THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 18, Issue of May 2, pp. 15905–15910, 2003
Printed in U.S.A.

TIP120A Associates with Cullins and Modulates Ubiquitin Ligase Activity*

Received for publication, December 23, 2002, and in revised form, February 11, 2003
Published, JBC Papers in Press, February 27, 2003, DOI 10.1074/jbc.M213070200

Kyoeng-Woo Min‡§, Ji-Won Hwang‡§, Jong-Sik Lee‡, Yoon Park‡, Taka-aki Tamura¶, and Jong-Bok Yoon‡‖

From the ‡Department of Biochemistry and Protein Network Research Center, Yonsei University, Seoul 120-749, Korea and the ¶Department of Biology, Faculty of Science, Chiba University, Chiba 263-8522, Japan

The cullin-containing ubiquitin-protein isopeptide ligases (E3s) play an important role in regulating the abundance of key proteins involved in cellular processes such as cell cycle and cytokine signaling. They have multisubunit modular structures in which substrate recognition and the catalysis of ubiquitination are carried out by distinct polypeptides. In a search for proteins involved in regulation of cullin-containing E3 ubiquitin ligases we immunopurified CUL4B-containing complex from HeLa cells and identified TIP120A as an associated protein by mass spectrometry. Immunoprecipitation of cullins revealed that all cullins tested specifically interacted with TIP120A. Reciprocal immunoaffinity purification of TIP120A confirmed the stable interaction of TIP120A with cullin family proteins. TIP120A formed a complex with CUL1 and Rbx1, but interfered with the binding of Skp1 and F-box proteins to CUL1. TIP120A greatly reduced the ubiquitination of phosphorylated IκBα by $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase. These results suggest that TIP120A functions as a negative regulator of SCF E3 ubiquitin ligases and may modulate other cullin ligases in a similar fashion.

The ubiquitin-dependent proteolysis provides a fundamental mechanism for regulating protein activity in various processes ranging from cell cycle and developmental switches to signal transduction (1). This process begins with the attachment of a multiubiquitin chain to a target protein and involves several enzymatic activities. A ubiquitin-activating enzyme (E1)[1] activates ubiquitin in an ATP-dependent reaction by forming a thioester bond with the C-terminal glycine of ubiquitin. The ubiquitin is then transferred to a specific sulfhydryl group on a ubiquitin-conjugating enzyme (E2). A ubiquitin-protein ligase (E3) transfers the activated ubiquitin from E2 to a lysine residue of a bound substrate, forming an isopeptide bond. Substrate specificity is determined mainly by E3s which bind both the protein substrate and the cognate E2. Once the multiubiquitin chain is assembled on a protein substrate by the cooperation of E1, E2, and E3 enzymes, the target protein is recognized and degraded by the 26 S proteasome (1–3).

In mammalian cells, a wide variety of E3s are found. The cullin family proteins play an important role in a group of multisubunit E3 ubiquitin ligases by associating with an Rbx1 (also known as ROC1 and Hrt1) family member of RING finger proteins to form the integral core (4). The SCF complexes are the best characterized ones of this class (5). They consist of CUL1, Rbx1, Skp1, and an F-box protein. Rbx1 contains the RING-H2 finger domain, forms a catalytic core with CUL1, and recruits the cognate E2 (6–8). Skp1 functions as an adaptor that links an F-box protein to CUL1 (9). Substrates of the SCF complexes are bound by F-box proteins, which contain the Skp1-binding F-box motif and a variable protein-protein interaction domain that directly interacts with substrates (9, 10). Since a large number of F-box proteins are encoded by eukaryotic genomes (11–13), a variety of proteins are expected to be substrates of the SCF complexes, assuming that most of the F-box proteins form functional SCF E3 ubiquitin ligases. So far, a few SCF complexes, including SCF^{Skp2}, $SCF^{\beta\text{-}TrCP}$, SCF^{Cdc4}, and SCF^{Grr1}, have been demonstrated to have E3 activities for specific substrates.

CUL2 and CUL5 can also assemble multisubunit E3 ubiquitin ligases that bear a striking resemblance to SCF-type complexes. CUL2/Rbx1 and CUL5/Rbx1 form a complex with the Elongin BC heterodimer that functions as an adaptor analogously to Skp1 in the SCF complexes. The Elongin BC complex binds to a large number of proteins including the von Hippel-Lindau (VHL) tumor suppressor protein (14–16) and members of the SOCS-box protein family (17, 18), each of which contains an Elongin BC-binding site and a diverse protein-protein interaction motif (19). As a component of the VHL ubiquitin ligase complex, the VHL protein targets the α subunits of the hypoxia-inducible transcription factors HIF1 and HIF2 for ubiquitination (20, 21). In the case of SOCS-1, Vav and JAK2 are known to be specific substrates of the E3 complex (22, 23). Most SOCS-box proteins may function as a substrate-binding subunit of an E3 ubiquitin ligase complex.

Studies on cullin/Rbx1-containing E3 ubiquitin ligases raise the possibility that other cullin family members could function as a component of ubiquitin ligases with distinct substrate specificities by forming multiprotein complexes with yet unidentified adaptors and/or substrate recognition subunits. In an effort to address this possibility, we have been purifying cullin-containing complexes from HeLa cells and identifying specifically associated proteins. In this report, we present purification of CUL4B-containing complexes and demonstrate that

* This work was supported in part by grants from the Korea Science and Engineering Foundation through Protein Network Research Center at Yonsei University and from the Korean Ministry of Science and Technology through 21C Frontier Project. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These two authors contributed equally to this work.
‖ To whom correspondence should be addressed. Tel.: 82-2-2123-2704; Fax: 82-2-392-3488; E-mail: yoonj@yonsei.ac.kr.

[1] The abbreviations used are: E1 or Uba, ubiquitin-activating enzyme; E2 or Ubc, ubiquitin-conjugating enzyme; E3, ubiquitin-protein isopeptide ligase; Ub, ubiquitin; GST, glutathione *S*-transferase; HA, hemagglutinin; SCF, Skp1-Cdc53/cullin-F box; SOCS, suppressor of cytokine signaling; VHL, von Hippel-Lindau; DTT, dithiothreitol; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; MS/MS, tandem mass mass spectrometry.

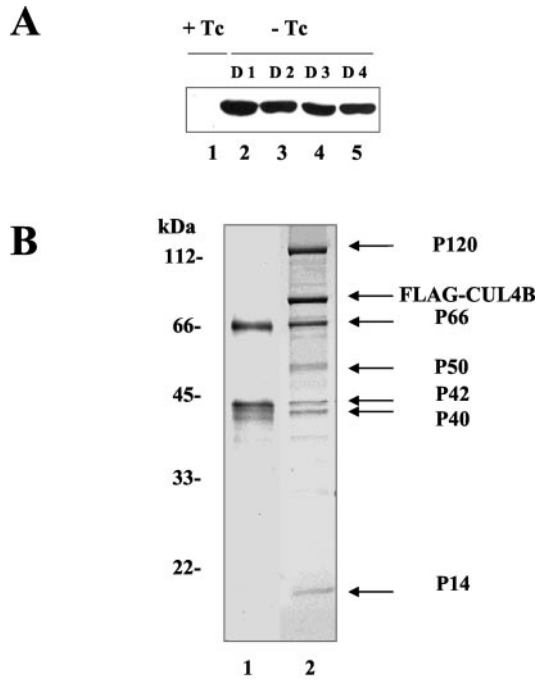

FIG. 1. **Immunoaffinity purification of FLAG-tagged CUL4B.** *A*, conditional expression of FLAG-CUL4B. Cells expressing FLAG-CUL4B in a tetracycline-controlled manner were established as described under "Experimental Procedures." Cells were grown in the presence (*lane 1*) or the absence (*lanes 2–5*) of tetracycline for the indicated time. FLAG-CUL4B expression was examined by immunoblotting of the cell lysates with anti-FLAG antibody. *B*, SDS-PAGE analysis of purified FLAG-CUL4B. Ten μl each of purified FLAG-CUL4B samples from uninduced and induced cells was separated by a 4–20% SDS-polyacrylamide gel and visualized by Sypro Ruby-Coomassie double staining. Protein size markers (in kilodaltons) are indicated on the *left*.

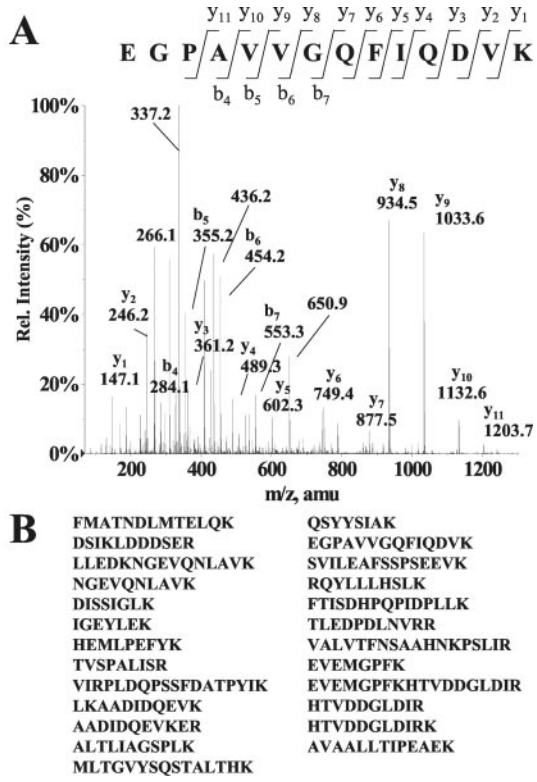

FIG. 2. **Identification of TIP120A as a CUL4B-interacting protein.** *A*, an MS/MS spectrum of a peptide derived from p120. MS/MS analyses were performed as described under "Experimental Procedures." *B*, sequences determined by MS/MS analyses of tryptic peptides obtained from p120. All peptides were identical to sequences in human TIP120A.

TIP120A specifically interacts with cullin family proteins and that it negatively regulates the activity of an SCF ubiquitin ligase by interfering with the binding of Skp1 to CUL1.

EXPRERIMENTAL PROCEDURES

Plasmids—The cDNAs encoding human TIP120A (GenBank™ accession number BE019018), CUL2 (GenBank™ AA206544), CUL4A (GenBank™ BC008308), and Rbx1 (GenBank™ H71993) were from Incyte Genomics Inc. CUL3 (GenBank™ accession number KIAA0617) and CUL4B (GenBank™ accession number KIAA0695) cDNA clones were a kind gift from Kazusa DNA Research Institute. Human CUL1, Skp1, and Skp2 cDNAs (kind gifts from Y. Xiong and H. Zhang) were described previously (24, 25). To construct plasmids for the expression of N-terminally FLAG- or HA-tagged proteins, cDNAs were amplified by PCR with appropriate primers and ligated into pcDNA3.1(+) vector (Invitrogen).

Stable Cell Lines, Extract Preparation, and Protein Complex Purification—HeLa Tet-Off (Clontech) derived cells stably expressing EBNA-1 were transfected with an episomal expression vector pYR-FLAG-hCUL4A or pYR-FLAG-hTIP120A that contained the gene of interest under the tetracycline-regulated promoter, oriP for episome replication, and the selection marker for hygromycin B. The cells were selected and maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 μg/ml G418 (Sigma), 300 μg/ml hygromycin B (Clontech), 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM L-glutamine, and with 2 μg/ml tetracycline (Sigma). To induce the expression of FLAG-tagged proteins, cells were grown without tetracycline for 2 days. Nuclear extracts and cytosolic S100 extracts were prepared as described previously (26). Nuclear extracts were dialyzed against buffer BC (20 mM Tris-HCl (pH 7.9), 15% glycerol, 1 mM EDTA, 1 mM DTT, 0.2 mM phenylmethanesulfonyl fluoride, 0.05% Nonidet P-40) containing 150 mM KCl (BC150) and rotated with anti-FLAG M2-agarose (Sigma) at 4 °C for 3–6 h. After extensive washes with BC150, proteins were eluted with 0.3 mg of FLAG peptide per ml in BC150.

Protein Identification by Mass Spectrometry—Immunopurified protein complexes were resolved on sodium dodecyl sulfate (SDS)-4–20% gradient polyacrylamide gels (Novex). After staining gels with Sypro Ruby (Bio-Rad) and subsequently with colloidal Coomassie Blue, protein bands were excised and digested with trypsin as described previously (27). In-gel tryptic digests of proteins were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using Voyager-DE STR (Applied Biosystems) and by nanoelectrospray ionization tandem mass spectrometry on API QSTAR Pulsar Q-TOF (Applied Biosystems). The mass spectral data were used to search the National Center for Biotechnology Information nonredundant and expressed sequence tag data bases.

Immunoprecipitations and Western Blotting—Transfection was carried out by the $CaPO_4$-DNA precipitation method using Hepes or BES buffer. After 36 h, cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2 mM phenylmethanesulfonyl fluoride, and 1.0% Nonidet P-40. Cell lysates were adjusted to 0.1% Nonidet P-40 and incubated with anti-FLAG or anti-HA antibody resin (Sigma) for 4 h at 4 °C. The immune complexes were recovered by low speed centrifugation, and the resin was washed extensively with the binding buffer with 0.1% Nonidet P-40 and then eluted with buffer containing 20 mM Tris-HCl (pH 8.0) and 2% SDS. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad) and visualized by Western blotting with the enhanced chemiluminescence reagents (Amersham Biosciences). For Western blotting, we used antibodies against FLAG (Sigma), HA (Babco), TIP120A (BD Biosciences), CUL1 (Lab Vision), Rbx1 (Lab Vision), Skp1 (Zymed Laboratories Inc.), and Skp2 (Zymed Laboratories Inc.).

Expression and Purification of Recombinant Proteins—His_6-tagged yeast Uba1, human Ubc3, and UbcH5A (Ref. 28, a kind gift from J. W. Conaway) were expressed in *Escherichia coli* strain BL21(DE3) and purified by Ni^{2+}-agarose beads (Qiagen). Ub containing a His_6 tag and a protein kinase C recognition site (a kind gift from Z.-Q. Pan) was purified as described previously (29). Rat TIP120A containing N-terminal FLAG and His_6 tags was expressed in Sf21 cells and purified as described previously (30). Generation of recombinant baculoviruses encoding human CUL1 with a His_6 tag, human β-TrCP with a FLAG tag, and human Skp1 with a His_6 tag (a generous gift from M. Pagano) were

described previously (31). Human Rbx1 linked with an N-terminal FLAG tag was subcloned into a baculovirus expression vector, pFastBac (Invitrogen), and expressed in Sf21 cells. Sf21 cells were cultured at 27 °C in TNM-FH (Sigma) with 5% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Sf21 cells were co-infected with the recombinant baculoviruses indicated in Fig. 5. Sixty hours after infection, cells were collected and lysed in ice-cold buffer containing 40 mM Hepes-NaOH (pH 7.9), 150 mM NaCl, 1 mM DTT, 0.5% (v/v) Triton X-100, 10% (v/v) glycerol, 5 μg/ml leupeptin, 5 μg/ml antipain, 5 μg/ml pepstatin A, and 5 μg/ml aprotinin. Lysates were clarified by centrifugation at 10,000 × *g* for 20 min at 4 °C. FLAG-β-TrCP/His-Skp1 complex was purified by applying the supernatant onto an M2-agarose column (Sigma) equilibrated with the lysis buffer. After extensive washing of the column with the lysis buffer, the bound proteins were eluted with the lysis buffer containing 0.3 mg/ml FLAG peptide (Sigma). His-CUL1/FLAG-Rbx1 was purified using Co^{2+}-agarose beads (Clontech) according to the manufacturer's instructions.

IκBα Ubiquitination Assays—$SCF^{\beta\text{-}TrCP}$ was reconstituted with recombinant His-CUL1/FLAG-Rbx1 and FLAG-β-TrCP/His-Skp1 and its activity of IκBα ubiquitination assayed using the procedure previously described (29, 32) with minor modifications. The Ub ligation reaction mixture (30 μl) contained 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM NaF, 10 nM okadaic acid, 2 mM ATP, 0.6 mM DTT, 0.8 μg of Ub, 50 ng of E1, 200 ng of E2, 500 ng of His-CUL1/FLAG-Rbx1, 300 ng of FLAG-β-TrCP/His-Skp1, and 1.2 μg of phosphorylated glutathione *S*-transferase (GST)-$I\kappa B\alpha^{1-54}$. Reaction mixtures were incubated at 37 °C for 20 min, terminated by adding 30 μl of 2× Laemmli loading buffer, and resolved by SDS-PAGE followed by autoradiography to visualize the ubiquitinated IκBα. For substrate preparation, 18 μg of purified GST-$I\kappa B\alpha^{1-54}$ was phosphorylated with HA-tagged, constitutively active $IKK\beta^{S177E/S181E}$ kinase purified from 293 cells following transfection. The reaction was carried out in the presence of 10 μCi of [γ-^{32}P]ATP at 37 °C for 20 min in a total volume of 30 μl of kinase buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 5 μM ATP, 2 mM NaF, 10 nM okadaic acid, and 0.6 mM DTT.

RESULTS AND DISCUSSION

Purification of CUL4B-containing Complex—To facilitate the purification of CUL4B-containing complex, we first established a HeLa-derived cell line that conditionally expressed FLAG-tagged CUL4B using an episomal expression vector. The tetracycline-regulated expression of FLAG-CUL4B in stable transfectants was tested by immunoblotting of cell extracts with anti-FLAG antibody. FLAG-CUL4B was induced upon removal of tetracycline, while it was not detected in cells grown in the presence of tetracycline (Fig. 1*A*). FLAG-CUL4B and its associated proteins were purified from extracts of induced and uninduced cells by a single-step immunoaffinity purification procedure, which had proved to be a gentle and efficient method (33). Several cellular proteins specifically co-purified with FLAG-CUL4B under the experimental conditions employed, as judged by SDS-polyacryamide gel electrophoresis of purified proteins from induced and uninduced cells (Fig. 1*B*). Bands corresponding to specifically interacting proteins were excised and analyzed by matrix-assisted laser desorption/ionization mass spectrometry and nanoelectrospray tandem mass spectrometry. The p14 band was identified as Rbx1 (data not shown), which forms a stable complex with all cullin family members (4). Another protein p50 turned out to be a novel protein of unknown function. However, since reciprocal immunoprecipitation experiments did not confirm the specificity of the interaction between p50 and CUL4B, p50 was not further studied. As shown in Fig. 2, mass spectrometric analyses of tryptic peptides derived from p120 revealed it as human TIP120A, which was shown to be a transcription factor that enhanced transcription by RNA polymerases I, II, and III (34, 35). The fact that TIP120A was found in CUL4B-containing complex raised the possibility that in addition to the reported function of TIP120A in transcription, it might be involved in regulation of E3 ubiquitin ligase and/or ubiquitin- dependent proteolysis.

TIP120A Interacts with Cullin Family Proteins—To address the specificity of CUL4B-TIP120A interaction, we tested other cullin family members for their binding to TIP120A. HeLa cells were transfected with expression constructs of FLAG-tagged CUL1, CUL2, CUL3, CUL4A, or CUL4B, and anti-FLAG immunoprecipitation was carried out on the cell lysates. Western blotting of immunoprecipitates with anti-TIP120A antibodies indicated that TIP120A associated with all cullins tested (Fig. 3*A*). Immunoprecipitates of an unrelated protein FLAG-Chk2kd did not contain TIP120A (*lane 1*), indicating that the interaction between TIP120A and cullins was specific. To confirm the association of TIP120A with cullins, FLAG-TIP120A was immunopurified using anti-FLAG antibodies from cells that conditionally expressed the protein. SDS-polyacryamide gel electrophoresis of purified proteins revealed several protein bands with their molecular masses of 80–90 kDa that were present only in the preparation derived from induced cells (Fig. 3*B*). Mass spectrometric analyses identified these proteins as CUL1, CUL2, CUL3, CUL4A, and CUL4B (data not shown), confirming that TIP120A specifically interacted with cullins. A protein band with the molecular mass of 14 kDa was identified as Rbx1, suggesting that TIP120A formed a trimeric complex with a cullin and Rbx1. Since TIP120A interacted with most, if not all, cullins, it might function as a global regulator of cullin-containing ubiquitin ligases.

TIP120A Interferes with Binding of Skp1 and F-box Proteins to CUL1—Since CUL1 was the best studied member of the

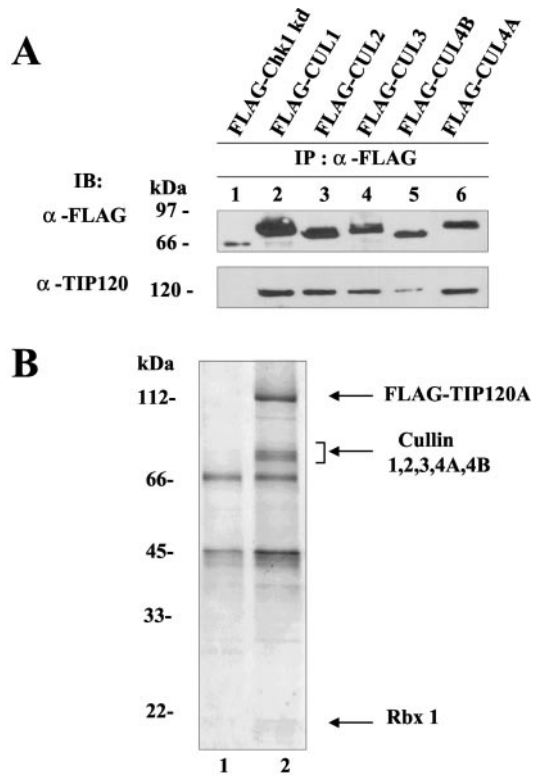

FIG. 3. **TIP120A interacts with members of the cullin family.** *A*, immunoprecipitations of cullins. HeLa cells were transiently transfected with expression constructs for FLAG-tagged CUL1, CUL2, CUL3, CUL4A, and CUL4B (*lanes 2–6*). As a negative control, an unrelated protein FLAG-tagged Chk2kd was expressed (*lane 1*). Cell lysates were immunoprecipitated with anti-FLAG antibody-coupled beads. The immunoprecipitates were subjected to SDS-polyacryamide gel electrophoresis and analyzed by Western blotting using anti-FLAG antibody to detect FLAG-tagged proteins (*lower panel*) and anti-TIP120 antibody to detect co-immunoprecipitated TIP120 (*upper panel*). *B*, immunoaffinity purification of FLAG-tagged TIP120A. Ten μl each of purified FLAG-TIP120A samples from uninduced and induced cells was separated by a 4–20% SDS-polyacrylamide gel and visualized by Sypro Ruby-Coomassie double staining.

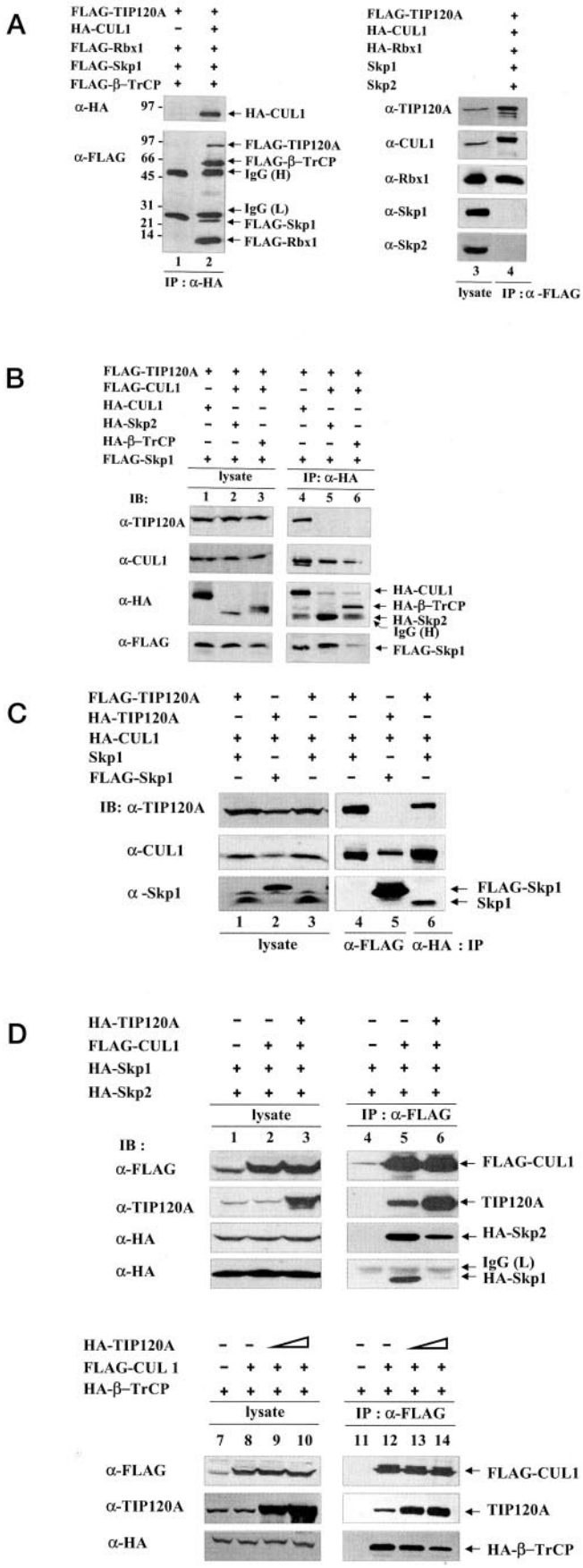

FIG. 4. **TIP120A interferes with binding of Skp1 and F-box proteins to CUL1.** *A*, immunoprecipitations of CUL1 and TIP120A. HeLa cells were transiently transfected with plasmid vectors expressing epitope-tagged or untagged TIP120A, CUL1, Rbx1, Skp1, Skp2, and β-TrCP proteins as indicated. Lysates were prepared 36 h after transfection and immunoprecipitated with α-HA (*lanes 1* and *2*) or α-FLAG antibodies (*lane 4*). The precipitates were separated by SDS-PAGE and immunoblotted with indicated antibodies. *B*, immunoprecipitations of Skp2 and β-TrCP. HeLa cells were transiently transfected with plasmid vectors expressing FLAG- or HA-tagged TIP120A, CUL1, Skp1, Skp2, and β-TrCP proteins as indicated. Immunoprecipitations with α-HA antibodies (*lanes 4–6*) and immunoblotting experiments were performed as described in the legend to *A*. *C*, association of CUL1 either with Skp1 or with TIP120A. HeLa cells were transiently transfected with plasmid vectors expressing epitope-tagged or untagged TIP120A, CUL1, and Skp1 proteins as indicated. Immunoprecipitations and immunoblotting experiments were performed as described in the legend to *A*. *D*, competition of TIP120A and Skp1 for the binding to CUL1. HeLa cells were transiently transfected with plasmid vectors expressing HA-TIP120A, FLAG-CUL1, HA-Skp1, HA-Skp2, and HA-β-TrCP proteins in combinations as indicated. Immunoprecipitations with α-FLAG antibodies and immunoblotting experiments were performed as described in the legend to *A*.

cullin family, we chose CUL1 to further characterize its interaction with TIP120A. To examine whether the interaction of TIP120A with CUL1 affects the SCF complex formation of CUL1, HeLa cells were transfected with expression constructs of epitope-tagged TIP120A, CUL1, Rbx1, Skp1, and an F-box protein Skp2 or β-TrCP. HA-CUL1 and FLAG-TIP120A were then immunoprecipitated with anti-HA and anti-FLAG antibodies, respectively, and co-purified proteins were probed by immunoblotting with suitable antibodies (Fig. 4*A*). FLAG-tagged TIP120A, Rbx1, Skp1, and β-TrCP were specifically detected in HA-CUL1 immunoprecipitates (*lanes 1* and *2*). In contrast, FLAG-TIP120A immunoprecipitates contained HA-CUL1 and HA-Rbx1, but not Skp1 and Skp2 (*lanes 3* and *4*), indicating that FLAG-TIP120A did not form a complex with Skp1 and Skp2. To confirm that F-box proteins Skp2 and β-TrCP do not interact with TIP120A, immunoprecipitation of HA-Skp2 or HA-β-TrCP was performed and associated proteins analyzed by immunoblotting (Fig. 4*B*). CUL1 and Skp1 were present in HA-Skp2 and HA-β-TrCP immunoprecipitates, but TIP120A was not pulled down in the precipitates (*lanes 5* and *6*). Since CUL1 directly associates with Skp1 (9, 36) and TIP120A (data not shown), interactions among CUL1, Skp1, and TIP120A were examined by coupled immunoprecipitation and immunoblotting (Fig. 4*C*). Whereas CUL1 was immunoprecipitated with both TIP120A and Skp1, the immunoprecipitates of FLAG-TIP120A contained only CUL1, but not Skp1, and those of FLAG-Skp1 contained only CUL1, but not TIP120A (*lanes 4–6*). Collectively, these data demonstrated that in cells existed at least two distinct CUL1-containing complexes either with TIP120A or with Skp1 and that the binding of TIP120A and Skp1 to CUL1 was mutually exclusive. To further test whether TIP120A and Skp1 compete for the binding to CUL1, HeLa cells were transfected with expression constructs of FLAG-CUL1, HA-TIP120A, HA-Skp1, and HA-Skp2. FLAG-CUL1 was immunoprecipitated with anti-FLAG antibodies, and HA-tagged TIP120A, Skp1, and Skp2 were probed by immunoblotting with anti-HA and anti-TIP120A antibodies (Fig. 4*D*). Overexpression of TIP120A reduced co-precipitation of Skp1 to 4–18% in triplicate experiments and Skp2 to 30–40% (*lanes 5* and *6*). In a similar competition experiment with TIP120A and β-TrCP, TIP120A inhibited the association of β-TrCP with CUL1 in a dose-dependent manner (*lanes 12–14*). These data showed that TIP120A interfered with binding of Skp1 and F-box proteins Skp2 and β-TrCP. It was noted that inhibition of Skp1 binding to CUL1 by TIP120A was greater than that of Skp2 or β-TrCP binding. Recent structural studies on SCF^{Skp2} showed that the F-box motif of Skp2 directly interacts with CUL1 as well as Skp1 (36). This Skp2-CUL1 interaction might not be affected by TIP120A binding

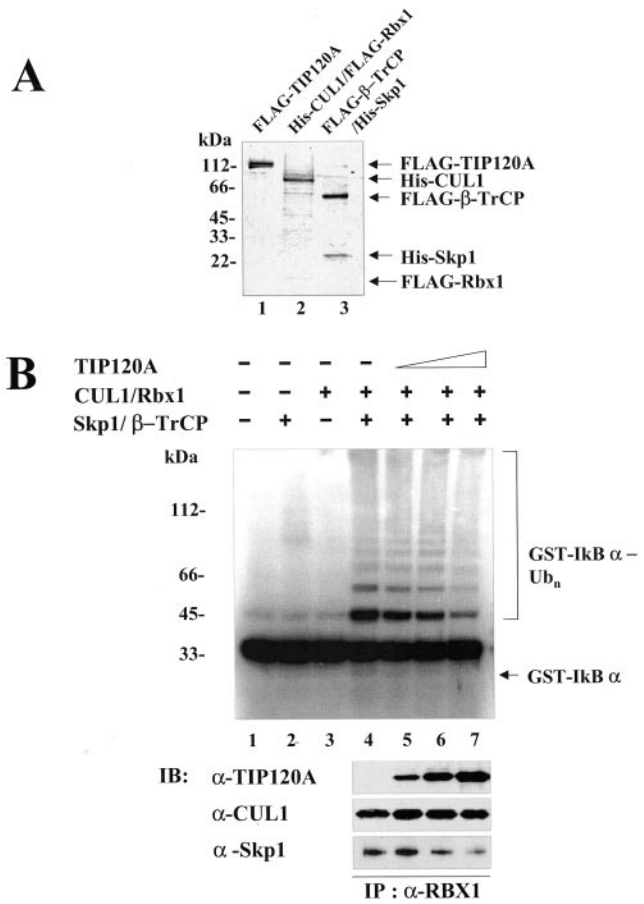

FIG. 5. **TIP120A inhibits ubiquitination of IκBα by SCF$^{β\text{-}TrCP}$.** *A*, SDS-PAGE analysis of purified FLAG-TIP120A, His-CUL1/FLAG-Rbx1, and FLAG-β-TrCP/His-Skp1. FLAG-TIP120A, His-CUL1/FLAG-Rbx1 and FLAG-β-TrCP/His-Skp1 were expressed in Sf21 cells and purified as described under "Experimental Procedures." Five μl each of purified proteins was separated by a 4–20% SDS-polyacrylamide gel and visualized by Coomassie Blue staining. Protein size markers (in kilodaltons) are indicated on the *left*. *B*, inhibition of IκBα ubiquitination by TIP120A. Purified GST-IκBα$^{1–54}$ was phosphorylated with IKKβ$^{S177E/S181E}$ in the presence of [γ-^{32}P]ATP and incubated with recombinant His-CUL1/FLAG-Rbx1(500 ng), FLAG-β-TrCP/His-Skp1 (300 ng), and FLAG-TIP120A (0.5, 1, and 2 μg in *lanes 5*, *6*, and *7*, respectively) as indicated. Reactions were performed at 37 °C for 20 min, terminated by adding Laemmli loading buffer, and resolved by SDS-PAGE followed by autoradiography to visualize the ubiquitinated IκBα ladders. For the immunoblotting experiments shown in the *lower panel*, reactions were performed without the substrate and ATP, and the CUL1/Rbx1 complex was immunoprecipitated with anti- Rbx1 antibodies.

and might contribute to less inhibition of Skp2 binding by TIP120A. Further investigation is required to clarify this point.

TIP120A Inhibits Ubiquitination of IκBα by SCF$^{β\text{-}TrCP}$—To determine the functional consequence of association of TIP120A with CUL1, we examined the effect of TIP120A on the substrate-specific ubiquitin ligase activity of SCF$^{β\text{-}TrCP}$ using IκBα as a substrate. To devise an *in vitro* reconstituted system for the ubiquitination of IκBα, we produced all components used in the ubiquitination reaction as recombinant proteins by *Escherichia coli* or the baculovirus system. Fig. 5*A* shows dye staining patterns of the purified subunits of SCF$^{β\text{-}TrCP}$ after separation by SDS-PAGE. GST-IκBα$^{1–54}$ was phosphorylated with IKKβ$^{S177E/S181E}$ in the presence of [γ-^{32}P]ATP and incubated in the Ub ligation reaction with recombinant CUL1/Rbx1 and β-TrCP/Skp1 (Fig. 5*B*). A high molecular weight ^{32}P-labeled protein ladder was formed, indicating that phosphorylated GST-IκBα$^{1–54}$ was polyubiquitinated. The ubiquitination of GST-IκBα$^{1–54}$ was dependent not only on E1 and E2 (data not shown) but on CUL1/Rbx1 and β-TrCP/Skp1 (*lanes 1–4*), showing that intact SCF$^{β\text{-}TrCP}$ complex was required for IκBα ubiquitination. Addition of increasing amounts of recombinant TIP120A greatly reduced the ubiquitination of IκBα by CUL1 complex in a dose-dependent manner (*lanes 4–7*). To test whether the reduction of IκBα ubiquitination by TIP120A is correlated with the decrease of Skp1 binding to CUL1, the CUL1/Rbx1 complex in the ubiquitination reactions was immunoprecipitated with anti-Rbx1 antibodies and bound TIP120A and Skp1 monitored by Western blotting. As the association of TIP120A with CUL1 increased, the binding of Skp1 to CUL1 decreased (Fig. 5*B*, *lower panel*). These results indicated that TIP120A inhibited ubiquitin ligase activity of CUL1 by interfering with the binding of Skp1 and β-TrCP to CUL1.

TIP120A was initially identified as a TBP-interacting protein using *in vitro* affinity purification procedures (34) and subsequently shown to function as a transcriptional activator (35). The results presented here demonstrate a novel function of TIP120A, namely, negative regulation of SCF E3 ubiquitin ligases by inhibiting Skp1 binding to CUL1. Since Skp1 is an adapter subunit of SCF complexes that links the F-box protein to CUL1 (9, 25, 37), inhibition of Skp1 binding by TIP120A results in inhibition of association of F-box proteins and reduction of functional SCF complexes. It has been reported that TIP120A expression was up-regulated during the differentiation process of certain cells. For instance, retinoic acid treatment of P19 mouse embryonal carcinoma cells, which induces differentiation and withdrawal from the cell cycle, elevated expression of TIP120A (38). In addition, it has been shown that overexpression of TIP120A in P19 cells arrested cell growth (38). Since SCF E3 ligases play a key role in progression of cell division cycle, it is tempting to speculate that induction of TIP120A down-regulates SCF E3 ligases which may, in turn, help cells exit from cell cycle.

The function of TIP120A is not likely to be restricted to negative regulation of SCF complexes. Recent evidence suggests that SCF ubiquitin ligases may directly deliver substrate proteins to the proteasome for degradation (39, 40). For instance, it has been shown that Skp1 interacts with an Snf1-related protein kinase (SnRK) and α4 subunit of the 20 S proteasome in *Arabidopsis* (39), suggesting that SnRK and/or α4 subunit may function as a docking site for the SCF complexes on the proteasome. Thus, competitive binding of TIP120A to CUL1 against Skp1 may help release of SCF ligases from a proteasomal docking site and unloading of the substrate. Our finding that TIP120A interacts not only with CUL1 but with other cullins suggests that TIP120A has a general role common to most, if not all, cullin-containing E3 ligases. Further structural and functional dissection of TIP120A-cullin complexes is expected to further clarify the role of TIP120A in regulation of ubiquitination and proteolysis.

Acknowledgments—We are grateful to Drs. M. Pagano, J. W. Conaway, Z.-Q. Pan, Y. Xiong, and H. Zhang for generous gifts of reagents.

REFERENCES

1. Hershko, A., and Ciechanover, A. (1998) *Annu. Rev. Biochem.* **67,** 425–479
2. Hochstrasser, M. (1996) *Annu. Rev. Genet.* **30,** 405–439
3. Pickart, C. (2001) *Annu. Rev. Biochem.* **70,** 503–533
4. Ohta, T., Michel, J. J., Schottelius, A. J., and Xiong, Y. (1999) *Mol. Cell* **3,** 535–541
5. Deshaies, R. J. (1999) *Annu. Rev. Cell Dev. Biol.* **15,** 435–467
6. Skowyra, D., Koepp, D. M., Kamura, T., Conrad, M. N., Conaway, R. C., Conaway, J. W., Elledge, S. J., and Harper, J. W. (1999) *Science* **284,** 662–665
7. Kamura, T., Koepp, D. M., Conrad, M. N., Skowyra, D., Moreland, R. J., Iliopoulos, O., Lane, W. S., Kaelin, W. G., Jr., Elledge, S. J., Conaway, R. C., Harper, J. W., and Conaway, J. W. (1999) *Science* **284,** 657–661
8. Seol, J. H., Feldman, R. M., Zachariae, W., Shevchenko, A., Correll, C. C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., Nasmyth, K.,

Deshaies, R. J., Shevchenko, A., and Deshaies, R. J. (1999) *Genes Dev.* **13,** 1614–1626
9. Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J., and Harper, J. W. (1997) *Cell* **91,** 209–219
10. Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., and Elledge, S. J. (1996) *Cell* **86,** 263–274
11. Kipreos, E. T., and Pagano, M. (2000) *Genome Biology* http://genomebiology.com/2000/1/5/reviews/3002
12. Winston, J. T., Koepp, D. M., Zhu, C., Elledge, S. J., and Harper, J. W. (1999) *Curr. Biol.* **9,** 1180–1182
13. Cenciarelli, C., Chiaur, D. S., Guardavaccaro, D., Parks, W., Vidal, M., and Pagano, M. (1999) *Curr. Biol.* **9,** 1177–1179
14. Kibel, A., Iliopoulos, O., DeCaprio, J. A., and Kaelin, W. G. (1995) *Science* **269,** 1444–1446
15. Duan, D. R., Pause, A., Burgess, W. H., Aso, T., Chen, D. Y. T., Garrett, K. P., Conaway, R. C., Conaway, J. W., Linehan, W. M., and Klausner, R. D. (1995) *Science* **269,** 1402–1406
16. Kishida, T., Stackhouse, T. M., Chen, F., Lerman, M. I., and Zbar, B. (1995) *Cancer Res.* **55,** 4544–4548
17. Kamura, T., Sato, S., Haque, D., Liu, L., Kaelin, W. G., Conaway, R. C., and Conaway, J. W. (1998) *Genes Dev.* **12,** 3872–3881
18. Zhang, J. G., Farley, A., Nicholson, S. E., Willson, T. A., Zugaro, L. M., Simpson, R. J., Moritz, R. L., Cary, D., Richardson, R., Hausmann, G., Kile, B. J., Kent, S. B., Alexander, W. S., Metcalf, D., Hilton, D. J., Nicola, N. A., and Baca, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 2071–2076
19. Hilton, D. J., Richardson, R. T., Alexander, W. S., Viney, E. M., Willson, T. A., Sprigg, N. S., Starr, R., Nicholson, S. E., Metcalf, D., and Nicola, N. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 114–119
20. Ohh, M., Park, C. W., Ivan, M., Hoffman, M. A., Kim, T. Y., Huang, L. E., Pavletich, N., Chau, V., and Kaelin, W. G., Jr. (2000) *Nat. Cell Biol.* **2,** 423–427
21. Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G., Jr. (2001) *Science* **292,** 464–468
22. De Sepulveda, P., Ilangumaran, S., and Rottapel, R. (2000) *J. Biol. Chem.* **275,** 14005–14008
23. Monni, R., Santos, S. C., Mauchauffe, M., Berger, R., Ghysdael, J., Gouilleux, F., Gisselbrecht, S., Bernard, O., and Penard-Lacronique, V. (2001) *Oncogene* **20,** 849–858
24. Zhang, H., Kobayashi, R., Galaktionov, K., and Beach, D. (1995) *Cell* **82,** 915–925
25. Michel, J. J., and Xiong, Y. (1998) *Cell Growth Differ.* **9,** 435–449
26. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489
27. Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T., and Mann, M. (1996) *Nature* **379,** 466–469
28. Kamura, T., Sato, S., Iwai, K., Czyzyk-Krzeska, M., Conaway, R. C., and Conaway, J. W. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 10430–10435
29. Tan, P., Fuchs, S. Y., Chen, A., Wu, K., Gomez, C., Ronai, Z., and Pan, Z. Q. (1999) *Mol. Cell* **3,** 527–533
30. Kayukawa, K., Kitajima, Y., and Tamura, T. (2001) *Genes Cells* **6,** 165–174
31. Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. (1999) *Nat. Cell Biol.* **1,** 193–199
32. Furukawa, M., Zhang, Y., McCarville, J., Ohta, T., and Xiong, Y. (2000) *Mol. Cell. Biol.* **20,** 8185–8197
33. Chiang, C. M., Ge, H., Wang, Z., Hoffmann, A., and Roeder, R. G. (1993) *EMBO J.* **12,** 2749–2762
34. Yogosawa, S., Makino, Y., Yoshida, T., Kishimoto, T., Muramatsu, M., and Tamura, T. (1996) *Biochem. Biophys. Res. Commun.* **229,** 612–617
35. Makino, Y., Yogosawa, S., Kayukawa, K., Coin, F., Egly, J. M., Wang, Z., Roeder, R. G., Yamamoto, K., Muramatsu, M., and Tamura, T. (1999) *Mol. Cell. Biol.* **19,** 7951–7960
36. 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. (2002) *Nature* **416,** 703–709
37. Lyapina, S. A., Correll, C. C., Kipreos, E. T., and Deshaies, R. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 7451–7456
38. Yogosawa, S., Kayukawa, K., Kawata, T., Makino, Y., Inoue, S., Okuda, A., Muramatsu, M., and Tamura, T. (1999) *Biochem. Biophys. Res. Commun.* **266,** 123–128
39. Farra, R., Ferrando, A., Jasik, J., Kleinow, T., Okresz, L., Tiburcio, A., Salchert, K., del Pozo, C., Schell, J., and Koncz, C. (2001) *EMBO J.* **20,** 2742–2756
40. Verma, R., Chen, S., Feldman, R., Schieltz, D., Yates, J., Dohmen, J., and Deshaies, R. J. (2000) *Mol. Biol. Cell* **11,** 3425–3439