Skp2 Contains a Novel Cyclin A Binding Domain That Directly Protects Cyclin A from Inhibition by p27[Kip1]*

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Skp2 is well known as the F-box protein of the SCF[Skp2]·Roc1 complex targeting p27 for ubiquitylation. Skp2 also forms complexes with cyclin A, which is particularly abundant in cancer cells due to frequent Skp2 overexpression, but the mechanism and significance of this interaction remain unknown. Here, we report that Skp2-cyclin A interaction is mediated by novel interaction sequences on both Skp2 and cyclin A, distinguishing it from the well known RXL-hydrophobic patch interaction between cyclins and cyclin-binding proteins. Furthermore, a short peptide derived from the mapped cyclin A binding sequences of Skp2 can block Skp2-cyclin A interaction but not p27-cyclin A interaction, whereas a previously identified RXL peptide can block p27-cyclin A interaction but not Skp2-cyclin A interaction. Functionally, Skp2-cyclin A interaction is separable from Skp2 ability to mediate p27 ubiquitylation. Rather, Skp2-cyclin A interaction serves to directly protect cyclin A-Cdk2 from inhibition by p27 through competitive binding. Finally, we show that disruption of cyclin A binding with point mutations in the cyclin A binding domain of Skp2 compromises the ability of overexpressed Skp2 to counter cell cycle arrest by a p53/p21-mediated cell cycle checkpoint without affecting its ability to cause degradation of cellular p27 and p21. These findings reveal a new functional mechanism of Skp2 and a new regulatory mechanism of cyclin A.

Skp2 is one of the founding members of the F-box protein family that serves as the ubiquitylation substrate recruiting subunit of the SCF[Skp2]·Roc1 complex (1). The best-established substrate of Skp2 is the cyclin-dependent kinase inhibitor (CKI)[5] p27 (2–4), although Skp2 can also promote ubiquitylation and degradation of other proteins including the p27 family members p21 (5) and p57 (6). Because p27 is a negative regulator of cell proliferation, the p27 ubiquitylation activity of Skp2 suggested that it is a proliferation-stimulating protein. Indeed, Skp2 exhibits proliferation-stimulating activity in various experimental assays and is found overexpressed in various human cancers (7).

Another property of Skp2 is its interaction with cyclin A. In fact, Skp2 was first identified and cloned as a cyclin A-associated protein in transformed cells (8, 9), and Skp2 associated with both cyclin A and Skp1 in stoichiometric amounts (9). If protein-protein interactions and the efficiencies of interactions are indications of protein functions, these early findings should suggest that Skp2-cyclin A interaction is an important aspect of Skp2 function. Because overexpression of Skp2 is a frequent event in cancer cells and cyclin A plays important roles in cell proliferation and survival, Skp2 may affect cyclin A function through this interaction as a tumorigenic mechanism. Surprisingly, however, very little has been learned about the Skp2-cyclin A interaction more than 10 years after its identification. Whether Skp2 belongs to the family of RXL-containing cyclin A-binding proteins remains uncertain, and the functional significance of Skp2-cyclin A interaction is controversial (see below). Currently, the role of Skp2 as a proliferation-stimulating protein has been exclusively attributed to p27 ubiquitylation and degradation.

In this study we determined that Skp2-cyclin A interaction represents a novel type of interaction between cyclin A and its binding proteins. Our investigation of the significance of the Skp2-cyclin A interaction reveals a new functional mechanism of Skp2 and a new regulatory mechanism of cyclin A.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and Peptides—Skp2, Skp2 N terminus (Skp2N), and p27 cDNAs were cloned into pGEX-2T. GST-p21 and GST-E2F1 are gifts from Anindya Dutta and Bill Kaelin, respectively. GST-Skp2N-AA45 and GST-Skp2N-AAA53 were generated using the QuikChange kit from Stratagene. GST moiety was cleaved off GST fusion proteins (GST-p27, GST-Skp2N, and GST-Skp2N-AA45) by thrombin for certain experiments. Myc-tagged cyclin A and mutant derivatives were

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5 The abbreviations used are: CKI, cyclin-dependent kinase inhibitor; GST, glutathione S-transferase; Cdk, cyclin-dependent kinase; BrdUrd, bromodeoxyuridine; GFP, green fluorescent protein; HP, hydrophobic patch; Skp2N, Skp2 N terminus.

generated by PCR. Various cDNAs were cloned under cytomegalovirus (CMV) promoter in lenti-CMV-GFP replacing GFP (10). Other plasmids were described previously (11).

Antibodies to Myc (9E10), Cdk2 (M-2 and D-12), p53 (DO-1), p21 (C-19), cyclin A (H432 and C-19), cyclin E (C-19), cyclin D1 (72–13G), Cdk4 (H-303), and Cul1 (H-213) were from Santa Cruz. Anti-FLAG (F7425 and F3165) were from Sigma. Anti-p27 was from was Pharmingen. Anti-Skp2 (51-1900 and 32-3300) was from Zymed Laboratories Inc. Peptides R*XL* (SACRNLFG), 4060 (KTSELLSGMGVSALE-KEE), and 4060AAAA (KTSEAASGMGVAAAEKEE) were from GenScript.

Cell Culture, Transfection, Lentivirus Production, and Infection—Cell lines U2OS, HeLa, and 293T were maintained in standard conditions. Transfection was performed with standard calcium phosphate protocol. To generate lentivirus stocks, 293T cells were transfected with various lentivirus vectors and packaging vectors (pMDLg/pRRE, pRSV-REV, and pMD2-VSVG) (10). Cell culture media at 48 h post-transfection were either used directly or after concentration for infection of U2OS cells at >90% efficiency.

Drug Treatment and Various Routine Assays—Actinomycin D (Sigma) treatment was for 20–22 h at 180 nM. Etoposide (Sigma) treatment was for 24 h at 10 μM. To arrest HeLa cells in mitotic phase, cells were treated with 100 ng/ml nocodazole (Sigma) for 16–17 h. Western blotting, immunoprecipitation, indirect immunofluorescence, and flow cytometry analysis to determine cell cycle profiles were all performed with standard protocols and as described previously (11).

GST Pull-down, p27 Ubiquitylation Assay, and Cyclin A Kinase Assay—GST fusion proteins were purified on GSH beads (Amersham Biosciences) that were then incubated with transfected cell extracts for 1 h in 4 °C with rocking. The beads were washed 3 times with lysis buffer (25 mM Hepes pH 7.6, 100 mM KCl, 0.1 mM EDTA, 10 mM MgCl$_2$, 0.1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol) before analysis by SDS-PAGE and Western blotting.

In vitro p27 ubiquitylation assay in Fig. 2B was performed as described (11). Briefly, 293T cells were transfected with cytomegalovirus expression vectors for FLAG-Skp2 (wild type or mutants), Myc-Roc1, Cul-1, and Skp-1, and the SCF^{Skp2}-Roc1 complex was immuno-purified with anti-FLAG. The immuno-complexes were washed 3 times in 1× ubiquitylation assay buffer, and the ubiquitylation reaction was performed by adding the following to the beads in 1× ubiquitylation buffer (20 mM HEPES pH 7.2, 10 mM MgCl$_2$, 1 mM dithiothreitol): 250 ng of phosphorylated purified p27, 1× <u>e</u>nergy <u>r</u>egeneration <u>s</u>ystem (ERS), 1 μg each E1 and E2(Cdc34) (Boston Biochem), 10 μg of ubiquitin (Sigma), 1 μM ubiquitin-aldehyde, 0.2 mM MG132, 10 ng of purified Cks1, and 1 μM okadaic acid. Reaction mixtures were incubated at 30 °C for 2 h. After incubation, p27 was immunoprecipitated from the reaction with polyclonal anti-p27 antibody, resolved by SDS-PAGE, and Western-blotted with a monoclonal anti-p27 antibody. *In vitro* p27 ubiquitylation assay in Fig. 2B was performed as described (12). Briefly, Roc1/HA-Cul1/His-Skp1/Skp2 (or Roc1/HA-Cul1/His-Skp1/Skp2ΔN; HA is hemagglutinin) and His-cyclin A-Cdk2 complexes were expressed in 5B insect cells and purified by nickel-agarose chromatography (2). 2 μl of *in vitro*

translated ^{35}S-labeled p27 were incubated at 30 °C for different times in 10 μl of ubiquitinylation mix containing 40 mM Tris. pH 7.6, 5 mM MgCl$_2$, 1 mM dithiothreitol, 10% glycerol, 1 μM ubiquitin aldehyde, 1 mg/ml methyl ubiquitin, 10 mM creatine phosphate, 0.1 mg/ml creatine kinase, 0.5 mM ATP, 1 μM okadaic acid, ~5 ng of purified recombinant SCF^{Skp2} complex (or SCF^{Skp2ΔN}), and 10 ng of purified Cks1 and 250 nM cyclin A-Cdk2 complex. Reactions were stopped with Laemmli sample buffer, and their products were run on protein gels under denaturing conditions. Polyubiquitinylated p27 forms were identified by autoradiography.

Cyclin A-Cdk2 kinase assays were performed as described previously (11). Both immuno-purified Myc-cyclin A-Cdk2 on beads and GST-purified and -eluted GST-cyclin A-Cdk2 (baculovirus vectors for GST-cyclin A and Cdk2 were provided by Brian Dynlacht) were used. The amounts of purified proteins were determined by comparing them with known amounts of standard proteins in Coomassie Blue-stained gels. Phosphorylation of GST-Rb-C was quantified with the ImageQuant software. The K_i of various proteins was determined by non-linear least squares analysis. To determine the amounts of various proteins in synchronized HeLa cells, extracts representing 7.5×10^4 HeLa cells were resolved with known amounts of purified proteins and compared in the same Western blots. Actual amounts of proteins in 7.5×10^4 HeLa cells were then determined (for example, the amounts of p27, Skp2, and cyclin A in 7.5×10^4 HeLa cells were determined to be about 0.1, 0.2, and 0.1 pmol, respectively, at 14 h post-replating and about 0.01, 0.1, and 1.0 pmol, respectively, at 19 h post-replating). Various molar ratios were then calculated and shown in Fig. 7G.

RESULTS

Identification of a Novel Cyclin A Binding Motif in the Skp2 N Terminus—Mapping of cyclin A binding sequences on Skp2 has been attempted previously with discrepant results (13, 14) (also see "Discussion"). Guided by the Skp2 crystal structure (15), we first divided the full-length Skp2 protein at residue 150 (the *NF* and *LRR* fragments shown in Fig. 1C) without disrupting α-helices or β-sheets. In cotransfection assays, Skp2NF bound cyclin A, whereas Skp2LRR did not (Fig. 1A). We then determined that the N-terminal 100 residues fused with GFP (Skp2N-GFP) were sufficient for cyclin A binding, indicating that the F-box is not required (*lanes 7* and *8*). We went on to use smaller deletions to map the amino acid residues necessary for binding cyclin A to between residues 40 and 60 in the context of full C-terminal sequences (Fig. 1, *B* and *C*).

A commonly used physical relationship between cyclins and non-Cdk proteins is mediated between the R*XL* motif in the interacting proteins (16, 17) and the hydrophobic patch (HP) on cyclins (the R*XL*-HP interaction) (18, 19). Interestingly, sequences between 40 and 60 of Skp2 do not contain a recognizable R*XL* motif (Fig. 1D). This sequence, however, is nearly completely conserved from human Skp2 to mouse and chicken Skp2 orthologs. When the alignment is extended to fly Skp2, four conserved residues can be identified. Mutation of these four conserved residues to alanine (the Skp2AAAA mutant) abolished the ability of the full-length Skp2 to bind cyclin A (Fig. 1E). These results suggest that Skp2 uses a novel, non-R*XL* cyclin A binding domain to interact with cyclin A.
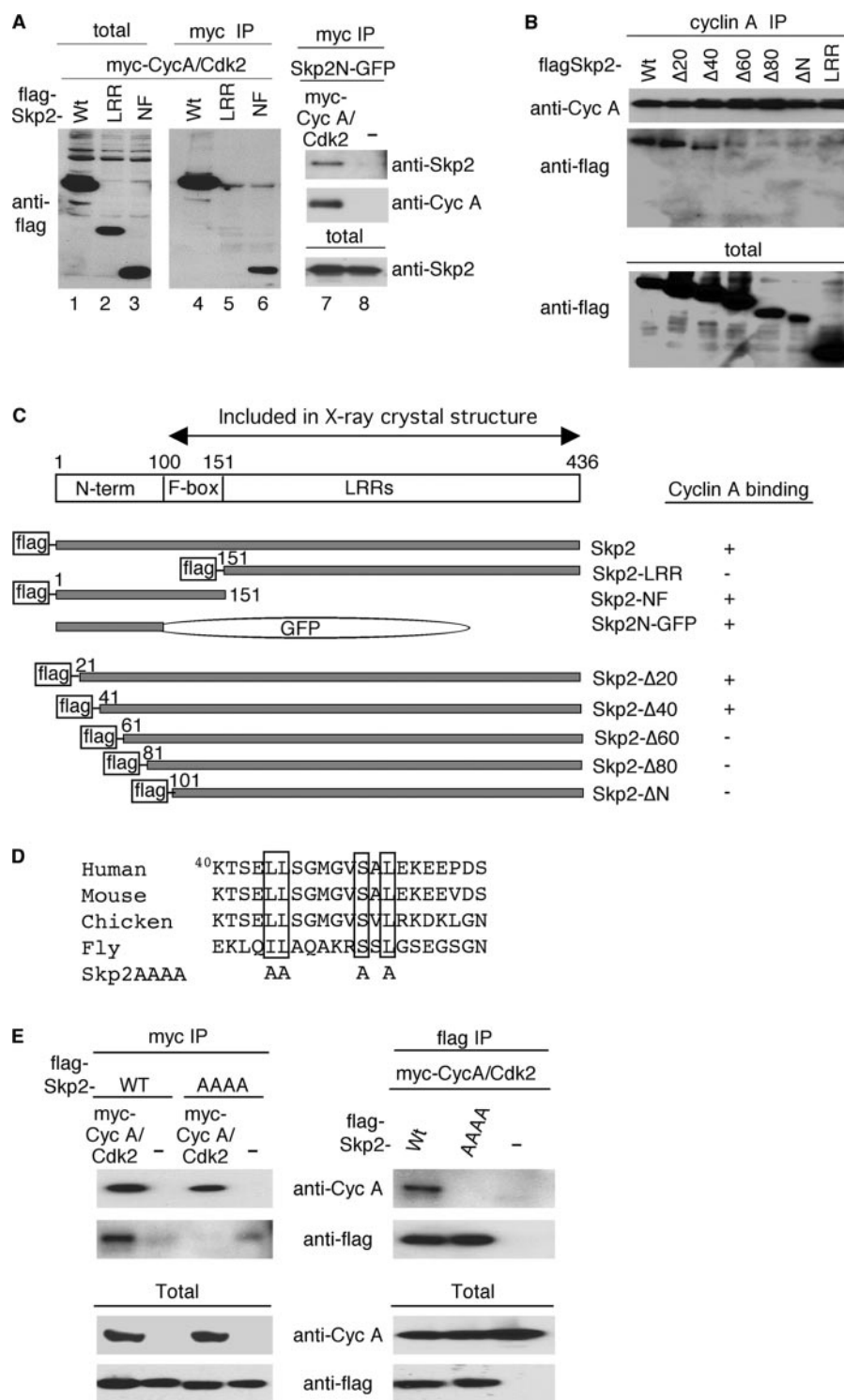
FIGURE 1. **Identification of Skp2 sequences required for binding cyclin A.** *A* and *B*, 293T cells were transfected with various expression vectors as indicated. Immunoprecipitation (*IP*)-Western blot assays were performed with extracts of transfected cells. *Wt*, wild type; *LRR*, leucine-rich repeat. *C*, a schematic drawing of Skp2 domain structure, various mutants, and their ability to bind cyclin A, as determined in *A* and *B*. Numbering is based on NCBI accession number U33761. *NF*, N terminus and F-box. *D*, alignment of human Skp2 sequence from amino acid residues 40–60 with corresponding sequences of Skp2 orthologs in different species. Identical residues are *boxed* and were mutated to alanine in Skp2AAAA. *E*, immunoprecipitation-Western assay with transfected 293T cell extracts, as in *A* and *B*.

biochemical properties of Skp2 by comparing Skp2 and Skp2AAAA. Because the cyclin A binding domain is located N-terminal to the F-box and an N-terminally truncated Skp2 (Skp2ΔN) could form complexes with Skp1-Cul1-Roc1 (15, 20), we predicted that Skp2AAAA should retain the ability to form the SCF[Skp2]·Roc1 complex. We tested this prediction by co-expressing the four components of SCF[Skp2]·Roc1 complex into 293T cells and immunoprecipitating Skp2 (or Skp2AAAA) via a FLAG tag on Skp2. The results show that Skp2AAAA was indeed capable of forming the SCF[Skp2AAAA]·Roc1 complex (Fig. 2*A*).
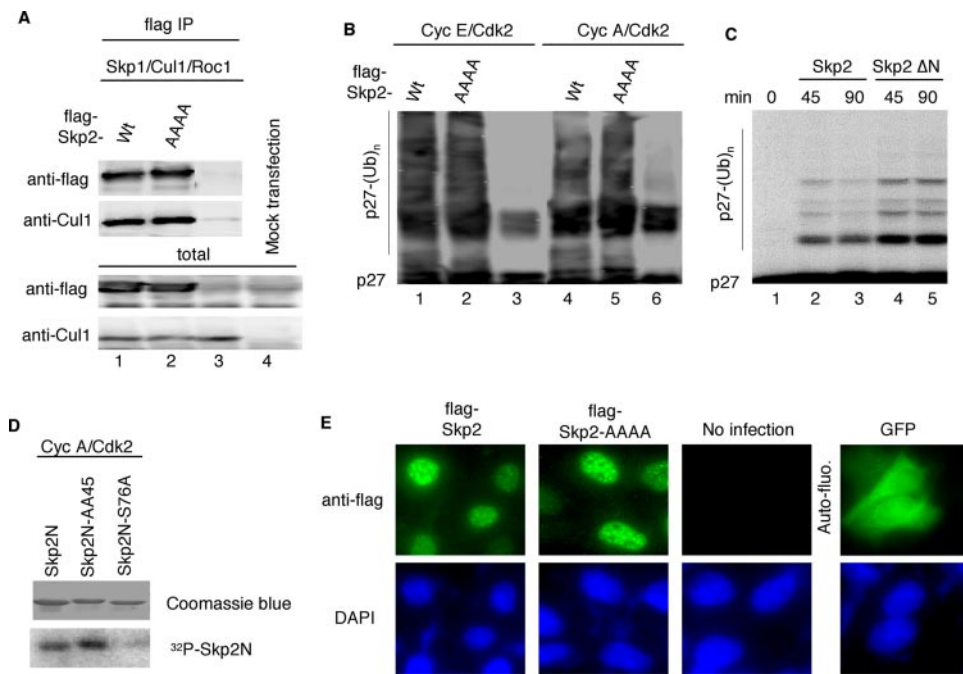
We next determined whether the SCF[Skp2AAAA]·Roc1 complex was active as an ubiquitin ligase for p27. Immunopurified SCF[Skp2]·Roc1 and SCF[Skp2AAAA]·Roc1 complexes supplemented with E1 and E2 were incubated with purified p27 that had been first phosphorylated by cyclin E-Cdk2 or cyclin A-Cdk2. Results shown in Fig. 2*B* demonstrate that Skp2AAAA retained the ability to ubiquitylate p27. No p27 ubiquitylation was observed when p27 was not first phosphorylated by cyclin E-Cdk2 or cyclin A-Cdk2 (data not shown). To confirm that the binding to cyclin A is not necessary for p27 ubiquitylation with another Skp2 mutant, wild-type Skp2 and Skp2ΔN were expressed in insect cells using a baculovirus system together with Skp1, Cul1, and Roc1 and then purified by nickel-agarose chromatography. Skp2ΔN was able to induce p27 ubiquitylation similarly to wild-type Skp2 (Fig. 2*C*). Similar results were obtained when Skp2ΔN and wild-type Skp2 were produced using a rabbit reticulocyte system[6] or with bacterial-purified Skp2ΔN.[7]

Ser-76 in the Skp2 N terminus can be phosphorylated by cyclin A-Cdk2 (14). We determined whether Skp2-cyclin A interaction

Skp2-Cyclin A Interaction Is Separable from Skp2 Ability to Mediate p27 Ubiquitylation—With the ability to disrupt Skp2-cyclin A interaction with point mutations in the Skp2 N terminus, we determined the role of this interaction in other known

serves a substrate recruitment function and, therefore, is

FIGURE 2. **Skp2-cyclin A interaction is separable from other known activities of Skp2.** *A*, immunoprecipitation-Western assay with transfected 293T cell extracts as in (Fig. 1, *A* and *B*) except expression vectors for SCF^{Skp2}·Roc1 subunits were used. Skp2 was omitted in transfection for *lane 3*. Cells in *lane 4* were mock-transfected. *Wt*, wild type. *B*, in vitro p27 ubiquitylation assay using the immuno-purified complexes as shown in *A*. Purified p27 was first phosphorylated by either purified cyclin E-Cdk2 or cyclin A-Cdk2 as indicated. *C*, in vitro ubiquitin ligation assay of ^{35}S-labeled in vitro translated p27 was conducted in the presence of purified recombinant Cdk2-cyclin A complex and purified recombinant SCF^{Skp2} (*lanes 2* and *3*) or SCF$^{Skp2\Delta N}$ (*lanes 4* and *5*). Similar results were obtained with purified recombinant Cdk2-cyclin E complex (not shown). Samples were incubated at 30 °C for the indicated times. *D*, various purified GST fusion proteins, as indicated, were incubated in kinase reactions with purified cyclin A-Cdk2. Coomassie Blue stain and autoradiograph of the same gel are shown. *E*, U2OS cells were infected with lentiviruses expressing GFP, FLAG-tagged Skp2, or FLAG-tagged Skp2AAAA. Expression and cellular localization of Skp2 was revealed by indirect immunofluorescence staining. *DAPI*, 4′,6-diamidino-2-phenylindole.

required for this phosphorylation. Results in Fig. 2*C* show that disruption of interaction between cyclin A and the Skp2N by point mutations (see also Fig. 3*C*) did not affect the phosphorylation of Ser-76 by cyclin A-Cdk2, suggesting that phosphorylation of Ser-76 does not require a stable association of Skp2 with cyclin A. Skp2 is a nuclear protein (13). We determined whether Skp2-cyclin A interaction is required for its nuclear localization. As shown in Fig. 2*D*, both Skp2 and Skp2AAAA are localized in the nucleus when expressed in U2OS cells. Based on these results, we conclude that Skp2-cyclin A interaction is separable from these known properties of Skp2.

Skp2 Binds Cyclin A as Efficiently as p27—We next examined the effects of Skp2-cyclin A interaction on cyclin A-Cdk2. p27 is the best-understood R*X*L protein that binds cyclin A-Cdk2 as a kinase inhibitor. As an approach to understanding the effects of Skp2 on cyclin A-Cdk2, we undertook to compare Skp2-cyclin A-Cdk2 interaction with p27-cyclin A-Cdk2 interaction.

We first compared the relative binding efficiencies of Skp2-cyclin A-Cdk2 and p27-cyclin A-Cdk2. We used FLAG-tagged Skp2 and p27 so that the amounts of these two proteins can be compared with anti-FLAG antibody on Western blots. When expressed at similar levels in transfected 293T cells (Fig. 3*A*, *total* gels), similar amounts of FLAG-p27 and FLAG-Skp2 were co-immunoprecipitated with the cotransfected cyclin A (Fig. 3*A*, *myc IP* gels). We next used GST pull-down assays to compare the relative binding efficiencies *in vitro*. We first showed

that the Skp2 N terminus (Skp2N) can bind cyclin A as efficiently as the full-length Skp2 by titrating down the input amounts of cyclin A-Cdk2 (Fig. 3*B*, *lanes 1–6*). When the binding efficiencies of Skp2N and p27 were compared in the titration experiments, relative binding efficiencies were again found to be similar (Fig. 3*B*, *lanes 7–15*). In Fig. 3*C* we demonstrate that point mutations of the conserved residues in the cyclin A binding sequence (40–60) abolished cyclin A binding of Skp2N. In this experiment we found that mutations of two conserved residues (either AA^{45} or AAA^{53}) can efficiently abolish cyclin A binding. Finally, purified Skp2N (but not Skp2NAA^{45}) and purified p27 are able to bind purified cyclin A-Cdk2 (Fig. 3*D*), indicating that Skp2-cyclin A interaction is also direct as the p27-cyclin A-Cdk2 interaction.

Skp2 and p27 Bind to Different Sequences on Cyclin A—Results in Fig. 1 demonstrated that Skp2 uses a novel non-R*X*L motif to interact with cyclin A. We next investigated whether the Skp2-cyclin A-Cdk2 interaction and the p27-cyclin A-Cdk2 interaction also differ on

the side of cyclin A-Cdk2. An important feature for p27-cyclin A-Cdk2 interaction is that, in addition to the R*X*L-HP interaction, p27 also has extended interaction with the N-terminal lobe of Cdk2, providing a structural basis for its inhibitory effects on cyclin A-Cdk2 kinase (18). To compare this aspect of p27 with Skp2, we expressed cyclin A and Cdk2 separately in 293T cells (levels of endogenous cyclin A or Cdk2 are not expected to be sufficiently high to form significant amount of complexes with transfected Cdk2 or cyclin A, respectively) and determined the binding behavior of GST-p27 and GST-Skp2N in these transfected extracts. As shown in Fig. 4*A*, GST-p27 binds cyclin A-Cdk2 complex as well as cyclin A or Cdk2 in singly transfected extracts. In the same conditions, GST-Skp2N binds cyclin A-Cdk2 and cyclin A alone but does not bind Cdk2 alone. The amount of Cdk2 is also less in GST-Skp2N-cyclin A-Cdk2 pull-down than in GST-p27-cyclin A-Cdk2 pull-down, which is consistent with p27, but not Skp2N, being also able to bind free Cdk2.

We then attempted to localize Skp2 interaction sequences in cyclin A in comparison with p27. Cyclin A contains two conserved cyclin-box sequences that form two cyclin folds in the cyclin A-Cdk2 crystal structure (21), which does not include the N-terminal 172-amino acid residues (Fig. 4*B*). The HP that mediates the interaction with the R*X*L motif of p27 is composed of residues ^{210}MRAILVDW in the α1 helix of the N-terminal cyclin fold (18). Based on this information, we designed the
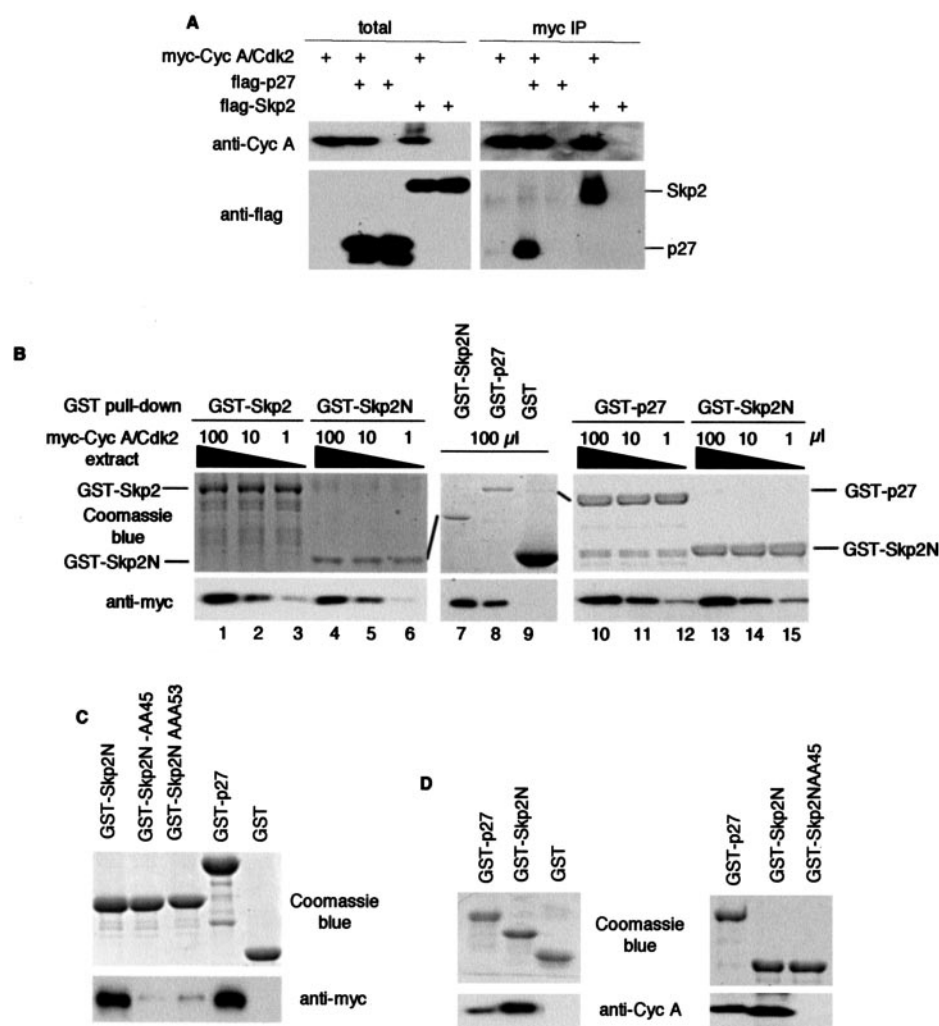
FIGURE 3. **Comparison of binding efficiencies of Skp2-cyclin A and p27-cyclin A.** *A*, Immunoprecipitation-Western assay of transfected 293T cell extracts as indicated. *B*, GST pull-down assays with GST, GST-p27, GST-Skp2, or GST-Skp2N and 293T cell extracts transfected with Myc-cyclin A and Cdk2. *C*, GST pull-down assays as in *B* except two GST-Skp2N mutants were included. *D*, GST pull-down assay with the indicated GST-fusion proteins and baculovirally produced and purified GST-cyclin A-Cdk2 complex after GST cleavage.

following cyclin A mutants to map and compare Skp2 and p27 binding sequences as shown in Fig. 4, *B* and *C*. Cyclin A sequences 1–303 (consisting of the N-terminal cyclin fold and the N terminus) bound to p27 and Skp2N to a similar extent as the full-length cyclin A (please compare the relative amounts in total and pull-down gels), whereas the C-terminal cyclin fold and the C terminus (the 309–432 fragment) did not bind p27 or Skp2N. Cyclin A with an N-terminal truncation to residue 173 (which was previously used for crystal structure analysis) bound to p27 to a similar extent as full-length cyclin A but barely detectably to Skp2N. This result is in agreement with the determination of HP to be the p27 binding site by crystal structure studies and indicates that a distinct site upstream of residue 173 provides major binding affinity for Skp2N. Consistent with this notion, cyclin A fragment 1–208 can bind to Skp2N but not p27. Identification of the cyclin A N terminus as the major binding site for Skp2N may provide a structural basis for the cyclin specificity of the Skp2-cyclin A interaction because, unlike the cyclin-box sequences, cyclin A N-terminal sequences are not conserved in other cyclins. Indeed, as shown

in Fig. 4, *D* and *E*, Skp2N did not interact with cyclin E-Cdk2 or cyclin D1-Cdk4, whereas p27 showed stable binding to these cyclins.
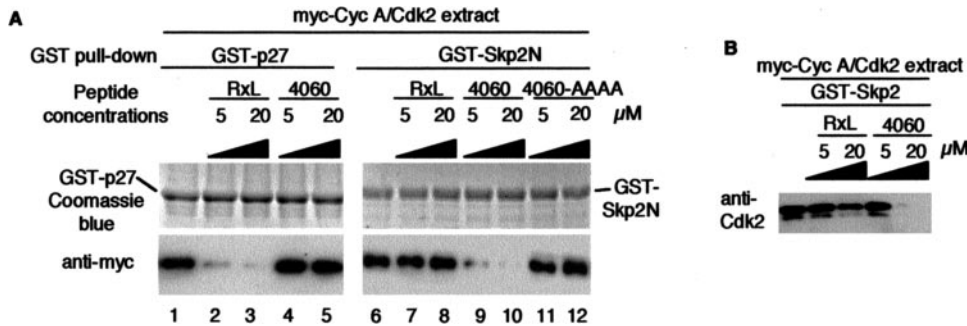
Skp2-Cyclin A Interaction and p27-Cyclin A Interaction Can Be Blocked by Distinct Peptides—Mutation analysis to map the required interaction sequences leaves open the possibility that certain mutations may alter binding indirectly through influencing general protein conformation rather than directly serving as the interacting site. An important aspect of R*X*L-HP interaction is that it can be blocked by a short R*X*L peptide (17). If Skp2-cyclin A-Cdk2 interaction and p27-cyclin A-Cdk2 interaction are mediated by different sequences on both sides of the interaction, as shown above, it can be predicted that the R*X*L peptide should not block Skp2-cyclin A interaction. We also determined whether an 18-residue peptide replica derived from Skp2 sequences between 40 and 60 (called the 4060 peptide) could block Skp2-cyclin A interaction but not p27-cyclin A interaction. Our results show that the 4060 peptide can block interaction between Skp2N and cyclin A but not between p27 and cyclin A (Fig. 5*A*, *lanes 9* and *10* and *lanes 4* and *5*). Mutations of the four conserved residues to alanine (the 4060AAAA peptide) abolished the blocking activity of the 4060 peptide (*lanes 11* and *12*). The R*X*L peptide has the opposite effects (blocking p27-cyclin A interaction but not Skp2N-cyclin A interaction) (*lanes 2* and *3* and *lanes 6* and *7*). Peptide concentrations required for their respective blocking activities were similar for R*X*L and 4060 peptides. The 4060 peptide, but not the R*X*L peptide, also blocked binding between full-length GST-Skp2 and cyclin A-Cdk2 (Fig. 5*B*). These results provide strong evidence that the Skp2 40–60 sequence contains a novel cyclin A interacting domain and Skp2-cyclin A interaction is distinct from the R*X*L-HP interaction.

Skp2 Competes with p27/p21, but Not E2F1, for Binding to Cyclin A—The original findings that transformed cells contained exclusively cyclin A-Cdk2-Skp2 complexes, whereas untransformed cells contained exclusively cyclin A-Cdk2-p21 complexes raised the possibility that these two complexes might be mutually exclusive (8, 9). It was further demonstrated that transfected p21 could reduce cyclin A-Cdk2-Skp2 interaction (14). Our determination that Skp2-cyclin A-Cdk2 interaction is distinct from p27-cyclin A-Cdk2 interaction prompted us to revisit this topic. We
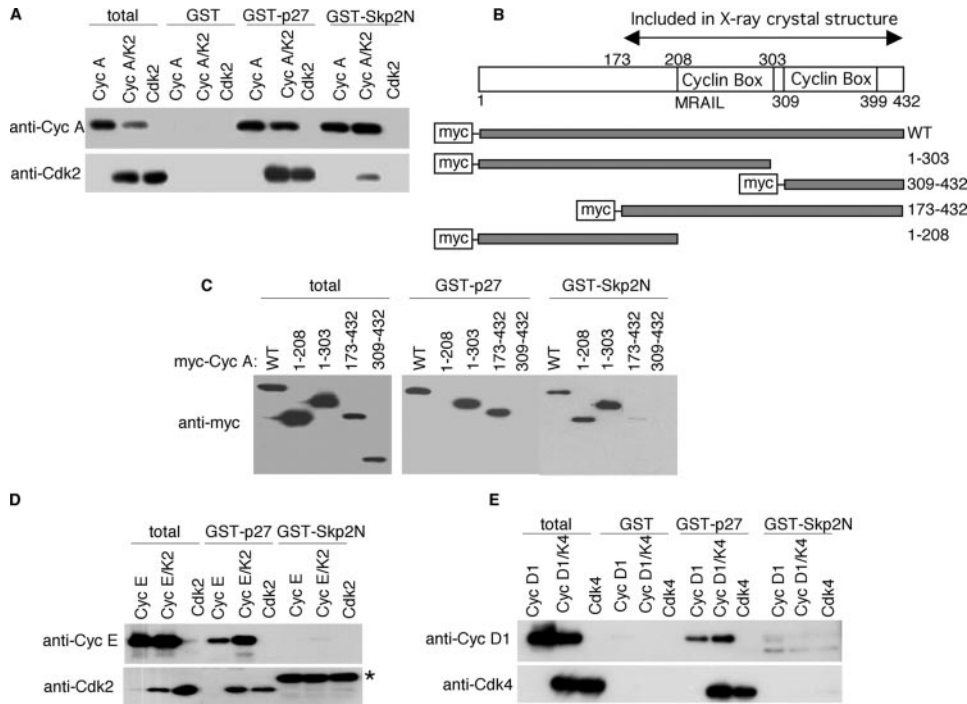
FIGURE 4. **Skp2 does not interact with Cdk2 alone and interacts with N terminus of cyclin A.** *A*, GST pull-down assay with indicated GST fusion proteins and 293T cell extracts transfected with indicated expression vectors. *B*, a schematic drawing of cyclin A domain structure and various mutants used in *C. C*, GST pull-down assay as in *A* with indicated GST fusion proteins and cell extracts transfected with various cyclin A mutants. *WT*, wild type. *D* and *E*, GST pull-down assays as in *A* except with cyclin E (*Cyc E*)-Cdk2 or cyclin D1-Cdk4-transfected cell extracts. The *asterisk* indicates background bands.

FIGURE 5. **Skp2-cyclin A interaction and p27-cyclin A interaction are blocked by distinct peptides.** *A*, GST pull-down assay with indicated GST fusion proteins and Myc-cyclin A-Cdk2-transfected 293T cell extracts in the absence and presence of various indicated peptides at indicated concentrations. *B*, same as in *A*, except GST-Skp2 was used.

in that it does not inhibit cyclin A-Cdk2 kinase activity and is mainly a phosphorylation substrate. As shown in Fig. 6*B*, by including progressively higher concentrations of Skp2N in a binding reaction with fixed amounts of GST-p21 and cyclin A-Cdk2, p21 binding to cyclin A was progressively diminished, confirming the previous finding (14). In parallel experiments we show that Skp2N also competed with GST-p27 for binding to cyclin A-Cdk2 (Fig. 6*C*). Interestingly, Skp2N did not affect E2F1-cyclin A binding, whereas p27 efficiently blocked it as expected (Fig. 6*D*). Thus, Skp2 binding to cyclin A does not block interaction of cyclin A with all R*X*L proteins, which is consistent with the fact that, unlike p27, Skp2 does not use the R*X*L-HP mechanism to interact with cyclin A. Finally, we show in Fig. 6*E* that Skp2N did not compete with p27 for binding to cyclin E-Cdk2, which is consistent with the finding in Fig. 4*D* that Skp2N did not bind cyclin E-Cdk2. How Skp2 competes with p21/p27 for cyclin A binding and how Skp2 discriminates between p21/p27 and E2F1 (and potentially other cyclin A binding proteins) for competition will require more detailed structural studies. Nevertheless, these binding characteristics apparently provide a molecular explanation for the regulatory effects of Skp2 on cyclin A-Cdk2 kinase activity described below.

Skp2-Cyclin A Interaction Directly Protects Cyclin A-Cdk2 from Inhibition by p27—Effects of Skp2-cyclin A interaction on cyclin A-Cdk2 kinase activity has remained

first determined whether the competition between Skp2 and p21 for cyclin A-Cdk2 binding could be observed *in vivo* with endogenous proteins under physiological conditions. For this purpose, we treated U2OS cells with actinomycin D to activate p53, which led to a significant increase in p21 protein levels. As shown in Fig. 6*A*, a significant increase in p21 levels after actinomycin D treatment in the assay conditions did not change protein levels of Skp2 and cyclin A (*lanes 1* and *2*) but decreased Skp2-cyclin A interaction to background levels (*lanes 4* and *5*) with increased binding of cyclin A to p21 (*lanes 6* and *7*), documenting that competition between p21 and Skp2 for cyclin A binding is a physiological process.

We next determined whether Skp2 competes with other R*X*L proteins for cyclin A binding. Using GST pull-down assays, we compared p21, p27, and another well established R*X*L protein E2F1 (22). E2F1 differs from p21/p27 as a cyclin A-binding protein

unclear and controversial. In the original report, Beach and co-workers (9) showed that Skp2-cyclin A-Cdk2 interaction did not inhibit nor stimulate cyclin A-Cdk2 kinase activity. To the contrary, Poon and co-workers (14) provided evidence that Skp2 could inhibit cyclin A-Cdk2 kinase activity. We reasoned that a better way to determine the effects of Skp2 on cyclin A-Cdk2 kinase activity is to compare Skp2 with a well established CKI-like p27 and relate the amounts and ratios of relevant proteins used *in vitro* to the respective amounts and ratios of the same proteins in cells. We also used purified proteins in soluble reactions to measure kinase activity under equilibrium conditions to avoid complications associated with immunoprecipitation of the kinases. These types of biochemical studies have been previously performed for p21, p27, and related CKIs (23).
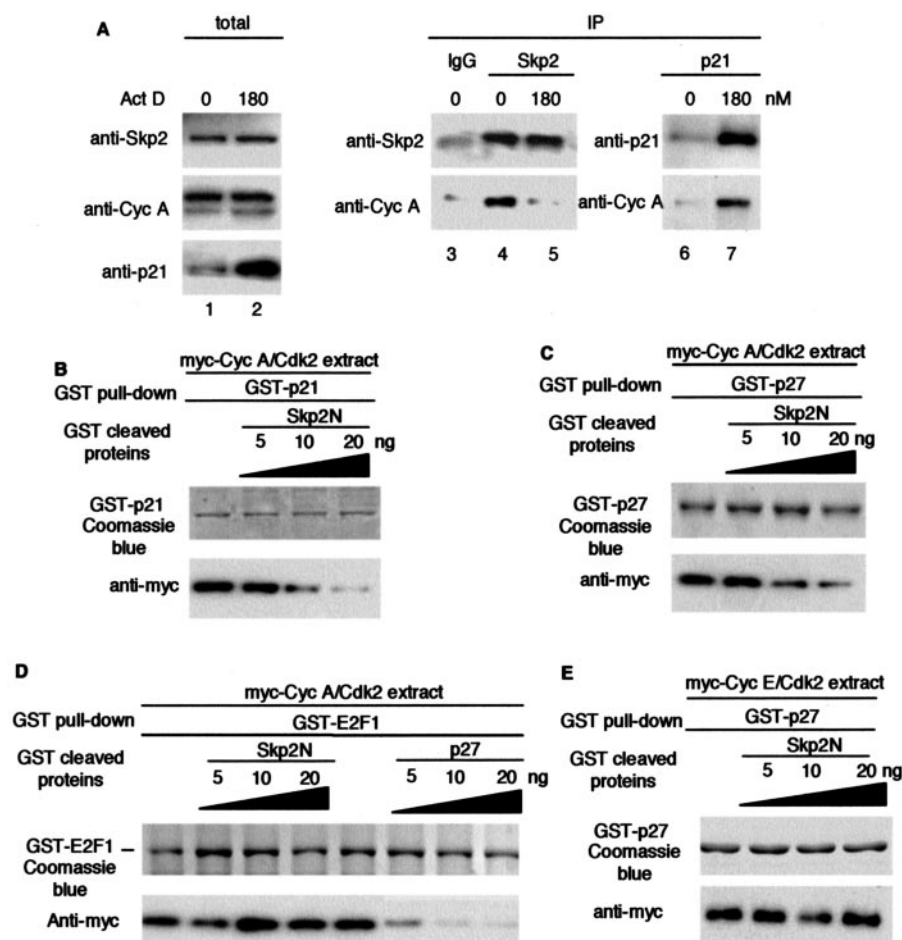
FIGURE 6. **Skp2 competes with p21/p27, but not E2F1, for binding to cyclin A.** *A*, U2OS cells were treated with 180 nm actinomycin D (*Act D*) for 20 h. Cell extracts were used for immunoprecipitation (*IP*)-Western blotting as indicated. *Cyc A*, cyclin A. *B*, GST pull-down assay with GST-p21 and Myc-cyclin A-Cdk2-transfected 293T cell extracts. Increasing amounts of eluted and GST-cleaved Skp2N were added as indicated. *C* and *D*, GST pull-down assay as in *B* except GST-p27 and GST-E2F1 were used as indicated. *E*, GST pull-down assay as in *C*, except extract expressing Myc-cyclin E-Cdk2 was used.

them with ratios used in kinase assays. Skp2 forms abundant complexes with cyclin A in tumor cells due to its frequent overexpression. We used HeLa cells, which contain higher levels of Skp2 compared with a number of commonly used cell lines (data not shown), to determine whether Skp2 to cyclin A ratios, that resulted in kinase inhibition in kinase assays, could be reached in cells. Because Skp2, p27, and cyclin A levels oscillate in the cell cycle, we determined their levels in synchronized cell populations obtained at various time points after release from mitotic arrest by nocodazole as shown in Fig. 7*G*. We quantified the quantities of p27, Skp2, and cyclin A in HeLa cells at various cell cycle stages by comparing them with known amounts of purified Skp2N, p27, and cyclin A in the same Western blots (see "Experimental Procedures"). We then determined the molar ratios of p27/cyclin A and Skp2/cyclin A at representative time points in the cell cycle. As presented in Fig. 7*G*, the p27/cyclin A molar ratio is ~1.0 at 14 h, when cyclin A is starting to accumulate, and ~0.01 at 19 h, when cyclin A has reached significant levels, whereas p27 levels decreased. Because the IC_{50} of p27 is ~2 nm in the kinase reaction con-

For kinase reactions we used insect cell-produced and -purified GST-cyclin A-Cdk2 at a concentration of ~3 nM, which was the lowest concentration that yielded readily detectible phosphorylation of GST-Rb-C in our assay. Purified p27, GST-Skp2, and Skp2N were titrated into the kinase reaction over a wide range of concentrations as shown in Fig. 7, *A–C*. Phosphorylation of Rb-C was measured and plotted, and the IC_{50} of these various proteins were determined by non-linear least squares analysis (Fig. 7*F*). Results show that p27 inhibited cyclin A-Cdk2 kinase activity with an IC_{50} of ~2 nM. In the same assay conditions, GST-Skp2 and Skp2N could also inhibit the kinase activity although their IC_{50} values of ~180 and ~520 nM, respectively, are significantly higher than that of p27. At this high concentration Skp2N may also inhibit cyclin A-Cdk2 kinase by a substrate competition mechanism since Ser-76 of Skp2 can be phosphorylated by cyclin A-Cdk2 (Fig. 2*D*). Results from this series of experiments suggest that previous discrepant results of Skp2 effects on cyclin A-Cdk2 kinase activity could be due to different amounts of Skp2 used in the kinase assays.

We wished to determine whether the observed inhibitory effects of Skp2 has physiological relevance by measuring the amounts and ratios of relevant proteins in cells and comparing

taining 3 nM cyclin A, these molar ratios suggest that p27 could function as an effective CKI at the 14-h time point but not at 19-h time points. When the same analysis was applied to Skp2, it was revealed that the highest Skp2-cyclin A molar ratio found at 3 h is about ~5, which is well below the molar ratio at the IC_{50} of Skp2 (~180 nM Skp2 over 3 nM cyclin A-Cdk2). Although it is formally possible that certain factors could affect or modify the activity of Skp2 *in vivo*, the large gap between Skp2-cyclin A molar ratios *in vivo* and that required to reach IC_{50} *in vitro* suggests that Skp2 is unlikely to function as an inhibitor of cyclin A-Cdk2 kinase activity.

Because Skp2 competes with p27 for binding to cyclin A and Skp2 IC_{50} is about 100-fold higher than the p27 IC_{50}, we reasoned that at concentrations well below its IC_{50}, Skp2 might be able to protect cyclin A-Cdk2 from inhibition by p27. To test this hypothesis we carried out the same cyclin A-Cdk2 kinase assay to determine the inhibitory activity of p27 in the presence of 80 nM Skp2N. The results in Fig. 7, *D* and *E*, show that inclusion of Skp2N, but not Skp2N-AA45, increased the IC_{50} of p27 from ~2 to ~80 nM, revealing such a protective effect of Skp2. Protein molar ratio analysis suggests that this protective effect of Skp2 could be effective at the 14-h time point when cyclin A
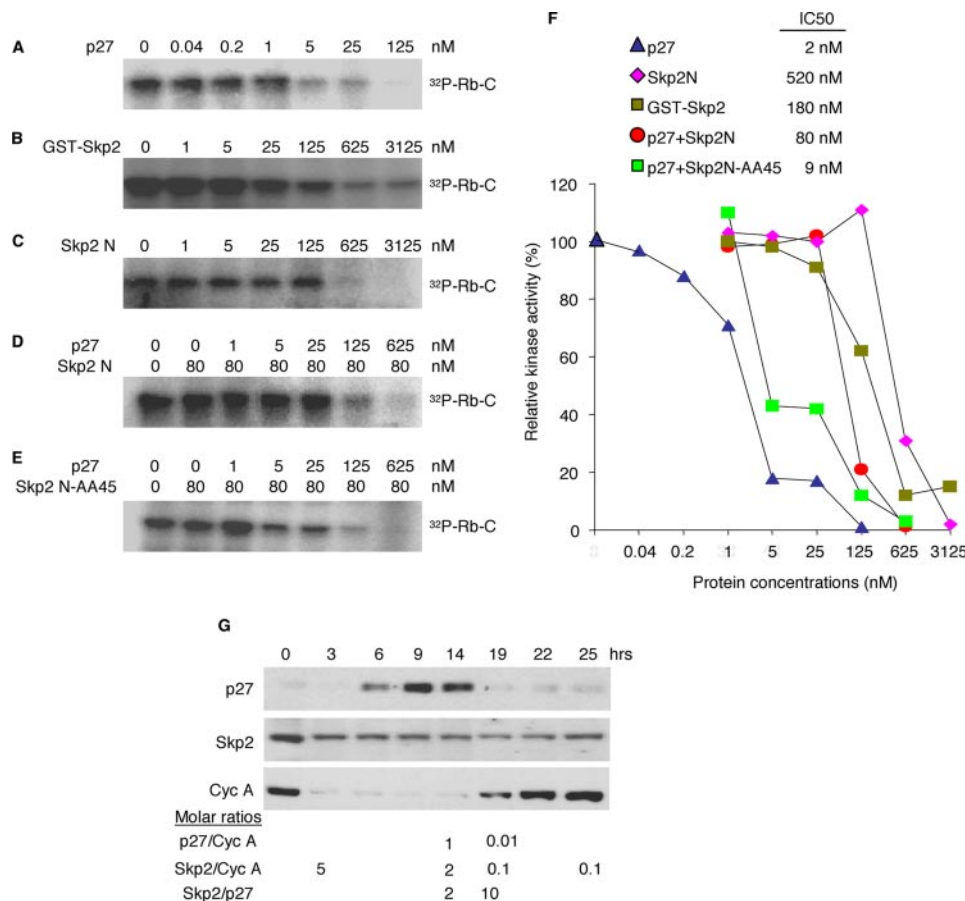
FIGURE 7. **Regulation of cyclin A-Cdk2 kinase activity by Skp2.** *A–E*, kinase reaction using purified cyclin A-Cdk2 at ~3 nM. Increasing concentrations of purified p27, GST-Skp2, Skp2N, and Skp2N-AA45 were included in the kinase reaction as indicated. *F*, phosphorylation of Rb-C was quantified and plotted to determine the K_i of various proteins. *G*, HeLa cells were arrested in mitotic phase with nocodazole treatment and released into fresh media at time 0. Fractions of cells were harvested at the indicated time points, and cellular protein levels of p27, Skp2, and cyclin A were determined with Western blotting. Determination of various molar ratios was described under "Experimental Procedures."

levels and increased cyclin A-associated kinase activity to similar degrees, consistent with the finding that Skp2AAAA was able to mediate p27 ubiquitylation *in vitro* (Fig. 2*B*). Overexpression of Skp2 also led to a slight (~4%) increase in S-phase populations (Fig. 8*D*). Overexpression of Skp2AAAA, however, did not have this effect.

In cells treated with actinomycin D, activation of p53, as demonstrated by increases in p53 protein levels, was not affected by overexpression of either Skp2 or Skp2AAAA (Fig. 8*A*). On the other hand, overexpression of Skp2 largely prevented the increase in p21 levels (which is consistent with previous findings that Skp2 can also target p21 for ubiquitylation (5)) (Fig. 8*A*) and the decrease in cyclin A-associated kinase activity after actinomycin D treatment (Fig. 8*B*). In comparison, overexpression of Skp2AAAA was fully competent in reducing p21 protein levels after actinomycin D treatment but was compromised in maintaining cyclin A-associated kinase activity. This defect in maintaining cyclin A-associated kinase activity correlated with more cyclin A-p21 association in cells expressing Skp2AAAA than in cells expressing Skp2 after actinomycin D treatment (Fig. 8*C*), which is consistent with results shown in Fig. 6*A*.
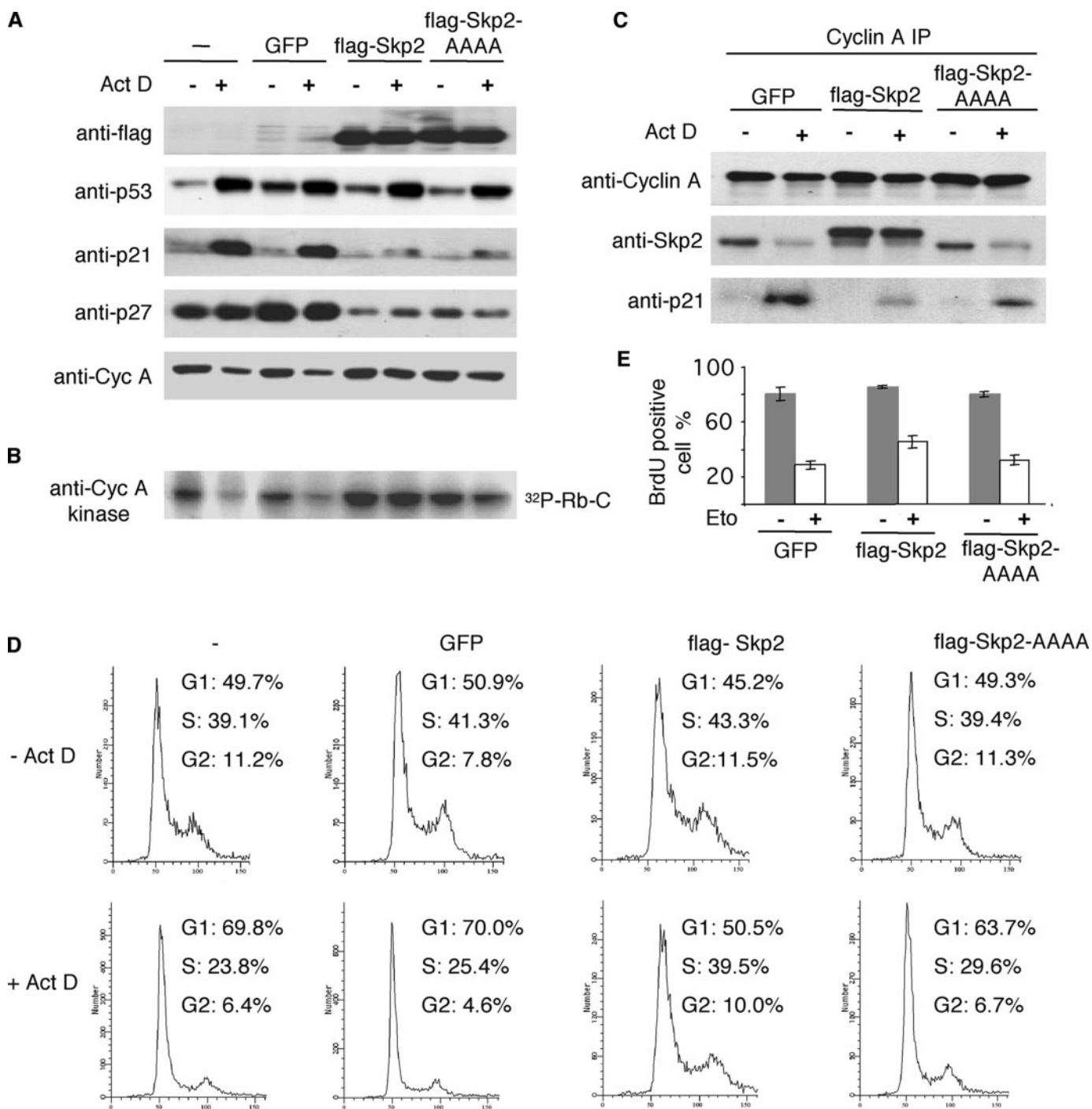
More importantly, Skp2AAAA exhibited a clear functional defect in countering G_1/S cell cycle arrest induced by actinomycin D treatment. As shown in Fig. 8*D*, overexpression of Skp2 largely prevented G_1/S cell cycle arrest after actinomycin D treatment, as manifested by only a 5% increase in G_1 cell population as compared with a 20% increase in G_1 cell populations in uninfected and GFP-infected cells. In contrast, actinomycin D treatment still led to a 15% increase in G_1 cell populations in the presence of overexpression of Skp2AAAA.

To determine and compare the ability of Skp2 and Skp2AAAA to counter p53/p21-mediated cell cycle arrest in another context, we treated cells with another commonly used DNA-damaging agent etoposide. Consistent with results obtained with actinomycin D treatment, treatment of U2OS cells with etoposide increased protein levels of cellular p53 and p21, and overexpression of Skp2 and Skp2AAAA similarly prevented the increases in p21 levels after etoposide treatment (data not shown). Treatment of GFP-infected U2OS cells with etoposide led to accumulation of cells in S phase by fluorescence-activated cell sorter analysis (data not shown) and inhibition of active DNA synthesis as demonstrated by a decrease in the fractions of BrdUrd-positive cells from 80 to 29%

levels start to rise and Skp2 is present in about a 2-fold excess over p27 (Fig. 7*G*).

The AAAA Mutation Compromises Skp2 Proliferation Stimulatory Activity without Affecting Its Ability to Cause Degradation of Cellular p27 and p21—Skp2 exhibits proliferation-promoting activities in various assays, which have been attributed to Skp2-mediated p27 ubiquitylation and degradation. With a better understanding of the Skp2-cyclin A interaction and the ability to disrupt this interaction with point mutations that did not affect the Skp2 ubiquitylating function, we next determined the role of Skp2-cyclin A interaction in the proliferation-stimulating activity of Skp2. Similar with the experiment shown in Fig. 6*A*, we treated U2OS cells with actinomycin D, which led to activation of p53, stimulation of p21 expression, and G_1 cell cycle arrest, manifested by a ~20% increase in G_1 phase cells (Fig. 8, *A* and *D*). We overexpressed Skp2 in U2OS cells to determine whether Skp2 could counter the G_1 cell cycle arrest mediated by this actinomycin D-triggered checkpoint.

In untreated cells overexpression of Skp2 led to a significant reduction of p27 levels (Fig. 8*A*, anti-p27 Western blot) and a marked increase in cyclin A-associated kinase activity measured with anti-cyclin A immunoprecipitates (Fig. 8*B*). Overexpression of Skp2AAAA to similar levels as Skp2 reduced p27

FIGURE 8. **The AAAA mutation does not affect Skp2-mediated degradation of p27 and p21 but significantly compromises Skp2 ability to counter a p53-p21 mediated G$_1$ cell cycle arrest.** *A*, U2OS cells were infected with various lentiviruses as indicated and treated with actinomycin D (*Act D*) for 20 h. Total cell extracts were analyzed by Western blotting. *Cyc A*, cyclin A. *B*, the same cell extracts as in *A* were used for immunoprecipitation (*IP*) with an anti-cyclin A antibody. The immunoprecipitates were used for a kinase reaction with GST-Rb-C as substrate. *C*, the same cell extracts were used for immunoprecipitation with an anti-cyclin A antibody followed by Western blotting as indicated. *D*, the same cells were stained with propidium iodide, and their DNA profiles were determined by flow cytometry. Results presented here are representatives of three separate experiments. *E*, U2OS cells infected with GFP, FLAG-Skp2, and FLAG-Skp2AAAA as indicated were treated with etoposide (*Eto*) for 24 h. Cells were labeled with BrdUrd for the last 2 h before fixation for BrdUrd indirect immunofluorescence staining. Nearly 200 cells were scored for BrdUrd staining. Results from three experiments were averaged with *error bars* as shown.

after a 24-h treatment (Fig. 8*E*). These effects suggested that, unlike actinomycin D, etoposide treatment of U2OS cells largely led to intra-S-phase arrest. In this cellular context, overexpression of Skp2 led to a net increase of 17% in the fraction of BrdUrd-positive cells over the 29% BrdUrd labeling index of etoposide-treated GFP-infected cells. In contrast, overexpression of

Skp2AAAA increased the BrdUrd labeling index over that of GFP-infected cells by only 4% (Fig. 8*E*). We conclude from these results that Skp2-cyclin A interaction has a significant role in Skp2 proliferation-stimulating activity in countering cell cycle arrest by p53/p21-mediated cell cycle checkpoint without affecting its ability to cause degradation of cellular p27 and p21.

DISCUSSION

Skp2-Cyclin A Interaction Reveals a Novel Way to Physically Interact with Cyclin A—Cyclin-Cdk as a core kinase complex interacts with other proteins for regulation and substrate recruitment. Interaction between the HP of a cyclin and an R*X*L motif of an interacting protein represents a paradigm of this relationship. Skp2 contains a potential R*X*L motif (the K*X*L[376] sequence), and mutation of K*X*L[376] to AAA[376] abolished its interaction with cyclin A, suggesting that Skp2 belongs to the R*X*L protein family (13). Interestingly, the AAA[376] mutation also abolished Skp2-Skp1 binding (13), suggesting that this mutation might have multiple effects. Subsequent x-ray crystal structure studies revealed that Skp2 (with N-terminal truncation of 100 residues) has a rigid structure shaped like a sickle (15). Skp2 F-box sequence and Skp1 together form the handle, whereas the K*X*L[376] sequence is buried inside the curved blade. Based on this structural feature, it is likely that the effects induced by the AAA[376] mutation could be due to more general protein conformational changes.

Results from our current study identify a non-R*X*L cyclin A binding domain in the Skp2 N terminus. Skp2 sequences between amino acid residues 40–60 are required for cyclin A binding and are highly evolutionarily conserved, and point mutations of the four most conserved residues are sufficient to disrupt Skp2-cyclin A interaction. Because the Skp2 N-terminal sequences were not included in the solved Skp2 structure, it is not known whether this 40–60 sequence is surface-exposed for protein-protein interaction. Nevertheless, our findings that the 4060 peptide, but not the R*X*L peptide, can block Skp2-cyclin A interaction, and vice versa, that the R*X*L peptide, but not the 4060 peptide, can block p27-cyclin A-Cdk2 interaction provide strong evidence for the presence of a non-R*X*L cyclin A-interacting sequence between Skp2 residues 40 and 60.

On the cyclin A side, Skp2-cyclin A interaction also differs from the R*X*L-HP interaction. When Skp2 and p27 were studied together, our mapping results show that the binding sites on cyclin A for these two proteins are distinct, with the Skp2 binding site located N-terminal to the HP site on the cyclin A N terminus. At the amino acid sequence level, mapping of the Skp2 interaction site to the N terminus provides a structural explanation for the cyclin specificity of the Skp2-cyclin A interaction since the N-terminal sequences of cyclin A are not highly conserved in other cyclins. This finding suggests an interesting possibility that the N termini of various cyclins could be exploited to subject them to specific regulation. In this respect, it will be interesting to understand with further studies the reasons for Skp2 to specifically target cyclin A. A clue may lie in the fact that cyclin A is unique in the cyclin family in that it plays important roles in both G_1/S and G_2/M phases of the cell cycle.

The R*X*L-HP interaction has been believed to be a general mechanism mediating relationships between cyclins and non-Cdk proteins. Determination that Skp2-cyclin A interaction represents a novel physical relationship between cyclin A and non-Cdk proteins broadens our view of how cyclins and non-Cdk proteins could interact, which should lead to new knowledge of how cyclins and non-Cdk proteins communicate and regulate each other.

Significance of Skp2-Cyclin A Interaction in Skp2-mediated p27 Ubiquitylation—In addition to phosphorylating p27 at Thr-187, cyclin A-Cdk2 complex (or cyclin E-Cdk2 complex) is also required to form trimeric complexes with p27 for its ubiquitylation by Skp2 (24). One clue to the nature of this requirement stemmed from the identification of Cks1 as an essential cofactor in p27 ubiquitylation by Skp2 (25, 26). Because Cks1 is a high affinity Cdk2-binding protein (27), interactions between Cks1 and Cdk2 may facilitate the recruitment of the trimeric cyclin A-Cdk2-p27 complex to Skp2. Structure and mutagenesis studies of Cks1 suggested that Cks1 is a three-faceted protein, and it uses its three binding interfaces to separately interact with Skp2, Cdk2, and phosphorylated p27 to organize these proteins into a super-complex (28). In this model phosphorylated p27 simultaneously interacts with cyclin A-Cdk2 (via the R*X*L-HP interface), Cks1 (via phosphorylated Thr-187 and the Cks1 anion-binding site), and Skp2. Interaction between Skp2 and cyclin A is mediated through Cdk2-Cks1, with Cks1 directly bound near the C terminus of Skp2 (29). The Skp2 N terminus is not involved, and no clear distinctions exist between the roles of cyclin A and cyclin E in this super-complex.

X-ray crystal structure studies have provided direct and more detailed evidence for the formation of a cyclin A-Cdk2-Cks1-Skp2-p27 super-complex as proposed above. An N-terminal-truncated Skp2 (Skp2ΔN) is able to form a SCF[Skp2ΔN]·Roc1 complex through the F-box (15, 20) and Skp2ΔN-Cks1-p27 complex through the C-terminal blade (30). Superimposing the available cyclin A-Cdk2, Cdk2-Cks1, Skp1-Skp2ΔN-Cks1-p27, and Skp2ΔN-Skp1-Cul1-Roc1 structures demonstrated that a super-complex of Skp1-Skp2ΔN-Cks1-Cdk2-cyclin A-p27 could indeed form on Skp2 (30). Skp2ΔN also coeluted with Skp1, Cul1, Roc1, Cks1, p27, Cdk2, and cyclin A from size-exclusion chromatographic columns, providing experimental evidence for such an eight-subunit complex in the absence of the Skp2 N terminus (30).

Interestingly, Koff and co-workers (31) recently reported that bacterially produced Skp2ΔN (which was used for x-ray crystal structure studies) was unable to bind cyclin A and ubiquitylate p27. This result suggested that the eight-subunit complex formed with Skp2ΔN would be functionally inactive in p27 ubiquitylation. A conceivable mechanism for the requirement for the N-terminal-bound cyclin A is that, whereas cyclin A can be tethered onto the Skp2 C terminus via Cks1-Cdk2-cyclin A interaction, an additional interaction between cyclin A and the Skp2 N terminus is needed to strengthen and/or reshape this super-complex for it to be active. Our finding that the Skp2 N terminus and p27 compete for binding to cyclin A-Cdk2 makes this scenario unlikely since when cyclin A-Cdk2 is bound to the Skp2 N terminus, it will not be able to bind p27 to form the trimeric complex. Our results that the Skp2AAAA mutant lost interaction with cyclin A but was able to ubiquitylate p27 provide experimental evidence that an N-terminal-bound cyclin A is not required for Skp2 to mediate p27 ubiquitylation. Importantly, Skp2ΔN was also able to mediate p27 ubiquitylation when coexpressed in 293T cells with Skp1-Cul1-Roc1 (11). We further found that baculovirus-produced and -purified Skp2ΔN is fully competent in mediating p27 ubiquitylation;

bacterially produced and purified Skp2ΔN also sustained robust p27 ubiquitylation.[7] Currently, we cannot explain the reasons of the discrepancies between Koff and co-workers' result and ours. Finally, like Skp2AAAA, Skp2ΔN was also active in promoting degradation of p27 and p21 in cells (32). Considered together, we are confident that cyclin A binding to Skp2 N terminus is not required for p27 ubiquitylation.

Skp2-Cyclin A Interaction Represents a New Regulatory Mechanism of Cyclin A-Cdk2 and a New Functional Mechanism for Skp2 Proliferation-stimulating Activity—With respect to regulation of cyclin/Cdk by a stably bound protein, the CKI p27 is the best-understood example. In addition to the R*X*L-HP interface, an N-terminal sequence (residues 85–90) of p27 inserts itself into the catalytic cleft of Cdk2 to block ATP binding (18). With this mechanism, the relative concentrations of p27 and cyclin-Cdk will determine the degree of kinase inhibition. Skp2-mediated p27 ubiquitylation and degradation can positively regulate cyclin-Cdk2 kinase activity by reducing p27 levels.

Our study of the Skp2-cyclin A interaction now reveals that the inhibitory effect of p27 on cyclin A-Cdk2 can also be regulated by Skp2 independent of p27 ubiquitylation and degradation. Skp2 competes with p27 for binding to cyclin A-Cdk2, but Skp2 itself does not inhibit cyclin A-Cdk2 at physiologically relevant concentrations. This property of Skp2 forms the mechanistic basis for Skp2 to exert a positive effect on cyclin A-Cdk2 whenever p27 is present. Our determination that the presence of the Skp2 N terminus at 80 nM in the kinase reaction shifted the IC_{50} of p27 rightward from 2 to 80 nM indicates that this protective effect of Skp2 N terminus is effective when the molar ratio of Skp2 over p27 is about 1 or larger. We further determined that the molar ratio of cellular Skp2 over p27 is about 2 at 14 h after HeLa cells were released from mitotic arrest when cyclin A protein levels are starting to rise. Although other factors such as cellular localization or compartmentalization can certainly affect the effective concentrations of Skp2 and p27 *in vivo*, we take our results to suggest that the protective effect of Skp2 could be physiologically relevant, at least when Skp2 is overexpressed in tumor cells. Our findings, therefore, add a new mechanism of regulation of cyclin A-Cdk2 kinase activity in that the outcome of regulation of cyclin A-Cdk2 kinase activity by p27 is determined not only by its levels but also by the levels of Skp2. This new mechanism is likely to be applicable to p27 family member p21 since Skp2 and p21 also compete for cyclin A binding. It is, however, important to note that whether this protective effect of Skp2-cyclin A interaction is an effective way to positively regulate cyclin A in normal cells (where Skp2 levels are significantly lower) will need to be determined with further studies, preferentially by knocking-in the AAAA mutation.

By revealing the functional significance of Skp2-cyclin A interaction, our study also adds a new functional mechanism for Skp2 proliferation-stimulating activity. In assays that measure the ability of Skp2 to counter cell cycle arrest by p53/p21-mediated cell cycle checkpoint, we found that point mutations that disrupt Skp2-cyclin A interaction but not p27 ubiquitylation and degradation significantly compromised the ability of Skp2 to counter cell cycle arrest, revealing the importance of Skp2-cyclin A interaction in Skp2 function in addition to p27 ubiquitylation and degradation in the context of overexpression.

The fact that Skp2 protein is often overexpressed and forms abundant Skp2-cyclin A complexes in various cancer cells suggests that Skp2 direct protective effects on cyclin A may play an important role in natural tumorigenesis as well. In this respect it is of note that whereas the inverse correlation of Skp2 and p27 levels have been found in a large number of cancer samples, cancer samples with high Skp2 but no reduction of p27 clearly exist (for prostate cancer as an example, see Refs. 33–35). In these cancer cells it is conceivable that certain steps for p27 ubiquitylation and/or degradation may have been disrupted to render Skp2 p27 ubiquitylation activity ineffective, but Skp2 direct protective effects of cyclin A should remain effective and could drive selection for Skp2 overexpression during tumorigenesis. In mouse models, ectopic expression of Skp2 in prostate epithelium induced a hyperplasia phenotype at 3–7 months of age (36), whereas p27 knock-out mice displayed similar hyperplasia only when aged to 14 months (37). Furthermore, ectopic expression of Skp2 induced dysplasia and low grade carcinoma in prostate epithelium (36), whereas such abnormalities have not been observed in p27 knock-out mice. New knowledge of the Skp2-cyclin A interaction should help shed light on these intriguing observations and, in the meantime, help reveal new targets for inhibiting proliferation of Skp2-overexpressing tumor cells.

REFERENCES

1. Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., and Elledge, S. J. (1996) *Cell* **86,** 263–274
2. Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. (1999) *Nat. Cell Biol.* **1,** 193–199
3. Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U., and Krek, W. (1999) *Nat. Cell Biol.* **1,** 207–214
4. Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H., and Zhang, H. (1999) *Curr. Biol.* **9,** 661–664
5. Bornstein, G., Bloom, J., Sitry-Shevah, D., Nakayama, K., Pagano, M., and Hershko, A. (2003) *J. Biol. Chem.* **278,** 25752–25757
6. Kamura, T., Hara, T., Kotoshiba, S., Yada, M., Ishida, N., Imaki, H., Hatakeyama, S., Nakayama, K., and Nakayama, K. I. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 10231–10236
7. Nakayama, K. I., and Nakayama, K. (2005) *Semin. Cell Dev. Biol.* **16,** 323–333
8. Xiong, Y., Zhang, H., and Beach, D. (1993) *Genes Dev.* **7,** 1572–1583
9. Zhang, H., Kobayashi, R., Galaktionov, K., and Beach, D. (1995) *Cell* **82,** 915–925
10. Follenzi, A., Sabatino, G., Lombardo, A., Boccaccio, C., and Naldini, L. (2002) *Hum. Gene Ther.* **13,** 243–260
11. Ji, P., Jiang, H., Rekhtman, K., Bloom, J., Ichetovkin, M., Pagano, M., and Zhu, L. (2004) *Mol. Cell* **16,** 47–58
12. Guardavaccaro, D., Kudo, Y., Boulaire, J., Barchi, M., Busino, L., Donzelli, M., Margottin-Goguet, F., Jackson, P. K., Yamasaki, L., and Pagano, M. (2003) *Dev. Cell* **4,** 799–812
13. Lisztwan, J., Marti, A., Sutterluty, H., Gstaiger, M., Wirbelauer, C., and

Krek, W. (1998) *EMBO J.* **17,** 368–383

14. Yam, C. H., Ng, R. W., Siu, W. Y., Lau, A. W., and Poon, R. Y. (1999) *Mol. Cell Biol.* **19,** 635–645

15. 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

16. Zhu, L., Harlow, E., and Dynlacht, B. D. (1995) *Genes Dev.* **9,** 1740–1752

17. Adams, P. D., Sellers, W. R., Sharma, S. K., Wu, A. D., Nallin, C. M., and Kaelin, j. W. G. (1996) *Mol. Cell Biol.* **16,** 6623–6633

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

19. Schulman, B. A., Lindstrom, D. L., and Harlow, E. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 10453–10458

20. Zheng, N., Schulman, B. A., Song, L., Miller, J. J., Jeffrey, P. D., Wang, P., Chu, C., Koepp, 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

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

22. Krek, W., Ewen, M. E., Shirodkar, S. Z., Arany, Z., Kaelin, W. G., and Livingston, D. M. (1994) *Cell* **78,** 161–172

23. Harper, J. W., Elledge, S. J., Keyomarsi, K., Dynlacht, B., Tsai, L.-H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., Swindell, E., Fox, M. P., and Wei, N. (1995) *Mol. Biol. Cell* **6,** 387–400

24. Montagnoli, A., Fiore, F., Eytan, E., Carrano, A. C., Draetta, G. F., Hershko, A., and Pagano, M. (1999) *Genes Dev.* **13,** 1181–1189

25. Ganoth, D., Bornstein, G., Ko, T. K., Larsen, B., Tyers, M., Pagano, M., and Hershko, A. (2001) *Nat. Cell Biol.* **3,** 321–324

26. Spruck, C., Strohmaier, H., Watson, M., Smith, A. P. L., Ryan, A., Krek, W., and Reed, S. I. (2001) *Mol. Cell* **7,** 639–650

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. Sitry, D., Seeliger, M. A., Ko, T. K., Ganoth, D., Breward, S. E., Itzhaki, L. S., Pagano, M., and Hershko, A. (2002) *J. Biol. Chem.* **277,** 42233–42240

29. Wang, W., Ungermannova, D., Chen, L., and Liu, X. (2003) *J. Biol. Chem.* **278,** 32390–32396

30. Hao, B., Zheng, N., Schulman, B. A., Wu, G., Miller, J. J., Pagano, M., and Pavletich, N. P. (2005) *Mol. Cell* **20,** 9–19

31. Zhu, X. H., Nguyen, H., Halicka, H. D., Traganos, F., and Koff, A. (2004) *Mol. Cell. Biol.* **24,** 6058–6066

32. Bashir, T., Dorrello, N. V., Amador, V., Guardavaccaro, D., and Pagano, M. (2004) *Nature* **428,** 190–193

33. Yang, G., Ayala, G., De Marzo, A., Tian, W., Frolov, A., Wheeler, T. M., Thompson, T. C., and Harper, J. W. (2002) *Clin. Cancer Res.* **8,** 3419–3426

34. Drobnjak, M., Melamed, J., Taneja, S., Melzer, K., Wieczorek, R., Levinson, B., Zeleniuch-Jacquotte, A., Polsky, D., Ferrara, J., Perez-Soler, R., Cordon-Cardo, C., Pagano, M., and Osman, I. (2003) *Clin. Cancer Res.* **9,** 2613–2619

35. Ben-Izhak, O., Lahav-Baratz, S., Meretyk, S., Ben-Eliezer, S., Sabo, E., Dirnfeld, M., Cohen, S., and Ciechanover, A. (2003) *J. Urol.* **170,** 241–245

36. Shim, E. H., Johnson, L., Noh, H. L., Kim, Y. J., Sun, H., Zeiss, C., and Zhang, H. (2003) *Cancer Res.* **63,** 1583–1588

37. Cordon-Cardo, C., Koff, A., Drobnjak, M., Capodieci, P., Osman, I., Millard, S. S., Gaudin, P. B., Fazzari, M., Zhang, Z. F., Massague, J., and Scher, H. I. (1998) *J. Natl. Cancer Inst.* **90,** 1284–1291

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