The Conserved RING-H2 Finger of ROC1 Is Required for Ubiquitin Ligation*

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ROC1 is a common component of a large family of ubiquitin E3 ligases that regulate cell cycle progression and signal transduction pathways. Here we present evidence suggesting that a conserved RING-H2 structure within ROC1 is critical for its ubiquitin ligation function. Mercury-containing thiol modification agents (β-hydroxymercurobenzoate and mercurochrome) irreversibly inhibit the ROC1-CUL1 ubiquitin ligase activity without disrupting the complex. Consistent with this, these reagents also eliminate the ability of the Skp1-CUL1-HOS-ROC1 E3 ligase complex to support the ubiquitination of IκBα. Site-directed mutagenesis analysis identifies RING-H2 finger residues Cys42, Cys45, Cys75, His77, His80, Cys83, Cys94, and Asp97 as being essential for the ROC1-dependent ubiquitin ligase activity. Furthermore, C42S/C45S and H80A mutations reduce the ability of ROC1 to interact with CUL1 in transfected cells and diminish the capacity of ROC1-CUL1 to form a stable complex with Cdc34 in vitro. However, C75S, H77A, C94S, and D97A substitutions have no detectable effect on ROC1 binding activities. Thus, the ROC1 RING-H2 finger may possess multiple biochemical properties that include stabilizing an interaction with CUL1 and recruiting Cdc34. A possible role of the RING finger in facilitating the Ub transfer reaction is discussed.

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ROC1 (also called Rbx1 and Hrl1) is a RING-H2 finger protein common to a large family of ubiquitin (Ub) E3 ligases that mediate the degradation of substrate proteins required for cell cycle progression, signal transduction, and the execution of tumor suppressor activities. It was initially isolated as a CUL4A-interacting protein by a yeast two-hybrid screen (1). ROC1 has also been biochemically purified as a common component of both the human (2) and yeast (3) SCF (Skp1-Cullin (Cdc53)-F-box protein) complexes as well as the native human von Hippel-Lindau (VHL)† tumor suppressor complex (4). In addition, the ROC1 homologue, ROC2 (also called SAG), was isolated as a redox agent-induced gene product that protects cells from apoptosis (5).

Yeast ROC1 encodes an essential gene whose reduced expression inactivates the Cdc34/SCF pathway that promotes the degradation of Sic1 and Clns required for the G1 to S transition (1, 3, 4, 6). It is a component of the SCF complexes that mediate the in vitro ubiquitination of Sic1 (by Cdc4; Refs. 3 and 4) and Clns (by Grr1; Ref 6). In vitro reconstitution experiments with human ROC1 (2) revealed that this RING-H2 finger protein is recruited by CUL1 to form the SCF^HOS/β-TRCP^ROC1 complex (with Skp1 and HOS/β-TRCP). The purified recombiant SCF^HOS/β-TRCP^ROC1 complex specifically binds IkKbphosphorylated IκBα and promotes its ubiquitination in the presence of Ub, E1, and Cdc34 as the E2 conjugating enzyme. These studies demonstrated that ROC1 is the fourth essential subunit of the SCF-ROC1 E3 protein complex that is both necessary and sufficient to activate the Cdc34-catalyzed substrate ubiquitination.

Further experiments involving the use of a sensitive 32P-Ub incorporation assay revealed an intriguing biochemical property of the human ROC1. In the absence of a substrate, the purified ROC1-CUL1 subcomplex promotes Ub polymerization in the presence of E1 and Cdc34 (2). In addition, missense mutations in ROC1 significantly reduced Ub ligase activity without affecting its interaction with CUL1 (1). Recently, the ROC1-binding site has been mapped to the C terminus of CUL1 (amino acids aa 324–776) and the ROC1-CUL1 (aa 324–776) complex is fully active in supporting Ub ligation (7). Consistent with these observations, Seel et al. (3) showed that the Cdc53-Hrl1 subcomplex is capable of activating the autoubiquitination of Cdc34. These studies substantiated the role of ROC1-CUL1 as a core Ub ligase. In keeping with this, the yeast Rbx1/Hrl1 (ROC1) was shown to directly interact with Cdc34 (3, 6).

It has been shown by co-transfection experiments that ROC1 binds four additional cullin family members (CUL2, CUL3, CUL4A, and CUL4B), whereas ROC2 preferentially interacts with CUL5 (1). ROC1 is found to be a component of the pVHL complex (4), whose additional subunits include pVHL, CUL2, and elongins C and B (8–10). Like the SCF-ROC1 complexes, the pVHL five-subunit complex supports substrate-independent Ub polymerization (11, 12). In support of these findings, it has been shown that the ROC1/CUL1 interaction is most likely mediated by a direct contact between ROC1 and a consensus region present in all cullin members (7). Furthermore, ROC1 shares extensive homology with APC11 (1, 3, 4), a subunit of the anaphase-promoting complex (13), which interacts with the cullin-related protein APC2 (1). Taken together, these observations suggest that the ROC/APC11 family members, through their combinatorial interactions with cullin/APC2, form a dimeric core component common to a large family of multisubunit Ub ligases. This core subcomplex functions to facilitate the
transfer of activated Ub from a cognate E2 to targeted substrates. RING finger structures have been postulated to mediate protein-protein interactions (14, 15). Here we present evidence from inhibition studies with mercury-containing agents and from site-directed mutagenesis experiments that demonstrate an essential role of the putative RING-H2 finger motif within ROC1 in Ub ligation.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids expressing HA-ROC1, CUL1, HOS/β-TRCP, and Skp1 were described previously (1, 2). Construction of the plasmid expressing both GST-ROC1 and CUL1 (aa 324–776) in bacteria will be described elsewhere. ROC1 point mutations in both the HA-ROC1 pcDNA or GST-ROC1 vector were generated by site-directed mutagenesis (Stratagene Quick-Change) and verified by DNA sequencing.

Enzymes—Human E1, mouse Cdc34 (mCdc34), and IkkK\textsubscript{G777S,M780R} were prepared as described previously (2).

Transfection, Metabolic Labeling, and Extract Preparation—Cells (293T) were grown on 20 ml/plate (150 × 25 mm) Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), 10% heat-inactivated FBS (Sigma), and 1% antibiotic-antimycotic (Life Technologies, Inc.). DNA(s) was transfected up to 40 μg/plate using the standard calcium phosphate precipitation method. For metabolic labeling, 40–48 h post-transfected cells were washed with 10 ml of phosphate-buffered saline and starved for 30 min with Dulbecco’s modified Eagle’s medium without L-methionine/L-cysteine (Life Technologies, Inc.; 6 ml/plate) and 100 μCi/ml Easy Tag Express-\textsuperscript{35}S Protein Labeling Mix from NEN Life Science Products. Labeling was allowed to occur for approximately 2 h.

Following harvesting of the transfected cells, pellets were reuspended in 0.4 ml of buffer A (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 0.5% Nonident P-40, 1 mM phenylmethylsulfonfluoride, 2 μg/ml antipain, 2 μg/ml leupeptin), and the resulting suspension was sonicated (7 repetitive 20-s treatments). Buffer B (20 mM Tris-HCl, pH 7.4, 1 mM NaCl, 0.2% Nonident P-40, 1 mM phenylmethylsulfonfluoride, 2 μg/ml antipain, and 2 μg/ml leupeptin) was then added (0.6 ml/plate). The mixture was rotated for 60 min at 4 °C, and supernatants following centrifugation (at 100,000 × g, 4 °C, 60 min) were saved.

Immunoprecipitation—Extracts, in amounts as indicated, were mixed with 10 μg of eHA (12CA5), and then protein A-agarose beads (10 μl; Upstate Biotechnology, Inc., Lake Placid, NY) were added. The mixture was rotated for 1 h at 4 °C. Beads were washed three times with 0.5 ml of buffer C (buffers A and B mixed in equal volumes) and then twice with 0.5 ml of buffer D (25 mM Tris- HCl, pH 7.5, 1 mM EDTA, 0.01% Nonident P-40, 10% glycerol, and 50 mM NaCl). Bound protein was released by boiling the beads for 3 min in the presence of 20 μl of SDS-loading buffer. Half of each reaction was used for SDS-PAGE followed by autoradiography.

Isolation of GST-ROC1-CUL1 (aa 324–776)—Bacteria expressing GST-ROC1-CUL1 (aa 324–776) complexes were induced at mid-log phase with 0.2 mM isopropyl-1-thio-galactopyranoside overnight at 25 °C. Following harvesting of cells, pellets were reuspended in 1/20 volume of buffer E (50 mM Tris-HCl, pH 8, 1% Triton X-100, 0.5 mM NaCl, 10 mM EDTA, 10% glycerol, 10 mM EGTA, 2 mM phenylmethylsulfonfluoride, 2 μg/ml antipain, 2 μg/ml leupeptin, and 5 mM DTT). The suspension was incubated on ice for 10 min, followed by sonication (seven to eight 20-s treatments). Supernatants following centrifugation (at 17,000 rpm, 4 °C, 30 min) were saved.

For GST pull-down, extracts, in amounts as indicated, were mixed with glutathione-Sepharose beads (15 μl; Amersham Pharmacia Biotech). The mixture was rotated for 1 h at 4 °C. Beads were washed three times with 0.5 ml of buffer E and then two times with 0.5 ml of buffer D. Bound protein was released by boiling the beads for 3 min in the presence of 20 μl of SDS-loading buffer. Half of each reaction was used for SDS-PAGE followed by Coomassie staining, as indicated.

Ub Ligation Assay—The immunoprecipitated recombinant ROC1-CUL1 or glutathione-Sepharose-bound GST-ROC1-CUL1 (aa 324–776) complexes, prepared as described above, were added to a Ub ligation reaction mixture (30 μl) that contained 50 mM Tris-HCl, pH 7.4, 5 mM MgCl\textsubscript{2}, 0.2 mM DTT, 2 mM ATP, \textsuperscript{32}P-Ub (200 pmol), E1 (0.2 pmol), and mCdc34 (7 pmol) in addition to the components as indicated. The reaction was incubated at 37 °C for 30 min and terminated with 7 μl of 4× Laemmli loading buffer (without DTT). Half of the mixture was then separated by 12.5% SDS-PAGE and analyzed by autoradiography.

Sulfhydryl Modifying Reagents—All reagents (Sigma) except N-ethylmaleimide (NEM) were prepared fresh. For the Ub ligation assay, immunoprecipitated HA-ROC1 and CUL1 were treated at 4 °C for 15 min in a mixture (90 μl) of buffer D containing each sulfhydryl modification reagent or DTT at the indicated final concentrations. Pretreatment of ρ-hydroxymercuribenzoate (PMB) or HgCl\textsubscript{2} with DTT was on ice for 5 min prior to the addition of HA-ROC1-CUL1. Post-treatment with DTT was at 4 °C for 10 min subsequent to PMB or HgCl\textsubscript{2} treatment. Mock-treated or sulfhydryl modification reagent-treated beads were washed twice with buffer D before incubation with the Ub ligation mixture described above.

Metabolically labeled extracts from 293T cells were used for examining HA-ROC1/CUL1 interaction in the presence of PMB and HgCl\textsubscript{2} or S\textsuperscript{C}P\textsuperscript{H565}-ROC1 interaction in the presence of HgCl\textsubscript{2}. Extracts were immunoprecipitated and treated with PMB or HgCl\textsubscript{2} as described above. Following treatment, the beads were washed with buffer D twice, and bound proteins were analyzed as described above.

For HA-HOS interaction with phosphorylated GST-LeBu (aa 1–54), S\textsuperscript{C}P\textsuperscript{H565}-ROC1 was anti-HA-immunoprecipitated. A \textsuperscript{32}P-GST-LeBu (aa 1–54) mixture (15 μl), as described below, was mixed with the beads that had been treated with or without HgCl\textsubscript{2}. The mixture was then rotated at 4 °C for 30 min. The resulting beads were then washed with buffer C twice and buffer D once followed by SDS-PAGE analysis.

In Vitro Ubiquitination of LeBu—The in vitro ubiquitination of LeBu was carried out as described previously (2) but with modifications. GST-LeBu (aa 1–54) (3.3 pmol) was phosphorylated by purified IkkK\textsubscript{G777S,M780R} (0.1 pmol) in a reaction mixture (30 μl) that contained 50 mM Tris-HCl, pH 7.4, 0.6 mM DTT, 5 mM MgCl\textsubscript{2}, 2 mM NaF, 10 mM okadaic acid, and 50 μM \textsuperscript{[γ\textsuperscript{32}P]}ATP. After incubation at 37 °C for 20 min, the mixture was added to anti-HA-antibody-protein A beads bound with CUL1, HA-HOS, Skp1, and HA-ROC1 derived from co-transfected extracts. ATP was also added at this stage to a final concentration of 4 mM. The second incubation was carried out by mixing at 4 °C for 15 min. Ub (300 pmol), E1 (0.6 pmol), and mCdc34 (60 pmol) were then added to the mixture, and the final incubation was at 37 °C for 60 min. The reaction was terminated by the addition of SDS-loading buffer (20 μl), and the mixture was boiled for 3 min. Aliquots of the reaction products (20 μl) were separated by 8.5% SDS-PAGE and analyzed by autoradiography.

Cdc34 Binding Assay—Glutathione-Sepharose-bound GST-ROC1-CUL1 (aa 324–776) complexes, prepared as described above, were incubated with purified mCdc34 (1 μg, 35 pmol) in buffer D (20 μl) for 60 min at 25 °C shaking. Beads were then washed twice with Buffer E and once with 1× phosphate-buffered saline. Bound protein was released by boiling the beads for 3 min in the presence of 20 μl of SDS-loading buffer. Half of each reaction was used for SDS-PAGE followed by Western blotting with a monoclonal α-Cdc34 antibody (Transduction Laboratories).

RESULTS

Inhibition of the ROC1-dependent Ub Ligase Activity by Mercury-containing Reagents—The role of sulfhydryl groups in the ROC1-CUL1 Ub ligase activity was examined through the use of sulfhydryl modification agents, including NEM, iodoacetamide (IAM), PMB, and HgCl\textsubscript{2}. For this purpose, a previously established \textsuperscript{32}P-Ub incorporation assay (2, 7) was used to measure the Ub ligase activity of the HA-ROC1-CUL1 complex, expressed and assembled in co-transfected 293T cells. As shown in Fig. 1A, HA-ROC1-CUL1 promoted the synthesis of \textsuperscript{32}P-labeled polyubiquitin chains (lanes 1 and 5), and this reaction required ATP, Cdc34, and E1 (lanes 2–4 and 6–8). The reaction products were visualized as a protein ladder (lanes 1 and 5) that reflected incremental additions of single \textsuperscript{32}P-Ub molecules (~12 kDa as the recombinant protein). To determine...
FIG. 1. Mercurial reagents irreversibly inhibit the ROC1-CUL1 Ub ligase activity. A, effects of sulfhydryl modification agents on the ROC1-CUL1 Ub ligase activity. The Ub ligation assay was carried out as described under “Experimental Procedures.” In the reactions shown in lanes 1–8, the HA-ROC1-CUL1 complexes (derived from 1 mg of transfected cell extract protein) bound to αHA antibody matrix were incubated with gel-filtrated 32P-Ub (1 μg) in the presence of ATP (lanes 1, 3–5, 7, and 8), E1 (lanes 1–3 and 5–7), and mCdc34 (lanes 1, 2, 4–6, and 8). Aliquots of the reaction were analyzed by both 12.5 and 7.5% SDS-PAGE to detect 32P-labeled monomeric Ub molecules as well as high molecular weight 32P-Ub conjugates. In the reactions shown in lanes 9–21, the HA-ROC1-CUL1 complexes (derived from approximately 0.3 mg of transfected cell extract protein) were treated without (lane 9) or with NEM (10 mM, lane 10), IAM (10 mM, lane 11), DTT (100 mM, lane 12), PMB (1 μM for lane 13; 10 μM for lanes 14–16), and HgCl2 (10 μM for lane 17; 100 μM for lane 18; 1 mM for lanes 19–21), as described under “Experimental Procedures.” In the reactions shown in lanes 15 and 20, excess DTT (100×) was mixed with PMB or HgCl2 prior to the addition of the HA-ROC1-CUL1 complexes. Following a brief incubation of the indicated compounds with ROC1-CUL1, excess DTT (100×) was added to the reactions shown in lanes 16 and 21. All treated beads were washed with buffer D prior to the addition of 32P-Ub (5 μg), E1, mCdc34, and other components as described under “Experimental Procedures.” Aliquots of the 32P-labeled reaction products were separated by 12.5% SDS-PAGE and analyzed by autoradiography. The positions of Ub monomers and dimers as well as Cdc34-S-Ub are indicated.

B, all sulfhydryl modification reagents inhibit Ub conjugation to Cdc34. Ub conjugation was carried out as described under “Experimental Procedures.” 32P-Ub was incubated with mCdc34 in the absence (lane 1) and presence (lanes 2–11) of E1. Various amounts of indicated compounds were used as follows: NEM (0.1 and 1 mM; lanes 3 and 4), IAM (1 and 10 mM; lanes 5 and 6), PMB (0.1 and 1 mM; lanes 7 and 8), and HgCl2 (0.1, 1, and 10 mM; lanes 9–11). Aliquots of the 32P-labeled reaction products were separated by 12.5% SDS-PAGE and analyzed by autoradiography. The positions of Ub monomers and dimers as well as Cdc34-S-Ub are indicated.

C, mercurial reagents do not disrupt the ROC1-CUL1 complex. Metabolically labeled extracts (0.3 mg of total protein) containing overexpressed HA-ROC1-CUL1 were immunopurified with anti-HA antibodies and incubated in the absence (lanes 1 and 3) or presence of PMB (10 μM, lane 2) and HgCl2 (1 mM, lane 4). Following washing, proteins that remained bound to the anti-HA-protein A matrix were released, separated by 12.5% SDS-PAGE, and analyzed by autoradiography.
the influence of sulfhydryl modification reagents in this reaction, NEM (lane 10), IAM (lane 11), PMB (lanes 13 and 14), and HgCl₂ (lanes 17–19) were mixed with the immobilized purified HA-ROC1-CUL1 prior to the addition of E1, Cdc34, and 32P-Ub. After a brief incubation, the unbound compound was removed by extensively washing the beads, and the ubiquitination reagents were then added to initiate Ub polymerization. The results indicate that while neither NEM nor IAM affected the ability of ROC1-CUL1 to catalyze Ub ligation (lanes 10 and 11), both PMB (lanes 13 and 14) and HgCl₂ (lanes 17–19) inhibited the Ub ligase activity in a concentration-dependent manner. Furthermore, the mercury-mediated inhibition was prevented by pretreating PMB (lane 15) or HgCl₂ (lane 20) with excess DTT, while the postaddition of excess DTT to the mercury-treated ROC1-CUL1 complex did not restore the Ub ligase activity (lanes 16 and 21). Taken together, these results demonstrate that the mercurial compounds irreversibly inhibit the ROC1-CUL1 Ub ligase. This effect is possibly achieved through the targeting of sulfhydryl groups that are inaccessible to NEM/IAM.

As shown in Fig. 1B, all four sulfhydryl modification agents used inhibited the E1-dependent conjugation of Ub to Cdc34. In addition, immunoprecipitation experiments indicated that neither PMB nor HgCl₂ affected the ability of ROC1 to interact with CUL1 (Fig. 1C). Thus, the mercurial compounds specifically inhibited the ability of ROC1-CUL1 to support Ub polymerization in the presence of Cdc34.

Mercurial Reagents Inhibit the SCFP²¹²⁻TRCP⁻ROC1-mediated Ubiquitination of Ikba—We have previously established that ROC1 is recruited by CUL1 to form the SCFP²¹²⁻TRCP⁻ROC1 E3 complex (with Skp1 and the HOS/β-TRCP F-box protein) that binds IKKβ-phosphorylated GST-IkBα (aa 1–54) and catalyzes its ubiquitination in the presence of Ub, E1, and Cdc34 (2, 7). To determine the effects of mercuric reagents on the ubiquitination of GST-IkBα (aa 1–54), the SCFP²¹²⁻TRCP⁻HA-ROC1 complex was assembled in co-transfected 293T cells and bound to anti-HA antibody-linked protein A beads. Following a brief incubation of the mercurial compounds with the E3 complex, the unbound compound was removed by extensively washing the beads, and then the IKKβ-phosphorylated 32P-labeled GST-IkBα (aa 1–54) and the ubiquitination agents (Ub, E1, and Cdc34) were added sequentially to initiate the reaction. In the presence of E1, Cdc34, E3, and Ub, multiple 32P-labeled high molecular weight ubiquitination products were formed (Fig. 2A, lanes 2 and 3). Omission of Cdc34 completely abolished ubiquitination (lane 4), demonstrating the dependence of
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Cdc34 in this reaction. In keeping with their inhibitory effects on the ROC1-CUL1 Ub ligase activity (Fig. 1A), both PMB (lane 5) and HgCl$_2$ (lane 6) blocked the SCF$^{HOS/\beta$-TRCP$}$-ROC1-catalyzed ubiquitination of GST-I$k\alpha$. As revealed by immunoprecipitation analysis, HgCl$_2$ had no effect on the assembly of SCF$^{HOS/\beta$-TRCP$}$-ROC1 (Fig. 2B) or its ability to interact with $^{32}$P-phosphorylated GST-I$k\alpha$ (aa 1–54) (Fig. 2C). We conclude from these experiments that mercurial reagents specifically inhibit the ROC1-CUL1 core Ub ligase that is required for the ubiquitination of I$k\alpha$.

Influence of Conserved RING-H2 Finger Mutations on Interactions between ROC1 and CUL1—Previous mutagenesis studies have demonstrated that none of the cysteine residues in Cdc34 (the CUL1 homologue in yeast) are essential for its biological function (16). Thus, it is likely that the observed mercury inhibition is mediated through its targeting on ROC1 cysteine residues. We employed site-directed mutagenesis to identify amino acid residues critical for the ROC1 interaction with CUL1 as well as for the ROC1-CUL1 Ub ligase activity. A panel of conserved ROC1 RING-H2 finger mutations was constructed, and the mutant proteins were expressed in both mammalian cells (Fig. 3A) and bacteria (Fig. 3B) and then analyzed for their ability to interact with CUL1. Cytomegalovirus-based vectors expressing wild type or mutant forms of HA-ROC1 were co-transfected with a CUL1 plasmid into 293T cells to allow for the assembly of HA-ROC1-CUL1 complexes. Following metabolic labeling, transfected cells were harvested, and the GST-ROC1-CUL1 (aa 324–776) complex, the same panel of conserved ROC1 RING-H2 finger mutations was introduced to GST-ROC1, since they reacted with anti-GST antibodies (data not shown). To examine the effects of the RING-H2 finger mutations on the formation of the GST-ROC1-CUL1 (aa 324–776) complex, the same panel of mutations as described above was introduced to GST-ROC1, and the mutant proteins were isolated from bacteria that simultaneously expressed CUL1 (aa 324–776). As shown, GST-ROC1 bearing substitutions at Cys$^{75}$ (lane 6), Cys$^{84}$ (lane 8), His$^{77}$ (lane 9), and Asp$^{97}$ (lane 12) maintained the full capacity of HA-ROC1 to interact with CUL1 as compared with the wild-type protein. Direct immunoblot analysis revealed similar levels of CUL1 protein present in extracts used for the above immunoprecipitation experiments, except for the H80A-containing lysates, where the level of CUL1 was 2-fold lower than the wild type.

We have recently mapped the ROC1 binding site to the C terminus of CUL1, which spans amino acid residues 324–776, and have demonstrated that ROC1 complexed with CUL1 (aa 324–776) is fully active in supporting Ub ligation (7). When GST-ROC1 was co-expressed with CUL1 (aa 324–776) in Escherichia coli, the two formed a complex that was isolated using a glutathione affinity purification step (Fig. 3B, lane 1). The ~33-kDa polypeptides co-purified were probably proteolyzed or prematurely terminated products of GST-ROC1, since they reacted with anti-GST antibodies (data not shown). To examine the effects of the RING-H2 finger mutations on the formation of the GST-ROC1-CUL1 (aa 324–776) complex, the same panel of mutations as described above was introduced to GST-ROC1, and the mutant proteins were isolated from bacteria that simultaneously expressed CUL1 (aa 324–776). As shown, GST-ROC1 bearing substitutions at Cys$^{75}$, His$^{77}$, Cys$^{84}$, and Asp$^{97}$ retained their full capacity to form complexes with CUL1 (aa 324–776) (Fig. 3B, lanes 3, 4, 7, and 8). In contrast to what was observed in transfected 293T cells, where C42S/C45S, H80A, and C83S mutations significantly reduced the ability of ROC1 to interact with CUL1 (Fig. 3A), these same mutations still support the formation of complexes between ROC1 and CUL1 (aa 324–776) (lanes 2, 5, and 6). However, the yield of the complex carrying H80A mutations was reproducibly found to be lower than that obtained with the wild type (lane 5). This
may reflect a reduced ability of this mutant to interact with CUL1 (aa 324–776) in bacteria. On the other hand, C42S/C45S and C83S behaved like the wild type in forming the ROC1-CUL1 (aa 324–776) complex (lanes 2 and 6). Taken together, these results demonstrate that none of the RING-H2 finger mutations examined eliminates the interaction between ROC1 and CUL1. However, C42S/C45S, H80A, and C83S may reduce the ability of ROC1 to interact with CUL1. The presence of high levels of these mutant proteins in bacteria may compensate for their low affinities in interacting with CUL1, thus promoting
The demonstration that each RING-H2 finger residue is essential for the ROC1 Ub ligation function. The observed effects on the ROC1-CUL1 Ub ligase activity by sulfhydryl modification agents are consistent with the proposed ROC1 RING-H2 finger structure. NEM and IAM do not affect the Ub ligase activity (Fig. 1A), which is, however, critically dependent on the ROC1 cysteine residues 42, 45, 75, 83, and 94 (Fig. 4). This is in keeping with the hypothesis that these sulfhydryl groups are not mediating a thiol-ester Ub linkage but are instead each bound to zinc ions and inaccessible to NEM/IAM. In support of this, the Ub ligase activity of the SCF and the Cdc53-Hrt1 (ROC1) subcomplex in yeast is insensitive to NEM treatment (3). In contrast to NEM/IAM, both PMB and HgCl2 effectively abolish the ROC1-CUL1 Ub ligase activity without disrupting the complex (Fig. 1). However, it

FIG. 6. A proposed “cross-brace” RING-H2 finger structure for the human ROC1. Residues postulated to coordinate two zinc binding sites are indicated.

C83S, C94S, and D97A did not affect the binding (lanes 5, 6, and 8–10), both C42S/C45S and H80A reduced the efficiency with which the GST-ROC1-CUL1 (aa 324–776) complex interacted with Cdc34 in vitro (lanes 4 and 7).

DISCUSSION

Through the use of extensive mutational analysis, we have identified the amino acids responsible for the ROC1 Ub polymerization function. Of the five conserved cysteine residues tested, Cys42, Cys45, Cys75, Cys83, and Cys94, all are essential for Ub ligation (Fig. 4). Previous studies suggest that the remaining three conserved cysteine residues, Cys53, Cys68, and Cys96, may not play a vital role in the ROC1-mediated ubiquitination reaction. A ROC1 mutant in which both cysteines at positions 53 and 56 are replaced by serines is still capable of interacting with Cdc53 and complementing the viability defect of a yeast ROC1 deletion strain (4). Furthermore, two amino acid substitutions in the yeast ROC1, Lys72 and Cys81 (corresponding to Cys88 in the human ROC1), to arginines rendered cells with a multibudded phenotype at a nonpermissive temperature (6), implying that under normal growth conditions, a Cys88 mutation does not significantly affect the ROC1 function. As revealed by this study, both conserved residues His77 and His80 are indispensable for mediating the ROC1-dependent Ub ligase function as well (Fig. 4). These results are consistent with the previous finding that the ROC1 mutant containing two alanine substitutions at positions Cys75 and His77 is deficient in Ub ligase activity (1). In addition, position 97, which contains a conserved aspartate in ROC1 but exists as a conserved cysteine in ROC2 and APC11 (1, 3, 4), is also required for Ub ligase activity. These results, taken together in conjunction with conservation of putative ligand spacing, suggest a “cross-brace” RING-H2 finger structure for the human ROC1 (Fig. 6). In this model, Cys42, Cys45, His80, and Cys83 form zinc-binding loop I, whereas Cys75, His77, Cys94, and Asp97 coordinate loop II to bind the second zinc ion. A precedent has been established that aspartate can substitute for cysteine to coordinate loop II to bind the second zinc ion. A precedent has been established that aspartate can substitute for cysteine to coordinate loop II to bind the second zinc ion.
remains to be determined with which residue(s) the mercuric ion interacts and whether this interaction induces a structural perturbation in ROC1-CUL1 that renders it inactive in supporting Ub ligation. It should be mentioned that in addition to its ability to interact with sulphydryl groups on cysteine residues, mercury is capable of binding to other amino acids as well, such as histidyl residues, thus possibly inhibiting enzymes through sulphydryl-independent reactions (17).

The absolute dependence on the intact ROC1 RING-H2 domain for Ub ligation indicates that the RING finger mediates critical biochemical processes. Is the ROC1 RING finger essential for the formation of the ROC1-CUL1 complex? In this study, we showed that the replacement of Cys42/Cys45, Cys83, or His80 residues with serine or alanine, respectively, reduced the ability of ROC1 to bind to CUL1 in co-transfected 293T cells (Fig. 3A), suggesting that alteration of the putative loop I (Fig. 6) may compromise the ability of ROC1 to interact with CUL1. Consistent with this, a ROC1 double mutant (F79A/H80A) showed reduced binding with CUL1 (18). In addition, Conaway’s group has shown that the Cys42/Cys45 substitution rendered Rbh1 inactive in complementing the viability defect of a yeast ROC1 deletion strain as well as in binding to the endogenous Cdc53 (4). However, ROC1 mutants harboring C42S/C45S, C83S, or H80A substitutions were still capable of forming a complex with the C terminus of CUL1 in E. coli (Fig. 3B). In keeping with this, a recent report from the Conaway group showed that the Cys42/Cys45 mutation still supported a Rbh1-Cdc53 complex assembled in baculovirus-infected insect cells (19). This discrepancy regarding the effects of the ROC1 RING-H2 mutations in supporting the ROC1-CUL1 complex formation may be reconciled by the significant difference in the level of protein expression in the different cell systems used. It is possible that the presence of high levels of ROC1 mutant proteins in either bacteria (Fig. 3B) or baculovirus-infected insect cells (19) may compensate for their deficiency in interacting with CUL1. These results have led us to suggest that the intact ROC1 RING-H2 finger may help to promote, but is not essential for, the formation of the ROC1-CUL1 complex.

The role of the ROC1 RING-H2 finger in recognizing an E2 has been previously postulated by the observations that the yeast ROC1 directly interacted with Cdc34 (3, 6). Consistent with this, we have shown in this study that the human ROC1-CUL1 (aa 324–776) was bound to Cdc34 in a specific and direct manner (Fig. 5). As shown in a GST pull-down assay, both the C42S/C45S and H80A mutations significantly reduced the ability of ROC1 to bind to Cdc34 (Fig. 5). However, mutations at Cys75, His77, Cys83, Cys94, or Asp97 did not affect the binding of ROC1-CUL1 to Cdc34 (Fig. 5). These results raise a question as to whether the integrity of the ROC1 RING-H2 finger is required for the interaction between ROC1-CUL1 and Cdc34. Mutagenesis studies have been carried out with other RING finger E3 ligases including Ubr1, AO7, and c-Cbl. Xie and Varshavsky (20) have extensively analyzed the interaction between Ubc2p and Ubr1p, an E3 ligase required for the N-end rule pathway, using a variety of methods, including yeast two-hybrid, GST pull-down, and immunoprecipitation approaches. They showed that the first cysteine of the RING-H2 is required for ubiquitination but is dispensable for Ubc2p binding. In contrast, Weissman’s group has shown that five individual substitutions of the AO7 RING finger residues eliminated both Ub ligation and UbcH5 binding activities (using a GST pull-down assay) (21). In agreement with this, Joazeiro et al. (22) have shown that replacement of a c-Bl RING finger cysteine with alanine rendered the ligase inactive to support Ub ligation and unable to bind to GST-Ubc4. However, Weissman noted that in some instances, there is no correlation between a RING finger E3 ligase’s ubiquitination activity and its capacity to bind an E2 (21). Taken together, these results support a general notion that at least some conserved residues in the RING-H2 motif are critical for the binding of a Ub ligase to an E2. However, the entire RING-H2 finger may not be required for the formation of stable E2-E3 complexes. It should be noted that future studies under equilibrium conditions are required to examine the E2-E3 interaction. Additionally, the role of the RING-H2 finger may be better evaluated using a thiol ester-linked E2 as a binding substrate.

The ROC1 RING finger may play a critical role in the Ub transfer reaction. Following its recruitment of Cdc34, the ROC1 RING-H2 finger may help to position the conjugate formed between the Cdc34 active site residue (Cys93) and Ub toward the Lys receptor residue of another Ub molecule or a given substrate that is anchored to the F-box protein, resulting in the covalent transfer of Ub. It has been recently shown that the Rbx1 mutants C42S/C45S, C75S, H82A/C83A, and C94S do not support the Rbx1-dependent Rub1 modification of Cdc35 (19), suggesting that Rbx1(ROC1) may utilize the same mechanism in promoting Ub polymerization and Rub1(Nedd8) modification.

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