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Cryo-EM of the pathogenic VCP variant R155P reveals long-range conformational changes in the D2 ATPase ring

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ABSTRACT

Single amino acid mutations in valosin containing protein (VCP/p97), a highly conserved member of the ATPases associated with diverse cellular activities (AAA) family of ATPases has been linked to a severe degenerative disease affecting brain, muscle and bone tissue. Previous studies have demonstrated the role of VCP mutations in altering the ATPase activity of the D2 ring; however the structural consequences of these mutations remain unclear. In this study, we report the three-dimensional (3D) map of the pathogenic VCP variant, R155P, as revealed by single-particle Cryo-Electron Microscopy (EM) analysis at 14 Å resolution. We show that the N-terminal R155P mutation induces a large structural reorganisation of the D2 ATPase ring. Results from docking studies using crystal structure data of available wild-type VCP in the EM density maps indicate that the major difference is localized at the interface between two protomers within the D2 ring. Consistent with a conformational change, the VCP R155P variant shifted the isoelectric point of the protein and reduced its interaction with its well-characterized cofactor, nuclear protein localization-4 (Npl4). Together, our results demonstrate that a single amino acid substitution in the N-terminal domain can relay long-range conformational changes to the distal D2 ATPase ring. Our results provide the first structural clues of how VCP mutations may influence the activity and function of the D2 ATPase ring.

1. Introduction

Valosin Containing Protein (p97/VCP) is a ubiquitous, chaperone-like, ATPase belonging to the ATPases Associated with diverse cellular Activities (AAA) protein family [1]. VCP is crucial for cell survival and has a variety of well-characterized functions in ubiquitin-proteasome-mediated protein degradation, membrane fusion, cell cycle regulation and autophagy [2,3]. Like other AAA proteins, VCP assembles into a barrel-shaped homohexamer with six protruding N-terminal domains attached to two stalked ATPase rings, D1 and D2, and a disorderly C-terminal region [4–6]. Flexibly linked to the D1 ATPase ring, the N-terminal domain is the primary region for substrate and cofactor binding [7,8].

Nucleotide binding to D1 contributes to VCP hexamerization and heat-induced activity *in vitro* [9,10], yet it is not required for trypanosome viability [11] and protein degradation [12,13]. VCP ATPase activity is mediated primarily by the D2 ATPase ring [9]. During the ATPase cycle, major conformational changes of the N-terminal domains have been observed [4,5]. These N-terminal conformational changes enable VCP to act as chaperone or 'molecular motor' to mediate substrate extraction/remodelling or macromolecular complex disassembly [3]. To execute these functions, VCP utilizes a plethora of adaptor proteins such as Ubiquitin fusion degradation-1 (Ufd1) and Nuclear protein localization-4 complex (Npl4), VCP–interacting protein (p47, VCIP) and VCP–interacting membrane protein (VIMP), which bind to the N-terminal domain [7] and channel its activity in specific cell biological pathways [14].

Hereditary and sporadic mutations in the VCP gene are linked to inclusion body myopathy, a progressive, degenerative disorder associated with Paget's disease of bone as well as frontotemporal dementia (IBMPFD-OMIM#167320) [15]. Missense mutations in *VCP* have also been associated with amyotrophic lateral sclerosis (ALS-OMIM#613954) [16]. The vast majority of IBMPFD and ALS-

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associated mutations consist of a single amino acid substitution at the N-D1 interface. Most mutations have no effect on the overall hexameric organization of VCP but they were found to increase the ATPase activity of the D2 ring [17–20]. A change of conformation of the N-terminal domains has been observed with a truncation mutant containing only the N-D1 domains and bearing IBMPFD mutations [21]. While the structural basis for the elevated ATPase activity remains unclear, it has been proposed that the conformation of the N-terminal domain in relation to the D1-D2 rings is directly linked to D2 ATPase activity [18]. It is plausible that VCP mutations confer conformational changes which are relayed to the D2 ring, affecting its activity. While the presence of D2 different conformations is supported by the increased proteolytic susceptibility of the D2 ring [17,22], a conformational difference within D2 has not yet been observed at the structural level.

For the purpose of this study, we analysed the impact of the most prevalent amino acid mutations found in IBMPFD patients on the structure of p97 and its ability to bind to cofactors. We determined the Cryo-EM structure at 14 Å resolution of the VCP variant R155P (substitution of an arginine by a proline at position 155). The arginine at position 155 (R155) is the most frequently mutated amino acid residue in IBMPFD patients. We expect that the substitution of the arginine by a proline (R155P) has the most measurable impact (similar but amplified compared to other IBMPFD mutations), due to its localization in a beta(β)-sheet structure and the potency of proline as a breaker of secondary structures. Our data shows that the local structural effects of the R155P mutation can be propagated over a long-distance to influence the conformation of the D2 ring. Consistent with a conformational change, biochemical analysis demonstrates that the VCP R155P variant disrupts cofactor interaction; especially the interaction of VCP with its well characterized cofactor Np14.

2. Materials and methods

2.1. Generation of recombinant expression constructs

The constructs containing murine VCP_{WT}, VCP_{R95G}, VCP_{R155P} and VCP_{A232E} are described elsewhere [17]. VIMP was cloned using the cDNA extracted from human embryonic kidney 293 cells (HEK293). Plasmids pET26-Ufd1-His, pET30-Npl4, pTrcHis-p47 and Flag-HA-VCPIP1 were acquired from Addgene (Cambridge, USA). All genes were recloned in pET101 (Invitrogen), introducing a His tag at the C-terminus. All the sequences were verified by full sequencing (Genome Quebec Innovation Center, Montreal, Canada) prior to starting the expression of proteins using a previously published technique [4,5,17].

2.2. Electrophoresis and isoelectric focussing

All electrophoresis was conducted using standard methods (see Supplementary Material). All gels were stained with Coomassie Brilliant Blue R-250.

2.3. Immunoprecipitation and western blotting

Each bacteria expressing plasmids were lyzed by sonication in immunoprecipitation buffer (IBP) containing 25 mM Tris–HCl pH 7.4, 200 mM KCl, 2mM$MgCl_2$ 1 mM DTT, 5% glycerol and 1% Triton X-100 and cleared by centrifugation at 6,000 g for 30 min. Supernatants of bacteria expressing each VCP were mixed separately with each of the supernatants of bacteria expressing VIMP, Ufd1, Npl4, p47 and VCIP in IPB. Polyclonal antibodies against VCP (Thermo Scientific at 1:1000 dilution) were added and incubated with the mixture of supernatants for 1 h at 4 °C. G-sepharose beads were added and the entire mixture incubated with rotation for 30 min at 4 °C. The mixture was pelleted and washed 3 times with IPB. Proteins were eluted with sample buffer and analysed by SDS-PAGE and Western blotting using a primary His-tag antibody (Pierce).

2.4. ELISA based protein binding assay

One microgram of purified cofactors was fixed in 96 wells plate in IPB Buffer. After saturation by 5% Bovine Serum Albumin (BSA), purified VCP (WT or variants) at different concentrations (from 0.01 pM to 100 μM) were added to the plates with fixed cofactors. After 6 washes with IPB buffer, polyclonal antibodies against VCP were added and incubated for 1 h at 4 °C. Samples were then washed 6 times and a secondary antibody bound to horseradish peroxidise (HRP) was added. Following several washes, labelling was detected using tetramethylbenzidine (TMB, ThermoFisher, Ottawa, Canada). The reaction was stopped after 10 min incubation in obscurity by adding 100 μl of 1 M sulphuric acid, and the plate was read at 405 nm. BSA was used as a negative control during the experiments. Three independent measurements were performed, each in duplicate, for each binding assay.

2.5. Cryo-Electron Microscopy

5 μL of 150 μg/ml VCP proteins were added to EM holey carbon grids (Quantifoil R2/2), blotted and frozen-hydrated by plunging into a bath of liquid ethane slush. The grids were stored in liquid nitrogen until transfer to a 626 Single Tilt Cryotransfer System (Gatan Inc.) and observed with a FEI G2 F20 microscope operated at 200 kV (FEI, Inc). Images were recorded on a Gatan Ultrascan 4 k × 4 k Digital (CCD) Camera System Camera at a nominal magnification of 50,000× corresponding to pixel size of 2.3 Å/pixel, at a defocus ranging from −1 to −3.5 μm.

2.6. Images analysis and three-dimensional reconstruction

Particle selection was conducted using Signature software [23]. Boxing was done in a centred manner to allow for subsequent alignment of the particles. Data was manually sorted in order to remove ice contamination, overlapping particles and particles too close to the frame edge. The defocus of the images was estimated using the program "ctffind3" [24]. Contrast transfer function (CTF) correction was performed during the projection matching refinement procedure. 19,700 and 15,800 images of individual VCP_{WT} and VCP_{R155P} particles were included in the 3D reconstructions respectively. The analysis was performed using the XMIPP software suite [25]. Two different starting models were used and led to the same result. The first starting model was derived from the high resolution structure of VCP-ADP solved by X-ray crystallography (pdb id: 3CF1) [6], converted to densities and low-pass filtered to 50 Å resolution. The second model was a cylinder. Several steps of projection matching refinement were repeated by progressively decreasing the angular sampling from 15° to 1°.

In addition to using two different starting models, the single particle reconstruction analysis was validated using the strategy of crossed models. The refinement procedure was repeated using the VCP_{WT} map as a starting model with the VCP_{R155P} data set and reciprocally by using the VCP_{WT} data set and the VCP_{R155P} as a starting model. This approach showed that the final maps were independent of the starting models.

Handedness of the maps was assigned by comparison with the crystal structures of VCP [6]. The resolution of both maps was estimated to be ~14 Å using the Fourier shell correlation criteria of 0.5. The 3D maps were visualised using Chimera [26].