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C-terminal region of USP7/HAUSP is critical for deubiquitination activity and contains a second mdm2/p53 binding site

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ABSTRACT

USP7, also known as the hepes simplex virus associated ubiquitin-specific protease (HAUSP), deubiquitinates both mdm2 and p53, and plays an important role in regulating the level and activity of p53. Here, we report that deletion of the TRAF-like domain at the N-terminus of USP7, previously reported to contain the mdm2/p53 binding site, has no effect on USP7 mediated deubiquitination of Ub_n-mdm2 and Ub_n-p53. Amino acids 208–1102 were identified to be the minimal length of USP7 that retains proteolytic activity, similar to full length enzyme, towards not only a truncated model substrate Ub-AFC, but also Ub_n-mdm2, Ub_n-p53. In contrast, the catalytic domain of USP7 (amino acids 208–560) has 50–700 fold less proteolytic activity towards different substrates. Moreover, inhibition of the catalytic domain of USP7 by Ubal is also different from the full length or TRAF-like domain deleted proteins. Using glutathione pull-down methods, we demonstrate that the C-terminal domain of USP7 contains additional binding sites, a.a. 801–1050 and a.a. 880–1050 for mdm2 and p53, respectively. The additional USP7 binding site on mdm2 is mapped to be the C-terminal RING finger domain (a.a. 425–491). We propose that the C-terminal domain of USP7 is responsible for maintaining the active conformation for catalysis and inhibitor binding, and contains the prime side of the proteolytic active site.

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Introduction

The ubiquitin–proteasome pathway plays an important role in regulating many biological processes, including cell cycle, differentiation, immune response, DNA repair, and apoptosis [1,2]. Ubiquitination is a highly dynamic and reversible process, regulated by multiple ubiquitin-conjugating enzymes and deubiquitinating enzymes. Formation of an isopeptide bond between the C-terminal carboxylate group of ubiquitin and the ε-amino group of a Lys residue in a substrate protein proceeds through a three-step cascade mechanism involving the ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3). Substrate proteins can form conjugates with a single ubiquitin or ubiquitin chains at a single or multiple Lys residues [1,2].

Ubiquitination of proteins can be reversed by the deubiquitinating enzymes (DUBs) [3–5]. DUBs have important functions, such as rescuing substrate proteins from proteasomal degradation, recycling Ub, and controlling protein trafficking. There are at least five distinct subclasses of DUBs, of which the Ub-specific proteases (USPs)[1] constitute the largest family with more than 50 family members [3–5]. USPs are cysteine proteases containing conserved regions in their amino acid sequence surrounding the Cys, His, and Asp/Asn residues that form the catalytic triad. Beyond the catalytic domain, USPs often encode N-terminal and C-terminal extensions that may be important for substrate recognition and subcellular localization. The substrate specificity and physiological functions of most USPs are not yet understood.

One of the best characterized USP is USP7 or HAUSP (herpes simplex virus associated ubiquitin-specific protease). USP7 was shown to directly bind to and deubiquitinate the p53 tumor suppressor protein [6]. Overexpression of USP7 resulted in the stabilization of p53 in cells. However, when USP7 was knocked out either by siRNA [7] or by homologous recombination [8],

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[1] *Abbreviations used*: Ub, ubiquitin; DUB, deubiquitinating enzyme; USP, ubiquitin-specific protease; HAUSP, hepes simplex virus associated ubiquitin-specific protease; Ub-AFC, ubiquitin 7-amino-4-trifluoromethylcoumarin; Ubal, ubiquitin aldehyde; TRAF, TNF receptor associated factors; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GST, glutathione-S-transferase; a.a., amino acid.

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stabilization of p53 was observed rather than the expected effect of destabilization. This paradox can be explained by USP7's preferential deubiquitination of mdm2, an E3 ubiquitin ligase which ubiquitinates p53 and thereby mediates its degradation. Knockdown of USP7 results in the stabilization of p53, induction of p53 responsive genes, and growth inhibition or apoptosis in cancer cells with functional p53 [7,8]. Because of the important role USP7 plays in regulating the p53 pathway, inhibition of USP7's protease activity or its interaction with mdm2 could be an effective way to reactivate p53 for the treatment of cancer.

USP7 is composed of 1102 amino acids and several distinct domains. The N-terminal domain (NTD) of USP7 has sequence homology to the TNF receptor associated factors (TRAFs) and was shown to bind to mdm2, p53, as well as the Epstein–Barr virus nuclear antigen 1 (EBNA1) [9–13]. Crystal structures and binding studies suggest that the mdm2 peptides bind to the same surface groove in USP7 as peptides derived from p53, but with more extensive interactions and enhanced affinity [10,12]. The C-terminal region of USP7(560–1102) contains regions required for interactions with Ataxin-1 [14] and the herpes virus protein ICP0 [11]. The catalytic domain was mapped to amino acids 208–560 [9]. Crystal structures of the catalytic domain in the presence and absence of a covalent inhibitor, ubiquitin aldehyde (Ubal), revealed that the active site of USP7 is misaligned in the apo structure. Formation of the covalent adduct of Ubal with the catalytic cysteine residue induces a drastic conformational change in the active site, realigning the catalytic triad for catalysis [9].

In the current study, we have identified novel mdm2/p53 binding sites on USP7 (a.a. 801–1050 and 880–1050 for mdm2 and p53, respectively), distinct from the N-terminal TRAF-like domain. We have also demonstrated that the catalytic domain of USP7 (a.a. 208–560) is significantly less active than the full-length USP7 or truncated USP7 (a.a. 208–1102). Moreover, inhibition of the catalytic domain of USP7 is also different from the full length or TRAF-like domain deleted proteins. The C-terminus of USP7 appears to be critical for its catalytic activity either by constituting part of the active site or by maintaining the active conformation for catalysis and for inhibitor binding.

Material and methods

Protein expression and purification of USP7, mdm2, and p53

Full-Length USP7 (NP_003461) was cloned from a human colon cDNA library using standard PCR techniques. It was subcloned into the vector pENTR/TEV/D-TOPO (Invitrogen K252520) and used as a template to generate different USP7 truncations. All USP7 constructs contained a 6× his tag and a tobacco etch virus (tev) protease cleavage site N-terminal of the USP7 coding region. Protein expression was carried out in Sf9 insect cells with baculovirus infection at 27 °C for 3 days.

Sf9 cells expressing various USP7 constructs were centrifuged and cell pellets were lysed in buffer A (20 mM Tris–HCl at pH 7.6, 2 mM TCEP, 5% glycerol) plus 250 mM NaCl, 20 mM imidazole and EDTA free protease inhibitor cocktail (Roche). The cellular extract was clarified by centrifugation at 17,000 rpm for 60 min at 4 °C, before loading onto a Ni–NTA column. After washing extensively with buffer A plus 250 mM NaCl containing 20 mM and then 40 mM imidazole, his-tagged USP7 was eluted with buffer A containing 250 mM imidazole and 250 mM NaCl. Subsequently, USP7 proteins were purified by size exclusion chromatography (superdex 200 in buffer A with 150 mM NaCl), followed by MonoQ chromatography (eluted in buffer A at 180 mM NaCl).

GST-mdm2 and its deletion mutants, GST- or strep-tagged p53 and GST-E1 were expressed and purified as described [15].

Generation and partial purification of Ub_n-p53 and Ub_n-mdm2

UbcH5b was conjugated to Cy5-labeled ubiquitin (Cy5-Ub-UbcH5b) as described [15]. Ubiquitinated p53 (Ub_n-p53) was generated using p53 and Cy5-Ub-UbcH5b in an enzymatic reaction catalyzed by mdm2. The reaction was carried out in buffer B (15 mM HEPES at pH 7.5, 5 mM NaCl, and 10 mM octylglucoside) containing 20 μM Cy5-Ub-UbcH5b, 5 μM strep-tagged p53, and 0.2 μM GST-tagged mdm2 at room temperature for 60 min. To get rid of the excess Ub-UbcH5b and mdm2, strep-tagged p53 species were partially purified from the reaction mixture using steptactin Sepharose resin (Genosys Biotech), washed with buffer C (50 mM Tris at pH 8.0, 150 mM NaCl, 10% glycerol, 0.1% triton X-100 amd 0.5 mM TCEP) with 1% triton X-100 followed by buffer C with 0.1% triton X-100, and eluted with 4 mM d-biotin. Partial purified Ub_n-p53 was desalted using a G-25 column into buffer C without triton X-100. Mdm2-catalyzed p53 ubiquitination usually results in multiple monoubiquitinated p53 (Ub_n-p53) containing mainly p53 with 1–5 ubiquitin conjugates [16]. Using a Cy5-Ub calibration curve, we analyzed the partially purified Ub_n-p53 by SDS–PAGE, and calculated the concentration of Ub_n-p53 based on the fluorescence intensity of Cy5-Ub that is attached to p53. We estimated that the partially purified Ub_n-p53 mixture contained ~24% ubiquitinated p53.

Ub_n-mdm2 was generated using the mdm2 auto-ubiquitination reaction carried in buffer B containing 20 μM Cy5-Ub-UbcH5b and 0.4 μM GST-tagged mdm2 for 4 h at room temperature. The reaction mixture was incubated with glutathione–Sepharose 4B resin (GE Healthcare), washed with buffer D (25 mM HEPES at pH 7.3, 150 mM NaCl, 10% glycerol, and 0.1 mM TCEP) with 1% followed by 0.1% triton X-100, and finally eluted with buffer D containing 0.1% triton X-100 and 20 mM glutathione. Partial purified Ub_n-mdm2 was desalted into a buffer containing 20 mM HEPES at pH 7.3, 1 mM DTT and 0.05% CHAPS using a G-25 column. Because mdm2 auto-ubiquitination usually results in a mixture of mdm2 with high molecular weight ubiquitin chains, we quantified the amount of mdm2 before and after the reaction using western blot analysis with a monoclonal anti-mdm2 antibody (BD Pharmingen, #556353). Our estimate is that ~70% of mdm2 has been converted to Ub_n-mdm2 under our reaction conditions. In our USP7 mediated deubiquitination assays using Ub_n-mdm2 as substrates, total protein concentration of Ub_n-mdm2 was indicated in the assays.

In vitro ubiquitination assays for USP7

We used three substrates to assess the activity of full-length USP7 and various truncated constructs: ubiquitin-7-amino-4-(trifluoromethyl)coumarin (ubiquitin-AFC, Boston Biochem), Ub_n-p53 and Ub_n-mdm2. Ubiquitin-AFC cleavage was monitored fluorometrically, similar to procedures described for Ub-AMC [17]. Reactions were carried out in buffer E (50 mM HEPES pH 7.5, 0.5 mM EDTA, 0.5 mM TCEP, 0.1 mg/ml BSA and 0.05% CHAPS) using low volume 384 black assay plates (GreinerBio) at room temperature. The reaction mixture contains Ub-AFC and different USP7 constructs at various concentrations. Cleavage of Ub-AFC by USP7 was monitored kinetically for 1 h using the analyst plate reader (Molecular Devices) for fluorescence intensity at excitation/emission wavelength of 405 nm and 520 nm, respectively. The initial velocity of the reactions was used for steady-state analysis and IC_{50} determination.

An SDS–PAGE assay was used to quantify USP7 mediated deubiquitination of Ub_n-mdm2 and Ub_n-p53. Reactions were carried out at room temperature using 2.65 μg/ml Ub_n-p53 or 1.68 μg/ml Ub_n-mdm2, and USP7 enzymes at various concentrations in buffer E. After 20 min, the reactions were stopped with 4× SDS-reducing sample buffer and separated using 4–12% NuPAGE Bis–Tris SDS–PAGE (Invitrogen). Fluorescence intensity

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