Substrate Recognition and Ubiquitination of SCF^{Skp2/Cks1} Ubiquitin-Protein Isopeptide Ligase*

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p27, an important cell cycle regulator, blocks the G1/S transition in cells by binding and inhibiting Cdk2/cyclin A and Cdk2/cyclin E complexes (Cdk2/E). Ubiquitination and subsequent degradation play a critical role in regulating the levels of p27 during cell cycle progression. Here we provide evidence suggesting that both Cdk2/E and phosphorylation of Thr^{187} on p27 are essential for the recognition of p27 by the SCF^{Skp2/Cks1} complex, the ubiquitin-protein isopeptide ligase (E3). Cdk2/E provides a high affinity binding site, whereas the phosphorylated Thr^{187} provides a low affinity binding site for the Skp2/Cks1 complex. Furthermore, binding of phosphorylated p27/Cdk2/E to the E3 complex showed positive cooperativity. Consistently, p27 is also ubiquitinicated in a similarly cooperative manner. In the absence of p27, Cdk2/E and Cks1 increase Skp2 phosphorylation. This phosphorylation enhances Skp2 auto-ubiquitination, whereas p27 inhibits both phosphorylation and auto-ubiquitination of Skp2.

p27 was first identified as an inhibitor for cyclin A, D, and E-dependent kinases (1, 2). It has now become clear that it positively regulates Cdk4/cyclin D while negatively regulating Cdk2/A^{2} and Cdk2/E (3). At the G1/S transition, phosphorylation of p27 on Thr^{187} leads to its ubiquitination and subsequent degradation, resulting in the activation of Cdk2/E or Cdk2/A and commitment of the cell to the S phase (4). Overexpression of p27 in certain cancer cells prevents DNA replication and tumor formation in nude mice (5), whereas the loss of p27 has been reported in many human cancers (6). Increased degradation of p27 is likely to be one of the mechanisms leading to abnormally low levels of the p27 protein in cancer cells. In several types of cancers, there is a strong correlation between the loss of p27 and induction of Skp2, a subunit of the SCF^{Skp2/Cks1} (Skp1, Cul1, Roc1, Skp2, Cks1 complex) ubiquitin E3 ligase that targets p27 for ubiquitination and degradation (7, 8). Skp2 itself has consistently been shown to be an oncogene, and increased expression of Skp2 has been reported in many human cancers (9, 10).

In the process of protein ubiquitination, the ubiquitin-activating enzyme E1 first forms a thiol ester bond with the C-terminal carboxyl group of Gly^{76} on ubiquitin via an active site cysteine, activating ubiquitin for nucleophilic attack (11). The activated ubiquitin is then transferred to an ubiquitin-conjugating enzyme (E2), forming a thiol ester bond between the C-terminal carboxyl group and an active cysteine on E2. An ubiquitin E3 ligase binds to both the substrate and E2 and facilitates the transfer of the ubiquitin from E2 to the substrate. Ubiquitin is attached to the substrate through an isopeptide bond formed between Gly^{76} of ubiquitin and the e-amino group of a substrate lysine residue. Polyubiquitin chains are formed via isopeptide bonds between the carboxyl group of Gly^{76} on one ubiquitin molecule and the e-amino group of Lys^{48} (or less frequently, Lys^{63}) on another ubiquitin molecule. The substrate specificity of ubiquitination is mainly provided by different E3 ligases. Substrate recognition by E3 is often the rate-limiting step for protein degradation in cells (8).

The SCF^{Skp2/Cks1} E3 ligase recognizes phosphorylated p27 in the p27/Cdk2/E complex and targets it for ubiquitination and degradation at the G1/S transition (12, 13). SCF^{Skp2/Cks1} is composed of five subunits: Cks1, Skp2, Skp1, Cul1, and Roc1. Cul1 serves as a scaffold connecting the catalytic core with the substrate-binding site. It is an elongated protein with a stalk at its N terminus and a globular domain at its C terminus (14, 15). The RING finger protein Roc1 binds the C-terminal globular domain of Cul1, forming a catalytic core that recruits the ubiquitin-conjugating enzyme E2. Skp1 binds to the N terminus of Cul1. Skp2 is connected to Cul1 by the interaction between an F box on Skp2 and an F-box recognition site on Skp1. The ten C-terminal leucine-rich repeats of Skp2 form a curved blade with a concave surface lined with β strands. A 39-amino acid tail C-terminal to the leucine-rich repeats packs into the concave surface and serves as a platform for binding Cks1 (16). The C terminus of Skp2 and Cks1 form the substrate recognition core of the SCF^{Skp2/Cks1} E3 ligase. Cks1 is a small Cdk2-binding protein essential for SCF^{Skp2}-mediated p27 ubiquitination (17, 18). Structural (16, 19) and mutagenesis (20, 21) studies indicate that Cks1 has a Cdk2-binding site, a Skp2-binding site, and a phosphate-binding site and that all three sites are required for...
p27 ubiquitination. Although the phosphate-binding site on Cks1 binds directly to phosphorylated Thr\textsuperscript{187} on p27, the Skp2/Cks1 interface provides another binding site for Glu\textsuperscript{185} on p27. Skp2 greatly enhances the affinity between Cks1 and a C-terminal p27 phospho-peptide (21), indicating that interaction of Glu\textsuperscript{185} with Skp2/Cks1 is also essential for substrate recognition by the SCF\textsuperscript{Skp2/Cks1} ubiquitin ligase.

In addition to phosphorylation on Thr\textsuperscript{187}, p27 must be in complex with Cdk2/E to be efficiently ubiquitinated by SCF\textsuperscript{Skp2/Cks1} (22). It has been shown that Cdk2/E or Cdk2/A plays a noncatalytic role in the ubiquitination of p27 by SCF\textsuperscript{Skp2/Cks1} (23, 24). Using an in vitro reconstituted p27 ubiquitination system with purified protein components, we found that a phospho-p27 peptide containing phosphorylated Thr\textsuperscript{187} can block p27 ubiquitination; however, the affinity of the phospho-p27 peptide is 1000-fold less than that of the phosphorylated p27 in complex with Cdk2/E. We developed an HTR-FRET assay to quantitatively measure the interaction between phosphorylated p27/Cdk2/E and the SCF\textsuperscript{Skp2/Cks1} complex. We found that phosphorylated p27/Cdk2/E binds to the Skp2/Cks1 complex in a cooperative manner with a K\textsubscript{D} of 44 nM. Both the phospho-p27 peptide and Cdk2/E can compete for Skp2/Cks1 binding to phosphorylated p27/Cdk2/E with different affinities. We also found that Cdk2/E phosphorylates Skp2 in a Cks1-dependent manner in vitro. This phosphorylation increases auto-ubiquitination of p27, which is inhibited by p27.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit anti-phospho-p27 (71–7700) was purchased from Zymed Laboratories Inc. (South San Francisco, CA). Anti-phospho-Kip1 (Thr\textsuperscript{187}) was purchased from Upstate Biotechnology, Inc. (catalog number 06-996). These antibodies recognize p27 phosphorylated on Thr\textsuperscript{187}. Mouse anti-p27\textsuperscript{Kip1} (554069) and mouse anti-Cdk2 (C18520) were purchased from BD PharMingen (San Diego, CA). Goat anti-human Skp2 antibody (N19), mouse anti-Cyclin E (SC-248), and horseradish peroxidase-labeled secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA). Goat anti-GST (catalog number 27-4577-01) and Cy5-labeled anti-GST (PA92002) were purchased from Amersham Biosciences (Piscataway, NJ). LANCE\textsuperscript{TM} Eu-labeled anti-Myc (AD0114) and anti-GST (AD0253) were purchased from PerkinElmer Life Sciences. XI665-labeled anti-FLAG antibody was purchased from Cis-US, Inc. (Bedford, MA). IRDye-labeled secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE). Ubiquitin was from Sigma. EZ-link\textsuperscript{TM} Sulfo-NHS-LC-Biotin was from Pierce. 2× SDS loading buffer for protein PAGE was purchased from Invitrogen. The phosphorylated and unphosphorylated forms of a p27 C-terminal peptide surrounding underlined Thr\textsuperscript{187} (Bio-6-aminohexanoic acid-AGGVEQTTPKPGGLRRQTSOH2) were synthesized by SynPep (Dublin, CA). GST-ubiquitin was purchased from Boston Biochem (Boston, MA). EZView red anti-FLAG M2 affinity beads were purchased from Sigma.

**Preparation of Protein Components**—His-E1 and His-UbcH3 were expressed in *Escherichia coli* and purified on Ni\textsuperscript{2+} chelate resin. The SCF\textsuperscript{Skp2} complex was affinity purified via glutathione agarose chromatography from Sf9 cells co-infected with baculoviruses expressing GST-Skp2, His-Skp1, His-Cul1, and Roc1. The baculoviruses for GST-Skp2, His-Skp1, his-Cul1, and Roc1 were kindly provided by Dr. Michele Pagano (New York University School of Medicine). p27/Cdk2/E, FLAG-p27/Cdk2/E, FLAG-p27 T187A/Cdk2/E, and Cdk2/E complexes were purified via Ni\textsuperscript{2+} chelate chromatography from Sf9 cells co-infected with baculoviruses expressing human p27 (with or without a N-terminal FLAG tag, YKDDDDK), Cdk2, and His-tagged cyclin E. In FLAG-p27 T187A, Thr\textsuperscript{187} was mutated to alanine through site-directed mutagenesis. Human Cks1 was expressed in *E. coli* with an S tag (KETA-AAKFERQHMD5) at the N terminus and a Myc tag (EQKLI-SEEDL) at the C terminus and purified via S rabbit affinity chromatography (Novagen). Subsequently, the S tag was removed from Cks1 by cleavage with thrombin. All of the above proteins were dialyzed into Ub dialysis buffer (30 mM Tris-HCl, pH 7.5, 20% glycerol, 1 mM DTT) and stored in small aliquots at −80 °C. Ubiquitin was labeled with biotin by incubating 100 mg of Ub (Sigma U6253) with 12.5 mg of EZ-link\textsuperscript{TM} Sulfo-NHS-LC-Biotin in phosphate-buffered saline on ice for 2 h. The average stoichiometry of labeling was determined by liquid chromatography/mass spectrometry to be 1–2 biotin group/ubiquitin molecule. The biotinylated ubiquitin was dialyzed three times against 4 liters of 10 mM Hepes, pH 8.0 for 2–4 h at 4 °C and stored in small aliquots at −80 °C.

**Mass Spectrometry Analysis of Phosphorylated p27/Cdk2/E—**Phosphorylated p27/Cdk2/E (phospho-p27) was prepared by incubating 0.1 mg/ml Cdk2/E (~1.25 μM) with 0.1 mg/ml (~1 mM) p27/Cdk2/E or FLAG-p27/Cdk2/E at room temperature for 2 h in kinase buffer (40 mM Tris-HCl, pH 7.5, 10 mM MgCl\textsubscript{2}, 1 mM DTT, 1 mM ATP). Aliquots were stored at −80 °C. For liquid chromatography/mass spectrometry analysis, p27/Cdk2/E or phosphorylated p27/Cdk2/E were precipitated by chloroform/methanol to remove detergent. Precipitated p27 was resuspended in 5% formic acid and 5% acetonitrile and injected onto a ZQ liquid chromatography/mass spectrometry system. To determine phosphorylation sites, p27 from different samples were subjected to SDS-PAGE. The gel was stained with SimplyBlue SafeStain (Invitrogen). p27 proteins were digested in-gel with trypsin as described (25). Tryptic digests were analyzed on an MS\textsuperscript{4} liquid chromatography system interfaced to an LTQ linear ion trap mass spectrometer (Thermo Electron) equipped with a nanotrap source (Michrom Bioresources). Tryptic peptides were first captured and desalted on a C18 trapping cartridge followed by separation on a MAGIC C18 0.1×150 mm nanotrap column. The peptides were eluted with a 120-min gradient from 5 to 65% acetonitrile in 0.1% formic acid and 0.005% heptafluorobutyric acid. During acquisition, continuous data dependent MS\textsuperscript{3} scanning was performed via the following steps: 1) survey MS scan of peptide ions, 2) MS/MS peptide fragmentation scan of the top five most intense ions, and 3) an MS\textsuperscript{3} scan (MS/MS/MS) of peptides triggered by neutral loss of 49 or 33 m/z values from the peptides selected for fragmentation in step 2. Raw MS files were processed with MASCOT Distiller then submitted to an internal MASCOT server (version 2.1) for data base searching using the Human IPI data base (June 1, 2006). Cyclin-dependent kinase inhibitor 1B (IP10006991) was the top protein identified. Relative phosphorylation ratios were calculated as percentages by determining...
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the ion current intensities from extracted ion chromatograms for the ions corresponding to phosphorylated and nonphosphorylated peptides.

\textbf{In Vitro Phosphorylation of Skp2—}The SCF\textsuperscript{Skp2} complex (1 \mu M) was incubated with Cdk2/E (1 \mu M), Cks1 (1 \mu M), and ATP (1 mm) in the kinase buffer described above at 30 °C for 2 h. In some experiments, 20 \mu M staurosporine was added to the reaction to inhibit the Cdk2/E activity.

\textbf{In Vitro Ubiquitination Assay—}Unless specifically indicated, p27 was ubiquitinated in a 15-\mu l reaction by incubating phosphorylated p27 (40 nM) with E1 (40 nM), E2 (5 \mu M), SCF\textsuperscript{Skp2/Cks1} (25 nM), Cks1 (25 nM), and Bio-Ub (27.8 \mu M) in Ub buffer (40 mM Tris-HCl, pH 7.5, 5 mM MgCl\textsubscript{2}) containing 1 mM DTT and 0.5 mM ATP at room temperature for 1 h. For peptide competition experiments, p27 ubiquitination was performed in the presence of phosphorylated or nonphosphorylated p27 peptide at various concentrations. For analysis of the ubiquitination of p27 by SDS-PAGE, the assay mixture was mixed with an equal volume of 2× SDS loading buffer containing 200 mM DTT. Following SDS-PAGE, the proteins in the gel were transferred to a polyvinyldene difluoride membrane and probed with anti-p27 or anti-Skp2 antibody. The signal on the membrane was detected and quantified by a densitometer or an Odyssey infrared imaging system from LI-COR Biosciences (Lincoln, NE).

\textbf{Measurement of Interaction between FLAG-p27 and GST-Skp2—}For interaction of FLAG-tagged p27 with Skp2 or Cks1, 20 nM of phosphorylated FLAG-p27/Cdk2/E, FLAG-p27/Cdk2/E, FLAG-p27 T187A/Cdk2/E, Cdk2/E, or buffer control was incubated with 20 nM of SCFSkp2 complex and 20 nM of Cks1 in the presence of 1 nM LANCE Eu Anti-Skp2 and 100 nM XL665 anti-FLAG. For competition experiments, 20 nM of phosphorylated FLAG-p27/Cdk2/E was incubated at room temperature for 2–4 h with 20 nM of SCFSkp2 complex and 20 nM of Cks1 in the presence of increasing amounts of phosphorylated p27/Cdk2/E, phospho- or nonphospho-p27 peptide, or Cdk2/E and 1 nM LANCE Eu anti-Skp2 and 40 nM XL665 anti-FLAG. The interaction between FLAG-p27 and GST-Skp2 was measured as above.

\textbf{Anti-FLAG Immunoprecipitation—}Phosphorylated FLAG-p27/Cdk2/E and unphosphorylated FLAG-p27/Cdk2/E (50 ng/\mu l, 100 \mu l) were incubated either on ice or at 78 °C for 20 min (heat inactivation). The heat inactivation samples were then centrifuged at 15,000 for 5 min at room temperature in a tabletop microcentrifuge. 90 \mu l of the supernatant for each sample was incubated with 20 \mu l of EZView anti-FLAG M2 affinity agarose for 2 h at 4 °C. The agarose beads were washed six times with phosphate-buffered saline containing 0.1% Tween 20 and subjected to SDS-PAGE and Western blotting with the indicated antibodies.

\textbf{RESULTS}

\textbf{Reconstitution of the Ubiquitination of p27 in Vitro—}An \textit{in vitro} p27 ubiquitination assay was first reported by Pagano \textit{et al.} (26) using cell extracts as a source of enzymes essential for p27 ubiquitination. Since then, all of the essential components for efficient \textit{in vitro} p27 ubiquitination have been identified (17, 18). All of these were recombinantly expressed and purified (Fig. 1A and Table 1). To generate a robust \textit{in vitro} p27 ubiquitination assay, p27 in complex with Cdk2/E (p27/Cdk2/E) purified from Sf9 cells was used as the substrate. We found that this complex was weakly ubiquitinated (Fig. 1B, lane 2) \textit{in vitro} even in the presence of Cks1. However, when extra Cdk2/E

\begin{table}[h]
\centering
\caption{Essential protein components of \textit{in vitro} p27 ubiquitination assay}
\begin{tabular}{|c|c|c|}
\hline
\textbf{Reagent name} & \textbf{Components} & \textbf{Expression system} & \textbf{Purification} \\
\hline
E1 & Hi他的 E1 & \textit{E. coli} or insect cells & Ni\textsuperscript{2+}-NTA column* \\
E2 & His-UbcH3 & \textit{E. coli} & Ni\textsuperscript{2+}-NTA column \\
SCF\textsuperscript{Skp2} complex & His-Skp1/His-Cul1/GST-Skp2/Roc1 & \textit{Insect cells} & \textit{GSH} column \\
p27/Cdk2/E & P27/Cdk2/His-Cyc E & \textit{Insect cells} & Ni\textsuperscript{2+}-NTA column \\
Cdk2/E & Cdk2/His-Cyc E & \textit{Insect cells} & Ni\textsuperscript{2+}-NTA column \\
Cks1 & S-Cks1-Myc & \textit{E. coli} & S column \\
Bio-Ub & Biotinylated ubiquitin & & \\
\hline
\end{tabular}
\footnotesize{*NTA, nitrilotriacetic acid.}
\end{table}

\textbf{FIGURE 1.} A, a schematic representation of the p27 ubiquitination complex. B, Cdk2/E significantly increases the ubiquitination of p27/Cdk2/E. GST-Ub was used in the assay. C, Cks1 is essential for p27 ubiquitination \textit{in vitro}. p27/Cdk2/E was ubiquitinated with or without Cdk2/E or Cks1 under standard conditions described under "Experimental Procedures." p27 was detected by Western blot with anti-p27 antibody (BD PharMingen).
Cdk2/E increased phosphorylation on Thr187 of p27 in a dose-dependent manner. Phosphorylated substrate was prepared for routine ubiquitination and binding assays by incubating p27/Cdk2/E (100 ng/μl) with additional Cdk2/E (100 ng/μl) in the presence of ATP and Mg2+. Mass spectrometry was used to further quantify p27 phosphorylation. Analysis of intact p27 protein as well as a peptide digestion of p27 in both the unphosphorylated and phosphorylated forms indicated that only a small portion of p27 in p27/Cdk2/E purified from insect cells was singly phosphorylated, whereas the majority of p27 was unphosphorylated. In contrast, p27 in the phosphorylated p27/Cdk2/E complex was either singly or doubly phosphorylated with the single phosphorylation species being the dominant form (data not shown). Data from peptide mapping indicated that Thr187 was ~90% phosphorylated in phosphorylated p27/Cdk2/E, whereas the corresponding peptides recovered from the digestion of unphosphorylated p27 were not phosphorylated. An additional phosphorylation site was identified at Ser106 in the phosphorylated p27 digestion, although it does not appear to be a consensus site for Cdk2 (27). Therefore, the p27 in the complex of p27/Cdk2/E purified from S9 cells was unphosphorylated; upon in vitro Cdk2/E phosphorylation under our experimental conditions, it became almost completely phosphorylated on Thr187.

Phosphorylation on Thr187 Is Essential but Not Sufficient for High Affinity Binding between p27/Cdk2/E and SCF^Skp2/Cks1—It has been shown that phosphorylation on Thr187 of p27 is essential for p27 ubiquitination (22). Previous studies have also revealed the importance of Cdk2/E or Cdk2/A for p27 ubiquitination (22–24). However, none of these studies had directly compared the impact of Thr(P)187 and Cdk2/E or Cdk2/A on the substrate recognition by the SCF^Skp2/Cks1 E3 ligase in a quantitative manner.

An HTR-FRET assay using FLAG-tagged p27 and GST-tagged Skp2 to measure the interaction between SCF^Skp2/Cks1 E3 ligase and phosphorylated p27/Cdk2/E was developed (Fig. 2). Phosphorylation of Thr187 on p27 by Cdk2/E. p27/Cdk2/E (100 ng/μl) was incubated with increasing amount of Cdk2/E at room temperature for 1 h. Total p27 and p27 phosphorylated on Thr187 were detected by Western blotting using anti-p27 antibody (BD Pharmingen) and anti-phospho-Kip (Thr187) (Upstate Biotechnology, Inc.) that specifically recognize phosphorylated Thr187.

It has been shown that p27 is heat stable, but the activity of Cdk2/E is heat-sensitive (1, 23). Phosphorylated FLAG-p27/Cdk2/E was incubated at 75 °C to heat-inactivate Cdk2/E. As shown in Fig. 3B, phosphorylated FLAG-p27/Cdk2/E interacted with Skp2 in the presence of Cks1 and produced a FRET signal; however, heat inactivation of phosphorylated FLAG-p27/Cdk2/E destroyed this interaction. The addition of fresh Cdk2/E to the heat-inactivated phosphorylated FLAG-p27 partially recovered the interaction. Although the levels of total p27, p27 phosphorylated at Thr187, Cyc E, and Cdk2 remained the same in samples following heat inactivation, Cdk2 and Cyc E no longer formed complex with FLAG-p27 after heat inactivation (Fig. 3, B and C). Thus, (i) phosphorylation on Thr187 is essential but not sufficient for the substrate binding of the SCF^Skp2/Cks1 E3 ligase and (ii) Cdk2/E in the p27/Cdk2/E complex provides the second binding site for the SCF^Skp2/Cks1 complex.

Cooperative Binding of Phosphorylated p27/Cdk2/E and SCF^Skp2/Cks1—To determine the dissociation constant (K_d) between phosphorylated p27/Cdk2/E and the SCF^Skp2/Cks1 complex, phosphorylated FLAG-p27/Cdk2/E was incubated with SCF^Skp2 and Cks1 at different concentrations. The data fit well to a sigmoidal binding curve (Fig. 4) but not to a hyperbolic binding curve (data not shown). The sigmoidal binding curve is a signature for positive cooperativity between two binding sites (28). This indicates that binding of one p27/Cdk2/E to the E3 ligase enhances the binding of another p27/Cdk2/E to the E3 ligase. There was no previous evidence suggesting that each Skp2/Cks1 complex binds to two p27/Cdk2/E complexes. Moreover, the structure of the Skp1-Skp2-Cks1-p27 peptide complex indicates that, although there are multiple sites contributing to the interaction between each p27/Cdk2/E complex...
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FIGURE 3. Phosphorylation on Thr\textsuperscript{187} is essential but not sufficient for the interaction of p27/Cdk2/E with the SCF\textsuperscript{Skp2/Cks1} complex. A, an HTR-FRET assay is used to detect interactions between FLAG-p27, GST-Skp2, and Cks1-Myc. FLAG-p27/Cdk2/E or FLAG-p27 T187A/Cdk2/E were preincubated with Cdk2/E and ATP in kinase buffer as described under "Experimental Procedures" (phosphorylated FLAG-p27/Cdk2/E and FLAG-p27 T187A/Cdk2/E, respectively). As controls, FLAG-p27/Cdk2/E or buffer alone were incubated with Cdk2/E under the same conditions in the absence of ATP (FLAG-p27/Cdk2/E and FLAG-p27 T187A/Cdk2/E, respectively). 20 nM of phosphorylated FLAG-p27/Cdk2/E, FLAG-p27 T187A/Cdk2/E, Cdk2/E, or buffer alone (−) were incubated with 20 nM of the SCF\textsuperscript{Skp2/Cks1} complex and 20 nM of Cks1 in the presence of 1 nM of LANCE Eu anti-GST and 20 nM of XL665 anti-FLAG (p27/Skp2 interaction), 1 nM LANCE Eu anti-Myc and 20 nM of XL665 anti-FLAG (p27/Cks1 interaction), or 1 nM LANCE Eu anti-Myc and 20 nM Cy5 anti-GST (Skp2/Cks1 interaction) at room temperature for 2–4 h. The interactions between p27 and Skp2, p27 and Cks1, and Skp2 and Cks1 were measured as a FRET signal (10,000 × fluorescence @ 665 nm/Fluorescence @ 620 nm) generated between Eu anti-Myc and XL665 anti-FLAG, Eu anti-GST and XL665 anti-FLAG, and Eu anti-Myc and Cy5 anti-GST. B, heat inactivation of phosphorylated FLAG-p27/Cdk2/E abolishes p27/Skp2 interaction. Left panel, phosphorylated FLAG-p27/Cdk2/E was preincubated either on ice (without heat inactivation) or at 75 °C (heat inactivation) for 20 min. 20 nM of pretreated phosphorylated FLAG-p27 was incubated with 20 nM of the SCF\textsuperscript{Skp2/Cks1} complex and 20 nM of Cks1 in the presence of 1 nM of LANCE Eu anti-GST and 20 nM of XL665 anti-FLAG in the presence or absence of 20 nM Cdk2/E at room temperature for 2–4 h. Right panel, the levels of total p27 p27 with phosphorylated Thr\textsuperscript{187}, Cdk2, and Cyc E in different samples were detected by Western blot with mouse anti-p27 (BD PharMingen), rabbit anti-phospho-p27 (Zymed Laboratories Inc.), mouse anti-Cdk2 (BD PharMingen), and mouse anti-Cyc E and each SCF\textsuperscript{Skp2/Cks1} complex, there is only one binding site for p27/Cdk2/E per Skp2/Cks1 complex. One explanation for the cooperativity is that the SCF\textsuperscript{Skp2/Cks1} complex forms a dimer and binding of phosphorylated p27/Cdk2/E to one E3 in the dimer increases the affinity of the other E3 for its substrate. The apparent \( K_m \) for phosphorylated p27/Cdk2/E with the SCF\textsuperscript{Skp2/Cks1} complex is ~40 nm. Consistent with previous observations, unphosphorylated p27 and the T187A mutant in complex with Cdk2/E have much lower affinities to SCF\textsuperscript{Skp2/Cks1}.

To determine the apparent \( K_m \) for substrate in the p27 ubiquitination assay (Fig. 4B), increasing amounts of phosphorylated p27/Cdk2/E were incubated with E1, E2, SCF\textsuperscript{Skp2/Cks1}, Cks1, and Bio-Ub as described under "Experimental Procedures." Ubiquitination of p27 was detected as a ladder of p27 with increasing molecular weights in the gel via Western blot using an anti-p27 antibody. The ubiquitinated p27 was quantified with a densitometer and plotted against the total p27 concentration (Fig. 4B, right panel). The concentration dependence of total p27 ubiquitination also displayed a sigmoidal curve, suggesting that substrate binding is the rate-limiting step in this p27 ubiquitination assay. The apparent \( K_m \) for phosphorylated p27/Cdk2/E was ~20 nm.

Cdk2/E Provides a Higher Affinity Binding Site than Thr(P)\textsuperscript{187}—Because both Cdk2/E and phosphorylation of Thr\textsuperscript{187} on p27 are essential for the substrate binding of the SCF\textsuperscript{Skp2/Cks1} complex, the binding affinity of each of the two sites was assessed using a competition assay. Increasing amounts of Cdk2/E, the C-terminal peptide of p27 with or without phosphorylation on Thr\textsuperscript{187}, phosphorylated p27/Cdk2/E, or p27/Cdk2/E were used to compete with the binding of phosphorylated FLAG-p27/Cdk2/E to the SCF\textsuperscript{Skp2/Cks1} complex (Fig. 5A). Phosphorylated p27/Cdk2/E was the most potent competitor with an IC\textsubscript{50} of 0.11 μM (Table 2). Cdk2/E and p27/Cdk2/E were less potent inhibitors than phosphorylated p27/Cdk2/E, with IC\textsubscript{50} values of 1.2 and 4.2 μM, respectively. Although the unphosphorylated C-terminal peptide of p27 did not inhibit the binding between FLAG-p27 and Skp2, the phosphorylated peptide showed weak inhibition with an IC\textsubscript{50} of 80 μM. These data indicate that Cdk2/E contributes the major binding strength, whereas the C-terminal phospho-p27 peptide alone has very weak binding to Skp2/Cks1. However, Thr(P)\textsuperscript{187} increases the binding affinity between p27/Cdk2/E and Skp2/Cks1 significantly.

The phospho-p27 peptide could block the ubiquitination of the phosphorylated p27/Cdk2/E (40 nm) in the ubiquitination assay with an IC\textsubscript{50} of 43 μM (Fig. 5B). Thus, although the phospho-p27 peptide could inhibit p27 ubiquitination, the affinity of the phospho-peptide to the SCF\textsuperscript{Skp2/Cks1} E3 ligase was 1000-fold less than that of the phosphorylated p27/Cdk2/E, supporting the hypothesis that Thr(P)\textsuperscript{187} is a weak binding element for the substrate recognition site of SCF\textsuperscript{Skp2/Cks1} E3 ligase.
Auto-ubiquitination of Skp2 Requires Cks1 and Cdk2/E but Is Inhibited by p27—Skp2 is weakly ubiquitinated in the presence of E1, E2, and SCFSkp2/Cks1 (Fig. 6A, lane 5). The presence of Cks1 or Cdk2/E alone did not significantly enhance its ubiquitination (Fig. 6A, lanes 6 and 7); however, the presence of both Cks1 and Cdk2/E significantly increased Skp2 ubiquitination (Fig. 6A, lane 2, and data not shown). p27 has no effect on Skp2 ubiquitination in the absence of Cks1 (Fig. 6A, lane 4); however, it significantly decreases Skp2 ubiquitination in the presence of Cks1, whereas p27 is strongly ubiquitinated (Fig. 6B, lane 1). These data suggest that p27 competes better for the ubiquitination machinery than does Skp2.

Cdk2/E Phosphorylates Skp2 in a Cks1-dependent Manner In Vitro—GST-Skp2 in the SCFSkp2 complex purified from S9 cells was detected as a single band after SDS-PAGE using either anti-GST (Fig. 7A, lane 8) or anti-Skp2 (data not shown). When it was incubated with Cdk2/E, Cks1, ATP, and MgCl₂, GST-Skp2 migrated as two bands (Fig. 7A, lane 2). One band migrated at approximately a similar rate to untreated GST-Skp2, whereas the other band migrated at a slower rate. This band shift of GST-Skp2 could be caused by Cdk2/E-mediated phosphorylation because it not only depends on the presence of ATP and MgCl₂ but also can be blocked by staurosporine, a kinase inhibitor (Fig. 7B). Mass spectrometry analysis of the GST-Skp2 bands confirms that the slowly migrating band contains one extra phosphate group at the N terminus of Skp2 (data not shown). Cks1 is required for the phosphorylation of Skp2 by Cdk2/E. This phosphorylation had a positive effect on Skp2 auto-ubiquitination because staurosporine, which blocked the Cks1-dependent phosphorylation of Skp2, also inhibited the Skp2 auto-ubiquitination (Fig. 8A, lanes 1 and 2). The presence of p27/Cdk2/E also inhibited both Skp2 phosphorylation and ubiquitination (Fig. 8A, lanes 5 and 6).

**DISCUSSION**

Cdk2/E Promotes p27 Binding to the SCFSkp2/Cks1 E3 Ligase Complex—Recognition of phosphorylated p27 by SCFSkp2/Cks1 E3 ligase is one critical step in p27 ubiquitination and therefore has been the focus of many studies. Previous studies have shown that Cdk2/E or Cdk2/A plays a noncatalytic role in p27 ubiquitination in addition to phosphorylating p27 at Thr₁⁸⁷. However, the relative role of Thr(P)₁⁸⁷ and Cdk2/E in the binding of phosphorylated p27/Cdk2/E to the SCFSkp2/Cks1 complex is not well understood.

In this study, we measured an apparent $K_d$ for the binding between phosphorylated p27/Cdk2/E and the SCFSkp2/Cks1 complex to be ~40 nM and an apparent $K_m$ for *in vitro* ubiquiti-
nation of phosphorylated p27/Cdk2/E to be ~20 nM. Using a quantitative HTR-FRET assay, we showed that phosphorylation of Thr(P)187 is essential but not sufficient for this high affinity association and confirmed that Cdk2/E provides another binding site. We further evaluated the relative contribution of Cdk2/E and Thr(P)187 to the substrate binding of the E3 complex in a competitive binding assay. Phosphorylated and unphosphorylated p27/Cdk2/E inhibit the binding between FLAG-p27 and Skp2/Cks1 with IC50 values of 0.11 and 4.2 μM, respectively, indicating that Thr(P)187 significantly increases the binding affinity. Cdk2/E and the phospho-p27 peptide inhibit the binding of the phosphorylated p27/Cdk2/E to the SCFSkp2/Cks1 complex with IC50 values of 1.2 and 80 μM, respectively. Therefore, Cdk2/E provides a stronger binding site, whereas the phospho-p27 provides a weaker binding site for the SCFSkp2/Cks1 complex. Consistent with this, the phospho-p27 peptide inhibits the ubiquitination of phosphorylated p27/Cdk2/E (40 nM) by the SCFSkp2/Cks1 E3 complex with an IC50 of 40 μM. The recent crystal structure of the Skp1-Skp2-Cks1-

| Competitor               | IC50 (μM) | Hill slope |
|-------------------------|----------|-----------|
| Phospho-p27 peptide     | 78.00 ± 7.07 | −0.75 ± 0.25 |
| Unphospho-p27 peptide   | >1500    | NAa       |
| Phosphorylated p27/Cdk2/E| 0.11 ± 0.05 | −1.20 ± 0.3  |
| p27/Cdk2/E              | 4.21 ± 3.70 | −0.68 ± 0.23 |
| Cdk2/E                  | 1.17 ± 0.72 | −0.75 ± 0.05 |

|       | A               | B               |
|-------|-----------------|-----------------|
|       | Phospho-p27     | Phospho-p27     |
|       | K2/E            | Cdk2/Cyc E      |
|       | Cdk2/Cyc E     | p27/Cdk2/E      |
|       | GST-Skp2/Skp1  | Cut1/Roc1       |
|       | +               | +               |
|       | +               | +               |
|       | +               | +               |
|       | +               | +               |
|       | +               | +               |
|       | +               | +               |
|       | +               | +               |
|       | +               | +               |
|       | +               | +               |

FIGURE 6. Ubiquitination of Skp2 is positively regulated by Cdk2 and Cks1 and negatively regulated by p27. The SCFSkp2 complex was incubated with the indicated components in the presence of E2 and Bio-Ub under the standard ubiquitination conditions. The ubiquitination of Skp2 (A) and p27 (B) were detected by Western blot using anti-Skp2 and anti-p27 antibodies, respectively.
phospho-Thr^{187}, which yields the high affinity complex required for efficient ubiquitination by the catalytic machinery of the SCFSkp2/Cks1 ligase. Therefore, we propose a mechanism whereby there is an anchoring complex formed between the Cdk2/E portion of the p27/Cdk2/E complex followed by binding the phospho-Thr^{187} residue for final positioning at the ubiquitination site (Fig. 9A). Via the above mechanism, unphosphorylated p27/Cdk2/E cannot be efficiently ubiquitinated, although it binds to the E3 ligase. Finally, we did observe the interaction between unphosphorylated p27/Cdk2/E and the SCFSkp2/Cks1 E3 ligase when binding studies were performed under low stringency conditions (data not shown).

How does Cdk2/E promote p27 binding to its E3 ligase? Our data, together with data from many other groups (16, 20, 21, 23, 24), provide sufficient evidence to indicate that Cdk2/E promotes p27 binding to its E3 via bridging the N terminus of p27 to Cks1 (Fig. 9A). This model is based upon the following evidence: (i) Cks1 binds to Skp2, Cdk2, and Thr(P)^{187} on p27 via three distinct sites, and all three binding sites are essential for SCFSkp2/Cks1-mediated p27 ubiquitination (16, 20, 21). (ii) An E63Q mutation of Cks1 destroys the Cks1 and Cdk2 interaction (19). This mutation has a minimal effect on the binding of Cks1/Skp2 to the C-terminal phospho-p27 peptide (21) but greatly reduces the binding of Cks1/Skp2 to phosphorylated full-length p27 (20), suggesting that the Cdk2/Cks1 interaction is critical for the binding of full-length p27 to E3 but not for the C-terminal phospho-p27 peptide. (iii) Interaction of the N terminus of p27 with Cdk2/E or Cdk2/A appears to be required for p27 ubiquitination (23, 24). (iv) Although the Cdk2/E or Cdk2/A complexes are essential for p27 ubiquitination, neither Cyc E nor Cdk2 alone is sufficient to promote the ubiquitination of p27 (23). (v) Structures of the Cdk2-Cyc E complex, Cdk2-Cyc A complex, and Cdk2-Cks1 complex indicate that Cyc E/A and Cks1 bind to distinct sites on Cdk2 and that Cdk2 provides the link between Cks1 and Cyc E/A (23, 30, 31). (vi) Cdk2/E provides a stronger binding site for substrate recognition than the C-terminal binding motif of p27 containing Glu^{185} and Thr(P)^{187}. Therefore, Cks1 recruits p27 to its E3 ligase by securing both the C terminus and N terminus of p27. Cks1 binds Thr(P)^{187} and Glu^{185} through its anion-binding site and a Skp2/Cks1 interface. It also brings the N terminus of p27 to the E3 ligase through the interactions between Cks1 and Cdk2, Cdk2 and Cyc E, and Cyc E and the N terminus of p27.

Thus, our work, as well as that of others, has demonstrated that substrate recognition of the SCFSkp2/Cks1 E3 ligase is an organized process in which simultaneous and cooperative interaction among multiple subunits are required for the highest affinity between the substrate and the E3 ligase.

**The SCFSkp2/Cks1 E3 Ligase Forms a Dimer**—The binding kinetics for phosphorylated p27/Cdk2/E to the SCFSkp2/Cks1 complex were found to be sigmoidal. This is recapitulated in the p27 ubiquitination assay. The sigmoidal binding curves indicate that the interaction of one site facilitates the binding at another site. Because there is one substrate-binding site per SCFSkp2/Cks1 complex, our data suggest that the SCFSkp2/Cks1 complex forms a dimer and that binding of one substrate to the E3 enhances the binding of the second substrate to another E3 in the dimer.
Based upon the crystal structures of SCF complexes (Skp1, Cul1, and F-box protein complexes), it was proposed that SCF facilitates the ubiquitination of its substrate through positioning of the substrate lysine(s) in proximity to the E2 active site and therefore increasing the effective concentration of the substrate at the E2 active site (32). However, the crystal structures have illustrated that there is a 32–50 Å gap between the substrate lysine and the active site on E2, which is a long distance for the E2 to span to transfer its charged ubiquitin to a substrate bound to Skp2/Cks1. If the SCFSkp2/Cks1 E3 ligase forms a dimer with Skp2/Cks1 homodimer with /H9252

\[ \text{E}_{12}^\text{dimer} \] bound to Skp2/Cks1. If the SCFSkp2/Cks1 E3 ligase forms a dimer, the catalytic core on one E3 complex may be brought into close proximity to the substrate bound on another E3 complex. Therefore, the ubiquitination process could be an intermolecular transfer of ubiquitin from one ligase complex to another dimer in the complex, rather than an intramolecular transfer within one ligase complex (Fig. 9B). In fact, another F box-containing protein, β-Trcp1, forms a homodimer or a heterodimer with β-Trcp2 (33). However, we have observed hyperbolic instead of sigmoidal kinetics for λSkp ubiquitination in vitro, suggesting that there is no cooperativity between the two substrate-binding sites in the SCFβ-Trcp1 E3 dimer (34).

**Ubiquitination and Phosphorylation of Skp2 Is Inhibited by p27**—Skp2 is phosphorylated and auto-ubiquitinated in vitro. The Skp2 phosphorylation is mediated by Cdk2/E and is dependent on Cks1. This phosphorylation is required for Skp2 auto-ubiquitination. p27, on the other hand, decreases both Skp2 phosphorylation and ubiquitination. The physiological significance of Skp2 phosphorylation and ubiquitination is not known yet. The protein level of Skp2 is regulated by ubiquitination during the cell cycle. In late mitosis and the G1 phase, Skp2 is maintained at a low level through APC/Cdh1-mediated ubiquitination (35). More cellular studies need to be done to find out whether Skp2 auto-ubiquitination plays a role in the down-regulation of the Skp2 protein level at the end of S phase before APC/Cdh1 is activated.

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