Hexamerization of p97-VCP is promoted by ATP binding to the D1 domain and required for ATPase and biological activities ☆

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Abstract

The 97-kDa valosin-containing protein (p97-VCP or VCP), a hexameric AAA ATPase, plays an important role in diverse cell activities, including ubiquitin–proteasome mediated protein degradation. In this report, we studied dissociation–reassembly kinetics to analyze the structure–function relationship in VCP. Urea-dissociated VCP can reassemble by itself, but addition of ATP, ADP, or ATP-γS accelerates the reassembly. Mutation in the ATP-binding site of D1, but not D2, domain abolishes the ATP acceleration effect and further delays the reassembly. Using hybrid hexamers of the wild type and ATP-binding site mutant, we show that hexameric structure and proper communication among the subunits are required for the ATPase activity and ubiquitin–proteasome mediated degradation. Thus, ATP-binding site in D1 plays a major role in VCP hexamerization, of which proper inter-subunit interaction is essential for the activities.
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VCP belongs to an ancient AAA (ATPases associated with a variety of cellular activities) family [1–7] whose members are characterized by having highly conserved ATPase domain(s) and diverse functions. The ∼250-amino acid ATPase domain contains the consensus Walker A and B motifs, which are responsible for ATP binding and ATP hydrolysis, respectively. In addition to sequence homology, the family members frequently form a ring-shaped oligomeric structure. The contrast between the functional diversity and the high sequence and structural similarities suggests that AAA proteins play a common and fundamental role, and the ring-shaped oligomeric structure is essential for such a role.

VCP is ubiquitous and abundant in cells, and is one of the most conserved proteins in eukaryotes. As other AAA proteins, VCP plays a role in many seemingly unrelated cell activities. Interestingly, almost all these activities are regulated by the ubiquitin–proteasome (Ub–Pr) degradation, an extremely important and intensively studied pathway. We previously demonstrated that VCP binds to the multiubiquitin chains and may act as a molecular chaperone that targets the ubiquitinated substrates to the proteasome for degradation. Thus, a link between VCP and the diverse functions is established: VCP acts as a ubiquitin-selective chaperone in the Ub–Pr pathway which then regulates these seemingly unrelated functions.

Although the detailed mechanisms of VCP functions are not clear, recent evidence indicates that it likely acts as a molecular chaperone that unfolds or unwinds macromolecules, and assembles or disassembles protein complexes. VCP has been reported to have a ring-shaped hexameric structure [8]. Presumably, the ring structure provides the mechanical basis for these activities, and the ATPase module-catalyzed ATP hydrolysis supplies the needed energy. Recent crystallography and electron microscopy studies [9,10] further suggested that VCP structure comprises two stacked hexameric rings made of the respective AAA modules. Each AAA module consists of two parts: an N-terminal domain

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with an α/β fold and a nucleotide binding pocket lying between protomers, and a C-terminal domain with at least three α-helices [10]. The helical domain facilitates protomer contacts in the ring and is important for communicating conformational changes in response to nucleotide binding [10]. However, the role of ATP in hexamerization of VCP is still unknown.

VCP molecule can be divided into three domains: the amino-terminal domain, N, and two ATPase domains, D1 and D2. We previously demonstrated that N is critical for substrate binding [11] and D2 is responsible for the major ATPase activity (Song et al, unpublished). In this study, we show that ATP binding to D1 accelerates the reassembly of dissociated VCP, thus D1 could be the major mediator for hexamerization. The reassembled hexamer is a functional ATPase and can mediate ubiquitin–proteasome dependent degradation. Further, hexameric structure and proper communication between subunits are critical for both the ATPase activity and biological function of VCP.

Materials and methods

Site-directed mutagenesis and bacterial strains. Site-specific mutants were generated using Quikchange Site-Directed Mutagenesis kit (Stratagene) with the wild-type pVCP-His [11] as a template. In A1 or A2 mutant, a lysine to threonine mutation was introduced in residues 251 in D1 or 524 in D2, respectively. For A1A2 mutant with double mutations, A1 was used as a template in the site-directed mutagenesis system. In B1B2 mutant, a glutamic acid was changed to glutamine in residues 305 in D1 and 578 in D2. All mutations were confirmed by DNA sequencing. All VCP constructs were cloned in a His-fusion expression vector, pQE60, and expressed as His fusion protein (through the C-terminus of VCP) in *Escherichia coli* strain M15 [pREP4] (QIAexpress system, Qiagen).

Purification of His-tagged VCP proteins. VCP-His$_6$ fusion protein was affinity-purified using Ni-NTA-agrose beads and dialyzed against dialysis buffer containing 50 mM Tris–HCl (pH 7.5), 140 mM NaCl, 5 mM KCl, 0.5 mM EDTA, and 1 mM DTT to remove residual phosphate and imidazole. Protein concentration was determined by BCA assay (Pierce).

Dissociation and reassembly assay. Purified wild type and mutant VCP proteins (0.2 mg/ml) were dissociated by mixing with the dialysis buffer containing 6 M of urea (final concentration) for 60 min at room temperature. After the removal of urea by Sephadex G-50 chromatography (Roche) [12], reassembly was carried out at room temperature in the dialysis buffer containing 5 mM MgCl$_2$ with or without 2 mM ATP. Reassembled proteins were analyzed by non-denaturing PAGE, Western transfer and immunoblotting [13] with anti-His antibody (Qiagen). All dissociation–reassembly experiments were repeated at least three times for each VCP variant purified from different preparations.

Trypsin digestion. Purified VCP (0.1 mg/ml) was incubated with 2.5 μg/ml trypsin (Roche) at 37 °C for various periods of time in buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM MgCl$_2$, and 1 mM DTT. The reactions were terminated by the addition of phenylmethylsulfonyl fluoride (0.5 mM) and SDS–gel sample buffer. The digestion products were analyzed by SDS–PAGE and immunoblotting with antiserum recognizing either the N- or C-terminus of VCP [13].

ATPase assay. The inorganic phosphate released from ATP hydrolysis was measured as described [14,15] with minor modifications, which will be reported elsewhere.

In vitro degradation assay. In vitro degradation assays were performed as previously described [11,13]. Human cyclin E was synthesized in in vitro transcription/translation system (Promega) in the presence of [^{35}S]methionine/cysteine and used as the test substrate. Untreated S100 (50 μg protein), which was isolated from actively growing CA46 cells [16], and differentially treated S100 preparations (see Results) were used as the enzyme sources. VCP was depleted by subjecting S100 to immunoprecipitation with VCP-4 antiserum [13]. In a typical VCP reconstitution experiment, 1–4 μg recombinant His fusion protein was used. The degradation reactions were stopped by boiling in SDS–gel sample buffer and analyzed by SDS–PAGE, Western transfer, and autoradiography.

Results

Purification of site-specific mutants

Wild type and mutant VCP-His fusion proteins were expressed in *E. coli* and affinity-purified to near homogeneity (Fig. 1). The A1 (K251T) and A2 (K524T) mutants harbor defective ATP-binding sites in the Walker A motif of D1 and D2, respectively. A1A2 mutant contains the same K → T mutations in D1 and D2 (Fig. 4A). B1B2 harbors E → Q mutations in the ATP-hydrolysis sites (Walker B motif) in D1 and D2 (Fig. 4A).

Self-assembly of urea-dissociated VCP and its acceleration by nucleotide binding

To explore the stability of VCP hexamers and how nucleotide affects VCP hexamerization, we studied dissociation and re-hexamerization of VCP. Unlike many AAA proteins, VCP hexamer is very difficult to dissociate into monomers. Among the numerous attempts, only treatment with 6 M (but not lower concentration) urea could successfully dissociate VCP into monomers (Fig. 2A). The monomeric VCP can reassemble into
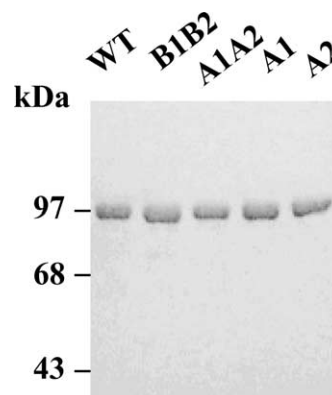

Fig. 1. Wild type and mutant VCP-His fusion proteins are purified. Wild type (WT) and mutant (as indicated on the top) VCP-His fusion proteins were expressed in *E. coli* and affinity-purified. The purified proteins (1 μg each) were resolved by SDS–PAGE and stained with Coomassie blue. The molecular size standards are shown on the left.

hexamers without the addition of nucleotides (Fig. 2B, left panel). But, the presence of ATP (middle panel), ADP (data not shown), or ATPγS (right panel), significantly accelerates the reassembly, which is evidenced by the early initiation (5 vs. 15–20 min) and completion (<60 vs. >90 min) of the reassembly. We noticed that although equal amounts of VCP were analyzed, a lower reactivity was detected in monomeric than hexameric form. This was observed in the immunoblots probed with either anti-His (Fig. 2) or anti-VCP antiserum (data not shown) and in gels stained with Coomassie blue (data not shown). This differential reactivity likely reflects the drastic conformational changes that resulted from the 6 M-urea treatment rather than unequal loading of the gel.

To further demonstrate the difference in the extent of reassembly with or without ATP, we analyzed trypsin sensitivity in untreated or differentially treated VCP. As expected, while the hexameric VCP is relatively resistant to trypsin digestion (Fig. 3, lanes 1–4), the urea-treated VCP is highly sensitive (lanes 5–8). Based on a silver-stained SDS–gel, the entire molecule was digested to fragments smaller than 18 kDa after 16 min (data not shown). We then carried out dissociation–reassembly experiments with or without ATP and analyzed the trypsin sensitivity of the reassembled VCP. The reassembly was performed specifically for only 30 min, when reassembly was still incomplete (see Fig. 2B), such that differences in trypsin sensitivity can be measured. As

shown in lanes 9–16, more full-length and near-full-length molecules are detected in the reactions with ATP (lanes 13–16) than without (lanes 9–12). Thus, the presence of ATP promotes the re-hexamerization (Fig. 2B), which renders resistance to trypsin digestion (Fig. 3).

Importance of ATP binding-, but not hydrolysis-, site in the stimulatory effect on reassembly

Because ATP, ATPγS, and ADP all stimulate the reassembly (Fig. 2B), it is likely that reassembly needs only nucleotide binding but not hydrolysis. To confirm this notion, the ATP-hydrolysis site mutant (B1B2) and the ATP-binding site mutant (A1A2) (Fig. 4A) were used. When the dissociation–reassembly assay was performed in B1B2, an accelerated (20 vs. 30 min) and more complete re-hexamerization (based on the relative level of hexamer to monomer) was detected in reactions with ATP than without (Fig. 4B). Again, ATP was not required for hexamer formation. In contrast, when A1A2 was subjected to the same assay, reassembly was severely hampered. No reassembled hexamer was detected within 90 min and even at 5 h the major population was still in a monomeric state (Fig. 4C). Therefore, the correct structure of ATP-binding site in the Walker A motif is essential for reassembly of urea-treated VCP, and ATP binding, but not hydrolysis, stimulates this reassembly.
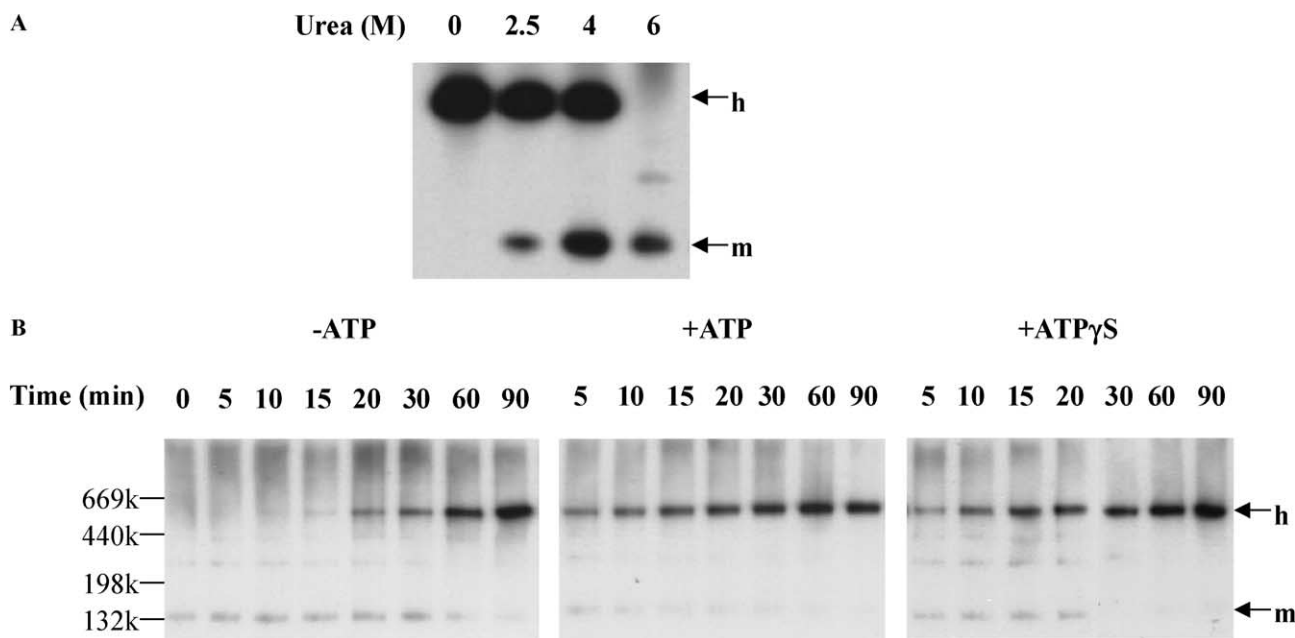

Fig. 2. Nucleotide binding stimulates the reassembly of dissociated VCP. (A) Six M urea dissociates VCP from hexamers to monomers. Purified wild-type VCP was treated with urea at the indicated concentration, then resolved on non-denaturing gel, and analyzed by immunoblotting with anti-His antibody. Hexameric (h) and monomeric (m) VCPs, confirmed by gel-filtration chromatography (not shown), are indicated. (B) Nucleotide binding accelerates VCP reassembly. VCP was dissociated with 6 M urea and then reassembled by removal of urea and addition of Mg^{2+} with the indicated nucleotide (2 mM). Reassembly was carried out for the indicated periods of time and analyzed by non-denaturing PAGE, Western transfer, and immunoblotting with anti-His antibody. Molecular size standards are indicated.
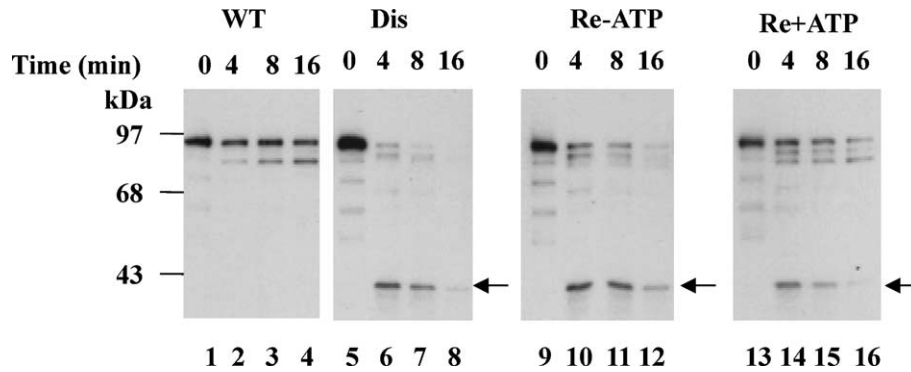
Fig. 3. VCP reassembled in the presence of ATP is more resistant to trypsin digestion. Wild-type VCP was either untreated (lanes 1–4) or treated (lanes 5–8) with 6 M urea and then digested with trypsin at 37 °C for the indicated periods of time. In parallel, VCP was dissociated with urea and reassembled for 30 min in the absence (lanes 9–12) or presence (lanes 13–16) of ATP before subjected to trypsin digestion. The proteolytic products were analyzed by SDS–PAGE, Western transfer, and immunoblotting with antiserum specific to the N-terminus of VCP. Molecular standards are marked on the left. The 40-kDa N-D1 intermediate is indicated by an arrow.
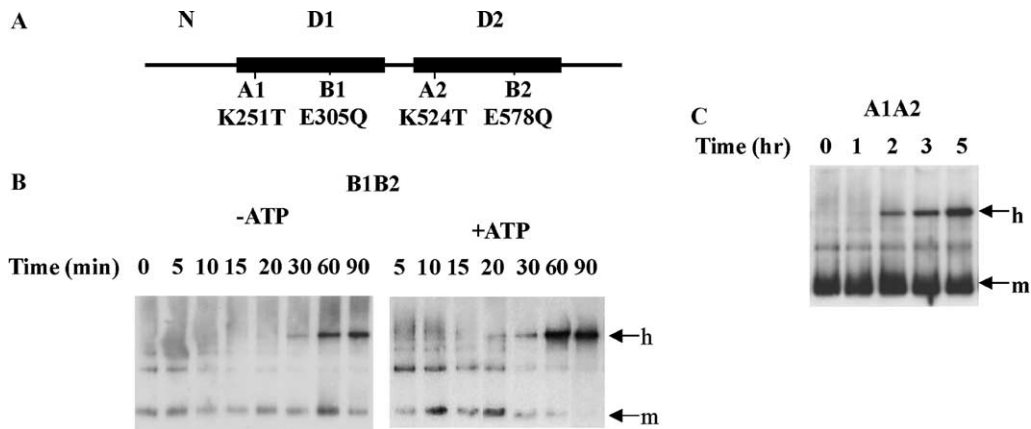

Fig. 4. Mutations in ATP-binding site, but not hydrolysis site, affect the reassembly process. (A) Mutations in ATP-binding and ATP-hydrolysis sites are depicted. Substitutions of lysine (K) by threonine (T) in Walker A (ATP binding) motifs and glutamic acid (E) by glutamine (Q) in Walker B (ATP hydrolysis) motifs are shown. (B) ATP stimulates the reassembly of B1B2 mutant. B1B2 mutant was dissociated with urea, reassembled in the presence or absence of 2 mM ATP, and analyzed as described in Fig. 2B. (C) Reassembly of A1A2 is severely delayed. A1A2 double mutant was subject to the dissociation–reassembly assay in the absence of ATP. Note the high abundance of monomeric form.

Critical role of D1 ATP-binding site in reassembly

Because both D1 and D2 of VCP are highly conserved, one major goal of this study was to determine which domain is responsible for the observed ATP stimulation effect. We used A1 (K251T) and A2 (K524T) mutants (see Fig. 4A) in dissociation–reassembly experiments. As shown in Fig. 5A, reassembly of A1 was not stimulated by ATP and was significantly delayed. The monomeric A1 was persistently detected throughout the time course and hexameric form was detected much later than that in the wild type (30 vs. 5 min with ATP). On the other hand, A2 molecules started to form hexamers at 5 or 20 min in the presence or absence of ATP, respectively (Fig. 5B), reflecting the kinetics similar to that of the wild type. Also contrary to A1, the reassembly was near completion in 90 min (Fig. 5B), as monomeric A2 was barely detectable. The results

demonstrate that the ATP-binding site in D1, rather than D2, is important for reassembly and responsible for the ATP stimulatory effect. This conclusion is further supported by the detection of a relatively stable, 40-kDa intermediate during the trypsin digestion (Fig. 3, indicated by an arrow). This intermediate is proven to be an N-D1 fragment because it is reactive with the antiserum specific to the N-terminus (lanes 5–16) and D1 (data not shown), but not to the C-terminus (data not shown). These data suggest that D1 domain has a more compact structure that is relatively resistant to trypsin digestion and is likely the key element in holding the higher structure of VCP.

Hexameric VCP as a functional ATPase

The biological function of VCP relies on its ATPase activity, which provides the energy needed in the chap-
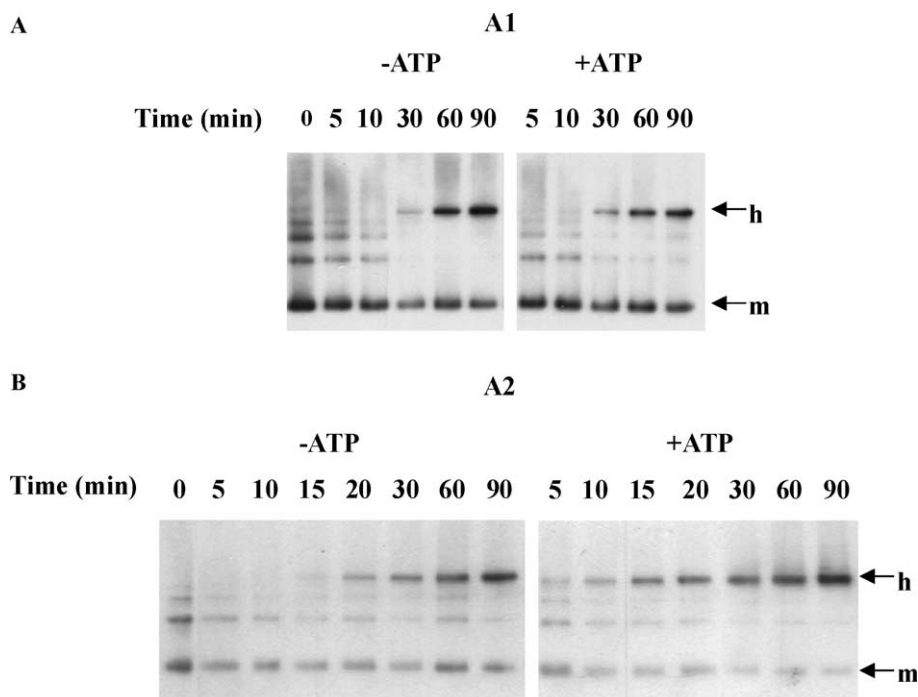
Fig. 5. ATP-binding site in D1 is more important for initiation of VCP reassembly than that in D2. (A) ATP does not stimulate the reassembly of A1 mutant. A1 was dissociated and reassembled in the presence or absence of 2 mM ATP and analyzed as described in Fig. 2B. (B) ATP stimulates the reassembly of A2 mutant. A2 was analyzed as described in (A).

erone functions. To examine whether the enzyme activity correlates with the hexameric structure, we monitored the ATPase activity during reassembly with or without ATP (Fig. 6A). The activity was barely detectable in dissociated VCP (0 min), indicating that the monomeric VCP is not a functional enzyme. As more hexamers were reassembled (as seen in Fig. 2B), the ATPase activity also increased. Moreover, while ATP accelerated the hexamerization of VCP (as seen in Fig. 2B), it also accelerated the regain of the ATPase activity.

Requirement for proper interactions between subunits for full ATPase activity and ubiquitin–proteasome mediated degradation

We further asked whether proper subunit interactions are required for the full ATPase activity. In other words, will a wild-type D2 neighbored by mutant D2 (as in the A2 mutant) in a hexamer still have ATPase activity? We performed disassociation-reassembly assay with a mixture of fixed amount of wild type and increasing amount of A2 and measured the ATPase activity of the reassembled hybrid hexamers (Fig. 6B). Since A2 mutant has no ATPase activity (Song et al, unpublished data), the total levels of functional D2, contributed by the wild type, are the same in all tested combinations. If a wild-type subunit neighbored by A2 mutants could still fully function, then the same ATPase activity would be expected in all tested mixtures. Clearly shown in Fig. 6B,

the activity decreased as the level of A2 increased in the mixture. The dominant negative effect of A2 suggests that in addition to the hexameric structure, the integrity of the hexamer and the proper interactions (allosteric communication) among subunits are also required for the full ATPase activity of VCP.

We previously demonstrated that VCP is necessary for ubiquitin–proteasome mediated degradation of a number of substrates [11,13]. Loss of VCP functions results in an inhibition of ubiquitin–proteasome mediated degradation and an accumulation of multi-ubiquitinated proteins [11]. Using an in vitro degradation system, we observed similar defects when VCP was depleted from mammalian cell extracts. Furthermore, when recombinant VCP was added back to the VCP-depleted extracts, the degradative activity was restored. In order to determine whether the reassembled VCP is biologically functional and whether proper subunit interactions are required in playing such a role, similar depletion and reconstitution experiments as previously reported [11] were carried out (Fig. 6C). As demonstrated, S100 extracted from cultured cells was capable of degrading ^{35}S-labeled cyclin E (S100, lanes 1–4) which was in vitro transcribed and translated; whereas VCP-depleted S100 lost such degradative ability (S100-VCP, lanes 5–8). As the reassembled wild-type VCP significantly restored the degradation (+WT$_{re}$, lanes 9–12), the reassembled wild type and A2 hybrid (at 1:1 ratio) restored less efficiently (+WT:A2$_{re}$, lanes 13–16). These
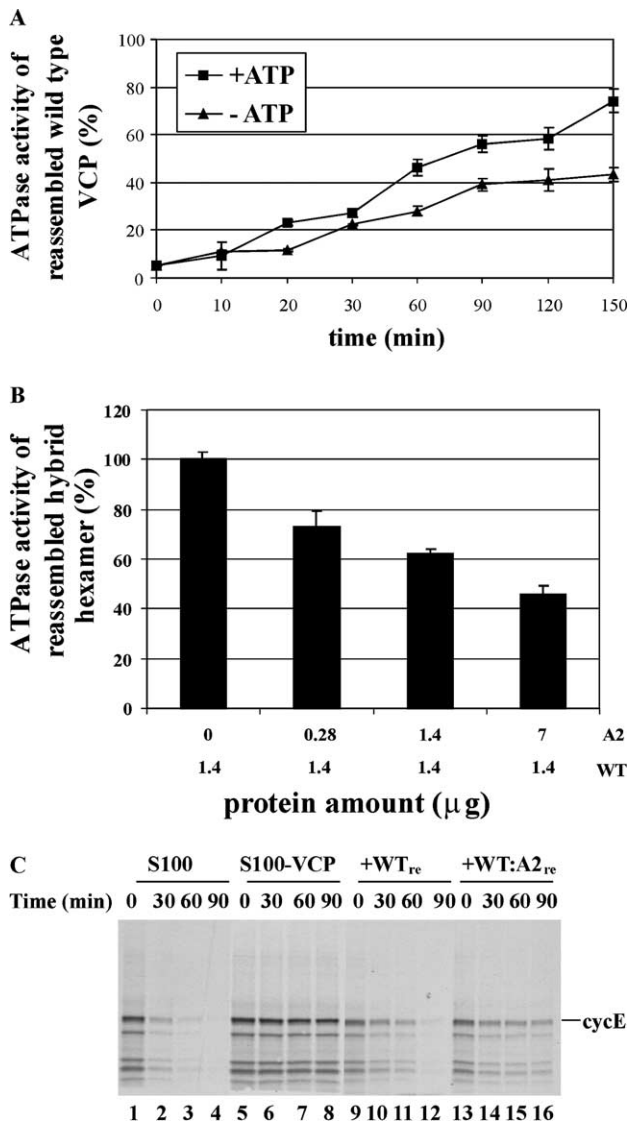
Fig. 6. Hexamerization and proper subunit interactions are required for the ATPase activity and biological function of VCP. (A) ATPase activity correlates with hexamerization. Dissociated wild-type VCP was reassembled in the presence or absence of 2 mM ATP for the various periods of time and the ATPase activity was assayed. The activity is presented as % of that of the untreated VCP. The representative experiment was performed in triplicates. (B) Proper subunit interactions of VCP are required for full ATPase activity. Different amounts of A2 mutant (indicated in μg) were added to a fixed amount (1.4 μg) of wild-type (WT) VCP and the mixture was subject to the dissociation–reassembly assay. The ATPase activity of the hybrid hexamer was assayed at 90 min after reassembly. The ATPase activity of the reassembled wild-type VCP is designated as 100%. The experiment was performed in triplicates and repeated twice. (C) Reassembled hexameric VCP can mediate ubiquitin–proteasome degradation. In vitro degradation assays were performed using radioactively labeled cyclin E as the substrate, and untreated or treated (as indicated) S100 as the source for enzymes of the ubiquitin–proteasome system. The reactions were terminated after the indicated periods of time and analyzed by SDS–PAGE, Western transfer, and autoradiography. Untreated S100 (S100, lanes 1–4), VCP-depleted S100 (S100-VCP, lanes 5–8), add back with the reassembled wild-type VCP (+WT$_{re}$, lanes 9–12), and add back with reassembled wild type and A2 hybrid at equal molar ratio (+WT:A2$_{re}$, lanes 13–16) are indicated.

results strongly suggest that only the hexameric form of VCP with full ATPase activity is biologically active and is necessary for supporting the ubiquitin–proteasome mediated degradation.

Discussion

Our studies show that VCP has a high propensity to form hexamer, which is essential for supporting the diverse functions of VCP. Harsh treatment with acid (pH 3), alkaline (pH 10), ethanol (30%), heat (65 °C), apyrase (an enzyme that destroys ATP and ADP), numerous detergents, and low protein concentration (0.01 mg/ml) has no impact on the hexameric structure (data not shown). These stress-tolerant properties of VCP underscore the nature of an important molecular chaperone. It is expected that VCP "self chaperones" its own refolding and reassembly under stress conditions, such that an essential hexameric structure is maintained.

Although hexamerization of VCP does not require the presence of nucleotides, nucleotide binding (Figs. 2–5), but not hydrolysis (Fig. 4B), accelerates the reassembly of urea-dissociated VCP. This is in agreement with a recent electron microscopy study that showed a major conformational change in p97-VCP upon ATP binding, and a relatively minor difference between the ATP- and ADP-bound forms [9]. Binding of nucleotide (ATP or ADP) likely allows VCP to quickly adopt a correct localized conformation. For example, nucleotide binding induces a conformational change which positions the hydrophobic residues involved in subunit interactions in the right context. The correctly positioned critical residues then facilitate the subsequent refolding and reassembly. Indeed, we found that hydrophobic interactions play a significant role in hexamerization of VCP. Urea-induced disassembly is more efficient when carried out on ice than at the room temperature (data not shown). Conversely, reassembly performed at 16 °C takes a much longer time than that at the room temperature (data not shown). This reduced interaction at lower temperature is one of the hallmarks of hydrophobic interactions [17].

While D1 and D2 of VCP share high sequence identity [18], they perform distinct functions. In this study, we show that mutation at the ATP-binding site in D1, but not D2, significantly delays the reassembly and abolishes the ATP-stimulatory effect (Fig. 5A). Thus, D1 plays a major role in hexamerization. Moreover, N-D1 intermediate accumulates in urea-dissociated VCP during trypsin proteolysis (Fig. 3), indicating that N-D1 is a more compact structure. While the less structured D2 ring is readily digested by trypsin, N-D1 serves as a structural core of VCP complex. In reassembly process, the more stable D1 domain probably serves as a nucleation site, from which subsequent refolding and physical

association can build on. This notion is consistent with the observation that N-D1 is relatively easier to crystallize than the full-length VCP [10].

Our recent experiments further showed that D2 is responsible for the major ATPase activity of VCP. Thus, similar to ClpA [19,20] and *T*ClpB [21], but opposite of NSF [22,23] and Hsp104 [24], D1 and D2 are major mediators for oligomerization and enzyme activity, respectively. Nevertheless, VCP variant with single mutation in D1 reassembles much more rapidly than the variant with mutation in both D1 and D2 (Figs. 5A and 4C). Thus, while D1 is the major mediator for oligomerization D2 may also play a minor role in the process.

Reassembled hexameric VCP is a functional ATPase and the proper interactions between subunits are also important for the full ATPase activity. Our reassembly experiment using the wild type/A2 mixed oligomers (Fig. 6B) indicated that once a defective D2 is incorporated into the hexamer, it practically "poisons" the neighboring subunits. This "poisoning effect" has at least two structural consequences: it eliminates the allosteric communication between adjacent subunits in the D2 ring and it also interferes the communication between D1 and D2 domains. As a result, a compromised ATPase activity is expected. The requirement for proper subunit interactions is evidenced in both the in vitro ATPase assay (Fig. 6B) and the functional degradation assay (Fig. 6C). Similar requirement has also been observed in other AAA proteins, such as Hsp104 [25], ClpA [20], and *T*ClpB [21]. The lower ATPase activity is also observed in wild type and B1B2 hybrid (data not shown), which explains the lethal effect in trypanosome when double hydrolysis mutant of TbVCP was overexpressed [26]. In conclusion, the integrity of VCP hexamer provides the structural basis for full ATPase activity, which fuels the chaperone functions.

The recently solved crystal structure of p97 N-D1 domains [10] showed significant interactions between subunits in the hexameric structure. Further biochemical and crystallographic studies of the full-length VCP and VCP complexed with substrates (such as ubiquitin chains) or adaptors will help to decipher the precise role of each domain and subunit during ATP binding–hydrolysis cycle, and to elucidate the molecular mechanism of this enzymatic machine.

Acknowledgments

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