Molecular and Biochemical Characterization of the Skp2-Cks1 Binding Interface

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SCF^{Skp2} is a multisubunit E3 ubiquitin ligase responsible for ubiquitination of cell cycle inhibitor p27. Ubiquitination of p27 requires an adapter protein, Cks1, to be in direct association with Skp2. The exact interface between Skp2 and Cks1 has not been elucidated. Here we have reported the definition of the critical functional interface between Skp2 and Cks1. We have identified eight amino acid residues in two discrete regions of Skp2 that are engaged in Cks1 binding. Mutation of any of these eight residues alone or in combination results in the loss of Cks1 association and negates Skp2-dependent p27 ubiquitination. These eight amino acid residues map on the same side of the Skp2 structure and likely constitute a functional binding surface for Cks1. Four of the eight amino acid residues are located in the largely unstructured carboxyl-terminal tail region of Skp2. These results uncovered the specificity of the Skp2-Cks1 interaction and reveal a critical function for the structurally flexible carboxyl-terminal tail region of Skp2 in Cks1 recognition and substrate ubiquitination.

Timed destruction of cell cycle inhibitors such as p27 by the ubiquitin-proteasome pathway plays a critical role in regulating normal cell cycle progression (1, 2). Aberrant degradation of p27 is one of the most common cellular events in the pathogenesis of numerous types of human cancers (3). The levels of p27 expression in cancer cells have proved to be a powerful prognostic indicator for tumor progression and patient survival (4, 5). Degradation of p27 in G1 and S phases is mediated by the SCF^{Skp2} ubiquitin ligase (6, 7). Consistent with this view was the finding that an elevated level of Skp2 was detected in several types of human tumors (8–11). Skp2 levels correlate with grade of malignancy and decreased p27 expression (3).

Ubiquitination and degradation of p27 by the SCF^{Skp2} ubiquitin ligase require phosphorylation of p27 at Thr-187. Cyclin E-Cdk2 has been shown to be the kinase that phosphorylates p27 at this site (12, 13). In addition, ubiquitination of phosphorylated p27 requires an accessory protein called Cks1 (14, 15). Cks1 binds to Skp2 directly, and this interaction is essential for p27 ubiquitination. The exact role of Cks1 in stimulating p27 ubiquitination is not very well understood. Available evidence suggests that Cks1 can either serve as an adapter to recruit p27 through the Cyclin E-Cdk2 complex or function as an allosteric effector to alter the conformation of Skp2 to enhance catalysis (14–17).

The F-box adapter protein in the SCF E3 ubiquitin ligase complex is primarily responsible for substrate recruitment (18). The F-box protein Skp2 contains ten leucine-rich repeats in addition to the F-box motif. Crystallographic analysis of Skp2 revealed that it forms a sickle-shaped structure (19). At the “handle” are the three helices that constitute the F-box motif. These helices mediate the interaction of Skp2 with Cks1. The highly conserved sequence motif (GxGxGxG) is a hallmark of F-box proteins. The C-terminal regulatory domain of Skp2 forms the “blade” of the sickle. The unstructured carboxyl-terminal tail region of Skp2 is essential for Cks1 binding and substrate recruitment. Therefore, our data indicate that the carboxyl-terminal tail of Skp2 is involved in direct recognition of the substrate as mutations in the basic amino acid residues on the surface of the leucine-rich repeats abolish substrate ubiquitination (20). It is not clear whether Skp2 uses a similar mechanism to recruit p27.

We have previously shown that Cks1 binds to the carboxyl-terminal region of Skp2 and that a negatively charged amino acid residue at position 331 of Skp2 is essential for the Skp2-Cks1 interaction (21). In this report, we defined the functional binding interface between Skp2 and Cks1. Four amino acid residues in the unstructured carboxyl-terminal region of Skp2 are required for Cks1 binding and substrate recruitment. Therefore, our data indicate that the carboxyl-terminal tail of Skp2 does not negatively regulate substrate recruitment; instead, the flexibility of this region is crucial for substrate recruitment through binding of the adapter protein Cks1 and subsequent ubiquitination of the substrate.

EXPERIMENTAL PROCEDURES

DNA Expression Constructs and Recombinant Proteins—Mammalian expression vector pEXL-Skp2 has been described previously (21, 22). All Skp2 deletion or point mutations were constructed by the QuickChange site-specific mutagenesis kit (Stratagene). Recombinant Cks1, Cdc34, and Myc proteins were purified as described previously (21).

Recombinant baculoviruses expressing GST-Cyclin E and Cdk2 were kindly provided by Dr. Wade Harper. Baculoviruses were amplified in SF9 cells, and infections were carried out in HiFive cells (Invitrogen). The Cyclin E-Cdk2 complex was purified using GST affinity chromatography and stored in QA buffer (20 mM Tris·HCl, 100 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂, and 1 mM dithiothreitol, pH 7.7) at −80 °C (21).

Binding Assays—Wild type and Skp2 mutants were synthesized and labeled with [35S]Met in the transcription and translation kit in vitro kit (Promega). To assess the ability of Skp2 and Cks1 to interact, binding assays were performed by incubating 10 μl of [35S]labeled Skp2 with 1 μg of GST-Cks1 or GST-Myc in 0.5 ml of NET buffer (20 mM Tris·HCl, 1 M KCl, 0.1 mM EDTA, and 1 mM dithiothreitol, pH 7.4).
pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40) at 4 °C for 1 h. GST beads were added to the mixture and incubated for 1 h prior to pelleting by brief centrifugation. The beads were washed three times with 0.5 ml of NETN buffer and boiled in 2/100 SDS loading buffer prior to SDS-PAGE analysis. The results of binding were expressed as the percentage of labeled proteins retained on the beads relative to total input. The binding efficiency of Skp2 mutants was expressed as the strength relative to the wild type Skp2.

Preparation of the Wild Type and Mutant SCFSkp2 Complex—The wild type Skp2 and various Skp2 mutants were amplified using PCR to introduce EcoRI and XhoI sites and subcloned into pFAST-HTb vector (Invitrogen) prior to transformation into DH10Bac cells (Invitrogen). Genomic DNA for each construct derived from DH10Bac cells that has undergone successful recombination was prepared according to the manufacturer’s instructions and subsequently transfected into Sf9 cells using Cellfectin (Invitrogen). Two days after transfection, supernatants containing infectious viruses were collected and amplified twice. High titer viral stocks were harvested and stored for coinfection experiments. To prepare indicated SCF complexes, recombinant wild type or mutant Skp2 viruses were coinfected in HiFive cells (Invitrogen) with baculoviruses encoding GST-Skp1, HA-Cul1, and Rbx1 (kindly provided by W. Harper, Harvard Medical School). Construction of these recombinant viruses has been described previously. Two days after infection, cells were harvested and lysed by three freeze/thaw cycles. SCF complexes were purified using glutathione beads (Amersham Biosciences).

In Vitro Ubiquitination Assay—Recombinant Skp2 and Skp2 mutant proteins were synthesized in rabbit reticulocyte lysate by in vitro transcription/translation. The amounts of Skp2 and Skp2 mutants produced in this system were measured by immunoblotting with an anti-Skp2 antibody. Expression of the endogenous rabbit Skp2 is virtually undetectable by immunoblotting in the control lysates. Reconstitution of the Skp2-dependent ubiquitination of p27 was accomplished by incubating in vitro translated 35S-labeled p27 that was phosphorylated by GST-Cyclin E-Cdk2 with 30/100 His-Cdc34, 1/100 g of ubiquitin, 1.5/100 g of methylated ubiquitin (Boston Biochem), 1 mM ubiquitin aldehyde, 2 g of MG 132, 2.5 g of 10 × energy regeneration system (ER), 1 mM dithiothreitol, and 1 g of Cks1. The reaction was incubated at 30 °C for 2 h. The products of the ubiquitination reaction were analyzed by SDS-PAGE and quantified by phosphorimaging analysis.

Immunoblotting Analysis—Recombinant proteins were synthesized using a coupled transcription and translation system (Promega). To measure the levels of recombinant protein in the reticulocyte lysates, a portion of the samples was resolved by 8 or 12% SDS-PAGE and electrophoretically transferred to nitrocellulose. Western blot analysis was performed using the anti-Skp2 (Santa Cruz Biotechnology), anti-Rbx1 (Neomarker), anti-GST (Santa Cruz), or anti-HA (Covance) antibodies. The proteins were detected using horseradish peroxidase-conjugated sheep anti-mouse or goat anti-
rabbit secondary antibody (GE Healthcare) with an ECL WestDura detection kit (Pierce).

**RESULTS**

**Requirement of the Carboxyl-terminal Tail Region of Skp2 for Cks1 Association**—Ubiquitination of phosphorylated p27 can be reconstituted in vitro using recombinant or purified E1, E2 (Cdc34 or UbcH5), and SCFSkp2 complex in the presence of Cyclin E-Cdk2. It has been shown that Cks1 binds to Skp2 in vitro and interaction between Cks1 and Skp2 is required for ubiquitination of p27 (14, 15, 17, 21). To understand the function of Cks1 in p27 ubiquitination, it is necessary to determine how Cks1 interacts with Skp2. Previous studies indicate that the carboxyl-terminal region of Skp2 is required for its association with Cks1 and this region may contain a specific binding site for Cks1 (Fig. 1) (21). To further define critical amino acid residues that are involved in the Skp2-Cks1 interaction, segments of Skp2 were removed by deletion and the resulting mutants were assayed for their ability to interact with Cks1. As shown in Fig. 1, deletion of the last 34 amino acid residues of Skp2 abolishes the Skp2-Cks1 interaction. In contrast, removal of the last 22 amino acid residues does not significantly affect the association. This result suggests that Skp2 amino acids 403–415 harbor important amino acid residues that mediate the Skp2-Cks1 interaction.

To identify critical amino acid residues that specify the interaction between Skp2 and Cks1, alanine scanning mutagenesis was performed. First, we constructed a series of double alanine substitution mutants covering the region between amino acid 403 and 415. Binding studies with these mutants indicate that three double alanine substitution mutants (AA for F405A,T406A or T407A,I408A or R410A,P411A) are defective in binding to Cks1 (Fig. 1C). To further evaluate the relative importance of each of the six amino acid residues in the Cks1 interaction, single alanine substitution mutants were also generated. Interestingly, mutation of Thr-407 and Pro-411 has no effect on Cks1 association and changes in Ile-408 and Arg-410 completely abolish Cks1 binding, whereas individual alteration of Phe-405 and Thr-406 only partially affects Cks1 association.

![Fig. 2. Skp2-dependent ubiquitination of p27 in vitro.](image)

Recombinant p27 was labeled with [35S]Met by in vitro translation and was incubated with or without Cyclin E-Cdk2 prior to the ubiquitination reaction. The ubiquitination reaction was performed under the conditions indicated. Lane 1, 10% of input [35S]-labeled p27 substrate alone. Lane 2, phosphorylated p27 incubated with E1, E2(Cdc34), Cks1, and 20 μl of recombinant Skp2 prepared by in vitro translation. Lane 3, the reaction mixture is identical to lane 2 except that p27 was not incubated with Cyclin E-Cdk2. Lane 4, p27 ubiquitination reaction in the absence of exogenous Cks1. Lane 5, p27 ubiquitination reaction in the absence of exogenous E1 enzyme. Lane 6, p27 ubiquitination reaction in the absence of exogenous E2 enzyme. Lane 7, p27 ubiquitination reaction in the absence of lysates containing recombinant Skp2. A control rabbit reticulocyte lysate was added in the reaction. Lane 8, p27 ubiquitination reaction in the absence of exogenous E1, E2, and Skp2.

![Fig. 3. Effects of Skp2 mutants on p27 ubiquitination in vitro.](image)

The deletion and double and single substitution Skp2 mutants in the carboxyl-terminal tail region of Skp2 were analyzed for their ability to ubiquitinate p27 in vitro, using the assay described in Fig. 2. B, D, and F, expression levels of various Skp2 mutants in the reticulocyte lysates as analyzed by immunoblotting experiment using an anti-Skp2 antibody. A and B, Skp2 deletion mutants. C and D, Skp2 double substitution mutants. E and F, Skp2 single substitution mutants.
in sharp contrast to the strong effects of the double mutant (Fig. 1D). Taken together, these data indicate that only four amino acid residues in the carboxyl-terminal tail region of Skp2 are involved in binding Cks1.

**Skp2-dependent p27 Ubiquitination in the Rabbit Reticulocyte System**—Now that we had defined critical amino acid residues involved in Cks1 binding in the carboxyl-terminal tail region of Skp2, it was essential to determine whether these mutations affect the ability of Skp2 to catalyze p27 ubiquitination. Such an analysis was typically done by expressing Skp2 variants in a baculovirus expression system and reconstituting the SCF complex by coexpression of other subunits of SCF in insect cells. The SCFSkp2 complex was affinity purified, and the ubiquitination reaction was carried out by incubating phospho-

**TABLE I**

| Skp2 mutants | Cks1 binding | p27 ubiquitination | Skp2 autoubiquitination |
|--------------|--------------|---------------------|-------------------------|
| Skp2 WT      | ++           | +++                 | +++                     |
| Skp2 (1–402) | +            | +                   | +                       |
| Skp2 (1–415) | +            | +                   | +                       |
| Skp2 SH–AA   | +            | +                   | +                       |
| Skp2 FT–AA   | –            | –                   | +                       |
| Skp2 TI–AA   | –            | –                   | NA                      |
| Skp2 RP–AA   | –            | –                   | ND                      |
| Skp2 TI–AA   | –            | –                   | ND                      |
| Skp2 GN–AA   | +            | +                   | ND                      |
| Skp2 F405 A  | +            | +                   | ND                      |
| Skp2 T406 A  | +            | +                   | ND                      |
| Skp2 T407 A  | ++           | ++                  | ND                      |
| Skp2 I408 A  | –            | –                   | +                       |
| Skp2 R410 A  | –            | –                   | +                       |
| Skp2 P411 A  | +            | +                   | ND                      |
| Skp2 W277 A  | –            | –                   | +                       |
| Skp2 D331 A  | ++           | ++                  | ND                      |
| Skp2 R356 A  | ++           | ++                  | ND                      |
| Skp2 Y358 A  | ++           | ++                  | ND                      |
| Skp2 F380 A  | ++           | ++                  | ND                      |

* ND, not determined.
No significant p27 ubiquitination was observed when \( \text{in vitro} \) translated Skp2 was omitted (Fig. 2, lane 7). In addition, efficient ubiquitination of p27 requires Cyclin E-Cdk2, Cks1, and to a lesser extent E2 and E1 (Fig. 2, lanes 5 and 6). It has been well documented that reticulocyte lysates contain significant amounts of E1 and E2 enzymes, and therefore adding exogenous E1 and E2 does not have a significant impact on p27 ubiquitination. The pattern and requirements for p27 ubiquitination are virtually identical to the reactions worked out using purified SCF complex from insect cells (21). Therefore, this assay allows faithful investigation of Skp2 function \( \text{in vitro} \).

To determine the effects of various Skp2 mutations in the carboxyl-terminal region on p27 ubiquitination, SCF complexes with Skp2 variants were assembled by \( \text{in vitro} \) translation. The expression levels of various Skp2 mutants in the reticulocyte lysates were comparable as determined by immunoblotting using an anti-Skp2 antibody (Fig. 3). To make sure that mutations introduced in Skp2 did not result in protein misfolding, each of the Skp2 mutants was analyzed for its ability to bind recombinant c-Myc. It has been shown previously that Skp2 binds c-Myc and that the region of Skp2 involved in c-Myc binding is located outside the carboxyl-terminal region of Skp2 (24, 25). All the Skp2 mutants constructed retained their ability to bind c-Myc, implying that none of these mutations appears to denature Skp2 (data not shown).

As shown in Fig. 3, mutant SCF complexes reconstituted in the reticulocyte lysates differ in their ability to ubiquitinate p27. Invariably, Skp2 mutants that failed to bind Cks1 efficiently are incapable of causing p27 ubiquitination efficiently, except Skp2 (1–415), which is able to bind Cks1 yet unable to support substrate ubiquitination. Three double mutants (F405A, T406A, T407A, I408A, R410A, P411A) are clearly defective in supporting p27 ubiquitination (Fig. 3, C and D). Results from analysis of single mutants indicate that T407A and P411A have no effect and F405A or T406A alone only caused a slight reduction in p27 ubiquitination in contrast to the dramatic effects observed with the double mutant (Fig. 3, E and F). Thus, functional analysis of Skp2 mutants clearly supports the im-

![Fig. 5. Locations of the critical amino acid residues that are involved in binding Cks1 mapped to the Skp2 structure.](image)

![Fig. 6. Assembly of the SCF complexes containing the wild type and mutant Skp2 in insect cells.](image)
portance of Phe-405, Thr-406, Ile-408, and Arg-410 in mediating the Skp2-Cks1 interaction.

Identification of a Second Cks1 Binding Interface in Skp2—Our previous study indicates that Asp-331 is critical for the Skp2-Cks1 association (21). A conservative change from Asp to Glu at this position does not affect the Skp2-Cks1 interaction, suggesting a negatively charged amino acid at this position is required. Charged amino acids are usually responsible for the specificity of protein-protein interactions but do not contribute to the binding energy. Surface-exposed hydrophobic amino acid residues often provide the main source of binding energy that drives protein-protein association. Thus, additional amino acid residues in close three-dimensional proximity are likely to be involved in mediating the Skp2-Cks1 interaction.

Because Asp-331 is located on the concave surface of Skp2 near the end of the S8 β-sheet, we reasoned that other residues that form the Cks1 binding surface in Skp2 may be located in the loops on the same side as Asp-331 and be surface exposed. Upon close examination of the Skp2 crystal structure, we identified a number of surface-exposed hydrophobic or charged residues that are likely candidates for mediating the Skp2-Cks1 interaction.

As demonstrated in Fig. 4, mutation of surface-exposed hydrophobic amino acid residues near Asp-331 has variable effects on the Skp2-Cks1 interaction. Whereas W277A completely abrogates the Skp2-Cks1 association, the impact of the F380A mutation is less dramatic and the Y358A mutation has no effect at all (Fig. 4, A and H). Because Asp-331 is located on the concave surface of Skp2, the loop regions of Skp2 are unstructured and the unstructured loop regions of Skp2 are engaged in this interaction.

Cks1 Binding-deficient Skp2 Mutants Can Be Efficiently Assembled into SCF Complex—The above studies indicate eight amino acid residues are important for Cks1 binding and p27 ubiquitination. However, it cannot be ruled out that these Skp2 residues may have a role in stabilizing the SCF complex and thus some of the mutations may not be able to assemble functional SCF complexes. To demonstrate that Skp2 mutants deficient in Cks1 binding are fully active in formation of SCF complexes, recombinant baculoviruses encoding all eight mutants in addition to two Skp2 deletion mutants were constructed and high titer infectious viral stocks were prepared. HiFive cells were coinfected with Skp2 virus and baculoviruses expressing levels of Skp2 in the insect cells except for Skp2 expression levels of Skp2 in the insect cells except for Skp2, are similar (lanes 1-8), even though the amounts of Skp2 mutants expressed in insect cells were quite variable. This is what we expected as the common components of the SCF complex, except for Skp2, are from the same stock mixture. The efficiency of SCF complex formation in insect cells was determined by affinity purification of Skp1, using glutathione beads and subsequent incubation with labeled Cks1 at 4 °C for 1 h on a tumbler. The beads were washed three times with cold NETN buffer, and labeled Cks1 retained on the glutathione beads was eluted by boiling in the 2× SDS sample buffer. Samples were analyzed by SDS-PAGE and phosphorimaging using a Typhoon scanner (GE Healthcare).

**Fig. 7.** Autoubiquitination of Skp2 and the Skp2 mutants assembled in the SCF complex. Recombinant SCF<sup>Skp2</sup> and SCF complexes loaded with mutant Skp2 were produced in HiFive cells and bound to GST beads for 1 h at 4 °C. Skp2 autoubiquitination assays were carried out by incubating 200 ng of E1, 5 μg of E2 (Cdc34), ubiquitin, methylated ubiquitin, and energy regeneration system with 2 μg of affinity-purified SCF complex as determined by Coomassie Blue staining at 30 °C for 1 h. The autoubiquitination reactions were terminated by adding 2× SDS sample buffer and analyzed by SDS-PAGE. Autoubiquitination of Skp2 was detected by immunoblotting with the Skp2 antibody (Santa Cruz).

**Fig. 8.** Association of Cks1 with SCF<sup>Skp2</sup> complex in vitro. Recombinant Cks1 was synthesized and labeled by in vitro translation in the presence of [35S]Met using transcription and translation reticulocyte lysate. Two micrograms of each Skp2-SCF complex made in insect cells was immobilized on the glutathione beads and subsequently incubated with labeled Cks1 at 4 °C for 1 h on a tumbler.
SCF<sub>Skp2</sub>. To determine whether any Skp2 mutants that are defective in Cks1 association are able to undergo autoubiquitination, we purified each SCF complex and incubated with the ubiquitination reaction mixture. As shown in Fig. 7, the wild type and mutant Skp2 were targeted to ubiquitination judging by the appearance of high molecular weight Skp2 bands. Appearance of these high molecular weight bands is E1- and E2-dependent. The efficiency of Skp2 autoubiquitination is comparable for Skp2 mutants. These data further suggest that each of the Skp2 mutants that is defective in Cks1 binding is capable of assembling into the SCF complex. To further confirm interaction between various SCF complexes and Cks1, each SCF complex was incubated with <sup>35</sup>S-labeled recombinant Cks1. Glutathione beads were subsequently added to recover the SCF complexes, and the amount of Cks1 retained on the beads was analyzed by SDS-PAGE. The result of this experiment showed good agreement with Cks1-Skp2 binding assay data (Fig. 8). Taken together, mutations introduced to Skp2 only affect Cks1 binding.

**DISCUSSION**

Ubiquitination of the cell cycle inhibitor p27 is catalyzed by a multisubunit E3 ubiquitin ligase SCF<sub>Skp2</sub> and requires cooperation of Cks1 as well as Cyclin E-Cdk2. The complicated molecular interactions among these players in orchestrating p27 ubiquitination have not been definitively sorted out. Here we investigated the interplay between Skp2 and Cks1, an interaction figuring prominently in substrate recruitment. In addition, we assessed the importance of nonstructural elements in Skp2 toward p27 ubiquitination. Our results suggest that a structural motif in Skp2 is involved in binding Cks1. At least eight amino acid residues are crucial for the Skp2-Cks1 association, and virtually all of them are located on the loop or unstructured region of Skp2. Mutation of any one of these disrupts the capacity of Skp2 to trans-ubiquitinate p27 but has no impact on the ability of Skp2 to form the SCF complex and engage in autoubiquitination. Thus, this constitutes a functional definition of the Skp2-Cks1 binding surface.

The absolute requirement for Cks1 in ubiquitination of p27 by SCF<sub>Skp2</sub> begs the question of what the role of Cks1 is in this process. Cks1 appears to have three binding interfaces, an anion-binding pocket, a Cdk2 binding surface that mediates its association with the carboxyl-terminal region of the kinase, and a Skp2 binding site (18). Two models have been proposed for the function of Cks1 in p27 ubiquitination. The first is an allosteric model wherein Cks1 functions as an allosteric effector of Skp2 (15, 16). Binding of Cks1 to Skp2 causes a conformational change in Skp2 and results in exposure of potential binding sites for the p27 substrate in Skp2. Skp2 adopts a relatively rigid structure except in the carboxyl-terminal tail region, which is unstructured. This region of Skp2 would be well suited to undergo a conformational change upon binding to Cks1. Of eight amino acid residues that are required for interaction with Cks1, four are located in the unstructured carboxyl-terminal tail. It is quite possible that upon Cks1 binding the carboxyl-terminal region becomes highly organized and structured. In this vein, our studies here suggest the targeted region for this allosteric effect to occur. The second is the adapter model. Cks1 can bind both Skp2 and Cdk2. Furthermore the substrate p27 binds the Cyclin E-Cdk2 complex, and ubiquitination of p27 requires the physical presence of the above complex. Therefore, it is conceivable that Cks1 serves as an adapter to bridge the F-box protein Skp2 with its substrate p27 (17). These two models are not necessarily mutually exclusive. In fact, the stringent requirement of the Cyclin E-Cdk2 complex and the fact that Thr-187-phosphorylated p27 cannot be ubiquitinated without the physical presence of the Cyclin E-Cdk2 complex strongly argue for the adapter model (26). We believe that Cks1 functions as an allosteric effector of Skp2 and adapter for p27 to be recruited to the SCF<sub>Skp2</sub> complex for ubiquitination.

The elucidation of critical residues involved in the Skp2-Cks1 interaction may have another important implication regarding the specificity of SCF<sub>Skp2</sub>. There are at least two isoforms of Skp2 (Skp2 and Skp2<sub>2CTV</sub>) described in the literature (27). The difference between these two isoforms lies in the last exon generated by alternative splicing. None of the four critical residues in the carboxyl-terminal region of Skp2 is present in Skp2<sub>2CTV</sub>. That these residues are indeed crucial to Skp2-Cks1 binding is predicated upon the fact that Skp2<sub>2CTV</sub> should be unable to bind Cks1 and mediate p27 ubiquitination. This is indeed the case. Therefore, Cks1 is designed to bind only one isoform of Skp2 to allow SCF<sub>Skp2</sub> to ubiquitinate specific substrates. What is the advantage to using Cks1 as an adapter for ubiquitination of p27 or p21 by SCF<sub>Skp2</sub>? One possible explanation could be the need to control the timing of ubiquitination of p27 and p21 during cell cycle progression. Skp2-dependent degradation of p27 and p21 occurs in the G<sub>1</sub>/S transition. The expression of Cks1 is regulated by cell cycle progression and growth factor treatment (28, 29). Cks1 binds the Cyclin E-Cdk2-p27 complexes and targets them to SCF<sub>Skp2</sub>. Thus, Cks1 couples cell cycle progression and ubiquitination of cell cycle inhibitors by SCF<sub>Skp2</sub>.

In summary, we have identified and characterized the binding interface between Skp2 and Cks1. Our data indicate that the unstructured carboxyl-terminal tail of Skp2 plays important roles in p27 ubiquitination and specificity of substrate recruitment through interaction with Cks1.

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**REFERENCES**

1. Sherr, C. J., and Roberts, J. M. (1999) *Genes Dev.* 13, 1501–1512.
2. Nakayama, K. I., Hatakeyama, S., and Nakayama, K. (2001) *Biochem. Biophys. Res. Commun.* 282, 853–860.
3. Bloom, J., and Pagano, M. (2003) *Semin. Cancer Biol.* 13, 41–47.
4. Loda, M., Cukor, B., Tam, S. W., Lavin, P., Fiorentino, M., Draetta, G. F., Jessup, J. M., and Pagano, M. (1997) *Nat. Med.* 3, 231–234.
5. Porter, P. L., Malone, K. E., Henggerty, P. J., Alexander, G. M., Gatti, L. A., Firpo, E. J., Daling, J. R., and Roberts, J. M. (1997) *Nat. Med.* 3, 222–225.
6. Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. (1999) *Nat. Cell Biol.* 1, 193–199.
7. Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U., and Krek, W. (1999) *Nat. Cell Biol.* 1, 207–214.
8. Gstaiger, M., Jordan, R., Lim, M., Catzaveflis, C., Mestan, J., Slingerland, J., and Krek, W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 5043–5048.
9. Yang, G., Ayala, G., Marzo, A. D., Tian, W., Frolov, A., Wheeler, T. M., Thompson, T. C., and Harper, J. W. (2002) *Clin. Cancer Res.* 8, 3419–3426.
10. Signoretti, S., Di Marcotullio, L., Richardson, A., Ramaswamy, S., Isaac, B., Rapp, M., Monti, F., Loda, M., and Pagano, M. (2002) *J. Clin. Invest.* 110, 633–641.
11. Latres, E., Chiarle, R., Schulman, B. A., Pavletich, N. P., Pellicer, A., Hinghirami, G., and Pagano, M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 2515–2520.
12. Sheaff, R. J., Grouding, M., Gordon, M., Roberts, J. M., and Clurman, B. E. (1997) *Genes Dev.* 11, 1464–1478.
13. Vlach, J., Hennecke, S., and Amati, B. (1997) *EMBO J.* 16, 5334–5344.
14. Gannot, D., Bernstein, G., Ko, T. K., Larsen, B., Tyers, M., Pagano, M., and Hershko, A. (2001) *Nat. Cell Biol.* 3, 321–324.
15. Spruck, C., Strohmaier, H., Watson, M., Smith, A. P., Ryan, A., Krek, T. W., and Reed, S. I. (2001) *Mol. Cell* 7, 639–650.
16. Xu, K., Belonis, C., Chua, W., Weber, D., Podlaski, F., Huang, K. S., Reed, S. I., and Vassilev, L. T. (2003) *Biochem. J.* 371, 957–964.
17. Sirthy, D., Seeligar, M. A., Ko, T. K., Gannot, D., Brewster, S. E., Itzhaki, L. S., Pagano, M., and Herskhow, A. (2000) *J. Biol. Chem.* 275, 42233–42240.
18. Harper, J. W. (2001) *Curr. Biol.* 11, R431–435.

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*Author's note:*

2 D. Ungermannova and X. Liu, unpublished observation.

3 W. Wang and X. Liu, unpublished observation.
Functional Interaction Between Skp2 and Cks1

19. Schulman, B. A., Carrano, A. C., Jeffrey, P. D., Bowen, Z., Kinnucan, E. R., Finnin, M. S., Elledge, S. J., Harper, J. W., Pagano, M., and Pavletich, N. P. (2000) Nature 408, 381–386
20. Hsiung, Y. G., Chang, H. C., Pellequer, J. L., La Valle, R., Lanker, S., and Wittenberg, C. (2001) Mol. Cell. Biol. 21, 2506–2520
21. Wang, W., Ungermannova, D., Chen, L., and Liu, X. (2003) J. Biol. Chem. 278, 32390–32396
22. Liu, X., Sun, Y., Constantinescu, S. N., Karam, E., Weinberg, R. A., and Lodish, H. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10669–10674
23. Koepp, D. M., Schaefer, L. K., Ye, X., Keyomarsi, K., Chu, C., Harper, J. W., and Elledge, S. J. (2001) Science 294, 173–177
24. Kim, S. Y., Herbst, A., Tworkowski, K. A., Salghetti, S. E., and Tansey, W. P. (2003) Mol. Cell 11, 1177–1188
25. 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., Kerpola, T. K., and Larsson, L. G. (2003) Mol. Cell 11, 1189–1200
26. Montagnoli, A., Fiore, F., Eytan, E., Carrano, A. C., Draetta, G. F., Hershko, A., and Pagano, M. (1999) Genes Dev. 13, 1181–1189
27. Ganiatsas, S., Dow, R., Thompson, A., Schulman, B., and Germain, D. (2001) Oncogene 20, 3641–3650
28. Wang, W., Ungermannova, D., Jin, J., Harper, J. W., and Liu, X. (2004) Oncogene 23, 1064–1075
29. Simon, K. E., Cha, H. H., and Firestone, G. L. (1995) Cell Growth Differ. 6, 1261–1269