Ubiquitination of p27<sup>Kip1</sup> Requires Physical Interaction with Cyclin E and Probable Phosphate Recognition by SKP2*†

Received for publication, September 28, 2004, and in revised form, June 23, 2005
Published, JBC Papers in Press, June 24, 2005, DOI 10.1074/jbc.M411103200

Dana Ungermannova, Yuefeng Gao, and Xuedong Liu‡

From the Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309

Cyclin-dependent kinase inhibitor p27 plays essential roles in controlling cell proliferation (1–4). In growth-arrested cells, p27 accumulates and inhibits the activity of Cyclin E-Cdk2 and Cyclin A-Cdk2 resulting in halting cell cycle progression. In proliferating cells, p27 is degraded during the G<sub>1</sub>/S transitions and thus releases inhibition of the Cdk2 kinase to drive cell cycle progression. The tight control of p27 degradation is abrogated in human cancer cells. Excessive proteolysis of p27 is a hallmark of many types of aggressive tumor cells (5, 6).

Proteolysis of p27 occurs mainly through the ubiquitin-mediated proteasome degradation pathway (7, 8). Ubiquitination of p27 is carried out by a multienzyme complex consisting of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). The E3 often controls the specificity and timing of substrate ubiquitination (9–12). Polyubiquitination of p27 during the G<sub>1</sub>/S transition is mediated by a multisubunit E3 enzyme complex, SCFSkp2 (9, 10). SCF is a RING-finger type E3 enzyme with four subunits including invariable components Rbx1, Cul-1, Skp1, and a variable F-box-containing substrate acceptor protein (13–15). Whereas Rbx1 contains a RING H2 domain that provides a docking site for conjugating enzymes (E2), Cul-1 is the scaffold protein that bridges Rbx1 and Skp1 (16). The latter recognizes the F-box motif existing in many F-box proteins. The F-box proteins typically contain a variety of protein-protein interaction domains that are involved in recruitment of various substrates for ubiquitination (15). Skp2 is the F-box protein that is responsible for p27 recognition (9, 10). There are 10 leucine-rich repeats (LRR) in Skp2 forming a “sickle” shaped structure when crystalized (17, 18). It has been assumed that these LRRs bind p27 and facilitate its ubiquitination.

Ubiquitination of p27 requires its phosphorylation at Thr<sup>187</sup> by Cyclin E-Cdk2 or Cyclin A-Cdk2 (19–21). Phosphorylation makes p27 competent to be a substrate for SCFSkp2. The exact role of phosphorylation is still not very clear but existing evidence suggests that acquisition of the phosphate group may increase binding of the substrate to the F-box protein, thus serving as a degradation signal (22). However, ubiquitination of p21 by SCFSkp2 can be phosphorylation-independent (23). Therefore, substrate phospho-ubiquitination is not absolutely required for all SCFSkp2 targets.

In addition to the requirement of substrate phosphorylation, ubiquitination of p27 also requires the presence of Cks1 and Cyclin E-Cdk2 (9–12, 24). Cks1 was originally identified as a subunit of the Cdk2 complex (25). Three independent binding sites exist in Cks1 to mediate its interaction with Cdk2, Skp2, and anion groups (26). Two models have been proposed for the role of Cks1 in p27 ubiquitination. The allosteric model suggests that Cks1 binds Skp2, causes a conformational change in Skp2, and allows phosphorylated p27 to dock onto Skp2 (12, 27). The adapter model favors the idea that Cks1 binds the Cyclin E-Cdk2-p27 complex and provides the substrate in proximity of Skp2 (11, 26). The anion binding site of Cks1 may recognize the phosphate group at Thr<sup>187</sup> of p27 (22, 26). These two models present contrasting views of p27 substrate recruitment to SCFSkp2 and lie at the heart of the question regarding how E3 enzymes recruit substrates in general.

In this report, we first set out to determine the role of Cyclin E-Cdk2 in p27 ubiquitination. We showed that Cyclin E-Cdk2 is absolutely required for ubiquitination of p27. Phosphorylated p27 cannot be ubiquitinated without direct physical contact with Cyclin E. The physical contact between p27 and Cdk2 is dispensable for p27 ubiquitination. In addition, we have identified a positively charged amino acid residue on the surface of the LRR in Skp2 that is required for ubiquitination of phosphorylated p27 but dispensable for ubiquitination of unphosphorylated p21. Our data suggest that p27 recruitment to SCFSkp2 is through the Cyclin E-Cdk2 complex via the adapter domain of Skp2.
protein Cks1. The phospho-epitope at Thr^{187} is probably recognized directly by Skp2 to allow the substrate to adopt a conformation competent for ubiquitination.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors and Recombinant Proteins—**Mouse p27 and human p21 were subcloned into pcDNA3. pcEXL-Skp2 was previously described (24). The Skp2 R306A/K367A, R306A, K307A, and R319A/R320A point mutations as well as p27-Cyclin E-Cdk2 contact mutants, p27T187A and p27 T187D, were generated by using the QuickChange site-directed mutagenesis system (Stratagene). These constructs were used as templates for in vitro translations using the Tnt kit from Promega. Human Cks1, purchased from ATCC, was cloned into pGEX4T-1 and purified as a GST-tagged protein as described previously (28). Recombinant E1 was produced in insect cells or purchased from EntrogenBiochem. Recombinant baculoviruses expressing GST-Skp1, HA-CUL1, Rbx, GST-Cyclin E, and Cdk2 were gifts of Dr. Wade Harper (Harvard Medical School), and catalytically inactive Cdk2 was a gift of Dr. David Morgan (University of California, San Francisco). To construct the SCF complex with mutant Skp2, wild type and mutant Skp2 were subcloned into pFAST-BacHT (Invitrogen) and transformed into DH10Bac. Genomic DNA for each construct derived from DH10Bac cells that had undergone successful recombination was prepared according to the manufacturer’s instructions and subsequently transfected into SF9 cells using Cellfectin (Invitrogen). All baculoviruses were amplified in SF9 cells, and the different SCF<sup>Skp2</sup> (Skp2 mutants) protein complexes and Cyclin E-Cdk2 complexes were generated by co-infection of Hi5 cells (Invitrogen) with the appropriate baculoviruses. GST affinity chromatography was used to purify the recombinant Cyclin E-Cdk2 complex (GE Healthcare). Human and bacterial cyclin A and Cdk2 (Boehringer Mannheim) expressed in bacteria and purified with nickel-nitrotriacetic acid-agarose (Qiagen). To produce recombinant p27, the coding region of p27 was cloned into the pGEX4T-1 vector using the SalI and NotI sites, transformed into BL21 cells (Novagen), and purified as a GST fusion protein. Native and taggated p27 were obtained by cleaving the purified protein with recombinant thrombin (Sigma).

**Purification of Phosphorylated p27—**Phosphorylated p27 was purified from E. coli coexpressed with bacterially purified p27 reporters of human p27 were purchased from Sigma: NAGSVEQTPKKPGLLRRQT (p27 phosphopeptide), which was generated by substituting the human p27 with Thr residue at position 8 (equivalent to human p27 Thr^{187}), which is normally phosphorylated by Cyclin E-Cdk2. The peptides were coupled to CNBr-activated Sepharose 4B as recommended by the manufacturer (GE Healthcare). In vitro binding reactions were performed in the presence or absence of the recombinant Cks1 as described (26). ImageQuant 5.2 was utilized to quantify the protein-protein interactions.

**Immunoblotting and Immunoprecipitation Assays—**Samples from the purified SCF complexes were resolved on 12% SDS-PAGE and electrophoretically transferred to nitrocellulose. The expression levels of Skp2 and HA-Cul1 were analyzed by immunoblotting with anti-Skp2 (Santa Cruz Biotechnology) or anti-HA (Covance) antibodies. Equal amounts of the wild type and mutant SCF complexes were incubated with 5 μl of in vitro translated Cks1 labeled with [35S]Met for 30 min at 4 °C. 2 μl of anti-HA antibody was added to each sample and incubated for 1 h. 20 μl of Protein A/G beads (Santa Cruz) were subsequently added to each sample and incubated for 1 h at 4 °C. Protein complexes on the beads were collected by brief centrifugation and analyzed by 12% SDS-PAGE.

**In Vitro p27 and p21 Ubiquitination Assays—**Wild type p27 or p27<sup>ΔN</sup>, or p27<sup>ΔK</sup> were labeled with [35S]methionine with a Tnt lysate system and phosphorylated by incubation with 100 ng of GST-Cyclin E-Cdk2 in a 20X energy regeneration system (10 mM ATP, 20 mM Hepes, pH 7.4, 1 mM MgOAc, 500 mM creatine phosphate, 0.5 mg/ml creatine phosphokinase). Reactions were incubated at 30 °C for 30 min. The ubiquitin ligation was performed in a 25-μl reaction containing pre-phosphorylated p27, 250 ng of E1, 1 μg of CDC34, 1 μg of SCF<sup>Skp2</sup> or SCF<sup>Skp2ΔN</sup> complex, or 7 μl of in vitro translated non-radioactively labeled Skp2 and Skp2 mutants, 0.1 μg of Cks1, 15 μg of methylated ubiquitin (Calbiochem), 1 μM ubiquitin aldehyde (BostonBiochem), 0.2 mM MG 132 (Calbiochem), and 2.5 μl of a 20X energy regeneration system. The reactions were incubated at 30 °C for 2 h, terminated by adding 2X SDS loading buffer, and resolved by SDS-PAGE. Phosphorimaging was used to analyze the results. Ubiquitination of p27 was performed in the same manner as p27 ubiquitination, except in vitro translated p27 was not preincubated with Cyclin E-Cdk2 and a 20X energy regeneration system.

**In Vitro Kinase Assay—**A 10-μl reaction system was used to phosphorylate recombinant p27. The mixture contained 1 μM Cyclin E-Cdk2, 2 μM p27, 100 μM unlabelled ATP, 10 μM [γ-<sup>32</sup>P]ATP, and 10 mM MgCl<sub>2</sub>. The reactions were incubated at 30 °C for 30 min. Heat treatment was applied to kinase reactions for 20 min using the indicated temperatures. Where necessary, the protein aggregates that resulted from the heat treatment were removed by centrifugation. Samples were quenched by addition of SDS sample buffer. Proteins were separated by SDS-PAGE and analyzed by Phosphorimaging (GE healthcare).

**RESULTS**

**Roles of Cyclin E-Cdk2 in p27 Ubiquitination—**Previous studies indicate that phosphorylation of p27 by Cyclin E-Cdk2 is pivotal for p27 ubiquitination (19–21). In addition, a trimeric complex containing Cyclin E-Cdk2 or Cyclin A-Cdk2 and p27 is also required (19). Because p27 forms a stable complex with Cyclin E-Cdk2, it is still not clear whether ubiquitination of phosphorylated p27 can occur in the absence of the Cyclin E-Cdk2 complex after p27 is phosphorylated. Previous studies indicated that p27 is heat stable (29). Heat-treated p27 (100 °C 3 min) retains its ability to bind Cyclin E-Cdk2 and inhibit the kinase activity (29). Our experimental strategy was to produce phosphorylated at Thr<sup>187</sup> and subsequently remove Cyclin E-Cdk2 by heat treatment. In this way, we could separate the phosphorylation function of Cyclin E-Cdk2 from the adapter function in p27 ubiquitination. As shown in Fig. 1, we carried out an in vitro kinase assay in which we used [γ-<sup>32</sup>P]ATP to phosphorylate bacterially purified p27. 30 °C is a standard temperature we use to perform kinase reactions. To assess stability of phosphorylated p27, we first carried out the kinase assay at the standard temperature followed by heat treatment that varied from 30 to 100 °C. Each reaction was treated at the given temperature for 20 min, and insoluble materials were separated by centrifugation following heat treatment. Phosphorylated p27 remains soluble in the range of temperatures indicated (Fig. 1A).

Next we determined the temperature at which Cyclin E-Cdk2 activity and stability can be destroyed. Using the same temperature range to heat treat the recombinant Cyclin E-Cdk2 complex and subsequently adding it to the in vitro kinase reaction, we found that the Cyclin E-Cdk2 complex denatures and loses its ability to phosphorylate p27 once the temperature was raised above 50 °C (Fig. 1B). Thus, we can remove Cyclin E-Cdk2 from phosphorylated p27 after the phosphorylation reaction by incubating the reaction mixture at 75 °C. This treatment will yield phosphorylated p27 without native Cyclin E-Cdk2. Whereas p27 ubiquitination occurs efficiently in a mixture containing Cyclin E-Cdk2, Cks1 and SCF<sup>Skp2</sup> (Fig. 1C, lane 2), no ubiquitin was found to be transferred to p27 if the p27 phosphorylation reaction mixture was treated at 75 °C for 20 min (Fig. 1C, lane 4). Conjugation of ubiquitin was rescued when we added the recombinant Cyclin E-Cdk2 back to the heat-treated reaction mixture, suggesting that the presence of active Cyclin E-Cdk2 complex restored the defects (Fig. 1C, lane 5).

If the Cyclin E-Cdk2 complex is also required to phosphorylate p27 substrate, we would expect that kinase
**FIG. 1. Cyclin E-Cdk2 is required for p27 ubiquitination complex formation.** 

A. p27 is heat stable and remains soluble at temperatures ranging from 30 to 100 °C (lanes 1–7). In this experiment, recombinant p27 was phosphorylated using an *in vitro* kinase assay. *In vitro* kinase reactions were then subjected to the indicated heat treatments for 20 min and insoluble materials were removed by centrifugation. The SDS sample buffer was added to the supernatant prior to SDS-PAGE. The results were analyzed by phosphorimaging. 

B. Cdk2 kinase activity is not heat stable. Prior to incubation with p27 and *in vitro* kinase components, recombinant Cyclin E-Cdk2 was heated to the indicated temperatures for 20 min (lanes 1–7). Lane 8, phosphorylation of p27 is not observed when wild type (wt) Cyclin E-Cdk2 is replaced with the mutant of Cdk2 that has lost its kinase activity. 

C. Presence of Cyclin E-Cdk2 is required for p27 ubiquitination complex formation. Lanes 1–9 and 10–11 represent two different experiments. Lane 1, p27 input; lane 2, ubiquitination of p27 (see “Experimental Procedures”); lane 3, ubiquitination of unphosphorylated p27; lane 4, p27 was phosphorylated by Cyclin E-Cdk2 and then heat treated (75 °C for 20 min). Recovered supernatant was mixed with ubiquitination components (E1, E2, SCF<sup>Skp2</sup> complex, methylated ubiquitin, MGI32, ATP, and recombinant Cks1); lane 5, same as lane 4 but with Cyclin E-Cdk2 added back; lane 6, p27 was incubated with dead kinase (Cyclin E-Cdk2<sup>m</sup>) and subject to p27 ubiquitination; lane 7, same as lane 4 but with Cyclin E-Cdk2<sup>m</sup>; lane 8, p27 T187A input; lane 9, ubiquitination of p27 T187A; lane 10, p27 T187D input, ubiquitination of p27 T187D. 

D. *In vitro* reconstitution of p27 ubiquitination with purified components and the requirement of Cyclin E-Cdk2 for ubiquitination. Untagged recombinant p27 was phosphorylated by incubation with GST-Cyclin E-Cdk2, which was subsequently removed by repetitive GST affinity chromatography after the reaction was terminated. Phosphorylated p27 was incubated with purified SCF<sup>Skp2</sup>, Cks1, and reaction mixture in the presence or absence of Cyclin E-Cdk2 or Cyclin E-Cdk2<sup>m</sup> and analyzed by SDS-PAGE. E, neither Cyclin E alone nor Cdk2 alone is sufficient for p27 ubiquitination. Phosphorylation of p27 was prepared as described for C. Recombinant GST-Cyclin E and catalytically inactive Cdk2<sup>m</sup> were prepared from insect cells by single infection. Ubiquitination of p27 was performed as in C with the exception of adding the components as indicated.
defective Cyclin E-Cdk2 would also be able to restore the ubiquitination of phosphorylated p27. This is indeed the case as adding catalytically inactive Cyclin E-Cdk2 to the heat-treated phosphorylation reaction restored the p27 ubiquitination (Fig. 1C, lane 7), whereas incubating Cyclin E-Cdk2 alone did not result in p27 ubiquitination. Therefore, these data suggest Thr187-phosphorylated p27 cannot be ubiquitinated by the SCFSkp2 complex without the presence of Cyclin E-Cdk2. Because the enzymatic activity of Cyclin E-Cdk2 is not required to promote the ubiquitination of Thr187-phosphorylated p27, Cyclin E-Cdk2 must be involved in presenting the sub-strate to the SCFSkp2 complex. Finally, ubiquitination of p27 is phosphorylation-dependent as the p27 T187A mutant failed to undergo ubiquitination (Fig. 1C, lane 9). However, when Thr187 was changed to Asp, which could partially mimic the phosphorylation, ubiquitination occurred in the absence of phosphorylation but still depended on the presence of Cyclin E-Cdk2 (Fig. 1C, lane 11). Heat treatment is considered to be a harsh procedure and may cause a structural change in Thr187-phosphorylated p27, even though heat-treated p27 retains a majority of its biological activity in vitro (29).

To definitively assess the role of Cyclin E-Cdk2 in p27 ubiquitination, we sought to prepare Thr187-phosphorylated p27 under native conditions. Untagged purified recombinant p27 from E. coli was phosphorylated by GST-Cyclin E-Cdk2. 32P-Labeled p27 free from the kinase complex was prepared as described previously (28). A ubiquitination reaction with this labeled substrate was performed in the presence or absence of Cyclin E-Cdk2 or catalytically inactive Cyclin E-Cdk2mut. As shown in Fig. 1D, 32P-labeled p27 is unable to undergo ubiquitination without either Cyclin E-Cdk2 complex (Fig. 1D, lane 2). Upon addition of either complex, ubiquitination of p27 was restored. The different ubiquitination patterns observed in Fig. 1, C versus D, are likely because of the relative amount of methylated ubiquitin in the system. The substrate p27 used in Fig. 1D is purified from E. coli, whereas 35S-labeled p27 in Fig. 1C was prepared from rabbit reticulocyte lysate that is known to contain fair amounts of endogenous ubiquitin. The same amount of methylated ubiquitin was included in both reactions to suppress polyubiquitination. In the pure reconstituted system in Fig. 1D, a higher methylated ubiquitin/ubiquitin ratio suppresses polyubiquitination more effectively resulting in predominantly mono-ubiquitinated p27.

To determine whether Cyclin E, Cdk2, and p27 function as a trimeric complex to interact with SCF30sp2, we attempted to reconstitute the p27 ubiquitination reaction in vitro with purified Cyclin E or Cdk2 alone. Phosphorylation of p27 was accomplished by incubation with the Cyclin E-Cdk2 kinase, which was subsequently removed by heat treatment. Ubiquitination of phosphorylated p27 in the presence E1, E2, and E3 (SCFSkp2) was performed by adding either the catalytically inactive Cyclin E-Cdk2mut complex or purified Cyclin E alone or catalytically inactive kinase Cdk2mut alone. As shown in Fig. 1E, whereas the catalytically inactive Cyclin E-Cdk2mut complex promotes p27 ubiquitination, neither Cyclin E alone nor Cdk2mut alone are sufficient to reconstitute ubiquitination of phosphorylated p27. Therefore, the SCF30sp2 complex likely recognizes a trimeric complex consisting of p27-Cyclin E-Cdk2. The Cyclin E-Cdk2 complex has a dual function in p27 ubiquitination: substrate phosphorylation and substrate presentation.

Requirement of Cyclin E but Not Cdk2 Contact for p27 Ubiquitination in Vitro—The crystal structure of p27 complexed with Cyclin A-Cdk2 revealed that p27 has dual contact with Cdk2 and Cyclin A (30). It has been demonstrated previously that the FNF motif in Cdk2 and the RXL motif in Cyclin E are required for interaction between p27, Cdk2, and Cyclin E, respectively (20). We next sought to understand the role of specific interactions between p27 and Cyclin E-Cdk2 in p27 ubiquitination in vitro. Targeted residues that interact with Cyclin E-Cdk2 are conserved among all cyclin kinase inhibitors, including p27, p21, and p57 (20, 30). When p27 lost a contact with Cdk2 or Cyclin E or both, in each case, the function of p27 as an inhibitor of Cdk2 was abolished (20). It was also demonstrated that p27 mutants deficient in their binding to Cyclin E or Cyclin E-Cdk2 remained stable in Rat1 cells, compared with rapid degradation of p27 that was devoid of the Cdk2 contact (20). Whether impairment of p27 contacts with Cdk2 or Cyclin E affects p27 ubiquitination by SCF30sp2 in vitro has not been systematically explored. We generated point mutants of p27 by replacing Arg30 and Arg32 with alanines to prevent binding to Cyclin E (p27C–), Phe62 and Phe64 with alanines to eliminate Cdk2 contact (p27K–), as well as a double mutant to disable the interaction with both the Cdk2 and Cyclin E subunits. First, we analyzed whether the mutants we constructed indeed affect association between p27 and the Cyclin E-Cdk2 complex. A binding assay was performed by incubating Cyclin E-GST-Cdk2 purified from insect cells with in vitro translated 35S-labeled p27 variants. As predicted, we observed the strongest association between Cyclin E-Cdk2 with the wild type p27 (Fig. 2A, lane 2). p27K– and p27C– showed decreased binding to Cyclin E-Cdk2 (lanes 4 and 6), whereas p27C– was incapable of forming the ternary complex (lane 8). The consequence of mutations on p27 ubiquitination was tested in the reconstituted in vitro ubiquitination assay (24). In vitro translated p27 proteins were added to the reaction containing Cyclin E-Cdk2 along with the energy regeneration system. The ubiquitin ligation was assayed by mixing the following with the phosphorylation reaction: E1, E2, SCF30sp2, Cks1, methylated ubiquitin, ubiquitin aldehyde, and MG132. This final reaction was incubated at 30 °C for 2 h. The results were quantified by phosphorimaging. As shown in Fig. 2B, ubiquitination was evident in wild type p27 and p27K– (Fig. 2B, lanes 2 and 4). Ubiquitination of p27C– and p27C– was completely absent (Fig. 2B, lanes 6 and 8). Thus, ubiquitination of p27 highly depends on a functional interaction with Cyclin E, and p27 is probably recruited to the SCF30sp2 complex through binding to Cyclin E but not to Cdk2.

Identification of Positively Charged Amino Acid Residues That Are Likely to Be Involved in Recognizing the Thr187 Phosphate Group of p27—The requirement for p27 phosphorylation at Thr187 for ubiquitination is puzzling, especially given the fact that p21, which is also a substrate of SCF30sp2, does not require substrate phosphorylation to be ubiquitinated (23). This raises a question as to whether Skp2 is directly involved in recognizing the phosphate group in the substrate. Phosphorylation of p27 could either change the conformation of the protein or yield a degradation signal to be recognized by SCF30sp2. These two hypotheses are not necessarily mutually exclusive. Surface-exposed positively charged amino acid residues are most likely to be involved in phosphate recognition. This proved to be the case in recognition of the phosphorylated Cyclin E degron by Cdc4 (31, 32). The crystal structure of Skp2 revealed that Skp2 is a sickle-shaped molecule with 10 leucine-rich repeats forming a concave surface (17). Previous studies implicate the concave surface of LRRs in involvement in recognizing phosphate moieties (33). Electrodenisty analysis of the concave surface of Skp2 suggests clustering of positively charged residues that is found in a few F-box proteins is missing in Skp2 (24). We therefore focused on assessing the role of surface-exposed, positively charged amino acid residues in recognition of phosphorylated p27. Five amino acid residues, Arg306, Lys307, Arg319, Arg320, and Arg356, were selected based
on the criteria that they are positively charged, localized in the loop region of Skp2 in close proximity to the Cks1 binding site we defined, and surface exposed (34). Mutation of these amino acid residues is unlikely to disrupt the overall folding and structure of Skp2.

Using site-specific mutagenesis, we constructed the following Skp2 mutants: R306A/K307A, R319A/R320A, and R356A. Because defects other than correct docking of phosphate can impair the ubiquitination process, we needed to confirm that the generated mutations of Skp2 maintained binding to Cks1. Each Skp2 variant was radioactively labeled and incubated with GST-Cks1, and the amount of proteins bound to Cks1 was determined by immunoblotting with an anti-Skp2 antibody. As shown in Fig. 4, the amounts of Skp2 and Skp2 mutants collected on the glutathione beads as Skp2 is tagged with GST. Because the same viral stock was used to express the core components of the SCF complex, it is expected that expression levels of these core components are similar among the various SCFSkp2 complexes. To determine whether Skp2 and Skp2 mutants can assemble into the SCF complexes with similar efficiencies, purified wild type and mutant SCF complexes were immunoprecipitated with an anti-HA antibody (CUL-1 was HA tagged) to bring down CUL-1 and associated SCF components (Rbx1, Skp1, and Skp2). The amounts of Skp2 and Skp2 mutants collected on the beads were determined by immunoblotting with an anti-Skp2 antibody. As shown in Fig. 4A, the levels of CUL-1-associated skp2 or mutant Skp2 were very similar suggesting that there is no significant difference between the wild type and mutant Skp2 in SCF assembly. Next, we tested the ability of these SCF complexes to bind Cks1. Equal amounts of SCF with or without Skp2, SCFSkp2, SCFSkp2R306A/K307A, and SCFSkp2R319A/R320A were incubated with [35S]Met-labeled Cks1. The amounts of Cks1 associated with each complex were very similar as determined by immunoprecipitation with the HA antibody (Fig. 4B). These data suggest that Skp2, Skp2 R306A/K307A, and Skp2 R319A/R320A bind Cks1 equally well. Finally, we determined the ability of these three SCF complexes to ubiquitinate p27. Consistent with the results we obtained with the reticulocyte
lysate experiment, SCP\textsuperscript{Skp2} and SCP\textsuperscript{Skp2R319A/R320A} are capable of ubiquitinating p27, with a slightly lower efficiency for the latter. SCP\textsuperscript{Skp2R306A/K307A} is unable to promote p27 ubiquitination, with a slightly lower efficiency for the functional SCF complex just as efficiently as the wild type Skp2 WT. This result indicates that Skp2 R306A/K307A can assemble ubiquitination in the presence of either Cyclin E-Cdk2 complex.

In Fig. 4, phosphorylation by the SCF complex can be phosphorylation-independent. An increase in association between Skp2 and p27 upon phosphorylation of p27 was observed with Skp2 R306A/K307A, Skp2 R356A, and Skp2 R356A (lanes 4, 5, and 8). Phosphorylation of p27 was assessed in the presence of Skp2 WT, Skp2 K307A, Skp2 R306A/K307A, and Skp2 K307A IVT (lanes 1, 5, 7, 9, 11, and 13). In vitro translated Skp2 or Skp2 mutants were incubated with beads bound either to GST or GST-Cks1. After binding, the beads were resolved by SDS-PAGE and visualized by autoradiography. Cks1 binds efficiently to Skp2 WT (lane 4), Skp2 R306A (lane 6), Skp2 K307A (lane 8), Skp2 R306A/K307A (lane 10), and Skp2 R319A/R320A (lane 12). Decreased binding was observed when Cks1 was incubated with Skp2 Y358A and Skp2 R356A (lanes 14 and 16). B, the effect of Skp2 LRR mutants on p27 ubiquitination. Skp2 WT or Skp2 LRR mutants were in vitro translated in reticulocyte lysate and expressed as nonradioactive proteins. p27 ubiquitination reactions in panel B were carried out by adding 10 μl of in vitro translated (IVT) Skp2, E1, E2, Cks1, methylated ubiquitin (Ub), Ub aldehyde, and MG132 to phosphorylated \textsuperscript{35}S-labeled p27. Results were evaluated using phosphorimaging. Lane 1, p27 input; lane 3, phosphorylated p27 was incubated with the ubiquitination reaction mixture and 10 μl of TnT lysate. p27 is ubiquitinated in the presence of Skp2 WT, Skp2 K307A, Skp2 R319A/R320A (lanes 2, 6, and 7, respectively). No ubiquitination of p27 was observed with Skp2 R306A/K307A, Skp2 R306A, and Skp2 R356A (lanes 4, 5, and 8). C, expression levels of recombinant wild type Skp2 and Skp2 mutants synthesized by in vitro translation by immunoblotting analysis using an anti-Skp2 antibody. Lane 1, in vitro translated (IVT) Skp2; lane 2, control IVT; lane 3, Skp2 R306A/K307A IVT; lane 4, Skp2 R306A IVT; lane 5, Skp2 K307A IVT; lane 6, Skp2 R319A/R320A IVT; lane 7, Skp2 R356 IVT.

**DISCUSSION**

Phosphorylation of p27 at Thr\textsuperscript{187} by Cyclin E-Cdk2 triggers its ubiquitination by SCP\textsuperscript{Skp2}. The exact role of phosphorylation and the requirement of intact Cyclin E-Cdk2 for the ubiquitination of p27 with or without phosphorylated Thr\textsuperscript{187} were made and coupled to CNB-Sepharose beads. In vitro translated \textsuperscript{35}S-labeled Skp2 or Skp2 R306A were incubated with beads loaded with either unphosphorylated or phosphorylated p27 peptide in the presence or absence of Cks1. Phosphorylation resulted in a modest increase in binding of Skp2 to p27 peptide in the presence of Cks1 (Fig. 4F, lane 3 versus 4). The increased association between Skp2 and phospho-p27 peptide is Cks1-dependent (Fig. 4F, lanes 4 and 5), suggesting a role of Cks1 in mediating this interaction. Previous studies indicated that \textsuperscript{35}S-labeled Cks1 does not bind the phospho-p27 peptide in the absence of the Skp2-Skp1 complex despite the fact that it has an anion binding site (26). Association between Cks1 and phospho-p27 peptide is significantly enhanced upon addition of Skp2-Skp1 and depends on the anion binding site of Cks1. However, direct association between Skp2 and the phospho-p27 peptide was not assessed in the previous study (26). Data presented here coupled with previous observations imply that either Cks1 or Skp2 or both could be involved in recognizing the phospho group. To determine whether Arg\textsuperscript{306} of Skp2 contributes to phospho group recognition, interaction between Skp2 R306A and phospho-p27 peptide was investigated. Unlike the wild type Skp2, which exhibits selective binding to phosphorylated p27 peptide, Skp2 R306A displays little specific binding to this peptide. This result suggests that Arg\textsuperscript{306} is required for tighter association between Skp2 and phosphorylated p27 peptide, suggesting this residue is directly involved in binding to the Thr\textsuperscript{187} phospho group.
phosphorylated p21, because p21 in mutants in the SCF complexes that were purified from Hi-Five insect cells. Lys307 of Skp2 were changed to alanines (lane 4) or when SCF complex lacked Skp2 (lane 3). The lower panel shows the expressions of Skp2 or Skp2 mutants in the SCF complexes that were purified from Hi-Five insect cells. D, Skp2 mutant at Arg306–Lys307 has no effect on p21 ubiquitination. The p21 ubiquitin (Ub) reactions were carried out in the same manner as ubiquitination of p27, except preincubation of [35S]p21 with Cyclin E-Cdk2 was omitted. Cyclin E-Cdk2 was added directly to the Ub mixture. Wild type Skp2 as well as the Skp2 mutants were able to transfer Ub onto p21 (lanes 2, 5, and 6). The lack of Skp2 abrogated Ub ligation completely (lane 3). The first two high molecular weight bands represent the phosphorylated p21, because p21 in lane 4 was only incubated with Cyclin E-Cdk2 and the energy regeneration system. E, mutation in Skp2 at Arg306–Lys307 has no effect on p21 (phosphorylation independent) ubiquitination. p21 ubiquitination was performed as above except Cyclin E-Cdk2 was substituted with dead kinase (Cyclin E-Cdk2m). The ubiquitination of p21 was sustained in every case (lanes 2–4) with the exception of adding SCF complex that lacked Skp2. F, Arg306 of Skp2 is required for tighter association between Skp2 and Thr187-phosphorylated p27 peptide. Unphosphorylated and phosphorylated p27 peptides were coupled to CNBr-Sepharose beads. [35S]Labeled Skp2 and Skp2 R306A mutant synthesized in TNT lysates were incubated with 10 μl of beads coupled with either peptides in the presence or absence of 100 μM Cks1. After a 2-h incubation at 4°C, beads were washed four times in the binding buffer by brief centrifugation. Proteins bound to the beads were eluted by boiling in SDS sample buffer prior to SDS-PAGE analysis. 10% of input proteins were loaded on the gel as controls for quantification using phosphorimaging.

SCI$	ext{C}_{58}$$\text{Skp2}^{R306A/K307A}$ abrogates ubiquitination of p27 but has no effect on p21 ubiquitin ligation. A, levels of Skp2 and Skp2 mutants assembled in the various SCF complexes. SCF complexes without Skp2 or with wild type (wt) Skp2, Skp2 R319A/R320A were assembled in insect cells by co-infection of each individual component. SCF complexes were immunoprecipitated with an anti-HA antibody. The amounts of Skp2 or Skp2 mutant in the SCF complexes were determined by immunoblotting with an anti-Skp2 antibody. B, binding of Cks1 to the wild type and mutant SCI$	ext{C}_{58}$$\text{Skp2}$ complexes. 5 μg of SCI$	ext{C}_{58}$$\text{Skp2}$, SCI$	ext{C}_{58}$$\text{Skp2}^{R306A/K307A}$, and SCI$	ext{C}_{58}$$\text{Skp2}^{R319A/R320A}$ complexes were incubated with 5 μl of [35S]Met-labeled Cks1 prepared by in vitro translation and immunoprecipitated with an anti-HA antibody. Bound proteins were boiled off the beads and analyzed by SDS-PAGE and phosphorimaging. C, Arg306 of Skp2 is required for p27 ubiquitination. p27 was radioactively labeled and incubated with phosphorylation/ubiquitination components and with recombinant SCI$	ext{C}_{58}$$\text{Skp2}$ mutant complexes. Ubiquitination of p27 was observed when wild type or Skp2 R319A/R320A was added (lanes 2 and 5). Ubiquitin was not ligated to p27 when Arg306 and Lys307 of Skp2 were changed to alanines (lane 4) or when SCI$	ext{C}_{58}$$\text{Skp2}$ lacked Skp2 (lane 3). The lower panel shows the expressions of Skp2 or Skp2 mutants in the SCF complexes that were purified from Hi-Five insect cells. D, Skp2 mutant at Arg306–Lys307 has no effect on p21 ubiquitination. The p21 ubiquitin (Ub) reactions were carried out in the same manner as ubiquitination of p27, except preincubation of [35S]p21 with Cyclin E-Cdk2 was omitted. Cyclin E-Cdk2 was added directly to the Ub mixture. Wild type Skp2 as well as the Skp2 mutants were able to transfer Ub onto p21 (lanes 2, 5, and 6). The lack of Skp2 abrogated Ub ligation completely (lane 3). The first two high molecular weight bands represent the phosphorylated p21, because p21 in lane 4 was only incubated with Cyclin E-Cdk2 and the energy regeneration system. E, mutation in Skp2 at Arg306–Lys307 has no effect on p21 (phosphorylation independent) ubiquitination. p21 ubiquitination was performed as above except Cyclin E-Cdk2 was substituted with dead kinase (Cyclin E-Cdk2m). The ubiquitination of p21 was sustained in every case (lanes 2–4) with the exception of adding SCI$	ext{C}_{58}$$\text{Skp2}$ complex that lacked Skp2. F, Arg306 of Skp2 is required for tighter association between Skp2 and Thr187-phosphorylated p27 peptide. Unphosphorylated and phosphorylated p27 peptides were coupled to CNBr-Sepharose beads. [35S]Labeled Skp2 and Skp2 R306A mutant synthesized in TNT lysates were incubated with 10 μl of beads coupled with either peptides in the presence or absence of 100 μM Cks1. After a 2-h incubation at 4°C, beads were washed four times in the binding buffer by brief centrifugation. Proteins bound to the beads were eluted by boiling in SDS sample buffer prior to SDS-PAGE analysis. 10% of input proteins were loaded on the gel as controls for quantification using phosphorimaging.

The SCI$	ext{C}_{58}$$\text{Skp2}^{R306A/K307A}$ complex is not only the kinase that is responsible for p27 phosphorylation at Thr187, but also is needed to present the phosphorylated p27 to SCI$	ext{C}_{58}$$\text{Skp2}^{R306A/K307A}$ complexes. Physical contact between p27 and Cyclin E is required for substrate recruitment. Furthermore, we demonstrated that Skp2 might be involved in directly recognizing the phosphorylated p27 as a positively charged amino acid residue, Arg106, in Skp2 is required for binding and ubiquitination of phosphorylated p27 but is dispensable for ubiquitination of unphosphorylated p21. Thus, Cyclin E-Cdk2 stably associates with p27 and brings it to the SCI$	ext{C}_{58}$$\text{Skp2}$ via Cks1. The Thr187 phosphate group in p27 is required for binding and ubiquitination of phosphorylated p27 peptide. Unphosphorylated and phosphorylated p27 peptides were coupled to CNBr-Sepharose beads. [35S]Labeled Skp2 and Skp2 R306A mutant synthesized in TNT lysates were incubated with 10 μl of beads coupled with either peptides in the presence or absence of 100 μM Cks1. After a 2-h incubation at 4°C, beads were washed four times in the binding buffer by brief centrifugation. Proteins bound to the beads were eluted by boiling in SDS sample buffer prior to SDS-PAGE analysis. 10% of input proteins were loaded on the gel as controls for quantification using phosphorimaging.

In this study, we identified that the Cyclin E binding motif in p27 is required for ubiquitination. This implies that the degradation signal for p27 is likely to be binary. The degradation signal created by phosphorylation does not appear to be the primary recognition site for the ubiquitin-conjugation machinery. Instead, association with Cyclin E-Cdk2 is an obligatory step for ubiquitination by SCI$	ext{C}_{58}$$\text{Skp2}$. In addition to phosphorylating p27, the Cyclin E-Cdk2 complex probably holds p27 in a conformation conductive to ubiquitination. Consistent with this notion was the observation that the Cyclin B-Cdk1 complex cannot promote p27 ubiquitination even though p27 can be phosphorylated by this complex but fails to associate with p27 tightly (19). The requirement for the Cyclin E-Cdk2 complex for substrate ubiquitination is not unique to p27 because another SCI$	ext{C}_{58}$$\text{Skp2}$ substrate, p21, showed a similar dependence (23). However, in the case of p21, either the Cdk2 binding or Cyclin E binding motif in p21 is sufficient for targeting the substrate for ubiquitination.2 Recently, studies by Koif and co-workers (29) indicate that Cyclin A-Cdk2 can substitute for Cyclin E-Cdk2 to promote p27 ubiquitination. Under their assay conditions, Cyclin A-Cdk2 appears to be more potent than Cyclin E-Cdk2 at promoting p27 ubiquitination. It was proposed that the interaction between Cyclin A and the N terminus of Skp2 may contribute to the differential stimulatory effects. Such stimulatory effects were negated when the Cyclin- and Cdk2-contact sites in p27 were mutated. Skp2 does not bind Cyclin E-Cdk2 directly in our investigations. Cks1 is responsible for
bridging Cyclin E-Cdk2 and Skp2. Thus, there may be differences in molecular contacts in the p27 ubiquitination complex between Cyclin E-Cdk2 and Cyclin A-Cdk2 that could translate into variations in p27 degradation rates and patterns at different cell cycle stages.

The requirement for direct physical contact between p27 and Cyclin E may have significant implications in tumorigenesis. Excessive proteolysis of p27 is prevalent in aggressive cancer cells. p27 has been shown to be a reliable prognostic marker for breast cancer progression. It is interesting to note that overexpression of Cyclin E is frequently observed in a majority of aggressive tumors as well. Recent data suggest that Cyclin E is a more powerful predictor of breast cancer outcome than proliferation (35, 36). It will be interesting to determine whether overexpression of Cyclin E drives p27 degradation and whether expression of Cyclin E and p27 is anti-correlated in aggressive tumor cells.

SCF<sub>Skp2</sub> is a prototype of a multisubunit E3 ubiquitin ligase. Understanding how SCF recruits substrate proteins for ubiquitination is essential for probing the mechanism of the E3 ubiquitin ligase. Crystallographic analysis of SCF<sub>Skp2</sub> yielded unprecedented insight into the organization and molecular interfaces of the SCF core subunit (17, 18). However, how the SCF<sub>Skp2</sub> complex recruits p27 remains elusive because no crystal structure of the SCF<sub>Skp2</sub> with its authentic substrate is currently available. Two recent crystallographic studies of the F-box protein Cdc4 complexed with a high affinity phosphopeptide from human Cyclin E and another F-box protein α-TrCP1 complexed with the doubly phosphorylated β-catenin destruction motif shed new light on the mechanisms of substrate recruitment by ubiquitin E3 ligases (32, 37). The WD40 repeats present in both F-box proteins are responsible for substrate recognition and there are a number of distinct positively charged amino acid residues involved in binding the phosphate groups in the substrate peptide (31). These studies provide convincing evidence that the phosphorylated short degron motifs are the ubiquitination signal.

Ubiquitination of p27 requires phosphorylation at Thr<sup>187</sup> by the Cyclin E-Cdk2 complex. The LRR repeats in the F-box protein Skp2 could in theory recognize the phosphorylated p27 degron and recruit it to the E3 ubiquitin ligase complex similar to Cdc4 or α-TrCP1. Previous studies with the yeast Grr1, another LRR-containing F-box protein, suggest that a cluster of positively charged amino acid residues on the concave surface of the LRR are likely to be involved in recognition of phosphorylated substrate (33). However, such a cluster of positively charged amino acid residues is absent on the concave surface of Skp2. Previous studies suggest that the Thr<sup>187</sup> phosphate group may be recognized by the anion binding pocket of Cks1 rather than Skp2 (26). Data presented here suggest that Arg<sup>306</sup> of Skp2 is likely to be involved in recognition of this phosphate group as this residue appears to be important for phosphorylation-dependent ubiquitination of p27 but not p21, and mutation of Arg<sup>306</sup> disrupts Cks1-induced Skp2 association with the Thr<sup>187</sup>-phosphorylated p27 peptide. Arg<sup>306</sup> localizes on the same side as the Cks1 binding surface we have previously defined (34) (Fig. 5A) and thus is very likely to be involved in substrate recognition. It is quite possible that both the anion binding site of Cks1 and certain positively charged residues in Skp2 coordinate to bind the Thr<sup>187</sup> phosphate group of p27. Thus, our data suggest that the LRR of Skp2 is probably a Thr(P) binding module (like FHA, WD-40, and Polo Box domain) in the presence of Cks1 (38). Definitive proof of this model requires co-crystallization of phosphorylated p27 with Cks1 and Skp2.

In summary, our data suggest the following model for p27 ubiquitination by SCF<sub>Skp2</sub> (Fig. 5B). Cks1 binds to the unstructured C-terminal region of Skp2 and likely induces a conformation change in Skp2. A trimeric complex consisting of p27-Cyclin E-Cdk2 is recruited to Skp2 via association between Cks1 and Cdk2. In the trimeric complex, p27 directly interacts with Cyclin E. The Thr<sup>187</sup> phosphate group is recognized by Arg<sup>306</sup> of Skp2 and the anion binding site of Cks1. It is likely that these interactions are cooperative resulting in correct positioning of substrate for the subsequent ubiquitination reaction. Other amino acid residues in Skp2 or Cks1 could also be involved in formation of this macromolecular complex. Further experiments are necessary to validate this model.

Acknowledgments—We thank Drs. Harper, Pagano, Sheaff, Amati, and Morgan for the generous supply of the reagents used in this study, Wei Wang for technical help, Mike Tyers and Michael Yaffe and members of the Liu laboratory for helpful discussions, and Dr. Robert Batey for help with the Pymol structure viewer program. We also thank Kristen Barthel for critically reading the manuscript.

REFERENCES
1. Toyoshima, H., and Hunter, T. (1994) Cell 78, 67–74
2. Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massague, J. (1994) Cell 78, 59–66
3. Koff, A., Ohnuki, M., Polyak, K., Roberts, J. M., and Massague, J. (1993) Science 260, 536–539
4. Sherr, C. J., and Roberts, J. M. (1999) Genes Dev. 13, 1501–1512
5. Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G. F., and Rolfe, M. (1995) Science 269, 682–685
6. Slingerland, J., and Pagano, M. (2000) J. Cell. Physiol. 183, 10–17
7. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
8. Pickart, C. M. (2001) Annu. Rev. Biochem. 70, 503–539
