Association of the Cell Cycle Regulatory Proteins p45\textsuperscript{SKP2} and CksHs1

**FUNCTIONAL EFFECT ON CDK2 COMPLEX FORMATION AND KINASE ACTIVITY**

Lidia Mongay, Susana Plaza, Elena Vigorito‡, Carles Serra-Pagès§§, and Jordi Vives¶

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From the Servei d’Immunologia, Hospital Clinic i Provincial de Barcelona, Villarroel 170, Barcelona 08036, Spain

In mammalian cells, CDK2 is part of a multiprotein complex that includes Cyclin A or E and cell cycle regulatory proteins such as p21\textsuperscript{Cip1}, PCNA, p27\textsuperscript{Kip1}, p45\textsuperscript{SKP2}, p19\textsuperscript{SKP1}, and CksHs1/CksHs2. While the role of some of these proteins has been well studied, the function of other proteins in the complex remains unclear. In this study, we showed that the carboxyl-terminal region of p45\textsuperscript{SKP2} associates directly with CksHs1 and that CksHs1 negatively regulated the interaction between p45\textsuperscript{SKP2} and CDK2. Moreover, we showed that overexpression of CksHs1 inhibited CDK2 kinase activity and that additional expression of p45\textsuperscript{SKP2} overcame this inhibition and restored CDK2 kinase activity. We proposed that the association of CksHs1 and p45\textsuperscript{SKP2} prevented CksHs1 from binding CDK2 and negatively regulating the CDK2 kinase activity.

Cell cycle events are tightly controlled by the sequential activation of the enzymes known as the cyclin-dependent kinases (CDKs). In mammalian cells, CDK2 complexed with Cyclin A acts as the main promoter for progression through the S phase of the cell cycle. Therefore the regulation of Cyclin A-CDK2 kinase activity is extremely important and is accomplished by several mechanisms.

CDK2 structure consists of a central deep cleft positioned between the amino-terminal and carboxyl-terminal lobes, which contains the catalytic residues, the substrate-binding site, and the ATP-binding site. Mechanisms of CDK2 regulation involve protein-protein interactions and phosphorylations, reviewed elsewhere (1). CDK2 kinase activation is mainly achieved by cyclin binding and phosphorylation of the conserved residue Thr-160. On the other hand, cyclin kinase inhibitors binding to the Cyclin A-CDK2 complex inhibit CDK2 kinase activity by direct blockade of the CDK2 substrate-binding site (2).

The active form of the Cyclin A-CDK2 enzyme complexes associates with other proteins such as p45\textsuperscript{SKP2}, CksHs1, and p19\textsuperscript{SKP1} (3), but the mechanism of action of these proteins as regulatory molecules of CDK2 kinase activity is not fully understood. Human p45\textsuperscript{SKP2} is an F-box/leucine-rich-containing protein that was originally identified by its association with Cyclin A-CDK2 (3), hence its designation as an S phase kinase-associated protein (SKP2). Levels of p45\textsuperscript{SKP2} protein are cell cycle regulated, increasing during G\textsubscript{1}-S phases, accumulating during S phase, and dropping toward M phase (4, 5). The level of p45\textsuperscript{SKP2} is also increased in many transformed cells (3). Moreover, p45\textsuperscript{SKP2} functional interference in cultured cells inhibits S phase entry (3) and p45\textsuperscript{SKP2} ectopic expression in quiescent fibroblasts causes mitogen-independent S phase entry (6).

As an F-box containing protein, p45\textsuperscript{SKP2} is involved in ubiquitin-mediated protein degradation by functioning as a substrate-specific receptor of the SCFSkp2 (p19\textsuperscript{SKP1}-CDC53 (CUL1)-p45\textsuperscript{SKP2}) ubiquitin-protein isopeptide ligase complex (E3). The SCF complexes function as the main ubiquitin ligases controlling the abundance of the cell cycle regulatory proteins at the G\textsubscript{1}-S transition; they consist of p19\textsuperscript{SKP1}, CUL1, and Rbx1/ROC1 as the invariant components and the F-box protein as the variable component and the substrate recognition subunit. The ubiquitin-dependent cell cycle regulatory role of p45\textsuperscript{SKP2} is growing in importance due to the number of cell cycle proteins found to interact with p45\textsuperscript{SKP2}. There is evidence that the cell cycle-regulated transcription factor E2F-1 binds to SCFSkp2 and is ubiquitinated by this complex (7). It is also described that p45\textsuperscript{SKP2} promotes p27\textsuperscript{Kip1} ubiquitination and degradation (6, 8, 9). p45\textsuperscript{SKP2} is also responsible for the ubiquitination and degradation of B-myb, a DNA-binding protein (10).

Taken together, the existing evidence suggests that p45\textsuperscript{SKP2} plays a prominent role in progression of cells through phases G\textsubscript{1}-S and S of the cell cycle. The requirement of p45\textsuperscript{SKP2} function may be partially explained by its F-box-dependent implication in cell cycle protein ubiquitination and proteasomal degradation. However, recent experimental evidence suggests involvement of p45\textsuperscript{SKP2} in several CDK2 regulatory functions, not only related to its amino-terminal F-box domain, but also to its carboxyl-terminal cyclin-protein kinase-binding domain, such as Cyclin A accumulation and Cyclin A- and Cyclin E-associated kinase activation (6).

Consistent with these observations is the hypothesis that p45\textsuperscript{SKP2} exerts other cell cycle regulatory functions involving protein-protein interactions through its carboxyl-terminal leucine-rich repeats. To investigate novel p45\textsuperscript{SKP2} protein-protein interactions, a p45\textsuperscript{SKP2} two-hybrid screening was performed. This study defines and characterizes the novel...
p45\(^{SKP2}\)-CksHs1 interaction and analyzes its effects on the CDK2 complex formation and the CDK2 kinase function.

**EXPERIMENTAL PROCEDURES**

**Interaction-trap Assay—**Plasmid DNAs, yeast strains, and the HeLa cell cDNA library used for the interaction-trap assay were provided by Dr. R. Brent and colleagues and used as described (11, 12). The human lymphocyte LexA cDNA library, derived from the HTLV-1-transformed T-cell line SLB-I, used for the interaction-trap assay was from CLONTECH Laboratories, Inc., (Palo Alto, CA). The various p45\(^{SKP2}\), CDK2, p19\(^{SKP1}\), and CksHs1 regions fused to LexA or the B42 transcription activator domain are shown in Fig. 1. The yeast strains EGY048 (MATa trp1 ura3 his3 LEU2::pLexAop6-LEU2) and EGY191 (MATa trp1 ura3 his3 LEU2::pLexAop2-LEU2), used as hosts for all the interaction assays, were kindly provided by Dr. E. Golemis. Both yeast strains contain the plasmid pSH18-34, which includes the reporter lacZ gene under the control of a modified Gal1 promoter.

**Plasmid Constructions—**The different expression constructs were made by using standard techniques and confirmed by DNA sequencing. pSK-p45\(^{SKP2}\) and pSK-p19\(^{SKP1}\) were a gift from Dr Drach. pCMV-CDK2 was a gift from Dr Drach. All the CksHs1 cDNA clones were isolated from the interaction-trap HeLa and human lymphocyte cDNA libraries. The CksHs1 full coding region was generated by PCR using the above mentioned lymphocyte cDNA library. CksHs1 and p45\(^{SKP2}\) deletion mutants were generated by PCR. For COS-7 cell transient transfections, different regions of p45\(^{SKP2}\), CDK2, p19\(^{SKP1}\), and CksHs1 cDNAs were cloned into pMT2 derived plasmids. These included pMT2-HA, which contains the glutathione S-transferase tag sequence immediately upstream of the cloning site (12), pMT2-myc, which encodes a myc epitope tag sequence immediately upstream of the cloning site (13) and pMT-GST, which contains the glutathione S-transferase gene coding sequence immediately upstream of the cloning site.

**Antibodies—**The anti-p45\(^{SKP2}\) rabbit polyclonal Ab was generated by immunizing rabbits with purified, Escherichia coli-expressed, amino acids 1–435 (14). For precipitation studies we used the rabbit polyclonal Ab anti-p45\(^{SKP2}\) (H-435) from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-HA mAb HA11 was from Berkeley Antibody Co. (Richmond, CA). The rabbit polyclonal Ab anti-CDK2 (sc-163) was from Santa Cruz Biotechnology. The rabbit polyclonal Ab anti-CksHs1 (FL-79) was from Santa Cruz Biotechnology. The anti-myc tag mAb 9E10 was described previously (14).

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**RESULTS**

**The Carboxyl-terminal Domain of p45\(^{SKP2}\) Interacts with CksHs1—**To identify novel proteins that interact with p45\(^{SKP2}\), the interaction-trap system was used (11). DNA encoding p45\(^{SKP2}\) (amino acids 3–436) was cloned into the pEG202 plasmid to create the LexA-p45\(^{SKP2}\) bait. This plasmid was transformed into the yeast strain EGY048, which contains the LEU2 and lacZ reporter genes under the control of the LexA operon. Unfortunately, this yeast strain could not be used for an interactor hunt, since the LexA-p45\(^{SKP2}\) bait was a weak transcription activator. The plasmid was then transformed into the less sensitive yeast strain EGY191, which contains one operator instead of three upstream of the LEU2 gene and was co-transformed with the lacZ reporter (pSH18-34). The resulting yeast strain was then transformed with a pJG4-5 based lymphoid cDNA library that conditionally expresses fusion proteins combining the B42 activation domain and the lymphoid proteins. A total of 10\(^6\) independent transformants of the lymphoid cDNA library were screened. One of the clones isolated that had the desired phenotype, namely Lex+, lacZ+ when grown using galactose, but not glucose, was identified as CksHs1. This clone encoded amino acids 19–79 of the full-length CksHs1 protein. Several other clones encoding the CksHs1 gene were also found to interact with p45\(^{SKP2}\) when we performed an interaction-trap screening with a HeLa cDNA library. A cDNA encoding full-length CksHs1 was cloned by PCR, and interaction-trap assays were also used to quantify the interactions between p45\(^{SKP2}\) and CksHs1 (Fig. 1A). The interaction-trap assays showed that p45\(^{SKP2}\) strongly interacted with full-length CksHs1 (698 β-gal units). p19\(^{SKP1}\) was previously known to interact with p45\(^{SKP2}\), and was also obtained in the present screening, was used as a positive control (161 β-gal units).

To further characterize the p45\(^{SKP2}\) and CksHs1 interaction, quantitative interaction-trap assays were performed using amino-terminal and carboxyl-terminal deletion mutants of p45\(^{SKP2}\). These deletions were cloned by PCR into the appropriate vector. One construct consisted of amino acids 3–232, which contains the F-box domain of p45\(^{SKP2}\) (Delta3–232), while the other consisted of amino acids 153–436, which contains the leucine-rich domain of p45\(^{SKP2}\). In the present screening, was used as a positive control (161 β-gal units).

Quantitative interaction-trap assays were also used to test the interaction between CDK2 and either p45\(^{SKP2}\) or CksHs1.
CksHs1 Is a p45SKP2-interacting Protein

As shown in Fig. 1B, CDK2 strongly interacted with CksHs1 (483 kDa) but not with p45SKP2 (2 kDa). p45SKP2 Interacts with CksHs1 in Vivo—To confirm the interaction between p45SKP2 and CksHs1 in vivo, co-precipitation assays were performed in the lymphoid cells line Jurkat and Cem (Fig. 2A). p45SKP2 was immunoprecipitated from unstimulated Jurkat and Cem cells using an anti-p45SKP2 antibody. The precipitates were resolved by SDS-PAGE, subjected to Western blotting, and the membranes probed using an anti-CksHs1 antibody. Both cell lines CksHs1 was specifically co-immunoprecipitated with p45SKP2. Similarly, co-precipitation experiments were done in COS-7 cells that were transfected with GST expression vectors encoding either CksHs1 or p45SKP2. The precipitates without the exogenous addition of His-CksHs1 were lysed in a Nonidet P-40-containing buffer, and proteins were immunoprecipitated using an anti-p45SKP2 Ab (lanes 4 and 6) or a control Ab (lanes 3 and 5). Immuno-precipitated proteins and total lysates (lane 5) were resolved by 15% SDS-PAGE and then transferred onto PVDF membrane.

Expression vectors encoding full-length CksHs1 and the four CksHs1 mutants mentioned above were fused to GST and were co-transfected in COS-7 cells with either myc-p45SKP2 or HA-tagged CDK2 (HA-CDK2, Fig. 3C). The lysates were incubated with glutathione-Sepharose beads, and the precipitates were immunoblotted using an anti-myc or anti-HA antibody, respectively. All the deletion mutants tested were able to bind p45SKP2, but only the full-length GST-CksHs1 and the mutant lacking the last 8 amino acids was able to bind CDK2. These results mapped the CksHs1-p45SKP2 binding region to CksHs1 (36–71) and confirmed that CksHs1 binds to p45SKP2 or CDK2 through different regions (Fig. 3).

CksHs1 Inhibits the Interaction between p45SKP2 and CDK2—To test whether CksHs1 has any effect on the p45SKP2-CDK2 interaction, a series of p45SKP2-CDK2 co-precipitation assays were performed in the presence of varying the amounts of CksHs1. Increasing amounts of HA-CksHs1 (0.4, 0.8, 1.6, and 3.2 μg) were expressed in COS-7 cells, together with fixed amounts of GST-CDK2 and HA-p45SKP2. HA-p45SKP2 co-precipitation with GST-CDK2 was detected by anti-HA immunoblotting of glutathione-Sepharose precipitates. As shown in Fig. 4A, less p45SKP2 is bound to CDK2 when higher amounts of CksHs1 are present. Similarly, a parallel co-precipitation assay was done using CksHs1-(19–79) instead of full-length CksHs1. CksHs1-(19–79), as shown in Fig. 3, binds to p45SKP2 but does not bind to CDK2. The results shown in Fig. 4B indicate that the presence of increasing amounts of CksHs1-(19–79) also resulted in less p45SKP2 bound to CDK2.

p45SKP2 and CksHs1 Complex with CDK2 in a Mutually Exclusive Way—The possibility that p45SKP2 binds to CDK2 indirectly through CksHs1 cannot be excluded based on either the previous data concerning p45SKP2 and CksHs1 interaction with CDK2 or our results about the interaction between p45SKP2 and CksHs1. Additionally, two-hybrid assays using CDK2 as bait did not result in its interaction with p45SKP2 (Fig. 1B), suggesting that a p45SKP2-CDK2 interaction is indeed indirect.

To further assess the involvement of CksHs1 in the interaction of p45SKP2 and CDK2, we tested the effect of increasing amounts of p45SKP2 on CksHs1 binding to CDK2. Increasing amounts of myc-p45SKP2 (0.4, 0.8, 1.6, and 3.2 μg) were expressed in COS-7 cells together with fixed quantities of GST-CDK2 and myc-CksHs1. Glutathione-Sepharose precipitates were subjected to anti-myc immunoblotting and showed that less CksHs1 is bound to CDK2 when higher amounts of p45SKP2 are present (Fig. 5A). This result argues against the hypothesis of CksHs1 acting like a bridge between p45SKP2 and CDK2. To further confirm this fact, glutathione-Sepharose precipitates containing GST-CDK2 or GST protein produced in COS-7 cells were incubated with saturating amounts of His-CksHs1 protein produced in bacteria. The resulting precipitates were then incubated with lysates from COS-7 cells transfected with HA-p45SKP2. These precipitates were resolved by SDS-PAGE and immunoblotted using an anti-HA antibody, which allowed the amount of p45SKP2 protein bound to CDK2 with and without the addition of His-CksHs1 protein to be determined. The results indicated that less p45SKP2 protein co-precipitated with His-CksHs1-saturated CDK2 precipitates in comparison with the p45SKP2 co-precipitated with CDK2 precipitates without the exogenous addition of His-CksHs1.
CksHs1 Is a p45SKP2-interacting Protein

FIG. 3. Mapping of sequences required for p45SKP2-CksHs1 binding. A, schematically shown are the regions of CksHs1 used for the experiments described below. Solid black bands represent the known CDK2 interacting regions. B, immunoblot analysis developed with an anti-myc mAb (upper and middle panels) or anti-GST Ab (lower panel). Nonidet P-40 cell lysates were prepared from COS-7 cells that were transiently transfected with pMT2 expression vectors encoding GST (lane 1), GST-CksHs1 (lane 2), GST-CksHs1(19–79) (lane 3), GST-CksHs1(36–79) (lane 4), GST-CksHs1(1–71) (lane 5), GST-CksHs1(36–71) (lane 6), and myc-p45SKP2 (lanes 1–6). Lysates were prepared 48 h after transfection and used in precipitation studies. Co-precipitation analysis was performed using glutathione-Sepharose beads (lanes 1–6, upper panel). Precipitated proteins (upper panel) and total lysates (middle and lower panels, ~5% of immunoprecipitated lysates) were resolved by 12% SDS-PAGE and then transferred onto a PVDF membrane. C, immunoblot analysis developed with an anti-HA mAb (upper and lower panels). Nonidet P-40 cell lysates were prepared from COS-7 cells that were transiently transfected with pMT2 expression vectors encoding GST (lane 1), GST-CksHs1 (lane 2), GST-CksHs1(19–79) (lane 3), GST-CksHs1(36–79) (lane 4), GST-CksHs1(1–71) (lane 5), GST-CksHs1(36–71) (lane 6), and HA-CDK2 (lanes 1–6). Lysates were prepared 48 h after transfection and used for precipitation studies. Co-precipitation analysis was performed using glutathione-Sepharose beads (lanes 1–6, upper panel). Precipitated proteins (upper panel) and total lysates (lower panels, ~5% of immunoprecipitated lysates) were resolved by 12% SDS-PAGE and then transferred onto a PVDF membrane.

(Fig. 5B). This result indicates that the exogenous His-CksHs1 blocks the interaction between CDK2 and p45SKP2, presumably by structural impediments, and that CksHs1 probably does not function as a bridge molecule between p45SKP2 and CDK2.

Overexpression of CksHs1 Inhibits CDK2 Kinase Activity, and Overexpression of p45SKP2 Restores Basal Kinase Activity—To examine whether CksHs1 or p45SKP2 can alter the CDK2 kinase activity in vitro, COS-7 cells were co-transfected with GST-CDK2 and either myc-CksHs1, HA-CksHs1(19–79), or myc-p45SKP2. Subsequently the CDK2 kinase activity of the GST-CDK2 precipitates was measured using histone H1 as a substrate. The results showed that overexpression of the full-length CksHs1 substantially inhibited CDK2 kinase activity in vitro, whereas overexpression of the mutant CksHs1(19–79) failed to inhibit the CDK2 kinase activity, consistent with its lack of binding to CDK2 (Fig. 6A). In a separate experiment, the construct encoding GST-CDK2 transfected with increasing amounts of either CksHs1 or CksHs1(19–79) further illustrated the inhibition that CksHs1 has over the CDK2 kinase activity (Fig. 6B). The presence of increasing amounts of full-length CksHs1 corresponded with a progressive inhibition of the CDK2 kinase activity, whereas in the case of CksHs1(19–79), there was no effect on kinase activity.

Once it was established that CksHs1 inhibited CDK2 kinase activity, we investigated whether the p45SKP2-CksHs1 interaction could modulate the effect of CksHs1 on the CDK2 kinase activity. The same CDK2 kinase assay was done in COS-7 cells transfected with GST-CDK2, an inhibitory concentration of myc-CksHs1 (0.8 μg), and increasing amounts of myc-p45SKP2 (0, 4, 0, 8 and 1, 6 μg). Our results showed that the increasing amounts of p45SKP2 were able to progressively relieve CDK2 from the inhibitory effect of CksHs1 and restore CDK2 kinase activity (Fig. 6C). It is noteworthy that overexpression of p45SKP2 alone slightly increased CDK2 kinase activity.

The same histone H1 kinase assay was performed in COS-7 cells transfected with GST-CDK2 adding His-CksHs1 protein produced in bacteria to the CDK2 precipitates. In this case CksHs1 failed to inhibit CDK2 kinase activity (Fig. 6D), suggesting that CksHs1 inhibits CDK2 kinase activity using an indirect mechanism.

DISCUSSION

In this study we provide evidence for the interaction between p45SKP2 and CksHs1. Both p45SKP2 and CksHs1 were known to be associated with the Cyclin A-CDK2 complexes, but the direct interaction between p45SKP2 and CksHs1 had never been reported. We identified CksHs1 as a candidate p45SKP2-interacting protein in an interaction-trap screening using p45SKP2 as a bait. The interaction was confirmed by co-precipitation studies in the lymphoid cell lines Cem and Jurkat and using COS-7 transfected with various constructs encoding p45SKP2 and CksHs1. This interaction has been mapped to the carboxy-terminal region of p45SKP2, which contains several leucine-rich repeats, while the amino-terminal F-box domain has proved not to be necessary for this association.

p45SKP2 is an F-box/leucine-rich repeat-containing protein
CksHs1 Is a p45\(^{SKP2}\) -interacting Protein

The interaction between CksHs1 and p45\(^{SKP2}\) and CDK2 as analyzed by co-precipitation studies indicated that different sites of CksHs1 interact with p45\(^{SKP2}\) and CDK2. While the CksHs1 residues found to interact with CDK2 agreed with previous studies (24, 33, 34), we mapped the CksHs1-p45\(^{SKP2}\) binding region to CksHs1 amino acids 36–71. Furthermore, our results indicate that CksHs1 modulates the interaction between p45\(^{SKP2}\) and CDK2. We have seen that excess amounts of either CksHs1 or CksHs1-(19–79), an amino-terminal deletion of CksHs1, that does not bind to CDK2 but does bind to p45\(^{SKP2}\), blocks the interaction between p45\(^{SKP2}\) and CDK2. This fact can be explained if p45\(^{SKP2}\) and CDK2 interact through CksHs1 or if CksHs1 blocks the CDK2-interacting region of p45\(^{SKP2}\). To clarify the situation, we performed the inverse experiment, showing that excess amounts of either CksHs1 or CksHs1-(19–79), an amino-terminal deletion of CksHs1, that does not bind to CDK2 but does bind to p45\(^{SKP2}\), blocks the interaction between p45\(^{SKP2}\) and CDK2. This result indicated that p45\(^{SKP2}\) can bind either CksHs1 or the CDK2 complex but not both simultaneously. In further confirmation of this hypothesis, CksHs1 exogenously produced in bacteria was added to a CDK2 precipitate, and the resulting complex reduced the amount of p45\(^{SKP2}\) pulled from a cell extract by the CDK2 alone, indicating that p45\(^{SKP2}\) competes for a binding site on the cyclin-CDK2 complex with CksHs1 or that the p45\(^{SKP2}\)-CksHs1 complex formation results in blocking of the

that belongs to both the ubiquitin-protein ligase complexes SCFSKP2 (4, 5, 18, 19) and the S phase Cyclin A-CDK2 complexes (3). The association between p45\(^{SKP2}\) and Cyclin A-CDK2 complexes has been well documented, but the exact nature of the interaction remains unclear. We have not found any evidence in favor of a direct protein-protein interaction, and in fact when we assayed this in an interaction-trap assay the results were negative. On the other hand, CksHs1 belongs to the Cks family of cell cycle regulatory proteins composed of small proteins (9–18 kDa). Cks proteins are bound to the mitotic cyclin-dependent kinase as described in yeast (20–22), human cells (23), and frog eggs (24). Human cells contain two isoforms of Cks proteins, namely CksHs1 and CksHs2, that can each bind to CDK2 (25). The crystallographic structure of CksHs1 as well as the human CDK2 kinase bound to CksHs1 has been described (17, 26, 27), but besides this knowledge in terms of sequence and structure, little is known about CksHs1 function (28). Even though Cks proteins have been related to the cyclosome/APC (anaphase promoting complex) (29–32), and p45\(^{SKP2}\) has been related to the SCFSKP2 E3 ubiquitin ligase, both CksHs1 and p45\(^{SKP2}\) have been related to the S phase Cyclin A-CDK2 complexes. Since both CksHs1 and p45\(^{SKP2}\) bind to the Cyclin A-CDK2 complexes, it is possible that their interaction is important in the structure and function of these complexes. Thus, once the CksHs1-p45\(^{SKP2}\) interaction was characterized, our studies focused on the role of this interaction in the structure and function of the S phase cyclin-CDK2 complexes. This does not preclude different roles for the...
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Fig. 6. CDK2 kinase assays in COS-7 cells transfected with p45SKP2 and CksHs1. A, analysis of CDK2 kinase activity using histone H1 as a substrate. COS-7 cells were transiently transfected with pMT2 expression vectors encoding GST (lane 1) or GST-CDK2 (lanes 2–5) and myc-p45SKP2 (lane 3), myc-CksHs1 (lane 4), or HA-CksHs1 (19–79) (lane 5). Lysates were prepared 48 h after transfection. Precipitation experiments were performed using glutathione-Sepharose beads, and the precipitates were incubated 10 min at 30 °C in the presence of 150 μCi of [γ-32P]ATP and histone H1. Precipitates were resolved by 12% SDS-PAGE followed by autoradiography. As a transfection control, an approximate 5% of the lysates was resolved by 12% SDS-PAGE, transferred onto a PVDF membrane, and then developed with an anti-CDK2 Ab (lower panel). B, analysis of CDK2 kinase activity using histone H1 as a substrate. COS-7 cells that were transiently transfected with pMT2 expression vectors encoding GST (lane 1) or GST fused to CDK2 (lanes 2–8) and pMT2-myc (lane 2), myc-CksHs1 at various concentrations (lanes 3–6), or myc-CksHs1319–79 at various concentrations (lanes 7–8). Lysates were prepared and processedт. As a transfection control, an approximate 5% of the lysates was resolved by 12% SDS-PAGE, transferred onto a PVDF membrane, and then developed with an anti-CDK2 Ab (lower panel). C, analysis of CDK2 kinase activity using histone H1 as a substrate. COS-7 cells were transiently transfected with pMT2 expression vectors encoding GST (lane 1) or GST-CDK2 (lanes 2–7) and pMT2-myc (lane 2), myc-CksHs1 (lanes 4–7), and myc-p45SKP2 at various concentrations (lanes 3 and 5–7). Lysates were prepared and processed as described in A. As a transfection control, an approximate 5% of the lysates was resolved by 12% SDS-PAGE, transferred onto a PVDF membrane, and then developed with an anti-CDK2 Ab (lower panel). D, shown is the analysis of CDK2 kinase activity using histone H1 as a substrate. COS-7 cells were transiently transfected with pMT2 expression vectors encoding GST (lane 1) or GST-CDK2 (lanes 2–6). Lysates were prepared 48 h after transfection. Precipitation studies were performed using glutathione-Sepharose beads, and the precipitates were incubated 10 min at 30 °C in the presence of 150 μCi of [γ-32P]ATP and histone H1 and various concentrations of protein His-CksHs1 produced in bacteria. Precipitates were resolved by 12% SDS-PAGE followed by autoradiography.

cyclin-CDK2-binding sites on each protein. Taken together, these results suggest the co-existence of at least two different cyclin-CDK2 complexes together with p45SKP2 or CksHs1. The two different cyclin-CDK2 complexes may be cyclin-CDK2-p45SKP2 and cyclin-CDK2-CksHs1, where p45SKP2 and CksHs1 bind to the complexes in a mutually exclusive way.

The structure of the cyclin-CDK2-CksHs1 complex is well determined, but little is known about the role CksHs1 binding plays in CDK2 regulation. Overexpression or deletion experiments performed on fission yeast, budding yeast, and Xenopus eggs implicate the Cks family of proteins in a wide variety of functions; namely entry into mitosis, exit from mitosis, and transition between G1 and S or between G2 and M (24, 33–35). According to most experiments, Cks proteins modulate the tyrosine phosphorylation state of the major mitotic CDK and may therefore have quite different effects on cell cycle progression depending upon when in the cell cycle the interaction occurs (24, 36). When CksHs1 function was tested in our experimental model, CksHs1 clearly inhibited CDK2 kinase activity in mammalian cells transfected with both CDK2 and CksHs1. This result demonstrates that CksHs1 complexed with cyclin-CDK2 exerts an inhibitory effect over the CDK2 kinase activity. However, there was no CDK2 kinase activity inhibition when exogenous CksHs1 protein was added to mammalian cells transfected with CDK2. This result clearly indicates that, in contrast to other CDK2 inhibitors like p21Cip1 and p27Kip1, which inhibit due to their direct interaction with the kinase (37, 38), CksHs1-mediated CDK2 kinase inhibition is probably through an indirect mechanism involving another regulatory protein.

Since binding between p45SKP2 and CksHs1 inhibits each of their respective interactions with cyclin-CDK2, the p45SKP2-CksHs1 complex also has functional implications regarding CDK2 activity. The results of our experiments show that p45SKP2 restores basal kinase activity after CksHs1-mediated CDK2 kinase inhibition by blocking CksHs1 binding to CDK2. Thus our findings attribute a new role to p45SKP2 in CDK2 kinase regulation and therefore S phase progression, which is different from the p45SKP2 function in the ubiquitin degradation pathway. Finally, our data also suggest that factors that regulate the CksHs1-p45SKP2 interaction may have an important role in cell cycle progression as they would regulate CksHs1-CDK2 interaction and hence kinase activity.

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REFERENCES

1. Morgan, D. O. (1995) Nature 374, 131–134
2. Morgan, D. O. (1996) Curr. Opin. Cell Biol. 8, 767–772
3. Zhang, H., Kobayashi, R., Galaktionov, K., and Beach, D. (1995) Cell 82, 915–925
4. Lissitwan, J., Marti, A., Sutterluty, H., Getaiher, M., Wirbelsauer, C., and Krek, W. (1998) EMBO J. 17, 368–383
5. Michel, J. J., and Xiong, Y. (1998) Cell Growth Differ. 9, 345–449
6. Sutterluty, H., Chatelain, E., Marti, A., Wirbelsauer, C., Senden, M., Muller, U., and Krek, W. (1999) Nat. Cell Biol. 1, 207–214
7. Marti, A., Wirbelsauer, C., Schaffner, M., and Krek, W. (1999) Nat. Cell Biol. 1, 14–19
8. Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. (1999) Nat. Cell Biol. 1, 193–199
9. Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H., and Zhang, H. (1999) Curr. Biol. 9, 661–664
10. Charrasse, S., Carena, I., Brondani, V., Klempnauer, K. H., and Ferrari, S. (2000) Oncogene 19, 2886–2895
11. Gyrnas, J., Golemis, E., Chertkov, H., and Brent, R. (1993) Cell 75, 791–803
12. Serra-Pages, C., Kedersha, N. L., Fazikas, L., Medley, Q., Debant, A., and Streuli, M. (1995) EMBO J. 14, 2827–2838
13. Medley, G. Q., Serra-Pages, C., Iannotti, E., Seipel, K., Tang, M., O’Brien, S. P., and Streuli, M. (2000) J. Biol. Chem. 275, 36116–36123
14. Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) Mol. Cell. Biol. 5, 3610–3616
15. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith,
CksHs1 Is a p45SKP2-interacting Protein

25036

J. A., and Struhl, K. (1987) *Current Protocols in Molecular Biology*, John Wiley & Sons

16. Bourne, Y., Watson, M. H., Arvai, A. S., Bernstein, S. L., Reed, S. I., and Tainer, J. A. (2000) *Struct. Fold Des.* 8, 841–850

17. Bourne, Y., Watson, M. H., Hickey, M. J., Holmes, W., Rocque, W., Reed, S. I., and Tainer, J. A. (1996) *Cell* 84, 863–874

18. Lyapina, S. A., Correll, C. C., Kipreos, E. T., and Deshaies, R. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 7451–7456

19. Yu, Z. K., Gervais, J. L., and Zhang, H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 11324–11329

20. Hayles, J., Beach, D., Durkacz, B., and Nurse, P. (1986) *Mol. Gen. Genet.* 202, 291–298

21. Brizuela, L., Draetta, G., and Beach, D. (1987) *EMBO J.* 6, 3507–3514

22. Hadwiger, J. A., Wittenberg, C., Mendenhall, M. D., and Reed, S. I. (1989) *Mol. Cell. Biol.* 9, 2034–2041

23. Draetta, G., Brizuela, L., Potashkin, J., and Beach, D. (1987) *Cell* 50, 319–325

24. Patra, D., and Dunphy, W. G. (1996) *Genes Dev.* 10, 1503–1515

25. Richardson, H. E., Stueland, C. S., Thomas, J., Russell, P., and Reed, S. I. (1990) *Genes Dev.* 4, 1332–1344

26. Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., and Pavletich, N. P. (1995) *Nature* 376, 313–320

27. Arvai, A. S., Bourne, Y., Hickey, M. J., and Tainer, J. A. (1995) *J. Mol. Biol.* 249, 835–842

28. Pines, J. (1996) *Curr. Biol.* 6, 1399–1402

29. Patra, D., and Dunphy, W. G. (1995) *Genes Dev.* 12, 2549–2559

30. Patra, D., Wang, S. X., Kumagai, A., and Dunphy, W. G. (1996) *J. Biol. Chem.* 271, 36539–36542

31. Kaiser, P., Moncollin, V., Clarke, D. J., Watson, M. H., Bertolaet, B. L., Reed, S. I., and Bailly, E. (1999) *Genes Dev.* 13, 1190–1202

32. Shteinberg, M., and Hershko, A. (1999) *Biochem. Biophys. Res. Commun.* 257, 12–18

33. Moreno, S., Hayles, J., and Nurse, P. (1989) *Cell* 58, 361–372

34. Tang, Y., and Reed, S. I. (1993) *Genes Dev.* 7, 822–832

35. Reynard, G. J., Reynolds, W., Verma, R., and Deshaies, R. J. (2000) *Mol. Cell. Biol.* 20, 5858–5864

36. Dunphy, W. G., and Newport, J. W. (1989) *Cell* 58, 181–191

37. Chen, J., Jackson, P. K., Kirschner, M. W., and Dutta, A. (1995) *Nature* 374, 386–388

38. Russo, A. A., Jeffrey, P. D., Patten, A. K., Massague, J., and Pavletich, N. P. (1996) *Nature* 382, 325–331