ATPase Activity of p97-Valosin-containing Protein (VCP)

D2 MEDIATES THE MAJOR ENZYME ACTIVITY, AND D1 CONTRIBUTES TO THE HEAT-INDUCED ACTIVITY

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The 97-kDa valosin-containing protein (p97-VCP) plays a role in a wide variety of cellular activities, many of which are regulated by the ubiquitin-proteasome (Ub-Pr)-mediated degradation pathway. We previously demonstrated that VCP binds to multi-ubiquitin chains and may act as a molecular chaperone that targets the ubiquitinated substrates to the proteasome for degradation. In this report, we show that although the ubiquitin chain-binding activity, carried out by the N-terminal 200 residues (N domain), is necessary for the degradation of proteasome substrates, it is not sufficient. Using in vitro degradation assays, we demonstrated that the entire VCP molecule, consisting of the N domain and two ATPase domains D1 and D2, is required for mediating the Ub-Pr degradation. The ATPase activity of VCP requires Mg2+, and is stimulated by high temperature. Under optimal conditions, VCP hydrolyzes ATP with a $K_m$ of $-0.33$ mm and a $V_{max}$ of $-0.52$ nmol P$_i$ min$^{-1}$ $\mu$g$^{-1}$. At a physiological temperature, mutation in D2 significantly inhibits the ATPase activity, while that in D1 has little effect. Interestingly, mutations in D1, but not D2, abolish the heat-stimulated ATPase activity. Thus, we provide the first demonstration that the ATPase activity of VCP is required for mediating the Ub-Pr degradation, that D2 accounts for the major ATPase activity, and that D1 contributes to the heat-induced activity.

VCP† is a member of the AAA (ATPases associated with a variety of cellular activities) family, which comprises a highly conserved group of molecular chaperones that regulate many cellular functions (reviewed in Refs. 1–4). The family members are characterized by the presence of either one (type I) or two (type II) conserved ATPase domains, also referred to as AAA modules. The 200–250-amino acid AAA module encompasses the consensus Walker A (GGxxxGKx, x = any amino acid) and Walker B (hhhhDExx, h = hydrophobic residue) motifs (5) that are critical for ATP binding and hydrolysis, respectively. It is generally thought that the AAA proteins act as molecular chaperones to assemble or disassemble protein complexes, unfold or unwind macromolecules, and transport cellular cargoes. These chaperone or chaperonelike activities are carried out by the conserved ATPase domains, which provide the needed energy through ATP hydrolysis.

VCP is a type II AAA-ATPase. It is highly abundant, accounting for about 1% of the total cellular proteins. It is present in all types of cells in a hexameric ring structure and localizes in the nucleus, cytoplasm, and perimembrane subcompartments (6). VCP is one of the most evolutionarily conserved genes, sharing high sequence homology from man to yeast and archaeabacteria (6–9). The high abundance and conservation suggest that VCP plays an important and fundamental role in cellular activities. As other AAA proteins, VCP and its orthologs have been shown to play a role in many seemingly unrelated cellular activities. Remarkably, almost all these activities have been shown to be regulated by the Ub-Pr degradation pathway, suggesting that VCP may play a fundamental role in the pathway, which in turn regulates the diverse activities. We previously showed that VCP is involved in the degradation of a number of Ub-Pr substrates (10–12), physically associates with the 26 S proteasome (12, 13), and binds the ubiquitinated substrates directly through the mult ubiquitin chains (10). Using in vitro degradation assays, we showed that depletion of VCP from cell extracts resulted in an inhibition of VCP in the degradation of proteins associated with the endoplasmic reticulum (ER). In both Ub-Pr-dependent and ER-associated degradation, VCP is believed to dissociate the ubiquitinated substrates from protein complexes or ER membrane, and the dissociation is presumably powered by the ATPase activity of VCP. As Ub-Pr and ER-associated degradation is the underlying cause of many diseases, it is of high importance to study the detailed mechanism of VCP functions, especially its ATPase activity.

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The VCP molecule can be divided into three parts: the N-terminal 200 residues, named N domain, and two conserved ATPase domains, D1 and D2. We previously showed that the N domain is responsible for mult ubiquitin chain binding, and this activity is necessary in mediating the in vitro degradation (10). Whether this substrate binding activity is sufficient for mediating the Ub-Pr degradation is not known. In other words, whether the D1 and D2 ATPase domains of VCP are also
required in Ub-Pr-mediated degradation has not been determined. Most type II AAA members have one ATPase domain with high sequence conservation, and the other with divergent sequences. It has been shown in NSF (N-ethylmaleimide-sensitive factor), a prototype AAA protein, that the highly conserved D1 domain is responsible for the ATPase activity, while the less conserved D2 domain is responsible for oligomerization (22). Interestingly, both D1 and D2 domains of VCP are highly similar to each other as well as to the D1 of NSF (23), implicating that both have the potential of being an active ATPase. It is, therefore, of high interests to characterize the ATPase activity of VCP and to identify the specific functions of the two ATPase domains.

In this study, we performed in vitro degradation assays to show that, in addition to the substrate-binding N domain, the ATPase domains of VCP are also required in mediating the Ub-Pr degradation. We systematically characterize the ATPase activity of VCP and provide the first evidence that the two domains are not catalytically equal: D2 is responsible for the major enzyme activity at physiological temperature, whereas D1 is involved in the regulation of heat-induced ATPase activity.

EXPERIMENTAL PROCEDURES

Generation of VCP Mutants—All VCP mutants were derived from the wild type (WT) VCP-His, fusion construct. NDI (kindly provided by A. Shaw) (24) and E (10) deletion mutants, containing only the D1 and D2 ATPase domain, respectively, have been described previously. All the site-specific mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing. Basically, two types of mutants were made. A-type mutant harbors a mutation at the ATP-binding site in the Walker A motif where an invariant lysine is replaced with a threonine. B-type mutant harbors a mutation at the ATP hydrolysis site in the Walker B motif where a glutamic acid is changed to a glutamine. Mutations were introduced according to the manufacturer. The induced cells were lysed in lysis buffer containing 50 mM sodium phosphate, pH 7.4, 1 M NaCl, 20 mM imidazole, 10% glycerol, 10 mM glutathione (GSH), and 1 mM dithiothreitol (DTT). Repeated affinity purification was often performed to obtain the high-purity product. The eluted fractions were analyzed by SDS-PAGE, Western assay (Pierce) and analyzed by SDS-PAGE and Coomassie Blue staining.

Purification of His-tagged Fusion Proteins—The plasmids carrying the purified proteins were dialyzed against 20 mM Tris-HCl, pH 8.0, 0.5 μM vCP for 15 min at 37 °C. The inorganic phosphate released by ATP hydrolysis was measured as previously described (26, 27) with modifications. Briefly, VCP was added to 50 μl of assay buffer, the reaction was carried out at 37 °C for 15 min, then 400 μl of dye buffer containing 6 mM ammonium heptamolybdate, 120 μM malachite green, 0.06% polyvinyl alcohol, and 4.25% sodium citrate was added. After 20 min of incubation at room temperature, 200 μl from each reaction was transferred to a 96-well plate, and the absorbance at 630 nm was measured using an ELx800 Universal Microplate Reader (Bio-Tek Instrument). Reactions performed without VCP were routinely included, and the activity, usually negligible, was subtracted from the respective experimental data. The inorganic phosphate released was calculated based on the absorbance standard curve established by KH₂PO₄ standards. VCP variants and His-NSF (28) were affinity-purified to near homogeneity (Fig. 1C, and E) and used as the test substrate. Untreated S100 (50 μg of recombinant VCP-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.

**ATPase Assay—**Except for specified variations, standard ATPase assays were carried out in the assay buffer containing 50 mM Tris-HCl (pH 8.0), 20 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 3 mM ATP, and 0.5 μM vCP for 15 min at 37 °C. The inorganic phosphate released by ATP hydrolysis was measured as previously described (26, 27) with modifications. Briefly, VCP was added to 50 μl of assay buffer, the reaction was carried out at 37 °C for 15 min, then 400 μl of dye buffer containing 6 mM ammonium heptamolybdate, 120 μM malachite green, 0.06% polyvinyl alcohol, and 4.25% sodium citrate was added. After 20 min of incubation at room temperature, 200 μl from each reaction was transferred to a 96-well plate, and the absorbance at 630 nm was measured using an ELx800 Universal Microplate Reader (Bio-Tek Instrument). Reactions performed without VCP were routinely included, and the activity, usually negligible, was subtracted from the respective experimental data. The inorganic phosphate released was calculated based on the absorbance standard curve established by KH₂PO₄ standards. VCP variants and His-NSF (28) were affinity-purified to near homogeneity and used in the assays. All assays were repeated at least three times, and the average activities with standard errors of measurement (± S.E.) were presented. Kinetic parameters, Kₘ, Vₘₐₓ, and Hill coefficient, were derived using the software Prism 3.0 (GraphPad Software, Inc.).

**RESULTS**

**Purification of Wild Type and Mutant VCP-His Proteins—**We generated a panel of VCP mutants in the form of His fusion proteins (Fig. 1A, also see Fig. 5 for gene structure). These include full-length, site-specific mutants and deletion mutants containing only the D1 (ND1 construct) or D2 (E construct) ATPase domain. Among the site-specific mutants, mutations were introduced into the Walker A or Walker B motif of each or both of the ATPase domains. In naming these mutants, alphabetical A or B designates the mutation in Walker A or B motif, while numerical 1 or 2 indicates the location of the respective mutation in the first or second ATPase domain. All mutants were confirmed by DNA sequencing, and all VCP-His fusion variants were expressed in E. coli and affinity-purified to near homogeneity (Fig. 1A).

**Requirement for ATPase Activity of VCP inUb-Pr-mediated Degradation—**We previously showed that the N domain of VCP directly binds to the multiquitin chain of proteasome substrates and is necessary for mediating the Ub-Pr degradation (10). In this study, we performed in vitro degradation assays, which used the S100 fraction isolated from cell extracts as the Ub-Pr enzyme source and in vitro synthesized cyclin E as the test substrate (10). As previously demonstrated, when VCP was depleted from S100, cyclin E degradation was inhibited, whereas adding back the recombinant WT VCP restored the degradation (Fig. 1B). In contrast, when the substrate-binding N domain (N200) and the site-specific mutants (A1A2, B1B2, and A2) were used in the reconstitution, no degradation was restored (Fig. 1B). These results indicate that although the substrate binding activity is necessary for an effective Ub-Pr degradation, it is not sufficient. Functions contributed by the two ATPase domains, D1 and D2, are also required. Interestingly, although adding back mutant A1 did not fully restore the degradation, it reproducibly resulted in significant restoration (Fig. 1B). These data suggest that A1 mutant may still possess partial activity in mediating the degradation. Based on the higher restoration obtained in A1 than in A2, we speculated that D2 domain may contribute more to the ATPase activity of VCP.

**Characterization of the ATPase Activity of VCP—**To directly study the ATPase activity, a colorimetric assay was used to measure the inorganic phosphate (P₀) released from ATP hydrolysis. Because recombinant VCP exhibited comparable ac-

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**ATPase Activity of p97-VCP**

**Characterization of the ATPase Activity of p97-VCP**

**Requirement for ATPase Activity of VCP in Ub-Pr-mediated Degradation**

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**RESULTS**

**Purification of His-tagged Fusion Proteins—**The plasmids carrying VCP variants were transferred into Escherichia coli strain M15[pREP4] (Qiagen), and protein expression was induced according to the manufacturer. The induced cells were lysed with lysis buffer containing 50 mM sodium phosphate, pH 7.4, 1 M NaCl, 20 mM imidazole, 10% glycerol, 10 mM β-mercaptoethanol, and protease inhibitor mixture for His-tagged proteins (Sigma, number P8849). The cleared cell lysates were mixed with nickel-nitrioltriacetic acid agarose beads for 1 h at 4 °C, and the fusion proteins were step-eluted with the elution buffer (lysis buffer containing 50, 100, 250, or 500 mM imidazole). Repeated affinity purification was often performed to obtain the high-purity product. The eluted fractions were analyzed by SDS-PAGE, and the purified proteins were dialyzed against 20 mM Tris-HCl, pH 8.0, with 10% glycerol. His-NSF fusion protein was expressed and purified with similar procedures. The purified proteins were quantified by BCA assay (Fierce) and analyzed by SDS-PAGE and Coomassie Blue staining.

**In Vitro Degradation Assay—**In vitro Ub-Pr-mediated degradation assays were performed as previously described (10, 12). Human cyclin E was synthesized in wheat germ lysate-based in vitro transcription/translation system (Promega) in the presence of [³²P]methionine/ cysteine and used as the test substrate. Untreated S100 (50 μg of protein) isolated from actively growing CA46 cells (25) and differentially treated S100 preparations were used as the enzyme sources. VCP depletion was carried out by subjecting S100 to immunoprecipitation with equal volume of VCP-4 antiserum (12). In a typical VCP reconstitution experiment, 1–4 μg of recombinant VCP-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.

**ATPase Assay—**Except for specified variations, standard ATPase assays were carried out in the assay buffer containing 50 mM Tris-HCl (pH 8.0), 20 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 3 mM ATP, and 0.5 μM vCP for 15 min at 37 °C. The inorganic phosphate released by ATP hydrolysis was measured as previously described (26, 27) with modifications. Briefly, VCP was added to 50 μl of assay buffer, the reaction was carried out at 37 °C for 15 min, then 400 μl of dye buffer containing 6 mM ammonium heptamolybdate, 120 μM malachite green, 0.06% polyvinyl alcohol, and 4.25% sodium citrate was added. After 20 min of incubation at room temperature, 200 μl from each reaction was transferred to a 96-well plate, and the absorbance at 630 nm was measured using an ELx800 Universal Microplate Reader (Bio-Tek Instrument). Reactions performed without VCP were routinely included, and the activity, usually negligible, was subtracted from the respective experimental data. The inorganic phosphate released was calculated based on the absorbance standard curve established by KH₂PO₄ standards. VCP variants and His-NSF (28) were affinity-purified to near homogeneity and used in the assays. All assays were repeated at least three times, and the average activities with standard errors of measurement (± S.E.) were presented. Kinetic parameters, Kₘ, Vₘₐₓ, and Hill coefficient, were derived using the software Prism 3.0 (GraphPad Software, Inc.).
tivity as purified cellular VCP (data not shown), wild type VCP-His fusion protein (WT in Fig. 1A) was used to characterize the enzyme activity. As shown in Fig. 2A, wild type VCP hydrolyzes ATP in a time-dependent manner, with free phosphate increasing linearly in the initial 30 min. The reaction is dependent on the concentration of ATP, and the enzyme activity is saturated when ATP is greater than 3 mM (Fig. 2B). The kinetics were modeled using Enzyme Kinetics template from software Prism 3.0 (GraphPad Software, Inc.). In the presence of 20 mM magnesium, VCP exhibited a max of 0.52 nmol Pi min$^{-1}$ mg$^{-1}$ of protein, a V$^{-1}$ of 0.52 nmol Pi min$^{-1}$ mg$^{-1}$, and a Hill coefficient of 1.2. Since VCP almost always adopts a stable hexameric ring structure (data not shown), the derived Hill coefficient probably suggests a cooperative nature in hydrolyzing ATP among the oligomers rather than a Mg$^{2+}$ and Co$^{2+}$ at low concentrations (Fig. 2E). Titration experiments further determine the optimum concentration for Mg$^{2+}$ to be 20 mM (Fig. 2F). Notably, at concentrations greater than 20 mM, the ATPase activity is markedly suppressed. This probably reflects a general inhibitory effect of the high salt concentration rather than a Mg$^{2+}$ specific phenomenon. The presence of NaCl (Fig. 2G) and KCl (data not shown) actually suppresses the ATPase activity, but does not significantly change the hexameric structure of VCP (data not shown). Based on the initial characterization of the ATPase reaction, 50 mM ATP, 3 mM Mg$^{2+}$, and a 15-min reaction time were routinely used in the rest of the study.

Heat-induced ATPase Activity—Recent studies have suggested an involvement of VCP/orthologs in stress tolerance pathways (15, 16, 18, 21, 29). We analyzed the in vitro ATPase profile of VCP under various conditions reflecting environmental stresses (see “Discussion”). Interestingly, the activity increased significantly at elevated temperatures and peaked between 50–55 °C (Fig. 2H), a temperature at which most enzymes would be inactivated. The observation is not unique to the low salt concentration (open circles) because experiments performed under physiological ionic strength condition also yielded similar results (filled squares). Comparison between the enzyme kinetics at 37 and 50 °C (Fig. 2I) revealed an ~60% increase in V$^{-1}$, a small decrease in K$^{-1}$ for ATP, and an ~35% increase in the Hill coefficient.

D2 as the Major ATPase Domain—The high sequence similarity between D1 and D2 and the sigmoid kinetic curve in VCP suggest that both D1 and D2 contribute to the ATPase activity. It has been shown in several type II AAA members that, although not in specific order, one domain is essential for forming the oligomers and the other responsible for the major enzyme activity. Since such structure-function relationship has not been demonstrated for VCP, we used the mutants to determine which domain is responsible for the major ATPase activity at physiological temperatures (Fig. 3A and summarized in Fig. 5). As negative controls, double mutants harboring mutations in ATP-binding sites (A1A2) or ATP hydrolysis sites (B1B2) in both domains exhibit near background ATPase activity. A1, B1, and A1B1 mutants, all containing a mutated D1 and an intact D2, exhibit an activity slightly lower than the wild type. By contrast, A2, B2, A2B2, and ND1 mutants, all containing an intact D1 but a mutation in D2, have a low but detectable activity. Moreover, deletion mutant E, which contains only the D2 and no D1 domain, displays a reduced but significant ATPase activity. Together these results suggest that the second ATPase domain, D2, is responsible for the major ATPase activity, a conclusion consistent with the in vitro degradation experiments (Fig. 1B).

NEM Inhibition of the ATPase Activity of D2—Another line of evidence supporting D2 as the major ATPase is provided by its sensitivity to N-ethylmaleimide (NEM), a cysteine-sensitive alkylating agent. It has been shown that VCP orthologs as well as homologous proteins, such as NSF, SEC18, etc., possess NEM-sensitive ATPase activity (6, 30, 31). As shown in Fig. 3B,
Fig. 2. Characterization of the ATPase activity of VCP. A, time dependence of ATPase activity of VCP. ATP hydrolysis was carried out at 37 °C for the indicated time (min). The released inorganic phosphate was quantified by malachite green assay. Activities were taken from the average of three independent experiments. B, ATPase activity of VCP at various ATP concentrations. Standard ATPase assay (as described under "Experimental Procedures") was carried out using 0.5 μM VCP and the indicated concentration of ATP. C, comparison of ATPase activity of VCP and NSF. Equal amount (0.5 μM) of VCP-His or His-NSF fusion protein was used in the standard in vitro ATPase assay. D, ATPase activity at various concentrations of VCP. Standard assay was carried out using the indicated amount of wild type VCP. E, requirement of divalent cations for the enzyme activity. ATPase activity was measured in the presence of different divalent cations at a concentration of either 2 mM (filled bar) or 20 mM (open bar). Control (Ctrl), no cation included. F, determination of the optimal Mg2+ concentration for ATPase activity. Standard assay was performed in the presence of indicated concentration of MgCl2. Note that the concentrations below and above 50 mM are presented in different scales. G, inhibitory effect of salt on ATPase activity. The ATPase activity was measured in the presence of the indicated concentration of NaCl at 37 °C. H, temperature effect on the ATPase activity of VCP. The enzyme activity was measured at the indicated temperatures with either 0 (open circle) or 135 mM NaCl (filled square). I, kinetic study at different temperatures. Reactions were carried out in the presence of varying amounts of ATP at either 37 (open circle) or 50 (filled circle) °C. The data represent an average of three independent experiments.
NEM markedly reduced the activity of wild type, A1, B1, A1B1, and E variants, all of which contain an intact D2 domain. For those variants containing only the wild type D1 domain (A2, B2, A2B2, and ND1), NEM did not further lower their activities. It is known that NEM sometimes causes proteins to be denatured and therefore inhibits ATPase activity. This is not the case for VCP, because gel filtration analysis showed that purified VCP variants (as shown in Fig. 1A) were not denatured and therefore inhibits ATPase activity. This is not the case because the ATPase assay using NSF preincubated with ATP (thus preassembled hexamer) did not yield significantly higher activity. It is likely that the presence of ATP rapidly facilitates the hexamerization of NSF in our assay. While both VCP and NSF play an essential role in mediating membrane fusion, it is not clear how the difference in ATPase activity translates into functional significance. Nevertheless, it is tempting to speculate that the involvement of VCP in the Ub-Pr pathway requires a higher enzyme activity than that in the membrane fusion. This is supported by the observation that p47, a cofactor required in membrane fusion events but not in Ub-Pr degradation, suppresses the enzyme activity of VCP.

The chaperone activity of AAA family proteins is often modulated by cellular environment. We tested the ATPase activity of VCP under conditions reflecting environmental stresses, including heat, pH, osmotic, alcohol, and oxidative stresses. We found that the activity is stimulated by high pH values, but inhibited by the presence of salt (Fig. 2G), ethanol, ADP, and hydrogen peroxide (data not shown). Most interestingly, the activity is significantly stimulated by high temperature (Fig. 2, H and I), peaking around 50–55 °C. Since most enzymes are inactivated at such temperature, the stabilization of VCP may be attributed to its own chaperone activity, namely the prevention of heat-induced denaturation of substrates (itself in this case) (34).2

Because of the essential nature and high conservation of VCP, it is important to characterize the specific functions of each domain. In this study, we report the first structure-func-
tion analyses in VCP. Our conclusion of D2 as the major ATPase domain is supported by several lines of in vitro evidence: 1) deletion and site-specific mutants containing only the intact D2 exhibit significant activity; 2) mutants lacking an intact D2 have severely reduced activity; 3) sequence comparison among VCP, NSF, and FtsH (a type I AAA-ATPase) reveals that the "Second Region of Homology" (35) in D2, but not in D1, of VCP shares high sequence similarity with those in the identified ATPase domain of NSF and FtsH; 4) depletion and add-back experiment shows that A1, but not A2, mutant restores the Ub-Pr-mediated degradation significantly; and 5) our recent studies further indicate that D1 of VCP plays a critical role in mediating hexamerization of VCP.3 The importance of D2 has also been demonstrated in in vivo studies. A yeast mutant harboring a point mutation in D2 exhibited typical apoptotic phenotypes (36). Expression of VCP with D2 mutation (equivalent to our A2 mutant) created massive cytoplasmic vacuoles and cell death in a neurodegenerative disease model (37). Moreover, a recent study on trypanosome showed

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that D2, but not D1, is essential for TbVCP activity and cell viability (38).

The identification of D2 as the major ATPase domain raised the question of how other domains of VCP influence the enzyme activity. Previous reports show that tyrosine phosphorylation at the C terminus of VCP correlates with nuclear translocation in a cell cycle-specific manner (39) and that phosphorylated VCP promotes its own release from associated membranes (40). These findings seem to suggest that the C-terminal domain of VCP may also regulate its biological/enzymatic functions. Nevertheless, mutation of these phosphorylation sites did not affect the ATPase activity (Ref. 41 and data not shown). Physical binding of specific antibody to the C terminus of VCP also did not impair the ATPase activity (data not shown). Therefore, tyrosine phosphorylation and the conformation of the C terminus of VCP likely do not play a significant role in regulating its ATPase activity. However, our recent trypsin digestion study suggested that the C-terminal ~100 residues of VCP form a loosely structured tail.3 Thus, it is formally possible that association of this relatively flexible C terminus with other cellular proteins could modify the overall enzyme activity of VCP.

Does the D1 oligomerization domain affect the ATPase activity at physiological temperature? It has been established in several AAA proteins that an oligomeric structure is a prerequisite to the ATPase activity and that these proteins require the presence of nucleotide to form oligomers (22, 33, 42, 43). Consistently, monomeric VCP does not have any enzyme activity (44).3 However, in contrast to these AAA proteins, VCP does not require the presence of nucleotide to form hexamers.3 The hexameric VCP is highly stable, and many attempts have failed to dissociate it into stable monomers. This unusually high preponderance to form functional hexamers in VCP further emphasizes its essential nature in the cell. This can also be observed in our mutants, A1, B1, and A1B1, all containing mutations in D1 yet still appearing to be hexamers.3 Nevertheless, these mutations do introduce conformational alterations to the ring structure. A lower ATPase activity in these mutants (relative to the wild type) (Fig. 3A) likely resulted from the lacking of a perfect hexameric structure and a proper intersubunit communication. Thus, under physiological conditions, the D1 domain plays a minor role in the steady-state kinetic behavior of the ATPase domain.

In contrast to the physiological condition, D1 probably plays an important role in mediating an elevated activity in heat-stressed condition (Figs. 4 and 5). As the wild type VCP exhibits its heat-induced activity, which peaks around 50 °C, mutation in D1 abolishes this heat-inducibility. On the other hand, mutants containing a mutated D2 and an intact D1 consistently exhibit a low activity at 37 °C but a significantly higher activity at a temperature greater than 50 °C. It is noted that when comparing the activity of the wild type with that of the sum of A1 and A2, the former is always greater than the latter, especially in the high temperature range (data not shown). This observation suggests that D1 and D2 rings communicate and cooperate with each other in hydrolyzing ATP (as discussed above), and this cooperative effect is more profound at elevated temperature. While the molecular basis for this heat response is unknown, we speculate that the structure of the two hexameric rings plays an important role. Previous studies revealed that it is relatively easy to obtain crystal structure of the oligomerization domain of type II AAA proteins, probably because the ring made up from such domains adopts a more stable and compact structure. In contrast, to this date, there is no crystal structure available for the ATPase domain, likely due to its more flexible and relaxed conformation. Since VCP undergoes significant conformational changes during ATPase cycles (45), it is conceivable that upon heat treatment, the originally accessible D1 ring expands and becomes more accessible to the solvent, thus facilitating ATP hydrolysis more readily.

Based on the heat-induced ATPase activity, VCP may be qualified as a heat-shock protein, and may have heat-stimulated chaperone activity which directly mediates the heat-shock responses. Interestingly, Hsp104, a two-domain-containing AAA super family chaperone, has been characterized to have many biochemical properties similar to those of VCP (46). The ATPase activity of Hsp104 is strongly influenced by factors that vary with cell stress, e.g., temperature, pH, and ADP. It is crucial for stress tolerance in Saccharomyces cerevisiae, and both of its ATPase domains are required in mediating the stress responses. Hsp104 is essential in protecting cells at extreme temperatures, e.g., 50 °C, and this protection is related to its ability to promote the solubilization and reactivation of damaged and aggregated proteins (47, 48). So far, no mammalian Hsp104 proteins have been found. Based on the importance of Hsp104 and the similar biochemical properties observed in the two chaperones, it will be of profound interest to examine whether VCP possesses similar biological functions.

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