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Development of an efficient *E. coli* expression and purification system for a catalytically active, human Cullin3-RINGBox1 protein complex and elucidation of its quaternary structure with Keap1

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Abstract

The Cullin3-based E3 ubiquitin ligase complex is thought to play an important role in the cellular response to oxidative stress and xenobiotic assault. While limited biochemical studies of the ligase's role in these complex signaling pathways are beginning to emerge, structural studies are lagging far behind due to the inability to acquire sufficient quantities of full-length, highly pure and active Cullin3. Here we describe the design and construction of an optimized expression and purification system for the full-length, human Cullin3-RINGBox 1 (Rbx1) protein complex from *E. coli*. The dual-expression system is comprised of codon-optimized Cullin3 and Rbx1 genes co-expressed from a single pET-Duet-1 plasmid. Rapid purification of the Cullin3-Rbx1 complex is achieved in two steps via an affinity column followed by size-exclusion chromatography. Approximately 15 mg of highly pure and active Cullin3-Rbx1 protein from 1 liter of *E. coli* culture can be achieved. Analysis of the quaternary structure of the Cullin3-Rbx1 and Cullin3-Rbx1-Keap1 complexes by size exclusion chromatography and analytical ultracentrifugation indicates a 1:1 stoichiometry for the Cullin3-Rbx1 complex (MW = 111 kDa), and a 1:1:2 stoichiometry for the Cullin3-Rbx1-Keap1 complex (MW = 280 kDa). This latter complex has a novel quaternary structural organization for cullin E3 ligases and it is fully active based on an *in vitro* Cullin3-Rbx1-Keap1-Nrf2 ubiquitination activity assay that was developed and optimized in this study.

Keywords

Cullin3; Keap1; Nrf2; Rbx1; codon-optimized; quaternary structure; stoichiometry

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Introduction

The E3 ubiquitin ligases are multi-subunit complexes that catalyze the third and final step in the conjugation of ubiquitin to target substrate proteins [1; 2]. During the addition of ubiquitin to a target protein, the cullin subunit serves as a scaffold that orients the adaptor-bound target protein for efficient ubiquitin transfer from the ubiquitin-charged E2 [3]. Seven different human cullin proteins form a subset of E3 ligases that each has its own unique set of substrate adaptors. This variety of substrate adaptors enables cullins to catalyze the ubiquitination of a wide range of proteins that are involved in numerous cellular processes including cell cycle progression, signal transduction, and transcriptional regulation [4; 5]. Ubiquitination can have various effects on target proteins such as moderating subcellular localization, changing protein stability and protein-protein interactions, or targeting a protein for proteasomal degradation [6].

Cullin3 (Cul3), and one of its substrate adaptors, Kelch-like ECH-associated protein 1 (Keap1), together play an important role in the signaling pathway that regulates the transcription of genes involved in combating oxidative stress and promoting cell survival. The Cullin3-Keap1 mediated signaling pathway controls the cellular levels of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which is a key protein involved in regulating the expression of numerous cytoprotective genes. Nrf2 is a critical determinant of a cell's ability to survive exposure to oxidative stress, toxic heavy metals, metabolic transformation of xenobiotics, and endogenous oxidized metabolites [7]. Under basal conditions, Nrf2 is constitutively expressed but maintained at low levels via ubiquitination by the Cullin3 E3 ligase complex and subsequent degradation by the proteasome [8; 9]. Under conditions of oxidative stress or xenobiotic assault, Nrf2 ubiquitination decreases and Nrf2 accumulates in the nucleus. Once in the nucleus, Nrf2 forms heterodimeric, transcription factor complexes with small Maf proteins, and these complexes bind to cis-acting DNA promoter sequences called antioxidant response elements (ARE) [10]. Binding to the ARE initiates transcription of a battery of antioxidant and detoxification genes that protect the cell against damage. Upregulation of these genes is a promising therapeutic strategy for the prevention of numerous diseases including cancer.

The core of the Cullin3 E3 complex is composed of the Cul3 protein bound to the small, 12 kDa protein RINGBox 1 (Rbx1) and the dimeric protein Keap1 [1; 4]. So far, the X-ray structures of Cul3, Keap1, or the complexes they form remain unsolved. However, the X-ray structure of Rbx1 has been determined in a complex with the protein Cullin1 (Cul1) [3]. The X-ray structure of Cul1-Rbx1 shows an extended rod-shaped protein complex with Rbx1 integrally bound within the C-terminal domain of Cul1. By analogy, the Cul3-Rbx1 structure is likely to adopt a similar rod-shaped tertiary structure to that of the Cul1-Rbx1 structure since Cul3 shares high sequence homology with Cul1. However, since Cul3 recruits entirely different protein substrates for ubiquitination than Cul1, this is likely where the analogy ends in terms of the formation of higher-order protein complexes. In addition, the stoichiometry of each protein, e.g. Cul3-Rbx1-Keap1-Nrf2, within the functional Cul3-based complex is unknown, yet it is the quaternary structure and the associated conformational changes within the complex that are believed to be an important feature of the ubiquitination mechanism of all cullin-based ligases [11].

In an effort to accelerate the structural and biochemical characterization of Cul3-based ligase complexes, we sought to develop a new, efficient expression and purification system for Cul3-Rbx1 that would enable the production of large quantities of soluble, full-length protein. In general, cullin proteins have been difficult to express in *E. coli* due to solubility issues. In the past, production of cullin proteins has been achieved via expression in insect cells or by a “Split-N-Co-express” approach utilizing *E. coli* as an expression host [12]. In the latter method, two separate fragments of Cul1 are expressed, which are then co-folded to produce a functional version of Cul1 but with a peptide-chain break [12]. Since neither method is currently able to easily produce large quantities of full-length Cullin protein, we sought to develop a more efficient expression and purification system that would be applicable to all cullin proteins. We therefore developed an *E. coli* expression and purification system for large-scale production of the full-length Cul3 protein in complex with Rbx1. Approximately 15 mg of highly pure and active Cullin3-Rbx1 protein from 1 liter of *E. coli* culture can be obtained for structure-function analyses.

Materials and Methods

Generation of Expression construct for Cul3/Rbx1 Co-expression

Full-length sequences of both proteins (Cul3: NP_003581, Rbx1: NP_055063) were codon-optimized for expression in *E. coli*, synthesized (BioBasic, Inc.), and cloned into a pUC57 vector. The two genes were then subcloned into the Novagen pET Duet-1 vector with BamHI and HindIII restriction sites for the Cul3 gene, and NdeI and XhoI restriction sites for Rbx1. The restriction sites within the resulting expression vector, pET-hCul3-Rbx1, position Rbx1 such that it expresses with an N-terminal His₆-tag for affinity purification.

Protein Expression and Purification

The expression plasmid pET-hCul3-Rbx1 was transformed into *E. coli* BL21 (DE3) cells via electroporation for protein over-expression. Transformed bacteria were plated onto Luria-Bertani (LB)-Amp (100 µg/mL) plates and grown overnight. Single colonies from plates were used to inoculate 10 mL LB-Amp (100 µg/mL) starter cultures that were grown for 8 hours at 37 °C. One milliliter of starter culture was then used to inoculate 1 L of LB-Amp (100 µg/mL) cultures that were grown at 25 °C until the optical density at 600 nm (OD_{600nm}) of the culture reached 0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 200 µM, and the cells were grown at 18 °C for an additional 8 hours.

The cells were harvested by centrifugation at 5,000 rpm (4,225 × g) for 15 minutes (Sorvall SLC-4000) and re-suspended to 0.33 g/mL of cell pellet in lysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, EDTA-free Protease Inhibitor Cocktail (Roche), 0.2 mg/mL lysozyme, and 0.002 mg/mL DNase I). The cellular suspension was homogenized manually and then lysed by sonication at 65% power for 0.6 seconds every 1.5 seconds for a total of 22.5 seconds. The cell debris was then centrifuged at 18,000 rpm (40,760 × g) for 45 min (Sorvall SA-600). The clarified cell lysate was loaded onto a 5 mL HisTrap affinity column (GE Healthcare), charged with Ni²⁺, and equilibrated with 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM imidazole, and 2 mM dithiothreitol (DTT). His₆-Rbx1 in complex with

Cul3 was eluted by a linear gradient of 0% – 50% imidazole in elution buffer (50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 0.5 M imidazole). The fractions containing both His₆-Rbx1 and Cul3, as judged by SDS-PAGE, were pooled and concentrated from 5 mg/mL to 30 mg/mL in a final volume of 5 mL. The entire 5 mL of protein was then injected onto a HiLoad 26/60 Superdex 200 prep grade size-exclusion column (GE Healthcare). The fractions (5 mL) containing pure His₆-Rbx1-Cul3 complex (Cul3/Rbx1 from here on) were pooled, concentrated to 10 mg/mL, and buffer-exchanged into storage buffer (50 mM Tris–HCl, pH 8.0, 0.25 M NaCl, 20% glycerol) using Amicon Ultra (Millipore) concentrator devices with a molecular weight cut-off of 30 kDa. The protein, in 500 μ L aliquots in 1.7 mL Eppendorf tubes, was flash-frozen in ethanol and dry ice for long-term storage at –80 °C.

Analytical Size-Exclusion Chromatography and Binding of Cul3/Rbx1 to Keap1

Purified Cul3-Rbx1 was subjected to analytical size-exclusion chromatography (SEC) in the absence and presence of Keap1 to assess its ability to form a stable complex and to determine the stoichiometry of the components of the Cul3/Rbx1:Keap1 complex. The experiment was performed at 4°C, using a 300 $\times$ 7.8 mm Bio-Silect® SEC 250-5 column (Bio-Rad), equilibrated with 50 mM Tris pH 8.0, 250 mM NaCl, and 2 mM DTT. Several molecular weight standards were used for calibration, including ovalbumin, conalbumin, aldolase, catalase, ferritin, thyroglobulin, and blue dextran. Cul3/Rbx1 and Keap1 were first run individually (50 μ L of 8 μ M Cul3/Rbx1 and 50 μ L of 16 μ M Keap1). The proteins were then mixed and incubated on ice for 30 minutes prior to injecting 50 μ L of 8 μ M complex on the column. The resulting fractions (500 μ L volumes) were visualized by SDS-PAGE. The retention volumes for each of the standards and samples were measured and used to calculate the partition coefficients, K_{av} :

$$K_{av} = (V_r - V_o) / (V_c - V_o)$$

where V_r is the retention volume, V_o is the void volume (calculated based on the retention time of the blue dextran standard), and V_c is the geometric bead volume for the column. The K_{av} for each standard was plotted against the log of the molecular weight in order to generate a standard curve, which was then used to calculate the approximate molecular weight for each experimental sample.

Analytical ultracentrifugation

The stoichiometry of binding of Cul3/Rbx1 to Keap1 was assessed by analytical ultracentrifugation. Purified Cul3/Rbx1 and Keap1 were first incubated on ice at three different molar ratios (2:1, 1:1, and 1:2) for 30 minutes. The samples were then subjected to sedimentation velocity experiments performed at a temperature of 20 °C and a rotor speed of 60,000 rpm using a Beckman Optima XL-A analytical ultracentrifuge equipped with 1.2 cm boundary-forming epon centerpieces in an An60TI rotor. After temperature and pressure reached the set points, loaded samples were allowed to reach thermal equilibrium before data collection began. Intensity data were collected at 280 nm as a function of radial position. Each centrifuge cell was scanned sequentially with 3-minute delay between scans until no further sedimentation was observed. The program Ultrascan 2.0 used for analysis of

the primary data as described by Demeler [13]. Solution density and viscosity were calculated from buffer composition as 1.00994 g/mL and 1.0363 cP. A value of 0.732442 mL/g was assumed for the partial specific volume. Intensity data was converted to pseudo-absorbance, which was used in a two-dimensional spectrum analysis (2DSA) with meniscus, and time invariant noise fitting was performed. The model with the lowest RMSD for each cell was chosen and its corresponding noise file was used for subtraction. Additional 2DSA analysis with radius invariant noise fitting was performed until no further decrease in RMSD was observed. Enhanced van Holde-Weischet analysis was performed on noise-subtracted data to determine sedimentation coefficients and distribution within each sample. Sedimentation coefficients plotted against the relative frequency distributions were exported to Excel (Microsoft) for graphing.

In vitro ubiquitination assay

In order to assess Cul3/Rbx1 activity, an *in vitro* ubiquitination assay was developed. Each ubiquitination reaction contained 65 nM human ubiquitin-activating enzyme 1 (UBE1), 5 μ M ubiquitin-conjugating enzyme H5 (UbcH5), 100 nM Cul3/Rbx1, 100 nM human Keap1, 100 nM human Nrf2, 46 μ M chicken egg white albumin, 75 mM Tris pH 8.0, 15 mM $MgCl_2$, and 7.5 mM ATP in 5.5 μ L, and was initiated with the addition of 1 μ L of ubiquitin to a final concentration of 169 μ M. The reactions were incubated at room temperature for 5, 10, 20, or 30 minutes and then quenched by the addition of 3.5 μ L of stop buffer such that a final concentration of 1X NuPAGE LDS sample buffer (Invitrogen), 1X NuPAGE reducing agent, and 3.5 mM EDTA was achieved. Reaction products were resolved by 4-12% Bis-Tris PAGE gels and transferred to PVDF membranes. The membranes were blocked with 5% dry milk in TBS-T (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 8) prior to incubation with rabbit anti-Nrf2 (Santa Cruz). Detection of bound antibodies was carried out by enhanced chemiluminescence using bovine anti-rabbit antibodies conjugated to horseradish peroxidase (Santa Cruz).

Results and Discussion

Design of Cul3/Rbx1 co-expression construct

The field of synthetic biology is ushering in a new generation of strategies for the design and synthesis of novel expression systems for proteins that are difficult to produce. Since synthesis of codon-optimized genes and construction of expression vectors is now mainstream and cost-effective, we decided to take advantage of this technology for the difficult-to-produce cullin proteins. BioBasic, Inc synthesized the full-length, codon-optimized genes of Cul3 and Rbx1, for expression in *E. coli*. Both genes were then inserted into the pET Duet-1 system for co-expression (Figure 1a). The Rbx1 protein, but not the Cul3 protein, was designed to have an intact hexahistidine tag for affinity purification. This strategy was more desirable than having both proteins tagged since the purified material would be used for structural characterization and crystallization trials. Highly mobile affinity tags decrease the likelihood of obtaining diffraction quality crystals. In addition, a thrombin cut site was engineered between the His₆-affinity tag and Rbx1. This allows the His₆ tag to be easily removed for crystallization. A stop codon was added to the Cul3 sequence so that

the native S-tag located within the pET Duet-1 vector would not translate with the Cul3 protein.

Protein over-expression and purification

Expression studies for Cul3/Rbx1 were performed to maximize co-expression and to obtain significant yields of purified protein. These studies consisted of varying incubation temperatures (18°C, 25°C, and 37°C), IPTG concentrations (200 µM to 1 mM), length of induction time (2, 4, 18 and 24 hours), and the use of different bacterial cell strains including BL21 (DE3) and Rosetta (DE3). The conditions leading to the highest expression were 25°C incubation temperature, 200 µM IPTG, and 18 hour induction time at 18°C.

The optimized expression conditions were adapted for large-scale expression and purification of the Cul3/Rbx1 complex. Cul3/Rbx1 was found to elute from the HisTrap and size-exclusion column as a single species, indicating that the Cul3 and Rbx1 proteins form a stable complex. The Cul3/Rbx1 complex, visible on a Coomassie-stained SDS-PAGE gel as two bands (88 kDa Cul3 and 12 kDa His₆-Rbx1), was highly purified after two chromatographic steps (Figure 1b). The Cul3/Rbx1 protein complex was found to be more stable at high NaCl concentrations; low salt concentrations led to precipitation of the protein. Therefore, the concentration of NaCl was maintained at 250 mM over the course of purification. The final optimized purification strategy yields on average about 15 mg of pure protein per liter of bacterial cell culture.

Size exclusion chromatography

The quaternary structure of the Cul3/Rbx1 complex was determined via analytical size-exclusion chromatography. Based on the retention times of the Cul3/Rbx1 protein when compared to the protein standards, the experimentally derived molecular weight for Cul3/Rbx1 is approximately 111 kDa, which corresponds to a heterodimeric complex of the proteins at a 1:1 ratio (Figure 2). The molecular weight is slightly greater than the *calculated* molecular weight of 100 kDa, most likely due to the predicted rod-like shape of Cul3/Rbx1 as observed in the Cul-Rbx1 crystal structure [3]. This experiment also shows the relatively tight interaction of Cul3 and Rbx1, as no free Cul3 or Rbx1 was detected.

The molecular weight of Keap1, which has recently been shown to be a homodimer via cryoelectron microscopy [14], was determined to be 198 kDa by analytical SEC (Figure 2). As observed for the Cul3/Rbx1 complex, the molecular weight of Keap1 is somewhat greater than the *calculated* molecular weight of 140 kDa. The cryo-EM structure of Keap1 indicates that Keap1 has an extended dimeric structure suggesting that the larger molecular weight derived from SEC may be the result of a larger hydrodynamic radius and stokes radii due to non-spherical, molecular geometry. Because Keap1 appears to exist as an obligate homodimer, anytime Keap1 is referred to in the text, it is used to describe the Keap1 dimer (K1₂).

Since the quaternary structure of the Cul3/Rbx1:Keap1 complex is also unknown we determined the approximate molecular size of this complex by analytical SEC. When the Cul3/Rbx1:Keap1 complex was formed at 1:1:2 ratios, the complex eluted from the SEC column as a single species with no other peak observed in the chromatogram (Figure 2).

Analysis of the retention time of this peak with protein standards indicates the Cul3/Rbx1:Keap1 complex has an *estimated* molecular weight of 280 kDa, which corresponds to a *calculated* molecular weight of 240 kDa. These results suggest that Cul3/Rbx1:Keap1 is a heterotetrameric complex consisting of one molecule each of Cul3 and Rbx1, and two molecules, i.e. two monomers or one dimer, of Keap1. From here on, this complex will be denoted as Cul3/Rbx1:Keap1₂. This quaternary structure represents a novel organization for cullin ligases since they usually form complexes having an overall dimeric structure with two cullin protomers present [11; 15; 16].

Analytical Ultracentrifugation

Sedimentation velocity experiments were performed to confirm the binding stoichiometry observed in the analytical size exclusion experiments, because the complex does not readily enter a native gel during electrophoresis. Sedimentation equilibrium experiments could not be performed, because Keap1 is not stable at room temperature over long periods of time. Sedimentation velocity experiments can readily show complex formation from subunits in a time frame that allows Keap1 to remain in solution. As shown from the data in Figure 3, a singular peak of Cul3/Rbx1:Keap1₂ was observed at 8.74S when Cul3/Rbx1 and Keap1 were mixed in a 1:2 molar ratio. When additional equivalents of Cul3/Rbx1 are added in a 1:1 or a 2:1 ratio with Keap1, another peak at 4.98S, representing excess, i.e. free, Cul3/Rbx1, is present. When excess Cul3/Rbx1 is present in the 1:1 and 2:1 ratio samples the complex peak shifts to 9.28S and 9.76S, respectively. This peak-shifting effect is most likely due to a rapid interconversion of the species during the time-course of sedimentation [17]. Together with the size exclusion experiments, these results conclusively show a 1:2 binding stoichiometry of one heterodimer of Cul3/Rbx1 bound to one dimer of Keap1.

In vitro ubiquitination assay

The Cul3/Rbx1 E3 ligase catalyzes Nrf2 ubiquitination in concert with other proteins from the ubiquitin cascade. We developed a discontinuous *in vitro* ubiquitination assay to ensure that co-expressed and purified Cul3/Rbx1 functions to bind and orient the Nrf2-bound substrate adaptor properly for ubiquitin transfer from UbcH5. This is the first reported assay to reconstitute Nrf2 ubiquitination entirely from purified proteins.

The transcription factor Nrf2 was found to be readily ubiquitinated by the recombinant Cul3/Rbx1:Keap1₂ complex in the presence of the other required ubiquitin-ligase components *in vitro* (Figure 4). The reaction proceeds rapidly and with higher-order Nrf2-ubiquitin conjugates forming and accumulating over time. The Nrf2-ubiquitin conjugates are visible as a short ladder of bands above Nrf2 (Figure 4, Lanes 2 to 4). Each band above Nrf2 represents an additional ubiquitin being added to Nrf2 through the catalytic efforts of the E3-ligase complex. The ladder of Nrf2-ubiquitin intensifies and higher molecular weight forms accumulate, the unmodified Nrf2 band decreases, corresponding to a decrease of free Nrf2 in the reaction.

Conclusions

An *E. coli* co-expression system has been developed for the purification of large quantities of full-length Cul3 and Rbx1 proteins as a tightly associating 1:1 complex. The resulting Cul3/Rbx1 protein binds a single dimer of Keap1 forming a complex unique to cullins, i.e., only one Cul3 molecule in complex with a dimeric substrate adaptor. The recombinant Cul3/Rbx1 protein is active in the *in vitro* ubiquitination assay indicating that functional studies can be performed with relative ease. The production of 15 mg of highly purified Cul3/Rbx1 protein per liter of cell culture should allow an increased number of structural and biophysical studies on this interesting and important system.

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- > A novel expression strategy was used to purify Cul3-Rbx1 from *E. coli*.
- >The Cul3-Rbx1 complex is fully active and catalyzes ubiquitination of Nrf2 *in vitro*.
- >Cul3, Rbx1, and Keap1 form a complex with unique stoichiometry of 1:1:2.

FIGURE 1.

A. Cloning site map showing the locations of synthesized Rbx1 and Cul3 genes within the pETDuet-1 co-expression vector **B.** SDS-PAGE analysis of the purification of Cul3-Rbx1. Lane 1 contains a standard protein marker. Lane 2 shows the insoluble protein content from the whole cell lysate. Lane 3 contains soluble proteins. Lane 4 shows the proteins contained in the HisTrap flow-through. Lane 5 is the pooled HisTrap elution fractions that contain Cul3/Rbx1. Lane 6 contains the final purified Cul3/Rbx1 after elution from the S200 SEC column.

FIGURE 2.

A. Analytical SEC chromatograph of Cul3/Rbx1, Keap1, and the *Cul3/Rbx1:Keap1₂* complex (*C3/R1: K1₂*) **B.** Table showing *calculated* molecular weight (*from amino acid sequence) versus *estimated* molecular weight (**experimentally-derived) for experimental proteins

FIGURE 3.

Sedimentation coefficient distribution plotted versus relative frequency for three molar ratios of Cul3/Rbx1 and Keap1.

FIGURE 4.

Anti-Nrf2 western blot showing *in vitro* ubiquitination of Nrf2. Addition of Cul3 catalyzes the ubiquitination reaction and Nrf2-ubiquitin conjugate formation increases in a time-dependent manner.