D/cdk4 provides the complex with a nuclear localization signal (NLS) [4] and blocks exposure of nuclear export sequences of cyclin D [5]. p21 has additional characteristics that assign it a unique role in cell cycle regulation. It is an important element in cell cycle arrest induced by DNA damage [6], differentiation and senescence [7]. It has also been implicated in inhibition of DNA re-replication [8–10] and in apoptosis protection [11]. The C-terminal half of p21 is different from the other CIP/KIP family members, and it binds to proliferating cell nuclear antigen (PCNA) and inhibits DNA replication in vitro [12,13]. Recently, another cyclin A/E and a cdk4 binding domain were found in the C-terminus region of p21 [14,15]. Furthermore, other proteins such as calmodulin [16], SET [17], GADD45 [18], c-myc [19], c/EBPα [20] and CARB [21] bind to the C-terminus of p21. Almost all these binding domains overlap with each other and with the PCNA binding domain [22].

Separate expression of the N- and C-terminal region of p21 showed that each domain inhibits the cell cycle independently [2]. The C-terminal half of p21 is also responsible for its nuclear localization and putative NLSs are present in this region. Nuclear translocation of p21 is positively modulated by calmodulin and negatively by AKT-dependent phosphorylation [16,23]. Although cell growth-inhibiting activity of p21 is strongly correlated with its nuclear localization [13,24], the evidence is not conclusive since all attempts to obtain cytoplasmatic p21 used p21-deleted mutants (p21$^{1–140}$). These mutants eliminated not only the putative NLSs within the C-terminal domain but also the binding domain to proteins that interact with the last amino acids of p21. Consequently, lack of inhibition could be due to the inability of p21 to bind some of these proteins. Here we identified which amino acids are essential for the nuclear translocation of p21. We then analyzed the cell growth inhibitory activity of p21 containing NLS mutations which blocked the nuclear entry.

2. Materials and methods

2.1. Plasmid construction and mutagenesis

Prediction of NLS sequences was performed using PSORTII program (http://psort.nibb.ac.jp). All recombinant p21 cDNA constructs were derived from the human cDNA (gift from Dr. Massagué, Memorial Sloan-Kettering Cancer Center, New York). Polymerase chain reaction (PCR) amplification was performed using a 32-mer initial forward oligonucleotide (5′-gTgggAATTCgCATATgTCAgAACCggCTggg-3′) and a 33-mer terminal reverse oligonucleotide (5′-ACgAggATCCAAgCTTTAgggCTTCCTCTTggA-3′) to amplify the full-length cDNA and a 30-mer middle forward oligonucleotide (5′-ggCCggAATTCgCATATgggAggCAggCgg-3′) and the same terminal reverse oligonucleotide to amplify the C-terminal half of the cDNA. Digestions with *Nde*I and *Hin*dIII allowed cloning into a modified pGEX-KG and digestions with *Eco*RI and *Bam*HI allowed cloning into pDsRed and pEGFP-C1 (GFP, green fluorescent protein) (Clontech). The p21AAA$^{161–163}$ mutant was obtained by single PCR amplification using a reverse 48-mer oligonucleotide (5′-ACgAggATCCAAgCTTTAggg**CgCCgCCgC**ggAgAAgATCAgCCggCg-3′) carrying the mutation (shown in bold). The p21AAA$^{140–142}$-AAA$^{161–163}$ and the p21AAA$^{140–142}$ were obtained by two-stage PCR using megaprimers. The first PCR reaction was performed using a 45-mer middle forward oligonucleotide (5′-ggACCTggAgACTCTCAgggT**gCggCCgCg**CggCAgACCAgCATg-3′) carrying the corresponding mutations (shown in bold) and a reverse terminal oligonucleotide wild type or

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Abbreviations: p21, p21^{cip1}; cdk, cyclin-dependent kinase; NLS, nuclear localization signal; GFP, green fluorescent protein

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carrying the $AAA^{161-163}$ mutation as shown above. The second PCR product was performed using the 32-mer initial forward oligonucleotide (for the full-length construct) or the 30-mer middle forward oligonucleotide (for the carboxy-terminal construct) and the first PCR product (megaprimer of 114 bp) as reverse mutated primer.

2.2. Cell culture and transfections

NIH3T3 and COS-7 cells were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% donor calf serum (DCS) or 5% fetal calf serum (FCS), respectively. Transient expression of the different p21 mutants was achieved by transfecting the cells using Effecten reagent (Qiagen) following the manufacturer's instructions.

2.3. Immunocytochemistry and confocal analysis

For intracellular localization analysis of DsRed and GFP fusion proteins, cells were grown on coverslips and fixed in 3% paraformaldehyde/phosphate-buffered saline (PBS) (140 mM NaCl, 5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) for 20 min at room temperature. Fluorescence was analyzed by confocal microscopy. For BrdU immunocytochemistry cells were first incubated for the indicated time with BrdU (10 μM) and then fixed as above, permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature, blocked for 20 min with 10% FCS in PBS and then incubated for 90 min with DNase (Boehringer, cat. #776785, 1:1 dilution in PBS) and then for 1 h at 37°C in a humidified atmosphere with monoclonal anti-BrdU antibody (Boehringer, cat. #1179376, 6 μg/ml). Fluorescein-conjugated secondary antibodies were used.

2.4. Protein expression and purification, pull-downs and cdk2 assay

pGEX-KG vectors containing either the different mutations of p21, cdk2, SET or cyclin A and the PET vector containing the PCNA-His tag were transformed into *Escherichia coli* BL21(DE3) strain carrying the pLysS plasmid. The expression and purification were performed using glutathione-affinity [17] or Ni-affinity chromatography according to the manufacturer (Novagen). GST was cut off from SET by digestion with thrombin protease according to the manufacturer (Sigma). Human recombinant CaM was also expressed in *E. coli* and purified as previously described [25]. Purified proteins were covalently bound to BrCN-activated Sepharose 4B, as indicated by manufacturers. Pull-downs were performed as follows: 1 μg of purified protein was incubated for 1 h at 4°C with 20 μl of either CaM-Sepharose (1:1 v/v), SET-Sepharose (1:1 v/v) or PCNA-Sepharose (1:1 v/v) in 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100 (the buffer also contained 0.1 mM $CaCl_2$ for CaM-Sepharose pull-downs). After centrifugation, unbound proteins were collected and bound proteins washed three times in the same buffer and eluted directly with sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis Laemmli loading buffer, electrophoresed and analyzed by Silver staining. Cdk2 activity was determined as previously described [17] using purified proteins. Where indicated purified wild-type or mutated 400 nM p21-GST (equimolar to cdk2) was added.

3. Results

3.1. Determination of p21 NLS

PSORTII analysis of $p21^{cip1}$ sequence gave three putative 'classical type' NLSs and three overlapping bipartite NLSs within the last 25 amino acids (Fig. 1). In order to identify which of the sequences was responsible for the nuclear localization of p21, site directed mutagenesis was performed to change the crucial amino acids. The following mutations were performed: $p21AAA^{140-142}$-$AAA^{161-163}$, $p21AAA^{140-142}$, $p21AAA^{161-163}$ (Fig. 1C). Wild-type p21 (p21WT) and the various mutations were fused to GFP or to DsRed and transfected to NIH3T3 (Fig. 2) and to COS (data not shown) cells in order to analyze subcellular localization. GFP-p21WT and DsRed-p21WT were all nuclear. Simultaneous mutation of $RKR^{140-142}$ and $KRK^{161-163}$ regions, $p21AAA^{140-142}$-$AAA^{161-163}$ mutant, induced an increase in cytoplasmatic protein in 95% of the cells when fused to GFP, and gave a

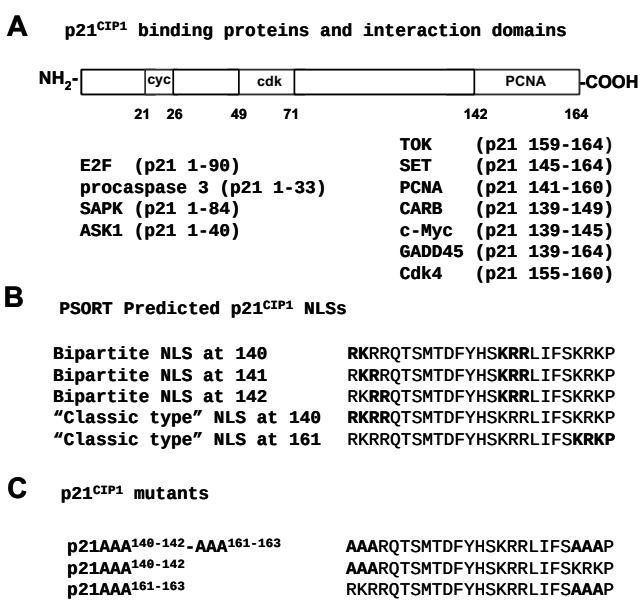

Fig. 1. $p21^{cip1}$ domains and mutants used. A: $p21^{cip1}$ binding proteins and interacting domains. B: PSORTII predicted NLS sequences in the carboxy-terminal region of $p21^{cip1}$. Putative essential amino acids are shown in bold. C: $p21^{cip1}$ mutants were generated as indicated in experimental procedures. Mutated amino acids are shown in bold. The rest of the protein sequence (not shown) was not modified.

completely cytoplasmatic protein in 95% of the cells when fused to DsRed. $p21AAA^{161-163}$ mutant fused to GFP or to DsRed was still nuclear while $p21AAA^{140-142}$ was more abundant in the cytoplasm when fused to GFP and totally cytoplasmatic in 98% of the cells when fused to DsRed. The differences between the subcellular localization of the DsRed- and GFP-fused mutants could reflect the fact that DsRed proteins oligomerize and in consequence the molecular weight of DsRed-p21 proteins is far from the limit for the passive diffusion through the nuclear pore, while p21-GFP might partially diffuse to the nucleus. These results showed that amino acids $RKR^{140-142}$ are essential for the nuclear localization of p21 while the $AAA^{161-163}$ are not. Thus, from now we will refer to $p21AAA^{140-142}$ as $p21NLS^{-}$ mutant.

3.2. $p21NLS^{-}$ preserves the capacity to inhibit cdk2 and to bind to PCNA, SET and CaM in vitro

p21WT and $p21NLS^{-}$ were purified as GST-fused proteins and incubated with cyclin A–cdk2 complexes. Cdk2 kinase activity was then determined by using histone H1 as substrate. As shown in Fig. 3A, the inhibitory activity of $p21NLS^{-}$ on cdk2/cyclin A was similar to the one of p21WT. Pull-downs were performed using PCNA-His, SET and CaM bound to Sepharose to analyze the capacity of $p21NLS^{-}$ mutant to bind to these proteins. As shown in Fig. 3B, mutation of amino acids $RKR^{140-142}$ to $AAA^{140-142}$ did not alter binding of p21 to either PCNA, SET or CaM.

3.3. Effect of cytoplasmatic p21 on cell proliferation

Transfections were performed with WT or NLS mutated p21, in NIH3T3 cells, and the effect on cell cycle was determined by 8 h BrdU incorporation into the cells followed by immunocytochemistry and quantification of the percentage of

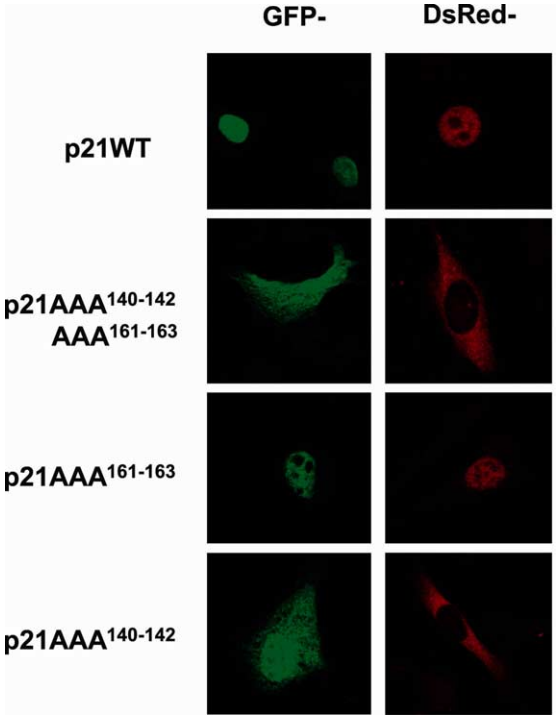

Fig. 2. Intracellular localization of the p21[cip1] mutants fused to GFP or to DsRed. NIH3T3 cells were transfected with p21WT, p21AAA$^{140-142}$-AAA$^{161-163}$, p21AAA$^{140-142}$, p21AAA$^{161-163}$ fused to either GFP or DsRed. Localization of expressed fusion proteins was analyzed by confocal microscopy. Examples of typical localizations of the various proteins are shown.

BrdU positive cells among transfected and non-transfected cells (Fig. 4). As previously described nuclear expression of p21 strongly inhibited cell proliferation. The proliferative inhibitory activity of p21 was highly reduced when the protein was cytoplasmatic. The remaining inhibitory activity could be due to a retention of cdk4/cyclin D1 in the cytoplasm as a consequence of its binding to the cdk and cyclin binding domains present in the amino-terminal half of p21NLS^{-}. In fact, retention of cyclin D1 in the cytoplasm was observed in p21NLS^{-}, compared with p21WT, transfected cells (data not shown). To rule out the possibility that binding of p21 C-terminal domain to cytoplasmatic proteins has an inhibitory effect on the cell cycle, NIH3T3 cells were transfected with C-terminal (amino acids 91–164) wild-type p21 (CTp21WT) or NLS mutated p21 (CTp21NLS^{-}) and cell

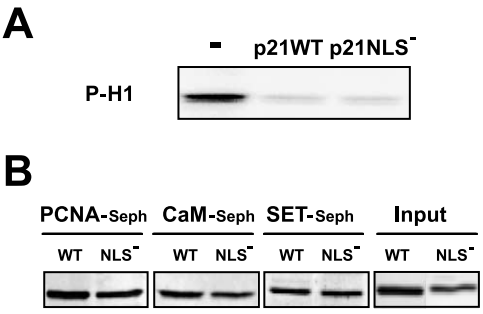

Fig. 3. Ability of p21NLS^{-} mutant to inhibit cdk2/cyclin A and to bind PCNA, SET and CaM. A: Inhibition of cdk2/cyclin A by GST-p21WT and GST-p21AAA$^{140-142}$. Cdk2/cyclin A activity was analyzed in the absence of p21 (−), or in the presence of either GST-p21 (p21WT) or GST-p21AAA$^{140-142}$ (p21NLS^{-}). B: Binding of GST-p21WT and GST-p21AAA$^{140-142}$ to CaM, SET and PCNA. Bound proteins and input (20%) were electrophoresed and silver stained.

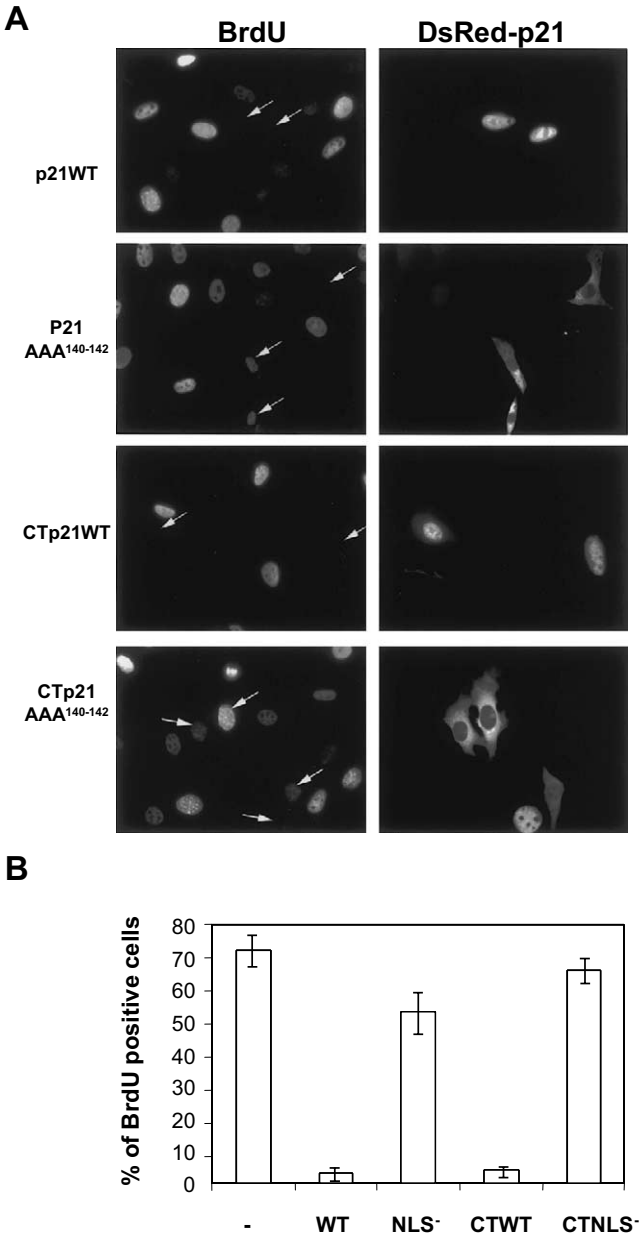

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Fig. 4. Effect of different p21[cip1] mutants on cell proliferation. NIH3T3 cells were transfected with either p21WT, p21AAA$^{140-142}$, CTp21WT or CTp21AAA$^{140-142}$, all fused to DsRed. Cells were trypsinized 24 h after transfection and grown on coverslips for a further 24 h at 30% confluence. During the last 8 h the cells were incubated in the presence of BrdU. They were then fixed and BrdU incorporation was analyzed by immunocytochemistry using an FITC-conjugated secondary antibody. The different DsRed fusion proteins are shown in the red channel and the BrdU incorporation in the green. A: Representative microscope image after transfection with the different DsRed-p21 constructs. Arrows show location of DsRed-p21 transfected cells. B: Percentage of BrdU positive cells for each p21 construct transfected cells or for non-transfected cells (−). Only cells with cytoplasmatic localization of p21 were counted for CTp21NLS^{-}. The experiment was performed in triplicate, and for each experiment at least 100 transfected cell were counted.

proliferation analyzed. While overexpression of nuclear C-terminal p21 inhibited cell cycle with the same efficiency as full-length p21, C-terminal cytoplasmatic p21 did not inhibit the cell cycle (Fig. 4). Interestingly, in 50% of the cells C-terminal NLS mutated p21 entered the nucleus and in those cells the inhibitory capacity was re-established. All these experiments were also performed with COS cells with similar results (data not shown).

4. Discussion

Here we identified the amino acids of p21 that are essential for its nuclear translocation and examined the consequences of their mutation. While the $RKR^{140-142}$ sequence was essential for nuclear localization of p21, the $KRK^{161-163}$ was not. Together with the PSORTII analysis, this indicates that either the bipartite NLS located in the sequence RKRRqtsmtdfyhsKRR$^{140-156}$ or the classic pattern of four amino acids, $RKRR^{140-143}$, is responsible for the nuclear translocation of p21.

The use of DsRed-fused mutants led us to this conclusion while the GFP-fused proteins gave confusing results. When we mutated the $RKR^{141-142}$ amino acids to $AAA^{140-142}$ and fused the p21 cDNA to GFP, the cytoplasmatic expression of GFP-fused protein increased significantly but the remaining nuclear localization suggested the presence of another NLS. In contrast, when the same mutated cDNA was fused to DsRed proteins, the nuclei were completely devoid of DsRed protein. The different results obtained with GFP- or DsRed-fused p21 mutants could be due to the fact that DsRed proteins oligomerize [26] while GFP do not. Thus, the molecular weight of GFP-p21 fusion proteins is close to the limit for nuclear diffusion through the nuclear pores, while DsRed-p21 proteins are much bigger. Although it could be argued that p21 has a low molecular weight and in consequence the GFP-p21 fusion is closer to the situation in vivo, the amount of free p21 in the cell may be small, and most of the p21 is found in high molecular complexes such as p21/cdk/cyclin, which will need an NLS for their nuclear translocation.

Although different data support the hypothesis that nuclear p21 inhibits proliferation while cytoplasmatic does not, studies were based on p21 deletions that compromised the last 25 amino acids, and in consequence the capacity to bind to various proteins [13,24]. The fact that the peptide spanning amino acids 139–164 exhibits cdk inhibitory activity when introduced to human cells [14,27] emphasizes the importance of this deleted domain in cell cycle regulation. Furthermore, those mutants were usually expressed fused to GFP or small tags, and consequently the proteins were still partially nuclear. The determination of the NLS of p21 and the construction of the DsRed-p21NLS$^-$ fused mutant allowed us to analyze the effect of cytoplasmatic versus nuclear p21 on cell proliferation.

In agreement with previous results we found that overexpression of nuclear p21WT strongly inhibits cell proliferation [2,13,24,28]. In contrast, cytoplasmatic p21-NLS$^-$ is less able to inhibit cell proliferation (only 20% inhibition with respect to nuclear p21WT). Because p21NLS$^-$ mutant inhibits cdk2/cyclin A activity in vitro, we conclude that p21 does not bind or inhibit cdk2/cyclin A when this complex is in the cytoplasm. The remaining cell proliferation inhibitory activity of p21NLS$^-$ mutant may be due to retention of cdk4/cyclin D1 complex in the cytoplasm as already described [28]. To rule out the possibility that binding of a cytoplasmatic protein to the C-terminal region of p21 partially inhibited cell proliferation, wild-type C-terminal p21 (CTp21WT) or C-terminal p21 with mutated NLS (CTp21NLS$^-$) were fused to DsRed. Interestingly, CTp21WT was completely nuclear and inhibits cell proliferation as strongly as the full-length protein, indicating an important role in cell cycle of the proteins that bind to the carboxy-terminus of p21. In contrast, CTp21NLS$^-$ was cytoplasmatic in 50% of the cells, in which cases it did not inhibit cell proliferation, confirming that the remaining inhibitory activity of p21NLS$^-$ was due to the N-terminal region. Mutations performed to obtain the NLS$^-$ mutant did not affect the amino acids essential for cdk4 [15] or cycE/A [14] binding to the C-terminal region. Furthermore we have shown that p21NLS$^-$ is able to interact with PCNA, CaM and SET. Thus, we also conclude that interaction with any of those proteins must take place in the nucleus if proliferation is to be inhibited. Confirming that, cell cycle was arrested in cells with CTp21NLS$^-$ in the nucleus. We do not know why a lower proportion of CTp21NLS$^-$ than full-length p21NLS$^-$ was exclusively cytoplasmatic, but it may indicate the presence of a cytoplasmatic retention sequence or a nuclear export signal in the N-terminal region.

We have identified the amino acids responsible for the nuclear translocation of p21 and shown that cytoplasmatic p21, although able to bind to PCNA, SET, CaM, cdk4 and cyclin A/E, has a reduced inhibitory effect on the cell cycle. Considering the different roles of nuclear and cytoplasmatic p21, it is not surprising that its entrance, and probably the exit, into and out from the cell nucleus are highly regulated [16,23]. Further analysis of these mechanisms promises to be relevant in cell proliferation, apoptosis and oncogenesis areas.

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