

ROC1, a Homolog of APC11, Represents a Family of Cullin Partners with an Associated Ubiquitin Ligase Activity

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Summary

We have identified two highly conserved RING finger proteins, ROC1 and ROC2, that are homologous to APC11, a subunit of the anaphase-promoting complex. ROC1 and ROC2 commonly interact with all cullins while APC11 specifically interacts with APC2, a cullin-related APC subunit. Yeast ROC1 encodes an essential gene whose reduced expression resulted in multiple, elongated buds and accumulation of Sic1p and Cln2p. ROC1 and APC11 immunocomplexes can catalyze isopeptide ligations to form polyubiquitin chains in an E1- and E2-dependent manner. ROC1 mutations completely abolished their ligase activity without noticeable changes in associated proteins. Ubiquitination of phosphorylated I κ B α can be catalyzed by the ROC1 immunocomplex *in vitro*. Hence, combinations of ROC/APC11 and cullin proteins potentially constitute a wide variety of ubiquitin ligases.

Introduction

Many regulatory proteins such as cyclins, CDK inhibitors, and transcription factors are regulated by the ubiquitin-dependent proteolytic pathway (Hochstrasser, 1996; King et al., 1996; Hershko, 1997). A cascade of enzymes, E1, E2, and E3, catalyze the attachment of ubiquitin (Ub) to substrates to form polyubiquitinated conjugates that are rapidly detected and degraded by the 26S proteasome. E1 and E2 both represent structurally related and well characterized enzymes that do not provide much substrate specificity. The E3 is ambiguously defined as a function containing two separate activities: an Ub ligase activity to catalyze isopeptide bond formation and a specific targeting activity to physically bring the ligase and substrate together. Elucidating the molecular nature and the regulation of E3s have become critical issues central to our understanding of regulated proteolysis.

Knowledge about E3 Ub ligases is very limited at present. The best characterized E3 ligase is the APC (anaphase-promoting complex or cyclosome), which plays a crucial role in regulating the passage of cells through anaphase (reviewed in King et al., 1996). Most proteins

known to be degraded by the APC contain a conserved 9-amino acid stretch, commonly known as the destruction box, that is necessary for their ubiquitination and degradation (Glotzer et al., 1991). Proteins that are degraded during G1 do not contain the conserved destruction box. Instead, substrate phosphorylation appears to play an important role in targeting their interaction with an E3 for ubiquitination. Genetic and biochemical analysis in yeast have identified an E3-like activity, the SCF, that plays a key role in regulating G1 progression. The SCF consists of at least three subunits, SKP1, CDC53/cullin, and an F box-containing protein, in which SKP1 functions as an adapter to connect CDC53 to the F box protein that binds directly to the substrate (reviewed in Hoyt, 1997).

Despite extensive investigations into the APC and SCF, the nature of E3 ligases still remains unclear. One inconsistency is that all *in vitro* reconstituted SCF ubiquitination reactions reported thus far have required the supplement of a cellular extract. This indicates that an essential protein component(s) or a critical modification was missing from the complexes assembled from isolated proteins. We report here the identification of a family of closely related RING finger proteins, ROC1, ROC2, and APC11. We present evidence that ROC1/APC11 are general cullin-binding proteins that have an associated ligase activity. Hence, ROC-cullins and APC11-APC2 may function as Ub ligases during interphase and mitosis, respectively.

Results

ROC1 Interacts Directly with All Cullins

In a yeast two-hybrid screen of a human HeLa pGAD-cDNA library using mouse cullin 4A as bait, we identified a gene encoding a RING finger protein, named ROC1 (regulator of cullins). ROC1 can also interact with cullins 1, 2, and 5 (Figure 1A). We then demonstrated that ROC1 interacts with the COOH-terminal 527 amino acid residues of CUL1, but not the NH₂-terminal 249 residues (Figure 1B). In contrast, SKP1 binds to the NH₂-terminal domain of CUL1. These results indicate that CUL1 contains at least two distinct domains, a NH₂-terminal SKP1-binding domain and a central/COOH-terminal ROC1-binding domain. Such structural separation suggests that it is unlikely that ROC1 competes with SKP1 for CUL1. Supporting this idea, we have detected ROC1 in the α -SKP1 immunocomplex, and SKP1 was reciprocally detected in the α -Roc1 immunocomplex when cells were cotransfected with ROC1, CUL1, and SKP1 (data not shown). Hence, ROC1 and SKP1 may coexist in the same protein complex with CUL1 to perform different functions.

To confirm the interaction between ROC1 and cullin proteins, Saos-2 cells were transfected with plasmids directing the expression of HA-tagged human ROC1 (HA-ROC1) together with CUL1 or other myc-epitope tagged cullins. Transfected cells were metabolically labeled with [³⁵S]methionine, and cell lysates were reciprocally immunoprecipitated with either α -HA, α -CUL1, or

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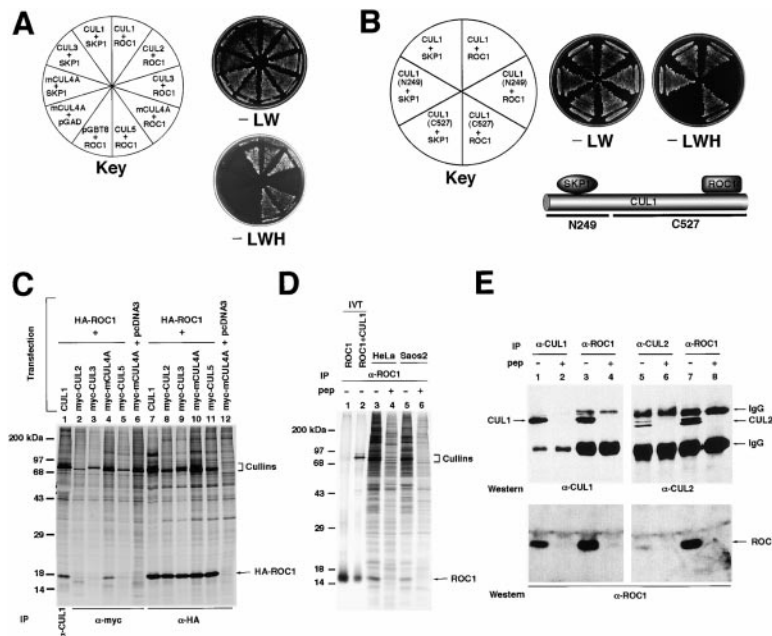


Figure 1. ROC1 Interacts with Members of the Cullin Family

(A) Yeast HF7c cells were cotransformed with plasmids expressing indicated proteins (key) and plated onto media lacking leucine and tryptophan (–LW) to verify the presence of both bait (Leu⁺) and prey (Trp⁺) plasmids; or onto media lacking leucine, tryptophan, and histidine (–LWH) to assay for interactions between bait and prey proteins.

(B) ROC1 interacts with the C-terminal portion of CUL1. HF7c yeast cells were cotransformed with plasmids expressing indicated proteins. Protein–protein interaction was assayed as described in (A).

(C–E) In vivo association of ROC1 with cullins. (C) ³⁵S-methionine-labeled lysates prepared from HeLa cells transfected with indicated plasmids.

(D) ³⁵S-methionine-labeled, in vitro translated ROC1 and CUL1, or cell lysates from HeLa and Saos-2 cells were immunoprecipitated with indicated antibodies and resolved by SDS-PAGE.

(E) Lysates prepared from HeLa cells were immunoprecipitated with indicated antibodies with (+) or without (–) competing antigen peptide. Proteins were resolved by SDS-PAGE and analyzed by Western analysis using indicated antibodies.

α-myc antibody. All five cullins coprecipitated with ROC1 by the α-HA antibody (Figure 1C; lanes 7–11). Reciprocally, HA-ROC1 was detected in α-CUL1 and α-myc-mCUL4A immunoprecipitations (lanes 1 and 4). The association between ROC1 and cullin 1 was not disrupted by buffer containing 0.1% SDS (data not shown), indicating that the ROC1–cullins association is very stable.

To obtain evidence for in vivo ROC1–cullins association under physiological conditions, we raised rabbit polyclonal antibodies specific to ROC1. This antibody is capable of precipitating both ROC1 and the ROC1–CUL1 complex as determined by the use of in vitro translated proteins (Figure 1D; lanes 1 and 2). From metabolically labeled HeLa and Saos-2 cells, the α-Roc1 antibody precipitated a protein of approximately 14 kDa (lanes 3 and 5). This protein corresponds to ROC1 as judged by its comigration with in vitro produced ROC1 and by competition using the antigen peptide (lanes 4 and 6). Several proteins that migrated in the 90 kDa range (indicated as “cullins” in Figure 1D) were also specifically competed by the antigen peptide. We immunopurified these proteins and determined their sequences by protein microsequencing. At least four cullin proteins (CUL1, CUL2, CUL3, and CUL4A or 4B) have been identified from this analysis thus far. HeLa cell lysate was then immunoprecipitated with antibodies to ROC1, CUL1, and CUL2, and precipitates were analyzed by Western blotting. As shown in Figure 1E, both CUL1 (lane 3) and CUL2 (lane 7) were readily detected in the ROC1 immunocomplexes and were specifically competed by ROC1 antigen peptide. Reciprocally, ROC1 was detected in both CUL1 (lane 1) and CUL2 (lane 5) complexes (lower panel, Figure 1E). Thus, ROC1 is a general cullin-interacting protein.

ROC1 Represents a Family of RING Finger Proteins Related to APC11

ROC1 encodes a 108-amino acid residue protein with a predicted molecular weight of 12265 D (Figure 2A). Database searches identified ROC1 as a highly evolutionarily conserved gene whose *S. cerevisiae* (ROC1-Sc), *S. pombe* (ROC1-Sp), and plant (ROC1-At) homologs share 67%, 88%, and remarkably 98% protein sequence identity with human ROC1, respectively, over the 82-amino acid region compared (Figure 2C). Database searches have also identified two additional genes, ROC2 in higher eukaryotes and APC11 in all eukaryotic species (Figures 2B and 2C), that are closely related to ROC1. ROC1 and ROC2 share an overall protein sequence identity of 51% with each other and 38% and 35% identity with APC11, respectively. Both ROC2 and APC11 are also highly evolutionarily conserved. Therefore, ROC1/ROC2/APC11 define a new family of proteins that are likely to carry out important cellular functions.

ROC/APC11 proteins contain two characteristic features: a RING finger and richness in tryptophan residues. The RING finger domain has been found in many eukaryotic proteins with diverse functions and is thought to mediate protein–protein interactions (Borden and Freemont, 1996). Three of the six highly conserved tryptophan residues in ROC1 are followed by an acidic amino acid residue (Asn, Glu, or Asp) that resembles the WD repeat indicating that they may be involved in mediating protein–protein interactions.

Selective Interaction between ROC2, APC11, and Cullin Family Proteins

We next determined whether ROC2 and APC11 also interact with cullins. Almost identical to ROC1, ROC2 interacted strongly with cullins 1, 2, 4A, and 5 (Figure

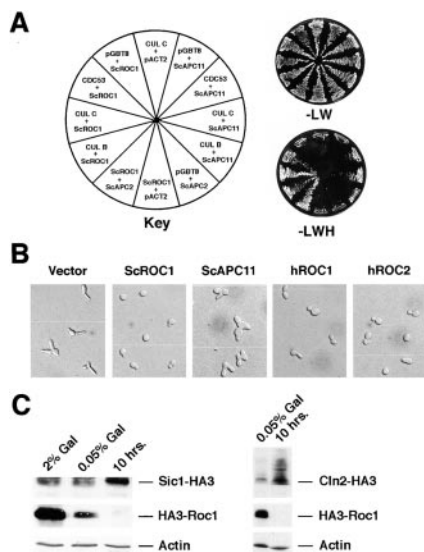


Figure 4. Function of ROC1 in Yeast

(A) ScRoc1p interacts with all yeast cullins. HF7c cells were cotransformed with plasmids expressing indicated proteins (Key). Protein-protein interactions were determined by the yeast two-hybrid assay as described in Figure 1(A).

(B) Human ROC1 and ROC2 can rescue the multibudded phenotype resulting from ScRoc1p depletion. His3MX6:P_{GAL}-HA3-ROC1 haploids were transformed with pADH-414 vector, pADH-ScROC1, pADH-ScAPC11, pADH-hROC1, or pADH-hROC2. Transformants were streaked onto selective plates containing 2% glucose and grown for 24 hr. Yeasts were formaldehyde fixed and sonicated before photography.

(C) Sic1p and Cln2p accumulate in yeast depleted of ScRoc1p. Haploid His3MX6:P_{GAL}-HA3-ROC1 SIC1-HA3:TRP1 and His3MX6:P_{GAL}-HA3-ROC1 CLN2-HA3:TRP1 yeast were grown in either 2% or 0.05% galactose plus 2% raffinose, or 2% glucose for 10 hr. Cell lysates were resolved on an SDS-PAGE gel, transferred to nitrocellulose, and blotted with anti-HA antibody to detect Sic1p-HA3, Cln2p-HA3, and HA3-Roc1p and with anti-actin antibody to verify equal protein loading.

ScROC1 was under the control of the galactose-inducible, glucose-repressible GAL1 promoter. Repression of ScROC1 by exposure to glucose caused the yeast to begin exhibiting a mutant phenotype at 9 hr and resulted in the accumulation of a multiply elongated budded yeast population with a single nucleus by 24 hr (Figure 4B and data not shown). This Roc1p depletion-induced phenotype is indistinguishable from those caused by temperature-sensitive mutations in the CDC53, CDC4, and CDC34 genes (Mathias et al., 1996). Taking advantage of this conditional phenotype, we determined the functional conservation and specificity of ROC family proteins. The multibudded phenotype incurred by Roc1p depletion can be completely rescued by the expression of yeast ROC1, but not vector control (Figure 4B), confirming that the level of Roc1p was the rate-limiting factor causing the multibudded phenotype. Ectopic expression of both human ROC1 and ROC2 also rescued the phenotype of ScRoc1p depletion. This indicates a functional conservation of the ROC gene family. Ectopic expression of yeast APC11, however, did not rescue the phenotype (Figure 4B) demonstrating a functional specificity between members of the ROC/APC11 family.

Roc1p Is Required for Sic1p and Cln2p Degradation
Phenotypic similarity between Roc1p depleted and cdc53 mutant cells and the interaction of ScROC1 with CDC53 prompted us to determine whether ScROC1 played a role in regulating protein degradation. Two critical substrates of the CDC53/SCF pathway are the G1 CDK inhibitor p40^{Sic1p} and the G1 cyclin Cln2p (Feldman et al., 1997; Skowyra et al., 1997). As such, we asked whether Sic1p and Cln2p were stabilized in yeast depleted of ScRoc1p as determined by Western blot analysis. We created two yeast strains in which either the SIC1 gene or the CLN2 gene was HA3 epitope tagged in a GAL-HA3-ScROC1 background. Yeast cells grown in a low concentration of galactose (0.05% plus 2% raffinose) expressed a reduced level of Roc1p but still exhibited a wild-type phenotype. After 10 hr of culturing in the presence of 2% glucose to deplete ScRoc1p, Sic1p and Cln2p accumulated (Figure 4C). Protein accumulation closely correlated with the appearance of multiply elongated buds. These results provide in vivo evidence that ROC1 functions in Ub-mediated proteolysis.

ROC1 and APC11 Complexes Contain Ub Ligase Activity

To test whether ROC1 immunocomplexes can function as Ub ligases, we immunoprecipitated ROC1 and CUL1 complexes from either 293T or HeLa cells and assayed for their ability to catalyze substrate independent Ub-Ub ligations (described in detail in Tan et al., 1999 [this issue of *Molecular Cell*]; Figure 5A). The ROC1 immunocomplex derived from both HeLa (lane 3) and 293T cells (lane 7) catalyzed the incorporation of ³²P-labeled Ub into a high molecular weight smear characteristic of an incremental Ub ladder in an E1- (lane 1) and E2- (lane 2) dependent manner. Inclusion of ROC1 antigen peptide in the ROC1 immunoprecipitation effectively blocked the ligase activity (lane 4), indicating that the polyubiquitination is catalyzed by the ROC1 immunocomplex. Similarly, the CUL1 complex also exhibited the Ub ligase activity (lane 6). Substitution of E2/CDC34 with E2/UbcH5c also supported ROC1-catalyzed Ub-Ub ligation (data not shown) and Ub-substrate ligation (see below), indicating that the ROC1 complex is capable of utilizing more than one E2 enzyme.

In contrast to ROC1, the anti-APC11 complex exhibited only background levels of ligase activity when similarly incubated with E1 and E2/CDC34 (Figure 5A, lane 5). A possible explanation is that ROC1 and APC11 selectively utilizes different E2s. To test this possibility, we substituted E2/CDC34 with UbcH5c, an isoform of E2/Ubc4 (97% identity), which was previously shown to be involved in ubiquitination of mitotic cyclin B by the APC (King et al., 1995). E2-UbcH5C supported Ub ligase activity of the α -APC11 immunocomplex (Figure 5B, lane 3). α -HA immunocomplexes derived from cells transfected with HA-APC11 and myc-APC2 (lane 6), but not with empty vector and myc-APC2 (lane 5), also exhibited high levels of Ub ligase activity. The immunocomplexes associated with the ligase activity predominantly contain HA-APC11 and myc-APC2 (compare lanes 7 and 8). These results demonstrate that ROC1/APC11 proteins play a role in the activity capable of linking together two Ub molecules by an isopeptide bond to form polyubiquitin chains.

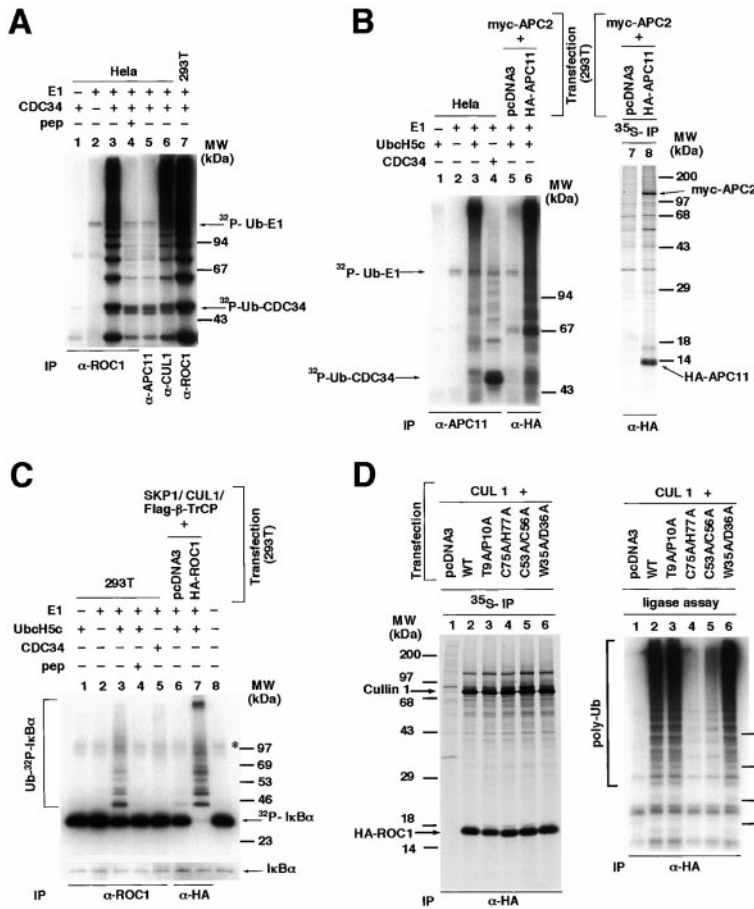


Figure 5. ROC1 and APC11 Constitute Ub Ligases with Cullin 1 and APC2

(A) Ub ligase activity of ROC1. Lysates (2 mg) from HeLa or 293T cells were immunoprecipitated with 2 μg of antibodies to either ROC1, APC11, or CUL1 as indicated. Immunocomplexes immobilized on the beads were washed and then mixed with purified E1, E2/CDC34 (unless otherwise indicated), ³²P-labeled Ub, and ATP. After incubation, the reactions were terminated by boiling the sample in Laemmli loading buffer and resolved by SDS-PAGE.

(B) Ub ligase activity of APC11. Lysates from untransfected HeLa or transfected 293T cells were immunoprecipitated with indicated antibodies. Ub ligase activity was assayed with either E2/CDC34 or E2/UbcH5C as described in (A). In vivo association of transfected APC11 and APC2 was confirmed by [³⁵S]IP (lanes 7 and 8).

(C) In vitro ubiquitination of IκBα by ROC1 ligase. Purified IκBα was phosphorylated with IKKβ and incubated with ROC1 immunocomplexes derived from untransfected or transfected 293T cells as indicated. Lane 8 represents a total input of the phosphorylated substrate IκBα used in each reaction. The faint band (denoted by an asterisk) may correspond to the autophosphorylated form of IKKβ. After autoradiography, the gel was stained with Coomassie blue to visualize IκBα (bottom panel).

(D) Protein complex formation and ligase activity of mutant ROC1. Two sets of 293T cells were cotransfected in parallel with a CUL1 expressing plasmid with either vector control or a plasmid expressing wild-type or mutant ROC1 as indicated. Thirty-six hours after

transfection, one set of cells was pulse labeled with [³⁵S]Met for 2 hr, lysed, and immunoprecipitated with HA antibody, and resolved by SDS-PAGE. The second set of transfected cells were lysed and immunoprecipitated with HA antibody under the same lysis and wash conditions and subjected to the Ub ligase activity assay.

Discovery of the Ub-Ub ligation catalyzed by α-Roc1 and α-APC11 immunocomplexes prompted us to determine whether they can also catalyze Ub-substrate ligations. We chose IκBα, an inhibitor of the NF-κB transcription factor, for this study, as it was recently reported that signal-induced phosphorylation of IκBα targets it for ubiquitination by cullin 1, SKP1, and the F box protein, β-TrCP (Yaron et al., 1998; Spencer et al., 1999; Winston et al., 1999). Incubation of the ROC1 complex immunoprecipitated from 293T cells with IKKβ-phosphorylated, ³²P-labeled IκBα resulted in an evident accumulation of a high molecular weight species (Figure 5C). Addition of a competing ROC1 antigen peptide reduced the IκBα ubiquitination to background levels (lane 4), confirming that IκBα ubiquitination is ROC1 dependent. Ubiquitination of IκBα by ROC1 is dependent on E1 (lane 1) and E2/UbcH5C (lane 2), and substitution of UbcH5C with CDC34 failed to catalyze IκBα ubiquitination (lane 5). Compared with ROC1 complexes derived from untransfected cells (lane 3), a much higher level of ligase activity toward IκBα was obtained with HA immunocomplexes derived from HA-ROC1/CUL1/SKP1/β-TrCP transfected cells (lane 7). Note that all of the phosphorylated IκBα was converted into a high molecular weight ubiquitinated form. Coomassie blue staining of the gel after

autoradiography showed that equal amounts of IκBα protein remained unubiquitinated after incubation with the ROC1 complex (Figure 5C, lane 7, bottom). This indicates that only the phosphorylated form of IκBα was ubiquitinated by the ROC1 complex.

ROC1 Is an Essential Subunit for Ligase Activity

Demonstration of ROC1-, CUL1-, and APC11-associated Ub ligase activities led us to seek direct evidence that ROC1 is an essential subunit for ligation. We mutated several amino acid residues that are highly conserved between members of the ROC family from different species (Figure 2C). HA-tagged wild-type or mutant ROC1 was cotransfected with CUL1 into two sets of 293T cells. While one set was employed for [³⁵S]IP analysis, the other set was analyzed for Ub ligase activity (Figure 5D). There is no detectable difference in the ROC1-CUL1 associated proteins between wild-type and the four mutant ROC1 complexes (Figure 5D, left panel). While double mutations of T9A/P10A (Figure 5D, right panel, lane 3) and W35A/D36A (lane 6) had no detectable effect on the ligase activity of ROC1, mutation of C53A/C56A dramatically reduced ROC1-associated ligase activity (lane 5), and mutation of C75A/H77A reduced the associated ligase activity to background levels (lane 4).

These results demonstrate that ROC1 is an essential subunit for the observed ligase activity.

Discussion

We have described an E3 ligase activity associated with a novel family of RING finger proteins, ROC/APC11, which complex with cullins to potentially constitute a large number of E3 Ub ligases. The detailed biochemical role the ROC/APC11 family plays in ubiquitination remains to be determined. Nonetheless, the presented experimental evidence supports the notion that ROC family proteins function as essential subunits of the ligase by forming heterodimers with cullins. ROC1 was shown to be an essential gene in yeast whose protein depletion caused an elongated, multibudded phenotype and concomitant accumulation of Sic1p and Cln2p (Figure 4). More convincingly, mutations in ROC1 completely abolished the associated ligase activity without a detectable alteration in the composition of ROC1-CUL1-associated proteins (Figure 5). Thus, strong evidence demonstrates that ROC1 is an essential subunit of the ligase. Though we have not definitively proven a cullin dependency for ROC1 associated ligase activity, it is likely that the cullin subunit is an obligate partner. We have demonstrated that ROC1 and ROC2 interact directly with cullins with a very high affinity, for both mammalian and yeast counterparts (Figures 1 and 4). Reinforcing this direct interaction is the *in vivo* association of a cullin-related protein, APC2, and a ROC protein, APC11 (Zachariae et al., 1998; Yu et al., 1998) (Figures 1 and 5B). Furthermore, in coupled transfection/ligase assays, CUL1 and ROC1 are the two predominant polypeptides in the ROC1 ligase complex. A critical issue that remains to be determined is whether the heterodimeric ROC/APC11-cullin complexes themselves contain intrinsic ubiquitin ligase activity or whether ROC/APC11 proteins act to bring the E2 and the cullins into close proximity for E2-mediated Ub-substrates ligations.

Discovery of ROC1 and APC11 as essential subunits for Ub ligation provides a clearer view of the ubiquitination pathway. In the case of the APC, identification of APC11 as a potential ligase subunit should help to determine how it is activated, inhibited, and targeted by other APC subunits. For the SCF complex, which consists of SKP1, CDC53/cullin 1, and an F box protein, it is now clear that SKP1 and the F box proteins are involved in substrate targeting. It should be pointed out that whether the SCF model can be generally applied to other cullins remains to be determined. Only cullin 1 interacts with SKP1 to be targeted by an F box protein to the substrate (Michel and Xiong, 1998), and whether higher eukaryotes contain additional SKP1-like cullin-interacting molecules is not clear. Conversely, cullin 1 and ROC1 are most likely required for ligase activity. Unlike SKP1, ROC1 and ROC2 interact commonly with all cullins that we have examined in both mammalian and yeast cells (Figures 1, 3, and 4). Hence, ROC1 and ROC2 are not just components of the SCF but essential subunits of all cullin-associated ubiquitination activities. Finally, both ROC/APC11 and cullin/APC2 represent multigene families. Among more than a dozen subunits identified, ROC/APC11 and cullin/APC2 are the only two

proteins common to both the APC and the SCF complexes. The variety of combinations that the ROC/APC11 family can form with different cullins point to a potentially large number of Ub ligases, and each may be involved in a specific cellular pathway as exemplified by the function of APC11-APC2 in mitosis, ROC1-CDC53 in yeast G1 control and ROC1-cullin 1 in NF- κ B/I κ B α -mediated transcriptional regulation.

Experimental Procedures

Plasmids Constructs

Mouse cullin 4A cDNA was described (Michel and Xiong, 1998). Human ROC2 and APC11 cDNAs were isolated by PCR amplification from a HeLa cDNA library and confirmed by DNA sequencing. The mouse APC2 EST cDNA clone (W13204) was used. ROC1 mutations were introduced by site-directed mutagenesis using Quick-Change kit (Stratagene) and verified by DNA sequencing.

Yeast cDNA sequences were amplified by PCR using lyticase-treated YEF473 genomic DNA and verified by DNA sequencing. CUL B, CUL C, and ScAPC2 were PCR amplified using the long template Expand kit (Boehringer Mannheim) following manufacturer's instructions. p414-ADH vector (CEN) was used for yeast rescue experiments. pcDNA3 (Invitrogen) was used for expression in mammalian cells. pGBT8, pGAD-GH, and pACT2 vectors were used for the yeast two-hybrid experiments.

Cell Culture

HeLa, Saos-2, and 293T were cultured in DMEM (10% FBS) in a 37°C incubator with 5% CO₂. Cell transfections were carried out using the LipofectAMINE reagent according to the manufacturer's instructions (GIBCO-BRL) or calcium-phosphate buffer (for 293T cells). For each transfection, 5 or 15 μ g of total plasmid DNA were used for each 60 mm or 100 mm dish.

Antibodies

Procedures for [³⁵S]methionine metabolic labeling, immunoprecipitation, and immunoblotting have been described previously (Jenkins and Xiong, 1995). The sequence of synthetic peptides used in generating rabbit polyclonal antibodies are as follows: anti-human ROC1N (CMAAMDVDTPSGTN, residues 1–14), anti-human ROC1C (CDNR EWEFQKYGH, residues 97–108), anti-human APC11 (CRQEWKFKE, residues 76–84), anti-human CUL2 (CRSQASADEYSYVA, residues 733–745). Antibodies to human CUL1 and SKP1 were previously described (Michel and Xiong, 1998). Monoclonal α -HA (12CA5, Boehringer-Mannheim) and α -myc (9E10, NeoMarker) antibodies were purchased commercially. Antibody to yeast actin was provided by Dr. J. Pringle. Coupled *in vitro* transcription and translation reactions were performed using the TNT kit following the manufacturer's instructions (Promega).

Yeast

All *S. cerevisiae* strains were derived from YEF473 (*a/α* ura3–52/ura3–52 his3 Δ -200/his3 Δ -200 trp1 Δ -63/trp1 Δ -63 leu2 Δ -1/leu2 Δ -1 lys2–801/lys2–801). Yeast were cultured per standard protocol (Guthrie and Fink, 1991). The procedure followed for lysing and immunoblotting has been described previously (Lamb et al., 1994). Yeast were fixed in 3.7% formaldehyde for 1 hr at 30°C, sonicated, and washed in 1 \times PBS.

Yeast strains were constructed using PCR-based gene deletion and modification by homologous recombination (Longtine et al., 1998). Primers for PCR products contained 40 bp of sequence homologous to the gene-specific sequence and 20 bp homologous to the vector template. PCR was performed using the Expand Long Template PCR System (Boehringer Mannheim) as described (Longtine et al., 1998). PCR products were transformed into diploid YEF473 yeast (to construct strains JM1 and JM5, see below) or into the haploid strain JM5 (to construct strain JM7 and JM8, see below)

using a standard protocol. To identify transformants that had integrated by homologous recombination, PCR was performed on genomic DNA. JM1: ROC1/roc1:kanMX6; JM5: ROC1/His3MX6:P_{GAL}-HA3-ROC1; JM7: MATA His3MX6:P_{GAL}-HA3-ROC1 SIC1-HA3:TRP1; JM8: MATA His3MX6:P_{GAL}-HA3-ROC1 CLN2-HA3:TRP1.

Ub Ligase Activity Assay

Human E1 was purified from HeLa cells as described (Hershko et al., 1983). Mouse E2/CDC34 was purified from insect cells infected with a mouse CDC34 expressing baculovirus, and human E2/UbcH5C were expressed in bacteria and purified using nickel beads (QIAGEN). Ub was prepared by subcloning full-length Ub as a fusion protein with a protein kinase C recognition site (LRRASV) and purified with nickel beads. Purified Ub was labeled with [³²P] by incubating with [³²P]ATP and cAMP kinase (Sigma) at 37°C for 30 min. For ubiquitination assays, immunocomplexes immobilized on protein A agarose beads were washed and added to an Ub ligation reaction mixture (30 μl) that contained 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 2 mM NaF, 10 mM Okadaic Acid, 2 mM ATP, 0.6 mM DTT, 0.75 μg [³²P]Ub, 60 ng E1, and 300 ng E2 protein. Reactions were incubated at 37°C for 60 min. For the IκBα ubiquitination assay, 8 μg of purified GST-IκBα (residues 1–54) were phosphorylated with 0.05 μg of IKKβ in the presence of 4 μCi of [³²P]ATP by incubating the reaction at 30°C for 30 min in a total volume of 40 μl of kinase buffer (20 mM HEPES [pH 7.7], 2 mM MgCl₂, 2 mM MnCl₂, 10 μM ATP, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na₂VO₄, and 1 mM DTT). Ubiquitination of ³²P-labeled GST-IκBα was performed as described above, except that 1 μg of ³²P-labeled GST-IκBα and 12 μg of unlabeled purified bovine Ub (Sigma) were used in place of [³²P]Ub.

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GenBank Accession Numbers

ROC1 (AF142059) and ROC2 (AF142060) sequences have been deposited into the GenBank database.