TIP120A Associates with Cullins and Modulates Ubiquitin Ligase Activity*

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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 immunoprecipitation 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 IkBα by SCFβ-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 multisubunit cullin chain to a target protein and involves several enzymatic activities. A ubiquitin-activating enzyme (E1) activations 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 multienzyme 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 20 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 Hrl1) 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 SCFβTrCP, SCFβ-TrCP, SCF-Cdc4, and SCF2γ, 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...
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.

EXPERIMENTAL PROCEDURES

Plasmids—The cDNAs encoding human TIP120A (GenBankTM accession number BE019018), CUL2 (GenBankTM AA206544), CUL4A (GenBankTM BC008308), and Rbx1 (GenBankTM H71993) 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-TIP120A 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 streptomycin, 1 mML -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 phenylmethylsulfonyl fluoride, 0.05% Nonidet P-40) containing 150 mM NaCl (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-Coomassie double staining, Protein size markers (in kilodaltons) are indicated on the left.

**Immunoprecipitations and Western Blotting—**Transfection was carried out by the CaPO4-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 phenylmethylsulfonyl 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 (Resigimmun) 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.).
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 Co2+–agarose beads (Clontech) according to the manufacturer’s instructions.

**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-CUL4B under the experimental conditions employed (37, 38). Several cellular proteins specifically co-purified with FLAG-CUL4B under the experimental conditions employed, as judged by SDS-PAGE and autoradiography to visualize the ubiquitinated [32]. With minor modifications. The Ub ligation reaction mixture (30 µl) contained 50 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 2 mM NaF, 10 mM 6-aminohexanoic acid, 4 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-Box)-S177E/S181E kinase purified from 293 cells following transfection. 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 [32]. For substrate preparation, 18 µg of purified GST-Box-S177E/S181E kinase purified from 293 cells following transfection. The reaction was carried out in the presence of 10 µCi of [γ-32P]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 MgCl2, 5 µM ATP, 2 mM NaF, 10 mM 6-aminohexanoic acid, and 0.6 mM DTT.

The specificity of CUL4B-TIP120A interaction, we tested other cullin family members for their binding to TIP120A. HeLa cells were transfected with expression constructs for FLAG-tagged CUL1, CUL2, CUL3, CUL4A, and CUL4B (lanes 2–6). As a negative control, an unrelated proteinFLAG-tagged Chk2kd was expressed (lane 1). Cell lysates were immunoprecipitated with anti-FLAG antibody-coupled beads. The immunoprecipitates were subjected to SDS-PAGE, blotted onto nitrocellulose membrane, and then probed with anti-TIP120A antibodies. The 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. 1A). 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-PAGE analysis 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. 3B). 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 Interferences with Binding of Skp1 and F-box Proteins to CUL1**—Since CUL1 was the best studied member of the cullin family, we investigated the specificity of CUL1-CUL120A interaction. To test other cullin family members for their binding to TIP120A, HeLa cells were transfected with expression constructs for 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. 3A). 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-PAGE analysis 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. 3B). 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. **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, or 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-PAGE, blotted onto nitrocellulose membrane, and then probed with anti-TIP120A antibodies. 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-PAGE analysis 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. 3B). 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.
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To further test whether TIP120A and Skp1 compete for the binding to CUL1, HeLa cells were transfected with expression constructs of FLAG- or HA-tagged TIP120A, CUL1, Skp1, and 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, Rbx1, Skp1, Skp2, and γ-TrCP proteins in combinations as indicated. Immunoprecipitations with α-FLAG antibodies and immunoblotting experiments were performed as described in the legend to A.

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 γ-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-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.
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of GST-IxBo\(^1-\)\(^{-54}\) was dependent not only on E1 and E2 (data not shown) but on CUL1/Rbx1 and \(\beta\)-TrCP/Skp1 (lanes 1–4), showing that intact SCF\(^{\Delta^\text{CIP}}\) complex was required for IxBo ubiquitination. Addition of increasing amounts of recombiant TIP120A greatly reduced the ubiquitination of IxBo by CUL1 complex in a dose-dependent manner (lanes 4–7). To test whether the reduction of IxBo 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. 5B, lower panel). These results indicated that TIP120A inhibited ubiquitin ligase activity of CUL1 by interfering with the binding of Skp1 and \(\beta\)-TrCP to CUL1.

TIP120A was initially identified as a TBP-interacting protein using \textit{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 \(\alpha\) subunit of the 26 S proteasome in \textit{Arabidopsis} (39), suggesting that SnRK and/or \(\alpha\) 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 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.

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