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Proteasome-Mediated Degradation of p21 via N-Terminal Ubiquitinylation

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Summary

We examined the mechanism responsible for the degradation of p21, a negative regulator of the cell division cycle. We found that p21 proteolysis requires functional ubiquitin and Nedd8 systems. Ubiquitinylated forms of p21 and p21(K0), a p21 mutant missing all lysines, are detected in vivo and in vitro, showing that the presence of lysines is dispensable for p21 ubiquitinylation. Instead, the free amino group of the N-terminal methionine of p21 is a site for ubiquitinylation in vivo. Although wild-type p21 is more abundantly ubiquitinylated than p21(K0) mutant due to the presence of internal lysine residues, their rates of proteolysis are indistinguishable. These results demonstrate that proteasomal degradation of p21 is regulated by the ubiquitin pathway and suggest that the site of the ubiquitin chain is critical in making p21 a competent substrate for the proteasome.

Introduction

The cyclin-dependent kinase inhibitor p21, the product of the *Cip1* gene, is critical for regulation of the cell cycle and DNA replication (Sherr and Roberts, 1999). The role for p21 as an inhibitor of the cell cycle is based on its ability to bind to cyclin/cdk complexes containing cdk2 and block their activity. p21 appears to have a further role at the G2 checkpoint by inhibiting the activity of cdk1 and also positively regulates G1 progression by aiding in the assembly of cdk4 and cdk6 with D-type cyclins. Additionally, p21 can act as an inhibitor of DNA replication through its association with PCNA, a protein required for processive DNA synthesis. Binding of p21 to PCNA affects the ability of PCNA to associate with other factors required for DNA replication.

Levels of p21 protein are regulated during the cell cycle at the levels of transcription and protein degradation. Transcriptional control of p21 has been well characterized, particularly in response to serum. In fact, although in quiescent cells, p21 is already expressed and bound to cdks, a further increase in p21 is induced by serum in early G1 via a serum-responsive element in its promoter to prevent premature activation of cdks and activation of DNA synthesis. Then later, for cells to enter S phase, p21 needs to be removed from the cdks that it has inhibited, so that the cell cycle can resume. However, p21 forms a stable interaction with cyclin/cdk complexes and to be removed from them it needs to be targeted to the proteasome, which in turn unfolds and degrades p21, releasing cdks untouched and active. Many questions remain as to the mechanism of p21 proteolysis by the 26S proteasome. Ubiquitinylated forms of both endogenous and exogenous p21 have been detected in vivo (Cayrol and Ducommun, 1998; Fukuchi et al., 2002; Maki and Howley, 1997; Maki et al., 1996; Rousseau et al., 1999; Sheaff et al., 2000) but the signals that induce proteasome-mediated degradation of p21 are still not well understood. Although there is evidence that during the progression through S phase the SCF^{Skp2} ubiquitin ligase is involved in the degradation of p21 (Bornstein et al., 2003), p21 is a short-lived protein also at times when Skp2 is absent. In addition, whether ubiquitinylation is necessary for p21 degradation remains unclear and it is actually believed that ubiquitinylation is dispensable for the proteasomal degradation of p21 (Sheaff et al., 2000). This conclusion was mostly based on the fact that a mutant of p21 with all of its six lysines mutated to arginines [p21(K0) mutant] is degraded at the same rate as p21 in vivo. Moreover, it was shown that p21 is able to bind the α7 subunit of the 20S proteasome, suggesting that this interaction eliminates the need for polyubiquitinylation of p21 for recognition by the 26S proteasome (Touitou et al., 2001). Taken together these results have led to the commonly accepted belief that the ubiquitinylation of p21 is unnecessary for its degradation, which is therefore considered to be ubiquitin-independent but proteasome-dependent.

We initiated studies to understand the mechanism by which p21 is degraded without prior ubiquitinylation but found that the ubiquitin system is indeed required for p21 proteolysis. Following is a description of our results.

Results

Wild-Type p21 and p21(K0) Mutant Are Ubiquitinylated and Degraded at Similar Rates In Vitro, and Their Degradation Is Mediated by a Functional Ubiquitin System

To investigate how the proteasome degrades p21, we developed an in vitro assay for the degradation of p21 similar to what we use for a related protein, namely p27 (Montagnoli et al., 1999). Wild-type p21 and p21(K0) mutant were in vitro translated using a rabbit reticulocyte lysate (RRL) in the presence of ^{35}S-methionine and then incubated with an ATP regenerating system in the absence or presence of purified 26S proteasomes. Since endogenous proteasomes are present in RRL, we expected to see some degradation in the absence of purified proteasomes. Indeed, we found that both wild-type p21 and p21(K0) mutant were degraded over time in the absence of added 26S proteasomes (Figures 1A and 1B, lanes 1–5) but that the supplement of exogenous 26S proteasomes enhanced their degradation (Figures 1A and 1B, lanes 6–10). RRL contains not only protea-

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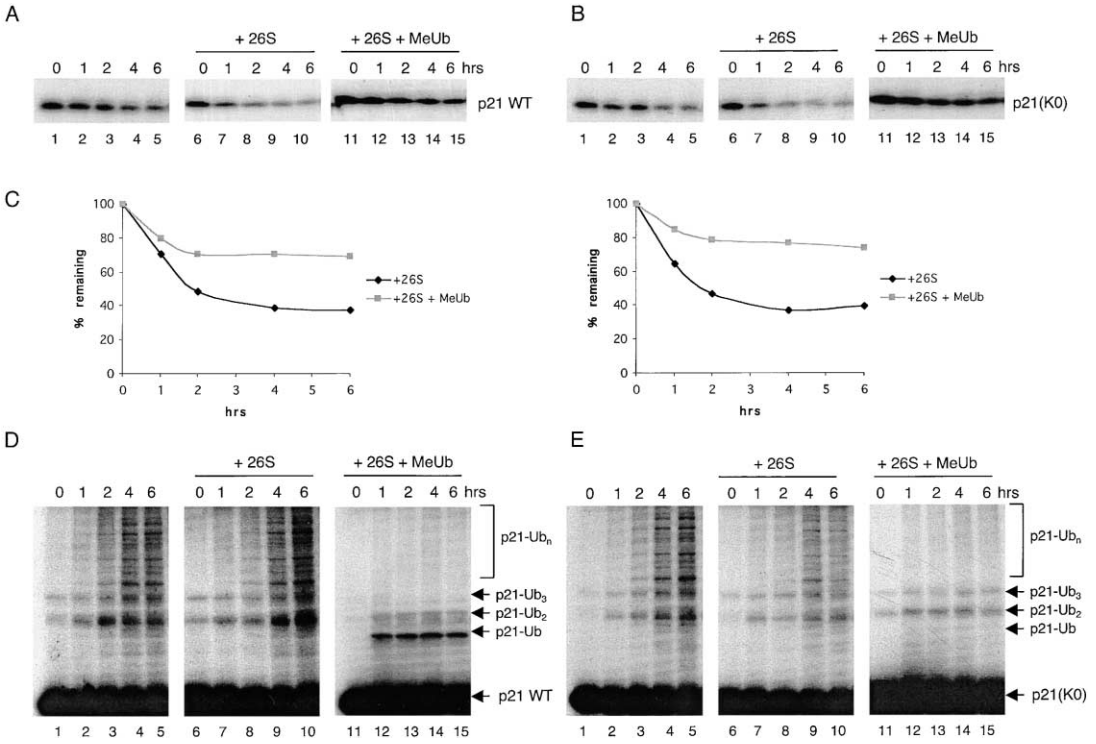

Figure 1. Wild-Type and p21(K0) Mutant Are Ubiquitinylated and Degraded In Vitro

(A–C) Inhibition of polyubiquitin chain formation induces p21 stabilization.

(A) Degradation of ^{35}S wild-type p21 was carried out in the absence (lanes 1–5) or presence (lanes 6–15) of purified 26S proteasomes (+26S) and methylated ubiquitin (MeUb) (lanes 11–15) for the indicated times. Reaction products were run on SDS-PAGE followed by autoradiography.

(B) Degradation of ^{35}S p21(K0) mutant was carried out in the absence (lanes 1–5) or presence (lanes 6–15) of purified 26S proteasomes (+26S) and MeUb (lanes 11–15) for the indicated times. Reaction products were run on SDS-PAGE followed by autoradiography.

(C) Quantification of the results shown in lanes 6–15 of (A) and (B).

(D–E) p21(K0) mutant is ubiquitinylated in vitro.

(D) Longer exposures of results shown in (A).

(E) Longer exposures of results shown in (B). The bracket on the left side of the images marks a ladder of bands >21,000 corresponding to polyubiquitinylated p21.

somes but is also an established source of ubiquitinylating enzymes (Hershko, 1988). We reasoned that if p21 is degraded by the proteasome in the absence of polyubiquitin chain formation, inhibition of polyubiquitin chain formation should not affect the degradation of in vitro translated p21. Thus, we included methylated ubiquitin, which is chemically modified to block all of its free amino groups and is therefore incapable of forming polyubiquitin chains, to the in vitro reaction. Surprisingly, in the presence of methylated ubiquitin, the half-lives of both wild-type p21 and p21(K0) mutant were significantly increased (Figures 1A and 1B, lanes 11–15, and Figure 1C). This result suggests that the in vitro degradation of p21 is dependent on polyubiquitin chain formation, which is not in agreement with the notion that p21 degradation is ubiquitin-independent. Importantly, longer exposures of films in Figure 1A revealed that polyubiquitinylated species of p21 accumulated over time (Figure 1D). Unexpectedly, these higher molecular weight forms were also visible with the p21(K0) mutant (Figure 1E), showing the availability of lysines is not required for p21 ubiquitinylation and suggesting that this mutant can be ubiquitinylated at the N terminus (see below). When methylated ubiquitin was added to the in vitro reactions, the high molecular weight forms of both wild-type p21 and p21(K0) mutant almost completely disappeared, and bands corresponding to mono-, di-, and triubiquitinylated species of p21 appeared. The di- and triubiquitinylated forms of p21 represent one ubiquitin molecule present in the RRL capped by a methylated ubiquitin or two endogenous ubiquitin molecules capped by a methylated ubiquitin, respectively. The fact that methylated ubiquitin changes the pattern of bands confirms that the higher molecular weight forms of p21 are indeed p21 polyubiquitinylated species. For reasons not yet understood, the monoubiquitinylated band was much less evident for p21(K0) mutant whereas bands corresponding to the di- and triubiquitinylated species were more apparent (compare lanes 11–15 in Figures 1D and 1E).

Taken together, these results show that a functional ubiquitin system is necessary for the polyubiquitinylation and efficient degradation of wild-type p21 and p21(K0) mutant in vitro.

Free p21 Is Degraded In Vitro by the 26S Proteasome in the Absence of Stable Binding to the Proteasome

It has been reported that purified recombinant p21 can be degraded in vitro by both 20S and 26S purified pro-

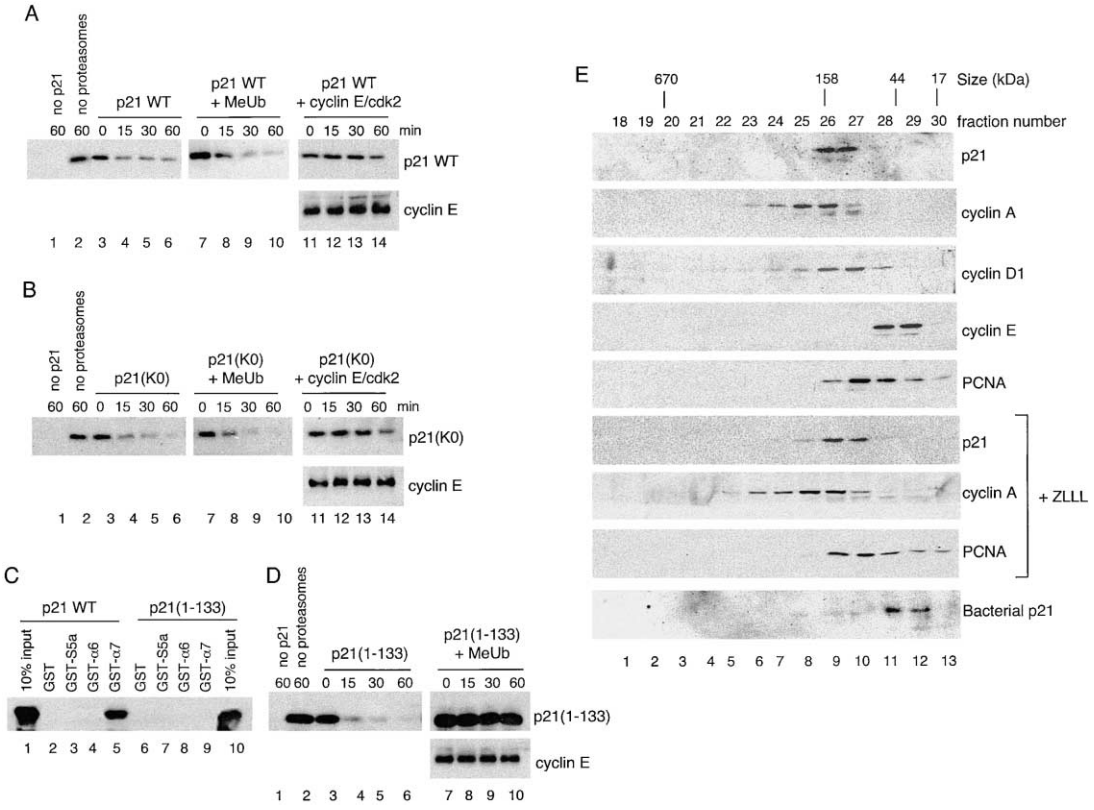

Figure 2. Free p21, but Not Bound p21, Is Degraded by the Proteasome Without Ubiquitinylation

(A–B) In vitro ubiquitin-independent degradation of free p21 by proteasomes is inhibited by its binding to cyclin E/cdk2.

(A) Degradation of purified wild-type p21 was carried out in the presence of purified 26S proteasomes either alone (lanes 3–6) or in the presence of methylated ubiquitin (MeUb) (lanes 7–10) or purified recombinant cyclin E/cdk2 complexes (lanes 11–14) at 37°C for the indicated intervals. In lane 1, the reaction was carried out in the absence of substrate and in lane 2, the reaction was carried out in the absence of purified 26S proteasomes.

(B) Degradation of purified p21(K0) mutant was carried out in the presence of purified 26S proteasomes either alone (lanes 3–6) or in the presence of MeUb (lanes 7–10) or purified recombinant cyclin E/cdk2 (lanes 11–14) at 37°C for the indicated intervals. The reaction shown in lane 1 was carried out in the absence of substrate and that in lane 2 was carried out in the absence of purified 26S proteasomes.

(C–D) Binding of p21 to the proteasome in not required for proteasomal degradation of p21. (C) Pulldown of wild-type p21 (lanes 2–5) or p21(1–133) (lanes 6–9) using GST alone (lanes 2 and 6), GST-S5a (lanes 3 and 7), GST-α6 (lanes 4 and 8) or GST-α7 (lanes 5 and 9). Ten percent input of wild-type p21 and p21(1–133) are shown in lanes 1 and 10, respectively.

(D) Degradation of purified p21(1–133) mutant was carried out in the presence of purified 26S proteasomes either alone (lanes 3–6) or in the presence of purified cyclin E/cdk2 (lanes 7–10) at 37°C for the indicated intervals. The reaction shown in lane 1 was carried out in the absence of substrate and that in lane 2 was carried out in the absence of purified 26S proteasomes.

(E) The large majority p21 is complexed in vivo. A Sepharose-6 column was used to separate p21 and its complexes present in human diploid fibroblasts untreated (upper images) or treated with the proteasome inhibitor ZLLL (lower images). The elution of purified bacterial p21 is shown in the bottom image. The elution of calibration standards is indicated at the top. Fractions were analyzed by immunoblotting with antibodies to p21 and to the indicated p21-interacting proteins.

teasomes (Liu et al., 2003). In light of the findings described in the previous section, we reexamined these results. Recombinant wild-type p21 and p21(K0) mutant were incubated with purified 26S proteasomes in the presence of an ATP regenerating system. Wild-type p21and p21(K0) mutant were rapidly degraded by 26S proteasomes (Figures 2A and 2B, lanes 1–6). In this purified system, we would not expect a dependency of p21 degradation on ubiquitin and, in fact, the addition of methylated ubiquitin did not block the degradation of wild-type p21 or p21(K0) mutant (Figures 2A and 2B, lanes 7–10). Since free p21 is an unstructured protein (Kriwacki et al., 1996), we sought to determine if p21 in complex with other proteins is degraded by 26S proteasomes. Wild-type p21 and p21(K0) mutant were preincubated with purified cyclin E/cdk2 complexes prior to the addition of purified proteasomes. The presence of cyclin E/cdk2 blocked the degradation of recombinant p21 by 26S proteasomes (Figures 2A and 2B, lanes 11–14). Abrogation of p21 degradation when complexed to cyclin E/cdk2 was not due to cyclin E, another proteasomal substrate, being degraded instead (Figures 2A and 2B, lanes 11–14). Based on this result, we proposed that purified recombinant p21 is degraded by the 26S proteasome since it is natively unfolded and as such can enter the proteasome pore by diffusion. When p21 is bound to another protein or protein complex, the access to the proteasome is inhibited and p21 cannot be degraded unless it is polyubiquitinylated. This interpretation is in agreement with the results presented in Figure 1, where at least 50% of p21 is bound to the rabbit cdk2 present in the RRL (data not shown) and where

the addition of methylated ubiquitin inhibited both p21 polyubiquitinylation and degradation. If our hypothesis were correct, then free p21 would be degraded by the proteasome independently of its ability to form a stable complex with it. It has been reported that p21 binds the α7 subunit of the proteasome through its extreme C terminus and it has been proposed that this domain is required for the ubiquitin-independent degradation of p21 (Touitou et al., 2001). We tested whether a p21 deleted at its C terminus was still degraded by the proteasome in vitro. Purified recombinant p21 C-terminal mutant missing the last 31 amino acids [p21(1–133) mutant], which is incapable of binding the α7 subunit of the proteasome (Figure 2C and Touitou et al., 2001), was incubated with 26S proteasomes in the presence of an ATP regenerating system. Similarly to wild-type p21, p21(1–133) mutant was rapidly degraded by purified proteasomes (Figure 2D), showing that an interaction with proteasomes is not a prerequisite for the degradation of free p21. When p21(1–133) mutant was preincubated with cyclin E/cdk2 complexes, its degradation by 26S proteasomes was blocked (Figure 2D) similarly to the wild-type protein.

Thus, we concluded that only free but not complexed p21 is degraded in vitro by the proteasome in a ubiquitin-independent manner. Since a stable interaction between p21 and the proteasome is not required for p21 degradation and since p21 is a natively disordered protein, the in vitro degradation of p21 in a purified system does not seem to be a regulated process but only relies on the ability of free p21 to access the catalytic core of the proteasome, an ability negated when p21 is complexed with other proteins.

In Vivo Degradation of p21 Is Dependent on a Functional Ubiquitin System

Since unbound p21 is degraded in vitro by the proteasome in the absence of ubiquitinylation, we asked whether free p21 exists in vivo. Extracts from normal human fibroblasts were subjected to Sepharose-6 gel filtration chromatography. In contrast to purified bacterial p21, which has an apparent molecular weight compatible with a monomeric form, cellular p21 is largely part of higher molecular weight complexes with the majority of p21 migrating in fractions 26 and 27 corresponding to complexes between the sizes of 44 kDa and 158 kDa (Figure 2E). Importantly, this is not due to the rapid degradation of free p21 in a ubiquitin-independent manner, because when cells were treated with the proteasome inhibitor ZLLL, p21 was still found in the same higher molecular weight fractions.

Since the majority if not all cellular p21 is present in a bound form, we excluded the possibility that p21 might access the proteasome just by diffusion and hypothesized that its degradation is a regulated process. Based on our in vitro results, we asked whether cellular p21 is degraded in a ubiquitin-dependent manner in vivo. 293T cells were either treated with the proteasome inhibitor ZLLL or transfected with a mutant ubiquitin with all of its lysines mutated to arginines [Ub(K0) mutant], which, similarly to methylated ubiquitin used in the in vitro experiments, cannot form polyubiquitin chains. In the presence of Ub(K0) mutant, endogenous p21 accumulated (Figure 3A), showing that inhibition of polyubiquitin chain formation does not allow for efficient degradation of p21. Since ZLLL reaches all treated cells, while only a fraction of cells is efficiently transfected with Ub(K0) mutant, it is not surprising that the effect of Ub(K0) mutant on the accumulation of p21 was slightly less pronounced than the effect of ZLLL treatment. We also tested the effects of ZLLL and the Ub(K0) mutant on exogenously expressed p21 by transfecting both wild-type p21 and the p21(K0) mutant in NIH-3T3 fibroblasts. Cell extracts were analyzed by immunoblotting with an antibody that recognizes human exogenous p21 but not murine endogenous p21. Similarly to previously published results (Sheaff et al., 2000), levels of both wild-type p21 and p21(K0) mutant cotransfected with wild-type ubiquitin increased in the presence of ZLLL as compared to the DMSO-treated control (Figure 3B), indicating that both are proteasomal substrates. Importantly, in the presence of Ub(K0) mutant, both wild-type p21 and p21(K0) mutant accumulated to levels similar to those with proteasome inhibition alone, showing that formation of polyubiquitin chains is essential not only for the degradation of wild-type p21 but also of the p21(K0) mutant. Previously published data showed that in the presence of Ub(K0) mutant, p21 did not accumulate (Sheaff et al., 2000) suggesting that ubiquitinylation is not required for the proteasomal degradation of p21. We reasoned that due to the abundance of endogenous ubiquitin, insufficient levels of Ub(K0) mutant may be unable to induce the increase in p21 levels. To determine if the effect of Ub(K0) mutant on p21 is dose-dependent, we transfected NIH-3T3 cells with p21 plus increasing amounts of the Ub(K0) mutant. We found that p21 accumulation induced by the Ub(K0) mutant is indeed dose-dependent (Figure 3C), suggesting that previously published experiments were performed in the presence of insufficient Ub(K0) mutant.

Next, we measured the half-lives of wild-type p21 and p21(K0) mutant to test whether the accumulation induced by inhibition of polyubiquitinylation is due to decreased degradation. Since p21 is not easily detectable in the presence of wild-type ubiquitin (see Figure 3B, lanes 1 and 4), to be able to measure its half-life, we transfected higher amounts of p21 cDNAs in the presence of wild-type ubiquitin than in the presence of the Ub(K0) mutant. This explains why the increase in p21 steady state levels in the presence of Ub(K0) mutant (Figure 3D, lanes 1 and 5) is not as pronounced as that in Figure 3B. Yet, in the presence of Ub(K0) mutant the half-lives of both wild-type p21 and p21(K0) mutant were significantly increased (Figure 3D). Taken together, these results implicate polyubiquitinylation as being critical for proteasomal degradation of p21.

In Vivo Degradation of p21 Is Dependent on a Functional Ubiquitin-Activating Enzyme and a Functional Nedd8-Activating Enzyme

To confirm that the ubiquitin pathway is necessary for the degradation of p21, we took advantage of the tsBN75 cell line that is temperature-sensitive for the ubiquitin-activating enzyme E1 (Nishimoto et al., 1980). We first examined endogenous levels of p21 in tsBN75 cells incubated at the permissive (34°C) or nonpermis-

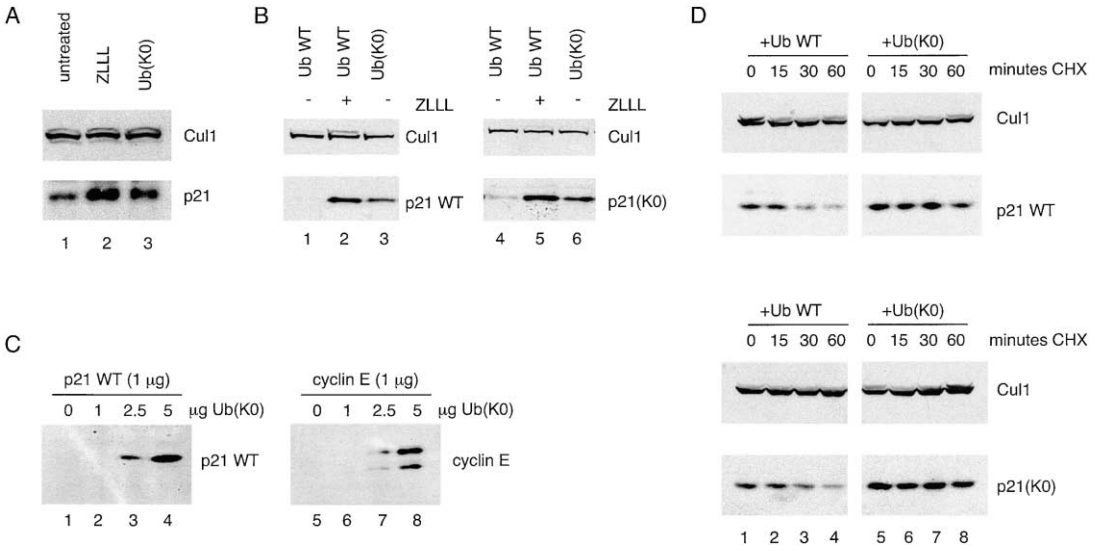

Figure 3. p21 Degradation Requires a Functional Ubiquitin System

(A–C) p21 is stabilized by both inhibition of proteasome function and inhibition of polyubiquitin chain formation.

(A) 293T cells were untreated (lanes 1 and 3) or treated with ZLLL (lane 2). 293T cells shown in lane 3 were transfected with Ub(K0) mutant. Protein extracts were analyzed by immunoblotting with the indicated antibodies.

(B) NIH-3T3 cells were transfected with wild-type p21 (lanes 1–3) or p21(K0) mutant (lanes 4–6) in combination with wild-type ubiquitin (lanes 1–2 and 4–5) or Ub(K0) mutant (lanes 3 and 6). Cells were untreated (lanes 1, 3, 4, and 6) or treated with ZLLL (lanes 2 and 5). Protein extracts were analyzed by immunoblotting with the indicated antibodies.

(C) NIH-3T3 cells were transfected with wild-type p21 (lanes 1–4) or cyclin E (lanes 5–8) in combination with increasing amounts of an expression vector encoding Ub(K0) mutant. Accumulation of p21 and cyclin E in the presence of Ub(K0) mutant was monitored by immunoblot analysis.

(D) NIH-3T3 cells were transfected with wild-type p21 (upper image) or p21(K0) mutant (lower image) in combination with wild-type ubiquitin (lanes 1–4) or Ub(K0) mutant (lanes 5–8). Cells were incubated in the presence of cycloheximide (CHX) for the indicated times. Degradation of wild-type p21 and p21(K0) mutant was monitored by immunoblotting.

sive (40°C) temperature by immunoblot analysis. At the nonpermissive temperature, p21 protein accumulated (Figure 4A) and was stabilized (Figure 4B); however, because p21 is a transcriptional target of p53, which also accumulated at the nonpermissive temperature (Figure 4A), we determined if the accumulation of p21 at the nonpermissive temperature was synthesis-independent. Therefore, tsBN75 cells were infected with retroviruses encoding wild-type p21 or p21(K0) mutant, which are under the control of a promoter no longer sensitive to transcriptional activation by p53 and incubated at the permissive or nonpermissive temperature for different times. We found that levels of exogenous wild-type p21 and p21(K0) mutant increased at the nonpermissive temperature (Figure 4C). These data show that inactivation of the E1 ubiquitin-activating enzyme and consequently of the ubiquitin pathway results in the stabilization of p21 protein regardless of the availability of internal lysines.

Next, we used the ts41 cell line that is temperature-sensitive for the Nedd8-activating enzyme APP-BP1 (Handeli and Weintraub, 1992). At the nonpermissive temperature (40°C), Nedd8 is no longer conjugated to cullins rendering them less active and all substrates of cullin-based ligases would be expected to accumulate. We found that after incubation at the nonpermissive temperature, there was an increase in endogenous p21 levels, which correlated with Cul1 deneddylation (Figure 4D). Since p53 is not a substrate of cullin-based ubiquitin ligases (Querido et al., 2001), it did not accumulate at the nonpermissive temperature (Figure 4D); therefore, the increase in p21 at the nonpermissive temperature is likely due to a stabilization of the protein and not due to increased synthesis. To determine if both wild-type p21 and p21(K0) mutant are stabilized when APP-BP1 is nonfunctional, we infected ts41 cells with retroviruses encoding wild-type p21 or p21(K0) mutant. We found that both proteins accumulated at the nonpermissive temperature (Figure 4E). These data show that p21 degradation is downstream of the Nedd8 pathway and suggest that p21 is a substrate of cullin-dependent ubiquitin ligases.

Lysines Are Not Required for p21 Ubiquitinylation In Vivo

The fact that a functional ubiquitin system is essential for the degradation of p21 and that p21(K0) mutant can be ubiquitinylated in vitro led us to examine the ubiquitinylation status of p21(K0) mutant in vivo. First, we expressed increasing amount of wild-type p21 or p21(K0) mutant in 293T cells in the absence or presence of the proteasome inhibitor ZLLL or in the presence of Ub(K0) mutant and analyzed p21 by immunoblotting. When higher levels of wild-type p21 or p21(K0) mutant cDNA were used for transfection, we were able to visualize high molecular weight species of p21 (Figure 5A, lanes 4 and 16) that correspond to ubiquitinylated forms (see below, Figure 5B). These bands and others of very high molecular weight became more evident in the presence of ZLLL (Figure 5A, compare lanes 4 and 8 and lanes 16 and 20), indicating that when proteasome function is

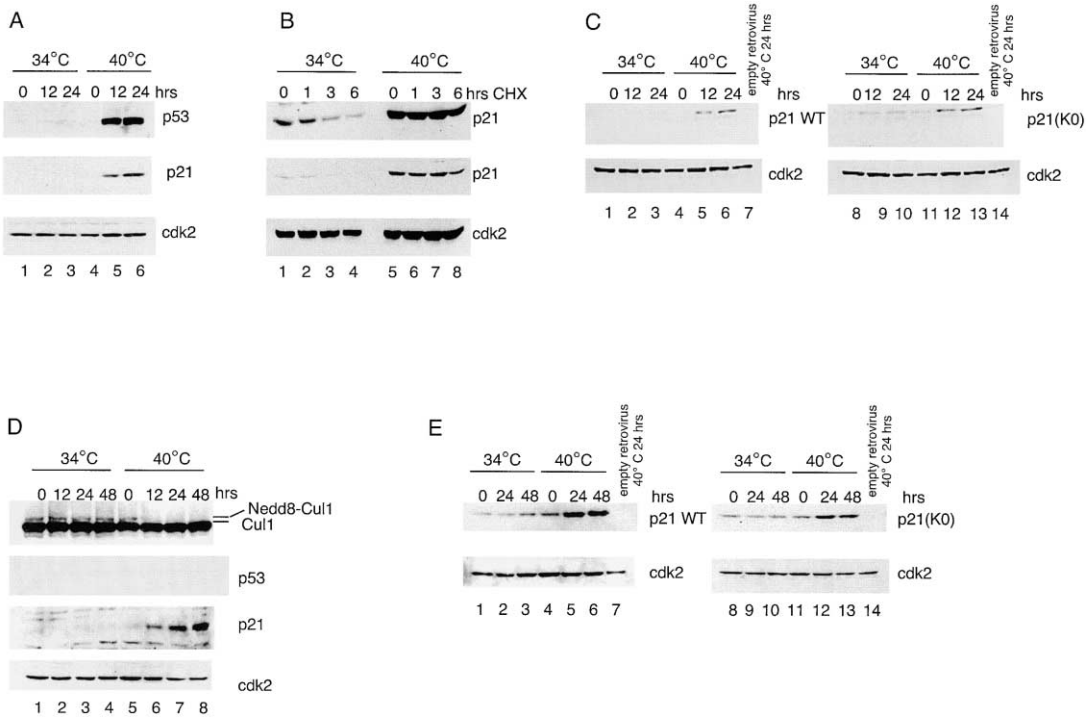

Figure 4. p21 Degradation Requires a Functional Ubiquitin-Activating Enzyme and Nedd8-Activating Enzyme

(A–C) p21 protein is stabilized when the ubiquitin-activating enzyme is not functional.

(A) tsBN75 cells were incubated at the permissive temperature (34°C; lanes 1–3) or nonpermissive temperature (40°C; lanes 4–6) for the indicated intervals. Protein extracts were analyzed by immunoblotting with the antibodies to the indicated proteins.

(B) tsBN75 cells were incubated at the permissive temperature (34°C; lanes 1–4) or nonpermissive temperature (40°C; lanes 5–8) for 18 hr. Cells were then incubated in the presence of cycloheximide (CHX) for the indicated times. Protein extracts were analyzed by immunoblotting with the antibodies to the indicated proteins. The middle image represents a short exposure time and the upper image represents a long exposure time.

(C) tsBN75 cells infected with retroviruses expressing wild-type p21 (lanes 1–6) or p21(K0) mutant (lanes 8–13) were incubated at the permissive temperature (34°C; lanes 1–3 and 8–10) or nonpermissive temperature (40°C; lanes 4–6 and 11–13) for the indicated intervals. Lanes 7 and 14 show extracts from tsBN75 cells infected with an empty retrovirus and incubated at the nonpermissive temperature for 24 hr. Protein extracts were analyzed by immunoblotting with the antibodies to the indicated proteins.

(D–E) p21 protein accumulates when the Nedd8-activating enzyme is not functional.

(D) ts41 cells were incubated at the permissive temperature (34°C; lanes 1–4) or nonpermissive temperature (40°C; lanes 5–8) for the indicated intervals. Protein extracts were analyzed by immunoblotting with the antibodies to the indicated proteins. A long exposure of Cul1 reveals that the Nedd8-conjugated form of Cul1 disappears at the nonpermissive temperature.

(E) ts41 cells infected with retroviruses expressing wild-type p21 (lanes 1–6) or p21(K0) mutant (lanes 8–13) were incubated at the permissive temperature (34°C; lanes 1–3 and 8–10) or nonpermissive temperature (40°C; lanes 4–6 and 11–13) for the indicated intervals. Lanes 7 and 14 show extracts from ts41 cells infected with an empty retrovirus and incubated at the nonpermissive temperature for 24 hr. Protein extracts were analyzed by immunoblotting with the antibodies to the indicated proteins.

abrogated, ubiquitinylated species of p21 accumulate. When p21 was cotransfected with Ub(K0) mutant, the very high molecular weight forms disappeared and lower molecular weight ubiquitinylated species were instead evident (Figure 5A, lanes 10–12 and 22–24). This indicates that the Ub(K0) mutant competes with endogenous ubiquitin to terminate chains generating short p21-ubiquitin conjugates, which accumulate since they are not efficiently degraded by the proteasome. In addition, this proves that p21 is modified by a canonical branched chain, not by a linear ubiquitin chain. To verify that slow migrating bands are indeed ubiquitinylated species, lysates from NIH-3T3 cells (Figure 5B, lanes 1–3) and 293T cells (Figure 5B, lanes 4–9) were subjected to a nickel-agarose pulldown to purify ubiquitinylated proteins through the histidine (His) tag on ubiquitin, which was cotransfected with HA-tagged p21. Immunoblotting of purified ubiquitinylated proteins with an antibody to p21 (Figure 5B, lanes 1–6) or to HA (Figure 5B, lanes 7–9) showed multiple higher molecular weight forms corresponding to ubiquitinylated wild-type p21 and p21(K0) mutant. Since in these transfections we used a His-myc-tagged ubiquitin, the ubiquitin chains bound to p21 contain different proportions of both exogenous ubiquitin (~10.5 kDa) and endogenous ubiquitin (~7.8 kDa). This explains why ubiquitinated species appear in some cases as a cluster of bands rather than a ladder of bands. To further prove that p21 is indeed ubiquitinylated in vivo, extracts from cells transfected with HA-tagged p21 were immunoprecipitated with an antibody to the HA tag under denaturing conditions to dissociate any proteins interacting with p21 that may also be ubiquitinylated. Immunoblot analysis of denatured immunoprecipitates with an antibody to ubiquitin specific for long polyubiqui-

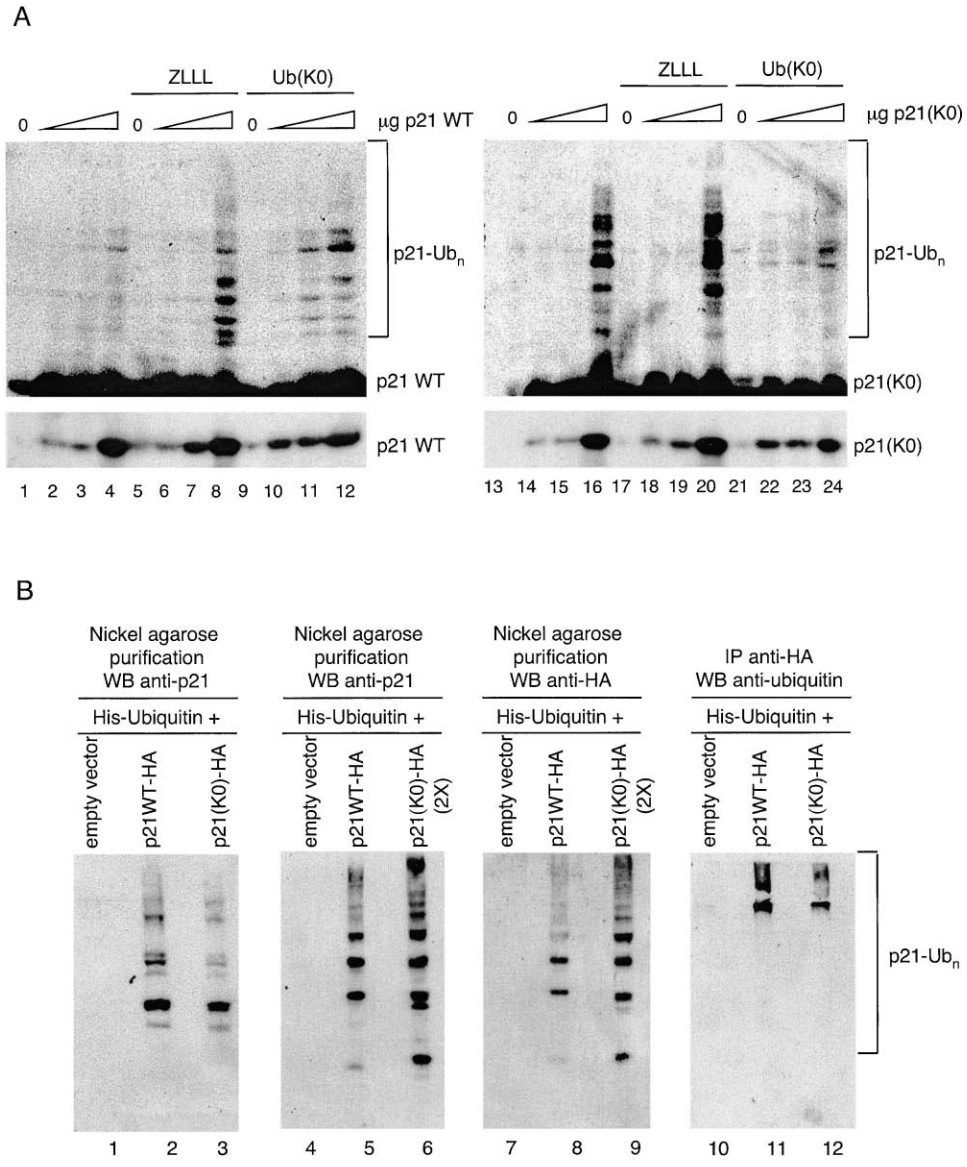

Figure 5. Wild-Type p21 and p21(K0) Mutant Are Ubiquitinylated In Vivo

(A) 293T cells were either untransfected (lanes 1, 5, 9, 13, 17, and 21) or transfected with increasing amounts of wild-type p21 (lanes 2–4, 6–8, and 10–12) or p21(K0) mutant (lanes 14–16, 18–20, and 22–24). Lanes 5–8 and 17–20 show extracts from cells treated with ZLLL, and lanes 9–12 and 21–24 contain extract from cells transfected with Ub(K0) mutant. Protein extracts were analyzed by immunoblotting with an antibody to p21. The lower image represents a short exposure time and the upper image represents a long exposure time. The blot in the upper right image was exposed longer than the blot in upper left image. The bracket on the left side of the images marks a ladder of bands >21,000 corresponding to ubiquitinylated p21.

(B) NIH-3T3 cells (lanes 1–3) or 293T cells (lanes 4–12) were transfected with His-tagged ubiquitin in the presence of an empty vector (lanes 1, 4, 7, and 10), wild-type p21 tagged at the C terminus with HA (lanes 2, 5, 8, and 11) or p21(K0) mutant tagged at the C terminus with HA (lanes 3, 6, 9, and 12). Cell extracts were subjected to nickel agarose purification (lanes 1–9) or immunoprecipitated with an anti-HA antibody (lanes 10–12). Where indicated, an amount of extract corresponding to twice the amount used for the wild-type p21 expressing extract was used [indicated as (2×)]. Precipitates were analyzed by immunoblotting with an antibody to p21 (lanes 1–6), an antibody against the HA tag (lanes 7–9) or an antibody specific for long polyubiquitin chains (lanes 10–12). The bracket on the left side of the images marks a ladder of bands >21,000 corresponding to ubiquitinylated p21.

tin chains (Montagnoli et al., 1999) confirmed that both wild-type p21 and p21(K0) mutant are in fact ubiquitinylated in vivo (Figure 5B, lanes 10–12).

In direct immunoblots, we noticed that p21(K0) mutant appeared quantitatively less ubiquitinylated than wild-type p21 (the blot shown in the right image of Figure 5A was exposed longer than that in the left image). This difference was confirmed by nickel-agarose pulldowns followed by immunoblotting (compare lanes 2 and 3, and lanes 11 and 12 in Figure 5B) prompting us to use in the pulldowns from 293T cells twice the amount of lysate from p21(K0) mutant expressing cells than from p21 wild-type expressing cells, to better compare the ubiquitinylation of the two p21 forms (Figure 5B, lanes

5–6 and 8–9). Thus, we concluded that despite having a similar half-life, wild-type p21 is ubiquitinylated more than p21(K0) mutant.

p21 Is Ubiquitinylated at the N Terminus In Vivo

Since p21 ubiquitinylation did not require the presence of lysine residues, we sought to examine the possibility that the N terminus of wild-type p21 and p21(K0) mutant is the target for ubiquitinylation in vivo. We generated constructs of wild-type p21 and p21(K0) mutant that after the first methionine contain an HA tag (which does not contain any lysine: Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) followed by a factor Xa protease cleavage site (Ile-Glu-Gly-Arg); these constructs were cotransfected in 293T cells together with His-tagged ubiquitin. Twenty-four hours after transfections cells were collected and each cell extract was divided in two aliquots, one of which was treated with factor Xa protease. Essentially, all of p21 had its N-terminal HA tag removed in the presence of factor Xa as shown by the increase in gel migration (Figure 6A compare lanes 3 and 5 to lanes 4 and 6). We then performed a nickel agarose pulldown with both the cleaved and uncleaved forms of wild-type p21 and p21(K0) mutant. If p21 is not ubiquitinylated at the N terminus, when factor Xa-treated extracts are pulled down with nickel agarose and immunoblotted with an antibody to HA, we should not be able to detect ubiquitinylated proteins since the HA tag has been cut away. If instead p21 is indeed ubiquitinylated at the N terminus, we should observe ubiquitinylated forms whether the extract is untreated or is treated with factor Xa since the N-terminal HA tag, although separated from p21, is still covalently bound to ubiquitin chains. In addition, for the extracts that were pretreated with factor Xa protease, we would expect to see a lower molecular band corresponding to the monoubiquitinylated form of the N-terminal HA tag, which would be absent for the uncleaved p21. After nickel agarose purification, we did observe ubiquitinylated species of both uncleaved p21 and the cleaved N-terminal HA tag (Figure 6B). In addition, only after treatment with factor Xa, we observed the appearance of a monoubiquitinylated form of the N-terminal HA tag (indicated in Figure 6B as HA-Ub_1), which had the same mobility as His-myc-tagged ubiquitin and was also recognized by an antibody to ubiquitin (data not shown). Significantly, taking in account the monoubiquitinylated form of the HA tag, the amount of ubiquitinylated HA tag derived from p21(K0) (lane 6) is comparable to the amount of ubiquitinylated p21(K0) (lane 5). In contrast, wild-type p21 can be ubiquitinylated on the N-terminal methionine as well as on multiple lysine residues, accounting for the lower levels of ubiquitinylation of the HA-tag derived from wild-type p21 (compare lanes 3 and 4). As indicated, to better compare the ubiquitinylation of the two p21 forms before cleavage (lanes 3 and 5) we used twice the amount of lysate from p21(K0) mutant expressing cells than from p21 wild-type expressing cells. Because of this, after cleavage (lanes 4 and 6) a difference between wild-type and mutant is evident since the total amount of N-terminal ubiquitinylation in these two forms is likely to be the same but we are using twice as much extract for the p21(K0) mutant.

The His-tagged ubiquitin we used also contains a myc tag and as a result has an apparent molecular mass of 10,500 Da; therefore, two His-ubiquitin moieties show the same migration in SDS-PAGE as p21. This explains why the pattern of bands of the ubiquitinylated uncleaved p21 and the polyubiquitinylated cleaved HA tag is similar. For example, the triubiquitinylated form of the HA tag is almost identical in size to the monoubiquitinylated form of uncleaved p21. Thus, the close electrophoretic mobility of ubiquitinylated p21 and ubiquitinylated HA tag explains the overall similarity between lanes with and without factor Xa (with the exception of the key presence of monoubiquitinylated HA-tag in cleaved samples). To further verify this explanation, we performed an identical experiment using a truncated p21 (1–133) mutant containing an HA tag followed by a factor Xa cleavage site. This mutant is missing 31 amino acids at the C terminus and therefore has a faster electrophoretic mobility. The result of this experiment (Figure 6C) demonstrates that the ubiquitinylated forms of the HA tag derived from both wild-type p21 and p21(1–133) mutant are very similar (see asterisks in lane 3 of Figure 6C and in lane 4 of Figure 6B) and they both resemble the pattern of bands corresponding to ubiquitinylated wild-type p21 (compare lane 3 to lane 2 in Figure 6C). Instead, the bands corresponding to ubiquitinylated wild-type p21 and p21(1–133) mutant differ in size, demonstrating that the cleaved samples do not contain any residual uncut ubiquitinylated p21 species but rather represent ubiquitinylated species of the HA tag.

These experiments show that both wild-type p21 and p21(K0) mutant are ubiquitinylated on the N-terminal methionine in vivo.

Addition of an N-Terminal Tag Stabilizes p21 In Vivo but Does Not Block Its Ubiquitinylation

As indirect evidence that certain proteins are ubiquitinylated at the N terminus, it has been shown that they are stabilized by the addition of seventy-eight amino acids (a 6×-myc tag represented by the following sequence repeated 6-fold: Met-Glu-Gln-Lys-Leu-Ileu-Ser-Glu-Glu-Asp-Leu-Asn-Glu) fused to their N-terminal methionine (Aviel et al., 2000; Breitschopf et al., 1998; Reinstein et al., 2000). This result was explained as the ability of the 6×-myc tag to block the recognition site for ubiquitin ligases or ubiquitin or both. We generated constructs of wild-type and p21(K0) mutant in which a 6×-myc tag was fused to the N-terminal methionine. When expressed in cells, these tagged versions of wild-type p21 and p21(K0) mutant were still able to inhibit cell proliferation and bind to cdk2, cdk4, cyclin A, cyclin B, and cyclin D1 (data not shown). Untagged or 6×-myc tagged versions of wild-type p21 and p21(K0) mutant were transfected into NIH-3T3 cells and their half-lives were measured. We found that while untagged wild-type p21 and p21(K0) mutant were degraded over time, 6×-myc tagged versions were more stable (Figure 7A) and were still not degraded after eight hours (data not shown). However, when we compared the ubiquitinylation status of untagged and 6×-myc tagged wild-type p21 and p21(K0) mutant, we found that the 6×-myc tagged versions were ubiquitinylated more than the untagged versions (Figure 7B). This is not surprising since the tag

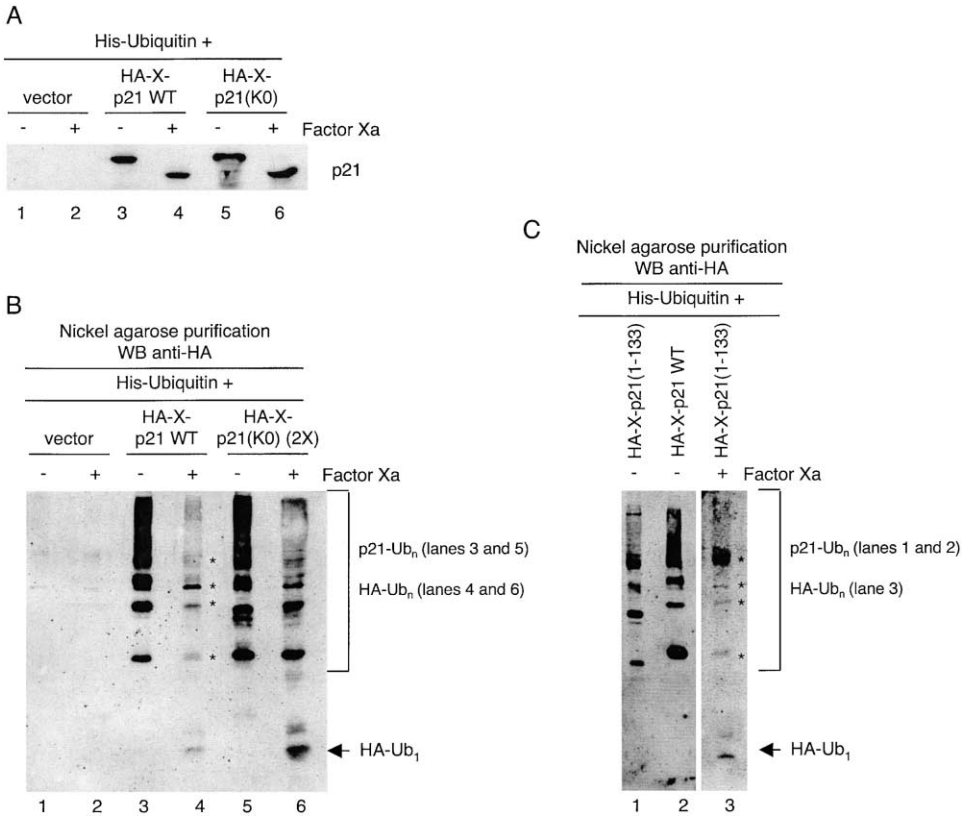

Figure 6. p21 Is Ubiquitinylated on the N Terminus In Vivo

(A) 293T cells were transfected with His-tagged ubiquitin plus empty vector (lanes 1 and 2), wild-type p21 with an N-terminal HA tag followed by a factor Xa cleavage site (HA-X-p21; lanes 3 and 4) or p21(K0) mutant with an N-terminal HA tag followed by a factor Xa cleavage site (HA-X-p21(K0); lanes 5 and 6). Extracts from transfected cells were left untreated (lanes 1, 3, and 5) or were treated with factor Xa protease (lanes 2, 4 and 6). Protein extracts were analyzed by immunoblotting with an antibody to p21.

(B) Cell extracts shown in (A) were subjected to nickel agarose purification and precipitates were analyzed by immunoblotting with an antibody against the HA tag. The bracket on the left side of the images marks a ladder of bands >21,000 corresponding to ubiquitinylated p21 (lanes 3 and 5) or polyubiquitinylated HA (lanes 4 and 6). The arrow on the left side marks the monoubiquitinylated form of the HA tag (lanes 4 and 6). Asterisks indicate the major polyubiquitinylated forms of the HA tag.

(C) 293T cells were transfected with His-tagged ubiquitin plus HA-X-p21(1–133) (lanes 1 and 3) or HA-X-p21 (lanes 2). Extracts from transfected cells were left untreated (lanes 1 and 2) or were treated with factor Xa protease (lane 3). Cell extracts were subjected to nickel agarose purification and precipitates were analyzed by immunoblotting with an antibody against the HA tag. The bracket on the left side of the images marks a ladder of bands >21,000 corresponding to ubiquitinylated p21 (lanes 1 and 2) or polyubiquitinylated HA (lane 3). Asterisks indicate the major polyubiquitinylated forms of the HA tag. The arrow on the left side marks the monoubiquitinylated form of the HA tag (lane 3).

contains six lysines and could at least theoretically be ubiquitinylated at the N terminus. What is surprising is that amply ubiquitinylated p21 is more stable. It is very unlikely that the 6×-myc tag causes a steric hindrance that interferes with unfolding by and/or translocation into the proteasome independent of the presence of the ubiquitin chain, since the same tag does not change the stability of other proteins (e.g., Emi1 in Guardavaccaro et al., 2003). In addition, ubiquitinylated p21 bound to the proteasome but not proteolyzed could get in the way of other proteins delaying their degradation. Instead, we found that other proteins analyzed (both endogenous and exogenous) are still unstable in cells transfected with 6×-myc tagged p21(K0) mutant (data not shown). Thus, p21 conjugated to polyubiquitin chains on the 6×-myc tagged is intrinsically incompetent to serve as an efficient substrate of the proteasome suggesting that the position of the chain plays an important role in the rate of proteasomal degradation.

Discussion

Can a Protein Be Degraded by the Proteasome in the Absence of Its Ubiquitinylation?

There is only one clear example of a protein that is targeted for degradation by the 26S proteasome without prior ubiquitinylation. The enzyme ornithine decarboxylase (Odc) is a short-lived protein whose degradation by the 26S proteasome is ATP-dependent but ubiquitin-independent. Yet, the proteolysis of Odc is a regulated process that depends on the binding of Odc to a specific cofactor, antizyme. Once bound to antizyme, Odc is recognized and degraded by the 26S proteasome with the release of antizyme (Li and Coffino, 1992). There are two reports in the literature proposing additional substrates for ubiquitin-independent degradation, namely p21 (Sheaff et al., 2000) and c-Jun (Jariel-Encontre et al., 1995). However, unlike for Odc, a clear targeting mechanism to the proteasome has not been described

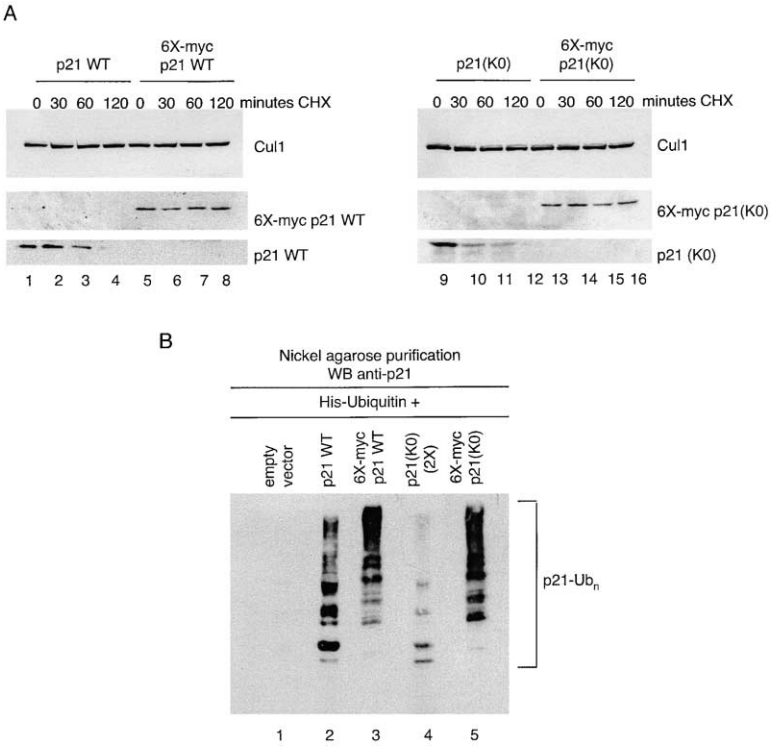

Figure 7. Modification of the N Terminus of p21 Blocks Its Degradation, but Not Its Ubiquitinylation In Vivo

(A) NIH-3T3 cells were transfected with wild-type p21 (lanes 1–4), 6×-myc-tagged wild-type p21 (lanes 5–8), p21(K0) mutant (lanes 9–12), or 6×-myc-tagged p21(K0) mutant (lanes 13–16). Cells were incubated in the presence of cycloheximide (CHX) for the indicated times. Protein extracts were analyzed by immunoblotting with the antibodies to the indicated proteins.

(B) 293T cells were transfected with His-tagged ubiquitin in the presence of an empty vector (lane 1), wild-type p21 (lane 2), 6×-myc-tagged wild-type p21 (lane 3), p21(K0) mutant (lane 4), or 6×-myc-tagged p21(K0) mutant (lane 5). Cell extracts were subjected to nickel agarose purification and precipitates were analyzed by immunoblotting with an antibody to p21. The bracket on the left side of the images marks a ladder of bands >21,000 corresponding to ubiquitinylated p21.

for either. It has been shown that p21 binds the α7 subunit of the proteasome (Touitou et al., 2001) and suggested that this physical interaction might target p21 to the proteasome for degradation. However, although p21 can bind to α7, it has not been possible to detect an interaction between p21 and α7 integrated in the proteasome, both in the absence and in the presence of proteasome inhibitors, whereas in the same experiments known proteasomal interactors such as Cul1 and Roc1 are readily detected (Touitou et al., 2001 and J.B. and M.P., unpublished data). More importantly, from a steric point of view it is unclear how the binding with α7 would facilitate the access of p21 to the 26S proteasome since α7 is distal from the entry to the proteasomal channel. Indeed we show herein that only free p21 and not p21 in complex with additional proteins can be degraded by purified 26S proteasomes (Figures 2A and 2B) and that p21 lacking the binding to α7 is also readily degraded by purified proteasomes when in its free form (Figures 2C and 2D). Accordingly, a p21(1–82) mutant has an in vivo half-life identical to the wild-type p21 protein (Rousseau et al., 1999), confirming that binding to α7 is not necessary for p21 proteolysis in vivo. p21 is a critical protein for regulating the cell cycle and DNA replication, and many of its functions are temporally and probably spatially regulated. As a result, stringent regulation of protein stability plays a role in controlling p21 functions. Indiscriminate degradation of p21 due to simple diffusion within proteasomes would be contrary to the necessity for rapidly regulating protein levels through proteolysis. p21 is mostly found in complexes (Figure 2E) and, based on our results, is not expected to be degraded by proteasomes in a ubiquitin-independent manner. Indeed, the results presented here demonstrate that regulation of p21 degradation both in vitro (Figure 1) and in vivo (Figures 3 and 4) is dependent on the ubiquitin system. In addition, a functional Nedd8 conjugation system is required for p21 degradation in vivo (Figure 4).

Are Lysines Necessary for Ubiquitinylation? And How Many Ubiquitin Chains Are Necessary For an Efficient Recognition and Degradation by the Proteasome?

Both in vitro and in vivo p21 can be targeted for degradation through the addition of ubiquitin to a nontraditional, nonlysine target residue (Figures 1, 5, 6, and 7). MyoD, the Epstein-Barr virus latent membrane protein 1 and the E7 human papillomavirus oncoprotein can be degraded in a ubiquitin-dependent and proteasome-dependent manner in the absence of lysine residues (Aviel et al., 2000; Breitschopf et al., 1998; Reinstein et al., 2000). It has been suggested that these proteins have the ubiquitin chain conjugated to the N-terminal methionine residue; however, evidence for this type of modification has thus far been indirect. Here, we provided direct proof that both wild-type p21 and the p21(K0) mutant are ubiquitinylated at the N terminus in vivo.

We found that both in vitro and in vivo, wild-type p21 is more ubiquitinylated than p21(K0) mutant (Figures 1D, 1E, and 5B). Yet, the rate of their degradation is very similar (Figures 1A, 1B, 3D, and 7A) suggesting that lysine-dependent ubiquitinylation does not increase the efficiency of the proteasome in degrading p21. Moreover, the addition of a 6×-myc tag to the N terminus of p21 stabilizes the protein in vivo (Figure 7A) although it does not inhibit, but actually increases, its ubiquitinylation. This result suggests that ubiquitinylation on the

N terminus is sufficient for degradation and that the N-terminal site for ubiquitinylation is a physiologically relevant site to make p21 a competent substrate for proteasomal degradation. Recent work has shown that the yeast cdk inhibitor, Sic1, can be ubiquitinylated on multiple lysine residues, but only ubiquitinylation on a specific single lysine residue can efficiently promote its degradation by proteasomes (Petroski and Deshaies, 2003). Our results are in agreement with this finding in that just one ubiquitin chain ligated to p21 efficiently targets this protein for degradation. However, a lysineless Sic1 mutant cannot be ubiquitinylated and is therefore stable, while p21(K0) mutant is ubiquitinylated on the N-terminal methionine and is rapidly degraded by the proteasome suggesting either that the enzyme responsible for the ubiquitin ligation of p21 has evolved properties not present in the analogous yeast enzyme, or that p21 can be ubiquitinated via both an SCF ligase (as Sic1) as well as a different ligase able to sustain the N-terminal ubiquitinylation.

It is possible that N-terminal ubiquitinylation of p21 is sufficient to target the protein for proteasomal proteolysis, while ubiquitinylation on lysine residues may have a different role in the regulation of p21 function. Alternatively, polyubiquitin chains attached to lysine residues are redundant to the chain at the N terminus. All together, our data show that p21 degradation by the proteasome occurs via the ubiquitin pathway and indicate that the position of the ubiquitin chain plays a critical role in the rate of proteasomal degradation.

Experimental Procedures

Cell Culture, Transient Transfections, and Retroviral Infections

NIH-3T3, 293T, IMR90, tsBN75, and ts41 cells were maintained as described (Carrano et al., 1999). 293T cells were transfected by the calcium phosphate method, and infections were performed as described (Carrano and Pagano, 2001).

Biochemistry

Protein extraction, immunoprecipitations and immunoblots were performed as described (Guardavaccaro et al., 2003). For gel filtration, precleared lysates were applied to Superose-6 PC 3.2/3.0 column (SMART system) and run in PBS plus 1 mM DTT. Rabbit polyclonal antibodies to Cul1, cyclin A, cyclin D1, and cdk2 and monoclonal antibody (Mab) to cyclin E were previously described (Carrano and Pagano, 2001). Mab to p21 was from BD-Transduction Laboratories, to ubiquitin from Zymed and to PCNA from Upstate-Biotechnology. Polyclonal antibodies to p21 and HA, and Mab to p53 were from Santa Cruz Biotechnology.

In Vitro Ubiquitinylation and Degradation

Ubiquitinylation and degradation of in vitro translated p21 was based on assays previously described (Montagnoli et al., 1999). Briefly, the reaction mix contained 0.5 μl of in vitro translated [^{35}S] p21, 1 μM ubiquitin aldehyde, and 2 μl of a 5× stock of a reaction mix (0.25 M Tris·HCl [pH 7.5], 25 mM MgCl2, 10 mM ATP, 50 mM phosphocreatine, and 17.5 U/ml creatine phosphokinase) ±50 nM purified 26S proteasomes. Following incubation at 37°C for various times, reactions were stopped by the addition of sample buffer and subjected to SDS-PAGE followed by autoradiography. For degradation of bacterial p21, 100 ng of purified p21, 1 μM ubiquitin aldehyde, 50 nM 26S proteasomes, and 2 μl of 5× reaction mix were incubated at 37°C for various times, after which reactions were stopped by the addition of sample buffer and subjected to SDS-PAGE and immunoblotting.

In Vivo Ubiquitinylation and Factor Xa Cleavage

293T and NIH-3T3 cells were cotransfected with His-myc-tagged ubiquitin and either wild-type p21 or p21(K0) mutant tagged at the C terminus with HA. Transfected cells were treated with 10 μM ZLLL for 5 hr and extracts were prepared in lysis buffer including 100 μM NEM and 20 μM ubiquitin aldehyde. Lysates (0.5 ml volume) were denatured by boiling for 10 min in the presence of 1% SDS. Lysates were then incubated with 100 μl of 10% Triton X-100 and 400 μl of lysis buffer on ice for 30 min prior to nickel agarose purification or immunoprecipitation. For Factor Xa cleavage, prior to nickel agarose purification, extracts were treated with 1 μg factor Xa protease for 16 hr at 25°C.

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