# Ubiquitination of p21$^{Cip1/WAF1}$ by SCF$^{Skp2}$: Substrate Requirement and Ubiquitination Site Selection†

Wei Wang,‡ Lucas Nacusi,§ Robert J. Sheaff,§ and Xuedong Liu*,‡

*Department of Chemistry and Biochemistry, University of Colorado−Boulder, Boulder, Colorado 80309, and Cancer Center, MMC 806, 420 Delaware Street SE, University of Minnesota, Minneapolis, Minnesota 55455*

*Received June 6, 2005; Revised Manuscript Received September 3, 2005*

ABSTRACT: Multiple proteolytic pathways are involved in the degradation of the cyclin-dependent kinase inhibitor p21$^{Cip1/WAF1}$. Timed destruction of p21$^{Cip1/WAF1}$ plays a critical role in cell-cycle progression and cellular response to DNA damage. The SCF$^{Skp2}$ complex (consisting of Rbx1, Cul1, Skp1, and Skp2) is one of the E3 ubiquitin ligases involved in ubiquitination of p21$^{Cip1/WAF1}$. Little is known about how SCF$^{Skp2}$ recruits its substrates and selects particular acceptor lysine residues for ubiquitination. In this study, we investigated the requirements for SCF$^{Skp2}$ recognition of p21$^{Cip1/WAF1}$ and lysine residues that are ubiquitinated *in vitro* and inside cells. We demonstrate that ubiquitination of p21$^{Cip1/WAF1}$ requires a functional interaction between p21$^{Cip1/WAF1}$ and the cyclin E−Cdk2 complex. Mutation of both the cyclin E recruitment motif (RXL) and the Cdk2-binding motif (FNF) at the N terminus of p21$^{Cip1/WAF1}$ abolishes its ubiquitination by SCF$^{Skp2}$, while mutation of either motif alone has minimal effects, suggesting either contact is sufficient for substrate recruitment. Thus, SCF$^{Skp2}$ appears to recognize a trimeric complex consisting of cyclin E−Cdk2−p21$^{Cip1/WAF1}$. Furthermore, we show that p21$^{Cip1/WAF1}$ can be ubiquitinated at four distinct lysine residues located in the carboxyl-terminal region but not two other lysine residues in the N-terminal region. Any one of these four lysine residues can be targeted for ubiquitination in the absence of the others *in vitro*, and three of these four lysine residues are also ubiquitinated *in vivo*, suggesting that there is limited specificity in the selection of ubiquitination sites. Interestingly, mutation of the carboxyl-terminal proline to lysine enables ubiquitin conjugation at the carboxyl terminus of the substrate both *in vitro* and *in vivo*. Thus, our results highlight a unique property of the ubiquitination enzymatic reaction in that substrate ubiquitination site selection can be remarkably diverse and occur in distinct spatial areas.

The cyclin-dependent kinase inhibitor p21$^{Cip1/WAF1}$ (referred to as p21 hereafter)[1] is an important regulator of cell-cycle progression, cellular responses to DNA damage, cell migration, and apoptosis (*1*, *2*). Originally identified as a protein interacting with cyclin-dependent kinase 2 (Cdk2) and as a component of a multiprotein complex consisting of cyclin−Cdk−PCNA, p21 is a potent inhibitor of cyclin E−Cdk2 kinase and a negative regulator of DNA synthesis (*3*, *4*). The ability of p21 to associate with key cell-cycle regulators makes it an ideal sensor for external signals. Indeed, the expression levels of p21 are highly modulated in response to extracellular stimuli by both transcriptional and post-transcriptional mechanisms (*2*). For example, p21 is a transcriptional target of the tumor suppressor p53. p53 activation following ionizing radiation triggers rapid up-regulation of the p21 transcript and accumulation of the p21 protein to shut down cell-cycle progression (*5*, *6*).

Steady-state levels of the p21 protein also depend upon its metabolic stability, and p21 undergoes rapid turnover in a variety of experimental settings. Both ubiquitin-dependent and ubiquitin-independent, proteasome-mediated degradation pathways have been implicated in p21 proteolysis (*7*, *8*). Polyubiquitinated p21 conjugates accumulate when cells are treated with proteasome inhibitors, implying a critical role for ubiquitin conjugation in p21 proteolysis (*7*). However, a p21 mutant devoid of lysine residues cannot be ubiquitinated; however, it is still rapidly degraded by the proteasome (*7*−*9*). The unstable nature of the lysine-less p21 mutant has been attributed to a direct physical interaction with the proteasome (*7*−*9*). A more recent study suggests that degradation of lysine-less p21 can occur through polyubiquitination at the N-terminal-free amino group of the substrate, suggesting yet another alternative pathway for p21 degradation (*9*). Therefore, it appears that multiple degradation systems are involved in controlling p21 stability.

The SCF$^{Skp2}$ ubiquitin E3 ligase mediates p21 degradation at the G1/S phase transition and in response to low doses of UV irradiation (*10*−*12*). Metabolic stability of p21 increases dramatically in Skp2-deficient mouse fibroblast cells (*11*). After low doses of UV exposure, p21 degradation accelerates

† This work is supported by grants from the National Institutes of Health (CA107098-01) and Colorado Tobacco Research Program (2R-45) to X.L. and by an Ellison Medical Foundation New Scholars Award in Aging to R.J.S. (AG-NS-0140-01).

* To whom correspondence should be addressed. Telephone: (303) 735-6161. Fax: (303) 735-6161. E-mail: xuedong.liu@colorado.edu.

‡ University of Colorado−Boulder.

§ University of Minnesota.

[1] Abbreviations: Cdk2, cyclin-dependent kinase 2; p21, p21$^{Cip/WAF1}$; SCF, Skp1/Rbx1/Cul1/F-box protein; SCF$^{Skp2}$, Skp1/Rbx1/Cul1/Skp2; Me-Ub, methylated ubiquitin; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase.

10.1021/bi051071j CCC: $30.25 
Published on Web 10/13/2005

and ubiquitinated p21 intermediates accumulate when these cells are treated with the proteasome inhibitor LLnL. Importantly, UV-induced degradation of p21 does not occur in Skp2-deficient MEF cells (*10*), indicating that $SCF^{Skp2}$ is likely involved in p21 ubiquitination in response to UV damage. It has been proposed that p21 elimination is essential for optimal DNA repair (*10*).

How E3 ubiquitin ligases recognize their substrates and catalyze ubiquitin conjugation to specific lysine acceptor residues are outstanding questions in elucidating the mechanism of protein ubiquitination. In the case of the SCF E3 complex, phosphorylation of protein substrates appears to play a major role in regulating the E3−substrate interaction. For example, ubiquitination of the cell-cycle inhibitor protein $p27^{Kip1}$ by $SCF^{Skp2}$ requires its phosphorylation at T187 by cyclin E−Cdk2 kinase (*13*−*15*). Similarly, phosphorylation of cyclin E by Cdk2 is essential for its ubiquitination by $SCF^{Cdc4}$ (*16*, *17*). However, not all SCF substrates require phosphorylation to interact with the E3 complex. Phosphorylation is dispensable for p21 recognition and subsequent ubiquitination by $SCF^{Skp2}$ *in vitro* (*11*), but the signal for p21 recognition by $SCF^{Skp2}$ and subsequent ubiquitination has yet to be defined.

In this study, we reconstituted a p21 ubiquitination system using purified proteins to address the requirement for $SCF^{Skp2}$ to ubiquitinate p21 and assess which lysine residues are selected for ubiquitination by the $SCF^{Skp2}$ complex. Our results indicate that the p21−cyclin E−Cdk2 interaction is obligatory for ubiquitination and that diverse lysine residues in p21 can be selected for ubiquitin conjugation.

## MATERIALS AND METHODS

*DNA Expression Constructs and Recombinant Proteins.* The human p21 expression vectors pCS2-p21, pCS-p21K6R, and pCS2-$(His)_6$-ubiquitin have been described previously (*7*). All p21 point mutations were made using the Quikchange site-specific mutagenesis kit (Stratagene) and confirmed by DNA sequencing. Recombinant Cks1 and E2−Cdc34 proteins were purified as described previously (*18*). Ubiquitin-activating enzyme E1 was purchased from Calbiochem or purified from insect cells. Recombinant baculoviruses expressing GST−cyclin E and Cdk2 were kindly provided by Dr. Wade Harper (Harvard Medical School). Catalytically inactive $Cdk2^m$ mutant was a gift of Dr. David Morgan (UCSF).

*Binding Assays.* Wild type and p21 mutants were synthesized and labeled with [$^{35}$S]Met using a TNT *in vitro* translation kit (Promega). Binding assays were performed by incubating 10 $\mu$L of radiolabeled p21 or p21 mutants with 1 $\mu$g of GST−cyclin E−Cdk2 prepared from insect cells in 0.5 mL of NETN buffer (20 mM Tris-HCl at pH 8.0, 1 mM EDTA, 150 mM NaCl, and 0.5% NP40) at 4 °C for 0.5 h. GST beads were added to the mixture and incubated for 30 min. The beads were collected by brief centrifugation and washed 3 times with 0.5 mL NETN buffer. Proteins bound to the beads were eluted by boiling in 2× SDS sample buffer and analyzed by SDS−PAGE, followed by visualization of the result by Phosphorimaging.

*In Vitro Ubiquitination Assay.* The $SCF^{Skp2}$-mediated p21 ubiquitination reaction was performed in a test tube containing 250 ng of recombinant E1 (Calbiochem), 0.1 $\mu$g of cyclin E−Cdk2 complex purified from insect cells, 3 $\mu$g of 6× His-Cdc34, 1 $\mu$g of ubiquitin, 120 $\mu$M methylated ubiquitin (Me-Ub) (BostonBiochem), 1 $\mu$M ubiquitin aldehyde (BostonBiochem), 5 $\mu$g of $SCF^{Skp2}$ complex, 0.1 mM MG-132, 2.5 $\mu$L of 20× ER (10 mM ATP, 20 mM HEPES at pH 7.4, 10 mM MgOAc, 300 mM creatine phosphate, and 0.5 mg/mL creatine phosphokinase), and 1 $\mu$g of Cks1 in a final volume of 20 $\mu$L. The reaction was incubated at 30 °C for 2 h. The reaction was terminated by adding 2× SDS sample buffer and boiled for 5 min before electrophoresis and phosphorimaging analysis.

*Cell Culture, Transfections, and Antibodies.* Human 293 cells were grown in DMEM and transfected as described previously (*7*). MG-132 (Calbiochem) and $\beta$-lactone (Calbiochem) were dissolved in DMSO. Approximately 24 h after transfection, cells were treated with inhibitors overnight (2 $\mu$M MG-132 and 10 $\mu$M $\beta$-lactone). Cells were lysed in RIPA buffer (10 mM Tris at pH 7.4, 0.15 M NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 10 g/mL each of aprotonin, leupeptin, and pepstatin, 50 mM NaF, and 1 mM Na vanadate), followed by scraping, sonication, and clarification by centrifugation. Wild-type and mutant p21 were cotransfected with His-Ub in 293 cells. Expression of p21 was detected by immunoblotting with a monoclonal anti-p21 antibody (Transduction Labs). Ubiquitinated p21 was purified with Ni-NTA agarose beads, eluted, and detected by immunoblotting with an anti-p21 antibody.

## RESULTS

*$SCF^{Skp2}$ Can Catalyze Ubiquitination of Four Lysine Residues in the Carboxyl Terminus of p21.* Recent studies have shown that ubiquitination of p21 can be reconstituted *in vitro* using the recombinant or purified E1, E2 (Cdc34 or UbcH5), and $SCF^{Skp2}$ complex in the presence of cyclin E−Cdk2 and Cks1 (*11*). In this study, we wished to further explore the requirement for $SCF^{Skp2}$ to ubiquitinate p21 and identify which lysines in p21 are targeted for ubiquitin conjugation. There are six lysine residues in p21 and two well-characterized cyclin- and Cdk2-binding motifs (Figure 1A). We have purified all of the components required for p21 ubiquitination and reconstituted the reaction in a cell-free system. Consistent with previous observations, p21 ubiquitination requires Cks1 and cyclin E−Cdk2 in addition to E1, E2, and E3 enzymes (Figure 1B). In the absence of Me-Ub, which prevents assembly of polyubiquitin chains, the labeled p21 substrate was reduced upon incubation with the E1, E2, and E3 complex in the presence of wild-type ubiquitin and high molecular weight, smeared labeled bands, which likely represent polyubiquitinated p21 (Figure 1B). When Me-Ub is included in the reaction mixture, we consistently observe four slower migrating bands on the gel (Figure 1B). The size of these bands is consistent with p21 mono-ubiquitinated at multiple sites. Two slower migrating bands close in size to input p21 are likely to be the phosphorylated forms, because these two bands are absent when cyclin E−Cdk2 was omitted from the reaction mixture (compare lane 5 versus the rest of lanes in Figure 1B). The appearance of these two bands also depends upon the kinase activity of Cdk2, because they were absent when p21 was incubated with the catalytically inactive kinase complex (Figure 7C). It is also interesting to note that Cks1 stimulates p21 phosphorylation by cyclin E−Cdk2 (compare lane 4

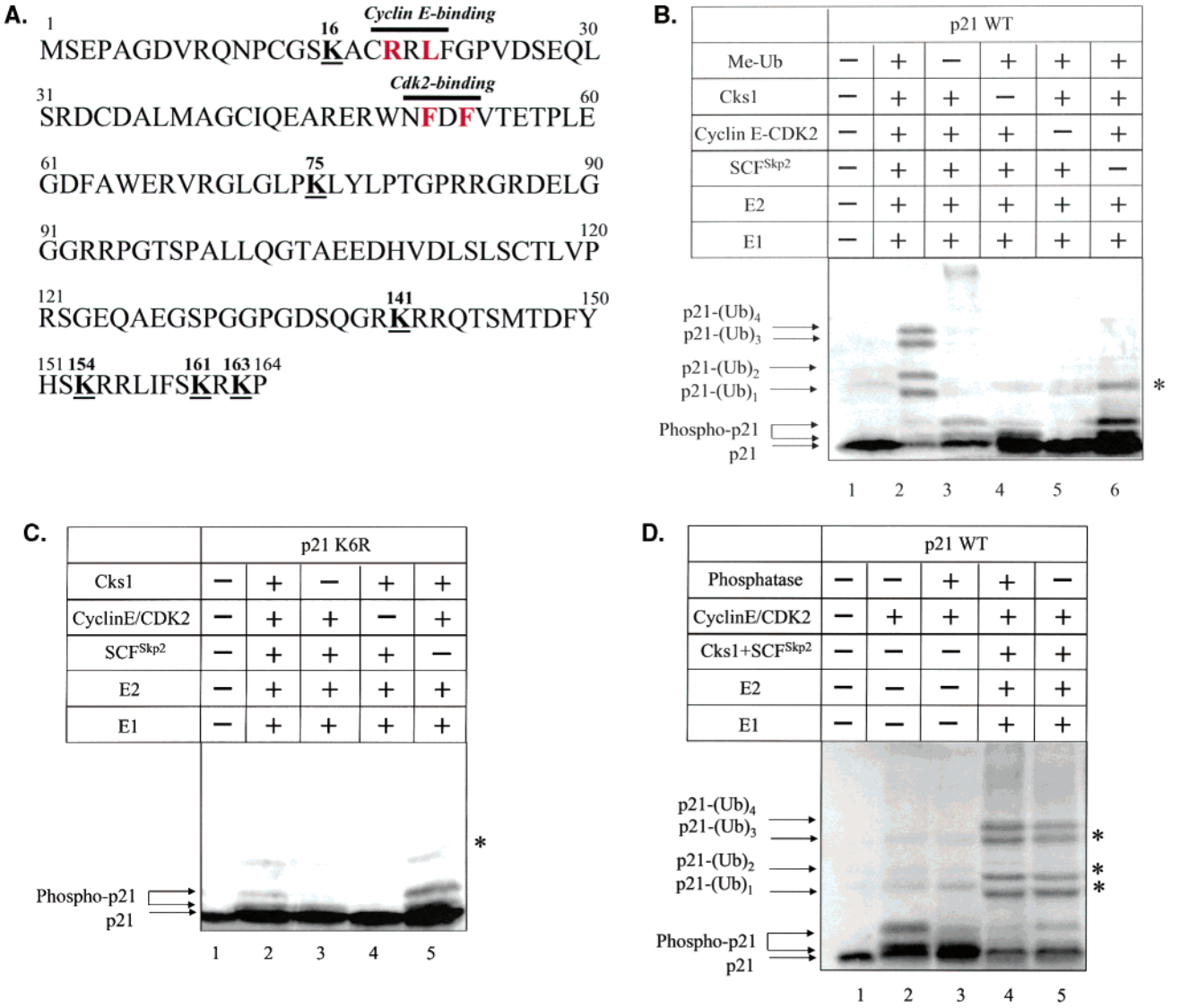


FIGURE 1: Ubiquitination of p21 *in vitro*. (A) Functional motifs and locations of lysine residues in p21. The cyclin E (RXL) and CDK2 (FNF) binding motifs are indicated by the line shown on the top. p21 contains six lysine residues located at positions shown in bold and underscored. (B) Ubiquitination of four lysine residues in p21 by SCF$^{Skp2}$. Recombinant WT and lysine-less (p21K6R) p21 were labeled with [$^{35}$S]Met by *in vitro* translation and incubated with or without cyclin E−Cdk2 or Cks1 prior to the ubiquitination reaction. The ubiquitination reaction was performed by adding recombinant E1, E2 (Cdc34), ATP, energy regeneration system, ubiquitin, and methylated ubiquitin in a reaction mixture as described in the Materials and Methods. (C) *In vitro* ubiquitination of lysine-less p21. The reaction is identical to B, except the p21K6R mutant was used as the substrate. (*) The faint band present in p21K6R, similar to the size of mono-ubiquitinated p21, is either a nonspecific background contamination or N-terminal ubiquitinated p21 because this band also exists in labeled protein alone without adding the reaction mixture. (D) Alkaline phosphatase treatment reduces the amounts of phosphorylated p21. Alkaline phosphatase was added to the reaction mixture containing labeled p21 after incubation with cyclin E−Cdk2 or cyclin E−Cdk2 plus the ubiquitination reaction mixture.

versus 6 in Figure 1B), consistent with previous observations (*19*). The intensity of these two bands was significantly decreased when the kinase reaction mixture or ubiquitination reaction mixture was treated with alkaline phosphatase prior to electrophoresis, providing additional support for the identity of these bands being phosphorylated forms of p21 (compare lane 2 versus 3 and 4 versus 5 in Figure 1D). If the four high molecular weight bands correspond to ubiquitin-conjugated p21, then a p21 mutant devoid of lysine residues should not generate slower migrating bands. As expected, the four major bands present in wild-type p21 are missing in a lysine-less mutant of p21(p21K6R) (Figure 1C). The faint band comigrating with the mono-ubiquitinated p21 is also present in the input p21 substrate and reactions without SCF$^{Skp2}$, suggesting that it is SCF$^{Skp2}$-independent. This band could be either a nonspecific contaminating protein from the *in vitro* translation reaction or N-terminal ubiquitinated p21, in light of a recent study suggesting that N-terminal ubiquitination activity is present in the rabbit reticulocyte lysates (*9*). Because Me-Ub was included in the reactions to generate mono-ubiquitinated species, the presence of multiple bands suggests that SCF is targeting multiple lysine residues on the same p21 molecule. To determine which of the six lysine residues in p21 can serve as the ubiquitin acceptor during conjugation, an array of p21 mutants with only one lysine residue was constructed. As shown in Figure 2A, only four of six lysine residues are capable of serving as ubiquitin acceptor sites. No ubiquitination is observed for the p21 substrate harboring K16 or K75 alone. Failure of ubiquitination does not appear to be a consequence of mutant p21 misfolding because they

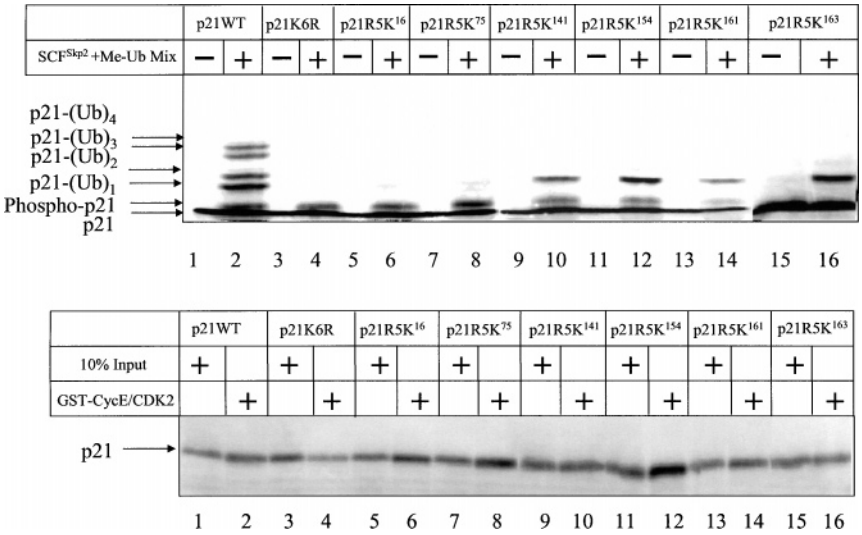


FIGURE 2: Ubiquitination of four of six lysine residues in p21 *in vitro*. (A) Wild-type p21, p21K6R, and p21 mutants containing single lysines were synthesized using *in vitro* translation, and their ubiquitination reactions were performed as described. (B) p21 mutants with a single lysine are still capable of binding to the cyclin E−Cdk2 complex. Recombinant p21 and p21 mutants were labeled with [$^{35}$S]Met and incubated with 2 $\mu$g of recombinant GST−cyclin E−Cdk2 purified from insect cells. Binding assays were performed, and p21 proteins bound to the GST−cyclin E−Cdk2 complex were analyzed by SDS−PAGE. The 10% input [$^{35}$S]Met-labeled p21 proteins were loaded on the gel as indicated.

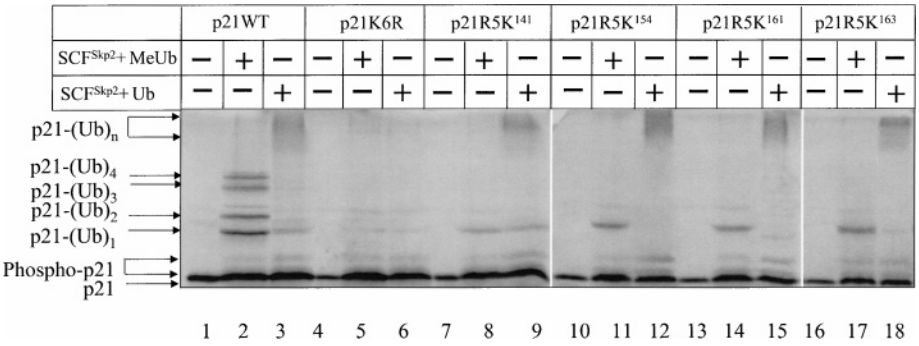


FIGURE 3: Polyubiquitination of p21 single-lysine mutants. Recombinant WT and mutant p21 were subjected to ubiquitination reactions by SCF$^{Skp2}$ in conjugation with cyclin E−Cdk2 and Cks1 in the presence or absence of 120 $\mu$M Me-Ub or Ub for 2 h and analyzed by SDS−PAGE and phosphorimaging.

all still bind cyclin E−Cdk2 (Figure 2B). Therefore, our data suggest that not all of the p21 lysine residues are targeted for ubiquitination. There is limited specificity in selecting the acceptor lysine residues by SCF$^{Skp2}$.

The above experiments clearly establish that four of the six p21 lysine residues can serve as acceptors for ubiquitin. However, one question still remains as to whether polyubiquitin chains can be assembled on any of the four lysine residues because Me-Ub was included in our reaction to suppress polyubiquitin chain formation. To determine whether any of the four lysine acceptors can be polyubiquitinated, Me-Ub was left out from the reactions. As shown in Figure 3, in the absence of methylated ubiquitin, mono-ubiquitin conjugates disappeared and were converted to high molecular weight polyubiquitinated conjugates. From this experiment, we concluded that SCF$^{Skp2}$ can catalyze polyubiquitin conjugation to any one of the four lysine residues at the carboxyl-terminal region of p21 *in vitro*.

*Ubiquitination of Multiple p21 Lysine Residues by SCF$^{Skp2}$.* To further investigate whether bands observed in the p21 ubiquitination reaction were a result of mono-ubiquitinating multiple lysine residues, we constructed p21 mutants with different combinations of lysine residues (Figure 4A). Our expectation was that the number of species of conjugated p21 should be the same as the number of lysine residues regardless of the exact combination if multiple lysine residues were simultaneously ubiquitinated. Indeed, this is exactly what we observed. Ubiquitination of p21R4K$^{141}$K$^{154}$ and p21R4K$^{161}$K$^{163}$ yielded two conjugated bands, whereas three bands were observed for p21R3K$^{141}$K$^{154}$K$^{161}$ and p21R3K$^{141}$K$^{154}$K$^{163}$ (Figure 4B). The intensity and pattern of conjugated bands were essentially the same as wild-type p21. All of the p21 mutants shown here also undergo polyubiquitination when Me-Ub is omitted from the reaction. Interestingly, K161 and K163 are only separated by one amino acid residue, and K163 is the next to last amino acid in p21 (Figure 1A). Both residues were conjugated to ubiquitins in the presence of SCF$^{Skp2}$. Therefore, physical proximity does not appear to prevent them both from being selected as ubiquitin acceptors. As expected, none of these p21 mutants is likely to be misfolded because all of them bind to cyclin E−Cdk2 as efficiently as the wild-type p21 (Figure 4C). The time course of p21 ubiquitination was also investigated. Shown in Figure 4D, ubiquitination of p21 appears to be stepwise in a time-dependent manner. When our results are taken together, they indicate that SCF$^{Skp2}$ can promote ubiquitination of multiple lysine residues in p21.

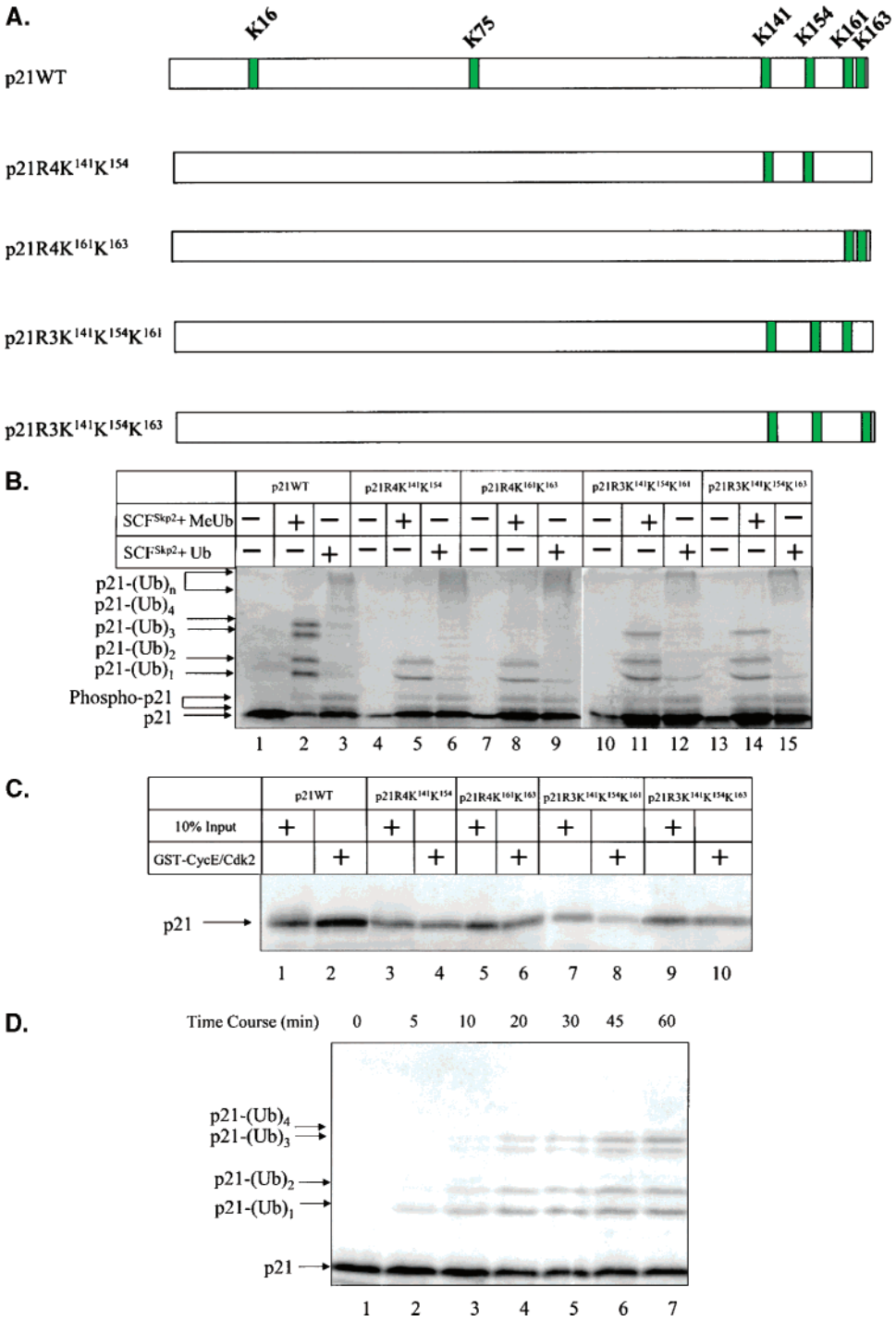


FIGURE 4: Selection of acceptor lysine residues in p21 for ubiquitination. (A) Schematic diagram of p21 mutants with the presence of various combinations of lysine residues in the indicated positions. (B) Analysis of ubiquitination patterns of p21 mutants with two or three lysine residues. p21 mutants indicated in A were incubated with the ubiquitination reaction mixture in the presence or absence of 120 $\mu$M Me-Ub and analyzed by SDS−PAGE. (C) Each of the p21 mutants was assayed for its ability to bind the cyclin E−Cdk2 complex. Binding assays were performed as described in Figure 2. (D) Time course of p21 ubiquitination *in vitro*.

Selection of ubiquitination sites does not appear to be ordered.

*Ubiquitination Site Selection in Cultured Cells.* Our *in vitro* ubiquitination data suggest that any one of the four lysine residues in the carboxyl terminal region of p21 can be targeted for ubiquitination. We have previously demonstrated that ubiquitinated endogenous p21 is difficult to detect because of the rapid removal of the ubiquitin chain, but exogenously expressed p21 showed a similar metabolic stability because the endogenous and exogenous expressed p21−ubiquitin conjugates were readily detectable (*7*). To demonstrate that these lysine residues are ubiquitinated in cells, we transfected the wild-type p21, the lysine-less p21 mutant (p21K6R), or p21 mutants containing only a single-lysine residue along with His-tagged ubiquitin (His-Ub) into 293 cells. The transfected cells were treated with proteasome inhibitor MG132 for 12 h. As reported previously, proteasome inhibition leads to the accumulation of mono- or polyubiquitinated p21 intermediates (*7*). Such intermediates are absent in the lysine-less p21 mutant (p21K6R), indicating

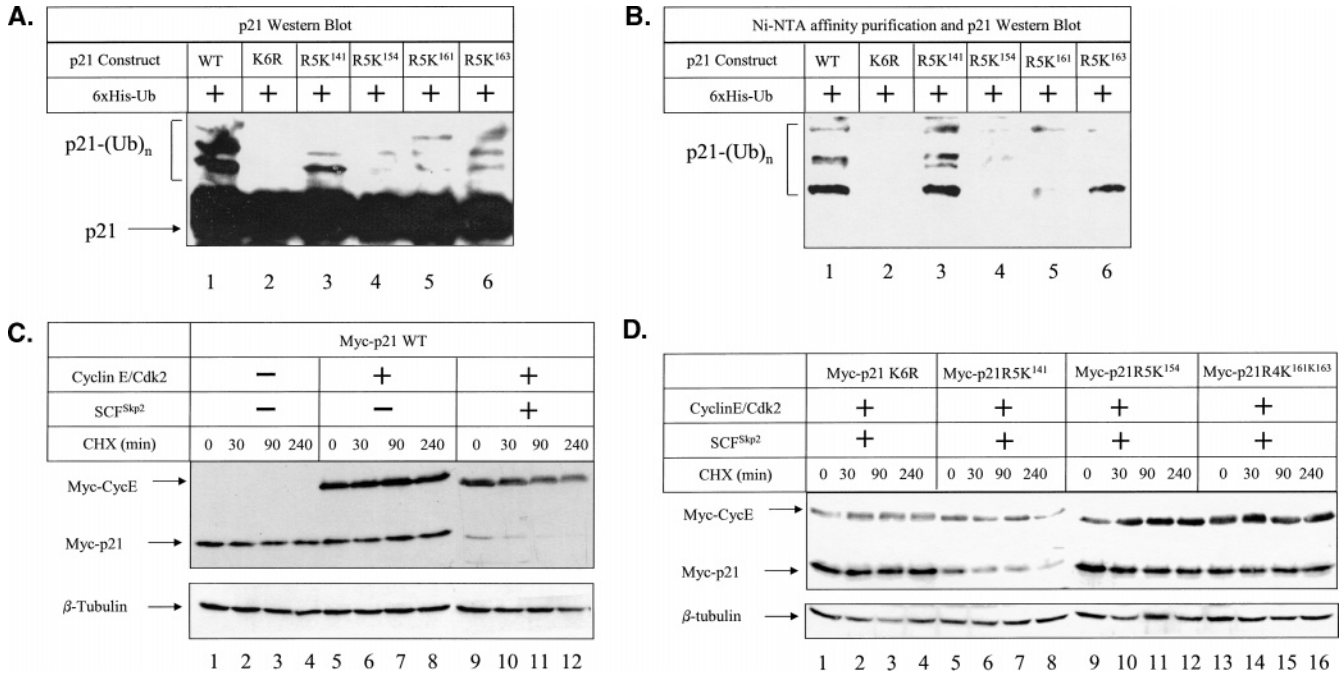


FIGURE 5: Ubiquitination and degradation pattern of p21 in human 293 cells. p21 expression constructs were cotransfected with His-Ub into H293 cells, and cells were harvested 36 h later. (A) Western blot with an antibody against p21 shows native and ubiquitinated forms of wild-type p21, p21R5K$^{141}$, p21R5K$^{161}$, p21R5K$^{154}$, and p21R5K$^{163}$. Whereas p21K6R is not ubiquitinated, ubiquitination of p21R5K$^{141}$ was very low or undetectable. p21R5K$^{161}$ was partially ubiquitinated. (B) His-ubiquitinated products in cell extracts were purified by a Ni-NTA affinity column. His-Ub products were eluted from the column and separated by SDS−PAGE, and Western blot was performed against p21. Only WTp21, p21R5K$^{141}$, p21R5K$^{161}$, and p21R5K$^{163}$ was detected after purification of His-Ub. (C) Destabilization of p21 by SCF$^{Skp2}$ in 293 cells. Myc-tagged wild-type p21 was transfected alone or in combination with cyclin E−Cdk2, Skp2, Skp1, Cul1, Rbx1, Cks1, and Cdc34 in 293 cells. A total of 24 h after transfection, cells were split and treated with cycloheximde (10 $\mu$g/mL) for indicated times before harvesting. Cell lysates were analyzed by immunoblotting with an anti-Myc antibody. As a loading control, $\beta$-tubulin was also blotted using an anti-$\beta$-tubulin antibody. (D) Stability analysis of p21 mutants using a cycloheximde chase experiment. The lysine-less p21 (p21K6R) is stable in the presence of the SCF complex. Myc-p21R5K$^{141}$ and Myc-p21R5K$^{154}$ are destabilized by SCF when compared to p21K6R, whereas Myc-p21R4K$^{161}$K$^{163}$ is not.

that the nature of these intermediates are related to ubiquitination of certain internal lysine residues of p21 (lane 2 in Figure 5A). Notably, the ubiquitination patterns of p21 mutants containing a single lysine residue at position 141, 161, or 163 are similar to the wild-type p21, except for a decrease in the levels of the mono-ubiquitinated form. Ubiquitination of p21R5K$^{154}$ is barely detectable, suggesting that this site is not the predominant lysine targeted for ubiquitination *in vivo*. To further prove that high molecular weight intermediates detected by the p21 antibody are indeed ubiquitinated p21, we purified His-ubiquitinated products using a Ni-NTA affinity column. Eluates from the column were subjected to SDS−PAGE, and Western blot analysis was performed using an anti-p21 antibody. As shown in Figure 5B, His-Ub conjugates were efficiently recovered for the wild type, p21R5K$^{141}$, and p21R5K$^{163}$ but not for p21R5K$^{161}$, for which only high molecular weight products were recovered. As expected, little His-Ub conjugates were recovered for p21R5K$^{154}$, consistent with our interpretation that this site is poorly selected *in vivo*. It is interesting to note that the p21 ubiquitination pattern as determined by the direct immunoblotting analysis of p21 is slightly different from the one in the His-Ub conjugates. It is very likely that endogenous ubiquitin could incorporate along with the exogenous His-Ub. Differences in the p21 ubiquitination pattern in Ni-NTA-purified conjugates could come from variations in the relative ratio of these two ubiquitin species in the transfected cells. The efficiency of ubiquitin conjugation to these three lysine residues inside cells may vary, but our data do suggest that three of the four lysine residues identified in our *in vitro* studies appear to be also targeted for ubiquitination *in vivo*.

Polyubiquitin chains assembled through lysine 48 of ubiquitin on protein substrates invariably act as the signal for targeting substrates to 26S proteasomes for degradation (*20*). To determine stability of p21 mutants in cells in the presence of the SCF complex and cyclin E−Cdk2 complex, we measured the decay of p21 and p21 mutants in 293 cells following cycloheximide treatment. In the absence of the SCF complex, the half-life of wild-type p21 exceeds 90 min in the absence or presence of the cyclin E−Cdk2 complex (lanes 1−8 in Figure 5C). Overexpression of the SCF complex significantly shortened the half-life of p21 to less than 30 min. Thus, SCF promotes degradation of wild-type p21 in this assay. In contrast, the lysine-less p21 mutant (p21K6R) is refractory to SCF and remains stable throughout the indicated time of treatment (lanes 1−4 in Figure 5D). The decay pattern of p21R5K$^{141}$ is reminiscent of that of wild-type p21, suggesting that lysine 141 is probably responsible for SCF-mediated p21 destruction. Destabilization of p21R5K$^{154}$ is also observed in the presence of SCF and the cyclin E−Cdk2 complex (compare lanes 9−12 in Figure 5D versus lanes 5−8 in Figure 5C) but to a much lesser degree than p21R5K$^{141}$, suggesting that lysine 154 is less efficient in SCF-mediated degradation, which is consistent with poor ubiquitination at this site observed in cells (parts A and B of Figure 5). Unexpectedly, p21R4K$^{161}$K$^{163}$ was very stable in the presence of the SCF complex (lanes

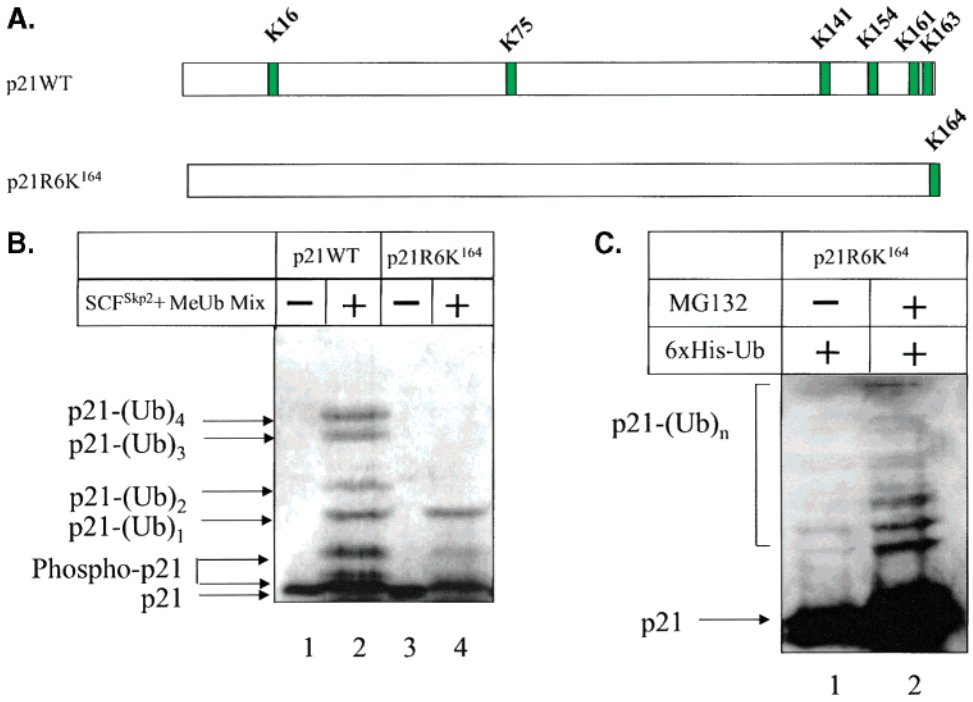


FIGURE 6: Ubiquitination of the carboxyl terminal lysine residue introduced into p21. (A) Schematic diagram of the p21 mutant in which P164 was substituted by a lysine residue. (B) Analysis of ubiquitination patterns of p21P164K mutants. Both the wild type and p21 mutant were incubated with the ubiquitination reaction mixture in the presence or absence of 120 $\mu$M Me-Ub and analyzed by SDS−PAGE as described in the Materials and Methods. (C) Ubiquitination of p21P164K in cells. p21P164K was cotransfected with His-Ub into H293 cells and incubated in the presence or absence of MG-132 prior to harvesting. Western blot analysis was performed with an antibody against p21.

13−16 in Figure 5D), given that ubiquitin was found to be conjugated at these two lysines in 293 cells (parts A and B of Figure 5). Considering that the carboxyl terminal region of p21 has been shown to be involved in binding to other cellular proteins such as PCNA (*21*, *22*), one plausible interpretation for this result is that polyubiquitination at those sites is not very efficient or processive because of these interactions. When our results are taken together, they suggest that the four lysine residues targeted by SCF for ubiquitination *in vitro* can also be ubiquitinated *in vivo* but at variable efficiencies. Other cellular factors may also influence ubiquitin site selection *in vivo*.

*Ubiquitination of a Carboxyl-Terminal Lysine Residue Introduced into p21.* Because multiple lysines in the C terminus can be ubiquitinated, we considered the possibility that their general location in the polypeptide chain rather than exact position was important. Ubiquitination has been shown to occur at the internal lysine residues or the N terminus of the protein (*9*). It is thus of considerable interest to test whether a C-terminus lysine residue can be targeted to ubiquitin conjugation. The carboxyl-terminal residue in p21 is a proline. We changed this proline to a lysine in the background of the p21 lysine-less mutant (p21R6K$^{164}$) (Figure 6A). Shown in Figure 6B, whereas ubiquitination of the wild-type p21 yielded four bands corresponding to four ubiquitin chains attached to the four individual lysine residues in p21, ubiquitination of p21R6K$^{164}$, which contains a single-lysine residue at the carboxyl terminus of p21 without any internal lysine residues, yielded only a single band corresponding to the size of mono-ubiquitinated p21. Note that p21R6K$^{164}$ is as efficiently ubiquitinated as any of the p21 mutants containing a single-lysine residue *in vitro*. To determine if p21R6K$^{164}$ can be targeted to ubiquitination *in vivo*, this mutant was expressed in 293 cells along with His-Ub by transient transfection. Transfected cells were treated with or without MG-132, and lysates were immunoblotted with p21 antibody. Proteasome inhibitor treatment leads to an increase in the steady levels of p21R6K$^{164}$, suggesting that the stability of this p21 mutant is also subject to proteasome regulation. The pattern of the high molecular weight intermediates accumulated in the presence of the proteasome inhibitor is identical to the wild-type p21 and other p21 mutants shown in Figure 5A. This result suggests that ubiquitination can occur at the carboxyl-terminal amino acid residue if it is lysine.

*Requirement of Cyclin E or Cdk2 Association for p21 Ubiquitination.* A previous study suggested that ubiquitin-independent degradation of p21 occurs via a C-terminal degradation signal that directly interacts with the C8 subunit of the 20S proteasome (*8*). However, the ubiquitin-mediated degradation signal in p21 remains poorly defined. The interaction between p21 and Cdk2 appears to be important for p21 degradation *in vivo* and *in vitro* (*8*, *11*). We set out to address whether the p21 interaction with the cyclin E−Cdk2 complex is required for its ubiquitination by SCF$^{Skp2}$. Sequence alignment of p21 and p27 revealed that there are Cdk2-binding and cyclin E-binding motifs in the N terminus of p21 (*14*) (Figure 1A). The putative cyclin E-binding motif (RXL) and Cdk2-binding motif (FNF) in p21 were mutated by site-directed mutagenesis. A double mutant in which both motifs were altered was also constructed. Mutation of only one of the two motifs has little effect on p21 ubiquitination, although there is a decrease in triple or quadruple conjugates when the Cdk2-binding site was altered. Neither of these mutations affects the phosphorylation of p21 by cyclin E−Cdk2, suggesting that the

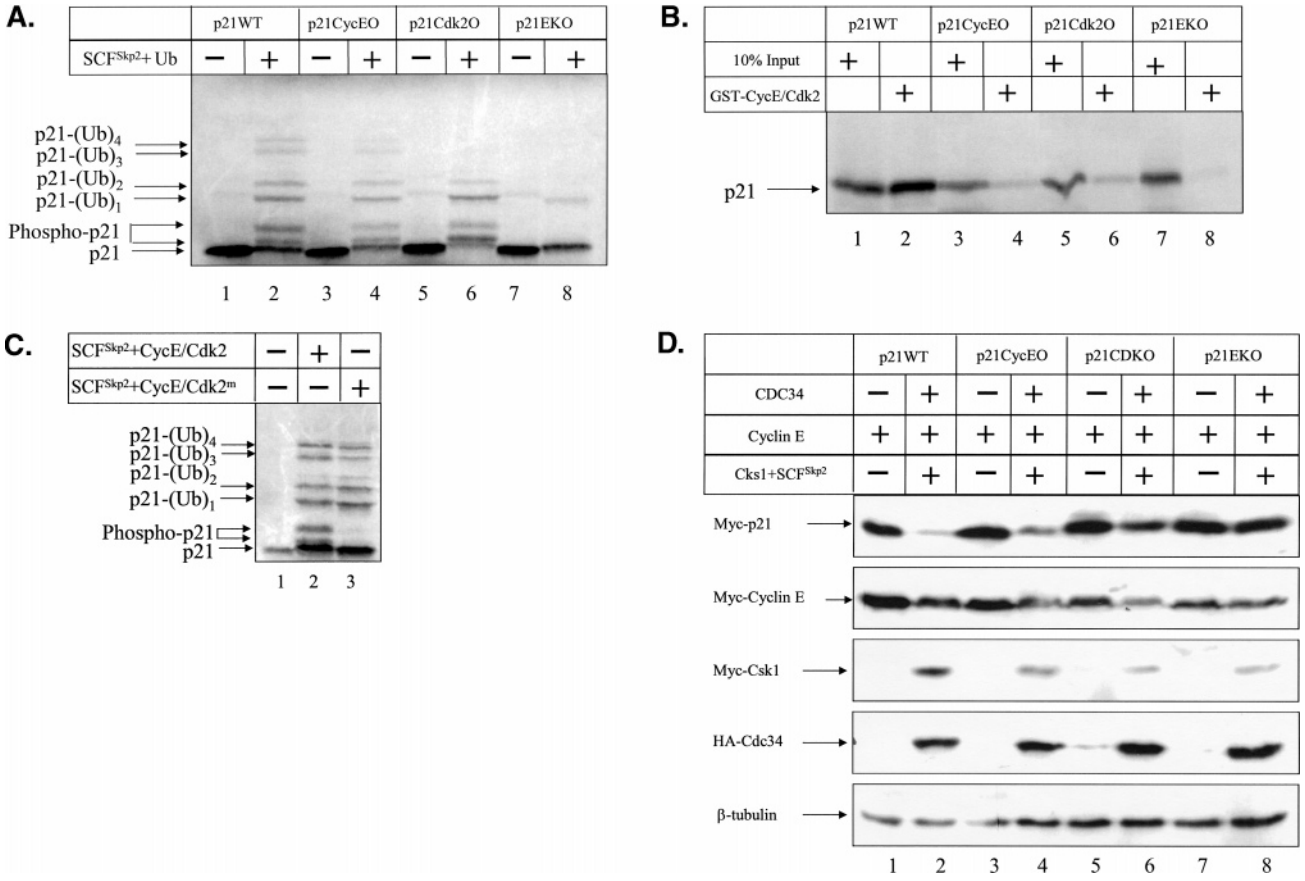


FIGURE 7: p21 ubiquitination requires stable association with either Cdk2 or cyclin E. (A) p21 mutants that are defective in cyclin E binding (p21CycEO), in Cdk2 binding (Cdk2KO), or in association with both (p21EKO) were constructed by mutating the cyclin E- and Cdk2-binding motifs indicated in Figure 1. Each mutant was *in vitro* translated and assayed for its ability to undergo ubiquitination by the SCF complex. (B) Association of p21 and p21 cyclin E- and Cdk2-binding motif mutants with GST−cyclin E−Cdk2. (C) Physical association but not kinase activity of cyclin E−Cdk2 is required for promoting p21 ubiquitination. Ubiquitination of p21 in the presence of catalytically inactive cyclin E−Cdk2$^m$ *in vitro*. (D) Destabilization of p21 by SCF requires physical contact with cyclin E−Cdk2 in cells. Wild type and p21 mutants deficient in cyclin E binding (p21CycEO), Cdk2 binding (Cdk2O), or both (p21EKO) were overexpressed in 293 cells along with cyclin E in the absence or presence of SCF components (Skp1, Skp2, Cul1, and Rbx1), plus Cdc34 and Cks1 by transient transfection. Levels of p21, cyclin E, Cks1, and Cdc34 were determined by immunoblotting with respective antibodies. $\beta$-Tubulin levels were used as loading controls.

presence of either of the two motifs is sufficient to promote p21 phosphorylation and ubiquitination by SCF$^{Skp2}$ (Figure 7A). However, when both of these two motifs were mutated, p21 failed to undergo ubiquitination in the presence of SCF$^{Skp2}$. To demonstrate that mutation of these two motifs affects p21 association with the cyclin E−Cdk2 complex, binding assays were performed using purified cyclin E−Cdk2 and *in vitro* translated p21 mutants. Mutation of either motif alone reduced the binding between p21 and the cyclin E−Cdk2 complex (compare lanes 4 and 6 versus 2 in Figure 7B). Association between p21 and cyclin E−Cdk2 was completely disrupted in p21 double mutants. Interestingly, the p21 double mutant is unable to be phosphorylated by cyclin E−Cdk2 (lane 8 in Figure 7A), suggesting that association between p21 and the cyclin E−Cdk2 complex is essential for substrate phosphorylation. Importantly, ubiquitination of the p21 double mutant was severely impaired (lane 8 in Figure 7A). Failure of p21 ubiquitination could have also been caused by the deficiency in p21 phosphorylation. To determine if p21 phosphorylation by cyclin E−Cdk2 is required for its ubiquitination by SCF$^{Skp2}$, we performed an *in vitro* p21 ubiquitination assay using the active and catalytically inactive cyclin E−Cdk2 complex (cyclin E−Cdk2$^m$). As shown in Figure 7C, although there is no obvious phosphorylation of p21 in the presence of cyclin E−Cdk2$^m$ judging by the absence of the phospho-p21 band present in the active cyclin E−Cdk2 (lane 2 versus 3 in Figure 7C), substrate ubiquitination was identical under either condition. Thus, phosphorylation of p21 is not obligatory for its ubiquitination, and p21 association with either Cdk2 or cyclin E is required for recognition by SCF$^{Skp2}$.

To determine whether association between cyclin E or Cdk2 and p21 is also important for SCF-mediated p21 destabilization in cells, p21 or p21 mutants defective in binding cyclin E, Cdk2, or both were cotransfected with or without the SCF complex in 293 cells. As shown in Figure 7D, overexpression of SCF causes a significant decrease in the levels of wild-type p21 (lane 1 versus 2 in Figure 7D). Reduction in the levels of p21 was impaired when the cyclin E- or Cdk2-binding sites were mutated in p21 (lane 3 versus 4 and lane 5 versus 6 in Figure 7D). There is little decrease in p21 expression when both binding sites were altered (lane 7 versus 8 in Figure 7D). Therefore, these results (Figure 7D) indicate that mutation of the CDK-binding site alone or in combination with the N-terminal cyclin E site prevents SCF$^{Skp2}$-mediated p21 elimination in cultured cells.

## DISCUSSION

The biological activity of p21 depends upon its expression levels. Because p21 is an unstable protein *in vivo*, post-

translational regulatory mechanisms play important roles in determining its steady-state levels under a variety of physiological settings. Both ubiquitin-dependent and ubiquitin-independent pathways have been implicated in regulating the stability of p21. Here, we studied mechanisms of p21 ubiquitination by $SCF^{Skp2}$. Our results indicate that there is limited specificity in selecting interior lysine residues for ubiquitination by $SCF^{Skp2}$. Only four of six lysine residues are targeted for ubiquitination *in vitro*, and three of these four are the predominant ubiquitination sites *in vivo*. Multiple lysine residues in a single p21 monomer can be selected to be ubiquitinated. In addition, SCF can catalyze ubiquitin transfer to the carboxyl terminal lysine if there is one available. The cyclin E-binding and Cdk2-binding motifs located at the N terminus of p21 are required for targeting it to the $SCF^{Skp2}$.

Ubiquitination occurs almost exclusively at the $\epsilon$-amino group of lysine residues, although the N-terminal-free amino group has been shown to be the site of ubiquitination for a few substrates (*23*). The rules for ubiquitination site selection are less well-defined. Evidence accumulated from studies of a number of substrates indicates that any single lysine residue is rarely essential for substrate ubiquitination and degradation (*24*−*26*). Mutation of a given lysine residue often results in selection of alternative ones. Multiple lysine residues need to be mutated to prevent substrate ubiquitination and degradation (*27*−*31*). This implies that either multiple lysines are capable of being ubiquitinated or selection of an alternative ubiquitination site when the predominant ubiquitination site was mutated. Our results with p21 ubiquitination support the notion that ubiquitination can occur at multiple lysine residues. It seems that the E3 enzymes are capable of lining up a number of sterically available or surface-exposed lysine residues for ubiquitination and hold the substrate to enable multiple rounds of ubiquitin transfer by recruiting ubiquitin-charged E2.

How does $SCF^{Skp2}$ target multiple lysine residues in distinct spatial area of substrates for ubiquitination? Studies with Cdc34/$SCF^{Cdc4}$ ubiquitination of Sic1 suggested that dynamic release of the ubiquitin-charged E2 enzyme (Cdc34) from the E3 enzyme may provide the spatial flexibility required to target multiple lysines on the substrate for ubiquitination (*32*). Consistent with this hypothesis, tighter association between Cdc34 and SCF is less active in promoting Sic1 ubiquitination. Our data on p21 ubiquitination can be explained by this "hit-and-run" model. However, the spatial flexibility in selecting a ubiquitination site can be also afforded by substrate conformation flexibility around target lysine residues. In this scenario, ubiquitin-charged Cdc34 does not have to diffuse away from the E3 enzyme as long as the uncharged Cdc34 can be readily dissociated from the E3 enzyme to allow for the charged Cdc34 to be recruited. The role of SCF is simply to ensure correct position of the incoming substrate to enable structurally flexible regions harboring lysines to be targeted for ubiquitination and to be accessible to the E2 enzyme stably bound to SCF (*33*). These two models are not mutually exclusive and may reflect two essential features of SCF E3 enzymes.

The potential lysine acceptor sites tend to be clustered in certain region of the substrate. p21 is a loosely folded protein, and the carboxyl-terminal region of the protein is rather flexible (*34*). Data presented here indicate that four lysine residues in the carboxyl-terminal region of p21 are lysine-acceptor sites for polyubiquitination. In contrast, ubiquitination of $\alpha$-synuclein occurs at four lysine residues in the N terminus of the protein (*31*). To further support the sterically available lysine residue to be chosen as the lysine-acceptor site, we found that a lysine residue introduced as the carboxyl-terminal residue can also be targeted for ubiquitination. However, not all lysine residues can serve as ubiquitin acceptor sites. For example, lysine 16 and lysine 75 of p21 are not ubiquitinated by $SCF^{Skp2}$. Consistent with this notion, only four bands were observed in the reconstituted p21 ubiquitination reaction *in vitro* in the presence of Me-Ub (Figure 1B). Mutagenesis analysis also supports the fact that four distinct lysine residues target for ubiquitin conjugation *in vitro* (parts B and C of Figure 4). The time course of the ubiquitin conjugation reaction *in vitro* indicates the conversion of mono-ubiquitinated species to multi-ubiquitinated species in a time-dependent fashion (Figure 4D). The pattern of ubiquitination site selection in p21 is quite different from ubiquitination of the S-phase CDK inhibitor Sic1 in which six lysine residues juxtaposed to the Cdk-binding sites are targeted for ubiquitination (*35*). It has been demonstrated that association of Sic1 with S-Cdk restricts ubiquitination to N-terminal lysines proximal to the Cdk site. However, in the case of p21, the opposite seems to be true. Lysines close to the Cdk-binding site in p21 are excluded from conjugation. Therefore, proximity to the Cdk-binding site does not appear to be the determinant for ubiquitination site selection for p21. Our data are consistent with the mode of lysine-acceptor site selection by availability. A survey of the preferred ubiquitination sites in yeast indicates that there is a preference for ubiquitin conjugation at lysine residues located in unstructured loop regions (*36*). Thus, lysines located in the flexible regions of proteins are prime candidates as ubiquitination sites in part because of their high probability to initiate attack of the E2−ubiquitin thioester bond. It is interesting to note that these four lysine residues are clustered in a region that was previously defined as the degradation signal for the ubiquitin-independent and proteasome-dependent pathway because this region is the binding site for the C8 $\alpha$ subunit of the 20S proteasome (*8*, *37*). It has been demonstrated that p21(1−133) is highly stable because it was no longer able to interact with C8 (*8*). In view of the findings presented here, the stability of p21(1−133) could be the result of disabling both ubiquitin-dependent and ubiquitin-independent pathways.

The four lysine residues in the carboxyl-terminal region of p21 are targeted for polyubiquitination by SCF at similar efficiencies *in vitro*. However, these four lysine residues do not appear to be equally ubiquitinated in cells. Lysine 141 is probably the predominant site for polyubiquitination *in vivo* because the amounts of polyubiquitinated conjugates that can be recovered are similar to that of the wild-type p21 and far more abundant than other single-lysine mutants *in vivo*. In further support of this notion is the finding that p21 with one single lysine at 141 exhibits metabolic stability similar to wild-type p21. Lysine 154 is poorly ubiquitinated, and destabilization of this mutant by SCF is less dramatic. Ubiquitin conjugation can be detected at lysine 161 and lysine 163 in cells; however, a p21 mutant with both lysine residues is refractory to SCF-mediated elimination. The reason for this is unclear at the moment given the fact that

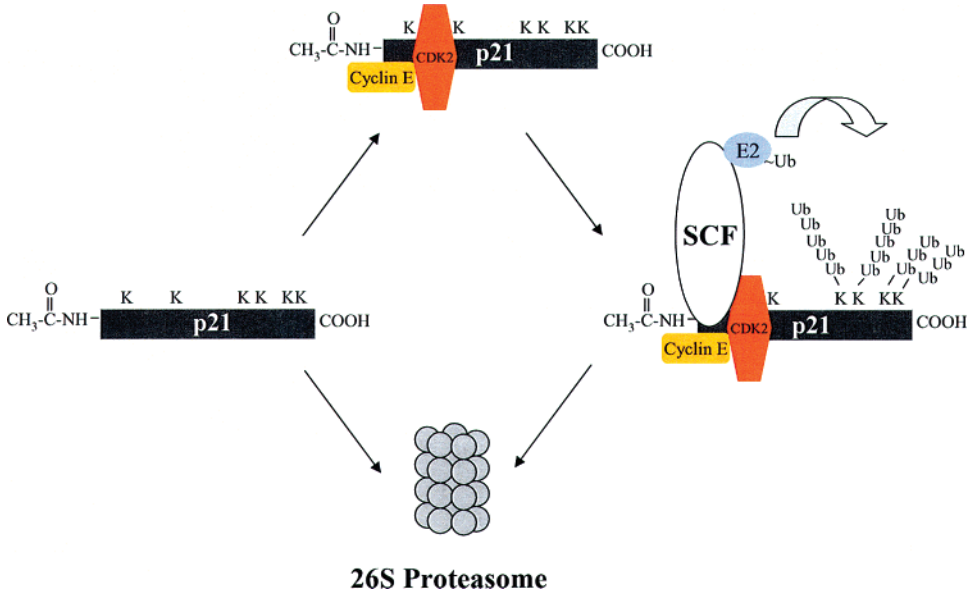


FIGURE 8: Model for ubiquitination site selection in p21 by $SCF^{Skp2}$.

both can be targeted for ubiquitination by SCF rather efficiently in the *in vitro* ubiquitination assay by SCF. A possible explanation for this observation is that the polyubiquitin assembled on these lysines may not be long enough to target the ubiquitinated substrate to the 26S proteasomes. Interestingly, this region of p21 has been previously shown to interact with PCNA (*21*, 22). This interaction could suppress polyubiquitination at these sites in cells. Alternatively, binding of PCNA or other cellular factors could also mask these lysine residues for ubiquitination. The difference in ubiquitination site selectivity between *in vitro* and *in vivo* contexts suggests that cellular factors other than SCF and cyclin E−Cdk2 may also dictate the choice of ubiquitination sites *in vivo*. Future studies are needed to elucidate mechanisms that can account for these differences.

There appear to be multiple pathways regulating p21 degradation (Figure 8). It is not uncommon for unstable proteins to harbor different destabilizing signals. There is a binding site for the C8 α subunit of the 20S proteasome in the carboxyl terminus of p21 that may mediate direct targeting of p21 to the proteasome (*8*). The presence of this binding site may explain in part why lysine-less p21 is still an unstable protein and subjected to proteasomal degradation (*7*, *8*). The complexity of p21 stability control *in vivo* prevents us from determining the contribution of each lysine residue in p21 metabolic stability in cells. Nevertheless, a plethora of data suggests that p21 stability is subjected to ubiquitin-mediated proteolysis. First of all, the metabolic stability of p21 is significantly increased in mouse cell lines containing homozygous deletion of Skp2 (*11*). Second, destabilization of p21 in response to UV irradiation requires Skp2 (*10*). Finally, a recent study suggests that the N terminus of p21 is completely acetylated and ubiquitination of endogenous p21 either at internal lysines or on the N terminus is unlikely to control its degradation by the proteasome (*38*). N-Terminal ubiquitination of p21 is inefficient in our system, and our result is more consistent with ubiquitination of internal lysine residues that may have profound effects on p21 degradation (Figure 8).

The specificity of ubiquitination is typically governed by the E3 enzymes. Thus far, $SCF^{Skp2}$ is the only E3 enzyme that has been shown to catalyze ubiquitin transfer from an E2 enzyme. How does p21 get recognized by this multisubunit E3 enzyme? Previous studies have highlighted the important role of Cks1 and the cyclin E−Cdk2 complex for p21 ubiquitination (*11*, *39*). Current studies revealed the importance of direct contact between p21 and cyclin E or Cdk2 for ubiquitination. Because Cks1 binds both the cyclin E−Cdk2 complex and Skp2, it is very likely that $SCF^{Skp2}$ recruits p21 through the Cks1−cyclin E−Cdk2 complex. In this regard, the F-box protein may not necessarily contact the substrate directly. Therefore, the Cdk2-binding motif or cyclin E-binding motif located in the N terminus of p21 can be regarded as the ubiquitination signal for degradation by the $SCF^{Skp2}$ E3 ligase.

There are many parallels between p21 and p27 when it comes to substrate recruitment to the $SCF^{Skp2}$ complex. Ubiquitination of both substrates requires Cks1 and cyclin E−Cdk2, in addition to $SCF^{Skp2}$ (*11*, *40*−*42*). However, there are some significant differences between them with regard to substrate recognition. Whereas p27 ubiquitination absolutely requires phosphorylation of p27 at T187 by the cyclin E−Cdk2 complex, phosphorylation of p21 is not strictly required for ubiquitination, although phosphorylation of p21 at S130 has been shown to increase the efficiency of ubiquitination (*11*). Another major difference lies in the requirement for cyclin E−Cdk2 contact for ubiquitination. Contact with either cyclin E or Cdk2 is sufficient for p21 recruitment to $SCF^{Skp2}$ (this paper); however, this is not the case for p27. The cyclin E-binding motif in p27 is absolutely required for p27 ubiquitination because the p27 mutant containing the Cdk2-binding motif alone is unable to be ubiquitinated by $SCF^{Skp2}$ (*43*). Therefore, $SCF^{Skp2}$ may recognize distinct features of the substrate to facilitate ubiquitin transfer from the E2 to its targets and underscores the idiosyncratic nature of the E3 enzyme in the ubiquitination reaction.

Aside from p27 and p21, other regulatory proteins have been reported to be potential substrates of $SCF^{Skp2}$. These include c-myc, b-myb, Cdt, hOrc1p, cyclin E, and p130 (*44*−*51*). The involvement of $SCF^{Skp2}$ in regulating the stability of these proteins has been demonstrated largely *in vivo*, and

*in vitro* ubiquitination of these substrates has not been reconstituted using a purified system. It will be interesting to determine whether $SCF^{Skp2}$ uses analogous mechanisms to target these substrates for ubiquitination.

If the cyclin E−Cdk2 complex targets p21 to ubiquitination, then how can the stability of p21 be regulated during cell-cycle progression, DNA damage, and cell differentiation? One obvious way is to regulate the availability of the E3 enzyme. The stability of Skp2 and Cks1 as well as the transcription of Cks1 are cell-cycle-regulated (*52*−*55*). Depletion of SCF components from cells will prevent p21 and p27 degradation. Given the importance of cyclin E−Cdk2 in p21 ubiquitination, the rise and fall of cyclin E expression is expected to have a significant impact on p21 stability. Another way of regulating p21 ubiquitination could be achieved by post-translational modification of the substrate. Phosphorylation is one of myriad modifications that alter the function of the protein. p21 has been shown to be a substrate for multiple protein kinases (*56*, *57*). It has been shown previously that phosphorylation of p21 by Akt/PKB inhibits p21 degradation (*57*). It is also possible that association of p21 with other cellular proteins may sequester it from the cyclin E−Cdk2 complex and prevent it from targeting $SCF^{Skp2}$. Therefore, degradation of p21 can be modulated in a variety of ways depending upon the intra- or extracellular signal inputs.

## ACKNOWLEDGMENT

We thank Drs. Wade Harper, David Morgan, Fukuchi, and Michele Pagano for the generous supply of reagents used in this study, members of Liu laboratory for helpful discussions, and Dana Ungermannova and Ethan Sanford for technical assistance. We also thank Kristen Barthel for critically reading the manuscript.

## REFERENCES

1. Coqueret, O. (2003) New roles for p21 and p27 cell-cycle inhibitors: A function for each cell compartment? *Trends Cell Biol. 13*, 65−70.
2. Sherr, C. J., and Roberts, J. M. (1999) CDK inhibitors: Positive and negative regulators of G1-phase progression, *Genes Dev. 13*, 1501−1512.
3. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases, *Cell 75*, 805−816.
4. Zhang, H., Xiong, Y., and Beach, D. (1993) Proliferating cell nuclear antigen and p21 are components of multiple cell cycle kinase complexes, *Mol. Biol. Cell 4*, 897−906.
5. el-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., Pietenpol, J. A., Burrell, M., Hill, D. E., Wang, Y., et al. (1994) WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis, *Cancer Res. 54*, 1169−1174.
6. Di Leonardo, A., Linke, S. P., Clarkin, K., and Wahl, G. M. (1994) DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts, *Genes Dev. 8*, 2540−2551.
7. Sheaff, R. J., Singer, J. D., Swanger, J., Smitherman, M., Roberts, J. M., and Clurman, B. E. (2000) Proteasomal turnover of p21Cip1 does not require p21Cip1 ubiquitination, *Mol. Cell 5*, 403−410.
8. Touitou, R., Richardson, J., Bose, S., Nakanishi, M., Rivett, J., and Allday, M. J. (2001) A degradation signal located in the C-terminus of p21WAF1/CIP1 is a binding site for the C8 α-subunit of the 20S proteasome, *EMBO J. 20*, 2367−2375.
9. Bloom, J., Amador, V., Bartolini, F., DeMartino, G., and Pagano, M. (2003) Proteasome-mediated degradation of p21 via N-terminal ubiquitinylation, *Cell 115*, 71−82.
10. Bendjennat, M., Boulaire, J., Jascur, T., Brickner, H., Barbier, V., Sarasin, A., Fotedar, A., and Fotedar, R. (2003) UV irradiation triggers ubiquitin-dependent degradation of p21(WAF1) to promote DNA repair, *Cell 114*, 599−610.
11. Bornstein, G., Bloom, J., Sitry-Shevah, D., Nakayama, K., Pagano, M., and Hershko, A. (2003) Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase, *J. Biol. Chem. 278*, 25752−25757.
12. Yu, Z. K., Gervais, J. L., and Zhang, H. (1998) Human CUL-1 associates with the SKP1/SKP2 complex and regulates p21(CIP1/WAF1) and cyclin D proteins, *Proc. Natl. Acad. Sci. U.S.A. 95*, 11324−11329.
13. Sheaff, R. J., Groudine, M., Gordon, M., Roberts, J. M., and Clurman, B. E. (1997) Cyclin E-CDK2 is a regulator of p27Kip1, *Genes Dev. 11*, 1464−1478.
14. Vlach, J., Hennecke, S., and Amati, B. (1997) Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27, *EMBO J. 16*, 5334−5344.
15. Montagnoli, A., Fiore, F., Eytan, E., Carrano, A. C., Draetta, G. F., Hershko, A., and Pagano, M. (1999) Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation, *Genes Dev. 13*, 1181−1189.
16. Strohmaier, H., Spruck, C. H., Kaiser, P., Won, K. A., Sangfelt, O., and Reed, S. I. (2001) Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line, *Nature 413*, 316−322.
17. Koepp, D. M., Schaefer, L. K., Ye, X., Keyomarsi, K., Chu, C., Harper, J. W., and Elledge, S. J. (2001) Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase, *Science 294*, 173−177.
18. Wang, W., Ungermannova, D., Chen, L., and Liu, X. (2003) A negatively charged amino acid in Skp2 is required for Skp2−Cks1 interaction and ubiquitination of p27Kip1, *J. Biol. Chem. 278*, 32390−32396.
19. Reynard, G. J., Reynolds, W., Verma, R., and Deshaies, R. J. (2000) Cks1 is required for G(1) cyclin-cyclin-dependent kinase activity in budding yeast, *Mol. Cell Biol. 20*, 5858−5864.
20. Hershko, A., and Ciechanover, A. (1998) The ubiquitin system, *Annu. Rev. Biochem. 67*, 425−479.
21. Gulbis, J. M., Kelman, Z., Hurwitz, J., O'Donnell, M., and Kuriyan, J. (1996) Structure of the C-terminal region of p21(WAF1/CIP1) complexed with human PCNA, *Cell 87*, 297−306.
22. Waga, S., Hannon, G. J., Beach, D., and Stillman, B. (1994) The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA, *Nature 369*, 574−578.
23. Pickart, C. M. (2001) Mechanisms underlying ubiquitination, *Annu. Rev. Biochem. 70*, 503−533.
24. King, R. W., Glotzer, M., and Kirschner, M. W. (1996) Mutagenic analysis of the destruction signal of mitotic cyclins and structural characterization of ubiquitinated intermediates, *Mol. Biol. Cell 7*, 1343−1357.
25. Hochstrasser, M., and Kornitzer, D. (1998) in *Ubiquitin and the Biology of the Cell* (Peter, J., Harris, J., and Finly, D., Eds.) pp 279−302, Plenum Press, New York.
26. Treier, M., Staszewski, L. M., and Bohmann, D. (1994) Ubiquitin-dependent c-Jun degradation *in vivo* is mediated by the δ domain, *Cell 78*, 787−798.
27. Scherer, D. C., Brockman, J. A., Chen, Z., Maniatis, T., and Ballard, D. W. (1995) Signal-induced degradation of I κ B α requires site-specific ubiquitination, *Proc. Natl. Acad. Sci. U.S.A. 92*, 11259−11263.
28. Baldi, L., Brown, K., Franzoso, G., and Siebenlist, U. (1996) Critical role for lysines 21 and 22 in signal-induced, ubiquitin-mediated proteolysis of I κ B-α, *J. Biol. Chem. 271*, 376−379.
29. Nishizawa, M., Furuno, N., Okazaki, K., Tanaka, H., Ogawa, Y., and Sagata, N. (1993) Degradation of Mos by the N-terminal proline (Pro2)-dependent ubiquitin pathway on fertilization of Xenopus eggs: Possible significance of natural selection for Pro2 in Mos, *EMBO J. 12*, 4021−4027.
30. Stroschein, S. L., Bonni, S., Wrana, J. L., and Luo, K. (2001) Smad3 recruits the anaphase-promoting complex for ubiquitination and degradation of SnoN, *Genes Dev. 15*, 2822−2836.
31. Nonaka, T., Iwatsubo, T., and Hasegawa, M. (2005) Ubiquitination of α-synuclein, *Biochemistry 44*, 361−368.
32. Deffenbaugh, A. E., Scaglione, K. M., Zhang, L., Moore, J. M., Buranda, T., Sklar, L. A., and Skowyra, D. (2003) Release of ubiquitin-charged Cdc34-S-Ub from the RING domain is essential for ubiquitination of the SCF(Cdc4)-bound substrate Sic1, *Cell 114*, 611−622.

33. Wu, G., Xu, G., Schulman, B. A., Jeffrey, P. D., Harper, J. W., and Pavletich, N. P. (2003) Structure of a $\beta$-TrCP1-Skp1-$\beta$-catenin complex: Destruction motif binding and lysine specificity of the SCF($\beta$-TrCP1) ubiquitin ligase, *Mol. Cell 11*, 1445−1456.
34. Kriwacki, R. W., Hengst, L., Tennant, L., Reed, S. I., and Wright, P. E. (1996) Structural studies of p21Waf1/Cip1/Sdi1 in the free and Cdk2-bound state: Conformational disorder mediates binding diversity, *Proc. Natl. Acad. Sci. U.S.A. 93*, 11504−11509.
35. Petroski, M. D., and Deshaies, R. J. (2003) Context of multiubiquitin chain attachment influences the rate of Sic1 degradation, *Mol. Cell 11*, 1435−1444.
36. Catic, A., Collins, C., Church, G. M., and Ploegh, H. L. (2004) Preferred *in vivo* ubiquitination sites, *Bioinformatics 20*, 3302−3307.
37. Fukuchi, K., Hagiwara, T., Nakamura, K., Ichimura, S., Tatsumi, K., and Gomi, K. (2002) Identification of the regulatory region required for ubiquitination of the cyclin kinase inhibitor, p21, *Biochem. Biophys. Res. Commun. 293*, 120−125.
38. Chen, X., Chi, Y., Bloecher, A., Aebersold, R., Clurman, B. E., and Roberts, J. M. (2004) *N*-Acetylation and ubiquitin-independent proteasomal degradation of p21(Cip1), *Mol. Cell 16*, 839−847.
39. Cayrol, C., and Ducommun, B. (1998) Interaction with cyclin-dependent kinases and PCNA modulates proteasome-dependent degradation of p21, *Oncogene 17*, 2437−2444.
40. Ganoth, D., Bornstein, G., Ko, T. K., Larsen, B., Tyers, M., Pagano, M., and Hershko, A. (2001) The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27, *Nat. Cell Biol. 3*, 321−324.
41. Spruck, C., Strohmaier, H., Watson, M., Smith, A. P., Ryan, A., Krek, T. W., and Reed, S. I. (2001) A CDK-independent function of mammalian Cks1: Targeting of SCF(Skp2) to the CDK inhibitor p27Kip1, *Mol. Cell 7*, 639−650.
42. Wang, W., Ungermannova, D., Chen, L., and Liu, X. (2004) Molecular and biochemical characterization of the Skp2−Cks1 binding interface, *J. Biol. Chem. 279*, 51362−51369.
43. Ungermannova, D., Gao, Y., and Liu, X. (2005) Ubiquitination of p27Kip1 requires physical interaction with cyclin E and probable phosphate recognition by SKP2, *J. Biol. Chem. 280*, 30301−30309.
44. Tedesco, D., Lukas, J., and Reed, S. I. (2002) The pRb-related protein p130 is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCF(Skp2), *Genes Dev. 16*, 2946−2957.
45. Kondo, T., Kobayashi, M., Tanaka, J., Yokoyama, A., Suzuki, S., Kato, N., Onozawa, M., Chiba, K., Hashino, S., Imamura, M., Minami, Y., Minamino, N., and Asaka, M. (2004) Rapid degradation of Cdt1 upon UV-induced DNA damage is mediated by SCFSkp2 complex, *J. Biol. Chem.*
46. Li, X., Zhao, Q., Liao, R., Sun, P., and Wu, X. (2003) The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation, *J. Biol. Chem. 278*, 30854−30858.
47. Liu, E., Li, X., Yan, F., Zhao, Q., and Wu, X. (2004) Cyclin-dependent kinases phosphorylate human Cdt1 and induce its degradation, *J. Biol. Chem. 279*, 17283−17288.
48. Kim, S. Y., Herbst, A., Tworkowski, K. A., Salghetti, S. E., and Tansey, W. P. (2003) Skp2 regulates Myc protein stability and activity, *Mol. Cell 11*, 1177−1188.
49. von der Lehr, N., Johansson, S., Wu, S., Bahram, F., Castell, A., Cetinkaya, C., Hydbring, P., Weidung, I., Nakayama, K., Nakayama, K. I., Soderberg, O., Kerppola, T. K., and Larsson, L. G. (2003) The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription, *Mol. Cell 11*, 1189−1200.
50. Yeh, K. H., Kondo, T., Zheng, J., Tsvetkov, L. M., Blair, J., and Zhang, H. (2001) The F-box protein SKP2 binds to the phosphorylated threonine 380 in cyclin E and regulates ubiquitin-dependent degradation of cyclin E, *Biochem. Biophys. Res. Commun. 281*, 884−890.
51. Charrasse, S., Carena, I., Brondani, V., Klempnauer, K. H., and Ferrari, S. (2000) Degradation of B-Myb by ubiquitin-mediated proteolysis: Involvement of the Cdc34-SCF(p45Skp2) pathway, *Oncogene 19*, 2986−2995.
52. Wei, W., Ayad, N. G., Wan, Y., Zhang, G. J., Kirschner, M. W., and Kaelin, W. G., Jr. (2004) Degradation of the SCF component Skp2 in cell-cycle phase G1 by the anaphase-promoting complex, *Nature 428*, 194−198.
53. Bashir, T., Dorrello, N. V., Amador, V., Guardavaccaro, D., and Pagano, M. (2004) Control of the SCF(Skp2-Cks1) ubiquitin ligase by the APC/C(Cdh1) ubiquitin ligase, *Nature 428*, 190−193.
54. Wang, W., Ungermannova, D., Jin, J., Harper, J. W., and Liu, X. (2004) Negative regulation of SCFSkp2 ubiquitin ligase by TGF-$\beta$ signaling, *Oncogene 23*, 1064−1075.
55. Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U., and Krek, W. (1999) p45SKP2 promotes p27Kip1 degradation and induces S phase in quiescent cells, *Nat. Cell Biol. 1*, 207−214.
56. Kim, G. Y., Mercer, S. E., Ewton, D. Z., Yan, Z., Jin, K., and Friedman, E. (2002) The stress-activated protein kinases p38 $\alpha$ and JNK1 stabilize p21(Cip1) by phosphorylation, *J. Biol. Chem. 277*, 29792−29802.
57. Li, Y., Dowbenko, D., and Lasky, L. A. (2002) AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival, *J. Biol. Chem. 277*, 11352−11361.

BI051071J