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Journal of Structural Biology 146 (2004) 44–57
Journal of Structural Biology
www.elsevier.com/locate/yjsbi

Molecular perspectives on p97–VCP: progress in understanding its structure and diverse biological functions

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Received 9 September 2003, and in revised form 22 October 2003

Abstract

The 97-kDa valosin-containing protein (p97 or VCP) is a type-II AAA (ATPases associated with a variety of activities) ATPases, which are characterized by possessing two conserved ATPase domains. VCP forms a stable homo-hexameric structure, and this two-tier ring-shaped complex acts as a molecular chaperone that mediates many seemingly unrelated cellular activities. The involvement of VCP in the ubiquitin-proteasome degradation pathway and the identification of VCP cofactors provided us important clues to the understanding of how this molecular chaperone works. In this review, we summarize the reported biological functions of VCP and explore the molecular mechanisms underlying the diverse cellular functions. We discuss the structural and biochemical studies, and elucidate how this sophisticated enzymatic machine converts chemical energy into the mechanical forces required for the chaperone activity.
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1. Introduction

The 97-kDa valosin-containing protein (p97 or VCP) is a member of the type II AAA (ATPases associated with a variety of activities) ATPases, which are characterized by the presence of two conserved ATPase domains, also called AAA domains (Neuwald et al., 1999; and reviewed in Confalonieri and Duguet, 1995; Maurizi and Li, 2001; Ogura and Wilkinson, 2001; Patel and Latterich, 1998; Vale, 2000; Zwickl and Baumeister, 1999). As are other AAA proteins, VCP is an enzymatic machine. It catalyzes ATP hydrolysis to generate energy and uses the energy to perform mechanical work in cells. VCP, also known as VAT in archaebacteria, CDC48 in yeast, TER94 in *Drosophila*, p97 in *Xenopus*, and VCP in plants and mammals, is one of the most highly evolutionarily conserved proteins (Frohlich et al., 1991; Koller and Brownstein, 1987; Pamnani et al., 1997; Peters et al., 1990). A 69% sequence identity is conserved between yeast Cdc48p and human VCP, and an even higher sequence similarity is preserved between the respective AAA domains (Frohlich et al., 1991). VCP is ubiquitous, essential, and highly abundant in cells, accounting for more than 1% of the total cellular protein. Because of its involvement in an unusually wide variety of functions, VCP has attracted a great deal of attention from researchers in different fields. In the last few years, emerging biochemical and genetic evidence has defined the diverse activities and identified specific cofactor/adaptor proteins in mediating these activities. While a recent commentary discussed the multiple identities of VCP (Woodman, 2003), this review aims to summarize the studies in which the mechanisms underlying the diverse functions were investigated and to elucidate the structure–function relationship in this essential protein.

2. Diverse biological functions

2.1. Ubiquitin–proteasome-mediated protein degradation

As observed with other AAA proteins, VCP acts as a molecular chaperone in many seemingly unrelated cellular activities, such as membrane fusion, cell cycle regulation, stress response, programmed cell death, B

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1047-8477/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.jsb.2003.11.014

and T-cell activation, transcriptional regulation, endoplasmic reticulum (ER)-associated degradation, and protein degradation (see reviews listed in previous section). Interestingly, almost all these activities are directly or indirectly regulated by the ubiquitin–proteasome (Ub–Pr) system. The Ub–Pr-mediated degradation pathway consists of two sequential steps. The destined substrate is first conjugated with poly-Ub chains, which tag it for proteolysis. The polyubiquitinated substrate is then moved, possibly guided by molecular chaperones, to the 26S proteasome for final degradation (reviewed in Hershko and Ciechanover, 1998). The involvement of both VCP and the Ub–Pr systems in similar cell activities suggests that VCP may act as a chaperone in the Ub–Pr pathway, thus regulating the various cellular functions through the Ub–Pr pathway. Both genetic and biochemical studies indicated that this is indeed the case. Mutations in the CDC48 gene result in cell cycle arrest (Moir et al., 1982) and the accumulation of polyubiquitinated proteins in yeast (Dai and Li, 2001). Depletion of VCP from cell extracts abolishes the Ub–Pr-mediated degradation of cyclin E, leading to the accumulation of polyubiquitinated proteins in mammalian cells (Dai and Li, 2001). The involvement of VCP in the Ub–Pr degradation pathway was further supported by the findings that VCP directly binds polyubiquitinated proteins, as well as other Ub–Pr pathway components, such as Ufd1, Ufd2, Ufd3 (proteins involved in the Ub fusion degradation pathway), and 26S proteasome (Dai et al., 1998; Ghislain et al., 1996; Kaneko et al., 2003; Koegl et al., 1999; Meyer et al., 2000; Verma et al., 2000). In the following section, we will summarize the reported diverse activities of p97/VCP/Cdc48p, with an emphasis on its connection to the Ub–Pr system.

2.2. Membrane fusion

In eukaryotic cells, the nuclear membrane, ER, and Golgi apparatus are fragmented at the onset of mitosis to ensure that the organelles are properly partitioned into daughter cells. At the end of mitosis, these membrane fragments undergo homotypic (self) fusion to reassemble in an organelle-specific manner (Rothman and Warren, 1994). During 1994–1995, several studies established that VCP/Cdc48p participates in the fusion of the ER membrane (Latterich et al., 1995; Zhang et al., 1994), and that VCP is required in the regrowth of the Golgi apparatus (Acharya et al., 1995; Rabouille et al., 1995). However, the molecular mechanism by which VCP regulates the membrane fusion remained unclear until an essential VCP cofactor, p47, was identified (Kondo et al., 1997). p47 and VCP form a stable complex which interacts with syntaxin5, a membrane-localized p47 receptor and a t-SNARE (target-soluble NSF attachment protein receptor) family member (Rabouille et al., 1998). When two membranes are brought close enough, the syntaxin5 on both membranes pair up and form a complex, leading to the fusion of the two membranes. How the VCP–p47 complex mediates the actual fusion event is not clear, nevertheless, it is suggested to act as a chaperone that "primes" the syntaxin5 molecules so that they can participate in the processes leading to membrane fusion. Recent evidence further suggested that another factor, VCIP135 (VCP-p47 complex-interacting protein, p135), is also required in the process (Uchiyama et al., 2002).

Nuclear envelope assembly also requires VCP (Hetzer et al., 2001). But, in addition to p47, another cofactor complex, Ufd1–Npl4 (a protein involved in nuclear protein localization), is needed to mediate the process. Specifically, two discrete and sequential steps are involved in nuclear envelope assembly: sealing and growth of the nuclear envelope. In the first step, VCP works with the Ufd1–Npl4 complex to mediate the formation of the closed nuclear envelope. While relatively more detail is known about how Ufd1–Npl4 participates in ER-associated protein degradation (see next section), the mechanism by which this complex regulates nuclear envelope formation is unknown, although the involvement of Ufd1–Npl4 provides a hint that the Ub–Pr system may play an important role. In the second step, VCP and p47 are responsible for feeding additional membranes into the growing nuclear envelope. It is expected that the underlying mechanism will be highly similar to that observed in ER or Golgi membrane fusion. The involvement of VCP in ER, Golgi, and nuclear membrane fusion has been taken to suggest that VCP is involved in all homotypic membrane fusion. However, this suggestion is challenged by a study showing that VCP is not required in the homotypic fusion of immature secretory granules during maturation (Urbe et al., 1998).

Although the involvement of VCP in membrane fusion had been established for a number of years, no formal connection between membrane fusion and the Ub–Pr system had been reported. Recent studies published by Meyer and colleagues shed light on this aspect of membrane fusion (Meyer et al., 2002; Ye et al., 2003). Using in vitro assay systems, these authors showed that VCP cofactors Ufd1, Npl4, and p47 all have Ub chain-binding activity, and cofactor binding to VCP further enhances the overall binding to ubiquitinated conjugates. While Ufd1 and Npl4 preferentially bind to poly-Ub chains, p47 appears to bind monoubiquitinated proteins (Meyer et al., 2002). It was further shown that p47 binds to mono-Ub through its UBA (Ub association) domain, which is required for VCP–p47-mediated reassembly of Golgi cisternae (Meyer et al., 2002). These results suggest that the Ub–Pr system, or at least the Ub system, may be involved in membrane fusion. However, the target protein for Ub conjugation (poly- or mono-) has not been identified in these membrane fusion events.

2.3. ER-associated degradation

Newly synthesized proteins in the ER of eukaryotic cells are subject to rigorous quality control, which consists of a sophisticated protein proofreading and elimination mechanism. When misfolded or improperly assembled proteins emerge in the lumen of the ER, they are exported back to the cytosolic side of the ER (an event called retrotranslocation), conjugated with polyubiquitin and subsequently degraded by the 26S proteasome. This process, named ERAD (ER-associated degradation), is capable of destroying both integral membrane proteins and lumenal proteins. In the past two years, a series of reports showed that VCP is required in ERAD and acts as a molecular chaperone that extracts the ubiquitinated proteins from the ER membrane before their final degradation by the proteasome (Bays et al., 2001; Hitchcock et al., 2001; Jarosch et al., 2002; Rabinovich et al., 2002; Rape et al., 2001; Ye et al., 2001). Recently, Ye et al. further reported that VCP is involved in retrotranslocation in both Ub-independent and Ub-dependent steps (Ye et al., 2003). They proposed a model of "dual substrate recognition by the VCP–Ufd1–Npl4 complex" to provide a mechanistic basis for this observation. In the initial step, an abnormal, possibly unfolded, polypeptide chain emerges from the ER, and the VCP–Ufd1–Npl4 complex is recruited to the membrane by an unknown receptor. This substrate then undergoes polyubiquitination catalyzed by the Ub system enzymes localized near the ER. Once the Ub chain reaches a certain length, it interacts with the VCP–Ufd1–Npl4 complex with a high affinity. Finally, VCP uses ATP hydrolysis to pull the polypeptide into the cytosol (Ye et al., 2003). In this model, VCP interacts weakly with non-ubiquitinated proteins at the early stage of retrotranslocation and more tightly with polyuiquitinated proteins at the later stage. The high-affinity binding results from the synergistic binding of VCP and Ufd1–Npl4 complex to the poly-Ub chains.

2.4. Transcription activation

VCP is involved in the activation of nuclear factor-κB (NF-κB) (Asai et al., 2002; Dai et al., 1998), a dimeric, sequence-specific transcription factor that regulates a large number of genes. In unstimulated cells, NF-κB is sequestered in the cytoplasm in an inactive form by physically associating with the inhibitory IκB proteins (reviewed in Ghosh et al., 1998; Karin and Ben-Neriah, 2000; Santoro et al., 2003; Silverman and Maniatis, 2001). In response to stimulation, NF-κB can be activated via many different pathways. In a well-characterized model system, IκBα is rapidly phosphorylated and polyubiquitinated after stimulation (reviewed in Karin and Ben-Neriah, 2000). VCP then binds the polyubiquitinated IκBα and likely dissociates it from the NF-κB complex (Dai et al., 1998). After dissociation, the NF-κB dimer translocates into the nucleus to regulate its target genes. In the meantime, VCP chaperones the polyubiquitinated IκBα to the 26S proteasome for irreversible degradation (Dai et al., 1998).

VCP not only participates in the activation of soluble transcription factors, such as NF-κB, but also in the activation of membrane-bound transcription factors. This was best demonstrated in the activation of Spt23 and Mga2, yeast homologs of mammalian NF-κB that control the expression of genes required for fatty acid metabolism (Hitchcock et al., 2001; Hoppe et al., 2000; Rape et al., 2001). Spt23p proteins are synthesized as inactive precursors, p120, which form dimers that anchor to the ER via the C-terminal transmembrane spans (Rape et al., 2001). Low levels of unsaturated fatty acids activate Spt23, whereby the Spt23p molecule is ubiquitinated (mostly monoubiquitination) and subjected to limited proteolysis by the proteasome (Rape et al., 2001). In this case, the 26S proteasome does not degrade p120 into small peptides and amino acids, rather it degrades only a portion of the substrate, leaving a truncated 90-kDa Spt23p, p90, intact. The processed p90, although lacking the transmembrane domain, is still bound to the inactive p120, and thus tethered to the ER membrane. Cdc48p, together with the Ufd1–Npl4 cofactor complex, binds the ubiquitinated p90, dissociates p90 from p120, and extracts p90 from the membrane. The activated p90 can then enter the nucleus and activate the transcription of the fatty acid desaturase gene OLE1 (Hoppe et al., 2000; Rape et al., 2001). Similar to Spt23, Mga2 and NF-κB precursors (p105 and p100) are also processed to truncated, activated forms by the 26S proteasome through limited digestion (Lin et al., 1998; Shcherbik et al., 2003). Presumably, a flexible, glycine-rich loop in the precursor protein is inserted into the channel of the proteasome and facilitates the limited proteolysis (Liu et al., 2003). It was recently shown that VCP/Cdc48p is indeed involved in Mga2 activation in the same manner described for Spt23 (Shcherbik et al., 2003). Although VCP is likely also involved in targeting NF-κB precursors p105 and p100 to the 26S proteasome, no direct evidence of this activity has not been reported.

2.5. Cell cycle control

The involvement of VCP/Cdc48p in cell cycle control was reported more than two decades ago. The yeast cold-sensitive *cdc48* mutant cells are arrested at the G2/M phase when cultured at a non-permissive temperature (Moir et al., 1982). In the mammalian system, stimulation of immune T cells induces a cascade of phosphorylation events, and VCP is one of the first proteins to undergo tyrosine phosphorylation upon activation (Egerton et al., 1992). Moreover, VCP is generally

expressed at a high level in rapidly dividing cells and cancer cells. These observations indicate that VCP/Cdc48p is required for normal growth control and is a pro-proliferation factor.

Although the involvement of VCP in cell cycle control has been appreciated for many years, the precise steps regulated by it have not been systematically studied. We previously showed that VCP is involved in the degradation of cyclin E both in vitro and in vivo (Dai and Li, 2001). A recent study showed that VCP interacts with DNA unwinding factor, DUF, in *Xenopus* egg extracts, and the VCP–DUF complex may produce a chromatin structure favorable for DNA replication (Yamada et al., 2000). Moreover, VCP physically associates with the BRCA1 protein, a breast/ovarian cancer susceptibility gene product, suggesting its potential involvement in DNA damage repair functions (Zhang et al., 2000a). A recent revelation of association between VCP and Werner protein (Joaquin Partridge et al., 2003), a member of the RecQ helicase family, also seems to support this notion. Although VCP is predominantly localized in the cytoplasm and is less abundant in the nucleus, VCP and Cdc48p have been reported to enter the nucleus during late G1 phase (Madeo et al., 1998; Zhang et al., 2000a). These studies suggest that VCP/Cdc48p regulates cell cycle transitions at multiple levels, and the regulation can be attributed to its involvement in the Ub–Pr degradation pathway. It is well known that cell cycle phase transitions depend on the destruction of specific Cdk activators (e.g., cyclins) and inhibitors (e.g., p21, p27) (reviewed in Glotzer et al., 1991; Sherr and Roberts, 1995, 1999). The loss of Cdc48p or VCP function results in an inhibition of Ub–Pr-mediated degradation of these critical cell cycle regulators, thus blocking phase transitions. This is evidenced by the accumulation of polyubiquitinated cyclins in *cdc48* mutant cells (unpublished result). During DNA replication, since large protein complexes are usually involved in the process, the energy generated from VCP-catalyzed ATP hydrolysis may be required for the construction or destruction of DUF-containing complexes. In this context, it is not clear whether the entire Ub–Pr pathway or only the chaperone activity of VCP is required.

2.6. Apoptosis

Apoptosis is a physiological cell suicide program that is essential for the development and homeostasis of all multicellular organisms. In the past few years, the involvement of the Ub–Pr pathway in regulating apoptosis has received increasing attention (reviewed in Jesenberger and Jentsch, 2002; Varshavsky, 2003; Yang and Yu, 2003). Important regulators of apoptosis, such as Bcl-2 family proteins, IAPs (inhibitor of apoptosis), NF-κB, and IκB kinase have been identified as new substrates of the Ub–Pr degradation pathway. Recent study further showed that apoptosis inhibitors XIAP and cIAP1 act as ring finger-type E3 Ub ligase to catalyze their own ubiquitination before their degradation by the proteasome (thus a pro-apoptotic effect) (Yang et al., 2000). The inhibitor cIAP2 also promotes the monoubiquitination of caspases -3 and -7, and regulates their levels in cells (Huang et al., 2000). Furthermore, proteasome inhibitors trigger apoptosis in most cell lines, indicating the tight connection between apoptosis and the Ub–Pr pathway.

The finding that VCP acts as a chaperone in the Ub–Pr pathway and the revelation that Ub–Pr pathway participates in the apoptotic process immediately suggested an involvement of VCP in apoptosis. Indeed, several lines of evidence showed that VCP is an anti-apoptotic agent. Using a cytokine stimulation model, Shirogane et al. (1999) showed that activation of the gp130 cytokine receptor induces the activity of Pim-1 kinase and c-Myc, ultimately leading to cell proliferation. They further identified VCP as a target gene for the Pim-1-mediated signal, and, importantly, expression of a mutant VCP resulted in apoptosis. Using zebrafish cells as a model system, Imamura et al. recently showed that cold treatment induces the expression of VCP, which leads to inhibition of apoptosis. In addition, a mutation introduced to the major tyrosine phosphorylation site in VCP inhibits cell proliferation and induces apoptosis at low temperature (Imamura et al., 2003). A more direct involvement of VCP in apoptosis was further demonstrated in *Caenorhabditis elegans*, in that the VCP homolog MAC-1 interacts with components of the apoptotic pathway, including CED3 (a caspase), CED4 (an apoptosis activator), and CED9 (a homolog of Bcl-2), and prevents apoptosis in the nematode (Wu et al., 1999). Furthermore, a yeast mutant, $cdc48^{S565G}$, exhibits morphological and molecular characteristics of apoptosis (Madeo et al., 1997). This result strongly supports the notion that Cdc48p has an anti-apoptotic activity and that the basic machinery of apoptosis is present in unicellular eukaryotic cells.

2.7. Molecular chaperone

Based on the functions described above, it is reasonable to propose that VCP plays a fundamental role in the Ub–Pr degradation pathway, which in turn regulates the diverse cellular activities. The exact role VCP plays in the Ub–Pr pathway has not been characterized in molecular detail. It appears that VCP, with the help of cofactors, specifically binds the ubiquitinated protein and prepares the protein before passing it to the 26S proteasome for degradation (Fig. 1). It is well known that unubiquitinated proteasome substrates are almost always complexed with other cellular proteins, which actually protect the substrates from proteolysis by proteases in the cell. After substrate ubiquitination, VCP

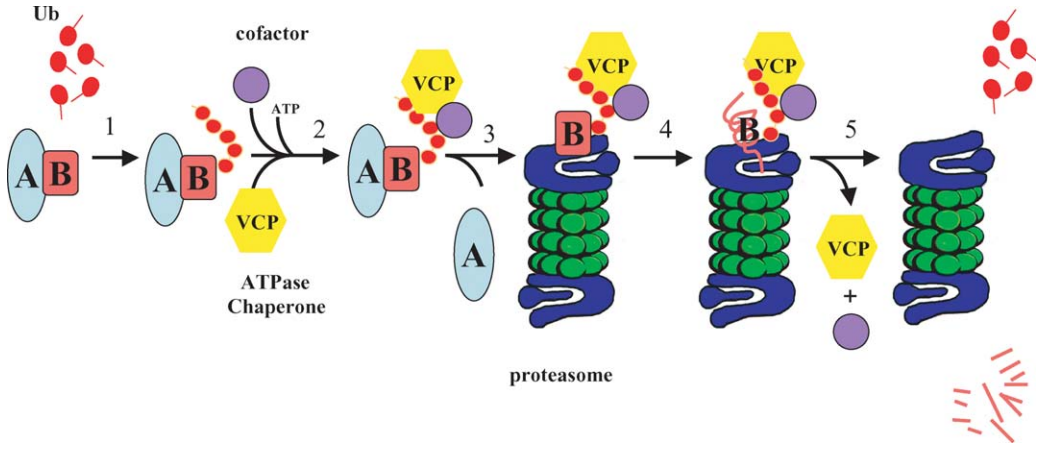

Fig. 1. VCP acts as a molecular chaperone in the Ub–Pr Pathway. Protein B undergoes polyubiquitination, which tags the protein for proteasomal degradation (step 1). Together with other cofactor(s), VCP binds to protein B via the N domain of VCP and the poly-Ub chain on protein B (step 2). The VCP/cofactor complex disassembles the protein A/B complex using the energy generated from VCP-catalyzed ATP hydrolysis (step 3). Dissociated protein B is then chaperoned to other intracellular locations (not shown) or to the proteasome for degradation (steps 4 and 5). It is not clear whether VCP further modifies the conformation of protein B (step 4), and if so, whether protein B is still conjugated with the poly-Ub chain. In this model, protein A and protein B may be cytosolic as well as membrane-bound.

likely uses the energy generated from ATP hydrolysis to disassemble the protein complex, singles the ubiquitinated protein, and presents it to the proteasome for degradation. This disassembly (or segregase) activity is demonstrated in the activation of the transcription factors NF-κB and Spt23/Mga2, as described in previous section. In each case, VCP specifically binds the ubiquitinated target protein, and disassembles the protein complex, thus "pulling" the target protein out of the complex. Similar disassembly activity is also observed when VCP extracts target proteins out of the ER membrane in ERAD. In these events, VCP catalyzes ATP hydrolysis and uses the energy to separate the target protein from other associated membrane proteins for subsequent translocation (Braun et al., 2002; Ye et al., 2003). Along the same line of reasoning, one can imagine that at the end of the Ub conjugation reaction the ubiquitinated protein is likely still associated with E2 and E3 enzyme complexes, and VCP may also act as a disassembly factor that isolates the specific target protein from the complex and further prepares the protein for proteasomal degradation. How much additional "preparation work" VCP actually performs on the substrate protein is an open question. A study showing that archaebacterial VAT is capable of mediating protein folding/unfolding suggests the possibility that VCP may also mediate further conformational changes of the substrate (Golbik et al., 1999).

Other than acting as a Ub-specific chaperone, VCP also exhibits characteristics of a general chaperone. A recent study showed that Cdc48p can distinguish between native and non-native proteins (Thoms, 2002). It binds the non-native proteins and prevents their aggregation, a classical duty of molecular chaperones. Our unpublished data also shows that VCP is capable of binding to denatured proteins and maintaining them in a soluble state. This general chaperone activity is likely conferred by a passive-binding activity of VCP because it does not need the ATPase activity of VCP (unpublished result).

3. Structural studies

3.1. VCP structure

The VCP molecule can be divided into N (1–187), D1 (209–460), D2 (481–761), and C (762–806) domains, with the N–D1 linker (188–208) and D1–D2 linker (461–480) joining the respective domains (DeLaBarre and Brunger, 2003; Zhang et al., 2000b). EM studies showed that VCP proteins form a barrel-like, homo-hexameric structure that comprises two ring-shaped layers made of the respective D1 and D2 AAA modules (Peters et al., 1992; Rouiller et al., 2000; Zhang et al., 2000b). While the N-terminal domain is mostly flexible, the C-terminal tail is highly disordered and thus undetectable by both EM and crystallography. Although a previous X-ray crystallography study provided informative details of the ADP-bound N–D1 domain (Zhang et al., 2000b), an overall crystal structure of full-length VCP has not been available until now. DeLaBarre and Brunger recently reported a 4.7 Å crystal structure of full-length VCP (residues 2–806) in which the D1 and D2 domains are bound to ADP and ADP–AlF_x, respectively (DeLaBarre and Brunger, 2003). Huyton et al. (in this issue) also presented a 3.6-Å partial structure of VCP (residues 17–700) in which D1 is bound to ADP and D2 is nucleotide-free. However, because of the highly disordered D2 domain, their final structure consists of residues

17–464, and a poly-alanine model applied for residues 465–700 (except for residues 612–615 and 665–668).

The crystal structures demonstrated that the two-layer VCP hexamer has a "flattened hourglass" (DeLaBarre and Brunger, 2003) or "mushroom" (Huyton et al., in this issue) shape with a diameter of ~160 Å at one end, ~120 Å at the other end, and a height of ~85 Å. A central pore, with an overall length of ~70 Å and a complex interior shape, runs through the entire span of the hexamer (DeLaBarre and Brunger, 2003). The N–D1 domain occupies the wider layer of the structure (i.e., the head of the mushroom), and has the N domains sticking out at positions ~30° from the apex of the D1 and D2 domains. D1 and D2 have highly similar shapes and are aligned above each other in a head-to-tail manner. In both crystal structures, the D1 ring is bound to ADP and is more stable than the D2 ring. These results are consistent with our previous biochemical study, which showed that the two rings are packed in a head-to-tail arrangement, and that the D1 ring is highly resistant to trypsin digestion and more compact than the D2 ring (Wang et al., 2003b).

As with all AAA modules, both D1 and D2 domains contain an N-terminal subdomain with an α/β fold, a C-terminal subdomain with α helices, and a nucleotide-binding site between the two subdomains (Zhang et al., 2000b). The structures of the α/β subdomains of D1 and D2 are similar to each other, but the respective helical subdomains show large differences. In comparison with the counterparts in D2 (DeLaBarre and Brunger, 2003), the helices α12 and α13 of D1 are tilted down toward the central axis of the hexamer. The tilt results in a close contact between helix α13 and the N domain, and thus may work as a molecular "latch" that regulates the freedom of N domain movement (also see Section 4.1). The D1 and D2 domains are joined by a 20-residue, highly flexible, and largely exposed linker that travels greater than 48 Å in distance. Both the N–D1 linker and D1–D2 linker are extended and make close contact to the nucleotide-binding site in D1 and D2, respectively (DeLaBarre and Brunger, 2003). This observation immediately suggests that the conformational change induced by nucleotide binding/release may be propagated through the flexible linker to other parts of the complex. This notion is supported by our biochemical study showing that intrinsic tryptophan fluorescence of Trp476 in the D1–D2 linker can monitor the nucleotide-binding state in the D2 domain (Wang et al., 2003b).

DeLaBarre and Brunger further reported that a Zn^{2+} ion blocks the central pore at the D1 end of the hexamer, where it meets the six His317 residues that face in toward the pore. Zinc-binding sites involving His residues have been previously reported in a number of proteases and in the N domain of ClpA, a well-studied type II AAA chaperone in *Escherichia coli* (Guo et al., 2002). The significance of zinc binding in VCP is not clear at present. Intriguingly, based on this novel finding, the authors speculated that substrate proteins possibly interact with the exterior of the VCP hexamer rather than through the central pore (DeLaBarre and Brunger, 2003).

3.2. Conformational changes during the ATPase cycle

Cryo-EM (Beuron et al., 2003; Rouiller et al., 2002) and biochemical (Wang et al., 2003b) analyses have been used to study the structure of VCP in different nucleotide states. Based on the molecular "snapshots" captured in these studies, models were proposed to depict the conformational changes of VCP during the ATPase cycle (see movie at http://www.scripps.edu/milligan/kubalek/p97_titles.mov). Cryo-EM analyses showed that D1 and D2, as well as the N domain, experience conformational changes during ATP binding, ATP hydrolysis, P_i release, and ADP release (Rouiller et al., 2002). The N domain is flexible in most nucleotide states except when VCP is bound to ADP–AlF_x, which simulates an ADP + P_i transition state in ATP hydrolysis. Both D1 and D2 rings become more compact upon ATP binding, while the conformational change in the D2 ring is more dramatic. During the ATPase cycle, the D2 ring rotates around the sixfold axis back and forth with respect to the D1 ring by a maximum of 10°. Along with these changes, the size of the axial openings on both rings fluctuates and the side protrusions observed at mid-length of the hexamer also rotate back and forth by a maximum of 6°(Rouiller et al., 2002). In agreement with these findings, using biochemical approaches such as intrinsic Trp fluorescence and limited proteolysis analyses, we previously demonstrated that the D1 ring is relatively stable, regardless of the nucleotide states, while the D2 ring undergoes dramatic conformational changes during ATP hydrolysis (Wang et al., 2003b).

The recently solved crystal structure of the full-length VCP provides valuable information on the structure of the D2 domain (DeLaBarre and Brunger, 2003), and comparing it with the cryo-EM results further offers insights into the mechanism of the conformational changes. The structure study clearly showed that the spike-like side protrusions consists of the α12′ helix and the subsequent loops from the helical subdomain of D2 (DeLaBarre and Brunger, 2003; Rouiller et al., 2002). Since the N-terminus of the α12′ helix directly points into the adenine ring in the D2 nucleotide-binding site, nucleotide states are expected to dictate the orientation of the helix and the subsequent loops. Indeed, comparison between the crystal and EM structures revealed that, during ATP hydrolysis, major conformational changes occur in the helical subdomains, particularly near the α12′ helix in D2, and no large motion is apparent in the α/β subdomains of D1 and D2 (DeLaBarre and Brunger, 2003). Therefore, the side protrusions

change from a disordered (ADP–AlF_x state) to an ordered (ADP or nucleotide-free state) conformation as detected in the cryo-EM study (Rouiller et al., 2002). The crystal structure also indicates that the conserved arginine finger (Arg635) in the D2 domain resides in a disordered loop, thus abrogating the strong ionic interaction with the adjacent subunit as observed in the corresponding arginine residue (Arg359) in D1 (DeLaBarre and Brunger, 2003). Moreover, the nucleotide bound to the D2 domain is capable of interacting with a number of elements from different regions, including the D1–D2 linker, the Walker A and B motifs, helix $\alpha6'$ and $\alpha12'$ (DeLaBarre and Brunger, 2003). Together, the weakened arginine finger and the multiple interactions provide a mechanism for the dramatic conformational changes in the D2 domain during ATP hydrolysis.

It should be noted that many variations of the nucleotide states of D1 and D2 may be present in vivo. Hence, the in vitro structural studies may not reflect a physiological condition and only provide a first insight into the possible movements of the complex. Indeed, isothermal titration calorimetry showed that only half of the nucleotide-binding sites in the VCP complex are available for ADP binding (DeLaBarre and Brunger, 2003). A recent study on native brain VCP also showed that only two to three binding sites are occupied per hexamer (Zalk and Shoshan-Barmatz, 2003). Therefore, the relative occupancy in the 12 nucleotide-binding sites and the specific nucleotide states in the VCP hexamer may play a functional role in vivo. A similar notion has been suggested for the HsIU AAA ATPase (Wang et al., 2001).

3.3. Models for VCP dynamics

It was previously shown that the VCP is highly stable in a hexameric structure, which is a prerequisite for its full ATPase activity (Peters et al., 1992; Wang et al., 2003a). Although D1 and D2 are responsible for distinct functions (see next section), there is significant communication and mutual dependence between the two domains as well as among the protomers. In essence, the mechanical force for VCP dynamics is provided through ATP hydrolysis along with the combination of conformational change of individual domains and allosteric communications among the 12 nucleotide-binding sites.

Basically two types of models have been proposed to elucidate the dynamics of VCP during the ATPase cycle. Similar to the mechanism proposed for the GroEL/ES chaperonin (reviewed in Bukau and Horwich, 1998), a ratchet model has been proposed for VCP by Freemont and colleagues (Zhang et al., 2000b). In this model, VCP acts as a molecular ratchet whereby the D1 and D2 rings are interdependent and counter-balanced in the ATP hydrolysis cycle. ATP hydrolysis in one hexameric ring (e.g., D1) promotes ADP release in the other (e.g., D2) ring, resulting in an ATP-ready (empty) conformation, and *vice versa*. In other words, D1 moves in toward the D1 pore while binding to ATP; meanwhile, D2 moves outward upon releasing ADP, thus generating an alternating "milking" movement. Such an anti-correlated motion may provide the mechanical force for the VCP complex to modify protein conformation, as in the case of GroEL/ES. Normal mode analysis of the recently solved crystal structure indeed suggests anti-correlated motions and different conformational states of the two AAA domains (Huyton et al., in this issue), thus providing support for the ratchet model. This model is further supported by a recent study showing that D1 and D2 rings alternate in ATP hydrolysis, and that the activities of both D1 and D2 are required for the function of VCP (Ye et al., 2003). Based on this model, it is envisioned that substrate proteins enter the central pore of the VCP hexamer and get remodeled in the interior chamber by the "milking" movement between the two AAA domains.

In the ratchet model, the actions of D1 and D2 are reciprocal and both domains are efficient in catalyzing ATP hydrolysis. However, our biochemical study (Song et al., 2003) and the recent full-length crystal structure (DeLaBarre and Brunger, 2003) suggested that D2 is responsible for the major ATPase activity, and D1 only exhibits minor activity. These new data prompted DeLaBarre and Brunger to propose that the nucleotide-binding α/β subdomains in both D1 and D2 rings are held constant, while motion in the more variable helical subdomains provides the force for VCP. More specifically, the D1 α/β subdomain serves as a fulcrum for motion in the hexamer, and ATP hydrolysis in D2 brings about dramatic conformational changes which generate force that is transmitted through the D1 helical subdomain to the N domain. Furthermore, because the opening of the hexamer is normally blocked by a zinc ion, substrate proteins possibly interact with the hexamer at the exterior of the complex rather than through the interior chamber. This model is supported by in vitro biochemical studies (Song et al., 2003) as well as in vivo studies, which showed that a mutation in D2, rather than D1, is detrimental to the biological functions of VCP in cells (Kobayashi et al., 2002; Lamb et al., 2001).

4. Structural–functional analyses of p97–VCP

4.1. N domain: responsible for substrate and cofactor binding

The N domain is the least conserved region in AAA family proteins, and is expected to be the determining factor for target specificity. Indeed, the N domain of VCP has been shown to be responsible for binding to the cofactors/adaptors, including p47 and the Ufd1–Npl4

complex (Meyer et al., 2000), and the ubiquitinated target proteins, especially those conjugated with poly-Ub chains (Dai and Li, 2001). p47 and the Ufd1–Npl4 complex bind to VCP in a mutually exclusive manner, indicating that they may bind to the same site in the N domain (Meyer et al., 2000). Whether the cofactors and poly-Ub chains also bind to the same site in the N domain has not been determined.

A previous crystal structure of ADP-bound N–D1 showed that the N domain is ordered and resides at the periphery of the D1 ring (Zhang et al., 2000b); however, recent cryo-EM studies indicated that the N domain is flexible in most nucleotide states except in ATP hydrolysis (Rouiller et al., 2002). The difference may be attributed to a consequence of crystal lattice packing restrictions or the absence of the D2 domain in the crystal structure. Cryo-EM studies also showed that while the N domains are not necessary for hexamerization or ATPase activity in VCP, they operate with D1 and D2 domains to generate nucleotide-induced conformational changes (Rouiller et al., 2002). Based on the recent full-length crystal study, a "latch" model was proposed to explain how the N domain coordinates with other domains to move about (also see Section 3.1). When D2 is in the $ADP + P_i$ state, the D1–D2 linker senses this specific nucleotide condition and transmits the information to the D1 helical subdomain (the molecular latch), which then tilts downward to touch the N domain, thus restricting the N domain to a fixed position. As D2 transits to the ADP state, the linker straightens up and releases the latch, freeing the N domain to survey the environment for target binding. How individual N domains move in the context of the hexamer has not been definitively determined. Based on the comparison between the cryo-EM density map and the N–D1 crystal hexamer, a "three in and three out" model has been proposed to depict the movement of the N domain (Beuron et al., 2003; Zhang et al., 2000b). In this model, the six N domains of the hexamer move as two distinct sets of three. While one set moves upwards, the other set moves downwards; thus, each individual N domain moves in the opposite direction of its neighbor. By contrast, the cryo-EM study clearly showed that six p47 molecules bind to the periphery of the VCP hexamer in the presence of ATP (Rouiller et al., 2000), implicating a concerted movement of individual N domains. Future high-resolution structural analyses on the VCP/ligand complexes are needed to resolve the contradiction.

Results from our laboratory indicated that the N domain is required in mediating in vitro Ub–Pr degradation (Dai and Li, 2001). Further, an intact and unmasked N-terminus of VCP is critical for the ability to bind Ub chains. In an in vitro system, while VCP fused to a His_6-tag through the C-terminus efficiently binds to poly-Ub chains (Dai and Li, 2001), VCP fused to a GST-, His_6-, or biotin-tag through the N-terminus fails to do so (unpublished result, and Doss-Pepe et al., 2003; Meyer et al., 2002). Based on the importance of the N domain, it is predicted that abnormality in this domain would result in pathological consequences. As expected, in the 5th international conference on AAA proteins, Watts et al. reported that five missense mutations in the N domain of VCP were identified in patients with Familial Inclusion Body Myopathy, and with Paget Disease of the Bone and Frontotemporal Dementia, now termed IBMPFD OMIM#605382 (personal communication). Whether these mutations disrupt cofactor or substrate binding, or change the overall enzyme activity in VCP is subject to future investigation.

4.2. D1 domain: responsible for the formation of stable hexamers

The AAA modules frequently oligomerize into ring-shaped hexameric structures, which provides the mechanical basis for the chaperone activity. Many AAA proteins, such as NSF, ClpA, Hsp104, and proteasome ATPase subunits, readily form oligomers in the presence of nucleotides, but dissociate into monomers or dimers in the absence of nucleotides (reviewed in Vale, 2000). In comparison, VCP is exceptionally stable in hexameric form, and many attempts have failed to dissociate it into stable monomers. Hexamerization of VCP does not seem to require the presence of nucleotide (Wang et al., 2003a,b) because: (1) almost all of the VCP molecules isolated from animal tissues or cultured cells exhibit a hexameric structure even without exogenous nucleotides, (2) bacterially expressed recombinant VCP readily forms hexamers without the addition of nucleotides (DeLaBarre and Brunger, 2003; Rouiller et al., 2000, 2002; Wang et al., 2003b), and (3) VCP mutants with defective ATP-binding sites still form hexamers (Wang et al., 2003a). The difference in nucleotide requirements between VCP and other AAA hexamers may be explained by the structural details revealed by the crystallography studies of VCP N–D1 (Zhang et al., 2000b), NSF D2 (Lenzen et al., 1998; Yu et al., 1998), and full-length ClpA (Guo et al., 2002). In NSF D2 and ClpA D1, but not in VCP D1, strong electrostatic interactions are present in the nucleotide-bound pocket; thus, the release of ADP results in electrostatic repulsion and disassembly of the hexamer. Therefore, hexamer formation is tightly coupled with nucleotide binding in both NSF D2 and ClpA D1, whereas the VCP hexamer is highly stable independent of the nucleotide condition.

Both crystallography and biochemical studies indicated that D1 is the major domain responsible for oligomerization in VCP (Wang et al., 2003a; Zhang et al., 2000b). Recent studies from our laboratory further showed that the N–D1-linker (1–481) domain could form hexamers in the absence of nucleotides

(Wang et al., 2003b). While the N domain is not necessary for hexamerization, both the D1 (209–460) and D1–D2 (461–480) linkers are required for this nucleotide-independent hexamerization. The requirement for the linker region explains why N–D1 (1–458) proteins only form hexamers in the presence of ADP (Zhang et al., 2000b). Exactly how this linker confers the nucleotide-independence to the hexamerization process is not understood at present.

4.3. D2 domain: responsible for the major ATPase activity

Both in vivo and in vitro evidence indicated that intact ATPase activity is required for the biological functions of VCP. As with all AAA proteins, VCP requires Mg^{2+} for its enzyme activity, and the activity is inhibited by NEM (*N*-ethylmaleimide) (Peters et al., 1990; Song et al., 2003). VCP is a weak ATPase, ~10-fold lower than the Na^+/K^+-ATPase (Song et al., 2003). Under optimal conditions, VCP hydrolyzes ATP with a K_m of ~0.33 mM and a V_{max} of ~0.52 nmol $P_i\,min^{-1}\,\mu g^{-1}$, in agreement with a K_m of 0.62 mM and V_{max} of 0.3 nmol $P_i\,min^{-1}\,\mu g^{-1}$ previously reported for the rat liver p97 (Meyer et al., 1998; Song et al., 2003). The high sequence similarity between D1 and D2 suggests that both domains contribute to the ATPase activity. However, in vitro assays showed that, at physiological temperatures, mutations in D2 significantly inhibit the ATPase activity, while similar mutations in D1 have less effect (Song et al., 2003; Ye et al., 2003). Consistent with this observation, expression of a VCP mutant with defective D2 results in cytoplasmic vacuoles, ERAD defects, and cell death in PC12 cells; whereas an identical mutation in D1 does not have the same impact (Kobayashi et al., 2002). Similarly, a D2, but not D1, mutation in Trypanosomal *Tb*VCP also led to cell death as a dominant negative phenotype (Lamb et al., 2001). Therefore, like many type II AAA proteins, D1 and D2 of VCP are not equal: while D1 is responsible for oligomerization, D2 accounts for the major enzyme activity. Together, these studies suggest that although D1 does play a role in the overall function of VCP, it is not the major player for ATP hydrolysis. Interestingly, a recent study by Ye et al. (2003) showed that both D1 and D2 have significant enzyme activity, presenting a discrepancy from that observed in the previous in vitro and in vivo studies. However, the discrepancy may be, at least partially, attributed to the different assay systems and the dissimilar mutant constructs used in the studies.

As would be expected of a molecular chaperone, the ATPase activity of VCP is modulated by many environmental factors. It is enhanced by high temperature and pH, but suppressed by salt, alcohol, ADP, and oxidizing agents (Song et al., 2003). Interestingly, as the wild-type VCP exhibits heat-enhanced ATPase activity, which peaks around 50–55 °C, mutations in D1, but not D2, abolish this heat-enhanced activity. 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 (Song et al., 2003). Thus, D2 accounts for the major ATPase activity, and D1 contributes to the heat-enhanced activity. While the molecular basis for this heat response is unknown, we speculate the structures of D1 and D2 rings play an important role. Both cryo-EM and biochemical studies indicated that the D1 ring is relatively stable, whereas the D2 ring undergoes dramatic conformational changes during ATP hydrolysis. It is conceivable that upon heat treatment, the originally compact D1 ring expands and becomes more flexible and accessible to the solvent, thus facilitating ATP hydrolysis more readily. Currently, it is not known whether heat treatment will induce the expression and/or activity of VCP in vivo. Another level of regulation of VCP activity is conferred by the binding of cofactors/adaptors. Both p47 (Meyer et al., 1998) and Ufd1 (unpublished result) inhibit the ATPase activity of VCP, with p47 being a stronger inhibitor. Curiously, tetra-Ub chains have no effect on the ATPase activity of VCP (unpublished result). These results seem to suggest that a low enzymatic activity of VCP is maintained through binding with cofactors under normal conditions. One can imagine that when these cofactors are replaced or released, perhaps as a result of environmental changes, the inhibition is relieved, resulting in an elevated ATPase activity.

4.4. C-terminal extension: tyrosine phosphorylation regulates membrane fusion and nuclear translocation

Compared with the rest of the VCP molecule, the C-terminal extension (residues 762–806) is much more elusive both structurally and functionally. It appears to be a highly flexible domain prone to protease digestion (Wang et al., 2003b), and is undetectable in crystallography and EM analyses. One striking characteristic of the C domain is the presence of a stretch of acidic residues at the very C-terminus, which also contains the major tyrosine (Tyr) phosphorylation site, Tyr805 (Egerton and Samelson, 1994). Tyrosine phosphorylation of VCP regulates transitional ER assembly in vitro. Lavoie et al. identified membrane-associated JAK-2 and PTPH1 as the relevant kinase and phosphatase, respectively, for VCP (Lavoie et al., 2000). They also demonstrated that Tyr dephosphorylation stabilizes the association between VCP and the ER membrane, hence promoting transitional ER assembly (Lavoie et al., 2000).

In *Saccharomyces cerevisiae*, nuclear import of Cdc48p is regulated by a cell cycle-dependent

phosphorylation event, whereby Cdc48p enters the nucleus during the late G1 phase (Madeo et al., 1998). It was shown that Tyr phosphorylation of the C-terminus causes a conformational change of the acidic stretch. This change leads to the exposure of the nuclear import signal sequence, at the N domain, thus causing nuclear translocation of Cdc48p (Madeo et al., 1998). It has been shown that VCP is one of the first proteins that undergo Tyr phosphorylation in response to T-cell stimulation (Egerton et al., 1992), and Tyr phosphorylation of VCP is highly sensitive to stimulation by sodium vanadate and hydrogen peroxide in B lymphocytes (Schulte et al., 1994). Moreover, Tyr-phosphorylated VCP was found to be one of the major phosphorylated proteins in capacitated human sperm (Ficarro et al., 2003). How Tyr phosphorylation is involved in these growth and differentiation-related functions is currently unknown. Since Tyr phosphorylation does not change the ATPase activity of VCP (Egerton and Samelson, 1994), we speculate that it serves as a localization signal by regulating the association of VCP with different adaptors or target proteins.

4.5. Entire hexameric VCP complex: responsible for the biological functions

It is generally accepted that the assembly into the oligomeric state of AAA proteins is a prerequisite for fulfilling biological functions. Several lines of evidence indicate that this is also true in VCP (Wang et al., 2003a), and the entire molecule of VCP is required for its biological functions (Song et al., 2003). In retrotranslocation, mutating the ATP-binding site in D1 of VCP eliminates substrate binding, and mutation in either D1 or D2 inhibits the extraction of substrates (Ye et al., 2003). VCP–p47-mediated membrane fusion also requires the intact VCP hexamer, because N–D1 domain is needed to bind p47 and both D1 and D2 are required for the full enzyme activity to dissociate the VCP/p47/syntaxin5 complex (Meyer et al., 2000; Uchiyama et al., 2002). Similarly, deletion mutants lacking one of the N, D1, and D2 domains failed to mediate the degradation of cyclin E in an in vitro Ub–Pr degradation system (Dai and Li, 2001; Song et al., 2003). The involved chaperone activities may include target binding, disassembly of protein complex containing the target, further remodeling of the target protein, and its final delivery to the proteasome. Whether each specific activity requires the presence of all domains of VCP is currently under investigation. It is known that the N–D1 domain is sufficient for binding to the polyubiquitinated substrates and cofactors. For binding to non-native proteins and prevention of aggregation, our preliminary study showed that only the hexameric D1 domain containing residues 200–481, and not ATPase activity, is needed (unpublished result). With respect to the disassembly and remodeling activity of VCP, in vitro structure–function studies have not been available to decipher the detailed relationship. Nevertheless, it is clear that these efficient chaperone activities require a tight coupling between ATP hydrolysis cycles and dynamic conformational changes. As mentioned in Section 3, two types of models have been proposed to elucidate the VCP dynamics. Based on these models, we speculate that VCP may work on different types of substrates with distinct mechanisms. For example, VCP may suppress protein

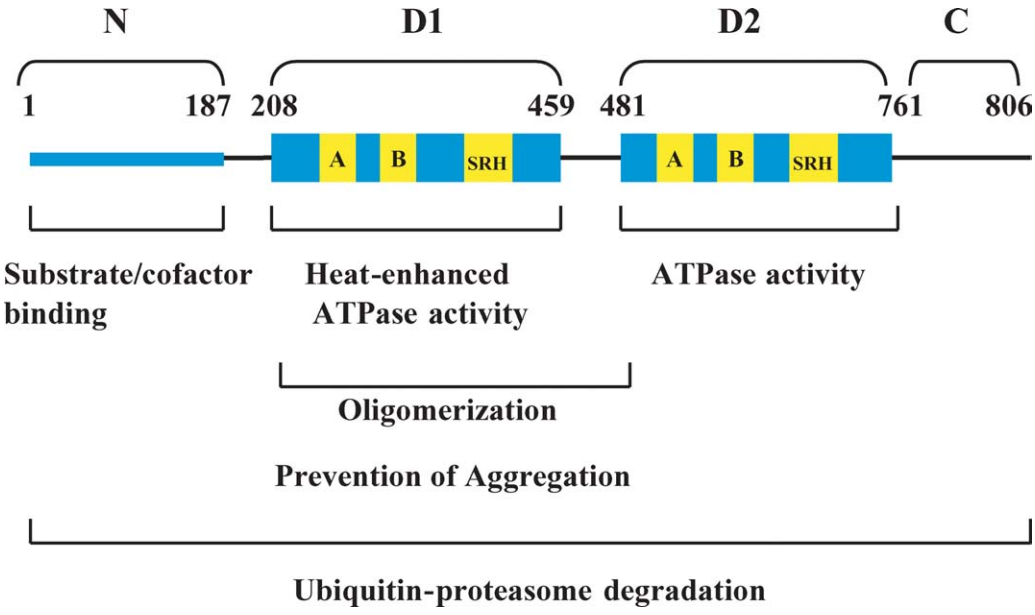

Fig. 2. Summary of the structural–function relationship in VCP. Based on the reported in vitro and in vivo studies, the major function of each domain in VCP is summarized. A, B, and SRH indicate the Walker A, Walker B, and the second region of homology motifs, respectively.

aggregation and disassemble protein complexes by interacting with non-native peptides at the exterior of the complex, as suggested by DeLaBarre and Brunger (2003). On the other hand, a protein may first bind to the N domain of VCP, then enter through the D1 pore, to be remodeled within the interior chamber before it is passed to the proteasome. The ratchet model proposed by Freemont and colleagues provides support for this scenario (Zhang et al., 2000b).

5. Perspective

In this review, we described the bewilderingly large variety of VCP functions and presented a general mechanism, whereby VCP acts as a molecular chaperone that cooperates with the Ub–Pr system in mediating the diverse activities (Fig. 1). We also summarized the current understanding of the structural dynamics of the VCP complex as well as the structure–function relationship (Fig. 2) in the complex. Although significant progress has been made during the past several years, many questions still need to be answered. It is anticipated that many additional cofactors/adaptors will be identified in the future. Biochemical and high-resolution structural studies of the VCP/adaptor complex during the ATPase cycle will undoubtedly provide valuable information to our understanding of how this enzymatic machine works. Presently, a great deal of effort is being devoted to investigate whether and how VCP further modifies the conformation of target proteins. Furthermore, how VCP cooperates with other molecular chaperones, such as Hsp70, 40, and 90, in performing its chaperone activities is another topic of profound interest.

Given the essential nature of VCP and the Ub–Pr system, altered expression or mutation of VCP is expected to have pathological consequences. Supporting this expectation, an elevated level of VCP is detected in many cancer cells and is associated with a poor prognosis in carcinoma (Yamamoto et al., 2003a,b). Moreover, because of the involvement of VCP in ERAD, dysfunction of VCP would seriously jeopardize protein quality control and secretory pathways (Higashiyama et al., 2002; Hirabayashi et al., 2001; Kobayashi et al., 2002; Nagahama et al., 2003). Indeed, recent reports have suggested an involvement of VCP in neurodegenerative diseases, which are often characterized by having Ub-positive protein aggregates. Since VCP interacts with polyubiquitinated proteins and is capable of suppressing protein aggregation, it may play a role in the formation or removal of these aggregates. Overall, these observations suggest that VCP may be a potential molecular target for the treatment of certain cancers and neurodegenerative disorders. It is conceivable that specific inhibitors or stimulators for the chaperone activity of VCP will prove to be chemopreventive and chemothrapeutic agents for human diseases.

Acknowledgments

We thank B. DeLaBarre and A. Brunger for sharing the preprint before its publication, and D. Bertollette, D. Ferris, and M. Maurizi for editing the manuscript. C.-C.L. would like to thank S. Gottesman, M. Maurizi and S. Wickner for their helpful discussions over the years. We apologize to colleagues whose important studies were not directly cited due to space limitation. This publication has been partially funded with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract #NO1-CO-12400.

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