Published in final edited form as:

Biochem Biophys Res Commun. 2015 July 31; 463(3): 453–457. doi:10.1016/j.bbrc.2015.05.111.

Valosin-Containing Protein (VCP/p97) is Capable of Unfolding Polyubiquitinated Proteins through Its ATPase Domains

Changcheng Song^{a,*}, Qing Wang^b, Changzheng Song^c, and Thomas J. Rogers^a

^aCenter for Inflammation, Translational and Clinical Lung Research, School of Medicine, Temple University, Philadelphia, PA 19140, USA

^bGraduate Center for Toxicology, University of Kentucky, Lexington, KY 40536, USA

^cErythrocrine Project of Translational Medicine, Shandong Academy of Medical Sciences, Jinan 250062, China

Abstract

Valosin-containing protein (VCP or p97) is required for the proteasomal degradation of polyubiquitinated proteins. However, the molecular mechanism for VCP to process the polyubiquitinated proteins remains unclear. Here, we show that VCP can unfold polyubiquitinated proteins. It preferably unfolds the pentaubiquitin- over monoubiquitin-conjugated dihydrofolate reductase (Ub5-DHFR or Ub-DHFR) in a dose dependent manner. In addition, the unfolding activity of VCP does not depend on its ATPase activity, on the contrary, ATP and its non-hydrolysable analogs suppress the unfolding of Ub5-DHFR. The structural and functional analysis showed that either D1 or D2 domain of VCP is sufficient to carry out this unfolding activity. The structure of the substrates also affects its unfolding by VCP. VCP is unable to unfold Ub5-DHFR in a tight structure when it binds with methotrexate, a folate analog with high affinity to DHFR. Thus, these results support that VCP is capable of unfolding polyubiquitinated proteins and suggest that VCP may facilitate the proteasomal degradation of polyubiquitinated proteins through its unfolding activity.

Graphical Abstract

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*Corresponding author. Fax: 1-215-707-4743. songc@temple.edu.

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Fig. VCP promotes chymotrypsin digestion of Ub5-DHFR. The ubiquitinated-DHFR (200 nM) mixed with increasing amount of VCP were incubated at 25°C for 10 min, then added 5 nM chymotrypsin and incubated at 37°C for 10 min. The proteins were detected by western blotting with anti-DHFR antibody.

Keywords

valosin-containing protein (VCP or p97); unfolding; ATPase; polyubiquitinated protein; structure and functional analysis

1. Introduction

Protein degradation is essential for maintaining the health of proteome in cells [1]. In eukaryotic cells, the ubiquitin proteasome system is responsible for the degradation of more than 80% cellular proteins [2, 3]. Valosin-containing protein (VCP or p97), a member of ATPase associate diverse cellular activities (AAA) family, is required for the proteasomal degradation of polyubiquitinated proteins [2, 4, 5]. Reducing the functionality of VCP either by siRNA in cells or immunodepletion in *in vitro* protein degradation assays suppresses the degradation of polyubiquitinated proteins [6, 7]. In addition, VCP is essential for the endoplasmic reticulum associated degradation (ERAD) in controlling the quality of ER produced proteins [8–14]. In this process, VCP through ubiquitin fusion degradation 1 (Ufd1) and nuclear pore localisation protein 4 (Npl4) interacts with polyubiquitinated proteins and retrotranslocates them from the ER lumen into the cytoplasm for proteasomal degradation [15, 16]. However, the role of VCP in mediating the proteasome degradation of polyubiquitinated proteins remains inadequately understood.

Recent studies showed that Cdc48 (also named VAT), a homolog of VCP, in the archaeon *Thermoplasma acidophilum* can interact with 20S proteasome to form a functional complex for the degradation of proteins conjugated with a 11-amino acid ssrA peptide (AANDENYALAA) [17, 18]. Cdc48 can unfold the ssrA tagged proteins and requires the aromatic residues lining in its central pore [19, 20]. VCP lacks these aromatic residues, however the mutation of two aliphatic residues of the D1 pore in VCP to tyrosines enables the N domain deletion-mutant of VCP (VCP-DeltaN) to unfold a yellow fluorescent protein (YFP) conjugated with ssrA [19]. It suggests that the structure of the ATPase domains of VCP may support the function of protein unfolding. Since the polyubiquitin chain, instead of the ssrA peptide, is required for VCP to mediate proteasomal degradation, VCP may unfold the proteins conjugated with polyubiquitin chains.

To examine whether VCP can unfold polyubiquitinated proteins, we choose dihydrofolate reductase (DHFR) conjugated with five ubiquitin moieties (Ub5-DHFR) as a model protein since at least four ubiquitin is required to form a proteasomal degradation signal [21], and this biochemically synthesized pentaubiquitinated protein can be degraded by the proteasome [22–24]. We employed a chymotrypsin digestion assay to analyze the conformational change of substrate proteins [22]. This method is based on that the allosteric alteration of a protein could expose or shield of chymotrypsin digestion sites to affect the rate of protein degradation. Here we showed that VCP can unfold proteins conjugated with

polyubiquitin chains and the unfolding activity is carried out through its ATPase domains in a non-ATP binding state.

2. Material and methods

2.1

ATP, ATP- γ S, AMP-PNP, methotrexate, heat shock protein 70 (Hsp70), and citrate synthesis were purchased from Sigma-Aldrich Inc. (St. Louis, MO).

2.2 Expression and purification of recombinant proteins

The recombinant proteins of VCP, Ufd1, and NSF were expressed and affinity-purified to apparent homogeneity as previously described [7].

2.3 Chymotrypsin digestion assay

The chymotrypsin digestion was employed to examine the conformational modification of substrates [25]. In each reaction, 200 nm ubiquitinated-DHFR (Kindly provided by Dr. Cecile Pickart) was diluted to a final volume of 100 μ l of buffer (100 mM Tris-HCl, pH 7.4, 3.5 mM MgCl₂, 10 mM KCl, and 0.01% Tween 20). Nucleotides or methotrexate were added to the reaction mixture at a final concentration of 3 mM or 200 nM, respectively. At time point 0, 5 μ l of 100 nm chymotrypsin was added to each sample and all samples were maintained at 37°C for the duration of the experiment. A 20- μ l aliquot was withdrawn immediately after chymotrypsin addition, quenched with 3 μ l of 50 mM phenylmethylsulfonyl fluoride, and placed on ice. At selected time points, 20- μ l aliquots were removed and treated in the same fashion as the time-point-0 sample. 10 μ l of 3 $\times$ SDS loading buffer was added to 15 μ l of each sample and heated for 5 min at 95°C. Samples were resolved on 4–12% Bis-Tris polyacrylamide gels (Invitrogen Inc.), and transferred to a membrane, followed by Western blotting with anti-DHFR antibody (Sigma-Aldrich Inc.). The protein bands were quantified using Image J 1.48 (<http://imagej.nih.gov/>) [26].

3. Results

3.1. VCP preferentially promotes the chemotrypsin digestion of Ub5-DHFR

To determine whether VCP can unfold the polyubiquitinated proteins, we employed a chymotrypsin digestion assay to determine the topological modification of ubiquitinated proteins by VCP. We utilized a mix of Ub5-DHFR and Ub-DHFR as substrates to distinguish whether the polyubiquitination is required for the unfolding activity of VCP. In the western blotting analysis with anti-Ub or DHFR antibodies, they appeared as a 59 and 33 kDa band, respectively, which equals to the calculated molecular weight of Ub5-DHFR and Ub-DHFR (Fig. 1A). The mix of Ub5-DHFR and Ub-DHFR were incubated with increased amount of VCP additive (Fig. 1B) following 10 min of incubation with chymotrypsin at 37°C and performed western blotting analysis using anti-DHFR antibody. The result showed that the intensity of Ub5-DHFR, but not Ub-DHFR band was decreased with the elevated concentration of VCP. The degradation rate was increased about 2 folds with 0.5 μ M VCP. The catalytic activity of VCP was saturated at 1 μ M with approximately a 3-fold increase in

the degradation rate. These results suggested that VCP could unfold the DHFR conjugated with polyubiquitin chains (Fig. 1B and 1C).

To determine whether the unfolding of polyubiquitinated proteins is a unique feature of VCP we examined whether other molecular chaperones, VCP cofactors, and AAA proteins also retain such unfolding activity. Hsp70 was selected as a representative chaperone since it is predominately involved in protein refolding [27]. Another AAA family member N-ethylmaleimide sensitive fusion protein (NSF) was chosen since its structure reassembles VCP, but mainly functions in the membrane fusion and protein complex disassembly [28]. The result showed that Hsp70, NSF and Ufd1 did not increase the digestion of Ub5-DHFR (Fig. 1D). On the contrary, the degradation of Ub5-DHFR was suppressed, suggesting these proteins cannot unfold polyubiquitinated proteins and may compete with Ub5-DHFR for chymotrypsin digestion.

3.2. The effect of nucleotide states of VCP on its mediated Ub5-DHFR chymotrypsin digestion

To determine whether the unfolding of polyubiquitinated proteins by VCP is affected by its ATPase activity, we first examined VCP-mediated chymotrypsin digestion of Ub5-DHFR in the presence of ATP and found that the addition of ATP to the reaction mixture significantly suppressed the proteolytic susceptibility of Ub5-DHFR (Fig. 2A and 2B, lanes 2, 4 and 6 vs 3, 5, and 7). As a negative control, citrate synthase (CS), did not promote the Ub5-DHFR degradation and also was not affected by the presence of ATP in the reaction (Fig. 2A and 2B, lane 8 vs 9). In addition, the effect of ATP on the unfolding activity of VCP was dose dependent. The intensities of Ub5-DHFR bands was higher with increased ATP concentration, and the effect of ATP on the VCP-mediated chymotrypsin digestion was saturated at 2.5 mM (Fig. 2C). These results show that VCP in the ATP-binding state cannot mediate the unfolding of polyubiquitinated substrates.

Then we examined the effect of other nucleotide states on the unfolding activity of VCP, and found that comparing to ATP, two non-hydrolysis analogs of ATP, ATP- γ S and AMP-PNP, also suppressed the chymotrypsin digestion of Ub5-DHFR, which supported that VCP in the ATP-bound state cannot modify the conformation of Ub5-DHFR. On the contrary, ADP and AMP-AlFx, which resembles ATP in its ground state, enhanced chymotrypsin digestion on Ub5-DHFR (Fig. 2D). These data indicate that the unfolding activity of VCP depends on its nucleotide-bound states.

3.3. Structural and functional analysis of VCP in unfolding Ub5-DHFR

To reveal the structure responsible for the unfolding activity of VCP, we determined the unfolding capability of VCP deletion mutants and found that the deletion mutants containing either the N and D1 domain (ND1) or D2 domain (D2) exhibit such activity, but the N domain (N) alone cannot (Fig. 3A). Then we examined the effect of ATP on the unfolding activity of these D1 and D2 domains, and observed that chymotrypsin digestion of Ub5-DHFR is suppressed by ATP in the reactions supplemented with VCP mutants bearing the ATP-binding site-specific mutations in D1 or D2 domain (A1 or A2), and the deletion

mutants ND1 or D2 (Fig. 3B). These results suggest that the ATPase domains of VCP carry out the unfolding activity in non-ATP bound form.

3.4. The effect of DHFR conformation on its unfolding by VCP

To determine the effect of the conformation of DHFR on the unfolding activity of VCP, we examined the chymotrypsin digestion of Ub5-DHFR in tight-up conformation with binding to its high-affinity ligand, methotrexate [29]. The result showed that in the presence of methotrexate, VCP-mediated chymotrypsin digestion of Ub5-DHFR was suppressed (Fig. 4, lane 3 and 4), suggesting that VCP is unable to unfold the substrates in a compact topological structure.

4. Discussion

Here, we found that VCP could unfold proteins conjugated with polyubiquitin chains. This activity is consistent with the function of VCP in the proteasomal degradation of polyubiquitinated proteins [7, 12, 30]. The 26S proteasome consists of a 20S proteolytic core with a 19S regulatory complex attached at each end of the core. Since the 20S core has a narrow opening that only allows the passage of unfolded polypeptides, the substrate proteins need to be unfolded before entering the 20S core [31–34]. In spite of the capability of the 19S regulatory complex in unfolding polyubiquitinated proteins, VCP is required for the proteasome degradation [7, 12, 30]. The unfolding activity of VCP may facilitate proteasomal degradation in three different ways. First, during dislocating aberrant proteins from in the ER lumen to the cytosol, VCP may unfold these substrates to ease their retrotranslocation from the membrane [16, 35]. Second, VCP is required for the preprocessing of highly folded, ubiquitinated substrates by the 19S regulatory complex of the proteasome [36]. In this process, VCP and the 19S regulatory complex may have a synergy in the substrate unfolding. Third, in response to proteasomal impairment, VCP can bind to 26S proteasome to form an additional hexameric ATPase ring to relieve repression [37], which could allow VCP to complement the deficient unfolding activity of the 19S regulatory complex. In these ways, VCP facilitates the proteasome degradation to meet the demand of various cellular activities.

We also found that the unfolding activity is carried out by the AAA domains, but not N domain, in VCP carry out. Although the N domain of VCP can bind to the polyubiquitinated proteins directly or through Ufd1/Npl4 or other cofactors [12, 16, 35], it cannot mediate the unfolding of its bound proteins. In a similar manner, the 19S regulatory complex, PA700 can mediate the unfolding of the polyubiquitinated proteins [22]. PA700 contains six AAA subunits at the base of its ring structure and attached to 20S proteasome, but the ubiquitin chain binding subunits is a non-AAA subunit S5a [38]. It suggests that the prerequisite binding of polyubiquitin chains to VCP and PA700 is not necessary for protein unfolding. The binding of polyubiquitin chains with N domain may allow its conjugated proteins to contact with the AAA domains for unfolding.

In addition, the polyubiquitin chain may also alter the structure of its attached proteins to prime the protein unfolding. Our data showed that VCP can unfold the polyubiquitinated, but not monoubiquitinated, proteins. Polyubiquitination may alter the structure of its

conjugated proteins to lower the energy threshold for further VCP-mediated protein unfolding. In consistence, the requirement of the ubiquitin chains for proteasomal degradation is dependent on the nature of the substrate. While most of the proteins require polyubiquitination, the proteasome even can degrade proteins with monoubiquitination such as Cks2, α -Synuclein and α -globin [39]. Moreover, the oxidized proteins do not need polyubiquitination for its proteasome degradation [40]. It is possible that these proteins are in loose structure so that the further unfolding is not necessary. Consistently, the “tightness” of protein structure can restrain the unfolding and consequent proteasome degradation. VCP and PA700 [22] cannot unfold Ub5-DHFR in a tight conformer when it is complexed with methotrexate, and the methotrexate-bound Ub5-DHFR cannot be degraded by the proteasome [41]. The assumption that polyubiquitination may decrease the unfolding effort of AAA domains in VCP is also consistent with the unfolding is independent of their ATPase activities. The ATPase activity of VCP or PA700, which is necessary for the proteasome degradation [7], may be required for transferring the unfolded proteins to the 20S proteasome.

The unfolding activity of AAA domains may carry out through the hydrophobic interaction with polyubiquitinated proteins since polyubiquitin chains can form a hydrophobic surface, which is required for its interaction with the proteasome [21, 42]. In addition, the D1 ATPase domain of VCP can bind misfolded proteins [43] and misfolded proteins expose hydrophobic regions which are buried inside of the native proteins. Thus, it is possible that the AAA domains may interact with both the substrate and polyubiquitin chains through hydrophobic interaction.

Acknowledgments

The authors thank Dr. Chou-Chi H Li for her fully support on initiation and development of this project, Dr. Xinhua Ji for critical reading of the manuscript and Dr. Di Xia for helpful comments. This study has been funded by 1R21ES023051 (C.S.) and DA-14230 (T.J.R.).

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- VCP can unfold polyubiquitinated proteins in non-ATP binding states.
- The unfolding activity is carried out by the AAA domains of VCP.
- VCP-mediated protein unfolding requires substrates in a loose structure.

Fig. 1.

VCP promotes chymotrypsin digestion of Ub5-DHFR. (A) The synthesized ubiquitinated-DHFR proteins were separated with 4–12% SDS-PAGE and analysed by western blotting with anti-ubiquitin and anti-DHFR antibodies. In addition, the ubiquitinated-DHFR (200 nM) mixed with increasing amount of VCP (B), or 2 μM Hsp70, NSF, or Ufd1 (D) were incubated at 25°C for 10 min, then added 5 nM chymotrypsin and incubated at 37°C for 10 min. The proteins were detected by western blotting with anti-DHFR antibody, and the relative intensities of Ub5-DHFR and Ub-DHFR proteins in each lane of (B) were quantified using Image J and presented in (C).

Fig. 2.

ATP suppresses VCP-mediated chymotrypsin digestion of Ub5-DHFR. (A) The ubiquitinated-DHFR (200 nM) mixed with increasing amount of VCP (A, lane 2 to lane 7) or citrate synthase (CS, A, lane 8 and 9) in the presence or absence of 3 mM ATP, was incubated at 25°C for 10 min, then added 5 nM chymotrypsin and incubated again at 37°C for 10 min. The proteins were analyzed by western blotting with anti-DHFR antibody. +, ++, and +++ represent the concentration of VCP or CS at 1, 2, and 3 μ M, respectively. The relative intensities of Ub5-DHFR and Ub-DHFR proteins in each lane of (A) were quantified using Image J and presented in (B). In addition, the ubiquitinated-DHFR (200 nM) was mixed with 2 μ M VCP with increasing ATP concentration (C), or 3 mM nucleotides as indicated (D), then the chymotrypsin digestion and western blotting detection of (C) and (D) were performed in the same manner as (A).

Fig. 3.

Both D1 and D2 domains can promote the chymotrypsin digestion of Ub5-DHFR. (A) The ubiquitinated-DHFR (200 nM) incubated with 1 μM wild-type (WT) VCP or 2 μM its variants ND1 (ND1 domain), D2 (D2 domain), or 1 μM N (N domain) was incubated at 25°C for 10 min, then added 5 nM chymotrypsin and incubated again at 37°C for 5 min. The Ub5-DHFR proteins were detected using western blotting with anti-DHFR antibody. (B) 1 μM A1, A2 (ATP-binding site mutation in D1 or D2 domain), ND1, or D2 were incubated with the ubiquitinated-DHFR (200 nM) in the presence or absence of 3 mM ATP. The chymotrypsin digestion and western blotting detection of Ub5-DHFR were performed in the same manner as (A).

Fig. 4. Methotrexate suppresses the unfolding of Ub5-DHFR by VCP. The ubiquitinated-DHFR (200 nM) was incubated with or without 1 μ M VCP, or with methotrexate (200 nM) or its solvent control DMSO at 25°C for 10 min, then add 5 nM chymotrypsin and incubated 37°C for 10 min; proteins were detected using western blotting with anti-DHFR antibody. The relative intensity of Ub5-DHFR in each lane of (A) were quantified using Image J and presented in (B).