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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 *Escherichia 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 L 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.

1. 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

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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 *Escherichia 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. Therefore, we 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 L of *E. coli* culture can be obtained for structure–function analyses.

2. Materials and methods

2.1. 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 His_6-tag for affinity purification.

2.2. Protein expression and purification

The expression plasmid pET-hCul3–Rbx1 was transformed into *E. coli* BL21 (DE3) cells via electroporation for protein overexpression. 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 h 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 h.

The cells were harvested by centrifugation at 5000 rpm (4225*g*) for 15 min (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 s every 1.5 s for a total of 22.5 min. 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 Ni^{2+}, and equilibrated with 50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 10 mM imidazole, and 2 mM dithiothreitol (DTT). His_6–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 His_6–Rbx1 and Cul3, as judged by SDS–PAGE, were pooled and concentrated from 5 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 His_6–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 μL aliquots in 1.7 mL Eppendorf tubes, was flash-frozen in ethanol and dry ice for long-term storage at −80 °C.

2.3. 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 × 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 μL of 8 μM Cul3/Rbx1 and 50 μL of 16 μM Keap1). The proteins were then mixed and incubated on ice for 30 min prior to injecting 50 μL of 8 μM complex on the column. The resulting fractions (500 μL 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.

2.4. 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, all 4 μM Keap1 for 30 min. 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