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Activation of UBC5 Ubiquitin-conjugating Enzyme by the RING Finger of ROC1 and Assembly of Active Ubiquitin Ligases by All Cullins*

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Protein ubiquitination plays an important role in regulating the abundance and conformation of a broad range of eukaryotic proteins. This process involves a cascade of enzymes including ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). E1 and E2 represent two families of structurally related proteins and are relatively well characterized. In contrast, the nature and mechanism of E3, proposed to contain activities in catalyzing isopeptide bond formation (ubiquitin ligation) and substrate targeting, remains inadequately understood. Two major families of E3 ubiquitin ligases, the HECT (for homologous to E6-AP C terminus) family and the RING family, have been identified that utilize distinct mechanisms in promoting isopeptide bond formation. Here, we showed that purified RING finger domain of ROC1, an essential subunit of SKP1-cullin/CDC53-F box protein ubiquitin ligases, was sufficient to activate UBCH5c to synthesize polyubiquitin chains. The sequence flanking the RING finger in ROC1 did not contribute to UBCH5c activation, but was required for binding with CUL1. We demonstrated that all cullins, through their binding with ROC proteins, constituted active ubiquitin ligases, suggesting the existence *in vivo* of a large number of cullin-RING ubiquitin ligases. These results are consistent with the notion that the RING finger domains allosterically activate E2. We suggest that RING-E2, rather than cullin-RING, constitutes the catalytic core of the ubiquitin ligase and that one major function of the cullin subunit is to assemble the RING-E2 catalytic core and substrates together.

Through a cascade of enzymes involving ubiquitin activating (Uba or E1),[1] conjugating (Ubc or E2), and ligating (E3) activities, the protein ubiquitination pathway catalyzes the formation of polyubiquitin chains onto substrate proteins via isopeptide bonds. Polyubiquitinated substrates are then rapidly delivered to and degraded by the 26 S proteasome (1, 2). Both E1 and E2 represent structurally related proteins and are relatively well characterized. Five E1s have been described for activating ubiquitin or ubiquitin-like modifiers (3): monomeric E1s Uba1, Apg7, and Uba4 that activate ubiquitin, Apg12, and Urm1, respectively; and two heterodimeric E1-like complexes Uba3-Ula1 and Uba2-Aos1, which activate Rub1/Nedd8 and SUMO-1, respectively. E2 ubiquitin-conjugating enzymes contain a conserved ~150-amino acid catalytic core and are present in the eukaryotic genome as a multigene family (13 in budding yeast, 29 in fruit fly, and at least 33 in human genomes). The E3 ubiquitin ligases, on the other hand, have been defined more ambiguously and operatively as an activity involved in both catalyzing isopeptide bond formation (ubiquitin ligation) and recruiting substrate. Currently, two major families of E3 ligases have been described. The homologous to E6-AP C terminus (HECT) family of E3s was discovered in the studies of ubiquitin-mediated degradation of tumor suppressor p53 in cells expressing papilloma viral oncoprotein E6 (4). E6 associates with a cellular protein, E6AP, to promote p53 degradation (5, 6). A domain of ~350 residues located at the C terminus of E6AP contains an active cysteine residue that can form thioester linkages with ubiquitin and whose mutation abolished the ligase activity of E6AP (4, 7). A large number of cellular proteins with otherwise diverse structures contain a domain homologous to E6-AP C terminus (2, 8). One biochemical mechanism underlying the physiological functions of these HECT domain-containing proteins is believed to be protein ubiquitination.

The second family of E3s contains a RING finger domain or subunit essential for their ubiquitin ligase activity (8–11). The RING finger motif was initially defined a decade ago as a novel cysteine-rich sequence present in several otherwise unrelated proteins (12). There exist a large number of RING finger-containing proteins in all eukaryotes (more than 350 in human genome), implicating a broad involvement of RING-dependent ubiquitination. The RING finger comprises eight cysteine and histidine residues that bind two atoms of zinc to form one unique three-dimensional structure referred to as the cross-brace rather than two separate mini-domains (13). Both the length and sequence between these eight conserved Cys and His residues vary significantly among different RING fingers. Based on the arrangement of Cys and His residues, RING fingers can be categorized into three subclasses: C3H2C3 (or RING-H2), C3HC4 (or RING-HC), and infrequently C2H2C4. Despite its presence in a wide range of proteins involved in various important cellular processes, the biochemical function

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[1] The abbreviations used are: E1 or Uba, ubiquitin-activating enzyme, E2 or Ubc, ubiquitin-conjugating enzyme; E3, ubiquitin-protein isopeptide ligase; APC, anaphase-promoting complex; GST, glutathione *S*-transferase; HA, hemagglutinin; HECT, homologous to E6AP C terminus; SCF, Skp1-Cdc53/cullin-F box; DTT, dithiothreitol.

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of the RING finger was unknown until the relatively recent discovery of its role in protein ubiquitination. Of a half-dozen well characterized RING finger proteins, investigations of ROC1 and APC11 have contributed significantly to our realization of RING finger domain mediated ubiquitination. APC11 is a subunit of the anaphase-promoting complex (APC or cyclosome) that is required for both entry into anaphase as well as exit from mitosis (14, 15). ROC1 (also known as Rbx1 and Hrt1) is an essential subunit of SKP1-cullin 1/CDC53-F box protein (SCF) complexes that function in regulating G_1 cell cycle control and signal transduction (16–20). Unlike most other RING finger-containing proteins, both ROC1 (108 residues) and APC11 (84 residues) are small proteins with the RING finger taking up most of the coding capacity. Various mutational analyses have demonstrated the requirement of each of the eight conserved Cys or His residues, and thus the integrity of RING finger, for the ubiquitin ligase activity.

A third emerging family of E3s contains a so-called U box, a domain of ~ 100 amino acid residues present in diverse proteins from all eukaryotic organisms. The prototype of U box proteins, yeast UFD2, was identified by its activity to collaborate with E1, E2, and a HECT domain protein UFD4 for an efficient synthesis of polyubiquitin chains (21). At least six U box proteins have been found to mediate polyubiquitination in the presence of E1 and E2, but absence of E3 (22). Despite lacking signature Cys and His residues in the U box, a computer-assisted data base search and three-dimensional structural threading revealed a similarity between the RING finger domain and the U box from various proteins, including UFD2 (23). These observations suggest that U boxes more likely represent a modified version of the RING finger that probably acts as a E3 via a mechanism similar to that of the RING finger family, rather than constitute a distinct fourth activity on the E1-E2-E3 ubiquitination pathway.

The exact biochemical mechanism underlying the function of RING fingers in protein ubiquitination remains incompletely understood. *In vivo*, the function of ROC1 and APC11 are dependent on their interactions with CUL1 and cullin-related APC2, respectively. ROC1 function has been linked to stabilize or bridge the interaction of E2 with cullins (18, 19), to promote cullin nuclear accumulation (24), and to facilitate covalent modification of cullins by a ubiquitin-like protein, NEDD8/Rub1 (16, 24). Surprisingly, APC11 alone, in the absence of cullin-like APC2, can interact with E2-UBC4 and is sufficient to promote E1- and E2-dependent polyubiquitin chain formation on protein substrates (25, 26), and our confirmatory results using human APC11 and UbcH5c). This finding raises the question on the role of RING fingers in E2 activation and substrate recognition. We report that ROC1 contains two domains, the C-terminally located RING finger that alone is sufficient to activate UBC5 and an N-terminal domain required for binding with CUL1. We demonstrate that cullins, through their binding with a ROC protein and the autonomous allosteric activation of E2 by the ROC RING finger, could assemble more than a dozen distinct ubiquitin ligases.

EXPERIMENTAL PROCEDURES

Plasmids and Purification of Recombinant Proteins—Full-length mammalian cullins, ROC1, ROC2, APC11, APC2, SKP1, and SKP2 expression plasmids were described in Refs. 17 and 27. β-TrCP clone was a gift from Dr. Yinon Ben-Neriah. ROC1 mutants were generated by site-directed mutagenesis using the QuikChange kit (Stratagene) and verified by DNA sequencing. Different E2s were amplified from a HeLa cDNA library by PCR and inserted into a T7 bacterial expression vector fused in-frame with a hexahistidine tag. Sequences for each ROC1 mutant and amplified E2 were verified by direct DNA sequencing. Purified rabbit E1 ubiquitin-activating enzyme (Affiniti, United Kingdom) and ubiquitin (Sigma) were purchased commercially. Hexahistidine-tagged ubiquitin (containing protein kinase C phosphorylation site) and E2 proteins were expressed in bacteria using the pET-3E-6xHis vector with isopropyl-1-thio-β-D-galactopyranoside induction, purified using nickel beads (Qiagen) according to the manufacturer's instructions, and stored with 10% glycerol at −80 °C. Hexahistidine-tagged mCDC34 was expressed using a baculovirus and purified from Sf9 insect cells. The concentrations of all the purified proteins were determined by Coomassie Brilliant Blue staining prior to the ubiquitination assay. GST-tagged ROC1 and ROC2 were expressed in BL21 (DE3) bacteria using the pGEX vector with isopropyl-1-thio-β-D-galactopyranoside induction and purified using glutathione-agarose (Sigma).

Cell Culture and Immunological Techniques—293T cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum in a 37 °C incubator with 5% CO_2. Cell transfections were carried out using calcium-phosphate buffer. For each transfection 15 or 5 μg of total plasmid DNA were used per 100- or 60-mm dish, respectively. Procedures for immunoprecipitation and immunoblotting have been described previously (28) with modification of the lysis buffer (15 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 1 mM Na_3VO_4, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 150 μg/ml benzamidine). Rabbit polyclonal anti-cullin 1 (27), anti-ROC1 (17), and anti-APC11 (29) antibodies have been described previously.

Ubiquitin Ligase Activity Assay—The procedure for ubiquitin labeling was described in Refs. 17 and 20. ROC and cullin immunocomplexes were precipitated from untransfected 293T cells with affinity-purified anti-ROC1 (1.5 μg) or anti-APC11 (3 μg) antibody, or from transfected cells with 3 μg of affinity-purified anti-CUL1, anti-HA, or anti-Myc antibody. For substrate-free ubiquitination assays, individual immunocomplexes were immobilized on protein A-agarose beads, washed three times with Nonidet P-40 cell lysis buffer, and washed twice with a buffer containing 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.01% Nonidet P-40, and 10% glycerol. Washed immunocomplexes were added to a ubiquitin ligation reaction (final volume 30 μl) containing 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, 0.75 μg of [^{32}P]ubiquitin, 60 ng of E1, and 300 ng of E2. Reactions were incubated at 37 °C for 30 min, terminated by boiling for 5 min with SDS-sample buffer containing 0.1 M DTT, and resolved by SDS-PAGE followed by autoradiography to visualize ubiquitin ladders.

RESULTS

ROC1 and APC11 Interact with Both CDC34 and UBCH5c—Discovery of ROC1 and ROC2 proteins, their combinatorial interactions with different cullins, and the function of cullin-ROC complexes in ubiquitination of various substrates raises issues on the role of ROC and cullin protein in ubiquitin ligation. Although only one E1 gene exists for activating ubiquitin, there are multiple E2s involved in ubiquitin conjugation (*e.g.* 13 in yeast) and an unknown number of E3s. Specific substrates may be selectively ubiquitinated by different E3s, and different E3s may preferentially interact with different E2s. This generates a potentially enormous complexity when assaying for *in vitro* ubiquitination of a specific substrate. Therefore, before testing *in vitro* ubiquitination of specific substrates, we first determined the interactions between different E2s and ROC1- and APC11-associated ligases in the absence of a substrate. This was made possible by the finding that the ROC1-CUL1 and APC11-APC2 complexes can promote ubiquitin-ubiquitin ligation in the absence of exogenously added substrate (20, 29). Ubc3/CDC34 and UbcH5c were expressed and purified from insect cell or bacteria, respectively, to near homogeneity (Fig. 1*A*), and incubated with either the ROC1 or the APC11 immunocomplex in the presence of purified E1 and ^{32}P-labeled ubiquitin. These two immunocomplexes represent a number of distinct ubiquitin ligases; the anti-ROC1 immunocomplex contains at least four different ROC1-cullin ligases (ROC1-CUL1, ROC1-CUL2, ROC1-CUL3, and ROC1-CUL4A), and the APC11 immunocomplex contains APC2 (29). Ubiquitin ligation was determined by incorporation of ^{32}P-labeled ubiquitin into a high molecular weight smear characteristic of an incremental ubiquitin ladder. Incubation of ROC1 immunocomplexes with CDC34 and UbcH5c resulted in the formation

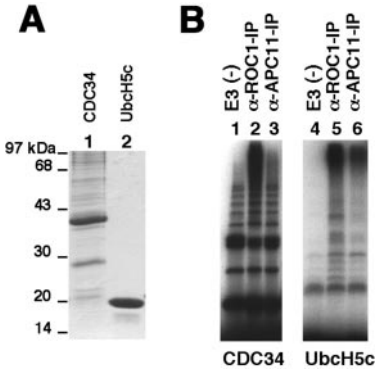

FIG. 1. **Activation of E2s by ROC1 and APC11 immunocomplexes.** *A*, His_6-tagged CDC34 was purified from Sf9 insect cells and UbcH5c was purified from bacteria using nickel beads. Each protein (2 μg) was resolved by SDS-PAGE followed by Coomassie Blue staining. *B*, immunoprecipitated ROC1 and APC11 complexes from 293T cells were incubated with the same amount of E1, ^{32}P-labeled ubiquitin, and 300 ng of CDC34 or UbcH5c. After incubating at 37 °C for 30 min, the reaction was terminated by adding sample buffer containing SDS (2%) and DTT (0.1 M), boiled for 5 min, and resolved by SDS-PAGE followed by autoradiography.

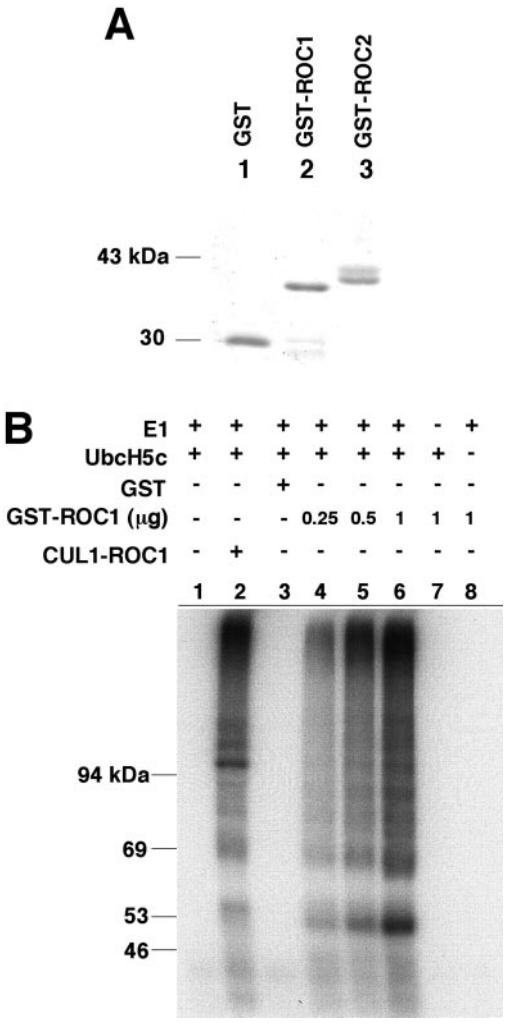

FIG. 2. ***In vitro*** **activation of UbcH5c by purified recombinant ROC1.** *A*, full-length human ROC1 and ROC2 were fused with GST moiety, expressed in bacteria, and purified using glutathione-agarose. Each purified protein was resolved by SDS-PAGE followed by Coomassie Blue staining. *B*, immunoprecipitated CUL1-ROC1 complexes, purified GST, or GST-ROC1 fusion proteins were incubated with the same amount of E1, ^{32}P-labeled ubiquitin, and 300 ng of purified UbcH5c. After incubating at 37 °C for 30 min, the reaction was terminated by adding sample buffer containing SDS (2%) and DTT (0.1 M), boiled for 5 min, and resolved by SDS-PAGE followed by autoradiography.

of a polyubiquitin chain (Fig. 1*B*). Distinctly, the APC11 immunocomplex promoted polyubiquitination when incubated with UbcH5c but exhibited very little polyubiquitination activity over the background when incubated with CDC34. This is consistent with the observation that CDC34 functions during the G_1 phase of the cell cycle, whereas the APC is most active during mitosis, an activity that is not affected by CDC34 mutation. UbcH5c shares 97% identity with Ubc4, an E2 that mediates APC-dependent cyclin B1 ubiquitination (30). Taken together, these results suggest that, as determined by the substrate-independent *in vitro* ubiquitination assay, ROC1-associated ligases can utilize CDC34 and UbcH5c, and the APC11-APC2 ligase can use UbcH5c.

Activation of UbcH5 by Purified Recombinant ROC1—Prompted by the finding that purified recombinant APC11 alone is sufficient to activate Hbc4 (25, 26), we tested whether purified recombinant ROC1 and ROC2 can activate UbcH5 and CDC34 *in vitro* in the absence of their cullin partners. GST-ROC1 fusion protein was expressed in bacteria and purified to near homogeneity (Fig. 2*A*) and incubated with purified UbcH5c in the presence of E1 and ^{32}P-labeled ubiquitin. As shown in Fig. 2*B*, recombinant GST-ROC1, but not control GST protein (*lane 3*), was capable of activating UbcH5c to synthesize polyubiquitin chains in a dose-dependent manner (*lanes 4–6*) to an efficiency similar to that observed with ROC1-CUL1 complexes derived from transfected 293T cells (*lane 2*). Omitting E1 (*lane 7*) abolished polyubiquitin chain formation, indicating that ROC1-mediated UbcH5c activation is E1-dependent.

The RING Finger Domain of ROC1 Is Sufficient to Activate UbcH5—We next determined the sequence within ROC1 that is necessary for activating UbcH5c. The human ROC1 protein contains 108 amino acid residues. The H2-RING finger occupies the C-terminal half of the protein and many mutations within the RING finger abolish its ligase activity. The function of the N-terminal half of ROC1 has not been determined. We generated two deletion mutants, $ROC1^{\Delta N23}$ and $ROC1^{\Delta N41}$, which removed the N-terminal 23 and 41 residues from ROC1, respectively (Fig. 3*A*), and determined their effect on binding with CUL1 *in vivo* and activating UbcH5c *in vitro*. Both deletion mutants, as well as a deletion mutant that removed the C-terminal 14 residues ($ROC1^{\Delta C14}$), and two double point mutants within the RING finger, were fused in-frame with an HA epitope tag and inserted into a cytomegalovirus-based mammalian expression vector. Individual plasmids expressing wild type ROC1 and individual ROC1 mutants were co-transfected with T7 epitope-tagged CUL1 into 293T cells. ROC1-CUL1 complex formation was examined by coupled immunoprecipitation and immunoblotting (Fig. 3*B*, *IP*, *WB*). Both ΔN23 and ΔN41 deletions severely reduced the association of ROC1 with CUL1 (*lanes 6* and *7*). In contrast, deletion of the C-terminal 14 residues and two double mutations within the RING finger did not detectably affect ROC1-CUL1 binding. Taken together, these results indicate that the N-terminal portion of ROC1 is required for the association with CUL1.

To determine whether the N-terminal domain of ROC1 is required for E2 activation, we expressed and purified GST fusion proteins containing these mutations. Both purified GST-$ROC1^{\Delta N23}$ and GST-$ROC1^{\Delta N41}$ activated UbcH5c *in vitro* at an efficiency similar to that reached by the wild type GST-ROC1 protein. In contrast, deletion of the C-terminal 14 residues or several double mutations within the RING finger completely abolished the E2 activation function of ROC1 (Fig. 3*C*). These results demonstrate that the RING domain alone is sufficient

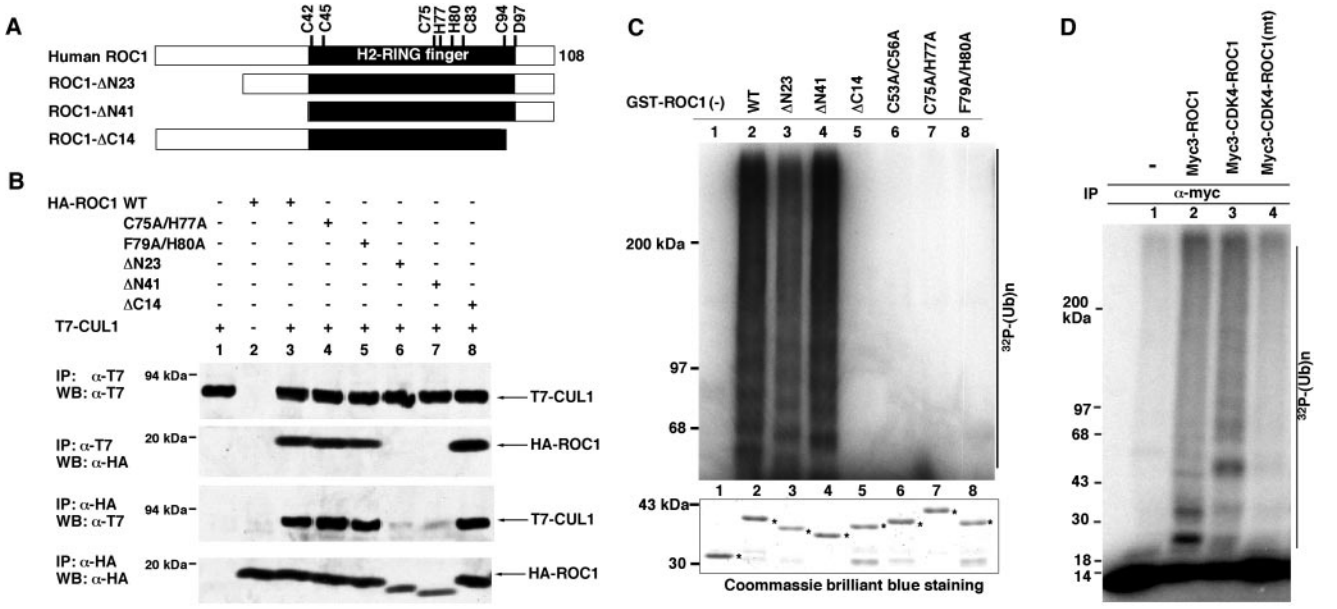

FIG. 3. **RING finger domain of ROC1 is sufficient to activate UbcH5c.** *A*, schematic diagram of wild type and mutant human ROC1 proteins. *Filled box* represents the RING-H2 finger. The positions of seven conserved Cys and His residues and an Asp residue located at the eighth zinc binding site are indicated. *B*, 293T cells were co-transfected with plasmids expressing HA-tagged ROC1 and T7-tagged CUL-1 expression plasmid as indicated. Cell lysates were precipitated with either anti-HA or anti-T7 antibodies, resolved by SDS-PAGE, and followed by immunoblotting. *C*, *upper panel*, purified GST or GST-ROC1 wild type or mutant fusion proteins were incubated with the same amount of E1, ^{32}P-labeled ubiquitin, and 300 ng of purified UbcH5c. After incubating at 37 °C for 30 min, the reaction was terminated by adding sample buffer containing SDS (2%) and DTT (0.1 M), boiled for 5 min, and resolved by SDS-PAGE followed by autoradiography. *Lower panel*, wild type full-length or mutant ROC1 were fused with GST moiety, expressed in bacteria, and purified using glutathione-agarose. Each purified protein was resolved by SDS-PAGE followed by Coomassie Blue staining. *D*, 293T cells were transfected with Myc-tagged ROC1 or in-frame fusion of Myc-tagged CDK4-ROC1-expressing plasmids. ROC1 or chimerical CDK-ROC1 complexes were recovered with anti-Myc antibody (9E10) and assayed for ubiquitin ligase activity by incubating with E1, ^{32}P-labeled ubiquitin, and UbcH5c. After incubating at 37 °C for 30 min, the reaction was terminated by adding sample buffer containing SDS (2%) and DTT (0.1 M), boiled for 5 min, and resolved by SDS-PAGE followed by autoradiography.

to activate UbcH5c and does not require the N-terminal sequence.

That the ROC1 alone is sufficient to activate an E2 to synthesize polyubiquitin chain formation in the absence of other ROC1-associated co-factors led us to test whether ROC1 can be crafted onto a heterologous protein and still retain its function to activate an E2. The entire sequence of ROC1 was fused in-frame with CDK4 and a Myc epitope tag. Myc-CDK4-ROC1 fusion protein was immunoprecipitated from transiently transfected 293T cells using anti-Myc antibody and incubated with ^{32}P-labeled ubiquitin in the presence of E1 and UbcH5c. Myc-CDK4-ROC1 exhibited a high level of activity similar to that seen with Myc-ROC1 to activate UbcH5c to synthesize polyubiquitin chains (Fig. 3*D*, *lane 3*). Introduction of a double point mutation, C75A/H77A, into the RING finger substantially reduced the polyubiquitin chain formation activity (*lane 4*). This result further supports the autonomous function of ROC1 in activating E2 and suggests the possibility of engineering RING finger-based chimerical ubiquitin ligases for targeting the ubiquitination and degradation of selected proteins.

ROC1 Alone Cannot Activate CDC34—We next determined whether purified recombinant ROC1 could activate other E2 CDC34. Purified recombinant ROC1, unlike ROC1 immunoprecipitated from mammalian cells, was unable to activate CDC34 (Fig. 4). The simplest explanation is that different E2s may interact distinctly with E3s and that ROC1 immunocomplexes derived from mammalian cells contain an additional factor(s) that is necessary for ROC1 to activate CDC34. One candidate factor is cullin proteins. Consistent with a requirement of cullins in ROC1-mediated CDC34 activation, HA-ROC1 immunocomplexes derived from cells co-transfected with CUL1 are more potent in activating CDC34 than those derived from cells singly transfected with ROC1 (Fig. 4*B*, compare *lane 3* with *lane 2*). Likewise, anti-HA immunocomplexes derived from cells co-transfected with CUL1 and CUL1-binding deficient HA-ROC1$^{\Delta N23}$ did not appreciably activate CDC34 (*lane 4*).

Activation of UbcH5c by Purified Recombinant ROC2—ROC1 has a closely related homologue, ROC2, that also encodes a small H2 type RING finger protein of 113 amino acid residues and interacts with cullins (17). To determine whether ROC2, like ROC1, alone can also activate UbcH5c in the absence of a cullin or other cofactors, GST-ROC2 fusion protein was expressed in bacteria, purified to near homogeneity (Fig. 2*A*), and incubated with purified CDC34 or Ubc5Hc proteins in the presence of E1 and ^{32}P-labeled ubiquitin. Similar to ROC1, recombinant GST-ROC2 efficiently activated UbcH5c, but not CDC34, to form polyubiquitin chains (Fig. 4*C*).

All Cullins and APC2 Form Active Ubiquitin Ligases with ROC and APC11 Proteins—Cullin 1/CDC53 represents a multigene family, containing six genes in mammalian cells (cullin 1, 2, 3, 4A, 4B, and 5 (Ref. 31)). Different cullins commonly interact with both ROC1 and ROC2 (17). The findings that ROC1 and ROC2 proteins can autonomously activate E2 suggest the possibility that all cullins, through their association with a ROC protein, could contain ubiquitin ligase activity. We tested this idea by assaying for ubiquitin ligase activity of individual cullin complexes as well as that of ROC2 complexes. We have previously characterized six full-length mammalian cullin cDNAs, CUL1, CUL2, CUL3, mCUL4A, CUL5, and mAPC2 (17, 27). HA-tagged ROC2 or Myc-tagged cullins were transfected into 293T cells. Cullin and ROC2 complexes were immunoprecipitated using either anti-Myc or anti-HA antibodies, respectively, and their associated ubiquitin ligase activities were assayed using CDC34 or UbcH5c as E2 (Fig. 5, *A* and *C*). Individual cullins were expressed uniformly in 293T cells, and

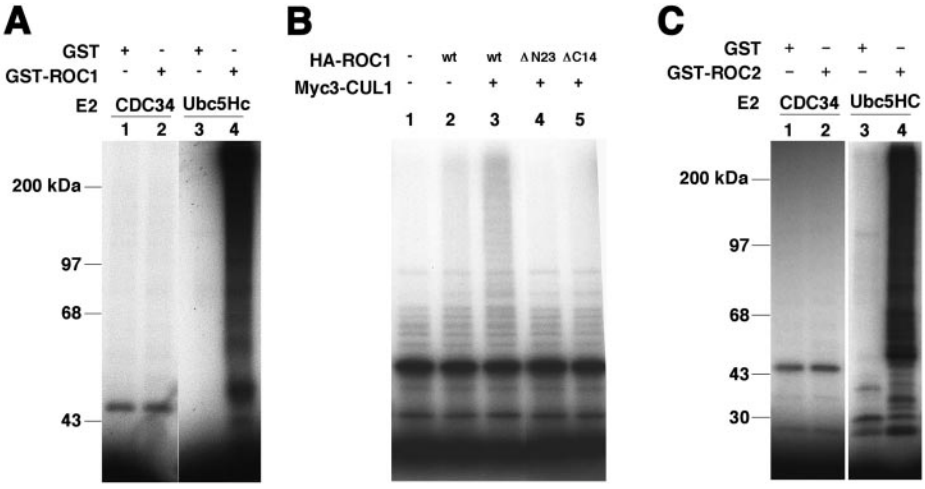

FIG. 4. **Activation of E2 by ROC1 and ROC2.** *A*, purified GST or GST-ROC1 fusion proteins were incubated with the same amount of E1, ^{32}P-labeled ubiquitin, and 300 ng of various purified E2s. After incubating at 37 °C for 30 min, the reaction was terminated by adding sample buffer containing SDS (2%) and DTT (0.1 M), boiled for 5 min, and resolved by SDS-PAGE followed by autoradiography. *B*, CUL1 facilitates ROC1 to activate CDC34. HA-tagged wild type and mutant ROC1 expression plasmid was co-transfected with Myc3-tagged CUL1 expression plasmid into 293T cells. Anti-HA immunoprecipitates were incubated with the same amount of E1, ^{32}P-labeled ubiquitin, and 300 ng of purified CDC34. After incubating at 37 °C for 30 min, the reaction was terminated by adding sample buffer containing SDS (2%) and DTT (0.1 M), boiled for 5 min, and resolved by SDS-PAGE followed by autoradiography. *C*, purified recombinant GST-ROC2 (Fig. 2*A*) was incubated with the same amount of E1, ^{32}P-labeled ubiquitin, and 300 ng of purified CDC34 or UbcH5c as indicated. After incubating at 37 °C for 30 min, the reaction was terminated by adding sample buffer containing SDS (2%) and DTT (0.1 M), boiled for 5 min, and resolved by SDS-PAGE followed by autoradiography.

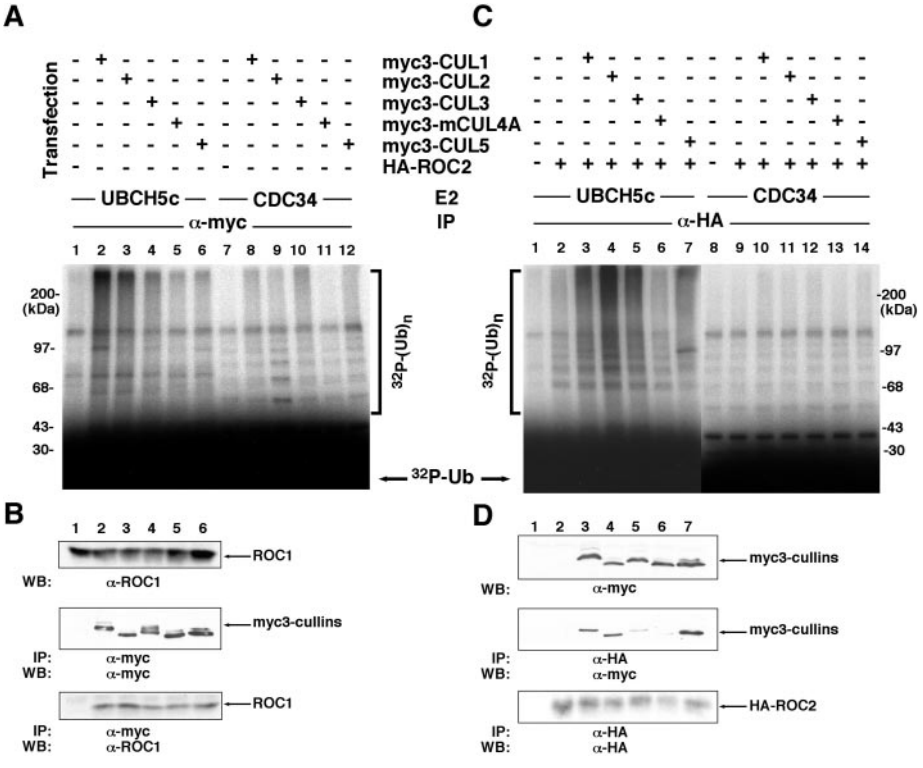

FIG. 5. **ROC1 and ROC2 form active ubiquitin ligases with all cullins.** *A* and *C*, 293T cells were co-transfected with indicated plasmids. Individual cullin and ROC2 complexes were recovered with indicated antibody and assayed for ubiquitin ligase activity by incubating with E1, ^{32}P-labeled ubiquitin, and either UbcH5c or Ubc3/CDC34. *B* and *D*, total cell lysates from transfected 293T cells or aliquots of the same immunoprecipitates from *A* and *C* were resolved by SDS-PAGE, followed by immunoblotting with indicated antibodies to determine the levels of expressed cullins and their complex with ROC1 and ROC2.

found to associate with similar amounts of endogenous ROC1 as determined by direct immunoblotting of total cell lysates and immunoprecipitation Western analysis (Fig. 5*B*). When assayed for UBCH5c activation, CUL1 complex exhibited the highest activity, followed by CUL2, CUL3, CUL5, and CUL4A complexes.

ROC2 protein displayed preferential association with CUL5, moderate binding with CUL1 and CUL2, and only low level interaction with CUL3 and CUL4A (Fig. 5*D*). These results suggest that, although ROC1 interacts with different cullins with similar affinity, ROC2 binding is more cullin-specific. ROC2-CUL2 complex appeared to activate UBCH5c most efficiently, followed by ROC2-CUL1, CUL3, and CUL5. Consistent with little association between ROC2 and CUL4A, ROC2 immunoprecipitate did not detectably activate UBCH5c when co-expressed with CUL4A. Somewhat unexpectedly, none of ROC2-cullin complex exhibited any activity when assayed for CDC34 activation. Together, these results demonstrate that all five members of the cullin family (CUL1, CUL2, CUL3, mCUL4A, CUL5) and ROC2 are assembled *in vivo* into complexes that contain ubiquitin ligase activity.

CUL1-ROC1, but Not Other Cullin-ROC1 Ligases, Promotes SKP1- and β-TrCP-dependent IκBα Ubiquitination—Cullin 1/CDC53-associated ubiquitin ligase is targeted to its substrate

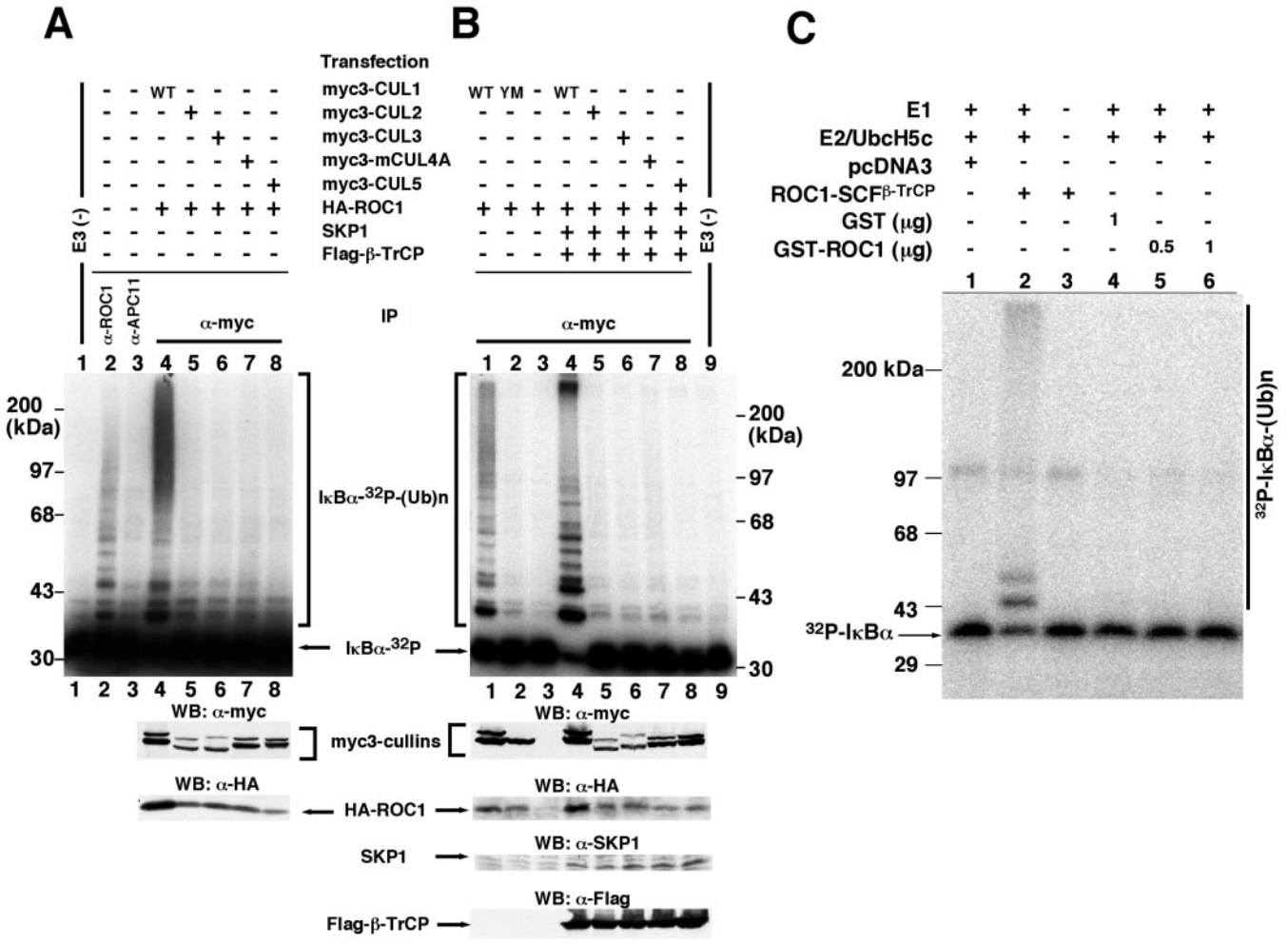

FIG. 6. **SKP1- and β-TrCP-dependent *in vitro* ubiquitination of IκBα by CUL1, but not other cullins.** *A*, 293T cells were co-transfected with indicated plasmids. Individual cullin, ROC, or APC11 complexes were recovered with indicated antibody and assayed for ubiquitin ligase activity using purified IκBα that was phosphorylated in the presence of [γ-^{32}P]ATP with IκBα kinase IKKβ. Reactions were incubated at 37 °C for 60 min, terminated by adding Laemmli loading buffer, boiled for 4 min, and resolved by SDS-PAGE followed by autoradiography to visualize the ubiquitinated IκBα ladders. The level of ectopically expressed HA-ROC1 and Myc3-cullins was examined by direct immunoblotting. Cullins were expressed as doublets, presumably as the result of covalent NEDD8 modification. *B*, overexpression of SKP1 and β-TrCP enhanced IκBα ubiquitination by CUL1, but not other cullins. 293T cells were co-transfected with plasmids expressing indicated proteins. Individual cullin ligases were recovered by anti-Myc immunoprecipitation and assayed for ubiquitin ligase activity using purified and phosphorylated IκBα as a substrate. Note that overexpression of SKP1 and β-TrCP substantially enhanced the IκBα ligase activity of CUL1, but had no detectable effect on IκBα ubiquitination by other cullins. *C*, activation of UbcH5c by ROC1 is not sufficient to ubiquitinate IκBα. Purified and phosphorylated IκBα was incubated with either ROC1-SCF$^{β\text{-TrCP}}$ or purified ROC1 in the presence of E1, UbcH5c, and ubiquitin (unlabeled). ROC1-SCF$^{β\text{-TrCP}}$ was prepared from 293T cells transfected with plasmids expressing HA-ROC1, CUL1, SKP1, and FLAG-β-TrCP by immunoprecipitation using HA antibody. *Lane 1* contains a negative control of an anti-HA immunocomplex derived from 293T cells transfected with empty pcDNA3 plasmid.

proteins via a complex containing two additional proteins, SKP1 and an F box protein. In this complex, CUL1/CDC53 simultaneously interacts with SKP1 via an N-terminal domain (27, 32, 33), with ROC1, and probably through ROC1 with E2 via a C-terminal domain (24). The SKP1 protein, in turn, interacts with the F box protein, which binds to the phosphorylated substrate protein. We previously found that only CUL1, but not other cullins, interacts with SKP1 (27), suggesting the possibility of selective utilization of SKP1 and F box protein in substrate targeting by CUL1. Detection of ubiquitin ligase activity of other cullins provides an opportunity to test this possibility more directly. Individual cullin-ROC1 ubiquitin ligases were purified from 293T cells co-transfected with HA-ROC1 and Myc3-cullins by anti-Myc immunoprecipitation and assayed using phosphorylated IκBα as substrate. As previously reported (17, 20), ROC1 immunocomplexes purified from 293T cells (Fig. 6*A*, *lane 2*), but not the negative control of endogenously expressed APC11 (*lane 3*), exhibited a readily detectable level of IκBα ubiquitin ligase activity. Overexpression of HA-ROC1 and Myc3-CUL1 substantially increased the IκBα ubiquitination activity of the Myc3-CUL1 immunocomplex (*lane 4*). In contrast, anti-Myc immunocomplexes derived from cells co-transfected with HA-ROC1 and other Myc-tagged cullins, including Myc3-CUL2 (*lane 5*), Myc3-CUL3 (*lane 6*), Myc3-mCUL4A (*lane 7*), and Myc3-CUL5 (*lane 8*), did not display detectable IκBα ubiquitination activity (*lanes 5–8*). A comparable level of ectopic expression of HA-ROC1 and individual Myc3-cullins was verified by direct immunoblotting with either HA or Myc antibodies (Fig. 6*A*, *bottom panels*).

The F box protein β-TrCP targets phosphorylated IκBα to CUL1-ROC1 ubiquitin ligase through SKP1 (34). Consistently, anti-Myc immunocomplexes derived from cells overexpressing Myc-CUL1, HA-ROC1, SKP1, and β-TrCP exhibited significantly higher levels of IκBα ligase activity than those derived from cells transfected with Myc-CUL1 and HA-ROC1 without SKP1 and β-TrCP (Fig. 6*B*, compare *lane 4* with *lane 1*). A double mutation in CUL1, Y42A/M43A, impaired CUL1's bind-

ing with SKP1[2] and diminished the IκBα ubiquitin ligase activity of CUL1 (Fig. 6*B*, *lane 2*). These results confirm the specificity of IκBα ubiquitination by $SCF^{\beta\text{-TrCP}}$ and also suggest that SKP1 and/or β-TrCP are rate-limiting factors for IκBα ligase activity when CUL1 and ROC1 are overexpressed. Stimulation of the IκBα ligase activity of CUL1 by overexpression of SKP1 and β-TrCP led us to determine whether other cullin ligases may be targeted by SKP1 and β-TrCP at a lower efficiency than CUL1 ligase, and whether they may be stimulated to ubiquitinate IκBα by the overexpression of SKP1/β-TrCP. To test this possibility, Myc-tagged individual cullins were co-expressed with HA-ROC1, SKP1, and β-TrCP, recovered by anti-Myc immunoprecipitation, and assayed for IκBα ligase activity *in vitro*. Under this more sensitive assay condition, no IκBα ligase activity was detected for all four cullins examined (Fig. 6*B*, *lanes 5–9*). We conclude that ubiquitin ligase activity of CUL2, CUL3, mCUL4A, and CUL5, unlike that of CUL1, cannot be targeted to IκBα by SKP1 and β-TrCP.

In vitro, APC11 is not only capable of activating Ubc4 to form polyubiquitin chains in the absence of a substrate, it can also promote E1- and E2-dependent substrate ubiquitination (25, 26). On the other hand, the specific requirement for the N-terminal SKP1-binding domain of CUL-1 in targeting IκBα ubiquitination predicts that activation of E2 by ROC1 to form a polyubiquitin chain may not necessarily be sufficient to ubiquitinate IκBα in the absence of CUL1-mediated SCF activity. To test this prediction, phosphorylated IκBα was incubated *in vitro* with purified recombinant GST-ROC1 in the presence of E1 and UbcH5c. Under the condition where phosphorylated IκBα can be readily ubiquitinated by a ROC1 immunocomplex derived from cells transfected with ROC1, CUL1, SKP1, and βTrCP (ROC1-$SCF^{\beta\text{-TrCP}}$; Fig. 6*C*, *lane 2*), purified recombinant GST-ROC1 cannot promote detectable ubiquitination of IκBα (*lanes 5* and *6*). This result reveals a distinction between ROC1- and APC11-mediated ubiquitination and differences between substrates of APC and SCF.

DISCUSSION

The RING Finger Domain of ROC1 Alone Is Sufficient to Activate E2—Previously, studies have established an essential function of ROC1 and the integrity of its RING finger for CUL1-mediated SCF ubiquitin ligase activity (16–20). We demonstrated in this paper that ROC1 contains two separate domains: the C-terminal RING finger that is sufficient to activate E2 UbcH5 ubiquitin-conjugating enzyme to synthesize a polyubiquitin chain and an N-terminal sequence that is required for binding with cullins. Deletion of the N-terminal cullin-binding sequence, leaving virtually only the RING finger domain, had no detectable effect on the ability of ROC1 to activate UbcH5c *in vitro* (Fig. 3). Mutations of metal-binding Cys and His residues, on the other hand, all disrupt the ligase activity of ROC1, but only some affect ROC1-CUL1 binding (*e.g.* C42S, C45C83S, and H80A (Refs. 29 and 35)). Several mutations of signature Cys and His residues in ROC1 preserve its binding with CUL1 (*e.g.* C53A/C56A and C75A/H77A (Ref. 17)). Taken together, these results indicate that the integrity of the RING finger is necessary for E2 activation and may also contribute to, but is not absolutely required for, CUL1 binding. Because there is little sequence conservation between the different RING fingers, it seems that eight conserved Cys and His metal binding residues likely contain all necessary information for interacting with and activating Ubc5.

A biochemical mechanism underlying the RING-E2-mediated polyubiquitination is not clear. One possible function of RING motif in E2 activation is to facilitate the binding of zinc ion to E2, as suggested by a recent finding that addition of zinc ion alone can active Ubc4 to synthesize polyubiquitin (36). Unlike HECT domain ligases, which forms a thioester bond intermediate with ubiquitin (7), ubiquitin ligase activity of ROC1-E2/CDC34 was not inhibited by sulfhydryl-modifying reagents such as *N*-ethylmaleimide and iodoacetamide, suggesting that this RING-E2 ligase does not require a reactive thiol linkage (18, 35). Crystal structural analysis of the c-Cbl RING finger domain and E2/UbcH7 revealed that the closest RING domain residue of c-Cbl is 15 Å away from the catalytic site Cys in Ubc7 (38). Unless conjugation of an ubiquitin with E2 dramatically changes the structure of RING-E2, the structure analysis of c-Cbl-Ubc7 suggests that the RING finger does not directly participate in the catalysis of ubiquitin transfer from E2 to the substrate and subsequent isopeptide bond formation. We suggest that the RING finger and E2 constitute the catalytic core of an E3 ubiquitin ligase in which the RING finger acts as an allosteric activating, rather than catalytic, subunit to activate E2 to synthesize polyubiquitin chains. Cullin-ROC and APC2-APC11 interactions may represent the primitive forms of RING family ubiquitin ligases in which the cullin or APC2 functions to bring together the separate RING-E2 catalytic core and substrates. During the course of evolution, many previous RING-interacting proteins might have incorporated the RING finger as a built-in domain and become a more efficient ubiquitin ligases.

Using CDC34 as an E2, it was found that ROC1/Hrt1 requires CUL1 for full E3 ligase activity (18). It is somewhat surprising to see that activation of E2 UbcH5c by ROC1 does not require a cullin. All ubiquitin-conjugating enzymes contain a core catalytic domain of ~150 amino acids. Based on the presence and location of additional sequences, E2s have been divided into four classes: class I consisting almost entirely of the conserved core domain, and class II, III, and IV containing additional sequence at the N-terminal, C-terminal, or both sides of the core domain (39). CDC34, a class III E2, contains a 13-residue insertion between two loops surrounding the ubiquitin-accepting cysteine residue and a 62-residue C-terminal sequence. Both sequences are absent from Ubc5 and most other class I ubiquitin-conjugating enzymes, suggesting that neither sequence is essential for the catalytic function of CDC34. Like CDC34, yeast Ubc7 also contains a 13-residue insertion between two loops. Atomic structure analysis indicated that this insertion alters the surface region of Ubc7, but does not significantly change its overall folding as compared with other class I E2s lacking this insertion (40–42). The unique C terminus of yeast Cdc34 is required for its *in vivo* function (43). The molecular basis for the requirement of this C-terminal sequence is not entirely clear, but it has been speculated to mediate the dimer formation of Cdc34 or the binding of Cdc34 with Cdc53/CUL1 and F box protein Cdc4 (43, 44). It will be interesting to determine whether either of these two sequences in CDC34 hinders its interaction with ROC1 and whether binding with CUL1 and CUL3 or CDC34 dimerization can release this inhibition.

Cullin and ROC Constitute a Potentially Large Family of Ubiquitin Ligases in Vivo—Cullin 1 and ROC1 both represent multigene families. We present direct biochemical evidence that, like ROC1, ROC2 also forms an active ubiquitin ligase with a cullin. We further demonstrate that all five members of the cullin family we examined constitute ubiquitin ligase activity with a ROC protein. Together with our previous report showing ubiquitin ligase activity of APC2-APC11 (17), these results demonstrate that ROC and cullin family proteins potentially constitute more than a dozen distinct ligases *in vivo*. There exist *in vivo* a large number of proteins whose levels are

[2] J. Liu and Y. Xiong, unpublished data.

known to be regulated by ubiquitin-mediated proteolysis, but their E3 ubiquitin ligases are yet to be identified. The challenge now is to identify specific substrates for individual ligases and elucidate the substrate targeting mechanism. One well characterized mechanism in targeting substrate to a cullin ligase involves the formation of SCF complex (45, 46). Thus far, however, the SCF-mediated substrate targeting mechanism appears to be utilized only by CUL1. In mammalian cells, both ROC1 and CUL1 complexes contain readily detectable IκBα ubiquitin ligase activity that can be further enhanced by the overexpression of SKP1 and the IκBα-targeting F box protein β-TrCP (see Ref. 17 and Fig. 6), and abolished by the mutations in CUL1 that disrupt CUL1-SKP1 binding (Fig. 6*B*). Under the same assay conditions, however, IκBα ubiquitin ligase activity was not detected in any of the other four cullin immunocomplexes purified from the same cell line (Fig. 6). In both mammalian (27) and in yeast cells,[3] SKP1 does not interact with other cullins. Because interaction of an F box protein and its associated substrate with CUL1 is mediated by SKP1 and does not appear to involve CUL1 itself, these findings indicate that the initially identified SCF pathway is utilized by perhaps only a small number of proteins whose ubiquitin-dependent proteolysis requires cullin 1 as a part of the E3 ligase. The ubiquitination of proteins by other cullin-ROC ligases may involve either a divergent SCF-like pathway such as the von Hippel-Lindau-elongin C/elongin B complex utilized by the CUL2 ligase (37, 47, 48), or a novel mechanism(s).

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