A Negatively Charged Amino Acid in Skp2 Is Required for Skp2-Cks1 Interaction and Ubiquitination of p27

Weixiang Wang, Dana Ungermannova, Lin Chen, and Xuedong Liu

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

Proteolysis of cyclin-dependent kinase inhibitor p27 occurs predominately in the late G1 phase of the cell cycle through a ubiquitin-mediated protein degradation pathway. Ubiquitination of p27 requires the SCF^Skp2 ubiquitin ligase and Skp2 F-box binding protein Cks1. The mechanisms by which Skp2 recognizes Cks1 to ubiquitylate p27 remain obscure. Here we show that Asp-331 in the carboxyl terminus of Skp2 is required for its association with Cks1 and ubiquitination of p27. Mutation of Asp-331 to Ala disrupts the interaction between Skp2 and Cks1. Although Asp-331 mutation negates the ability of the Skp1-Cullin-F-box protein (SCF) complex to ubiquitylate p27, such a mutation has no effect on Skp2 self-ubiquitination. A conservative change from Asp to Glu at position 331 of Skp2 does not affect Skp2-Cks1 interaction. Our results revealed a unique requirement for a negatively charged residue in the carboxyl-terminal region of Skp2 in recognition of Cks1 and ubiquitination of p27.

Proliferation of eukaryotic cells requires sequential activation of cyclin-dependent kinases (1, 2). The activity of these enzymes is regulated positively and negatively by transcriptional, translational, and post-translational mechanisms. p27 is a negative regulator of Cdk2-cyclin E and Cdk2-cyclin A, the activities of which are required for G1 to S transition and for triggering DNA replication (3). The levels of p27 fluctuate during cell cycle progression. In quiescent cells, p27 is metabolically stable and accumulates. When quiescent cells begin to divide and enter the cell division cycle, p27 is rapidly degraded, thereby releasing Cdk2-cyclin E (4). Increased Cdk2-cyclin E activity drives cells from G1 phase into S phase of the cell cycle.

Numerous studies have established that the stability of p27 is primarily controlled by the ubiquitin proteasome pathway. Polyubiquitinated p27 is targeted to the 26 S proteasome and degraded (4). Ubiquitination of p27 occurs when it is phosphorylated by Cdk2-cyclin E kinase at Thr-187 during the G1 to S transition (5, 6). Ubiquitination of proteins is carried out by a multi-enzyme complex consisting of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) (7, 8). It has been shown previously that the SCF^Skp2 complex containing Skp1, Skp2, Cul1, Rbx/Roc1 is responsible for recognizing phosphorylated p27 and catalyzing its ubiquitination (9, 10). Skp2 was originally identified as a protein associated with the S phase promoting kinase Cdk2-cyclin A. Skp2 activity is required for S phase entry because a neutralizing antibody against Skp2 blocks cell cycle progression (11).

Subsequent studies revealed that Skp2 is a member of a diverse family of proteins that share a common sequence motif named the F-box. Skp1 directly binds to the F-box motif and mediates assembly of the SCF^Skp2 ubiquitin ligase complex (12). The specificity of SCF^Skp2 E3 substrate recognition is dictated by the F-box protein (13). The crystal structure of the SCF^Skp2 complex solved by Pavletich and coworkers (14, 15) offers an unprecedented view of the molecular organization of this complex. At the center of the complex is the cullin, which interacts with all three subunits and serves as a scaffold. Skp2, which contains a carboxyl-terminal leucine-rich repeat (LRR) domain that has been implicated in binding to the substrate phosphorylated p27, is located ~50 Å away from the charged E2 enzyme based on the structure model (15). It remains unclear how substrates are recognized and ubiquitin chains are formed.

One way to fill this gap is to recruit an “adapter” protein to bridge the E3 and substrates (1, 16). Indeed, ubiquitination of phosphorylated p27 requires an accessory protein called Cks1 (17, 18). Cks1 was originally discovered as an essential gene product in yeast that forms a complex with Cdk5 (19, 20). In addition to binding to Cdk and regulating substrate selection and extent of substrate phosphorylation (21–23), Cks1 has been shown to be required for ubiquitination of p27 (17, 18). Cks1, but not its closely related protein Cks2, binds to Skp2 (18). Furthermore, Hershko and coworkers (24) have recently shown that Cks1 harbors three independent binding surfaces that can simultaneously bind to Cdk2, Skp2, and p27. They have identified two essential amino acid residues in Cks1 that are required for interaction with Skp2 and ubiquitination of p27. However, the regions of Skp2 that are involved in binding to Cks1 remain unknown. Here we have used deletion and a site-specific mutagenesis approach to elucidate the regions of Skp2 that are required for Cks1 interaction and ubiquitination of p27. Our results indicate that Cks1 binds to the carboxyl-terminal region of Skp2 and that Asp-331 is essential for binding. Mutation of Asp-331 to Ala abrogates Skp2-Cks1 interaction. An Asp-331 to Glu mutation does not affect Skp2-Cks1 interaction, suggesting that the specificity of this interaction depends on the presence of a negatively charged amino acid at this position.

EXPERIMENTAL PROCEDURES

Cell Lines and Transfection—Human 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with l-glutamine, penicillin, streptomycin, and 10% fetal bovine serum (Invitrogen). 293T
FIG. 1. Deletion mapping of the region of Skp2 that is required for binding to Cks1. A, diagram of the structure of Skp2; regions of Skp2 that were removed are indicated. B, the carboxyl-terminal region of Skp2 is required for binding to Cks1. The wild type or various deletion mutants were synthesized and labeled by in vitro translation in the presence of [35S]Met using the TNT reticulocyte lysate. Reombinant Cks1 or Cks2 were purified as GST fusion proteins from E. coli. One microgram of Cks1 or Cks2 was incubated with 10 μl of the wild type Skp2 or various Skp2 mutants in 0.2 ml of NETN buffer at 4 °C for 1 h. Cks1 or Cks2 complexes were retrieved by incubating with GST beads for 0.5 h at 4 °C and eluted by boiling in SDS sample buffer. The amount of Skp2 that binds Cks1 or Cks2 was analyzed by SDS-PAGE and phosphoimaging. The binding efficiency was estimated by comparing with 10% of input-labeled proteins for each mutant. Lanes 1–3, Skp2 wild type; lanes 4–6, Skp2 (Δ332–436); lanes 7–9, Skp2 (Δ417–436); lanes 10–12, Skp2 (Δ2–107); lanes 13–15, Skp2 (Δ1109–1151).

Interaction between Skp2 and Cks1

|       | Cks1 | Cks2 | Cks1 | Cks2 | Cks1 | Cks2 | Cks1 | Cks2 |
|-------|------|------|------|------|------|------|------|------|
| input% |      |      |      |      |      |      |      |      |
| 1     | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    |
| Skp2 WT |      |      |      |      |      |      |      |      |
| Skp2 (Δ332–436) |      |      |      |      |      |      |      |      |
| Skp2 (Δ417–436) |      |      |      |      |      |      |      |      |
| Skp2 (Δ2–107) |      |      |      |      |      |      |      |      |

[35S]Met-p27 were phosphorylated by incubation with 0.5 μg of GST-Cdk2-cyclin E in 10X ER (10 mM ATP, 20 mM HEPES, pH 7.4, 10 mM MgCl2, 300 mM creatine phosphate, 0.5 mg/ml creatine phosphokinase) at 30 °C for 45 min. GST-Cdk2-cyclin E was affinity-purified from insect cells that had been infected previously with the recombinant baculovirus. The ubiquitination reaction was carried out by adding the following to the phosphorylation reaction in a final volume of 30 μl: 250 ng of E1 (Boston Biochemical), 3 μg of 6X-His-cdc43, 5 μg of SCFwt complex, 1 μg of ubiquitin, 1.5 μg of methylated ubiquitin (Boston Biochemical), 1 μg ubiquitin aldehyde, 2 μl of MG 132, 2.5 μl of 10X ER, 1 mM dithiothreitol, 1 μg of Cks1. The reaction was incubated at 30 °C for 2 h. SDS-PAGE and phosphoimaging analyses were used to evaluate the results.

**RESULTS**

**The Carboxyl Terminal Region of Skp2 Is Involved in Binding to Cks1 in Vitro**—Previous studies have established that human Cks1 binds to Skp2 in vitro and that this binding interaction with Cks1 and Skp2 is required for ubiquitination of p27 (17, 18, 24). To determine the region of Skp2 that is involved in binding to Cks1, we constructed several deletion mutants lacking various regions of Skp2. These deletion mutants were synthesized and labeled with [35S]Met-p27 by in vitro translation and incubated with recombinant GST-Cks1 or GST-Cks2 prepared in bacteria. As shown in Fig. 1, wild type Skp2 shows strong binding to Cks1 but not to Cks2, in good agreement with previous reports (17, 18, 24). Although deletion of the amino-terminal region of Skp2 or the F-box had little effect on their association with Cks1, removal of the last 105 amino acids completely abolished Skp2-Cks1 interaction. This experiment suggests that the carboxyl-
image

Asp-331 Is Required for Skp2-Cks1 Interaction in Vitro—To identify the potential Cks1 binding surface in Skp2, we studied the crystal structures of the Skp1-Skp2 complex determined by Tainer and coworkers (27, 28). The concave side of Skp2 appears to be an ideal surface binding based on observations in other systems (29, 30). Another striking feature of Skp2 structure is the unusual distribution of surface electrostatic potential. A patch of surface located near the carboxyl terminus of Skp2 is significantly more electronegative than the rest of the protein surface (Fig. 2A). Coincidentally, this negative patch is located in the area of the concave surface of Skp2. The amino acids that contribute to this negative patch are two negatively charged aspartates (Asp-328 and -331). Both of them are on the surface and exposed to solvent in the crystal structure (Fig. 2B). Alignment of Skp2 from different species indicates that these two residues are highly conserved (Fig. 2C), raising the question whether the electronegative nature of the surface is important for its function. To determine whether these two negatively charged aspartates are important for Skp2-Cks1 association, we mutated these two residues to alanines (DLSD → ALSA). As shown in Fig. 3 and Table I, mutation of these two aspartates completely abolished the interaction between Cks1 and Skp2. Next, to test whether either one of the two aspartates or both are required for binding, single amino acid substitution mutants were generated. Generation of Asp-328 to Ala caused a slight drop in binding efficiency; on the other hand, mutation of Asp-331 abrogates Skp2 binding to Cks1 just like the double mutant (Fig. 3 and Table I). Mutation of other residues in this area such as Ser-330 does not affect Cks1-Skp2 interaction (Fig. 3, lanes 16–18). Therefore, Asp-331 appears to play an important role in Cks1-Skp2 interaction.

Asp-331 Is Required for Skp2-Cks1 Interaction in Vivo—The above experiment established the importance of Asp-331 in Skp2-Cks1 interaction in vitro. To test whether this region is also important for association between Skp2 and Cks1 in cultured cells, we transfected the wild type and various Skp2 mutants along with FLAG-Cks1 into 293T cells. Co-immunoprecipitation experiments were performed using cell lysates prepared from transiently transfected cells. As shown in Fig. 4B, all Skp2 mutants expressed at a level virtually identical to Skp2 as determined by immunoblotting with the anti-Skp2 antibody. When immunoprecipitated using an anti-FLAG antibody, both transiently transfected and endogenous Skp2 were able to associate with FLAG-Cks1 (Fig. 4, lane 1 versus lane 2). However, the Skp2 carboxyl-terminal deletion mutant and the Skp2 D328A/D331A double mutant fail to precipitate efficiently (lanes 2 and 3). Only the endogenous Skp2 was precipitated in this assay (compare lanes 2 and 3 versus lane 9). Asp-331 single mutant showed a decrease in Cks1 association in vivo but not as dramatic as observed in vitro (lane 6 and Fig. 3, lane 11). This could be attributed to other factors influencing Skp2-Cks1 interaction in vivo. Just as we observed in vitro, the Asp-331 to Glu change has no effect on Skp2-Cks1 binding in vivo (lane 7). Therefore, the co-immunoprecipitation experiment not only confirms the binding results in vitro but also suggests that this interaction was maintained in intact cells.
by binding assays described in Fig. 1. Mutants and Cks1 or Cks2 was measured translation. Association between Skp2 mutants and Cks1 or Cks2 was measured by binding assays described in Fig. 1.

Table I

| Cks1 binding | WT | D328A/D331A | D328A | D328E | D331A | S330A |
|--------------|----|-------------|-------|-------|-------|-------|
|              | +++| --          | +++   | +     | +++   | +++   |

Table II

| Skp2 binding | Cks1 | Cks2 | Cks1S41E | Cks1N45R |
|--------------|------|------|----------|----------|
|              | +++  | --   | ++       | --       |

Key amino residues of Skp2 involved in binding to Cks1

Key amino residues of Cks1 involved in binding to Skp2

Fig. 3. Asp-331 is required for Skp2-Cks1 interaction in vitro. Skp2 mutants were constructed by the Quick-Change XL mutagenesis kit (Stratagene) and labeled with [35S]Met by in vitro translation. Association between Skp2 mutants and Cks1 or Cks2 was measured by binding assays described in Fig. 1.

Significance of Skp2 Asp-331 in p27 Ubiquitin Ligation—Previous studies indicate that Cks1-Skp2 interactions are required for p27 ubiquitination by SCF<sup>Skp2</sup> in a reconstituted system. SCF complex was expressed and purified using the baculovirus expression system. Recombinant p27 was phosphorylated by a recombinant Cdk2-cyclin E complex purified from Hi-Five cells prior to incubation with E1, E2 (Cdc34), and SCF<sup>Skp2</sup>. As shown in Fig. 5B, ubiquitination of p27 depended on phosphorylation by cyclin E/Cdk2 and occurs in the presence of Cks1 but not Cks2. Addition of S41E Cks1 can partially support ubiquitination of p27, but not the N41R mutant (Fig. 5B, lanes 3 and 4). These results are consistent with previous data suggesting that the interactions between Cks1 and Skp2 are required for p27 ubiquitination.

To determine the effects of Skp2 Asp-331 mutation on assembly of the SCF complex and its ability to cause p27 ubiquitination, we prepared SCF<sup>Skp2DLSD</sup>—<sup>ALS</sup>A by co-expressing Cul1, Rbx1, Skp1, and the Skp2 ALSA mutant in insect cells. First, we determined whether the Skp2 double mutant affects the ability of Skp2 to assemble the SCF complex. As shown in Fig. 6A, recombinant mutant SCF complex prepared from insect cells is indistinguishable from its wild type counterpart (Fig. 6A, lane 1 versus 2). Judging from Skp1 affinity purification and immunoblotting experiments (Fig. 6B), mutant Skp2 is still able to interact with Skp1 and form a core complex with Cul1 as well as Rbx/Roc1. As a control, a complex lacking Skp2 expression was also prepared (Fig. 6A, lane 3). Equal amounts of the wild type SCF or mutant SCF or Skp2-deficient SCF were incubated with phosphorylated p27 in the presence of E1 and E2. As shown in Fig. 6C, the wild type SCF was able to catalyze strong ubiquitination of p27, whereas the mutant SCF or Skp2-deficient SCF failed to convert any p27 at appreciable levels (Fig. 6C, lanes 2–4). Taken together, these data suggest that Asp-331 is required for SCF E3 activity and this activity is correlated with its ability to interact with p27.

Asp-331 of Skp2 Is Not Required for Skp2 Self-ubiquitination—Skp2 can undergo self-ubiquitination in the absence of substrates (31). It has been postulated that self-ubiquitination of the F-box is a mechanism for facilitating dynamic exchange of the SCF complex (32, 33). To determine whether Asp-331 of Skp2 plays a role in self-ubiquitination, we measured the efficiency of the wild type or mutant Skp2 ubiquitination in the SCF complex. As shown in Fig. 7, the wild type Skp2 is capable of undergoing self-ubiquitination as judged by the presence of N45R mutation completely disrupted the interaction between Cks1 and Skp2 (Fig. 5A, lane 5); however, the S41E mutant still bound Skp2 but at a reduced level (Fig. 5A, lane 4).

To determine the effect of the Cks1-Skp2 interaction on p27 ubiquitination, we performed in vitro p27 ubiquitin ligation assays using a reconstituted system. SCF complex was expressed and purified using the baculovirus expression system. Recombinant p27 was phosphorylated by a recombinant Cdk2-cyclin E complex purified from Hi-Five cells prior to incubation with E1, E2 (Cdc34), and SCF<sup>Skp2</sup>. As shown in Fig. 5B, ubiquitination of p27 depends on phosphorylation by cyclin E/Cdk2 and occurs in the presence of Cks1 but not Cks2. Addition of S41E Cks1 can partially support ubiquitination of p27, but not the N41R mutant (Fig. 5B, lanes 3 and 4). These results are consistent with previous data suggesting that the interactions between Cks1 and Skp2 are required for p27 ubiquitination.
Fig. 5. Critical amino acid residues of Cks1 involved in association with Skp2 and ubiquitination of p27. A, in vitro binding of Skp2 to GST-Cks1, Cks2, Cks1S41E, and Cks1N45R recombinant proteins. Ten percent of the [35S]Met-labeled Skp2 was directly loaded as a control (lane 1). Lane 2, Cks1; lane 3, Cks2; lane 4, Cks1S41E; and lane 5, Cks1N45R. B, ubiquitination of p27 by SCF complex in the presence of Cks1 (lane 2) or Cks1S41E (lane 3) or Cks1N45R (lane 4) or Cks2 (lane 5) or without prior phosphorylation of p27 by Cdk2-cyclin E complex. SCF complex and Cdk2-cyclin E complex were prepared as described under “Experimental Procedures.” [35S]Met-labeled p27 was prepared by in vitro translation. Approximately 1 ng of recombinant p27 was used in each ubiquitination reaction and was phosphorylated by 0.5 μg of recombinant Cdk2-cyclin E for 30 min prior to addition to the ubiquitination reaction.

Fig. 6. Requirement of Asp-331 in Skp2 for ubiquitination of p27. Hi-Five insect cells (Invitrogen) were infected with baculovirus expression vectors that express Skp2 wild type, Skp2ALSA mutant, or mock together with GST-Skp1, Cul1, and Rbx1. Forty-eight hours after infection, lysates were prepared by three freeze-thaw cycles. A, aliquots of cell lysates were incubated with GST agarose beads for 1 h at 4 °C, and bound proteins were analyzed by SDS-PAGE followed by staining with Coomassie Blue. The composition of SCFSkp2ALSA wild type (lane 1) is very similar to SCFSkp2ALSA. B, Skp2 mutation does not appear to disrupt the overall structure of Skp2 and its interaction with Skp1. Recombinant SCFSkp2ALSA, SCFSkp2ALSA, and SCF without Skp2 complexes were produced in Hi-Five cells and bound to GST beads for 1 h at 4 °C, bound proteins were analyzed by SDS-PAGE followed by immunoblotting with Skp2 antibody. C, Asp-331 of Skp2 is essential for SCF activity. In vitro translated p27 was phosphorylated by Cdk2-cyclin E and incubated with various recombinant SCF complexes in the presence of 1 μg of Cks1 as indicated. Polyubiquitylated p27 is indicated by the bracket on the right.

Fig. 7. Asp-331 is not required for Skp2 self-ubiquitination. Recombinant SCFSkp2 and SCFSkp2ALSA complexes were produced in Hi-Five cells and bound to GST beads for 1 h at 4 °C. Skp2 self-ubiquitination assays were carried out by adding 280 ng of E1 (Boston Biomed), 5 μg of E2 (Cdc34), and energy regeneration system as described under “Experimental Procedures.” Lane 1, SCFSkp2 and E1 and E2; lane 2, SCFSkp2ALSA without E1; lane 3, SCFSkp2 without E2; lane 4, SCFSkp2ALSA with E1 and E2; lane 5, SCFSkp2ALSA without E1; and lane 6, SCFSkp2ALSA without E2.

Discussion

Self-ubiquitination of Skp2 requires the activity of E1 and E2, because reactions without these two components did not exhibit significant modification of Skp2. The Skp2 double mutant SCF is also capable of self-ubiquitination in a manner indistinguishable from the wild type. This experiment suggests that Asp-331 is only required for SCFSkp2 to transubiquitylate p27 but not required for Skp2 self-ubiquitination. In addition, this provides independent proof that Asp-331 mutation does not adversely affect the integrity of SCFSkp2.

Activation of cyclin-dependent kinases is required for cell cycle progression. Removal of the inhibitors of cyclin-dependent kinase such as p27 through ubiquitin-mediated proteolysis is one of the major mechanisms that eventually leads to kinase activation. It has been well established that the SCFSkp2 E3 ubiquitin ligase complex recognizes phosphorylated p27 and catalyzes its polyubiquitination prior to targeting degradation by the 26 S proteasome. The molecular mechanism underlying p27 recognition and entrance into the SCF complex remains unclear. Here we identified a key amino acid residue in Skp2 that is required for p27 ubiquitination. We showed that Asp-331 in Skp2 mediates physical interaction with Cks1, an essential adapter protein for bringing in phosphorylated p27. In addition, our results suggest that a negatively charged amino acid at this position is important for binding specificity between Cks1 and Skp2. Thus, substrate recognition specificity of a given E3 ubiquitin ligase may be governed by additional interactions between the F-box protein and an accessory protein.

Skp2 is a member of a large family of F-box proteins whose primary function is thought to be mediating ubiquitination of a variety of substrates that are destined for degradation. In addition to the three helices that constitute the F-box motif, Skp2 also contains 10 LRR that form a sickle-shaped concave structure and a 30-amino acid unstructured carboxyl-terminal tail. It has been suggested that the concave LRR of another protein. Grr1, may be involved in recognition of the...
substrate (30). Potential roles of the concave surface of Skp2 in recognition of other proteins have not been defined. Here we provide evidence that the Asp-331 located on the surface of the concave LRR is required for Skp2 recognition of Cks1. Mutation of Asp-331 to alanine negates the interaction. It is unlikely that the effect we observed is due to disruption of the general structure of Skp2. First, Asp-331 → Ala or Asp-328 → Ala/Asp → Ala double mutants are expressed at levels indistinguishable from the wild type Skp2 in vivo. Second, these mutants are still able to bind Skp1 and can assemble into the SCF complex in Hi-Five cells similarly to the wild type protein, indicating mutation of these residues does not appear to affect molecular interactions within the SCF core complex. Third, Skp2 with mutations of Asp-328 and -331 to alanine undergoes self-ubiquitination just as the wild type. Together these provide strong arguments for the structural and functional integrity of Skp2 mutants.

Crystallographic analysis of Cks1 and closely related homologs indicates that the Cks1 family of proteins forms a four-stranded β-sheet surface. They all contain an anion-binding site, which could potentially bind to a phosphate, sulfate, or to acidic residues in proteins (27, 28). Using a site-directed mutagenesis approach, Hershko and coworkers (17, 24) have mapped the potential Skp2 binding sites in Cks1. Ser-41 and Asn-45, Leu-31 and Ala-29 appear to be involved in direct binding to Skp2. These amino acids are located on a convex surface of Cks1 formed by the α2- and α1-helices (24). We have constructed similar mutations in Cks1 in this study. In agreement with the results of Sitry et al. (24), the N45R mutation completely abolishes the interaction between Skp2 and Cks1; however, the S41E mutation displays much higher affinity than previously reported and still is capable of supporting ubiquitination of p27 in vitro at a reduced efficiency (Fig. 5, lane 3). Despite this minor discrepancy, our data are consistent with the view that the α2-helix of Cks1 may be directly involved in binding to Skp2.

Extensive mutagenesis analysis near Asp-331 indicates that this amino acid appears to be the only one that has a significant impact on Skp2-Cks1 interaction. In support of our original hypothesis that the electronegative surface potential near Asp-331 is important for protein-protein interaction, we found that a conservative change of Asp-331 → Glu has apparently no effect on Skp2-Cks1 interaction. It is very likely that this negatively charged amino acid in Skp2 is recognized directly by Cks1. Assuming this could be the case, it is tempting to speculate that the anion-binding site in Cks1 could be involved in recognizing Asp-331. Further experiments are needed to test this hypothesis.

Our identification of the essential residues involved in the Skp2-Cks1 interaction was aided by examination of the surface electrostatic properties of the Skp2 crystal structure utilizing the GRASP program (34). The impact of our mutations on the Skp2 surface electrostatic potential was also investigated by modeling Fig. 8. Although the D328A mutation in Skp2 has a modest and localized effect on the surface electrostatic potential, D331A mutation appears to have a much more dramatic effect, changing the surface potential from negative to positive relative to the rest of the protein surface. (The comparison of surface electrostatic potential is made on the same scale in the modeling). This modeling is consistent with the experimental observation that D331A mutation had a much more pronounced effect on Skp2 function. It would be interesting to test whether this type of analysis is generally applicable to design mutagenesis strategies to dissect the mechanism of protein-protein interaction.

We have identified a critical amino acid that is required for Skp2-Cks1 interaction. It is possible that this negatively charged amino acid is responsible for the specificity of the
interaction. Other regions of Skp2, in particular, hydrophobic amino acids on the surface of Skp2, may provide the main source of binding energy. We are working on identifying additional amino acid residues that are required for the Skp2-Cks1 interaction.

Acknowledgments—We thank Drs. Wade Harper, Michele Pagnao, and Pengbo Zhou for the generous supply of the cDNA clones and reagents used in this study, Jim Goodrich and Ming Lei for advice on baculovirus expression and recombinant protein purification, and members of the Liu laboratory for helpful discussions. We also thank Natalie Ahn, James Goodrich, and Rob Kutchta for critically reading the manuscript.

REFERENCES
1. Harper, J. W. (2001) Curr. Biol. 11, R431–R435
2. Sherr, C. J., and Roberts, J. M. (1999) Genes Dev. 13, 1501–1512
3. Nakayama, K. I., Hatakeyama, S., and Nakayama, K. (2001) Biochem. Biophys. Res. Commun. 282, 855–860
4. Slingerland, J., and Pagnan, M. (2001) J. Cell Physiol. 183, 10–17
5. Sheaff, R. J., Groudine, M., Gordon, M., Roberts, J. M., and Clurman, B. E. (1997) Genes Dev. 11, 1464–1478
6. Vlach, J., Hennecke, S., and Amati, B. (1997) EMBO J. 16, 5334–5344
7. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
8. Pickart, C. M. (2001) Annu. Rev. Biochem. 70, 503–533
9. Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U., and Krek, W. (1999) Nat. Cell Biol. 1, 207–214
10. Carrano, A. C., Eytan, E., Hershko, A., and Pagnan, M. (1999) Nat. Cell Biol. 1, 193–199
11. Zhang, H., Kobayashi, R., Galaktionov, K., and Beach, D. (1995) Cell 82, 915–925
12. Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., and Elledge, S. J. (1996) Cell 86, 263–274
13. Winston, J. T., Koepf, D. M., Zhu, C., Elledge, S. J., and Harper, J. W. (1999) Curr. Biol. 9, 1180–1182
14. 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
15. Zheng, N., Schulman, B. A., Song, L., Miller, J. J., Jeffrey, P. D., Wang, P., Chu, C., Koepf, D. M., Elledge, S. J., Pagano, M., Conaway, R. C., Conaway, J. W., Harper, J. W., and Pavletich, N. P. (2002) Nature 416, 703–709
16. Bartek, J., and Lukas, J. (2001) Nat. Cell Biol. 3, E95–8
17. Ganoth, D., Bornstein, G., Ko, T. K., Larsen, B., Tyers, M., Pagano, M., and Hershko, A. (2001) Nat. Cell Biol. 3, 321–324
18. Saspruch, C., Strohmaier, H., Watson, M., Smith, A. P., Ryan, A., Krek, T. W., and Reed, S. I. (2001) Mol. Cell 7, 639–650
19. Hayles, J., Beach, D., Durkacz, B., and Nurse, P. (1986) Mol. Gen. Genet. 202, 291–293
20. Hadwiger, J. A., Wittenberg, C., Mendenhall, M. D., and Reed, S. I. (1989) Mol. Cell Biol. 9, 2034–2041
21. Reynard, G. J., Reynolds, W., Verma, R., and Deshaies, R. J. (2000) Mol. Cell Biol. 20, 5858–5864
22. Patra, D., and Dunphy, W. G. (1998) Genes Dev. 12, 2549–2559
23. Patra, D., Wang, S. X., Kumagai, A., and Dunphy, W. G. (1999) J. Biol. Chem. 274, 36839–36842
24. Silry, D., Seelig, M. A., Ko, T. K., Ganoth, D., Brevard, S. E., Itzhaki, L. S., Pagano, M., and Hershko, A. (2002) J. Biol. Chem. 277, 42233–42240
25. 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
26. Liu, X., Constantinescu, S. N., Sun, Y., Bogan, J. S., Hirsch, D., Weinberg, R. A., and Lodish, H. F. (2000) Anal. Biochem. 280, 20–28
27. Bourne, Y., Watson, M. H., Hickey, M. J., Holmes, W., Rocque, W., Reed, S. I., and Tainer, J. A. (1996) Cell 84, 863–874
28. Bourne, Y., Watson, M. H., Arvai, A. S., Bernstein, S. L., Reed, S. I., and Tainer, J. A. (2000) Structure Fold. Des. 8, 541–550
29. Graham, T. A., Weaver, C., Mao, F., Kinelman, D., and Xu, W. (2000) Cell 103, 885–896
30. Hsiung, Y. G., Chang, H. C., Pellequer, J. L., La Valle, R., Lanker, S., and Wittenberg, C. (2001) Mol. Cell Biol. 21, 2506–2520
31. Wirbelauer, C., Sutterluty, H., Blondel, M., Gstaiger, M., Peter, M., Reymond, F., and Krek, W. (2000) EMBO J. 19, 5362–5375
32. Zhou, P., and Howley, P. M. (1998) Mol. Cell 2, 571–580
33. Deshaies, R. J. (1999) Annu. Rev. Cell Dev. Biol. 15, 435–467
34. Nicholls, A., Sharp, K. A., and Honig, B. (1991) Proteins 11, 281–296
A Negatively Charged Amino Acid in Skp2 Is Required for Skp2-Cks1 Interaction and Ubiquitination of p27 Kip1

Wei Wang, Dana Ungermannova, Lin Chen and Xuedong Liu

J. Biol. Chem. 2003, 278:32390-32396.
doi: 10.1074/jbc.M305241200 originally published online June 16, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M305241200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 34 references, 11 of which can be accessed free at http://www.jbc.org/content/278/34/32390.full.html#ref-list-1