Multifunctional Roles of the Conserved Arg Residues in the Second Region of Homology of p97/Valosin-containing Protein*

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The 97-kDa molecular chaperone valosin-containing protein (VCP) belongs to a highly conserved AAA family and forms a hexameric structure that is essential for its biological functions. The AAA domain contains highly conserved motifs, the Walker A, Walker B, and the second region of homology (SRH). Although Walker A and B motifs mediate ATP binding and hydrolysis, respectively, the function of the SRH in VCP is not clear. We examined the significance of the SRH in VCP, especially the conserved Arg359 and Arg362 in the first AAA domain, D1 and Arg635 and Arg638 in the second AAA domain, D2. We show that Arg359 and Arg362 in D1 are critical for maintaining the hexameric structure and the ability to bind the ubiquitin chains. Although the rest of the tested SRH mutants retain the hexameric structure, all of them exhibit severely reduced ATPase activity. Tryptophan fluorescence analysis showed that all of the tested mutants can bind to ATP or ADP. Thus, the reduced ATPase activity likely results from the hampered communications among protomers during hydrolysis. Moreover, when the ATPase-defective mutant R635A or R638A is mixed with the Walker A mutant of D2, the ATPase activity is partially restored, suggesting that Arg635 and Arg638 can stimulate the ATPase activity of the neighboring protomer. Interestingly, mutation of Arg359 and Arg362 uncouples the inhibitory effect of p47, a VCP co-factor, on the ATPase activity of VCP. Therefore, the Arg residues allow D1 to take on a specific conformation that is required for substrate binding and co-factor communications. Taken together, these results demonstrate that the conserved Arg residues in the SRH of both D1 and D2 play critical roles in communicating the conformational changes required for ATP hydrolysis, and SRH in D1 also contributes to substrate binding and co-factor communications.

The AAA⁺ (ATPases associated with a variety of activities) family members act as molecular chaperones in many cellular activities in which they use the energy generated from ATP hydrolysis to remodel the structure of the “client” proteins. These AAA⁺ proteins frequently form an oligomeric complex, with a ring-shaped structure, that provides the mechanical basis for the enzymatic and biological activities. The AAA family proteins are characterized by having either one or two ATPase domains also called the AAA domains. The ~250-amino acid ATPase domain contains highly conserved sequence motifs, such as Walker A and Walker B motifs that are responsible for ATP binding and ATP hydrolysis, respectively. In addition, the second region of homology (SRH), is only present in a subfamily of AAA⁺, named the AAA family (Ref. 1; reviewed in Refs. 2 and 3).

The 97-kDa VCP (named p97 or VCP in animal or Cdc48 in yeast) is an ancient member of the AAA family and is present ubiquitously in all cells at a high level. Like other AAA proteins, VCP plays a critical role in many seemingly unrelated cellular activities, such as membrane fusion, cell cycle regulation, stress response, programmed cell death, B and T cell activation, endoplasmic reticulum-associated degradation, and protein degradation (reviewed in Refs. 3–10). Interestingly, all of these activities have been shown to be regulated, directly or indirectly, by the ubiquitin-proteasome degradation pathway. It suggests that VCP may play a fundamental role in the degradation pathway that underlies all of these seemingly unrelated functions (4). The VCP molecule comprises an N-terminal domain, two AAA domains (D1 and D2), and a C-terminal domain. We previously showed that the entire VCP molecule is required for the ubiquitin-proteasome-mediated degradation of cyclin E in vitro (11) and the N-terminal domain binds to the polyubiquitin chain and is responsible for substrate recognition (11). In agreement with the diverse functions of VCP, it has been shown that in addition to the polyubiquitin chains the N-terminal domain can bind to a number of co-factors and membrane proteins, including p47 (12), Ufd1 (13), VCP135 (12), SVIP (14), and VIMP (15). In most of the proteins containing two AAA domains, only one of the domains is highly conserved, referred to as the bona fide AAA domain. Interestingly, unlike most of these family members, VCP possesses two bona fide AAA domains. Despite sharing a high sequence similarity, the two domains are not functionally equal. Although D2 is responsible for the major enzymatic activity at physiological temperature, D1 mediates a heat-enhanced ATPase activity (16). Our recent study further showed that D1 (residues 208–459) plus the linker region (residues 460–481) between D1 and D2 is responsible for the nucleotide-independent hexamORIZATION. During the ATPase cycle, D1 stays as a relatively compact and protease-resistant hexameric ring, but D2 only does so when it binds to nucleotides (17). After ADP is released at the end of the ATP hydrolysis, the D2 ring is destabilized and adopts a relatively flexible and open structure (17).

The consensus SRH sequence in the AAA family proteins, X(T/S)(N/
VCP SRH Critical for Hexameric Structure and ATP Hydrolysis

S. XXXXX DAXXXRXXRX(D/E), contains two perfectly conserved Arg residues (18). A few available studies revealed that the conserved Arg residues play different roles in various AAA proteins. In FtsH, a protease containing a single AAA module in prokaryotes, Arg312 and Arg315 in the SRH are crucial for ATP hydrolysis but not for nucleotide binding (19). A different function is observed in NSF, a chaperone containing two AAA modules involved in membrane fusion events. In its bona fide ATPase domain D1, the conserved Arg359 and Arg362 in the SRH are not required for ATPase activity but rather serve as nucleotide state sensors (20). This again demonstrates the confusing contrast between the extreme sequence conservation within the domain and the diversity of function of the AAA proteins. Because VCP has two conserved AAA domains, it would be interesting to study the function of the SRH in each domain.

Available structural studies of AAA proteins suggest that SRH resides at the interface between the neighboring protomers and the two conserved Arg residues in SRH are of crucial importance. The N-terminal crystal structure of VCP showed that Arg359 is available for interacting with the terminal phosphate of the bound nucleotide and that Arg362 forms a salt bridge with Glu306 from a neighboring protomer (21). Thus, Arg359 and Arg362 are considered the trans-element of the ATP-binding pocket of the D1 domain. The orientations of these two arginines are significantly different in the crystal structures obtained from ND1 and full-length constructs (21, 22). Because the two structures are crystallized at different nucleotide states, whether these two Arg residues are functionally interchangeable during the ATPase cycle is not clear. Recently, DeLaBarre and Brunker (23) reported that Arg359 can interact with the terminal phosphate of ADP bound to the neighboring protomer. Moreover, the corresponding Arg residues in the D2 domain, Arg363 and Arg368, are not well ordered in the full-length crystal structure (22). Previous studies on the GTPase-activating proteins identified an "arginine finger" that can stimulate the GTPase activity of GTP-binding protein (24). Similarly, the conserved Arg residues in VCP may act as arginine fingers with respect to the ATPase activity of VCP. Therefore, the functional roles of these conserved elements in mediating the activities of VCP need to be investigated.

The published mutagenesis studies of VCP have focused on the alterations in Walker A and B motifs, which are required for ATP binding and hydrolysis, respectively. However, the completion of the ATPase cycle is far more complicated than the steps of nucleotide binding and hydrolysis. To better understand how the communication between adjacent protomers influences the enzymatic and biological activities of the hexameric VCP complex, it is necessary to examine the function of the SRH in VCP. In this report, we mutated the conserved Arg residues in the SRH of D1 (Arg359 and Arg362) and D2 (Arg363 and Arg368) and assessed the impact of these mutations on the functions of VCP such as hexameric structure, ATPase activity, nucleotide binding, substrate binding, and co-factor communication. We found that the Arg residues are critical for maintaining the hexameric structure and ATP hydrolysis but not for ATP binding, and they likely play a role in communicating between protomers. The His-tagged wild type and mutant VCP proteins were purified as previously described (17). To generate the hybrid hexamer constituting K524A and SRH mutants, we first generated K524A-Tag100 construct. Wild type VCP was cloned into pQE-100 vector (Qiagen) and a Lys to Ala mutation was introduced to obtain the pHis-K524A-100 construct. Then the N-terminal His tag was removed using the mutagenesis kit with the following primer: 5’-CATTTAAAAGGAAAATTTAAC-TATGGCCTCTGGAGCAGTTTC (the coding sequence of VCP is underlined, and the complementary strand is not shown). The resulting pK524A-Tag100 was co-transformed with either pR635A-His or pR638A-His into M15, and the expressed VCP protein (presumably heterohexamer) was purified with Ni-NTA-agarose. The identification of the heterohexamer was confirmed by Western blot with anti-Tag100 antibody (Qiagen).

Oligomeric Status Analysis—The oligomeric status of VCP variants was analyzed by both native gel electrophoresis using 10% Tris-glycine gel (Invitrogen) and gel filtration chromatography using Superose 6 column (Amersham Biosciences) (17). The fractions collected from the gel filtration column were resolved on SDS gels. VCP proteins on the native gel or the SDS gel were electrophoretically transferred to the polyvinylidene difluoride membrane and detected by Western blot analysis as described previously (17).

ATPase Activity Assay—The ATPase activity of the wild type and VCP mutants were carried out as previously described (16). To test the allosteric effect of the Arg in SRH on ATPase activity, VCP heterohexamers containing the His-tagged SRH mutants and the Tag100-fused K524A were used in the ATPase assay. To test the effect of p47 on the activity of VCP, p47 (2.5 µg) and VCP variants (2.5 µg each) were incubated in the reaction buffer for 15 min at room temperature prior to the standard ATPase assay.

Fluorescence Spectroscopy—Fluorescence spectra of tryptophan were measured with a Jasco FP6500 spectrometer equipped with a microcell (Jasco Inc., Japan). The excitation wavelength was set at 295 nm, and the tryptophan emission was monitored between 305 and 450 nm with bandwidths of 3 and 5 nm, respectively. The concentration of the test proteins was approximately 100 µg/ml (1 µM). The experiments were carried out at room temperature in a buffer containing 50 mM Tris-HCl, pH 7.5, 80 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM MgCl2, and 4 mM nucleotide (if desired).

p47 Binding Assays—The GST-p47 expressed from plasmid of pGEX-2T-p47 (a gift from Dr. Mitsuo Tagaya, Tokyo University of Pharmacy and Life Science, Tokyo, Japan) on glutathione-Sepharose 4B beads was incubated with individual VCP-His variants at 4°C for 2 h with gentle rotating in the binding buffer (20 mM Tris-HCl, pH 7.9, 150 mM NaCl, 10 mM MgCl2, 5 mM KCl, 0.05% bovine serum albumin, 0.5% gelatin, 0.1% Tween 20, 0.25 mM dithiothreitol, and 4 mM ATP) (11). The beads were sedimented in a microcentrifuge and washed three times with the same buffer. The complex pulled down with the beads was analyzed by SDS-PAGE, Western transfer, and immunoblotting with a VCP antiserum recognizing the C terminus of VCP (25).

Polyubiquitin Chain Binding—The polyubiquitin chain (Affiniti Research Products Ltd., U88860) binding assay was carried out as described previously (11). All of the reactions contained 4 mM ATP, and the reaction components were separated onto 4–20% Tris-glycine gel (Invitrogen).

Western Blotting—Western blot analysis was performed as described previously with a 1:1,000 dilution of the antibodies specific to VCP C terminus (25), ubiquitin (DAKO), His (Qiagen), and Tag100 (Qiagen).

MATERIALS AND METHODS

Mutagenesis and Protein Purification—Site-specific mutants were generated with the QuikChange site-directed mutagenesis kit (Stratagene) using wild type pVCP-His (11) or p[1–481]-His (17) as the templates. All of the mutations were confirmed by DNA sequencing. All of the VCP variants were cloned into His6 fusion expression vector pQE60 and expressed as His6 fusion proteins (through the C terminus of VCP) in Escherichia coli strain M15 (pREP4) (QIAexpress system; Qiagen).
**RESULTS**

*Generation and Purification of SRH Mutants of VCP—*Sequence alignment shows that several amino acids in the SRH, including Asn, Asp, and two Arg residues, are almost 100% conserved among the AAA family proteins (Fig. 1A). Crystallographic studies showed that the two Arg residues in SRH are *trans*-elements for the ATP-binding pockets of VCP. We therefore generated a variety of VCP mutants (summarized in Fig. 1B), including the full-length VCP variants and the truncated [1–481] variants, all of which harbor single or double mutations at the conserved Arg residues in the D1 (Arg359 and Arg362) or D2 (Arg635 and Arg638) domain. Specifically, the positively charged Arg residue was systematically mutated to a similarly charged Lys residue (Arg to Lys), or a neutral Ala (Arg to Ala) or a negatively charged Glu (Arg to Glu). For comparison, two additional conserved residues in SRH, Asn348 and Asp354, which are *cis*-elements of the ATP-binding pocket of D1 domain, were also mutated for the study. All of the site-specific mutations were confirmed by DNA sequencing. All of the variants were expressed as fusion proteins with a His6 tag fused at the C terminus of VCP. The expressed proteins were subjected to consecutive affinity chromatography and purified to apparent homogeneity (partially shown in Fig. 1C).

*Arg to Glu Mutations in the SRH of D1, but Not D2, Disrupt the Hexameric Structure of VCP—*To examine the functional roles of the SRH in VCP, we first studied the oligomeric status of the double
mutants, in which both Arg are changed to Glu. In gel filtration analysis, although almost all of the D2 double mutant (R635E/R638E) was detected in a hexameric form, D1 mutant (R359E/R362E) was predominantly in a monomeric form or in an oligomeric form smaller than hexamers (Fig. 2A and summarized in Fig. 1B). In accordance, the native gel electrophoresis showed that the D1 mutant was detected in three types of complexes corresponding to hexamers, trimers, and monomers (Fig. 2B, lane 3), whereas the D2 mutant was present predominantly as hexamers (lane 2). We then asked whether changing Arg to an uncharged Ala or a similarly charged Lys impacts the hexamer stability. As shown in Fig. 2C, mutating Arg to Ala in all four conserved positions, in the context of either single or double mutations, did not affect the hexamerization. Similar results were obtained with Lys substitution (data not shown). Together, these results demonstrate that 1) the Arg residues in the SRH of D1, but not D2, are critical for hexamerization, 2) substitution of Arg with Ala or Lys at any of the four positions does not impact the overall hexameric structure, and 3) only changing Arg to Glu at both positions 359 and 362 in D1 disrupts the hexamer.

**Arg<sup>362</sup> Is More Important than Arg<sup>359</sup> in Maintaining the Hexameric Structure**—To determine whether Arg<sup>359</sup> and Arg<sup>362</sup> are of equal importance in maintaining the structure, we further examined the oligomeric status of the single mutants, R359E and R362E. As shown in Fig. 3A, the hexamer was partially disrupted in R362E but intact in R359E mutant (also summarized in Fig. 1B). This result suggests that although both Arg<sup>359</sup> and Arg<sup>362</sup> are trans-elements in the ATP-binding pocket, Arg<sup>362</sup> is more important in holding the hexamer together.

We previously reported that the first 481 amino acids of VCP ([1–481]), comprising the ND1 domain and the subsequent 20-amino acid linker, are sufficient to form hexamers in the absence of nucleotides (17). To further substantiate the significance of Arg<sup>359</sup> and Arg<sup>362</sup> in hexamerization, the Arg to Ala substitutions were introduced into [1–481], designated [1–481]R359A and [1–481]R362A. Because VCP can form hexamers in the absence of nucleotides, a similar mutation at the ATP-binding site Lys<sup>251</sup> was also generated ([1–481]K251A) for comparison. As revealed by the native gel electrophoresis, [1–481] and the majority of [1–481]K251A migrated as hexamers, but [1–481]R359A and [1–481]R362A failed to form a hexamer (Fig. 3B). Again, the oligomeric structure was disrupted more severely in [1–481]R362A than in [1–481]R359A mutant. In summary, the conserved Arg<sup>359</sup> and Arg<sup>362</sup> in D1 domain are crucial for maintaining the hexameric structure in VCP, and Arg<sup>362</sup> plays a more important role than Arg<sup>359</sup>.

**Impact of Arg Mutations on Polyubiquitin Chain Binding**—Our previous study showed that a hexameric structure is critical for binding to the polyubiquitinated proteins (11). Because D1 SRH is essential for the hexameric structure, it is expected to play an important role in substrate binding. To test this, an *in vitro* ubiquitin chain binding assay was carried out. VCP variants containing Arg mutations in D1 or D2 domain were attached to Ni-NTA beads and used in polyubiquitin chain pull-down assays. As evidenced in Fig. 4, D2 mutants, including R635A, R635K, R638A, and R638K, were all capable of binding the ubiquitin chains. But the D1 mutants are dissimilar: whereas R359K and R362K

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**FIGURE 2. Negatively charged substitutions of Arg of SRH in D1, but not D2, disrupt the hexameric structure of VCP.** A, gel filtration analysis of R359E/R362E and R635E/R638E mutants. Purified SRH mutants, R359E/R362E (upper panel) and R635E/R638E (lower panel), were separated by Superose 6 chromatography, and the eluted fractions were analyzed by SDS-PAGE, Western transfer, and immunoblotting with anti-His antibody. Thyroglobulin (669 kDa) and ferritin (440 kDa), catalase (232 kDa), and albumin (66 kDa) were analyzed in parallel as standards. B, native gel electrophoretic analysis of R359E/R362E and R635E/R638E. Purified VCP variants were resolved on nondenaturing gel in the absence of nucleotides, electrophoretically transferred, and analyzed by immunoblotting with an antibody recognizing the C terminus of VCP. Hexamer (6×), trimer (3×), and monomer (1×) are indicated. C, substituting Arg with Ala does not affect the hexameric structure. The hexameric status of Arg mutants was analyzed as described for B. Substituting Arg with Lys gives the same result (not shown). The molecular standards are marked on the left. WT, wild type.

**FIGURE 3. Conserved Arg residues in the D1 domain play a critical role in hexamer formation, and Arg<sup>362</sup> is more important than Arg<sup>359</sup>.** A, native gel electrophoretic analysis of R359E and R362E mutants. The purified mutants were resolved by native gel electrophoresis in the absence of nucleotides and analyzed as described in the legend to Fig. 2B. B, hexameric status of the truncated [1–481] variants. Purified mutant proteins were resolved by native gel electrophoresis in the absence of nucleotides and analyzed by Western blotting with anti-His antibody. The hexamer (6×) and monomer (1×) are indicated. WT, wild type.
retain the binding ability, R359A and R362A do not. This indicated that the specific conformations of R359K and R362K are different from those of R359A and R362A, although they are all capable of forming hexamer (Fig. 2C) and binding to nucleotides (Fig. 5C and D). Based on these results, we conclude that the extended positively charged side chains at positions 359 and 362 in D1 SRH allow the N-terminal domain to adopt a specific conformation for substrate binding.

All Arg Mutations in SRH Severely Reduce the ATPase Activity of VCP—In addition to binding to the substrates, an intact hexameric structure is also a prerequisite to the efficient ATPase activity of VCP (26). It is therefore not surprising that the R359E/R362E mutant barely exhibits ATPase activity (TABLE ONE and summarized in Fig. 1B). Although most other Arg mutants retain the hexameric structure, all have severely compromised ATPase activities. Even with the conservative change from Arg to Lys, the ATPase activity was abolished in R635K and R638K mutants and decreased ~80% in R359K and R362K mutants (TABLE ONE). It was also noted that mutations in the D1 domain resulted in a smaller decrease in ATPase activity than the analogous mutations in the D2 domain. This is consistent with our previous report that D2 is the major ATPase domain in VCP (16); thus the corresponding mutation in D2 inflicts more drastic impacts. The activity of another D1 SRH mutant, N348Q, was also reduced to ~25%, comparable with that observed in the Arg mutants of D1 domain (TABLE ONE).

The activity of another D1 domain mutant, N348Q, was also reduced to ~25%, comparable with that observed in the Arg mutants of D1 domain (TABLE ONE). This result is consistent with the crystallographic data that Arg<sup>359</sup>, Arg<sup>362</sup>, and Asn<sup>348</sup> residues are all involved in protomer interactions (21). However, mutating Asp<sup>354</sup>, which is not directly involved in protomer interactions, only reduced the activity by ~50%. Therefore, an efficient ATPase activity requires the conserved SRH residues, especially those directly involved in protomer contacts. Previously, we reported that although D2 is the major ATPase domain, D1 domain mediates the heat-induced activity of VCP (16). We compared the ATPase activity measured at 50 °C versus 37 °C and found that although the wild type, N348Q, and D354E exhibited nearly 2-fold increases, the D1 Arg mutants have a much smaller increase (TABLE ONE). It suggests that

**FIGURE 4.** Impact of arginine mutations on polyubiquitin chain binding. Arginine mutants (1 μg each) were immobilized onto Ni-NTA beads and incubated with polyubiquitin chains as described under “Materials and Methods.” The bound ubiquitin chains were analyzed by SDS-PAGE (4–20% Tris-glycine gel) and detected by Western blotting with anti-ubiquitin antibody. WT, wild type.

**FIGURE 5.** Nucleotide binding is not affected in Arg mutants of both domains. The Trp fluorescence spectra of wild type (WT), K524T, and Arg mutants are shown. The steady-state fluorescence spectra of the wild type (A), K524T (B), R359A (C), R362A (D), R635A (E), and R638A (F) in the presence of ATP or ADP were compared with the nucleotide-free state.
VCP SRH Critical for Hexameric Structure and ATP Hydrolysis

TABLE ONE
ATPase activity and its stimulation by heat

|                   | ATPase activity at 37 °C | Stimulation by heat |
|-------------------|--------------------------|---------------------|
|                   | %                        | fold                |
| Wild type         | 100                      | 1.9                 |
| R359E             | 13.7                     | 1.2                 |
| R359A             | 11.7                     | 1.3                 |
| R359K             | 22.1                     | 1.4                 |
| R362E             | 17.0                     | 1.2                 |
| R362A             | 21.5                     | 1.3                 |
| R362K             | 22.2                     | 1.3                 |
| R359E/R362E       | 5.1                      | 1.2                 |
| R359A/R362A       | 8.9                      | 1.2                 |
| R635A             | ND                       | ND                  |
| R635K             | ND                       | ND                  |
| R638A             | ND                       | ND                  |
| R638K             | ND                       | ND                  |
| N348Q             | 25.0                     | 2.0                 |
| D354E             | 54.4                     | 1.7                 |

* ND, not detectable at the protein concentration used in the experiments here.

these two trans-elements are also important for mediating the heat-stimulated activity.

Arg to Ala Substitution in D1 or D2 SRH Does Not Affect the Nucleotide Binding to D2—The decrease of enzyme activity in hexameric SRH mutants may result from an incapability of nucleotide binding and/or an inefficient hydrolysis. To differentiate the two possibilities, intrinsic Trp fluorescence spectra were analyzed. We previously showed that binding of ATP or ADP to VCP induces a characteristic Trp fluorescence change (Fig. 5A) specifically resulting from residue Trp676 (17). This fluorescence change was completely abolished in mutant K524T, which is defective in ATP/ADP binding in the D2 domain (Fig. 5B). As shown in Fig. 5 (C–F), the fluorescence of R359A, R362A, R635A, and R638A increased upon binding to ATP or ADP to an extent similar to that of the wild type, indicating that these mutants can effectively bind nucleotides. Similar results were obtained in all of the Arg to Lys mutants and in N348Q and D354E (data not shown). Therefore, the decrease in ATPase activities cannot be attributed to a defective nucleotide binding but rather to a compromised ATP hydrolysis. These results suggest that during ATP hydrolysis the long and flexible side chains of the two Arg residues are responsible for mediating the conformational changes between protomers but not for nucleotide binding. Hence, the Arg to Ala or Lys substitutions are sufficient to interrupt the communications, resulting in a decreased enzymatic activity.

Mixing R635A or R638A with K524A Partially Restores the ATPase Activity—It was recently reported that a conserved Arg in RuvB, an AAA− ATPase, acts as an allosteric effector by stimulating the ATPase activity of the adjacent subunit in a hexamer. The RuvB mutant harboring mutation at this Arg or Walker A motif is completely defective in ATPase activity. However, the activity is partially restored when these two mutants are mixed at a 1:1 ratio (27).

To test whether the Arg residues in VCP SRH have similar functions, we generated the hybrid hexamers from Arg mutant and ATP-binding site mutant. Because VCP forms stable hexamers even in the absence of nucleotides, we co-expressed K524A-Tag100 with R635A-His or R638A-His (Figs. 1B and 6A) and purified the VCP hexameric complex using Ni-NTA-agarose. The purified His-containing complex was further shown to also contain Tag100 (Fig. 6B), thus confirming the identity of the hybrid hexamer. If the trans-elements of the ATP-binding pocket, Arg635 and Arg638, do mediate the allosteric communication by stimulating the activity of an adjacent protomer in the hexamer, the ATPase activity should be enhanced in the hybrid. Indeed, we observed that, contrary to the undetectable ATPase activity in His-K524A-100 (data not shown), R635A, and R638A mutants (TABLE ONE), the activity of the hybrids R635A/K524A and R638A/K524A was partially restored to levels of ~20 and 10%, respectively, of that of the wild type (Fig. 6C). This result supports that the conserved Arg residues in the SRH of D2 do contribute to the ATP hydrolytic process by stimulating the activity of the adjacent subunits.

Mutation of Arg in D1 SRH Uncouples the Inhibitory Effect of p47 on the ATPase Activity of VCP—Because VCP co-factors also bind to the N-terminal domain of VCP, does the conformational alteration in D1 SRH mutants also affect the co-factor communications? To address this question, the effect of p47 (Fig. 1C, lane 10), a VCP co-factor, on the ATPase activities of these mutants was tested. It has been reported that p47 suppresses the ATPase activity of VCP (Fig. 7A and Ref. 28), and this inhibitory effect requires intra- and intermolecular communications at many levels: e.g. among the N-terminal, D1, and D2 domains within the same protomer, among protomers within the VCP hexamer, and among the hexameric N domains, D1 ring, and the D2 ring. We first examined whether the SRH mutants bind to p47. For comparison, the SRH mutants carrying the mutations at the cis-elements in the ATP-binding pocket of D1, N348Q, and D354E, were also tested. In contrast to unequal binding to the ubiquitin chain, all of the tested SRH mutants bind p47 similarly as the wild type does (Fig. 7A). As expected, p47 did not bind to the [200–806] mutant, which lacks the N-terminal domain (Fig. 7A). Therefore, the extended positively charged side chains at positions 359 and 362 in D1 SRH are not required for binding to p47.

We then tested the ATPase activity and found that p47 suppressed the activity of the wild type, N348Q, and D354E but stimulated that of R359A/K and R362A/K (Fig. 7B). Moreover, the stimulation was higher in R362 than in R359 mutants. Because there is no binding between p47 and the [200–806] mutant (Fig. 7A), p47 did not have any effect on the ATPase activity. When the activities obtained in the presence of p47 are compared against the activity of the wild type without p47, we obtain the following result: wild type, 50%; N348Q, 19%; D354E, 36%; R359A, 16%; R362A, 60%; R359K, 30%; and R362K, 40%. Remarkably, in the presence of p47, the activities of R362 mutants are increased to levels similar to that of the wild type. Based on these results, we conclude that the trans-elements Arg359 and Arg362 play critical roles in interprotomer communications that are translated into domain-domain interactions required for the p47-induced inhibitory effect on the ATPase activity of VCP (see “Discussion”). Because the ATPase activity of D2 SRH mutants cannot be detected, we did not perform similar assays on D2 mutants.

**DISCUSSION**

In this report, we investigated the functions of the SRH in both AAA domains of VCP. Different from the reported AAA proteins FtsH and NSF, VCP has two bona fide AAA modules that append more complexity in dissecting the role of SRH in the activities of VCP. The results indicate that the highly conserved Arg residues in the SRH of both domains are not required for binding to ATP/ADP but are critical for mediating the concerted conformational changes during ATP hydrolysis. In agreement with the previously identified major functions of D1 and D2 domains (16, 17), the SRH in D1 is crucial for holding the structure, whereas that in D2 is more vital for ATP hydrolysis. The conformational alterations in Arg mutants of D1 also influence the substrate binding and the communication with co-factors. Mutational studies further suggest that SRH is involved in the interprotomer communica-
tions that are required for the enzymatic and biological activities of VCP.

The SRH in D1 Plays a Critical Role in Maintaining the Structure—Compared with other AAA proteins, VCP has a highly stable hexameric structure, and many attempts have failed to disrupt the VCP hexamer (29). Here, we demonstrated for the first time that mutating the conserved arginines (Arg359 and Arg362) to Glu in SRH of D1 domain almost completely disrupts the hexamer into monomers (Fig. 2, A and B). Adding nucleotides to this mutant (R359E/R362E) cannot restore the hexamer (data not shown). Moreover, the single Arg to Glu mutation at residue 362 destabilizes the hexamer, whereas that at residue 359 does not (Fig. 3A). In this regard, the side chains of these two Arg are not functionally interchangeable, and Arg362 plays a more important role than Arg359. The crystal structure of VCP ND1 shows that Arg359 interacts with the bound nucleotide and that Arg362 interacts with Glu305 in the ATP-binding pocket of the neighboring protomer (21, 22). Therefore, Glu at residue 362 produces an electrical repulsion against Glu305 of the adjacent protomer, resulting in destabilization of the hexamer. Furthermore, when both Arg359 and Arg362 are substituted by Glu the repulsion is even stronger, hence dissociate the hexamer.

Although the ND1 domain (1–481) including ND1 and the downstream linker is the major region to hold the hexameric structure, when the corresponding residues in D2, Arg635 and Arg638, are mutated to Glu, the VCP hexamer is slightly disturbed (Fig. 2, A and B). This suggests that although D2 is not the major domain for maintaining the hexamer, D2 contributes to the stabilization of the structure. The finding can be explained by the fact that both D1 and D2 the SRH is in the α/β subdomain, which is the major protomer–contacting region in VCP hexamer (22). The contribution of D2 in stabilizing the hexamer also explains why Arg to Ala substitution at 359 and 362 barely affects the full-length hexamer (Fig. 2C) but disrupts the structure of [1–481] hexamer (Fig. 3B). Because the crystal structure of VCP revealed that the backbone electron density for the loop containing Arg359 and Arg362 (D1) is well ordered, whereas that of Arg635 and Arg638 (D2) is not (22), the interaction between the adjacent D2 domains is weaker than that in the D1 domains. Consequently, Arg635 and Arg638 mutations result in less disruption of the hexamer. In summary, whereas the Arg residues in both D1 and D2 SRH are involved in protomer interactions, those in D1 play a critical role in maintaining the hexameric structure.

The SRH in D2 Contributes More to the ATPase Activity—When less drastic mutations (e.g. Arg to Ala or Lys) are introduced to the Arg residues in both domains of VCP, the overall hexameric structure is maintained (Fig. 2C). But is the ATPase activity affected? Enzyme assays show that D1 mutants (R359A/K and R362A/K) still have an ATPase activity close to 20% of the wild type, but D2 mutants completely lose the activity (TABLE ONE). This result indicates that the SRH in D2 plays a more critical role in enzyme activity and is consistent with our previous finding that D2 is the major ATPase domain (16). The tryptophan fluorescence spectra further show that these hexameric SRH mutants all bind nucleotides efficiently (Fig. 5). Therefore, the decrease in ATPase activity is a consequence of compromised ATP hydrolysis. This catalytic
role of the conserved Arg in VCP resembles that in FstH, a single AAA domain protein (19). This similarity is also supported by the crystallography studies showing a high similarity between the structures of the AAA domain in FstH and VCP-D1 (30).

Although the D2 SRH of VCP is critical for the ATPase activity, the SRH of AAA proteins is not always required for ATP hydrolysis. It has been reported that Arg359 and Arg362 in the SRH of NSF-D1, the bona fide AAA domain, are important for sensing the nucleotide state during the disassembly of soluble NSF attachment protein receptors rather than for hydrolysis (20).

Arginine Finger—The presence of conserved arginines at the active sites and protomer interfaces is not limited to AAA proteins. The functions of the conserved Arg residues among different AAA+ subgroups are recently summarized and reviewed by Ogura et al. (18). Mutational analyses of several AAA+ proteins including NtrC (31), γ complex (32), MCM (33), HsIU (34), and RuvB (27) indicate that the conserved Arg is essential for ATP hydrolysis but not for ATP binding and is believed to function as an arginine finger that was originally identified in GTPase-activating protein (24). A recent study further showed that the Arg finger in RuvB (Arg174) functions as an allosteric effector for the ATPase activity of the adjacent protomer in a hexamer. This is based on the observation that when R174A mutant is mixed with Walker A mutant, the ATPase activity can be partially restored (27). The arginine finger of GTPase-activating protein, serving as a trans-element, completes the active sites of the GTPase and contributes to the GTPase activity by stabilizing the transition states during hydrolysis (24).

By analogy, the Arg in the SRH of VCP can be categorized as arginine finger because they are all trans-elements of the ATP-binding pocket of the adjacent protomer, and the ATPase activity is abolished when they are mutated (TABLE ONE). Our result further showed that the heterohexamers composed of Walker A (K524A) with R635A or R638A partially restored ATPase activity (Fig. 6C). Thus, as in Ruv B, the extended, flexible, and positively charged side chain of Arg may mediate the long range communications in proteins connecting disparate residues. Although DeLaBarre and Brunger (23) recently reported that both Arg359 and Arg362 point away from the nucleotide that bound to the neighboring protomer and into their own protomer in the ATP state, it does not rule out the potential transient interactions between Arg359 and Arg362 with the neighboring nucleotide.

It is noted that the restored activity shown in Fig. 6C depends on the relative amount of the heterohexamer in the purified protein and the ratio of the two variants in the heterohexamer. Theoretically, our purification procedure can isolate both homo- and heterohexamers. A higher activity would be detected if a higher amount of the hetero-form is present in the purified protein. In addition, it is expected that the restored activity detected in R635A/K524A and R638A/K524A heterohexamers is higher if the ratio of the two variants in the hybrid is closer to 1. However, a quantitative evaluation of the above-mentioned criteria is beyond the limit of the current system.

The conserved Arg-mediated connection can contribute to both the structure and the ATPase activity. As in the case of HsIU, mutating Arg325 to Glu disrupts the hexamer and abolishes the ATPase activity, although the complex is still able to bind nucleotide (34). Similarly, the D1 mutant of VCP, R359E/R362E, can effectively bind to ATP (data not shown) but exists mainly as monomer (Fig. 2, A and B). In addition, superposition of the crystal structures of full-length VCP and HsIU hexamer suggests that Arg362 in VCP corresponds to Arg325 in HsIU (18). Clearly, Arg residues in both domains of VCP are involved in maintaining the protomer-protomer interactions as well as in ATP hydrolysis.

Arg359 and Arg362 Are Important for Substrate Binding and Co-factor Communication—Besides the functional role in stabilizing the structure and in ATP hydrolysis, Arg359 and Arg362 are also important for substrate binding. It has been shown that polyubiquitinated proteins...
and polyubiquitin chains bind to the N-terminal domain of VCP, and the binding occurs in the presence of ATP but not ADP (11). Because D1 is directly connected to N-terminal domain, the conformational alterations in D1 during ATP hydrolysis must also change the conformation of the N-terminal domain, thus affecting the binding property. Our mutant analyses show that Arg to Ala and Arg to Glu mutations abrogate the polyubiquitin chain binding, but the Arg to Lys mutant retains the activity (Fig. 4). Apparently, the positively charged side chain of Arg and Lys in D1 SRH is crucial for the N-terminal domain to adopt a specific conformation for substrate binding.

In addition, conserved Arg residues in D1 SRH also play critical role in communicating with the co-factors. It has been demonstrated that association of VCP with co-factor p47 results in an inhibition of the ATPase activity of VCP (28), and this inhibitory effect requires proper p47 binding as well as correct communication during the ATPase cycle. Strikingly, our results showed that although p47 binding to the wild type VCP leads to a reduction in the ATPase activity, p47 binding to mutant R359A/K or R362A/K results in an increase in the activity (Fig. 7B). Because the SRH mutants are capable of binding to p47 (Fig. 7A), the alteration in the inhibitory effect of p47 is likely resulted from changed communications within the VCP complex. This observation can be rationalized by the recent crystallographic study. The recently solved co-crystal of VCP and p47 reveals that a loop (S3/S4) in the ubiquitin communications within the VCP complex. This observation can be altered in the inhibitory effect of p47 is likely resulted from changed communications within the VCP complex. This observation can be rationalized by the recent crystallographic study. The recently solved co-crystal of VCP and p47 reveals that a loop (S3/S4) in the ubiquitin

ATP hydrolytic process, we need to have a better understanding of the structures of VCP at different nucleotide states.

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