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Steady-state kinetic studies reveal that the anti-cancer target Ubiquitin-Specific Protease 17 (USP17) is a highly efficient deubiquitinating enzyme

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ABSTRACT

USP17 is a deubiquitinating enzyme that is upregulated in numerous cancers and therefore a drug target. We developed a robust expression, purification, and assay system for USP17 enabling its enzymatic and structural characterization. USP17 was expressed in *E. coli* as inclusion bodies and then solubilized, refolded, and purified using affinity and size-exclusion chromatography. Milligram quantities of pure USP17 can be produced that is catalytically more efficient (k_{cat}/K_m = 1500 (x10^3) $M^{-1}sec^{-1}$) than other human USPs studied to date. Analytical size-exclusion chromatography, analytical ultracentrifugation, and dynamic light scattering studies suggest that the quaternary structure of USP17 is a monomer. Steady-state kinetic studies show that USP17 efficiently hydrolyzes both ubiquitin-AMC (k_{cat} = 1.5 sec^{-1} and K_m = 1.0 μM) and ubiquitin-rhodamine110 (k_{cat} = 1.8 sec^{-1} and K_m = 2.0 μM) substrates. Ubiquitin chain cleavage assays reveal that USP17 efficiently cleaves di-ubiquitin chains with Lys^{11}, Lys^{33}, Lys^{48} and Lys^{63} linkages and tetra-ubiquitin chains with Lys^{11}, Lys^{48} and Lys^{63} linkages but is inefficient in cleaving di-ubiquitin chains with Lys^{6}, Lys^{27}, or Lys^{29} linkages or linear ubiquitin chains. The substrate specificity of USP17 is most similar to that of USP1, where both USPs display higher specificity than other characterized members of the USP family.

1. Introduction

Deubiquitinating enzymes (DUBs) reverse the process of ubiquitination by hydrolyzing ubiquitin from the protein substrate to which it is conjugated [1,2]. Thus far, approximately 100 DUBs have been identified in the human genome and they are involved in regulating a number of cellular processes and disease states [1,2]. DUBs are classified into six families, of which the largest family is the Ubiquitin Specific Protease (USP) family with roughly 60 members [1,2]. The USPs are multi-domain enzymes that can range in size from ~40 kDa, in the case of USP12, up to ~400 kDa, in the case of USP34 [1]. Each USP contains a catalytic domain, which shares the highly conserved papain-like fold composed of a catalytic triad of cysteine, histidine, and aspartate residues [1,2]. Other domains within the USPs are often important for protein-protein interactions and/or substrate recognition. A detailed review of the domains and their functions has been described elsewhere [2,3].

USP17 was originally identified as a member of the murine hematopoietic specific genes as DUB3 [4]. Expression of USP17 within the cell is cytokine-inducible and is required for cell-cycle progression through the G_1-S and G_2-M checkpoints. A well-characterized substrate of USP17 is the cell division cycle 25A (CDC25A) phosphatase. In a normal cell, CDC25A is responsible for dephosphorylation of Cdk1 and activation of Cdk1-CyclinB complex for the progression of the cell-cycle (Fig. 1A) [5]. Cellular levels of CDC25A are regulated by the ubiquitin-proteasome system through

Abbreviations: DUB, deubiquitinating enzyme; USP, Ubiquitin Specific Protease; Ub, ubiquitin; CDC25A, cell division cycle 25A; RCE1, Ras converting enzyme 1; IPTG, isopropyl β-D-1-thiogalactopyranoside; βME, β-mercaptoethanol; AMC, 7-amino-4-methylcoumarin; Rho110, rhodamine110; AFU, arbitrary fluorescence units; DTT, dithiothreitol; BSA, bovine serum albumin; SEC, size-exclusion chromatography; AUC, analytical ultracentrifugation; DLS, dynamic light scattering; *Sf9*, *Spodoptera frugiperda 9*; TCEP, Tris(2-carboxyethyl)phosphine.

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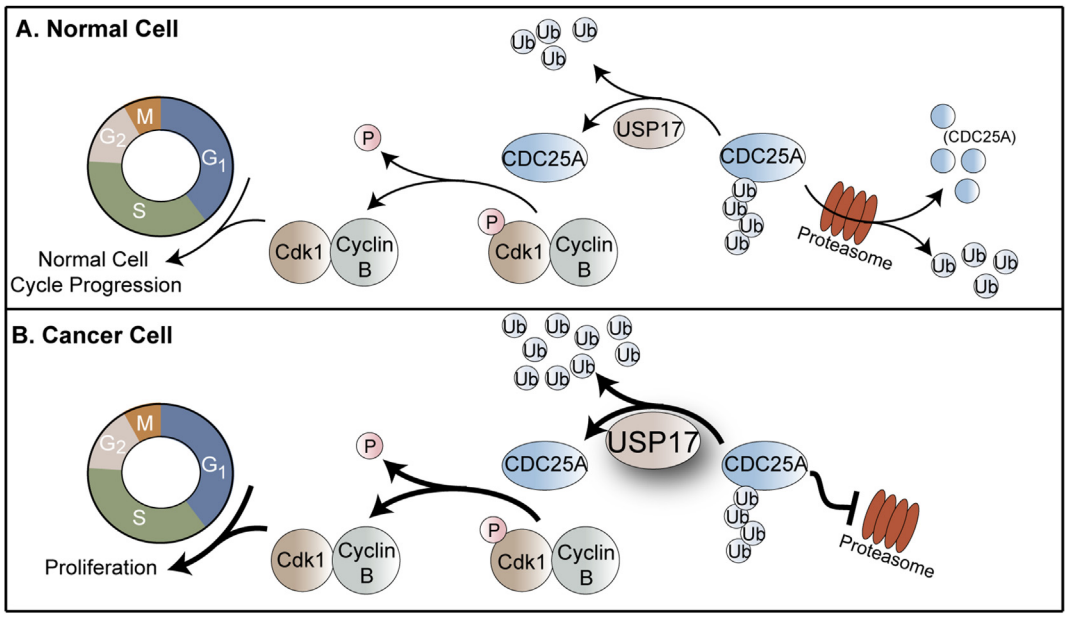

Fig. 1. Role of USP17 in cell-cycle regulation and cancer. CDC25A is a cell-cycle phosphatase that promotes cell-cycle progression by dephosphorylating Cdk1. CDC25A cellular levels are regulated through ubiquitination that signals for CDC25A degradation. **(A)** In a normal cell, USP17 deubiquitinates and stabilizes CDC25A to promote cell-cycle progression [5]. **(B)** In cancer cells, USP17 levels are upregulated which results in increased deubiquitination and stabilization of CDC25A. The increased levels of CDC25A therefore result in cancer cell proliferation [5].

Lys^{48} ubiquitin chains. During the G_1-S and G_2-M checkpoints, USP17 is expressed to deubiquitinate and stabilize CDC25A in order to promote cell-cycle progression through these checkpoints [5]. In numerous cancer phenotypes, expression of USP17 is upregulated, resulting in its expression outside of these checkpoints which causes an excess of stabilized CDC25A that applies stress to the cell-cycle resulting in unregulated cancer cell proliferation (Fig. 1B) [5–7]. Other identified substrates of USP17 include the Ras converting enzyme 1 (RCE1) and the histone deacetylase dependent Sin3A co-repressor complex component, SDS3, both of which also play a role in cell-cycle regulation [8–10].

USP17 is a 58 kDa protein that has its catalytic domain located near the N-terminus as well as two hyaluronan binding motifs predicted to reside within the C-terminal region (Fig. 2A) [4,11]. A sequence alignment of the catalytic regions of USP17 against three well-studied USPs is shown in Fig. 2C [12]. As with other USPs, there are three highly conserved residues (Cys89, His334 and Asp350) that form the catalytic triad. Experimental binding studies by Ramakrishna and coworkers have shown the two predicted hyaluronan binding motifs bind to hyaluronan, a polysaccharide that is responsible for numerous cellular processes, including the regulation of cell division [11]. Many USPs are predicted to contain hyaluronan binding motifs, including the well-studied USP7, however the interactions between the USPs and hyaluronon itself is poorly understood [13]. In the case of USP17, the hyaluronan binding motifs have been shown to be important for the interaction of USP17 with its substrate, SDS3 [14].

McFarlane and coworkers have shown that the persistent overexpression of USP17 applies stress to the cell-cycle which results in proliferation of both breast and prostate cancers [4,6]. Combining the results of this study with the role of USP17 in CDC25A-driven cellular proliferation elucidated by Pereg and coworkers, strongly suggests that inhibitors of USP17 may serve as anti-cancer drugs. While previous work has been able to define the role of USP17 *in celluo*, without the ability to express and purify USP17, further characterization of USP17, as well as the identification of USP17 inhibitors, is severely limited. We describe here a reliable and robust method for the expression and purification of recombinant USP17 which enables the production of highly pure and active USP17 that is amenable to enzymatic and structural characterization, as well as inhibitor identification.

2. Materials and methods

2.1. Design of expression constructs

The human gene of *usp17* (GenBank: Q0WX57.2) was codon optimized for *E. coli* expression, synthesized, and inserted into the pET11a expression vector by BioBasic Inc. The coding region for USP17 was subcloned into the pEV-L8 expression vector by methods described by Báez-Santos et al. [15].

For baculovirus expression, BioBasic Inc. codon optimized *usp17* (GenBank: Q0WX57.2) for *Spodoptera frugiperda 9* (*Sf9*) expression, synthesized the gene, and inserted the gene into a modified pVL-1932 vector which includes a dual N-terminal 10His and a Green Fluorescent Protein (GFP) tag.

2.2. Site-directed mutagenesis

The catalytic mutant USP17-Cys^{89}Ser pEV-L8 vector was prepared following the QuikChange site-directed mutagenesis protocol (Stratagene) and transformed into *E. coli* BL21 (DE3) cells for protein expression.

2.3. Expression, solubilization, refolding and purification of USP17

Five liters of *E. coli* BL21 (DE3) cells harboring the USP17 pEV-L8 expression vector were grown from five separate one liter cultures of LB medium, each supplemented with 50 μg/mL Carbenicillin in 2 L Fernbach flasks. Shaking was performed at 37 °C and at 200 rpm in an Infors Multitron shaker until an A_{600} of 0.6 is reached. Cultures were then cooled for 30 min at 4 °C and USP17 expression was induced with a final concentration of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures were incubated at 25 °C