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Cul3 Ubiquitin Ligase and Ctb73 Protein Interactions

By

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Introduction

Ubiquitination

Proper regulation of proteins in the cell is important for cellular processes such as cell cycle and division, DNA transcription and repair, and differentiation and development.

Figure 1. Ubiquitin, isopeptide linkage and chain conformation variations (Traub et. al, 2007).

A. Seven lysines available for conjugation of Ubiquitin.

B. Isopeptide bond formation of K48 and K63.

Ubiquitination is a fundamental biochemical process, which controls numerous aspects of protein function, such as degradation, protein-protein interaction and subcellular localization (Sadowski et.

al, 2010). Ubiquitin (Ub) is a highly conserved 76-amino-acid-polypeptide small regulatory protein containing 7 lysines found only in eukaryotic cells (Figure 1A). Ubiquitin attachment to certain lysine residues generates diverse substrate-ubiquitin structures, targeting proteins to different cellular

compartments and affecting cell physiology (Hicke, 1999). Poly-ubiquitin chains are built by formation of an isopeptide bond between Glycine 76 of one ubiquitin to the epsilon amide group of one of the seven potential lysines (K6, K11, K27, K29, K33, K48 or K63) of the preceding ubiquitin (Figure 1B). The ability to generate diverse substrate-ubiquitin structures is important for targeting proteins to different fates. For example, monoubiquitination can regulate DNA repair and gene expression. Polyubiquitination through Ub K48 generally targets proteins for

proteasomal degradation, while K63-linked Ub chains can regulate kinase activation, DNA damage tolerance, signal transduction and endocytosis (Sadowski, et. al, 2010).

The ubiquitination process occurs in three steps (Figure 2): ubiquitin activation via an E1 activating enzyme, transfer to E2 conjugating enzyme, and covalent attachment via E2 and the target substrate. It has been shown that E3 ligases function as scaffold proteins, specifically binding substrate proteins and specific E2s; that ubiquitin is then transferred directly from E2s to substrates (Scheffner et. al, 1999). It has also been shown that the chemical properties of the active site of each E3 ligase function in a very specific way, targeting specific lysine residues and catalyzing a specific ubiquitination pathway and specific substrates. It is the specificity in the E2/E3 combinations that result in the formation of linkage specific ubiquitin chains.

Figure 2. Ubiquitin Pathway (Hellman et. al, 2002).

E3 Ligase Families

There are two main classes of E3 ligases that differ based on their E2 binding domains and mechanism of action: RING (Really Interesting New Gene) E3 ligases act as scaffolds for E2 enzymes, facilitating the direct transfer of ubiquitin on to the substrate; HECT (Homologous to

E6-AP C-terminus) E3 ligases covalently attach to ubiquitin before it is transferred to the substrate. The RING E3 ligases are further sub-divided into the monomeric or simple RING type and the multimeric type, which includes the Cullin family (Wilkie et. al, 2012). Most Eukaryotic genomes encode at least six cullins (Cul1, Cul2, Cul3, Cul4A, Cul4B, and Cul5)

Figure 3. A schematic representation of cullin family ubiquitin ligases and substrate adaptors. Cul1 binds F-box proteins (A) while Cul3 binds BTB proteins (D). (Bosu et al., 2008).

and are crucial for a variety of biological processes (Chen et al., 2009). Cullins share a homologous C terminus that binds the RING-finger protein Rbx1, but are heterogenous when

it comes to the N-terminal substrate recruiting domain. The best understood of the Cullin superfamily is Cul 1 and its involvement in the Cul1-Skp1-F-box protein complex. Skp1 serves as an adaptor that simultaneously binds sequences near the N-terminus Cul1 and the F-box motif of an F-box protein which, in turn, recruits substrates through additional protein-interaction domains and facilitating ubiquitination via the C-terminus of Cul1 (Figure 3). Other Cullins such

as Cul2 and Cul5 protein complexes are known to share a similar organization, varying in the protein specificity at the N-terminus (Zhuang et al., 2009).

Cul3 and BTB Proteins

Cul3 is just beginning to be understood and has been found to be involved in a variety of critical cellular processes such as regulation of the cell cycle. The Cul3 ubiquitin ligase is known to target proteins for poly-ubiquitination that play a critical role in the cell cycle (Cummings et al., 2008). A protein domain that has been highly conserved and found in many proteins that function as substrate adaptors for Cul3 is the BTB (Brick-Brack, Tram-Trak, and Broad Complex) domain (Romero et al., 2013). Like all Cullins, Cul3 binds to an Rbx1 protein on its C terminus but unlike other Cullins, Cul 3 binds the BTB domain of the BTB containing protein on its N terminus (Figure 3). BTB is a protein interaction/dimerization domain that is structurally homologous to the cullin- binding region of Skp1 and that binds Cul3 via motifs analogous to those in the Skp1-Cul1 complex (Zhuang et al., 2009). Altogether, this forms the BTB-Cul3-Rbx1 (BCR) complex. There have been more than 200 BTB proteins characterized in humans (Singer et al.,) and these proteins harbor additional protein-protein interaction modules signifying the importance of BTB domain containing proteins (Chen et al., 2009). BTB protein family can be subdivided into BTB-zinc finger (BTB-ZF), BTB-BACK-kelch (BBK), MATH-BTB, BTB-NPH3, Kelch family (KLHL), and Kelch repeat and BTB domain- containing proteins (KBTBD) subfamilies of proteins, among others; it is through the multiplicity in protein interacting domains that contributes to their ability to act as substrate adaptors for Cul3 mediated ubiquitination (Anderica-Romero et al., 2013).

Ctb73

A novel BTB domain containing protein that has been shown to interact with Cul3 is Ctb73, or BTBD. Ctb73 has a BACK and Kelch domain (Figure 4), belonging it to the BBK class of BTB domain containing proteins. The BACK (**B**TB and **C**-terminal **K**elch) domain was identified as a conserved sequence motif in proteins containing both BTB and Kelch motifs; based on its predicted secondary structure and proximity to the BTB domain, the BACK domain is hypothesized to be involved in substrate orientation for ubiquitin conjugation within BTB-Cul3 ubiquitin ligase complexes. It is possible that the BACK domain functions to position the kelch-motif beta propeller and its bound substrate in the Cul3 E3 complexes. BBK proteins are found predominantly in vertebrates and there are 53 found in humans (Stogios et. al., 2004).

Figure 4. Architecture of BBK proteins (Stogios et al., 2004).

Some members of the BTB-Kelch domain subfamily are known to interact directly with actin or to regulate the actin cytoskeleton, thereby affecting cell-cell interactions, cell-substrate

interactions, and cell migration (Gray et al., 2009). Since Cul3 is the most recently identified and least understood of the Cullin family ubiquitin ligases and are known to interact with an array of BTB domain containing proteins, it is important to study the interactions between Cul3 and BTB proteins; specifically how those proteins function as adaptors for substrate ubiquitination. BTB proteins work as Cul3 substrate adaptors; since there are approximately 200 BTB proteins

encoded in the human genome, this implies the vast array of possible substrates for Cul3 ubiquitination (Cummings et al., 2008). This ubiquitination process is highly orchestrated; every protein involved in the complex is critical for the regulation of specific substrates. In order to understand the importance of Cul3, we must be able to understand all of their protein interactions.

A novel BTB domain containing protein has been found that contains a BACK and kelch domain, Ctb73 or BTBD. It is not known whether or not Ctb73 works as a substrate adaptor for Cul3 or how it binds to Cul3. The purpose of this experiment is to determine if Ctb73 binds to Cul3 through any of the protein-interacting domains. In order to do this, HEK 293 cells will be co-transfected with 3X FLAG-tagged Cul3 and one of the following Myc- tagged Ctb protein plasmids: Ctb73 wildtype (WT), Ctb73 Δ BTB with its BTB domain mutated, Ctb73 Δ BACK with its BACK domain mutated, and Ctb73 Δ BTB-BACK with its BTB and BACK domains mutated. The cells expressing these proteins will be harvested and lysed 48 hours later. Expression levels will be determined using immunoblotting for Myc and FLAG antibodies. A FLAG antibody immunoprecipitation assay and a Myc antibody Western blot will be performed in order to detect binding of the Ctb 73 proteins and Cul3. Since it has been shown that the BTB domain is the domain in a BTB protein that interacts with Cul3, both of the Ctb73 proteins with a mutated BTB domain are not expected to bind to Cul3. However, it is expected that Ctb73 wildtype and Ctb73 with mutated BACK domain only will bind to Cul3. This will be shown by the absence of banding patterns in the lanes correlating to Cul3 plus Ctb 73 Δ BTB and Cu3 plus Ctb73 Δ BTB-BACK in the FLAG immunoprecipitation assay and Myc Western blot.

Methods

Plasmid Preparation and Purification

3X-FLAG tagged Cul3 transformed ampicillin resistant *E. coli* were grown in lysogeny broth (LB) media overnight. Bacterial cells were harvested and lysed, followed by plasmid DNA purification.

Transfection of HEK 293 Cells

Cells from the human embryonic kidney 293 cell line (HEK 293) were allowed to grow to confluency on p90 plates then split for transfection on p60 plates. The cells were co-transfected by calcium phosphate precipitation with purified plasmids containing a 3X-FLAG tagged Cul3 and either one of the Myc-tagged Ctb73 plasmids: Ctb73 WT, Ctb73 Δ BTB, Ctb73 Δ BACK, or Ctb73 Δ BTB-BACK, incubated for 48 hours before harvest and cell lysis.

Immunoprecipitation and Immunoblotting

An immunoprecipitation assay was used to precipitate or “pull down” FLAG tagged Cul3 and whatever protein is bound to Cul3 for each cell extract. To see if there was a Ctb73 protein bound to Cul3, Western blotting by immobilizing the proteins on a membrane and probing them by complexing them with primary antibodies followed by horseradish peroxidase (HRP) conjugated secondary antibodies. The Western blot was developed using an enhanced chemiluminescence reaction (ECL).

Results

Figure 5. FLAG Immunoprecipitation assay and Myc Western blotting was performed to detect protein binding.

Expression levels of Cul3 and Ctb73 plasmids were detected using western blot analysis using mouse Flag and mouse Myc antibodies, respectively. An immunoprecipitation assay using mouse FLAG antibodies precipitated out Cul3 proteins then binding of Ctb73 proteins to Cul3 was analyzed via probing of Western blot using rabbit Myc antibodies. It was shown that all Ctb 73 proteins bound to Cul3. Expression levels of Cul3 were much lower, as shown in figure 5, than that of all the Ctb73 plasmids; due to this, the results from the mouse FLAG immunoprecipitation assay and rabbit Myc Western blot are unreliable.

Discussion

Ubiquitination is a fundamental biochemical process, which controls numerous aspects of protein function, such as degradation, protein-protein interaction and subcellular localization (Sadowski et. al, 2010). This occurs through a series of steps and involves an ubiquitin-activating enzyme, E1 forms a thioester bond with ubiquitin, a highly conserved 76-amino acid protein. This reaction allows subsequent binding of ubiquitin to a ubiquitin conjugating enzyme, E2, followed by the formation of an isopeptide bond between the carboxy-terminus of ubiquitin and a lysine residue on the substrate protein by a ubiquitin ligase, E3.

The focus of this experiment was to determine protein interactions between an E3 ubiquitin ligase, Cul3 and a BTB protein, Ctb73. BTB proteins have been found to work as substrate adaptors of Cul3, interacting with the substrate and Cul3; facilitating the ubiquitination process. There have been more than 200 BTB proteins found, implying the significance of these proteins in normal cell functioning. Ctb73 is a novel BTB protein that contains a BACK and Kelch domain. BTB domain containing proteins are known to bind to Cul3 via the BTB domain; whether binding occurs through other domains is not yet clear. In order to determine whether or not Ctb73 binds to Cul3 through the BACK domain, located in the middle of the BTB and Kelch domains, HEK 293 cells were transfected on p60 plates with 3X-FLAG Cul3 and one of the Myc-tagged Ctb 73 plasmids: WT, ΔBTB, ΔBACK, and ΔBTB-BACK. Expression levels of Cul3 and Ctb73 proteins were determined by probing Western blots with mouse FLAG antibody and mouse Myc antibody, respectively. Mouse FLAG antibody immunoprecipitation assay was used to bring down Cul3 of cell lysates and a Western blot was probed using rabbit

Myc antibody to detect Ctb73 binding to Cul3 (Figure 5). It was expected that, since BTB domain of BTB domain containing proteins has been shown to bind Cul3, there would not be binding between Cul3 and both the Ctb73 BTB domain mutants, Ctb73 Δ BTB and Ctb73 Δ BTB-BACK. However, it was expected that the Ctb73 WT and Ctb73 Δ BACK proteins would bind to Cul3. The results of this experiment show that all Ctb73 proteins bind to Cul3. It is believed that since Cul3 expression levels were much lower than that of Ctb73 expression levels, this result is due to high levels of background and therefore, not conclusive data.

For future experiments, adjusting levels of protein expression could obtain more reliable results by either changing the amount of DNA transfected or the amount of cell lysate used for immunoprecipitation assay. Even levels of expression are important for accurate binding detection. Once protein interactions of Cul3 and Ctb73 have been determined, the next step would be to determine binding specificity between Ctb73 and Cul3 specific substrates. HEK 293 cells could be transfected with differently tagged plasmids then immunoprecipitation assays and Western blots would be used to detect binding in the same way as the current experiment. Another step towards the understanding of Cul3 and Ctb73 protein interactions would be cellular localization studies to determine where in the cell Cul3 and Ctb73 interact. This would further our knowledge of possible implications and functions of Cul3 and Ctb73 and their importance for canonical cellular functioning.

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