Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation

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The cellular abundance of the cyclin-dependent kinase (Cdk) inhibitor p27 is regulated by the ubiquitin–proteasome system. Activation of p27 degradation is seen in proliferating cells and in many types of aggressive human carcinomas. p27 can be phosphorylated on threonine 187 by Cdks, and cyclin E/Cdk2 overexpression can stimulate the degradation of wild-type p27, but not of a threonine 187-to-alanine p27 mutant [p27(T187A)]. However, whether threonine 187 phosphorylation stimulates p27 degradation through the ubiquitin–proteasome system or an alternative pathway is still not known. Here, we demonstrate that p27 ubiquitination (as assayed in vivo and in an in vitro reconstituted system) is cell-cycle regulated and that Cdk activity is required for the in vitro ubiquitination of p27. Furthermore, ubiquitination of wild-type p27, but not of p27(T187A), can occur in G_1-enriched extracts only upon addition of cyclin E/Cdk2 or cyclin A/Cdk2. Using a phosphothreonine 187 site-specific antibody for p27, we show that threonine 187 phosphorylation of p27 is also cell-cycle dependent, being present in proliferating cells but undetectable in G_1 cells. Finally, we show that in addition to threonine 187 phosphorylation, efficient p27 ubiquitination requires formation of a trimeric complex with the cyclin and Cdk subunits. In fact, cyclin B/Cdk1 which can phosphorylate p27 efficiently, but cannot form a stable complex with it, is unable to stimulate p27 ubiquitination by G_1 extracts. Furthermore, another p27 mutant [p27(CK^-)] that can be phosphorylated by cyclin E/Cdk2 but cannot bind this kinase complex, is refractory to ubiquitination. Thus throughout the cell cycle, both phosphorylation and trimeric complex formation act as signals for the ubiquitination of a Cdk inhibitor.

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The eukaryotic cell cycle is regulated by the sequential activation of cyclin-dependent kinases (Cdks) (for review, see Pines 1998; Sheaff and Roberts 1998). Cdk activation is regulated by phosphorylation of the catalytic subunit and by binding to activating (cyclins) and inactivating subunits (Cdk inhibitory proteins, or Ckis). Whereas the cellular levels of Cdk subunits appear to vary little through the cell division cycle phases, the cellular abundance of cyclins and Cki subunits can change rapidly in response to transcriptional or post-transcriptional events, including degradation through the ubiquitin–proteasome pathway (for review, see Pagano 1997).

The Cki p27 specifically inhibits cyclin E/Cdk2 and cyclin A/Cdk2, two kinases necessary for DNA replication to occur. It was shown originally that in response to mitogenic stimuli, the levels of p27 decrease allowing Cdk2 activation and entry into S phase. Previously, we demonstrated that the p27 half-life is longer in quiescent than in proliferating cells (Pagano et al. 1995). Furthermore, we showed that inhibition of the proteasome in intact cells leads to the accumulation of p27 and polyubiquitinated p27 species. Accordingly, p27 accumulates at the restrictive temperature in the murine *ts20TG^R* cell line that bears a mutation in the ubiquitin-activating enzyme *E1* gene. Several lines of evidence show that degradation of p27 can be recapitulated in an in vitro system (Pagano et al. 1995; Brandeis and Hunt 1996; Millard et al. 1997) and that it requires both ubiquitination and the activity of the proteasome (Pagano et al. 1995; Loda et al. 1997). First, extracts from proliferating cells degrade p27 faster than extracts from quiescent cells; second, incubation of p27 with proteasome-depleted extracts results in a block of its degradation and upon re-addition of purified proteasome particles, p27 degradation is restored completely; third, ATP depletion pre-

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vents the appearance of p27 polyubiquitinated species and inhibits its proteolysis; fourth, addition of ATP-γ-S, an ATP analog that can be hydrolyzed by the E1 ubiquitin-activating enzyme but not by the proteasome, leads to a substantial decrease in p27 proteolysis and to the accumulation of p27 polyubiquitinated species.

Phosphorylation plays a major role in ubiquitin-mediated proteolysis. In *Saccharomyces cerevisiae*, phosphorylation of G_1 regulatory proteins is necessary for their recognition by a specific ubiquitin protein ligase and for their consequent ubiquitination and degradation (for review, see Pagano 1997). Degradation of p27 is stimulated by Cdk2-dependent phosphorylation (Muller et al. 1997; Sheaff et al. 1997; Vlach et al. 1997). The vast majority, if not all, of Cdk-dependent phosphorylation of p27 is on threonine 187 (T187) (Alessandrini et al. 1997; Sheaff et al. 1997; Vlach et al. 1997). In fact, (1) the phosphorylation site containing T187 is the only Cdk-consensus site in human and mouse p27, (2) amino acid analysis of p27 phosphorylated in vitro by either Cdk2 or Cdk1 shows phosphorylation exclusively on threonine, (3) p27(T187A) is no longer a Cdk-substrate, and (4) coexpression of cyclin E/Cdk2 and p27, stimulates T187 phosphorylation of p27. However, it is unclear whether T187 phosphorylation acts as a signal for p27 ubiquitination or whether it controls an alternative degradation pathway. In fact, p27 (Levkau et al. 1998) and other substrates of the ubiquitin pathway such as p53 (Kubbutat and Vousden 1998), cyclin D1 (Choi et al. 1997), β catenin (Brancolini et al. 1997), IκBα (Cuervo et al. 1998), and others are also substrates of other proteolytic systems (e.g., caspases, calpain, lysosomal proteases, etc.), especially under conditions of cellular stress and upon overexpression of these substrates. In addition, for at least one protein, ornithine decarboxylase, it has been shown that proteasome-mediated degradation can occur in the absence of ubiquitination (for review, see Pickart 1997). Thus, understanding whether p27 phosphorylation regulates its ubiquitination is fundamental to determining which pathway regulates the degradation of p27 occurring in late G_1 phase of the cell cycle. Here we present studies on p27 ubiquitination that address these crucial points.

Results

Cdk-dependent phosphorylation on T187 is required for p27 ubiquitination

We have developed an in vitro p27 ubiquitination assay that requires human cell extracts as a source of the enzymes necessary to conjugate ubiquitin to p27 and ATP (Pagano et al. 1995). We now asked whether ATP was necessary solely for the activation of ubiquitinating enzymes or also for protein phosphorylation. Charging of the E1 ubiquitin-activating enzyme with ubiquitin requires the hydrolysis of the α–β bond of ATP, whereas protein phosphorylation reactions involve the transfer of the γ phosphate of ATP. We tested whether a β–γ nonhydrolyzable ATP analog (AMP–PNP), which can replace ATP in the E1-charging reaction but not in a kinase reaction, would allow p27 ubiquitination to occur. ATP was depleted by the addition of hexokinase and deoxyglucose and then AMP–PNP or ATP and an ATP regeneration system were added. In the absence of ATP, p27 was not ubiquitinated (Fig. 1A, lanes 1,2) (Pagano et al. 1995). Readdition of ATP but not of AMP–PNP was able to support p27 ubiquitination (lanes 3,4). To confirm that phosphorylation is necessary for p27 ubiquitination, the reaction was performed in the presence of staurosporine, a potent broad-specificity kinase inhibitor, which inhibited p27 ubiquitination completely (lane 5).

Degradation of p27 is stimulated by its phosphorylation on threonine 187 (T187), and the vast majority, if not all, of Cdk-dependent phosphorylation of p27 is on

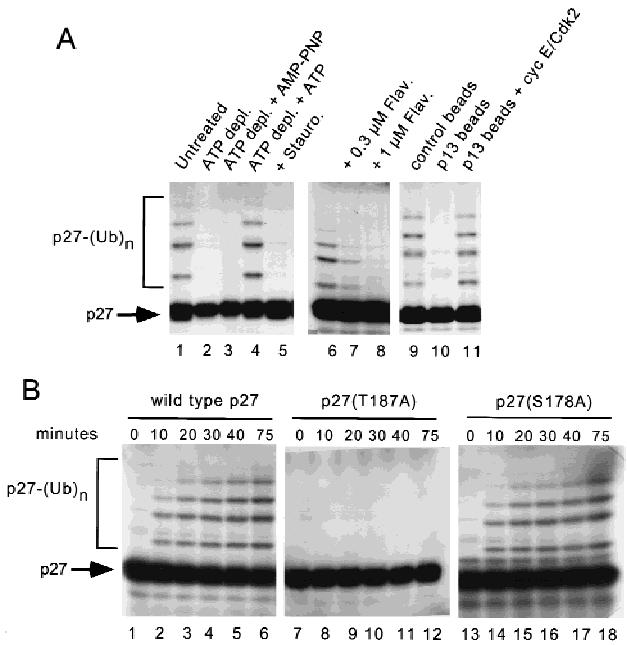

Figure 1. p27 polyubiquitination depends on Cdk-dependent phosphorylation on T187. (*A*) Human *p27* cDNA was transcribed and translated in vitro in the presence of [^{35}S] methionine and subjected to a ubiquitination reaction with a HeLa extract, as described in the Materials and Methods. Samples shown in lanes *2–4* were incubated with hexokinase and deoxyglucose to deplete extract from ATP (ATP depl.). Then, AMP–PNP (lane *3*) or ATP and an ATP regeneration system (lane *4*) were added. Sample in lane *5* was incubated with staurosporine (Stauro.). Samples in lanes *7* and *8* were incubated with the indicated concentrations of flavopiridol (Flav.), used as described in Materials and Methods. Samples in the last three lanes were depleted with control beads (lane *9*) or p13 beads (lanes *10,11*). Then, purified recombinant cyclin E/Cdk2 complex was added (lane *11*). The reaction products were analyzed by SDS-PAGE and autoradiography. The bracket (*left*) marks a ladder of bands >27,000 corresponding to polyubiquitinated p27. (*B*) In vitro-translated wild-type p27 (lanes *1–6*), p27(T187A) (lanes *7–12*), or p27(S178A) (lanes *13–18*) was incubated for the indicated times in the presence of a HeLa extract. The reaction products were analyzed by SDS-PAGE and autoradiography. The bracket (*left*) marks a ladder of bands >27,000 corresponding to polyubiquitinated p27.

T187 (Muller et al. 1997; Sheaff et al. 1997; Vlach et al. 1997; data not shown). We therefore asked whether Cdk activity and the integrity of T187 were also essential for p27 ubiquitination in vitro. First, we used the Cdk-specific kinase inhibitor flavopiridol (Meijer 1995; Carlson et al. 1996) and found that this compound inhibited p27 ubiquitination (lanes 6–8). We then depleted Cdks from cell extracts using p13 beads (Brizuela et al. 1987). Extracts depleted with p13 beads but not with control beads were unable to sustain p27 ubiquitination unless reconstituted with recombinant purified cyclin E/Cdk2 complex (lanes 9–11). Finally, we compared wild-type p27, p27(T187A), and another proline-directed phosphorylation site p27 mutant, p27(S178A), for their ability to be ubiquitinated in vitro. p27(T187A), but not p27(S178A), failed to be conjugated with ubiquitin (Fig. 1B).

In summary, our results show that in vitro ubiquitination of p27 requires its Cdk-dependent phosphorylation on T187.

p27 ubiquitinating activity is cell-cycle regulated

Compared to proliferating or S-phase cells, G_0 and G_1 cells contain much lower levels of p27 proteolytic activity when assayed either in vitro (Pagano et al. 1995; Brandeis and Hunt 1996; Millard et al. 1997) or in vivo (Pagano et al. 1995; Hengst and Reed 1996). We asked whether this difference in degradation ability reflects a difference in ubiquitination-specific degradation activity. We incubated p27 with extracts made from either proliferating HeLa cells or from HeLa cells enriched in the G_1 population with lovastatin, or alternatively with extracts made from either G_1 or quiescent (G_0) human diploid fibroblasts. Extracts from proliferating cells were able to sustain p27 ubiquitination, whereas the G_0 or G_1 extracts were not active in p27 ubiquitin conjugation (Fig. 2, lanes 1,2,10,11). In contrast, both the proliferating cell extract and the G_1 extract were active in a cyclin B in vitro ubiquitination assay (Fig. 2, lanes 8,9), in agreement with fact that the cyclin B-specific ligase is active in G_1 (Amon et al. 1994). The addition of recombinant purified cyclin E/Cdk2 to G_1 extracts enabled ubiquitination of wild-type p27 to an extent similar to that observed in proliferating extracts (Fig. 2, lanes 3,12). Similarly, cyclin E/Cdk2 stimulated the in vitro degradation of p27 by G_1 extracts (data not shown). In contrast, p27(T187A) mutant was not ubiquitinated (Fig. 2, lanes 6,7) or degraded (data not shown) even in the presence of recombinant purified cyclin E/Cdk2. Staurosporine inhibited the stimulation of ubiquitination by cyclin E/Cdk2 (Fig. 2, lane 4). This effect was likely caused by the inhibition of cyclin E/Cdk2 kinase activity, because when cyclin E/Cdk2 was first incubated with p27 and then added to the reaction in the presence of staurosporine, p27 ubiquitination in a G_1 extract still occurred efficiently (Fig. 2, lane 5).

These results show that the enzymatic activity missing from G_1 extracts can be provided by cyclin E/Cdk2. In contrast, quiescent-cell extracts were rescued very poorly by the addition of cyclin E/Cdk2 (Fig. 2, lane 13) indicating that yet another activity, perhaps that of a ubiquitin ligase, is missing from those extracts.

p27 phosphorylation on T187 and p27 ubiquitination are cell-cycle regulated

As the half life of p27 is longer in G_1 cells than in proliferating ones (Pagano et al. 1995; Hengst and Reed 1996), and its ubiquitination requires its phosphorylation on T187, we asked whether p27 phosphorylation on T187 and p27 ubiquitination were cell-cycle regulated. We generated a phospho-T187 site-specific p27 antibody using a phosphopeptide that spans the phosphorylated T187 residue of p27. After affinity chromatography using both phospho- and nonphosphopeptide columns, the purified antibody recognized recombinant p27 only when

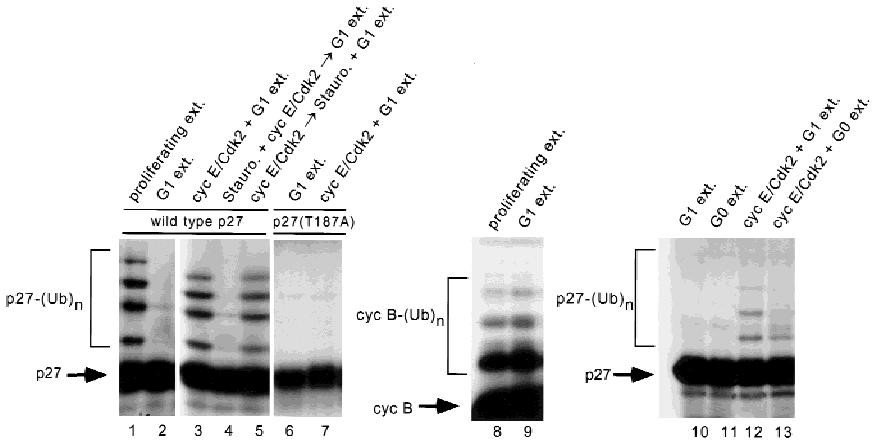

Figure 2. p27 ubiquitinating activity is cell-cycle regulated. In vitro-translated wild-type p27 (lanes *1–5* and *10–13*), p27(T187A) (lanes *6,7*), or ^{125}I-labeled cyclin B (lanes *8,9*) were incubated in the presence of extracts (ext.) from proliferating HeLa (lanes *1,8*), G_1 HeLa (lanes *2–7,9*), G_1 IMR-90 (lanes *10,12*), or G_0 IMR-90 cells (lanes *11,13*). Purified recombinant cyclin E/Cdk2 complex (80 ng) was added to the samples contained in lanes *3–5*, *7*, *12*, and *13*. Samples in lanes *4* and *5* were incubated in the presence of staurosporine (Stauro.) added to the ubiquitination reaction either before (lane *4*) or after p27 incubation with the cyclin E/Cdk2 complex (lane *5*). The reaction products were analyzed by SDS-PAGE and autoradiography. The bracket (*left*) marks a ladder of bands >27,000 corresponding to polyubiquitinated p27.

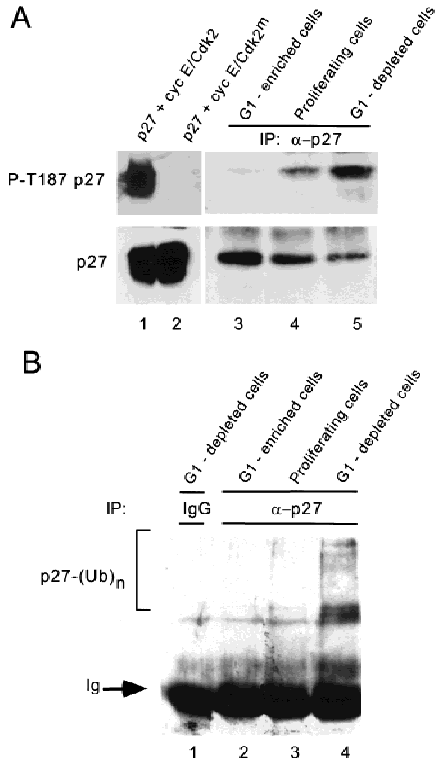

Figure 3. Phosphorylation on T187 and ubiquitination of cellular p27 are cell-cycle regulated. (*A*) Recombinant purified p27 incubated in a kinase reaction with either recombinant purified cyclin E/Cdk2 (lane *1*) or cyclin E/Cdk2^m (lane *2*). Sample in lane *3* is from G_1-enriched cells (75% in G_1, 6% in S, and 19% in G_2/M); sample in lane *4* is from proliferating cells (58% in G_1, 21% in S, and 21% in G_2/M); and sample in lane *5* is from G_1-depleted cells (4% in G_1, 35% in S, and 61% in G_2/M). Lanes *3–5* represent immunoprecipitations with a mouse anti-p27 monoclonal antibody. (*Top*) samples were immunoblotted with a rabbit phospho-T187 site-specific p27 antibody (P-T187 p27); (*bottom*) samples were immunoblotted with a goat anti-p27 antibody. (*B*) Samples in lanes *1* and *4* are from G_1-depleted cells; sample in lane *2* is from G_1-enriched cells; and sample in lane *3* is from proliferating cells. (Lane *1*) Immunoprecipitation with purified rabbit IgG (IgG); (lanes *2–4*) immunoprecipitations with a mixture of a rabbit phospho-T187 site-specific p27 antibody and a rabbit anti-p27 antibody. Samples were then immunoblotted with a mouse monoclonal antibody to ubiquitin. The bracket (*left*) marks a ladder of bands corresponding to polyubiquitinated p27; (→) the IgG heavy chains (Ig).

phosphorylated by cyclin E/Cdk2 (Fig. 3A, lanes 1,2). To determine whether p27 is phosphorylated on T187 in a cell-cycle-regulated manner, we immunoprecipitated total p27 from G_1-enriched, proliferating, and G_1-depleted HeLa cells (prepared as described in Materials and Methods), and then we immunoblotted with the phospho-T187 site-specific p27 antibody. The phospho-T187-specific p27 antibody detected phosphorylated p27 only in the immunoprecipitates from proliferating and G_1-depleted cells (Fig. 3A, top panel, lanes 3–5), whereas an anti-p27 antibody detected p27 in all three samples, with the highest levels found in the G_1-enriched population (Fig. 3A, bottom panel, lanes 3–5). In a parallel experiment, the G_1-enriched, proliferating, and G_1-depleted HeLa cell extracts were denatured to dissociate p27 from any interacting proteins, and immunoprecipitated with a mixture of the phospho-T187-specific p27 antibody and an anti-p27 antibody. Immunoblotting of the immunoprecipitates with an antibody to ubiquitin detected ubiquitinated p27 in G_1-depleted but not in G_1-enriched cells (Fig. 3B).

Our results demonstrate that endogenous cellular p27 is phosphorylated on T187 and that this phosphorylation, along with p27 ubiquitination, is cell-cycle regulated, being absent in G_1 and present in proliferating cells.

p27 ubiquitination requires its stable binding to a cyclin/Cdk complex

The experiments described above show that G_1-enriched extracts are unable to sustain p27 ubiquitination unless cyclin E/Cdk2 is added to the reaction. Surprisingly, cyclin E in a complex with an inactive catalytic mutant of Cdk2 [Cdk2^m (Desai et al. 1992)] was able to stimulate p27 ubiquitination although to a lesser extent than wild-type cyclin E/Cdk2 (Fig. 4A, lanes 2,3). This stimulation was not caused by the presence of cyclin E in the cyclin E/Cdk2^m complex, as cyclin E alone did not stimulate conjugation of ubiquitin to p27 (Fig. 4A, lane 5). We speculated that the stimulation of p27 ubiquitination by cyclin E/Cdk2 is caused by both p27 phosphorylation and the formation of a p27/cyclin/Cdk complex, which allows its recognition by the ubiquitin ligase. This is the case for other proteins that require phosphorylation and assembly with other subunits for ubiquitination [e.g., IκBα (Yaron et al. 1997)]. If this hypothesis is correct, the stimulation of p27 ubiquitination by cyclin E/Cdk2^m could be caused by complex formation and phosphorylation by low levels of the cyclin E/Cdk2 present in G_1 extracts.

All p27 ubiquitin conjugation reactions were performed in the presence of okadaic acid, a potent phosphatase inhibitor able to stabilize p27 phosphorylated forms (data not shown). Removal of okadaic acid from the reaction mix completely blocked the stimulation of p27 ubiquitination by cyclin E/Cdk2^m (Fig. 4A, lane 4). We then performed a p27 phosphorylation reaction with cyclin E/Cdk2^m followed by a ubiquitination reaction using G_1 cell extracts that had been preincubated with flavopiridol. Under these conditions cyclin E/Cdk2^m was unable to induce p27 ubiquitination even in the presence of okadaic acid (Fig. 4A, lanes 6–8), indicating that cyclin E/Cdk2^m stimulation of p27 ubiquitination requires a kinase present in the cellular extract. Cdk2 activity in these G_1-enriched extracts is present in sufficient amounts to phosphorylate some p27 in the presence of okadaic acid (data not shown). As 0.3 μM flavopiridol shows high specificity towards Cdk2 and Cdk4 (Meijer 1995), it is very likely that the kinase present in the G_1 extract necessary to stimulate p27 ubiquitination in the presence of cyclin E/Cdk2^m is a G_1 Cdk, and is

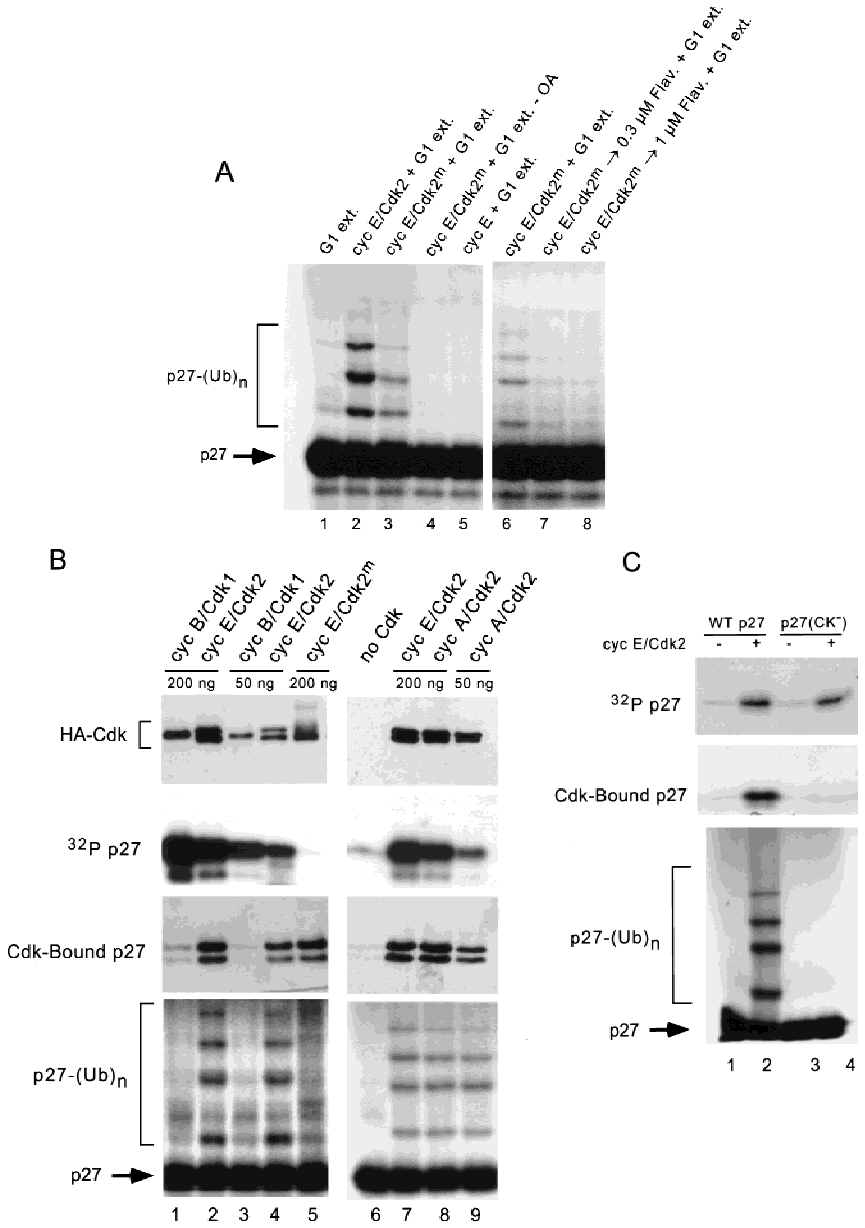

Figure 4. p27 polyubiquitination requires its stable binding to a cyclin/Cdk complex. (*A*) In vitro-translated p27 was incubated in the presence of extracts from G_1 HeLa cells (G1 ext.) and 80 ng of purified recombinant cyclin E/Cdk2 complex (lane *2*), 80 ng of purified recombinant cyclin E in complex with catalytic inactive Cdk2 mutant ($Cdk2^m$) (lanes *3*,*4*,*6*–*8*), 80 ng of purified recombinant cyclin E alone (lane *5*), or buffer (lane *1*). Sample in lane *4* lacked okadaic acid (–OA); samples in lanes *7* and *8* were incubated in the presence of the indicated concentrations of flavopiridol (Flav.). The reaction products were analyzed by SDS-PAGE and autoradiography. The bracket (*left*) marks a ladder of bands >27,000 corresponding to polyubiquitinated p27. (*B*) Comparison of the abilities of cyclin E/Cdk2, cyclin E/$Cdk2^m$, cyclin B/Cdk1, and cyclin A/Cdk2 to phosphorylate, bind, and stimulate the ubiquitination of p27. The amounts of purified cyclin/HA-tagged Cdk complexes were measured by colorimetric methods and confirmed with an anti-HA antibody (HA-Cdk). (Lane *1*) Cyclin B/Cdk1 (200 ng); (lane *2*) cyclin E/Cdk2 (200 ng); (lane *3*) cyclin B/Cdk1 (50 ng); (lane *4*) cyclin E/Cdk2 (50 ng); (lane *5*) cyclin E/$Cdk2^m$ (200 ng); (lane *6*) no kinase added; (lane *7*) cyclin E/Cdk2 (200 ng); (lane *8*) cyclin A/Cdk2 (200 ng); (lane *9*) cyclin A/Cdk2 (50 ng). Lanes *1*–*5* and *6*–*9* are from two separate experiments. The phosphorylation of p27 by different amounts of cyclin/Cdk complexes was performed as described in Materials and Methods. Phosphorylated p27 was detected by autoradiography (^{32}P p27). The binding of p27 to different cyclin/Cdks was assayed by incubating in vitro-translated p27 with His-tagged cyclin/Cdk complexes and then purifying them with nickel-agarose. Bound p27 was then detected by immunoblot with an anti-p27 monoclonal antibody (Cdk-Bound p27). The stimulation of p27 ubiquitination by different amount of cyclin/Cdk complexes was assayed by incubating in vitro-translated p27 with these complexes and extracts from G_1 HeLa cells. The reaction products were analyzed by SDS-PAGE and autoradiography. The bracket (*left*) marks a ladder of bands >27,000 corresponding to polyubiquitinated p27. (*C*) Phosphorylation, binding, and ubiquitination of wild-type p27 (WT p27) and p27(CK^-) in the presence (lanes *2*,*4*) or in the absence (lanes *1*,*3*) of 200 ng of cyclin E/Cdk2. In vitro-translated wild-type p27 (lanes *1*,*2*) and p27(CK^-) (lanes *3*,*4*) were immunoprecipitated with an anti-p27 antibody and then subjected to a phosphorylation reaction in the presence or in the absence of cyclin E/Cdk2. Phosphorylated p27 was detected by autoradiography with a cellophane screen that blocked the ^{35}S but not ^{32}P (^{32}P p27) (*top*). The binding to cyclin E/Cdk2 was assayed by incubating in vitro-translated wild-type p27 and p27(CK^-) with His-tagged cyclin E/Cdk2 complex and then purifying the complex with nickel–agarose. Bound p27 was then detected by autoradiography (Cdk-Bound p27) (*middle*). The ubiquitination reaction was performed by incubating in vitro-translated wild-type p27 and p27(CK^-) with extracts from G_1 HeLa cells in the presence and in the absence of cyclin E/Cdk2 (*bottom*). The reaction products were analyzed by SDS-PAGE and autoradiography. The bracket (*left*) marks a ladder of bands >27,000 corresponding to polyubiquitinated p27.

most likely Cdk2 as Cdk4 is inactive in HeLa cell extracts (Tam et al. 1994).

To demonstrate that p27 ubiquitination requires both Cdk-dependent phosphorylation as well as its binding to the kinase complex, we used cyclin E/Cdk2, cyclin B/Cdk1, and cyclin A/Cdk2 complexes which, although having different affinities for p27 (Polyak et al. 1994; Toyoshima and Hunter 1994), were all able to phos-

phorylate wild-type p27 on T187 (as detected with a phospho-T187 site-specific p27 antibody) but not p27(T187A) (data not shown). We tested cyclin E/Cdk2, cyclin E/Cdk2^m, cyclin B/Cdk1, and cyclin A/Cdk2 for their ability (1) to phosphorylate p27, (2) to bind p27, and (3) to induce p27 ubiquitination. All three of these kinases were able to phosphorylate p27 (Fig. 4B, second panel) but cyclin B/Cdk1 was unable to stably interact with p27 (Fig. 4B, third panel). These results are in agreement with published reports showing that the affinity of p27 for cyclin B/Cdk1 is lower than that for cyclin E/Cdk2 and cyclin A/Cdk2 (Polyak et al. 1994; Toyoshima and Hunter 1994). In contrast to cyclin A/Cdk2 and cyclin E/Cdk2, cyclin B/Cdk1 did not bind or stimulate p27 ubiquitination at kinase concentrations which allowed efficient p27 phosphorylation (Fig. 4B, bottom panel, lanes 1,3). Similarly, cyclin E/Cdk2^m which bound, but did not phosphorylate p27, was unable to stimulate p27 ubiquitination efficiently (Fig. 4B, bottom panel, lane 5) unless incubated in the presence of either cyclin E/Cdk2 or cyclin B/Cdk1 (data not shown).

To study further the possibility that a stable interaction of cyclin E/Cdk2 with p27 was required for ubiquitination, we used a p27 mutant that cannot stably interact with both cyclins and Cdks [p27(CK$^-$)], but is still a substrate of cyclin E/Cdk2 (Vlach et al. 1997). We confirmed that p27(CK$^-$) could be phosphorylated by cyclin E/Cdk2 but did not bind stably to this complex (Fig. 4C, top and middle panels). Importantly, p27(CK$^-$) was refractory to ubiquitin conjugation (Fig. 4C, bottom panel).

In conclusion, using three different approaches we demonstrate that efficient p27 ubiquitination requires phosphorylation on T187 and its stable interaction with a cyclin/Cdk complex.

Discussion

In agreement with the notion that Cdk2 needs to be active for DNA replication to occur (Pagano et al. 1992, 1993; van den Heuvel and Harlow 1993; Ohtsubo et al. 1995) the levels of the Cdk inhibitor p27 are high in G_1 and decrease as cells approach S phase. Transcription of p27 occurs at a similar rate throughout the different phases of the cell-cycle and, although there is translational control of p27 levels (Hengst and Reed 1996), this does not appear to be the main mechanism for the reduction in p27 abundance observed at the G_1/S transition (Millard et al. 1997). Rather we have shown that the half-life of p27 is cell-cycle regulated, being much longer in quiescent fibroblasts than in proliferating cells (Pagano et al. 1995). Likewise, the half-life of p27 in contact-inhibited and lovastatin-blocked cells is longer than in asynchronous and thymidine-blocked cells, respectively (Hengst and Reed 1996). In addition, we have demonstrated that p27 is ubiquitinated both in vivo and in vitro (Pagano et al. 1995).

p27 is phosphorylated on threonine 187 by cyclin E/Cdk2 and overexpression of this complex in mammalian cells induces degradation of transfected p27 (Muller et al. 1997; Sheaff et al. 1997; Vlach et al. 1997). However, it had not been clear whether T187 phosphorylation targets p27 for ubiquitin-mediated degradation or for other degradation pathways. In fact, it has been shown that, in addition to cell-cycle arrest, p27 overexpression induces apoptosis (Katayose et al. 1997) and that during apoptosis p27 is cleaved by caspases (Levkau et al. 1998). We now have demonstrated that Cdk-mediated phosphorylation of p27 on threonine 187 is indeed required for p27 ubiquitination and consequent degradation. Accordingly, it has been shown recently that the cell-free degradation of p27 is dependent on Cdk2 activity (Nguyen et al. 1999). Furthermore, we have established that ubiquitination of p27 bound to a cyclin/Cdk complex is more efficient than the ubiquitination of uncomplexed p27. Importantly, the phosphorylation on threonine 187 and the ubiquitination of cellular p27, as well as p27 ubiquitinating activity (as assayed in vitro) were found to be regulated collectively during the cell cycle, being present in proliferating cells but not in G_0- and G_1- arrested cells.

Increased turnover of p27 starts in late G_1 and continues until mitosis. It is conceivable, therefore, that different Cdks are responsible for p27 phosphorylation on T187 at different points of the cell cycle. Cdk2 and Cdk1 are the candidate kinases for p27 phosphorylation/binding and subsequent ubiquitination. Cdk3 is also a good candidate as its function is requested for the G_1/S transition and has several properties similar to those of Cdk2 and Cdk1 (van den Heuvel and Harlow 1993). In contrast, Cdk4 does not seem to play a role in inducing p27 proteolysis since co-expression of cyclin D1 and Cdk4 does not result in p27 elimination (Sheaff et al. 1997); however, the cyclin D1/Cdk4 complex does regulate p27 activity by sequestration (Blain et al. 1997; Cheng et al. 1998). In agreement with these data, we found that the cyclin D3/Cdk4 complex does not stimulate p27 ubiquitination (Montagnoli and Pagano, unpubl.). The published p27/cyclin A/Cdk2 X-ray crystallography data show that p27 inserts itself within the Cdk catalytic cleft, likely preventing ATP transfer (Russo et al. 1996). This would exclude therefore that p27 can be phosphorylated in an intracomplex reaction. Our results that p27 bound to an inactive cyclin E/Cdk2^m complex can be phosphorylated by an unbound cyclin B/Cdk1 complex (Montagnoli and Pagano, unpubl.), rather demonstrate that an intercomplex phosphorylation of p27 is possible.

p27 inhibits different Cdks with different affinities (Polyak et al. 1994; Toyoshima and Hunter 1994; Blain et al. 1997). We have shown here that the abilities of Cdk2 and Cdk1 to phosphorylate p27 on T187 and to induce p27 ubiquitination do not correlate. Therefore, although phosphorylation is necessary for p27 ubiquitination, it is not sufficient. In fact, cyclin B/Cdk1 in amounts sufficient to phosphorylate but not bind efficiently p27, does not stimulate p27 ubiquitination. Similarly, p27(CK$^-$), a mutant unable to bind cyclin E/Cdk2 complex is still phosphorylated by this kinase but cannot be ubiquitinated. Finally, a cyclin E/Cdk2^m complex that binds p27 but cannot phosphorylate it, stimulates p27 ubiquitination by relying on a Cdk (Fig. 4A, lanes 6–8, flavopiridol-

sensitive) present in the G_1 extract. It is conceivable that p27 phosphorylation by and binding to a cyclin/Cdk complex expose a domain in p27 that is recognized by a specific ubiquitin ligase, or, alternatively, the cyclin/Cdk complex binds and targets this ligase to p27.

The inability of cyclin E/Cdk2 to stimulate p27 ubiquitination in extracts from quiescent (G_0) cells suggests that another factor, perhaps a cell-cycle-regulated ubiquitin ligase necessary for p27 ubiquitination, is limiting. In yeast, phosphorylation of G_1 regulators (e.g., Ckis, cyclins) allows their recognition by ubiquitin ligases called SCFs because they are formed by three subunits: S-phase kinase-associated protein 1 (Skp1), CulA, and one of many F-box proteins (for review, see Pagano 1997). Whereas CulA interacts directly with a ubiquitin-conjugating enzyme, the F-box protein subunit recruits specific phosphorylated substrates. We and others have shown that, as in yeast, the human homolog Skp1/Cul1 complex forms a scaffold for multiple F-box proteins, including Skp2 (for review, see Patton et al. 1998), β-transducin repeat-containing protein Trcp, also called Slimb or Fbp1 (Latres et al. 1999; Winston et al. 1999), and other novel F-box proteins (C. Cenciarelli, D.S. Chiaur, S. Murthy, D. Guardavaccaro, M. Loda, G. Inghirani, W. Parks, M. Vidal, D. Demetrick, and M. Pagano, in prep.). These different SCF complexes potentially target different substrates for ubiquitin-mediated degradation. We are currently assessing whether a specific F-box protein targets p27 for ubiquitination.

The p27/cyclin/Cdk complex is very stable and even when phosphorylated on T187, p27 does not appear to dissociate from the cyclin/Cdk complex. We have reported here that Cdk-dependent phosphorylation of p27 at T187 together with the formation of a stable p27/cyclin/Cdk trimeric complex are both required for ubiquitination to occur. Thus, it is possible that p27, in response to specific mitogenic stimuli, is removed physically by its ubiquitination and/or degradation from the complex which it inhibits. p27 could be regulated similarly to IκBα, whose ubiquitination requires both its phosphorylation and its stable binding to NF-κB (Yaron et al. 1997).

In summary, the timely regulation of p27 cellular abundance and activity derives from a combination of several factors, which include the ability of certain cyclin/Cdk complexes to interact stably with p27, the cellular abundance and specific activity of Cdk complexes which phosphorylate p27, as well as the availability of yet-to-be-identified p27-specific ubiquitin ligase.

Materials and methods

Protein extraction for in vitro ubiquitination assay

Approximately 4 ml of HeLa S3 cell pellets were suspended in 6 ml of ice-cold buffer consisting of 20 mM Tris-HCl (pH 7.2), 2 mM DTT, 0.25 mM EDTA, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. The suspension was transferred to a cell nitrogen-disruption bomb (Parr, Moline, IL) that had been rinsed thoroughly and chilled on ice before use. The bomb chamber was connected to a nitrogen tank and the pressure was brought slowly to 1000 psi. The chamber was left on ice under the same pressure for 30 min, and the pressure was released slowly. The material was transferred to an Eppendorf tube and centrifuged in a microcentrifuge at 10,000*g* for 10 min. The supernatant (S-10) was divided into smaller samples and frozen at –80°C. This method of extract preparation based on the use of a cell nitrogen-disruption bomb extract preserves the activity of in vitro ubiquitinate p27 better than the method described previously (Pagano et al. 1995) (data not shown).

In vitro ubiquitination assay

In vitro-translated ^{35}S-labeled p27 (1 μl) was incubated at 30°C for different times (0–75 min) in 10 μl of ubiquitination mix containing 40 mM Tris at pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 1 μM ubiquitin aldehyde, 1 mg/ml methyl ubiquitin, 10 mM creatine phosphate, 0.1 μg/ml creatine kinase, 0.5 mM ATP, 1 μM okadaic acid, and 20 μg of HeLa cell extract. In the indicated samples, purified active His–cyclin E/Cdk2 was added. In the experiments in which flavopiridol effects were tested, ATP was used at the concentration of 5 μM. Reactions were stopped with Laemmli sample buffer containing β-mercaptoethanol and their products were run on protein gels under denaturing conditions. Polyubiquitinated p27 forms were identified by autoradiography. Ubiquitin aldehyde was added to the ubiquitination reaction to inhibit the isopeptidases that would remove the chains of ubiquitin from p27. Methyl ubiquitin was added because it competes with the ubiquitin present in the cellular extracts and terminates p27 ubiquitin chains that can be appreciated as discrete bands instead of a high molecular smear. These shorter polyubiquitin chains have lower affinity for the proteasome and therefore are more stable. In the absence of methyl ubiquitin, p27 degradation activity, instead of p27 ubiquitination activity, could be measured (data not shown). In addition to the fact that p27 mutant lacking all 13 lysines (a gift of B. Amati, Swiss Institute for Experimental Cancer Research, Switzerland) was not ubiquitinated, a further formal demonstration that p27 high-mobility species are ubiquitinated was obtained using an immunoprecipitation with an antibody to p27 followed by a subsequent immunoprecipitation with an anti-ubiquitin antibody (data not shown).

Reagents and antibodies

Ubiquitin aldehyde (Hershko and Rose 1987), methyl-ubiquitin (Hershko and Heller 1985) and p13 beads (Brizuela et al. 1987) were prepared as described. AMP–PNP, staurosporine, hexokinase, and deoxyglucose were from Sigma; lovastatine form Merck; flavopiridol was from Hoechst Marion Roussel. The preparation, purification, and characterization of a mouse monoclonal antibody (mAb) to human p27 (clone 4FIIDII) and a polyclonal against human p27 were performed in collaboration with Zymed. In the indicated case, a goat anti-p27 from Santa Cruz was used. Ubi-1-1510 mAb to ubiquitin was from Zymed and Rat anti-HA antibody was from Boehringer Mannheim. The phospho-site p27-specific antibody was generated in collaboration with Zymed by injecting rabbits with the phospho-peptide NAGSVEQT*PKKPGLRRRQT, corresponding to the carboxyl terminus of the human p27 with a phosphothreonine at position 187 (T*). The antibody was then purified from serum with two rounds of affinity chromatography using both phospho- and nonphosphopeptide chromatography.

p27 mutants

All *p27* constructs were derived from the human cDNA sequence. Point mutations described in the text were generated by

oligonucleotide-directed mutagenesis using the polymerase chain reaction of the QuikChange site-directed mutagenesis kit (Stratagene). All mutants were sequenced in their entirety. Mouse p27 wild type and mouse p27(CK$^-$) mutant (R30A, L32A, F62A, F64A) (Vlach et al. 1997) used in Figure 3C were obtained from B. Amati.

Recombinant proteins

Baculoviruses expressing human His-tagged cyclin A, His-tagged cyclin B, His-tagged cyclin E, HA-tagged Cdk2, HA-tagged Cdk1, HA-tagged catalytic inactive Cdk2 (K33T, K34S) (Cdk2^m) were supplied by D. Morgan (Desai et al. 1992). Recombinant viruses were used to infect 5B cells as described (Hannon 1995) and assayed for expression of their encoded protein by immunoblotting. Cyclin–Cdk complexes and uncomplexed cyclin subunits were purified by nickel-agarose chromatography (Invitrogen) according to the manufacturer's instructions.

Cell synchronization and extract preparation

HeLa S3 cells and human lung fibroblasts, IMR-90, were obtained from the American Type Culture Collection. G_1-enriched HeLa cells were obtained with a 24-hr lovastatin treatment (O'Connor and Jackman 1995), and G_1-depleted cells with an 11-hr treatment with nocodazole (40 ng/ml). Synchronization was monitored by flow cytometry (percent of cells in G_1, S and G_2/M is reported in the Fig. 3 legend). IMR-90 were synchronized in G_0 by serum starvation for 48 hr, and in G_1 by serum starvation followed by serum readdition for 8–10 hr. Cell-cycle analysis by flow cytometry showed that after serum starvation, ~90% of the cells had a 2N DNA content. Conditions for protein extraction have been described previously (Pagano et al. 1993).

Immunoprecipitation and immunoblotting

Cell extracts were prepared with a 0.1% Triton X-100 lysis buffer as described (Pagano et al. 1995). Proteins were first denatured with 1% SDS and then diluted with 1% Triton X-100 lysis buffer prior to addition of the antibody. Protein extract (3 mg) was immunoprecipitated as described (Pagano et al. 1995) with either 15 µg/ml affinity purified (AP) anti-p27 monoclonal antibody or a mixture of AP anti-p27 antibody (5 µg/ml) and AP phosphosite p27-specific antibody (15 µg/ml). Conditions for immunoblotting have been described previously (Pagano et al. 1995).

Kinase assay

In vitro-translated p27 (1 µl) immunoprecipitated with an anti-p27 antibody or 1 µg of recombinant purified p27 was incubated for 1 hr at 30°C in the presence of 50 mM Tris-Cl at pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 30 µM ATP, 5 µCi [γ-^{32}P]ATP, and 50–200 ng of recombinant purified cyclin B/Cdk1 or cyclin E/Cdk2 complex.

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