C-terminal phosphorylation controls the stability and function of p27kip1

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Entry of cells into the cell division cycle requires the coordinated activation of cyclin-dependent kinases (cdks) and the deactivation of cyclin kinase inhibitors. Degradation of p27kip1 is known to be a central component of this process as it allows controlled activation of cdk2-associated kinase activity. Turnover of p27 at the G1/S transition is regulated through phosphorylation at T187 and subsequent SCF^{skp2}-dependent ubiquitylation. However, detailed analysis of this process revealed the existence of additional pathways that regulate the abundance of the protein in early G1 and as cells exit quiescence. Here, we report on a molecular mechanism that regulates p27 stability by phosphorylation at T198. Phosphorylation of p27 at T198 prevents ubiquitin-dependent degradation of free p27. T198 phosphorylation also controls progression through the G1 phase of the cell cycle by regulating the association of p27 with cyclin–cdk complexes. Our results unveil the molecular composition of a pathway, which regulates the abundance and activity of p27kip1 during early G1. They also explain how the T187- and the T198-dependent turnover systems synergize to allow cell cycle progression in G1.

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Introduction

The cyclin kinase inhibitor p27kip1 plays a pivotal role in the regulation of the mammalian cell cycle. High levels of p27 expression are found in quiescent cells and the protein contributes to the maintenance of the quiescent state (Coats *et al*, 1996; Sherr and Roberts, 1999). Upon sufficient mitogenic stimulation p27 levels drop, thus allowing the activation of cyclin E- and cyclin A-associated cyclin-dependent

kinase 2 (cdk2) (Coats *et al*, 1996). The levels of p27 expression throughout the cell cycle are controlled by transcriptional, translational and post-translational mechanisms (Millard *et al*, 1997; Kolluri *et al*, 1999; Montagnoli *et al*, 1999; Medema *et al*, 2000; Gopfert *et al*, 2003). Phosphorylation-induced degradation of p27 by the skp2-dependent SCF (skp-cullin-f-box) E3 ligase has been studied in detail. Phosphorylation of p27 at a conserved c-terminal threonine (T187) creates a binding site for the F-box protein skp2, which in concert with the SCF complex allows poly-ubiquitylation and subsequent proteasomal degradation of p27 (Sheaff *et al*, 1997; Vlach *et al*, 1997; Carrano *et al*, 1999; Sutterluty *et al*, 1999). Mouse fibroblasts derived from mice expressing a nonphosphorylatable form of p27 (p27T187A) reaccumulate p27T187A at the G1/S transition due to impaired turnover of the mutant form (Malek *et al*, 2001). However, the downregulation of p27 observed after mitogenic stimulation of quiescent cells was not impaired in the T187A cells, thereby indicating that a degradation system independent of this phosphorylation site is required for the turnover of p27 in early G1. Loss of the F-box protein skp2 in the mouse leads to a severe defect in tissue homeostasis involving multiple organs including the liver, kidney, lung and testes of homozygous skp2 knockout mice (Nakayama *et al*, 2000). Interestingly, while several proteins have been shown to be substrates of the skp2-dependent SCF complex, loss of p27 reverts the phenotype of the skp2 knockout mouse back to normal (Nakayama *et al*, 2004). Moreover, loss of skp2 prevents the degradation of p27 in primary mouse fibroblasts and liver cells after mitogenic stimulation, thereby indicating that some cell types might require skp2 for the turnover of p27 in early G1 (Kossatz *et al*, 2004). Meanwhile, a second ubiquitin ligase for p27, named KPC1/2, was shown to regulate the stability of p27 (Kamura *et al*, 2004). Degradation of p27 by the KPC ubiquitin ligase requires nucleo-cytoplasmic transport (Kamura *et al*, 2004); however, not all degradation of p27 takes place in the cytosol (Ishida *et al*, 2000; Boehm *et al*, 2002). In fact, confining p27 to the nucleus leads to a decrease in its stability indicating that nuclear degradation systems take part in the turnover of p27 (Rodier *et al*, 2001). In this work, our goal was to identify post-translational modifications on p27, which contribute to the regulation of p27 stability specifically in early G1. As our initial characterization of this early degradation system pointed towards a phosphorylation-dependent system (Malek *et al*, 2001), we speculated that phosphorylation of p27 at a site different from T187 might induce degradation of the protein upon mitogenic stimulation of quiescent cells. In this work, we identify the c-terminal threonine T198 as a phosphorylation site, which controls several important aspects of p27 function including binding to cyclin–cdk complexes, subcellular localization and protein stability. Interestingly, cyclin–cdk binding and protein stability are intimately linked processes, which cooperate to allow cell cycle-regulated expression of the p27 protein.

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Results

Phosphorylation at T198 controls the stability of p27kip1

We began our study with a systematic analysis of point mutations in known or predicted p27 phosphorylation sites. To determine the consequences of these changes on p27 stability, we transfected the mutant p27 proteins into HEK

293 cells and measured their steady-state levels. Figure 1A shows the expression levels of a subset of p27 phosphosite mutants. Surprisingly, changing the threonine in position 198 into alanine (T198A) significantly decreased the steady-state expression levels of the mutant p27 protein (Figure 1A). To assay p27 phosphorylation *in vivo*, we raised a phospho-specific antibody against phospho-T198. As part of the characterization of this antibody, we first immunoprecipitated

Figure 1 Loss of T198 phosphorylation leads to ubiquitylation and degradation of p27kip1. **(A)** Expression analysis of a series of p27 phosphosite mutants after transfection into HEK 293 cells. **(B)** After transfection into 293 cells, the immunoprecipitated protein was either treated with alkaline phosphatase or left untreated. Phosphatase inhibitors were added (lane 3) to ensure the specificity of the reaction. Expression levels of p27 and phosphorylation status at T198 were analyzed by Western blotting. **(C)** After transfection of 293 cells with wild-type or T198A mutant p27, expression levels of p27 and phosphorylation status at T198 were determined by Western blotting. **(D)** The half-life of wild-type or T198A p27 was determined after transfection into Rat1a cells. The graph shows a half logarithmic display of the band intensities as detected by a p27-specific antibody. **(E)** 293 cells were transfected with p27 wild-type or the T198A mutant plasmids and increasing amounts of an UbR7-expressing plasmid. **(F)** 293 cells were transfected with wild-type or T198A mutant p27 and treated with the proteasome inhibitor MG132 (25 μ M) for 8 h. Expression levels of wild-type and mutant p27 were analyzed by Western blotting. **(G)** 293 cells were transfected with p27 wild-type or the T198A mutant form with or without His-tagged ubiquitin. Ubiquitylated proteins were purified on a Co-column and detected by Western blotting with a p27-specific antibody. The graph shows a quantification of the amount of ubiquitylated p27 and p27T198A protein in relation to input protein levels. **(H)** HeLa cells were transfected with siRNA against skp2 and expression plasmids for p27 and p27T198A. Expression levels of p27, skp2 and actin levels were compared in siRNA-treated and untreated cells. **(I)** HeLa cells were transfected with siRNA against KPC1 and expression plasmids for p27 and p27T198A. Expression levels of p27, p27T198A and actin levels were compared in siRNA-treated and untreated cells. Efficient knockdown of KPC1 was verified by RT-PCR in wild-type, p27T198A and vector-transfected cells. Untransfected HeLa cells were used as positive control.

p27 from asynchronously growing 293 cells and showed that the phospho-T198 antibody recognizes wild-type p27 (Figure 1B). Treatment of the immunoprecipitated material with alkaline phosphatase led to a complete disappearance of the phospho-T198 signal, which was maintained when phosphatase inhibitors were added to the reaction before the addition of phosphatase. To ensure that the phospho-T198 antibody recognizes only phosphorylated T198, we transfected 293 cells with wild-type and p27T198A and showed that the phospho-specific antibody is only detecting the wild-type form of the p27 protein but not the p27T198A mutant when equal amounts of p27 protein were loaded (Figure 1C). (For more information on the characterization of the antibody, refer to Supplementary Figure 1.)

To determine whether the reduced expression levels of the p27T198A mutant were due to increased turnover, we measured the half-life of p27T198A in comparison to wild-type p27 and found it to be significantly shortened (Figure 1D). Next, we tested whether the increased turnover at steady-state levels was due to increased ubiquitylation by cotransfecting HEK 293 cells with a mutant ubiquitin (Ubr7), which prevents ubiquitin chain elongation. As shown in Figure 1E, Ubr7 lead to a dose-dependent increase in the levels of wild type and p27T198A. To exclude an indirect effect of Ubr7 on the stability of p27T198A, we directly measured the amount of His-ubiquitin incorporated into wild-type and mutant p27. As shown in Figure 1G, significantly more His-ubiquitin was incorporated into p27T198A when compared with wild-type p27 after normalization for protein input levels. Furthermore, treatment of transfected cells with the proteasome inhibitor MG132 stabilized p27T198A leading to expression levels comparable to wild-type p27 (Figure 1F). To determine whether the F-box protein skp2 is involved in the turnover of T198A mutant p27, we transfected wild-type or T198A p27 into continuously proliferating HeLa cells and reduced the expression of skp2 by siRNA-mediated knockdown. As shown in Figure 1H, reduction of skp2 expression led to an increase in the levels of wild-type and T198A mutant p27. However, the relative differences between p27 wild type and T198A protein levels were maintained under conditions of skp2 knockdown, indicating that p27T198A is still degraded by the SCF skp2-dependent degradation system, but that in addition to this pathway a second skp2-independent mechanism must regulate the abundance of the nonphosphorylatable protein in proliferating cells. Similar results were observed in skp2 KO MEFs transfected with Flag-tagged versions of wild-type and T198A p27 (data not shown). Recently, a second ubiquitin ligase named KPC1 was shown to regulate the abundance of p27 in early G1 (Kamura *et al*, 2004). We therefore tested whether loss of KPC1 would result in stabilization of p27T198A. As shown in Figure 1I, transfection of HeLa cells with siRNA directed against KPC1 led to a complete loss of KPC expression as detected by RT-PCR without a significant change in p27T198A levels. In summary, these results point towards a previously unknown mechanism that stabilizes p27 through phosphorylation at T198, thereby preventing ubiquitylation and proteasomal turnover.

p27T198A phosphorylation controls p27 levels during the G0 and G1 phase

Our transient transfection assays suggested that loss of T198 phosphorylation decreases the stability of p27 in

asynchronously proliferating cells. In order to analyze the pattern of p27 phosphorylation at T198 throughout the cell cycle, we synchronized human fibroblasts in G0 using a combined serum starvation, contact inhibition protocol. As expected, p27 immunofluorescent staining was detectable in almost all cells during G0 and gradually decreased as cells progressed through G1 and S phase (Figure 2A). To determine what percentage of cells that stained positive for p27 also stained positive for phospho-T198, we performed double stainings with antibodies against p27 and phospho-p27T198. Phosphorylation at T198 was strongly detectable in approximately 60% of all quiescent cells and increased further as these cells progressed into the cell cycle (Figure 2B). Nevertheless, the remaining cells stained positive for p27 but did not show significant phospho-T198 staining. This result could suggest that not all quiescent cells express the factors required to efficiently phosphorylate p27 at T198, which might reflect different arrest stages or even the heterogeneity of the cell lines used in these experiments. However, it remains possible that the level of phosphorylation at T198 in the nonstaining cells is simply below the detection limit of the phospho-antibody.

We therefore decided to test the physiological significance of p27 phosphorylation at T198 in an *in vivo* model. To this end, we mutated a 7.7 kb fragment derived from the genomic mouse p27 locus at position T198 (Figure 2C) and stably integrated it or the wild-type p27 fragment into p27 knockout MEFs. To ensure that our transgenic lines recapitulate the activity of the endogenous p27 promoter, we measured p27 mRNA levels by RT-PCR. As shown in Figure 2D, all cell lines (p27 wild type and p27T198A) express p27mRNA at levels identical to unmodified wild-type MEFs. However, in agreement with our transfection data, we found that p27T198A protein was expressed at significantly lower levels than wild-type p27 in the transgenic cell lines (Figure 2E). This decrease in expression levels is due to a significantly shortened half-life of p27T198A as compared to wild-type p27 (Figure 2F). Given these observations, we concluded that phosphorylation at T198 is required to stabilize p27 in proliferating cells by preventing its proteasomal turnover.

Next, we arrested wild-type and T198A cell lines in G0 and measured the expression levels of wild type and p27T198A as cells progressed through the cell cycle after mitogen stimulation. As expected, wild-type p27 was strongly expressed in G0 cells (0 h time point) and was downregulated after restimulation with mitogens (Figure 2H). However in contrast to wild-type cells, arrested p27T198A cells showed greatly reduced levels of p27T198A (Figure 2H) in G0. This reduction in protein expression was again caused by rapid degradation of the mutant protein as shown by a significantly shortened half-life (Figure 2G) of the p27T198A protein. Thus, phosphorylation at T198 protects p27 against proteolytic degradation in asynchronously proliferating and in quiescent cells. From this data we also conclude that the fact that not all quiescent cells stained positive for T198 phosphorylated p27 is most likely due to the detection limit of our antibody.

Unexpectedly however after cell cycle re-entry, p27T198A levels increased continuously and peaked 12–16 h after serum stimulation. At this time point, p27T198A overall levels were similar to what is normally detected in quiescent wild-type cells (Figure 2H). This pattern was reproducibly observed in different p27T198A clones and also in pools of

Figure 2 Generation and characterization of p27T198A fibroblast cell lines. **(A)** Human fibroblasts were synchronized in G0 and released into the cell cycle. To ensure specificity, we used a p27T198A fibroblast cell line (see below) as a negative control. The synchronicity of the cells was verified by flow cytometry (data not shown). At the indicated time points, cells were fixed and the number of p27-positive cells was determined by immunofluorescence staining using a p27-specific antibody. The graph shows average values of three independent experiments (in each experiment, a minimum of 300 cells were counted). A representative example of p27, and phospho-T198 immunofluorescent staining in a synchronization experiment is shown in Figure 3A. **(B)** Human fibroblasts were synchronized in G0 and released into the cell cycle. At the indicated time points, cells were fixed and the numbers of p27 and p27T198 double-positive cells were determined by immunofluorescence double staining using a p27 and a phospho-T198-specific antibody. The graph displays the percentage of p27-positive cells which stained also positive for phospho-p27T198 derived from several independent experiments. p27/phospho-T198 double stainings are shown in Supplementary Figure 2. **(C)** Introduction of a 7.7 kb genomic fragment encoding the mouse p27 locus (cartoon) into p27 knockout MEFs. **(D)** Analysis of the mRNA expression levels of the p27 wild-type, p27T198A mutant (T198A), wild-type MEFs (3T3 p27 WT) and p27 knockout (p27 KO) cell lines by semiquantitative RT-PCR using the GAPDH gene as an internal control. **(E)** The levels of p27 or p27T198A expression in the respective cell lines (two independently derived wild-type and p27T198A lines) were measured by Western blot analysis. **(F)** Half-life measurement of p27 in asynchronously growing wild-type and T198A mutant cells. The graph shows a quantification of p27 band intensities of a representative experiment. **(G)** Identical experiment as in (E) but the cells were arrested in G0. **(H)** Wild-type or p27T198A mutant cell lines were arrested in G0 and then released into the cell cycle. At the indicated time points, expression levels of actin, p27, cyclin A and the retinoblastoma protein were measured by Western blotting analysis. **(I)** The graphs show the percentage of BrdU-positive cells detected at the indicated time points after serum readdition in wild-type and T198A mutant p27-expressing cell lines.

p27T198A transgenic cell lines (data not shown). The reaccumulation of p27T198A led to an 8 h delayed entry into S-phase compared to p27 wild-type cells as measured by BrdU labeling of synchronized MEFs (Figure 2I). This delay in

the passage through the G1 phase paralleled a delay in Rb phosphorylation and in the expression of the cyclin A protein, indicating that the reaccumulation of p27T198A had a significant impact on cell cycle progression (Figure 2H and I).

Stability and subcellular localization of p27 are independently regulated processes

T198 phosphorylation by AKT or RSK of p27 had previously been shown to promote nucleo-cytoplasmic export of the protein (Fujita *et al*, 2002, 2003; Ishii *et al*, 2004). As subcellular localization of p27 has been shown to influence its stability (Rodier *et al*, 2001; Kamura *et al*, 2004), we analyzed whether changes in the distribution of wild-type p27 and p27T198A throughout the cell cycle might have caused the observed differences in protein expression and

hence cell cycle progression. To answer this question, we first determined the subcellular expression of T198-phosphorylated p27 throughout the cell cycle in synchronized human fibroblasts using the T198 phospho-specific antibody. These experiments showed that T198-phosphorylated p27 was strongly expressed in quiescent cells (see also Figure 2B) where it is predominantly localized to the nucleus, while after mitogenic stimulation as cells passed through the G1 and S phase the T198 phospho-specific staining recognized mainly cytoplasmic p27 (Figure 3A).

Figure 3 Subcellular localization of p27 is regulated through phosphorylation at T198. **(A)** Human fibroblasts were released into the cell cycle after 72 h of serum deprivation. At the indicated time points, the subcellular localization of p27 and T198-phosphorylated p27 was determined by immunofluorescence staining. p27 knockout fibroblasts were used as a negative control for the p27 staining. p27T198A fibroblasts were used as a negative control for the phospho-p27T198 staining. **(B)** The graphs display the percentage of wild-type or T198A mutant cells staining positive for p27 in either the nucleus or cytoplasm after release from quiescence. The localization of p27 in these cells was determined by immunofluorescence microscopy. At least 300 stained cells were counted per time point. Results shown are representative for at least three independent experiments. **(C)** Proliferating or quiescent p27 wild-type and T198A mutant cell lines were treated with MG132 or DMSO for 8 h. The subcellular localization of p27 was determined by immunofluorescence staining. **(D)** The indicated mutants were transfected into asynchronously growing 293 cells and the expression levels of p27 were measured by Western blotting.

To determine if the loss of T198 phosphorylation affects the subcellular localization of p27, we synchronized p27 wild-type or the T198A mutant cell lines in G0. Immunofluorescence staining of p27 in G0-arrested cells revealed that both p27T198A and wild-type p27 are expressed exclusively in the nucleus (Figure 3B). However in G0 arrested cells, total protein levels of p27T198A are significantly lower than those of wild-type p27 (compare 0 h time point in Figure 2H). These results indicated that during G0, wild-type and mutant p27 are both correctly localized to the nucleus but that due to its decreased stability p27T198A is expressed at greatly reduced levels. To exclude the possibility that p27T198A, which is detected in the nucleus, only represents a stable subfraction of the total p27T198A present in the cell, we treated G0 arrested p27 wild-type and p27T198A cell lines with MG132 to prevent degradation of a putatively unstable cytoplasmic pool of the protein. As shown in Figure 3C, MG132 treatment did not increase the staining of cytoplasmic p27T198A while at the same time the total number of cells that express p27T198A in the nucleus increased (data not shown). This result therefore suggests that mislocalization is not the reason for the increased turnover of the mutant form in quiescent cells.

While G0 arrested cells degrade p27T198A in the nucleus, it remains possible that delayed nuclear exit of the nonphosphorylatable form in mitogen-stimulated cells contributes to its increased stability in early G1. In this model, a cytoplasmic ubiquitin ligase would turnover p27 after its export from the nucleus, which is impaired in the p27T198A cell line. Indeed when we stimulated quiescent cells with mitogens, we observed a significant delay in the export of p27T198A (Figure 3B). Within the first 12 h of stimulation, most of the mutant p27T198A remained nuclear while at the same time its protein levels increased significantly compared to wild-type p27, which exited the nucleus within the first 12 h (Figure 3B). Between 16 and 20 h after stimulation, however 80% of the p27T198A-expressing cells showed a cytoplasmic staining while p27T198A protein levels were still significantly higher than in wild-type cells (Figures 2H and 3B). Furthermore, when we treated asynchronously proliferating wild-type and p27T198A cells with MG132 to prevent proteasomal turnover, we observed a strong increase in the cytoplasmic staining of wild-type p27 while p27T198A remained predominantly nuclear (Figure 3C). These results suggested that in quiescent and proliferating cells, p27T198A is primarily degraded in the nucleus. To test whether the predominant nuclear localization in proliferating cells is a prerequisite for the degradation of p27T198A, we constructed p27 wild-type and T198A mutants which are defective in their nuclear localization signal. Even though loss of the NLS led to a predominant cytoplasmic localization of wild-type and T198A p27 (Supplementary Figure 3A), this change in subcellular localization did not lead to a stabilization of p27T198A compared with wild-type p27 (Figure 3D) nor did it stabilize Δ NLSp27T198A compared to p27T198A (see half-life measurement in Supplementary Figure 3B). We therefore conclude that while p27T198A is predominantly degraded in the nucleus, changing its subcellular localization is not influencing the turnover of the mutant form, that is, nucleo-cytoplasmic export and degradation of p27T198A are independent processes.

T198 phosphorylation controls distribution of p27 into cyclin/cdk complexes

At this point of our analysis, we had shown that cell lines expressing p27T198A under the control of the genomic p27 promoter show extremely low levels of the mutant protein during G0 and in asynchronously proliferating cells. However, in synchronized cells, p27T198A accumulates during the G1 phase to levels comparable to what is regularly found in quiescent wild-type cells. Given that the expression of p27T198A during G1 had a significant impact on the start of S-phase, we wondered if p27T198A might have accumulated in cyclin/cdk complexes, which resulted in impaired Rb phosphorylation and the observed delay in G1 phase progression. We therefore measured the relative amount of p27 or p27T198A, which was bound to either cdk2 or cdk4 complexes after cells exited from quiescence and progressed through the cell cycle. For this, we immunoprecipitated p27 or p27T198A at different time points after release from quiescence and measured the amount of bound cdk2 or cdk4. Figure 4A shows a quantification of the relative amount of p27 bound into cdk2- or cdk4-containing complexes from several independent experiments (for raw data, see Supplementary Figure 4A). As expected, we found increased binding of wild-type p27 into cdk2-containing complexes after the cell enters into the G1 phase (Figure 4A). Interestingly however, at the same time, namely 12–16 h after serum stimulation, a period in the G1 phase where p27T198A levels reach their maximum, we found even more p27T198A bound into cdk2 complexes than in wild-type cells. The accumulation of p27T198A in cdk2 complexes especially between the 12 and 16 h time points led to a reduction in total cdk2- and cyclin A-associated kinase activities compared to the wild-type control cells (Figure 4B). The observed delay in cell cycle progression during the G1 phase in p27T198A cells (Figure 2I) can, therefore, be explained by a delay in cdk2 kinase activation due to the increased levels of p27T198A bound into these complexes in early G1. Given the increase in total p27 levels in p27T198A cell lines during early G1, we expected to find more p27T198A bound to cdk4 complexes. Surprisingly, however, while wild-type p27 did efficiently form complexes with cdk4, no increase in p27T198A/cdk4 complex formation was seen in the p27T198A-expressing cell lines (Figure 4A) after release from quiescence.

The observation that p27T198A did efficiently bind to cdk2 complexes while the binding to cdk4-containing complexes was significantly reduced led to the question whether p27T198A was also preferentially bound into cdk2 complexes in asynchronously growing cells. To answer this question, we immunoprecipitated wild-type and p27T198A p27 from asynchronously proliferating cells and measured the amount of bound cdk2. Figure 4C shows that relative to the amount of immunoprecipitated p27, significantly more cdk2 is immunoprecipitated from T198A mutant-expressing cell lines than from wild-type p27-expressing cell lines. At the same time, we observed a reduction in the amount of cdk4 that was immunoprecipitated in complex with p27 from T198A cell lines as compared to wild-type p27-expressing cells (Figure 4C). Figure 4D shows an alternative representation of these findings, namely the ratio of p27T198A bound to cdk2 versus cdk4 compared to the same ratio in wild-type cells. As shown in Supplementary Figure 4B, binding of p27 to active cyclinD1/cdk4 complexes was not changed when

Figure 4 p27T198 phosphorylation controls binding to cyclin/cdk complexes. (A) Wild-type or T198A mutant cell lines were made quiescent by serum starvation for 48 h and released into the cell cycle through readdition of serum. At the indicated time points, cells were lysed and p27 was immunoprecipitated from the extracts using a p27-specific antibody. The amount of bound cdk4 or cdk2 was determined by Western blotting and normalized against the p27 input (intensity p27 bound cdk2/p27 intensity and intensity p27 bound cdk4/p27 intensity). Results were normalized against the 0 h time point which was set as 1. The graphs show the quantification of several independent experiments in different wild-type and p27T198A cell lines. Points represent average values and standard deviations are shown. (B) cdk2 and cyclin A associated kinase activity was measured in synchronized cell lines of the indicated genotypes. (C) The amount of cdk2 and cdk4 bound to p27 wild type or p27T198A was measured by Western blot analysis after immunoprecipitation against p27. Graphs display quantifications and standard deviations of band intensities from multiple experiments and Western blots display a characteristic experiment. (D) The graph shows an alternative representation of the experimental data namely the ratio (Figure 4C) of p27T198A bound to cdk2 versus cdk4 compared to the same ratio in wild-type cells.

p27 was phosphorylated at T198 by AKT suggesting that the defect in cdk4 binding we observe *in vivo* is not eminent on preassembled cyclin D1/cdk4 complexes. However in support of our *in vivo* findings, p27 phosphorylation at T198 is required for efficient assembly of monomeric cyclin D1 with cdk4 *in vitro* (J Slingerland, personal communication). Together, these results suggest that phosphorylation of p27 at T198 regulates the distribution of p27 between cyclin-cdk complexes. While loss of this phosphorylation site impairs binding to cdk4, it promotes binding to cdk2-containing complexes.

Phosphorylation at T198 stabilizes free cyclin unbound p27

At this point of our analysis, we had shown that in addition to its function in controlling the stability of p27 in quiescent and proliferating cells, phosphorylation at T198 also regulates the

distribution of p27 between cdk2- and cdk4-containing complexes. While it was possible that both functions are independent, we also considered the alternative explanation, namely that binding to cdk2 complexes might have stabilized the otherwise unstable T198A mutant. In an extension of this idea we reasoned that p27 which is not bound to cyclin/cdk, that is, 'free p27' needs to be phosphorylated at T198 to prevent ubiquitylation and subsequent turnover. It is necessary to state that 'free p27' only refers to the fact that p27 is not bound to cyclin or cdks, but that other protein interactions might still exist. This model would predict that free p27 should be phosphorylated at T198 and that loss of this site would lead to destabilization of the mutant form. We therefore tested if a cyclin-cdk nonbinding form of p27 (p27C-K-) is phosphorylated at T198 and whether its stability depends on phosphorylation at T198. As shown in Figure 5A, p27C-K- was indeed strongly phosphorylated at T198. Interestingly on

Figure 5 Phosphorylation at T198 stabilizes free p27. **(A)** 293 cells were transfected with expression plasmids encoding wild-type p27, p27T198A, non-cyclin and cdk-binding p27 (C-K-) and p27C-K-T198A, p27 expression levels were analyzed by Western blotting with either p27 (upper panel) or phospho-T198 p27 antibodies (lower panel). **(B)** 293 cells were transfected with expression plasmids encoding wild-type p27, p27T198A, non-cyclin and cdk-binding p27 (C-K-) and p27C-K-T198A and treated with CHX 48 h after transfection for the indicated times. The graph displays a quantification of the results of at least three independent experiments. **(C)** MCF7 cells were transfected with the indicated expression plasmids and increasing amounts of cyclin E and dnckd2 (0.5–2 μ g) expression plasmids. p27 levels were analyzed by Western blotting.

higher percentage polyacrylamide gels p27C-K- runs as a doublet, which was also detected by the phospho-specific p27T198 antibody indicating that p27C-K- must be phosphorylated at T198 and additional phosphorylation sites. Additionally and in agreement with previous reports (Besson *et al*, 2006), we found that the half-life of p27C-K- was significantly increased as compared to wild-type p27 in

asynchronously growing cells (Figure 5B). Importantly however, mutation of T198 into alanine in p27C-K- (p27 T198AC-K-) converted the stable p27C-K- protein into a highly unstable protein (Figure 5B). In fact, the half-life of the p27T198AC-K- form was even shorter than what was measured for p27T198A. These results supported the idea that free cyclin unbound p27 is only stable as long as it is phosphorylated at T198.

Our data therefore lead to a model suggesting that p27 stability is largely dependent on its ability to form complexes with cyclin-cdk partners. However, if p27 is no longer able to bind to these complexes like in the p27C-K- mutant, it needs to be phosphorylated at T198 to prevent degradation. Previous studies had convincingly shown that in asynchronous cells p27 is primarily bound into cdk4-containing complexes (Poon *et al*, 1995) making these complexes the main reservoir for p27. Given that loss of T198 phosphorylation impairs cdk4 binding, the reservoir for p27 binding and hence stabilization would become considerably smaller. Based on this reasoning, we tested if by artificially increasing cyclin-cdk binding partners, we could stabilize p27T198A. We chose dominant negative cdk2 (dnckd2) for these experiments because p27T198A is still subject to degradation by the T187-dependent pathway (see experiments described in Figure 6). In agreement with our hypothesis that binding to cyclin-cdk complexes stabilizes p27, coexpression of dnckd2 with p27 or T198A led to a dose-dependent stabilization of both wild-type and mutant p27 (Figure 5C). Our observation concurs with previous studies, which had shown that dnckd2 allows formation of trimeric complexes but prevents the phosphorylation of p27 at T187, thereby inhibiting T187-dependent turnover (Montagnoli *et al*, 1999). It is important to note that even though mutating T187 in the T198A mutant to create p27T187A/T198A led to an increase in the steady-state levels of the protein, this increase was comparable to the increase in steady-state levels comparing p27 wild type and p27T187A (Figure 5C). Therefore, the greatly reduced stability of the p27T198A mutant, while still subject to the skp2/T187-dependent turnover mechanism, cannot be explained solely through degradation by this pathway. In support of this conclusion we found that coexpression of dnckd2 with p27T198A/T187A led to an even stronger increase in the levels of the double mutant form (Figure 5C). Conversely, no stabilization was observed when p27T198AC-K- was expressed together with cyclin E and dnckd2 (Figure 5C), indicating that the observed stabilization of p27T198A does indeed depend on direct binding of the mutant form to cyclin/cdk complexes.

T187-dependent phosphorylation is independent of phosphorylation at T198

From these experiments we concluded that the changes in the stability of p27T198A throughout the cell cycle were primarily regulated through the association of the mutant form with cyclin E/cdk2 complexes. This model also predicts that p27T198A should become unstable when cdk2 kinase activity increases, thereby leading to phosphorylation of p27 at T187. Given that the levels of cdk2 associated p27 dropped between 16 and 20 h after mitogen stimulation (Figure 5A), we wondered if the downregulation of p27 correlates with increased phosphorylation at T187. To this end we monitored phosphorylation at T187 by immunofluorescence staining using a

Figure 6 p27 T198A is still subject to T187-dependent degradation at the G1/S transition. **(A)** MCF7 cells were transfected with the indicated expression plasmids and increasing amounts of cyclin E and active cdk2 (0.5–2 μ g) expression plasmids. p27 levels were analyzed by Western blotting. **(B)** Cell lines expressing wild-type or the T198A mutant form of p27 were made quiescent through serum starvation and then stimulated to re-enter the cell cycle through addition of 10% serum-containing media. The expression of T187-phosphorylated p27 was determined by fluorescence microscopy using a T187 phospho-specific antibody. At least 300 cells were counted per time point. Mouse fibroblasts derived from a T187A knock-in strain were used as a negative control. **(C)** Quantification of the results from (B), scoring cells with phospho-T187 staining as positive.

phospho-p27T187-specific antibody (Figure 6B and C), the specificity of which was tested using a p27T187A knock-in cell line (Figure 6B). We found that the drop in p27T198A levels at later time points (20–24 h) corresponded to phosphorylation of p27T198A at T187 (Figure 6B) and to the full activation of associated cdk2 kinase activity (Figure 4B). However, even though p27T198A was maximally phosphorylated at T187 between 20 and 24 h after serum stimulation at which points the levels of cdk2 associated p27T198A had dropped, the overall levels of p27T198A declined less than in the wild-type control cells (Figure 3G). This observation suggests that p27T198A either accumulated in complexes with other proteins (Aleem *et al*, 2005) as the cells entered S phase or that the degradation system which turns over unphosphorylated p27 is not active during this stage of the cell cycle. The elevated levels of p27T198A however did not interfere with S phase progression. This result is in agreement with our previous observations in T187A-deficient cells

(Malek *et al*, 2001; Kossatz *et al*, 2004) in which reaccumulation of p27T187A at the G1/S transition did not slow down S-phase progression. Together, these observations suggest that phosphorylation of p27 at T187 relieves the block to cell cycle progression caused by the accumulation of p27T198A in cdk2 complexes and thus the T198- and T187-dependent turnover mechanisms are independent systems.

Discussion

To allow p27 to efficiently control cell cycle progression, it must be expressed at high levels in quiescent cells and decrease in response to mitogenic stimulation to allow timely activation of cyclin E and cdk2 complexes. This pattern of p27 expression is essentially opposite to what is seen for the expression and activation of cyclin E–cdk2 complexes, which are inactive in quiescent cells and during early G1 and become activated as the cell progresses towards S phase.

In this work, we describe the identification of a mechanism that controls the levels, localization and activity of the cyclin kinase inhibitor p27 through phosphorylation at T198. During the G0 phase of the cell cycle when cyclin/cdk levels are generally low, p27 is preferentially in its monomeric form (Poon *et al*, 1995; Soos *et al*, 1996). Our work shows that in order to prevent proteolytic degradation of this free, cyclin unbound form, p27 needs to be phosphorylated at T198. However, phosphorylation at this site serves a second function as cells enter the G1 phase. Under normal conditions, p27T198 phosphorylation increases after mitogenic stimulation of quiescent cells and the majority of the protein associates with cyclin D/cdk4 complexes. By studying the distribution of p27T198A in cdk2- and cdk4-containing complexes using p27T198A-expressing cell lines, we found that when both complexes are highly expressed during early and mid G1 most p27T198A is found in cdk2 complexes and very little is associated with cdk4. Also in asynchronous cells significantly more p27T198A is bound into cdk2 complexes than in wild-type control cells. Loss of T198 phosphorylation therefore prevents binding to cyclin D/cdk4 complexes and instead promotes the binding of p27T198A into cyclin E/cdk2 complexes. This shift in complex formation has two consequences: first, it prevents timely activation of cdk2-associated kinase activity. Loss of cdk2 kinase activation impairs the phosphorylation of Rb and is responsible for the observed delay in cell cycle progression in p27T198A-expressing fibroblast cell lines.

Secondly, the increased binding of p27T198A into cyclin E/cdk2 complexes stabilizes the mutant form of the protein as shown in cell synchronization experiments using p27T198A and wild-type cells. These data indicate that phosphorylation at T198 is necessary to allow downregulation of p27 after mitogen stimulation of quiescent cells. We realize that the increase in T198 phosphorylation at p27 when cells exit from quiescence and the stabilization of the p27T198A mutant during exactly this time of the cell cycle could be taken as evidence for a direct role of T198 phosphorylation in turnover of p27 during early G1. However at this point of our analysis, our data argues for a passive stabilization of p27T198A in cdk2-containing complexes as opposed to a direct defect in a turnover system. Nevertheless, it remains possible that T198-phosphorylated p27 that is bound to cyclin D/cdk4 complexes is recognized as part of this multimeric complex by an ubiquitin ligase. A similar mechanism has been shown for T187-phosphorylated p27 bound to cyclin E/cdk2 complexes, which is recognized by the F-box protein skp2 and ubiquitinated by the SCF E3 ligase (Montagnoli *et al*, 1999).

To date three kinases have been shown to phosphorylate p27 at T198 namely AKT, RSK1 and RSK2 (Fujita *et al*, 2002, 2003). The activation of these kinases in response to mitogen stimulation could be responsible for the increase in the expression of T198-phosphorylated p27. We show in this work that T198 phosphorylation is required for binding to cdk4-containing complexes and exit from the nucleus. Although we were unable to detect differences in binding of T198-phosphorylated p27 compared to nonphosphorylated p27 to pre-assembled cyclin D/cdk4 complexes *in vitro*, our results do not rule out the possibility that phosphorylation at T198 promotes cyclin D/cdk4 complex formation. In fact, previous studies have shown differences in the phospho-

rylation pattern of p27 bound to cyclin D- or E-containing complexes throughout the cell cycle (Ciarallo *et al*, 2002) and T198-phosphorylated p27 was preferentially detected in cyclin D/cdk4 complexes (J Slingerland, personal communication). In fact, p27 also serves as an assembly factor for cyclin D complexes (LaBaer *et al*, 1997) by helping to activate cyclin D/cdk4 kinase activity, which in turn promotes Rb phosphorylation. Interestingly, AKT activity itself is under the control of E2F-dependent transcriptional control (Chaussepied and Ginsberg, 2004). AKT phosphorylation of p27 at T198 could therefore promote cyclin D/cdk4 complex formation, which in turn helps to activate AKT thereby forming a positive feedback loop that promotes progression through early G1. AKT has been shown to be dysregulated in many human cancers (Testa and Tsichlis, 2005) and specifically breast cancers with high constitutive AKT activity express p27T198 predominantly in the cytoplasm and show reactivity with a T198-specific antibody (Viglietto *et al*, 2002; Motti *et al*, 2004). It is therefore conceivable that tumor cells abuse this mechanism to exclude p27 from the nucleus and sequester it into cdk4 complexes thereby allowing early activation of cdk2 kinase activity. Phosphorylation of p27 at T198 might, therefore, present a means to inactivate p27 by several different mechanisms in tumor cells.

By proposing this new regulatory mechanism, we consider two possible pitfalls. First, we considered the possibility that changes in the subcellular localization of p27T198A might have influenced its stability. Previous reports had shown that p27 phosphorylation at T198 by AKT and RSK facilitates its nucleo-cytoplasmic transport and promotes cytoplasmic anchoring of p27 (Fujita *et al*, 2002, 2003; Motti *et al*, 2005). Based on these results, we tested the possibility that changes in p27T198A subcellular localization might have caused the dramatic decrease in its stability. As shown by immunofluorescent staining of quiescent cells, p27T198A and wild-type p27 are exclusively localized to the nucleus in the G0 phase. Inhibition of proteasomal turnover in these cells did result in an increase in the number of stained nuclei but did not lead to an increase in cytoplasmic p27T198A staining, which indicates that turnover of p27T198A during the G0 phase is exclusively taking place in the nucleus. Therefore, differences in subcellular localization are not responsible for the increased turnover of the mutant form in quiescent cells. After release from quiescence, we find T198 phosphorylation to be required for the timely exit of p27 from the nucleus, which is in agreement with the cited earlier reports (Fujita *et al*, 2002, 2003; Motti *et al*, 2004). By comparing subcellular localization and expression levels of p27 and the p27T198A protein, we show however that even under conditions when p27T198A is almost entirely cytoplasmic, its levels are still significantly higher than in wild-type cells. Also, changing p27T198 subcellular localization to the cytoplasm by deleting the NLS did not stabilize the mutant protein compared to wild-type p27. We therefore conclude that while nucleo-cytoplasmic transport is clearly impaired in p27T198A-expressing cells, it is not the cause of the changes in p27 stability and cell cycle progression in these cells.

Second, our observation that p27T198A is preferentially bound into cdk2-containing complexes could also result in an increased turnover by the well-documented SCFskp2 and T187-dependent degradation system (Sheaff *et al*, 1997; Vlach *et al*, 1997; Montagnoli *et al*, 1999), especially in

proliferating cells. Indeed, mutating T187 in the T198A mutant to create p27T187A/T198A led to an increase in the steady-state levels of the double mutant, but did not restore expression levels back to wild-type p27. Wild-type levels were however reached when the amount of cyclin E/cdk2 was artificially increased. Also, lowering the levels of the F-Box protein skp2, which is essential for T187-dependent turnover of p27, did not result in a stabilization of p27T198A, a result in agreement with the observation that p27T198A is unstable in quiescent cells where skp2 is not expressed. p27T198A is however still subject to skp2-dependent degradation at the G1/S transition, a function which is essential to allow progression of p27T198A cells into S phase. The kinetics of T187 phosphorylation on p27T198A corresponded directly to the release of p27T198A from cdk2 complexes, Rb phosphorylation and the onset of S phase. Given that T187 phosphorylation although delayed is still clearly detectable in p27T198A-expressing cell lines, we conclude that phosphorylation at T198 and at T187 are independent events, which regulate the turnover of p27 during the G1 and S phase of the cell cycle sequentially.

Materials and methods

Cell culture, drug treatment and RNA interference

HEK 293, Rat1a, MCF7, primary human fibroblasts (HK1) and immortalized MEFs were cultivated in DMEM supplemented with 10% FCS and 2 µg/ml penicillin/streptomycin. HEK 293 and MCF7 cells were transfected by the calcium phosphate method, and Rat1a and MEFs were transfected with FuGene6 (Roche) according to the instructions.

Serum starvation and release experiments were carried out as previously described (Malek *et al*, 2001). For p27 half-life measurements, cycloheximide (10 µg/ml) was added to the cells for the indicated time. p27 protein levels were determined by immunoblotting.

For RNAi studies, 21-nucleotide short interfering RNA (siRNA) duplexes specific for skp2 (5'-AAG CAU GUA CAG GUG GCU GUU-3' Dharmacon) (von der Lehr *et al*, 2003) and KPC1 (ID 133486, 133487 Ambion) were obtained and transfected into HeLa cells using HiPerFect transfection reagent (Qiagen) and 0.05 µg of the indicated plasmid DNA. p27 and skp2 levels were determined by Western blotting. RT-PCR was carried out as described before (Kossatz *et al*, 2004) using the indicated primer sequences:

KPC forward: 5'-CAG CCT CAG AGA GAG AGC AA-3'
KPC backward: 5'-GCT GTC CCA GGA GAT AGC A-3'

Antibodies, Western blots, immunoprecipitations and immunofluorescence staining

A phospho-specific antibody against T198-phosphorylated p27 was generated at the MRC Protein Phosphorylation Unit, University of Dundee, Dundee, Scotland, as described in Supplementary Figure 1. Antibodies were used as recommended by the manufacturers: anti-p27 mouse monoclonal (Transduction Labs), anti-p27 rabbit polyclonal (Santa Cruz), cyclin A (Santa Cruz), Rb (BD Pharmingen), cdk2 (Santa Cruz), cdk4 (Neomarkers), phospho-T187 (Santa Cruz), actin (ICN) and Flag epitope (Sigma). For detection of cdk4 and Cyclin D1 on Western blots after pull down assays, cdk4 C-22 (Santa Cruz) and cyclin D1 72-13G (Santa Cruz) antibodies were used. Western blots and immunoprecipitations were essentially carried out as described (Malek *et al*, 2001). Band intensities were measured on autoradiographs of Western blots using the

QuantityOne software (BioRad). Kinase and phosphatase assays were carried out as described before (Malek *et al*, 2001).

For immunofluorescence, cells were grown on glass coverslips and fixed using 3.7% paraformaldehyde. After incubation in blocking buffer (5% BSA in TNT plus goat serum (1:100)) for 30 min, cells were stained overnight in a humidified chamber using the indicated primary antibodies (phospho-p27T198, phospho-p27T187 (Santa Cruz), p27 (Transduction Labs)). The cells were stained with the following secondary antibodies (1:40) (rabbit anti-sheep IgG-Texas Red, (Santa Cruz) for phospho-p27T198, donkey anti-rabbit IgG Rhodamine Red, (Dianova) for phospho-p27T187 and donkey anti-mouse IgG Rhodamine Red, (Dianova) or Cy2 donkey anti-mouse (Jackson Immuno) for p27) for 30 min after which the cells were washed with PBS and mounted. For double staining experiments, anti-mouse FITC-labeled antibody was used. Images were acquired on a Leica DM 5000B microscope.

Plasmid constructs

Point mutations in the p27 expression plasmid pCS2⁺-p27 and p27C-K- were generated by using the Quick Change site-directed mutagenesis kit (Stratagene). Mutagenesis was confirmed by sequencing. pCMV-His₆-ubiquitin was kindly provided by Dirk Bohmann, p27C-K-, pCS2cyclin E, pCS2CDK2 and pCS2dnCDK2 were kindly provided by Jim Roberts. For generation of p27 in bacteria, the His₆-p27 expression plasmid pET21b-p27 was used.

In vivo ubiquitination assay

The ubiquitination assay was performed as described in Campanero and Flemington and the amount of incorporated His-ubiquitin measured by the established protocol (Campanero and Flemington, 1997).

Generation of stable cell lines

To create stable cell lines expressing p27 wild type and the T198A mutant under the control of the murine p27 promoter, a vector carrying nucleotide -2724 to +4967 from the murine p27 locus (gift of Arnaud Besson) was generated. To generate this construct, a *HindIII/KpnI* fragment encoding Exon 2 of the p27 locus was cloned into pBS (Stratagene) and used as a template for site-directed mutagenesis using the Quick Change system (Stratagene), thereby introducing the T198A mutation which was subsequently verified by sequencing. After mutagenesis, an *EcoRI/HindIII* fragment carrying the 5' region with exon 1 and the p27 promoter up to nucleotide -2724 was cloned in front of the *HindIII/KpnI* fragment in pBS. Finally a *KpnI/EcoRV* fragment with exon 3 and the 3' part up to nucleotide +4967 was cloned downstream of the *HindIII/KpnI* fragment to generate pBSgenomic-p27wild type or pBSgenomic-p27T198A.

The pBSgenomic-p27wildtype or T198A constructs were co-transfected with pBabe-puro into p27^{-/-} MEFs. Stably expressing clones were selected for 2 weeks with 2 µg/ml puromycin and tested for p27 expression by immunoblotting and RT-PCR as described (Li *et al*, 2003).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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