Three Different Binding Sites of Cks1 Are Required for p27-Ubiquitin Ligation*

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Previous studies have shown that the cyclin-dependent kinase (Cdk) inhibitor p27Kip1 is targeted for degradation by an SCFSkp2 ubiquitin ligase complex and that this process requires Cks1, a member of the highly conserved Suc1/Cks family of cell cycle regulatory proteins. All proteins of this family have Cdk-binding and anion-binding sites, but only mammalian Cks1 binds to Skp2 and promotes the association of Skp2 with p27 phosphorylated on Thr-187. The molecular mechanisms by which Cks1 promotes the interaction of the Skp2 ubiquitin ligase subunit with p27 remained obscure. Here we show that the interaction of Cks1 with p27 requires the phosphorylation on Thr-187. Phosphorylation of some cell cycle regulatory proteins by Cdks has been proposed that by docking Cdks to partially phosphorylated proteins, Cks/Suc1 may promote the multiple phosphorylation of the molecular mechanisms of p27 degradation is of considerable interest.

Previous work has shown that p27 is degraded by the ubiquitin pathway (3). The ubiquitination and degradation of p27 require its phosphorylation on Thr-187 (4, 5). Phosphorylated p27 is recognized by an SCF (Skp1-Cullin 1-F box protein) ubiquitin ligase complex, which contains Skp2 (S-phase kinase-associated protein 2) as the specific substrate-binding F box protein (6–8). SCF complexes are a large family of ubiquitin ligases, whose variable F box protein subunits recognize a variety of phosphorylated substrates (9). Skp2 is unique among known mammalian F box proteins in that its levels fluctuate in the cell cycle, being very low in G1/G0, and increasing in entry of cells into the S-phase (10, 11). Skp2 is oncogenic (12, 13), and high levels of Skp2 have been observed in several types of human cancers (14, 15). The crystal structure of the SCFSkp2 complex has been solved (16, 17).

An interesting feature of the SCFSkp2 ubiquitylating machinery is that it requires an accessory protein, Cks1 (Cdc kinase subunit 1/18, 19). Cks1 belongs to the Cks/Suc1 (suppressor of cdc2) family of small (9–18 kDa) proteins, conserved in eukaryotic evolution. They were originally discovered in fission (20) and budding (21) yeast as essential gene products, which interact with Cdks. They are involved in several cell cycle transitions, but the molecular basis for their action remained obscure (reviewed in Ref. 22). The crystal structure of the yeast Suc1/Cks proteins (23, 24) and of the two human orthologues, Cks1 (25, 26) and Cks2 (27), showed that they all share a four-stranded β-sheet surface involved in binding to Cdk (26). In addition, they all have an anion-binding site, which can bind phosphate, sulfate, or acidic residues in proteins (22, 26). It has been proposed that by docking Cdks to partially phosphorylated proteins, Cks/Suc1 may promote the multiple phosphorylation of these proteins (22). Indeed, Cks-assisted multiphosphorylation of some cell cycle regulatory proteins by Cdks has been observed (28–30).

The role of mammalian Cks1 in the ubiquitylation and degradation of p27 was established by both biochemical reconstitution (18) and gene knockout (19) approaches. The requirement for Cks1 appears to be specific for the SCFSkp2 complex, as no such requirement has been found with some other SCF ubiquitin ligases (18). On the other hand, it is not specific for p27, because the ubiquitylation of cyclin E by SCFSkp2 also

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The abbreviations used are: Cdk, cyclin-dependent kinase; Cks, Cdk2 kinase subunit; DTT, dithiothreitol; SCF, Skp1-Cullin1-F box protein; Skp1 and Skp2, S-phase kinase-associated proteins 1 and 2; Suc1, suppressor of cdc2; IVTT, In vitro transcription and translation.

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Replication. The levels of p27 are high in quiescent cells. Following stimulation by mitogenic agents, p27 is rapidly degraded, allowing Cdk2 action to drive cells into the S-phase (1). p27 is destabilized in human cancers (2). Thus, the understanding of the molecular mechanisms of p27 degradation is of considerable interest.
requires Cks1.2 Interestingly, levels of Cks1 mRNA (21) and protein also fluctuate in the cell cycle in parallel with levels of Skp2. This may provide an additional control mechanism for the timely degradation of substrates of the SCFSkp2 complex in the cell cycle. It was surprising to find that Cks1 cannot be replaced for p27-ubiquitin ligation by the closely related human orthologue Cks2 (19, 18), even though it is functionally similar to Cks1 in other processes (21). This was explained by the observation that Cks1, but not Cks2, binds to Skp2 (18, 19). Furthermore it was found that Cks1 promoted the binding of Skp2 to Thr-187-phosphorylated p27 (18, 19). However, the molecular mechanisms by which Cks1 facilitates the interaction of the Skp2 ubiquitin ligase subunit to its substrate remained obscure. Although it was known that Thr-187-phosphorylated p27 has to be presented to the ubiquitin ligase in trimeric complex with Cdk2-cyclin A/E (4), it was proposed that the action of Cks1 is independent of its binding to Cdk (19). In the present investigation, we have used site-directed mutagenesis to identify the Skp2-binding site of Cks1 and to show that all three sites of Cks1 are required for its action to promote Skp2-p27 binding. By the use of a phosphorylated C-terminal peptide of p27 as a model substrate, we could distinguish between different steps in which each binding site of Cks1 contributes to the high affinity binding of this ubiquitin ligase subunit to its substrate protein.

EXPERIMENTAL PROCEDURES

Proteins—His6-Skp1/Skp2, his6-Cul1/Roc1, and his6-cyclin E/Cdk2 were produced by co-infection of 5B insect cells with baculoviruses encoding the corresponding proteins and were purified by nickel-agarose chromatography, as described previously (4, 6). The approximate concentrations of these preparations are as follows (in mM): Cul1/Roc1, 1; Skp1, 7; Skp2, 0.4; cyclin E/Cdk2, 0.5. Site-directed mutagenesis of Cks1 was carried out by the Quikchange kit (Stratagene). Mutations were confirmed by DNA sequencing in all cases. All mutants to be expressed in bacteria were constructed as his6-Cks1 fusions, and all mutants to be expressed by in vitro transcription-translation or by transient transfection of cells were constructed as Cks1-FLAG fusions in pcDNA3. Control experiments showed that the C-terminal FLAG extension did not impair the activity of wild-type Cks1 in p27-ubiquitin ligation. Cks1 proteins were expressed in Escherichia coli BL21 DE3 strain and were purified by nickel affinity chromatography, removal of the His tag by thrombin cleavage and size exclusion chromatography, as described (31). Because of the construction and thrombin cleavage procedure, all bacterially expressed Cks1 proteins had two extra amino acid residues (GS) at the N terminus. Control experiments showed that the activity of wild-type Cks1 in p27 ubiquitylation was not affected by the GS N-terminal extension. All bacterially expressed proteins were >95% homogenous, as judged by SDS-PAGE/Comassie staining and mass spectrometry. The thermodynamic stability of all bacterially expressed Cks1 proteins used in this study was essentially similar to that of wild-type Cks1, as estimated by equilibrium unfolding (31).

35S-Labeled Cks1-FLAG proteins, p27, Skp2, and Cdk2 were produced by in vitro transcription-translation, using the Tnt Quick kit (Promega) and [35S]methionine (Amersham Biosciences). The following proteins were prepared as described previously: ubiquitin-activating enzyme (32), his6-Cdc34 (18), methylated ubiquitin (33), and ubiquitin aldehyde (34).

Binding to Skp2—The reaction mixture contained the following in a volume of 40 ml: 40 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 10% (v/v) glycerol, 1 mM DTT, 10 mM phosphocreatine, 100 mM KCl, 10% (v/v) glycerol, 1 mM DTT, 10 mM phosphoreactivase, 100 μM/ml creatine kinase, 0.5 mM ATP, 1 mg/ml soybean trypsin inhibitor, 1 mM ubiquitin aldehyde, 1 mg/ml methylated ubiquitin, 1 pmol of ubiquitin-activating enzyme, 50 pmol of Cdc34, 0.15 μM Skp2/Skp1, 0.05 μM Cdc34, 0.05% (v/v) DMSO, 0.3 μM of unlabeled p27, and Cdk2 protein as specified. Methylated ubiquitin was used in this assay because its conjugates with p27 are more resistant to degradation by the 26 S proteasome than those of native ubiquitin (4, 6). Following incubation at 30 °C for 60 min, samples were subjected to SDS-PAGE. Results were quantified by PhosphorImager analysis. A small amount of p27-ubiquitin conjugates formed without added Cks1 was subtracted, and the results were expressed as the percentage of 35S-p27 converted to ubiquitin conjugates. Assays were carried out in the range linear with Cks1 concentrations, which were up to ~40% of p27 ligated to ubiquitin.

Binding Assays—To determine the binding of Cks1 to Skp2, 35S-labeled Cks1 (1 μM) was incubated with Skp2/Skp1 (1 μM) in a 10-μl reaction volume containing 40 mM Tris-HCl (pH 7.6), 2 mM mg/ml bovine serum albumin, 5 mM MgCl2, and 1 mM DTT. When the binding to Skp2 of different 35S-labeled Cks1 proteins was compared, the assay was normalized by adding mutant proteins at amounts containing radioactive similarity to that of 1 μM of wild-type 35S-Cks1. Following incubation at 30 °C for 30 min, 6 μl of Affi-prep protein A beads linked to rabbit polyclonal anti-Skp2 antibodies (18) were added with 30 μl of phosphoantibodies. The samples were rotated for 2 h, and then the beads were washed 4 times with 1 ml portions of RIPA buffer (35). Following elution with SDS electrophoresis sample buffer, samples were subjected to SDS-PAGE and PhosphorImager analysis. Results were expressed as the percentage of labeled protein bound to beads. The binding of 35S-p27 to Skp2 was determined in a similar assay, except that the reaction mixture contained 35S-p27 (1 μM), Cdk2/cyclin E (1 μM), Skp2/Skp1 (1 μM), and ATP, phosphocreatine, and creatine phosphokinase at concentrations similar to those described for the p27-ubiquitin ligation assay. Where indicated, 10 μM of wild-type or mutant bacterially expressed Cks1 proteins were supplemented. Subsequent immunoprecipitation with anti-Skp2 antibodies was as described for the former assay.

To determine the binding of 35S-Cdk2 to Cks1 proteins, bacterially expressed wild-type or mutant Cks1 proteins were covalently linked to cyanogen bromide-activated Sepharose 4B (Sigma) at ~1.5 mg/ml beads. Samples of 10 μl of Cks1 beads were mixed with 20 μl of a reaction mixture similar to that described for the binding of 35S-Cks1 to Skp2/Skp1 but with 35S-Cdk2 instead of the latter two components. Following rotation at room temperature for 60 min, beads were washed 4 times with RIPA buffer as above.

To determine the binding of 35S-Skp2 or 35S-Cks1 to C-terminal peptides of p27, we have used two synthetic peptides (Sigma) of sequence similar to that of the 19 C-terminal amino acid residues of p27. These were NAGSVEQTPKKPGLRRRQT for the unphosphorylated peptide, and a similar sequence with phosphorylated Thr at position 8 (corresponding to Thr-187 in full-length p27) for the phosphopeptide. The peptides were covalently linked to cyanogen bromide-activated Sepharose 4B at a concentration of 7 mg/ml beads. To determine the binding of Skp2 to p27 peptide beads, 35S-Skp2 (3 μl) was first incubated in the presence or absence of bacterially expressed Cks1 proteins (at concentrations indicated in the figures), in a 10-μl reaction mixture containing 40 mM Tris-HCl (pH 7.6), 2 mM mg/ml bovine serum albumin, 5 mM MgCl2, 1 mM DTT, and 1 μg okadaic acid. Okadaic acid was added to minimize dephosphorylation of the phosphorylated peptide by phosphatases present in reticulocyte lysates. Following incubation at 30 °C for 15 min, 10 μl of Skp2 peptide or p27 phosphopeptide beads were added. Samples were rotated at 4 °C for 2 h, washed 4 times in RIPA buffer, and processed as described above for the other binding assays. The binding of 35S-Csk1 to p27 peptide or phosphopeptide beads was determined by a similar procedure, except that 35S-labeled Cks1 proteins (normalized to contain radioactivity similar to that of 1 μM of wild-type 35S-Cks1) were incubated with or without unlabeled Skp2/Skp1 (1 μM). All results were quantified and expressed as the percentage of 35S-labeled protein bound to beads.

RESULTS

Identification of Amino Acid Residues of Cks1 Involved in Binding to Skp2—It was shown previously (18, 19) that human Cks1 is required for p27-ubiquitin ligation by the SCFSkp2 complex, binds to Skp2, and promotes the binding of Thr-187-phosphorylated p27 to Skp2. A closely related human homologue, Cks2, which is 81% identical and 85% similar to Cks1, has none of these activities. We have therefore set out to identify the Skp2-binding site of Cks1 by mutagenesis of specific amino acid residues in which non-conservative substitutions between Cks1 and Cks2 occur. As shown in Fig. 1A (red letters), there are 12 such amino acid residues in the 79-amino acid human Cks1 proteins. We have mutated most of these amino acid residues in Cks1 to those present in Cks2; the resulting derivatives were designated “Cks1→Cks2 mutants.” Because the crystal structures of human Cks1 (25, 26) and
Binding Sites of Cks1 for p27-Ubiquitin Ligation

p27-ubiquitin ligation was determined as described under "Experimental Procedures" in the presence of 3 nm bacterially expressed and purified Cks1 proteins (left column) or 0.1 μl (normalized by 35S-radioactivity) of in vitro translated (IVTT) Cks1 proteins (right column). Results are expressed as the percentage of activity obtained with a similar amount of wild-type Cks1. ND, not determined.

Table I

| Mutations                          | Purified | IVTT |
|------------------------------------|----------|------|
| Δ75–79                             | ND       | 100  |
| E16H                               | ND       | 104  |
| K26R                               | 75       | ND   |
| L31Q                               | 75       | 74   |
| A29S, L31Q                         | 48       | 57   |
| S41E                               | 5        | 7    |
| N45R                               | 1        | 3    |
| E63Q                               | 12       | 15   |
| E63A                               | 24       | ND   |
| P62A                               | 47       | ND   |
| K11A                               | 80       | ND   |
| R20A                               | 2        | 0    |
| R20K                               | ND       | 31   |
| W54F                               | 29       | 31   |
| R71A                               | 13       | 10   |

Cks2 (27) are very similar, it seemed reasonable to expect that these mutations would nor impair much protein structure and stability. Indeed, the thermodynamic stability of all Cks1 → Cks2 mutants, estimated by urea-induced equilibrium denaturation (31), was not significantly decreased as compared with that of wild-type Cks1 (data not shown). Most mutants were expressed by the following two methods: (a) bacterial expression, followed by purification to >95% homogeneity; (b) in vitro transcription and translation (IVTT) of 35S-labeled Cks1 derivatives in reticulocyte lysates. The second method was required to produce radiolabeled Cks1 mutants, required for some of the assays, and to confirm the results obtained with bacterially expressed Cks1 proteins.

We have first examined the influence of different Cks1 → Cks2 mutants on p27-ubiquitin ligation in the presence of purified components of the SCF<sup>Skp2</sup> complex. A representative experiment is shown in Fig. 1B. It may be seen that ubiquitylation of 35S-labeled p27, assayed by its conversion to higher molecular weight derivatives, was strongly stimulated by wild-type Cks1 but to a much lesser extent by certain Cks1 → Cks2 mutants, most notably the S41E and N45R mutants. Essentially similar results were obtained with either bacterially expressed, purified Cks1 derivatives (Fig. 1B, lanes 1–6) or with the same mutants produced by IVTT (lanes 7–12), indicating the validity of results obtained with both types of Cks1 preparations. The results of similar experiments with a variety of Cks1 → Cks2 mutants are summarized in Table I, part A. All results were obtained with low, limiting concentrations of Cks1, in the linear range of the p27-ubiquitin ligation assay. Results were quantified and were expressed as the percentage by protein kinase Cdk1/cyclin B. Unphosphorylated cyclosomes were partially purified from extracts of HeLa cells synchronized in the S-phase and were incubated with MgATP and okadaic acid, as described (42). Where indicated, 250 units of Cdk1/cyclin B (immunodepleted of residual Suc1, as described (30)) or 50 nm of the different Cks1 proteins were supplemented. Following incubation at 30 °C for 30 min, samples were separated on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and blotted with a monoclonal anti-Cdc27 antibody (Transduction Laboratories). Cdc27-P indicates the position of multiphosphorylated Cdc27.
of activity obtained with a similar concentration of wild-type Cks1. The small amount of p27-ubiquitin conjugates formed without Cks1 (see Fig. 1B, lanes 1 and 7) was subtracted. We have first deleted the four C-terminal amino acid residues of Cks1, because three of these are different between Cks1 and Cks2 (Fig. 1A). However, the resulting ΔTVT–79 mutant was as active as wild-type Cks1 in p27 ubiquitylation (Table I, part A). Similarly, mutations in residues 16, 26, and 27 had either no influence or only moderately decreased activity. There was some decrease in p27-ubiquitin ligation activity of the L31Q mutant, and this was more noticeable with the A29S, L31Q double Cks1→Cks2 mutant. Drastic decrease of activity was observed with the S41E and N45R Cks1→Cks2 mutants (Fig. 1B and Table I, part A).

Human Cks1 and Cks2 are functionally similar in their ability to replace the homologous protein in the yeast Saccharomyces cerevisiae (21). The only known functional difference between human Cks1 and Cks2 is the ability of the former to bind to Skp2 and to promote Skp2-mediated ubiquitylation. Therefore, it seemed reasonable to assume that the defect of specific Cks1→Cks2 mutants in p27-ubiquitin ligation activity is due to their inability to bind to Skp2. We have tested this notion by direct assay of the binding of different, 35S-labeled Cks1 mutants to Skp2. This assay included incubation of 35S-labeled Cks1 derivatives in the presence or absence of Skp2/Skp1, followed by immunoprecipitation with an antibody directed against Skp2. We used S2p2/Skp2 complex instead of Skp2, because without Skp1, recombinant Skp2 is not expressed well in a soluble form in insect cells (6). By using this assay, it was shown previously (18) that wild-type Cks1 binds strongly to Skp2-Skp1 complex but not to Skp1. We now find that the S41E and N45R Cks1→Cks2 mutants, which are defective in p27-ubiquitin ligation (Table I), are also greatly decreased in binding to Skp2 (Fig. 1C). The A29S, L31Q double Cks1→Cks2 mutant, which has partially decreased activity in p27-ubiquitin ligation, has also partially decreased binding to Skp2. The specificity of the binding assay is indicated by the observation that the E63Q and R20A mutants of Cks1, which are severely affected in the Cdk-binding and anion-binding sites, respectively (see below), bind well to Skp2 (Fig. 1C).

Similar results were observed in vivo, following expression of various Cks1 mutants in 293T cells; binding was abolished with the S41E and N45R mutants but not with the R20A mutant (Fig. 1D and data not shown). These results suggest that amino acid residues 41 and 45, and less essentially residues 29 and 31, are parts of the Skp2-binding site of Cks1. These residues are located at or near α2- and α1-helices of Cks1 (26).

It was possible that drastic loss of function of the S41E and N45R mutants in the above assays was due to some drastic change in protein structure induced by the mutations. We therefore tested the activity of these mutants in a different function of Cks proteins. Various Cks1/Suc1 proteins stimulate Cdk-dependent multiple phosphorylation of some proteins, such as subunits of the APC/cyclosome. This is possibly due to Cks-mediated increase in the binding of Cdk to partially phosphorylated substrate protein (29, 30). As shown in Fig. 1E, wild-type Cks1 stimulated the multiphosphorylation of the Cdc27 subunit of the cyclosome by protein kinase Cdk1/cyclin B, as indicated by shift to slower migrating disperse forms in SDS-PAGE. The S41E and N45R Cks1→Cks2 mutants were nearly as effective as wild-type Cks1 in stimulation of multiple phosphorylation of Cdc27. By contrast, mutations in the Cdk-binding site (E63Q) or anion-binding site (R20A) of Cks1 did not promote significantly multiple phosphorylation of Cdc27 (Fig. 1E), suggesting that the latter two sites are required for this process. These results indicate that Cks1→Cks2 mutants in residues 41 and 45 are selectively affected in Skp2-related functions but are functional in another Cks-stimulated process.

Role of Cdk-binding Site of Cks1 in p27-Ubiquitin Ligation—p27 binds tightly to Cdk2/cyclin A/E (36). It has been shown that p27 phosphorylated on Thr-187 is presented as a substrate for the ubiquitin ligase only when it is in trimeric complex with Cdk2-cyclin A/E (4). Cks1, like other Cks/Suc1 proteins, has a Cdk-binding surface that includes all four β-strands (22). Thus, it appeared reasonable to assume that the binding of Cks1 to the trimeric complex via its Cdk-binding site may increase the affinity of SCF(Skp2) to the phosphorylated p27 substrate. However, it has been suggested by Spruck et al. (19) that the action of Cks1 to stimulate p27 ubiquitylation is independent of Cdk binding. This suggestion was based on the observation that Cks1 E63Q mutant, which is defective in binding to Cdk2 (26), stimulated p27-ubiquitin ligation by the purified SCF(Skp2) complex only slightly less efficiently than did wild-type Cks1 (19). We have re-examined this problem, using this and other mutants in the Cdk-binding site of Cks1. We have first tested the effects of these mutations on binding to Cdk2. In the experiment shown in Fig. 2A, the binding of 35S-labeled Cdk2 to various immobilized Cks proteins was tested. The binding of Cdk2 to the E63Q mutant of Cks1 was greatly reduced, as compared with the wild-type protein. Other mutations in Glu-63, such as E63A (Fig. 2A) or E63G (not shown), and in the neighboring conserved Pro-62 residue of the Cdk-binding site, also decreased Cdk2 binding, although not as drastically as did the E63Q mutation. The specificity of the assay was indicated by the observation that 35S-Cdk2 bound almost normally to R20A and W54F anion-binding site mutants of Cks1 (Fig. 2A).

We have next determined the effects of these mutations in the Cdk-binding site of Cks1 on p27-ubiquitin ligation. As shown in Table I, part B, activity was greatly reduced, although not absent, with the E63Q mutant. Similar results were obtained with either bacterially expressed, purified E63Q or with the same mutant produced by IVTT, in the range of 10–15% of the activity of wild-type Cks1. Activities of other Cdk-binding site mutants were somewhat higher, in correspondence with their higher residual binding to Cdk2 (compare Table I, part B with Fig. 2A). Trying to explain the difference between our results and those reported by Spruck et al. (19) for the same E63Q Cks1 mutant, we noted that their assay was not done in the range linear with enzyme or Cks1 concentrations; essentially all free p27 was converted to ubiquitin conjugates with wild-type Cks1, and only a small amount of free p27 remained with the E63Q mutant (see Ref. 19, Fig. 6A, lanes 6 and 7). By contrast, we carried out all p27-ubiquitin ligation assays in the range linear with Cks1 concentrations, in the presence of slight excess of SCF(Skp2) components (see “Experimental Procedures”). We have therefore examined the effects of increasing Cks1 or SCF(Skp2) concentrations on the apparent efficiency of E63Q Cks1 mutant in p27-ubiquitin ligation. When the concentrations of wild-type and E63Q mutant Cks1 were increased in the presence of a limiting amounts of SCF(Skp2) enzyme, a marked difference in activities remained even at high Cks1 concentrations (Fig. 2B, left panel). However, when the concentration of SCF(Skp2) enzyme was increased in the presence of high concentrations of Cks1 proteins, the difference between wild-type and E63Q Cks1 in the formation of p27-ubiquitin conjugates was minimized at high enzyme concentrations (Fig. 2B, right panel). This was due to the fact that with wild-type Cks1, essentially all p27 was converted to ubiquitin conjugates with a moderate increase in enzyme concentration, although with the E63Q mutant the formation of ubiquitin conjugates continued to rise with increasing enzyme concentrations. These observations underscore the importance of determination of the
activity of mutant proteins in the linear range of enzymatic assay.

We examined further the role of the Cdk-binding site of Cks1 to promote the interaction of phosphorylated p27 with Skp2. In this assay, 35S-labeled p27 was first phosphorylated by incubation of Cdk2/cyclin E in the presence of ATP, and then binding of 35S-p27 (in trimeric complex with Cdk2-cyclin E) to Skp2 was estimated by immunoprecipitation with a Skp2-specific antibody. By using this assay, we have found previously (18) that wild-type Cks1 greatly increased the association of phosphorylated p27 to Skp2. A representative experiment on the effects of different types of Cks1 mutants on p27-Skp2 interaction is shown in Fig. 2C, and results from several quantitative assays are summarized in Table II. As could be expected, the binding of phosphorylated p27 to Skp2 was greatly diminished with Cks1→Cks2 mutants defective in Skp2 binding, such as the S41E,N45R and A29S,L31Q double mutants (Fig. 2C, lanes 4–6, and Table II, part A). The binding of phosphorylated p27 to Skp2 was also markedly decreased with Cdk2-binding site mutants such as E63Q, E63A, and P62A (Fig. 2C, lane 3 and Table II, part B). It is notable that the binding of p27 to Skp2 with the Cdk-binding site mutants is more drastically decreased than the residual activity of the same mutants in p27-ubiquitin ligation (cf. Table II, part B, with Table I, part B). This difference may be due to the high affinity required for the demonstration of p27-Skp2 binding in this assay. These results suggest that the high affinity binding of phosphorylated p27 (in complex with Cdk2-cyclin E) to Skp2 requires both Cdk2- and Skp2-binding sites of Cks1.

Involvement of Anion-binding Site in Cks1 Action—An anion-binding site is highly conserved in all known Cks1/Cdc5 proteins. It was discovered in crystallographic studies of these proteins as a site that binds anions, such as sulfate, vanadate, phosphate, chloride ions, or to glutamic acid residues in dimeric Cks structures (23–26). We have tested the possible role of the anion-binding site of Cks1 in p27-ubiquitin ligation by site-directed mutagenesis of its four highly conserved amino acid residues (see Refs. 22 and 26): Lys-11, Arg-20, Trp-54, and Arg-71. As shown in Table I, part C, all these mutations also decreased activity in p27-ubiquitin ligation, although different residual activities were observed with different anion-binding site mutants. Thus, p27-ubiquitin ligation activity was most severely affected in the R20A mutant; even the conservative R20K mutation in this site resulted in considerable loss of activity. Some residual activity was observed with the R71A mutant, more with the W54F mutant, and only a slight decrease in activity was observed with the K11A mutant. It thus appears that the contribution of the different amino acid residues in the anion-binding site of Cks1 on SCFpromoted p27-ubiquitin ligation is not equal. Essentially similar results were obtained with either bacterially expressed, purified anion-binding site mutants of Cks1 or with the same mutants expressed by IVTT (Table I, part C). The anion-binding site of Cks1 is required for the binding of phosphorylated p27 to Skp2, as shown by the considerable decrease in this interaction with the anion-binding site mutants of Cks1 (Fig. 2C, lanes 7 and 8, and Table II, part C). It is notable that the order of the magnitude of residual activities of these mutants in p27-Skp2 binding is similar to that in p27-ubiquitin ligation (cf. Table II, part C, with Table I, part C), suggesting a similar role of the anion-binding site amino acid residues in these processes. The R20A mutant, which is most severely affected in these functions, is not significantly impaired in direct binding to Skp2 (Fig. 1C, lane 7), suggesting that the anion-binding site is involved in another interaction necessary for p27-ubiquitin ligation.
To examine further the specificity of the effects of the above-described mutations in the different binding sites of Cks1, we have also tested the possible effects of a variety of mutations in regions of Cks1 different from its three binding sites. The following Cks1 point mutants had close to wild-type activity in p27-ubiquitin ligation: 16V, S9A, E18A, V22A, K30G, V32A, S39A, S51A, V55A, and H65A (data not shown). A notable exception was a Y8A mutant that had greatly decreased activity (∼15% of wild-type Cks1). Y8A had no significant folding defect, as estimated by equilibrium unfolding (data not shown). Amino acid residue Tyr-8 is spatially very close to the anion-binding site of Cks1 (26).

Roles of the Different Binding Sites in the Interaction of Cks1-Skp2 Complex with C-Terminal Phosphorylated Peptide of p27—In order to gain further insight into the roles of the different binding sites of Cks1 in the interactions of the SCF(Skp2) complex with the phosphorylated p27 substrate, we made use of a synthetic peptide corresponding to the C-terminal 19 amino acids of p27 with phosphorylated threonine at position 187. We have previously observed that wild-type Cks1 increased the binding of 35S-labeled Skp2 to a similar, immobilized p27 phosphopeptide (18). Examination of the dose-response curve of this interaction (Fig. 3A) showed that it required concentrations of Cks1 much higher than those effective in p27-ubiquitin ligation or in binding to Skp2 of phosphorylated full-length p27 in the presence of Cdk2/cyclin E. Thus, the concentration of wild-type Cks1 required for half-maximal stimulation of the binding of Skp2 to the C-terminal p27 phosphopeptide was ∼50 versus ∼5 nM for the latter processes. This decreased affinity for Cks1 may be due to steric occlusion, caused by immobilization of peptide on beads, or due to the lack of the Cdk-binding region of p27, which is not present in the C-terminal peptide, and possibly to the lack other regions of p27 required for high affinity interactions. By using suitably high concentrations of mutant Cks1 proteins, we observed that the stimulation of binding of Skp2 to phosphopeptide requires the Skp2-binding site, because there was no significant stimulation of binding (over that observed without added Cks1) with the S41E and N45R mutants (Fig. 3B). As may be expected, the Cdk-binding site E63Q Cks1 mutant is fully active in this assay (Fig. 3B, lane 5), because Cdk binding is not involved in this low affinity interaction. On the other hand, the anion-binding site of Cks1 is still required, as indicated by the observations that the R20A mutant does not stimulate significantly the binding of 35S-Skp2 to the p27 phosphopeptide, and the activity of the W54F mutant is significantly reduced as compared with that of the wild-type protein (Fig. 3B, lanes 6 and 7).

We next examined the opposite binding reaction and found that the binding of 35S-Cks1 to p27 phosphopeptide was also greatly increased by Skp2/Skp1 (Fig. 3C, lane 4). A control incubation showed that binding to non-phosphorylated peptide was only slightly stimulated by Skp2/Skp1 (Fig. 3C, lane 2). The Skp2-stimulated binding of Cks1 to phosphopeptide requires a functional Skp2-binding site of Cks1, as indicated by the very low binding of 35S-labeled S41E and N45R Cks1 mutants, and significant decrease of binding of the A29S,L31Q double mutant (Fig. 3C, lanes 5–7). Here again, Skp2-assisted binding of Cks1 to p27 phosphopeptide does not require a functional Cdk-binding site, as indicated by the normal binding of the E63Q mutant (lane 8). However, it does require a functional anion-binding site, indicated by the lack of binding of 35S-labeled R20A mutant, and the reduced binding of W54F mutant (Fig. 3C, lanes 9 and 10). It is concluded that Cks1 and Skp2 mutually assist each other’s binding to the C-terminal region of phosphorylated p27, and these interactions require functional Skp2-binding and anion-binding sites of Cks1. A likely explanation is that the Cks1-Skp2 complex binds more tightly to the phosphopeptide than each separate component, either by conformational change in some component(s) or by the formation of a joint substrate-binding site. The anion-binding site may bind directly to the phosphate group of p27 or to some other anionic group essential for these interactions (see “Discussion”).

**DISCUSSION**

Although the role of Cks1 to promote the binding of p27 to Skp2 has been established, the molecular mechanisms of this process remained unknown (reviewed in Refs. 37 and 38). In this study we have used site-directed mutagenesis to map the Skp2-binding site of Cks1 and to assess the role of this and of other binding sites of Cks1 in SCF(Skp2)-mediated p27-ubiquitin ligation. Fig. 4A shows the amino acid residues of the three binding sites of Cks1, and Fig. 4B shows the location of these residues in the structure of this protein. As may be seen, residues Ser-41, Asn-45, Leu-31, and Ala-29 of the Skp2-binding site are located on a convex surface of Cks1, composed of parts of the α2-helix, the α1-helix, and their immediate vicinity. Fig. 4B also shows that the Skp2-binding surface of Cks1 (red) is well separated from the Cdk-binding surface (green).
Our results indicate that all three binding sites of Cks1 are involved in its action to promote SCF<sub>Skp2</sub>-mediated p27-ubiquitin ligation. This is suggested by the effects of mutations in all three sites on p27-ubiquitin ligation (Table I) and on similar effects of these mutations on the binding of Cdk-bound, phosphorylated p27 to Skp2 (Table II). That the effects of these mutations are not secondary to some impairment of the general structure of the Cks1 protein is suggested by the following observations. (a) All mutants used in this study had thermodynamic stabilities comparable with that of wild-type Cks1 (data not shown). (b) Mutants produced either by bacterial expression or by in vitro translation in reticulocyte lysates had similar activities (Table I). (c) The S41E and N45R mutants, which are nearly inactive in all Skp2-related functions, have close to wild-type activity in the promotion of multiphosphorylation of the Cdc27 subunit of the cyclosome/APC, a process that requires the action of the two other sites of Cks1 (Fig. 1E).

Further insight into the mechanisms by which Cks1 promotes the interaction of Skp2 with the substrate was gained by the use of the C-terminal phosphorylated peptide of p27 as the model substrate. Cks1 and Skp2 mutually promote the binding of each other to the phosphopeptide, and both processes require functional Skp2-binding and anion-binding sites of Cks1 (Fig. 3, B and C). These findings indicate that the affinity of the Skp2-Cks1 complex for the C-terminal region of Thr-187-phosphorylated p27 is much higher than that of each separate component. Based on these observations and on other data presented in this paper, we propose the model shown in Fig. 4C for the interactions by which Cks1 promotes the high affinity binding of the phosphorylated p27 substrate to the SCF<sub>Skp2</sub> ubiquitin ligase. The binding of Cks1 to Skp2 via the Skp2-binding site of Cks1 (Step 1) creates an initial substrate-binding site, which interacts with the C-terminal region of phosphorylated p27 (Step 2). This second process requires the anion-binding site of Cks1; the possible roles of the anion-binding site in this interaction are discussed below. The affinity of the Skp2-Cks1 complex to p27 is further increased by the binding of Cks1 to Cdk2/cyclin E (with which p27 is associated) via the Cdk-binding site of Cks1 (Step 3). The following evidence suggests the involvement of Cdk binding in this process. (a) Phosphorylated p27 is a good substrate for the ubiquitin ligase only in trimeric complex with Cdk2/cyclin A/E (4). (b) The rate of p27-ubiquitin ligation by the SCF<sub>Skp2</sub> complex is strongly reduced (although not absent) when wild-type Cks1 is replaced by mutants defective in Cdk binding, such as the E63Q Cks1 mutant, produced either in bacteria or by IVTT (Table I). Previous observations from another laboratory (19) showing only slight reduction in this process with the same mutant were presumably due to the use of a large excess of SCF<sub>Skp2</sub> enzyme (Fig. 2B). (c) The binding of full-length, Thr-187- phosphorylated p27 to Skp2 is greatly reduced by mutations in the Cdk-binding site of Cks1 (Fig. 3C and Table II, part B). By contrast, the binding of the C-terminal phosphopeptide of p27 to Skp2 does not require a functional Cdk-binding site of Cks1 (Fig. 3B). It is noteworthy that the concentrations of Cks1 required for the binding of the phosphopeptide to Skp2 are ~10-fold higher than those required for the binding of full-length, phosphorylated p27 to Skp2 in the presence of Cdk2/cyclin E (Fig. 3A). The increased affinity of Cks1 for the latter interaction may reflect the formation of a tight complex consisting of Cks1, Skp2, phosphorylated p27, and Cdk2/cyclin E, strengthened by the additional contact between Cks1 and Cdk2. We suggest that although the initial binding of the Skp2-Cks1 complex to the C-terminal region of phosphorylated p27 does not require binding to Cdk, the subsequent binding of Cks1 to Cdk2 further increases enzyme-substrate interaction and thus facilitates the efficiency of p27 ubiquitylation. It should be noted that the order of events may be different from that shown in Fig. 4C, so that Cks1-Cdk binding may precede the other interactions.

The mechanism by which the interaction between Cks1 and Skp2 creates the initial substrate-binding site remains to be

![Fig. 4. The three binding surfaces of Cks1 and their proposed roles in Skp2-p27 interaction.](image)

A. The amino acid residues of the Skp2-binding (red), Cdk-binding (green), and anion-binding (blue) sites of human Cks1 are indicated. B. The three binding surfaces in the structure of Cks1 are shown in colors similar to those in A. The Skp2-binding site (red) and the Cdk-binding site (green) are on opposing surfaces of the protein. C. Proposed model for the role of the different sites of Cks1 in promoting the binding of Skp2 ubiquitin ligase subunit to phosphorylated p27 substrate. See the text. Cks1 may also bind to Cdk2 prior to the formation of the Skp2-Cks1 complex. The schematically indicated conformational changes in Skp2 and Cks1 are possible but not necessary for the model.
elucidated. It is possible that binding to Cks1 induces a conformational change in Skp2, which exposes a substrate-binding site of Skp2, as proposed by Spruck et al. (19). It is also possible, however, that a conformational change is induced in Cks1, which increases its affinity to phosphorylated p27. A further possibility is that the initial substrate-binding site of the Skp2-Cks1 complex is formed by adjacent surfaces of both Skp2 and Cks1. Such extended surface of a composite binding site may have greatly increased affinity for the substrate. The observation that the C-terminal domain of Skp2 is necessary for its interaction with Cks1 and the structure modeling and site-directed mutagenesis suggested the existence of a phosphopeptide-binding site composed of a cluster of several basic amino acids (40, 41). In the case of the yeast F box protein Grr1, it has been suggested that the putative phosphate-binding site is on a concave surface of a horse-shoe-like structure formed by its leucine-rich repeats (40). The leucine-rich repeats of Skp2 form a similar horseshoe-like structure, but there is no obvious clustering of basic amino acid residues and an ~30-amino acid C-terminal tail is packed to the concave surface (16). It is thus possible that Skp2 is unique among F box proteins in its interaction with the auxiliary protein, Cks1, which in turn has the phosphate-binding site. However, it cannot be concluded from the present data that the anion-binding site of Cks1 actually binds directly to phosphorylated Thr-187 of p27. It is possible that the anion-binding site of Cks1 interacts with some negatively charged amino acid residues of the p27 substrate or of some other component of the enzyme-substrate multienzyme complex. Further studies employing crystallographic or biophysical methods are needed to resolve these remaining problems.

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