Structural dynamics of AAA+ ATPase Drg1 and mechanism of benzo-diazaborine inhibition

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Abstract

The AAA+ ATPase Drg1 is a ribosome assembly factor in yeast, and functions to release Rlp24, another assembly factor, from the pre-60S particle just exported from nucleus to initiate its further cytoplasmic maturation. Being a type II AAA+ protein with two ATPase domains (D1 and D2), its activity in ribosome assembly can be inhibited by a drug molecule diazaborine. In human, mutations of Drg1 homologue has been linked to a disease condition called epilepsy, hearing loss, and mental retardation syndrome. Although the general structure of Drg1 hexamer was recently reported, its complete structure and dynamic conformational rearrangements driven by ATP-hydrolysis are poorly understood. Here, we report a comprehensive structural characterization of Drg1 hexamers in different nucleotide-binding and benzo-diazaborine treated states. Our data show that Drg1 hexamers transits between two extreme conformations, characterized by a planar or helical arrangement of its six protomers. By forming covalent adducts with the ATP molecules in the active centers of both D1 and D2, benzo-diazaborine locks Drg1 hexamers in a more symmetric and non-productive conformation. In addition, we obtained the structure of a mutant Drg1 hexamer (Walker B mutations) with a polypeptide trapped in the central channel, representing a 3D snapshot of its functional, substrate-processing form. Conserved pore loops on the ATPase domains of Drg1 form a spiral staircase to interact with the substrate through a sequence-independent manner. These results suggest that Drg1, similar as Cdc48/p97, acts as a molecular unfoldase to remodel pre-60S particles, and benzo-diazaborine inhibits both the inter-protomer and inter-ring communication to disable the conformational cycling of Drg1 protomers required for the unfolding activity.

Introduction

AAA+ ATPases (ATPases-associated with diverse cellular activities) are a superfamily of proteins with various functions in remodeling their substrates in different subcellular localization, ranging from protein quality control, membrane trafficking to DNA/RNA unwinding ¹⁻⁶. Many AAA+ ATPases are dedicated to the unfolding of proteins and the disassembly of macromolecular complexes. These protein-remodeling AAA+ ATPases generally consist of two types: Type I, such as Katanin and Spastin which contribute to microtubule dynamics ⁷, contains an N-terminal domain (NTD) and an ATPase domain, whereas Type II, such as *N*-ethylmaleimide sensitive factor (NSF), p97/Cdc48 and ClpB/HSP104 ⁸⁻¹⁴, contains an NTD and two ATPase domains (D1 and D2). The NTD often binds to adaptor proteins, which are responsible for substrate recognition ^{5,15}. The adaptors can localize, regulate and determine the specific functions of AAA+ ATPases ¹⁶⁻¹⁸. Over recent years, based on extensive structural and functional studies on several type II AAA+ proteins, the mechanistic understanding of AAA+ proteins in protein unfolding has been greatly advanced ^{2,8,9,11,12,14,19-22}. Especially, the structural studies on AAA+ ATPase hexamers with their native substrates revealed the molecular basis of substrate recognition and processing with a hand-over-hand mechanism ^{2,5,23}.

Eukaryotic ribosome biogenesis is a complicated and energy-consuming process that requires more than 200 ribosome assembly factors^{24–27}. Three AAA+ ATPases are involved in the assembly of the large ribosomal subunit, including Rix7, Rea1, and Drg1, which are located in nucleolus, nucleoplasm, and cytoplasm, respectively²⁸. Rea1, as a member of dynein-related AAA+ ATPase subfamily, is involved in two steps of the 60S maturation, including the release of Nop7 and Rsa4 in nucleolus and nucleoplasm, respectively^{29,30}. Both Rix7 and Drg1 belong to type II AAA+ proteins. Rix7 was proposed to act as the earliest AAA+ ATPase in the maturation pathway to catalyze the removal of Nsa1 from the nucleolar pre-60S ribosomal particles³¹. Drg1 initiates the cytoplasmic pre-60S maturation, and is necessary for releasing several shuttling factors Nog1, Rlp24, Tif6 and Mrt4 and export factors Mex6/Mtr2 from the cytoplasmic pre-60S particles^{32,33}. Specifically, the NTD of Drg1 was reported to interact with the C-terminal domain (CTD) of Rlp24, and this interaction in turn enhances the ATPase activity of Drg1³². The function of Drg1 may also require a nuclear pore protein Nup116, which was identified as a Drg1 interactor through a yeast two-hybrid analysis³². Nup116 might have a potential adaptor function for the release of Rlp24 from pre-60S particles. Presumably, the removal of Rlp24 directly by Drg1 triggers the simultaneous or subsequent dissociation of other biogenesis factors that interact with Rlp24 on pre-60S particles³⁴.

Drg1 is an essential protein in *Saccharomyces*³⁵, and its mammalian homologue SPATA5 is associated with spermatogenesis by regulating mitochondrial functions^{36,37}. SPATA5 mutations in human have been linked to a disease condition, Epilepsy, Hearing Loss and Mental Retardation Syndrome³⁸. Yeast *AFG2* encoding Drg1 was also identified as one of the genes related to the sensitivity of the cells towards diazaborine treatment³⁹. Diazaborine is a heterocyclic boron-containing compound targeting fatty acid and phospholipid biosynthesis in Gram-negative bacteria^{40,41}. Diazaborine inhibits the ATP hydrolysis of Drg1 and therefore leads to accumulation of shuttling proteins on the pre-60S particles in the cytoplasm^{32,33,42,43}. Importantly, diazaborine resistance mutations were found to locate close to the D2 ATPase site of Drg1³³. Recently, two cryo-EM studies have reported the structural characterization of yeast Rix7 and Drg1^{22,44}, revealing a conserved hexameric architecture for the two proteins ATPases, highly similar as those of Cdc48/p97^{8,11,12,14}. The structure of Drg1 was solved in the presence of diazaborine which explained the structural basis of the drug action on the D2 domain⁴⁴. However, neither of them provided the high-resolution structures for the NTDs of these two proteins, and the structure of Drg1⁴⁴ was not in the substrate-processing states.

In the present study, we provide a comprehensive structural characterization of yeast Drg1 hexamers in the presence of ADP, AMPPNP, a mixture of ADP/AMPPNP/benzo-diazaborine or a mixture of ATP/benzo-diazaborine. We also obtained a structure of a mutant form of Drg1 (E346Q/E617Q) hexamer, featuring a polypeptide substrate in the central channel with staggered interactions with the pore loops of Drg1 ATPase domains. These structures provide insights into the general mechanisms of substrate unfolding and translocation for type II AAA+ proteins, and the molecular basis for understanding the specific function of Drg1 in ribosome assembly. In addition, our results show that benzo-diazaborine binds to the

ATPase sites of both D1 and D2 domains, and locks the hexamer in a more symmetrical conformation. As one of the highly potent inhibitors in the diazaborine family compounds against bacteria ⁴⁵, our data thus provide critical information for improvement of benzo-diazaborine chemicals as anti-fungal drugs ⁴⁶.

Results

Structural determination of Drg1 hexamers in different nucleotide binding states

To understand the structural dynamics of *S. cerevisiae* Drg1 hexamer, we assembled Drg1 hexamers *in vitro* in the presence of excessive ADP or AMPPNP (a non-hydrolysable analogue of ATP). To obtain possible hybrid nucleotide bound states that best mimic the ATPase hydrolysis cycle of Drg1 and to illustrate the inhibition mechanism of benzo-diazaborine, we also incubated Drg1 with a mixture of ADP, AMPPNP and benzo-diazaborine, as well as with a mixture of ATP and benzo-diazaborine. These samples were subjected to electron microscopic analysis. Negative staining electron microscopy confirmed the hexameric forms of all these Drg1 samples (Supplementary Fig. 1). The structures of Drg1 hexamers (Fig. 1) from these different samples were subsequently determined using cryo-EM at resolutions of 3.5 to 5.6 Å (Supplementary Figs. 2-5). In general, Drg1 hexamers are high dynamic and exhibit different modes of motion. All structures display a typical three-layered architecture, similar to those of type II AAA+ hexamers, such as p97/VCP and Cdc48 ^{8,19}, and the NTD, D1 and D2 rings show an apparent deviation from a perfect six-fold symmetry. Multiple rounds of 3D classifications suggest that the conformational changes of Drg1 hexamers are likely continuous, as different 3D classes tend to have comparable numbers of particles (Supplementary Figs. 2-5). Comparisons of these structures indicate that Drg1 hexamers transit between two extreme conformations, a planar and a helical state. While the six subunits in the planar or planar-like states are arranged more symmetrically, the subunits in the helical states adopt a spiral configuration to varying extents. In summary, these cryo-EM analyses provide an ensemble of structures that reflect intrinsic properties of Drg1 hexamers.

Next, we prepared a mutant form of Drg1 with two mutations (E346Q and E617Q) located in Walker B motif of the D1 and D2 domains, respectively ¹. These two mutations abolish ATP hydrolysis but not ATP binding to the active centers of the D1 and D2 domains ³². Mutant Drg1 hexamers were incubated with excessive ATP for stable hexamer formation before cryo-EM sample preparation. The cryo-EM structure of mutant Drg1 hexamers was determined at a much-improved resolution of 3.5 Å (Fig. 2). Similar as in the structural study of Rix7 ²², the introduction of the Walker B double mutation allowed the capture of an endogenous peptide within the central channel of Drg1 hexamer during purification. Upon the placement of the substrate in an upright position, the six protomers clearly form spiral staircases around the peptide (Fig. 2d). This substrate binding should have stabilized the hexamer and likely reduced its conformational space. This is probably the reason why the structure of the mutant hexamers could be resolved at a higher resolution, allowing the atomic modelling of most of the Drg1 sequence.

Based on the high-resolution map of the mutant hexamer, we built an atomic model of Drg1 from residues 29 to 777 (Fig. 3). The residues 29-230, 244-509 and 515-777 constitute the NTD, D1 and D2, respectively. A surprising finding is that the NTD structure of Drg1 is highly similar to those of p97 and Cdc48 (Fig. 4b-f), despite of their low sequence homology (Fig. 4a). Compared to Cdc48, the orientation of the NTD relative to the D1 domain in the Drg1 hexamer is different, displaying a large rotation (Fig. 4g-i).

The D1 and D2 domains can be divided into two subdomains (an α/β core and an α -helical lid subdomain). The α/β core subdomain is composed of a five-stranded β -sheet and five α -helices in the order of $\alpha 0$ - $\beta 1$ - $\alpha 1$ - $\beta 2$ - $\alpha 2$ - $\beta 3$ - $\alpha 3$ - $\beta 4$ - $\alpha 4$ - $\beta 5$. The α -helical lid is composed of four α -helices ($\alpha 5$ - $\alpha 6$ - $\alpha 7$ - $\alpha 8$) (Fig. 3a). The functional motifs of AAA+ domains, such as Walker A, Walker B, Sensor I, Sensor II, arginine finger (AF) and pore loops I and II (PL-I, PL-II), are all well resolved (Fig. 3b-d). The models of Drg1 hexamers treated with ADP, AMPPNP, ADP/AMPPNP/benzo-diazaborine and ATP/benzo-diazaborine were similarly obtained, by rigid-body fitting of multiple copies of the Drg1 model (with manual adjustment and rebuilding when necessary).

Structures of Drg1 hexamer in the absence of substrate

A distinct feature for Drg1 hexamers from other AAA+ hexamers, such as p97/Cdc48 and NSF is that the NTDs do not show a large-scale, nucleotide-dependent axial displacement (Figs.1-2). For Cdc48 and p97, their NTDs form a plane with the D1 domains in the presence of ADP (co-planar with the D1 ring) and turn into an “up” conformation upon binding to ATPyS^{8,11,12,19}. In all our structures, the NTD rings of Drg1 hexamers are always in “up” conformations (Figs. 1-2, Supplementary Figs. 2-5).

To better understand the structural dynamics of Drg1 hexamers related to the nucleotide hydrolytic cycle, we analyzed the structures of Drg1 hexamers obtained from three different samples (ADP, AMPPNP, ADP/AMPPNP/benzo-diazaborine). In these datasets, only a small fraction of particles displays an approximate six-fold symmetry (Supplementary Fig. 2-5). Most 3D classes adopt asymmetrical conformations, with one or two subunits more flexible than others. In addition, a few 3D classes also show highly flexible D2 rings (Supplementary Figs. 3c and 4c). In contrast, for the ATP/benzo-diazaborine treated hexamers, most of the 3D classes are more symmetrical (Fig. 1e and Supplementary Fig. 5c).

Taking the dataset of ADP/AMPPNP/benzo-diazaborine as an example, we performed deep 3D classification and analyzed the conformational differences between its planar and helical states (Fig. 1c-d; Supplementary Fig. 4 and 6). While the NTD and D1 rings are roughly superimposable, the D2 rings display a very large rotation between two states (Supplementary Fig. 4c and 6). These results indicate that the conformational cycling of Drg1 hexamers would generate a large-scale inter-ring rotation, in addition to the axial displacement of individual subunits.

Distinct nucleotide occupation of the D1 and D2 rings in Drg1 hexamers

An intriguing observation is that the nucleotide binding pockets of D1 domains in the structures of different datasets are all occupied, either by ATP or AMPPNP (Supplementary Fig. 7a-10a), even for the Drg1-ADP sample (Supplementary Fig. 7a). Although the structure from the Drg1-ADP dataset was not resolved at atomic resolution, but the local densities suggest the presence of ATP in these D1 sites. Therefore, this co-purification of ATP with Drg1 implies that the D1 ATPase site likely has a high affinity for ATP and a low hydrolysis activity. A similar observation was also made on p97: its D1 domain has a higher nucleotide affinity than the D2 domain ^{47,48}. Although the nucleotide affinity and the ATPase activity of the D1 and D2 domains of Drg1 are not explicitly examined, some data did reveal sharply different enzymatic properties for the two domains ^{32,33,49}.

There is no obvious nucleotide occupation in the D2 domains of the structures from the ADP and AMPPNP treated samples (Supplementary Fig. 7b and 8b). The nucleotide binding to the D2 domains was only seen in structures from benzo-diazaborine treated samples, including the ADP/AMPPNP/benzo-diazaborine and ATP/benzo-diazaborine datasets (Supplementary Fig. 9b and 10b). While the six D2 ATPase sites in the structure of the ATP/benzo-diazaborine dataset are fully occupied by ATP, the six D2 sites in the structures from the ADP/AMPPNP/benzo-diazaborine dataset (Supplementary Fig. 9b and 10b) are not saturated and appear to be partially occupied by ADP. These observations again suggest different affinity and complex regulation on the ATP/ADP binding to the D1 and D2 domains. Nevertheless, these results are in accordance with previous functional data that Drg1 D1 mutant (E346Q) does not affect growth, and has little effect on the release of shuttling proteins from pre-60S particles, whereas Drg1 D2 mutation (E617Q) is lethal ³². Therefore, like many type II AAA hexamers, the primary role of one high-affinity ATP binding site (D1 in p97, D2 in NSF, and D1 in Drg1) is likely to facilitate the nucleotide-dependent hexamer formation (reviewed in ref ⁵). In summary, our data suggest a complex regulation on the nucleotide binding to the 12 ATPase sites in a single hexamer.

Structure of Drg1 hexamer captured in processing peptide substrate

In the structure of the mutant Drg1 hexamer (Drg1^{E346Q/E617Q}-ATP), a substrate was found in the central channel, which passes through the central pores of Drg1 hexamers formed by the PLs of the D1 and D2 rings (Fig. 2 and Fig. 5). This peptide should be a non-specific substrate of *E. coli* origin that was processed by Drg1 hexamers in *E. coli* cells and therefore co-purified with mutant Drg1 hexamers. The density of the main chain of this substrate was well resolved such that we could build a poly-alanine model (23 residues) for it. The peptide spans about 65 Å length of the pore (Fig. 5d), and is similar to the one in the Rix7 structure which also contains a threaded peptide in the central channel ²².

The six protomers of Drg1 form a spiral staircase to accommodate the substrate. Among the six protomers of Drg1 (named P1-P6), five are apparently more stable and display a higher resolution in the density map (Fig. 2 and Supplementary Fig. 5f-j). Each protomer was then subjected to a mask-based 3D refinement. The improved local maps of P1 to P6 confirm that P6 is relatively unstable compared to the other five. Thus, P6 of the mutant Drg1 hexamer should be the “seam” subunit reported in the structures of Cdc48/p97 and Rix7 in substrate-engaged states (Supplementary Fig. 11) ^{2,11,12,14,22}. Although the

density of P6 is not well resolved at atomic resolution, it is clear enough to conclude that P6 is relatively detached from the peptide and other protomers in the hexamer (Fig. 2 and 5b-c).

When the trapped substrate is used as a vertical ruler, the six spirally arranged protomers display different axial displacements (Supplementary Fig. 12a), with P5 being the lowest. When the six protomers were superimposed with the D1 domains as reference, a few principles regarding the inter-domain conformational changes of Drg1 subunits could be derived. First, the connection between the NTD and D1 domains is relatively rigid, as the NTD-D1 displacements are always very small in these pair-wise comparisons (Supplementary Fig. 12b). Second, compared with the D2 domain of P1, the D2 domains of P2, P3, P4 and P5 display a similar rotational movement; but the D2 domain of P6 is in a distinct position, sharply different from other protomers (Supplementary Fig. 12b). Third, when the D2 domains were used as references of alignment, the same conclusion could be drawn that P6 is in an outlier conformation from the rest five (Supplementary Fig. 12c). Therefore, Drg1 protomers could take at least three major conformations, represented by P1, P2-P5 and P6, during the substrate processing cycle.

The pore loops (PL-I and PL-II) form upward spiral steps around the substrate and display an anticlockwise rotation pattern viewed from the D1 ring. The overall vertical displacements of the PLs in the D1 and D2 rings are about 22 Å and 28 Å, respectively (Fig. 5e), indicating that the PLs of two ATPase rings process the substrate with functional synergy. The PL-I of D1 and D2 domains each contain a conserved aromatic motif (K318-Y319-L320 for D1 and K589-Y590-V591 for D2), and they show distinct patterns of substrate interacting at the D1 and D2 domains. Specifically, while the substrate is held by PL-I from four protomers (P2-P5) at the D1 layer, the substrate is clamped by PL-I from five protomers (P1-P5) at the D2 layer (Fig. 5b-c and 5e-f). The same observation holds true for the PL-II of the two rings. In general, while P2, P3, P4 and P5 show consistent interactions with the substrate at both the D1 and D2 layers, P1 only directly contact the substrate at the D2 ring. Therefore, in line with previous functional models of the pore loops^{50,51}, our data clearly show that the pore loops of Drg1 hexamer grip and move the substrate in a collaborative manner to facilitate substrate processing and translocation.

The D1 domains of all six protomers are in the ATP-bound state (Supplementary Fig. 13a-f), which is similar to the substrate engaged structures of Cdc48 and Rix7^{11,12,22}. The AFs were proposed to coordinate ATP binding, nucleotide hydrolysis and conformational changes between adjacent protomers⁵². The AFs of the D1 domains (R401 and R404) were clearly resolved in P1-P5 (Supplementary Fig. 13a-f). The D1 AFs of P2, P3, P4 and P5 are in close to the bound ATP on the adjacent protomer. R404 residues in P3, P4 and P5 show a strong interaction with the γ -phosphate of ATP. In contrast, in P1 and P2, the γ -phosphate interaction is mainly mediated by R401, instead of R404. Also, as a seam subunit, the D1 AFs of P6 show no interaction with the adjacent ATP on P5.

In the D2 ring, protomers of P1 to P4 are in the ATP-bound state (Supplementary Fig. 13g-l). The D2 domain of P6 that is away from substrate is in the nucleotide-free state (Supplementary Fig. 13l). The D2 ATPase center of P5 is also occupied, although not fully resolved, and should be ATP as well (Supplementary Fig. 13k). The D2 AFs (R671 and R674) of P2, P3, P4 and P5 are close to the adjacent

ATP (Supplementary Fig. 13g-j). Because P6 is relatively detached from the rest protomers, the D2 AFs of P6 show relatively larger distance from the bound ATP in the D2 domain of P5 (Supplementary Fig. 13k).

Altogether, these results demonstrate that the nucleotide binding states of the two ATPase domains determine the conformations of Drg1 protomers, and that the subtle conformational changes at the protomer interface during ATP hydrolysis cycle could be translated into large-scale inter-protomer and inter-ring motions to drive the progressive movement of the substrate within the central channel.

Benzo-diazaborine binds to the active centers of both the D1 and D2 domains

The cryo-EM map of Drg1 hexamers treated with ATP/benzo-diazaborine was resolved at a resolution of 3.8 Å (without symmetry imposed) (Fig. 6 and Supplementary Fig. 1 and 5a-e). Notably, compared with structures from other samples, this structure is more symmetrical at both the D1 and D2 rings, and the six protomers are roughly in a same conformation (Fig. 6d). Importantly, analysis of the benzo-diazaborine occupancy in the hexamers of the planar and helical states from the ADP/AMPPNP/benzo-diazaborine dataset led to a similar observation. Benzo-diazaborine was detected in the six D1 ATPase sites of the planar state (Supplementary Fig. 9), which is more symmetrical, whereas all the ATPase sites in the helical hexamer show no trace of benzo-diazaborine. Therefore, it is apparent that the treatment of Drg1 hexamers with benzo-diazaborine led to the formation of more symmetrical structures, indicating that the chemical also directly modulates the conformation of Drg1 hexamer, in addition to the inhibition of the ATP hydrolysis.

Benzo-diazaborine is docked in equivalent sites of the D1 and D2 domain (Fig. 6f-g). On the D1 domain, benzo-diazaborine is surround by I422, T458, R297, and R429 (Fig. 6f). Importantly, benzo-diazaborine contains an extra benzyl group, which is coordinated by R429 through a cation- π interaction. Since thieno-diazaborine does not have this aromatic ring, this may explains why only the D2 domains are occupied by thieno-diazaborine in the previous Drg1 structure⁴⁴. The binding position of benzo-diazaborine on the D2 domain similar as the one reported for thieno -diazaborine⁴⁴, and agrees with previous genetic data that yeast strains harboring Drg1 mutations of A569V, C561T, and V725L/E are resistant to diazaborine treatment³³. In the D2 domain, the equivalent position of R429 is K695, which might also contribute to the stabilization of the benzyl group. The boron atom of diazaborine forms a covalent bond with the 2'-OH of the ribose of NAD⁺ in the structure of FabI-diazaborine complex^{53,54}. Therefore, benzo-diazaborine should have also formed covalent bonds with the 2'-OH of the ribose of the ATP molecules in the ATPase centers of both the D1 and D2 domains (Fig. 6f-g).

Next, we performed pairwise comparison of the subunit of the Drg1-ATP/benzo-diazaborine (Drg1-ATP/B-Dia) complex with the six protomers of the double mutant Drg1-ATP complex. While the NTD and D1 remain largely as a rigid body in all these comparisons, the D2 domain of the Drg1-ATP/B-Dia complex displays a distinct conformation that is dissimilar to any of the six protomers from the mutant Drg1-ATP complex (Supplementary Fig. 14). These results suggest that benzo-diazaborine has locked the ATPase centers of Drg1 in a specific conformation and limited its conformational transition. Mechanistically,

benzo-diazaborine probably has impaired both the intra-ring and inter-ring communications of Drg1 hexamers.

In summary, benzo-diazaborine blocks the ATP hydrolysis cycle and hinders the conformational changes of the D1 and D2 rings, thereby disabling the conformational cycling of Drg1 subunits required for substrate processing.

Discussion

Drg1 is a type II AAA+ ATPase that plays an essential role in the maturation of the cytoplasmic pre-60S particles. Through the purification of a double mutant form of Drg1, we accidentally captured a form of Drg1 hexamer in processing a substrate. The structure shows that Drg1 hexamer displays a spiral conformation, with six protomers arranged in a staircase-like configuration. Five of the six protomers directly interact with the substrate, with the seam subunit P6 relatively detaches from the substrate (Fig. 2 and 5). Substrate interactions are mediated by the four sets of PLs on the D1 and five sets of PLs on the D2 domains (Fig. 5). The structural similarity between Drg1 and Cdc48/p97 hexamers indicates that Drg1 protomers employ a conserved mechanism to grip and pull the substrate one after another during the ATPase cycle. In general, when the ATP in the P5 nucleotide binding pocket is hydrolyzed to ADP, its conformation will be converted to the P6 state and detaches from the substrate, ready to bind to ATP. When P6 binds to ATP, it will be converted to the P1 state and binds with an axial rise to the substrate.

These observations suggest that Drg1 is highly likely a protein unfoldase, rather than a disassemblase such as NSF, which relies on large-scale NTD movement to separate SNARE complex²⁰. This hypothesis is also supported by the fact that the NTD of Drg1 closely resembles that of Cdc48/P97 despite their low sequence homology. Therefore, it is possible that removal of Rlp24 from the pre-60S particles by Drg1 also involves a threading of Rlp24. The NTD of Cdc48 binds to diverse adaptor proteins to recognize different substrates in multiple physiological processes⁵⁵. Therefore, Drg1 likely works similarly as Cdc48 does and requires an adaptor protein. Previously, it was reported that several proteins of nuclear pore complex, including Nup116, could interact with Drg1 and Nup116 facilitates the release of Rlp24 from pre-60S particles *in vitro*³². Whether any of them is a bona fide adaptor merits further investigation.

Diazaborine was first discovered as an antibacterial compound that blocks the biosynthesis of fatty acids and phospholipids, thereby preventing bacterial proliferation^{40,41}. Diazaborine inhibits the activity of enoyl reductase by forming a covalent bond between the 2' hydroxyl of nicotinamide ribose and boron atom (Baldock et al., 1996). Later, it was found that *S. cerevisiae* was also sensitive to diazaborine and a few potential target genes including *PDR1*, *PDR3*, and *DRG1* were identified³⁹. More recently, a diazaborine derivative was demonstrated to be a potent and selective inhibitor against human neutrophil elastase, which is a promising therapeutic target for many inflammatory diseases⁵⁶. This broad-spectrum activity of diazaborine compounds highlights its high therapeutic potential. Over years, there has been an continuous effort of synthesizing and evaluating new classes of diazaborines for different

purposes⁵⁷. There are several types of diazaborines, including benzene, naphthalene, thiophene, furan and pyrrole. In this work, we discovered that benzo-diazaborine binds to both the D1 and D2 domains of Drg1. Benzo-diazaborine contains a benzyl ring instead of propyl group in thieno-diazaborine, and this aromatic group indeed form a strong cation- π interaction with R429 of the D1 domain (Fig. 6). This readily explains why benzo-diazaborine could also bind to the D1 domain, whereas thieno-diazaborine has a preference for the D2 domain⁴⁴.

More importantly, our data show that the binding of benzo-diazaborine to the D1 and D2 sites inhibits the nucleotide-driven conformational changes on the Drg1 hexamer, locking the individual subunits in a conformation different from those typical ones seen in the substrate-processing hexamer (Supplementary Fig. S14). The conformational modulation benzo-diazaborine on the Drg1 hexamer is that it allosterically impairs the inter-subunit and inter-ring communications, resulting in a more symmetrical configuration for the hexamer. Similarly, UPCDC30245 is a conformation-selective inhibitor that preferentially binds the ADP state of p97 at the interface of the D1 and D2 domains, and the mechanism of UPCDC30245 action was reported to inhibit D1-D2 conformational communication⁸. The same observation was also found in the structure of p97 bound with a different inhibitor CB-5083⁵⁸. Being an ATP analogue competing for the ATP binding to the D2 domain of p97, CB-5083 also blocks inter-subunit and inter-domain communications to exert its function⁵⁸. Our data show that benzo-diazaborine could bind to both D1 and D2 domains, suggesting that it might be a more potent anti-fungal agent than other diazaborine classes. Thus, further development could be focused on the modification benzyl group to increase its affinity for Drg1 to treat fungi infection. Additionally, our structural data and previous studies on the structures of diazaborine derivatives bound with different target proteins^{44,53,56,59} together lay a foundation for tailoring diazaborine compounds to target various enzymes including eukaryotic AAA+ ATPases for therapeutic or other purposes.

Method

Protein expression and purification

S. cerevisiae genome was isolated from yeast strain BY4741 using LiOAc-SDS method. Full-length *AFG2* that express Drg1 was cloned into pET28a vector with a N-terminal His6-SUMO tag followed by a TEV protease cleavage sequence. The expression plasmid of wild type Drg1 was used as template to prepare the double mutant E346Q/E617Q by site-specific mutagenesis. The proteins were overexpressed in *E. coli* Transetta (DE3) cells (TRANS). Protein purification methods were adapted from a previous work¹⁹. Briefly, cells were grown at 37 °C to an OD₆₀₀ of 0.6–0.8 in LB medium with 100 µg/ml ampicillin and 25 µg/ml chloramphenicol and induced with 0.5 mM IPTG at 18 °C overnight. Cells were harvested and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM KCl, 5 mM MgCl₂, and 40 mM imidazole). The suspension was supplemented with 1 mM PMSF and 1% TritonX-100, and cells were lysed by sonication. Lysates were cleared by centrifugation in a JA-25.50 rotor (Beckman) for 75 min at 16,000 r.p.m. and the supernatants were incubated with Ni-NTA agarose beads (GE Healthcare) for 2 h at 4 °C.

Beads were transferred into gravity column and washed with lysis buffer. Subsequently, the resins were washed with lysis buffer containing 80 mM imidazole, and proteins were eluted in elution buffer (50 mM Tris-HCl, pH 8.0, 300 mM KCl, 5 mM MgCl₂, and 500 mM imidazole). The eluates were supplemented with 5 mM DTT and TEV protease, and then incubated at 18 °C for 2 h to remove His6-SUMO tags. Protein solutions were loaded onto a Mono Q column (GE Healthcare). Proteins were eluted with a linear gradient to 500 mM KCl, pooled and concentrated using 100-kDa-cutoff spin concentrator (Millipore). To prepare different samples, proteins were incubated with 5 mM nucleotide (ADP, AMPPNP, ADP/AMPPNP for Drg1^{WT}, and ATP for Drg1^{E346Q/E617Q}) for 2 h at room temperature before size-exclusion chromatography, which was pre-equilibrated with 50 mM HEPES-KOH, pH 8.0, 300 mM KCl, 5 mM MgCl₂, and 5 mM DTT. Peak fractions were first examined by negative staining electron microscopy, pooled and concentrated for cryo-EM. To obtain the benzo-diazaborine bound state of Drg1, Drg1^{WT} proteins were incubated in a solution containing 5 mM ATP, 2 mM benzo-diazaborine (CAS#22959-81-5) (prepared from 200 mM stock solution in DMSO), 50 mM HEPES-KOH, pH 8.0, 300 mM KCl, 5 mM MgCl₂ and 5 mM DTT for 2 h at room temperature before gel filtration.

Cryo-EM sample preparation and data collection

The sample homogeneity was first examined by negative-staining with 2% uranyl acetate. Protein samples at a concentration of 0.02 mg/ml were applied onto carbon-coated copper grids, and examined with an FEI Tecnai T20 TEM at 120 kV. Initial cryo-EM sample preparation found that Drg1 hexamers suffered from a severe dissociation. Therefore, GraFix⁶⁰ was used to counteract the sample dissociation during cryo-grid preparation. Fractions from gel filtration containing intact Drg1 hexamers were pooled and concentrated to a volume of 150 µl and applied onto a 2 ml glycerol gradient (10–30%) containing glutaraldehyde (0–0.05%) in a buffer (50 mM HEPES-KOH pH 6.8, 150 mM KCl, 5 mM MgCl₂, 1 mM DTT, 0.5 mM nucleotide). For the inhibitor-treated samples, 2 mM benzo-diazaborine was present in the gradient buffer and throughout the complete process. The gradient was centrifuged in a TLS55 rotor (Beckman) for 13 h at a speed of 38,000 r.p.m. at 4 °C. Fractions were collected manually, and the crosslinking reactions were quenched by adding 1 M Tris-HCl (pH 7.4) to a final concentration of 100 mM. Fractions containing Drg1 hexamers were examined by negative staining electron microscopy, pooled and concentrated to remove glycerol. The final samples were exchanged into buffer A (50 mM HEPES-KOH pH 6.8, 150 mM KCl, 5 mM MgCl₂, 1 mM DTT) containing 1 mM nucleotide for cryo-EM analysis.

All cryo-grids were prepared with holey-carbon gold grids (Quantifoil, R1.2/1.3, 300 mesh). The grids were glow-discharged for 30 sec in a plasma cleaner prior to sample freezing. Cryo-grids were prepared with an FEI Vitrobot Mark IV with the chamber set at 4 °C and 100% humidity. Grids were screened with an FEI Talos Arctica (FEI Ceta camera) before data collection. All the images were collected with FEI Titan Krios TEM (Gatan K2 summit camera) operated at 300 kV with GIF Quantum energy filter (Gatan). The movies were acquired using the SerialEM program⁶¹ and with a magnification of 130,000× (defocus -1.0 to -2.5 µm). The statistics of data collection was summarized in Supplementary Tables S1-S2.

Image processing

Motion correction and electron-dose weighting were performed using MotionCor2⁶² and the contrast transfer function (CTF) parameters were estimated with the Gctf⁶³. RELION (versions 3.0 and 3.1)⁶⁴ were used for particle picking, 2D and 3D classification and structural refinement. 2D templates used for particles auto-picking was generated by 2D classification of a small set of manually picked particles. The initial model was generated by RELION program from selected particles and was used as reference for the following image processing procedures.

For the sample of Drg1-ADP, 1,593,028 autopicked particles were subjected to 2D classification, resulting in a dataset of 936,387 particles for further processing. After two rounds of 3D classification, one class with more symmetrical features (class 5) was subjected to another round of 3D classification, which retained 51,928 particles for 3D refinement. The final density maps were determined at 5.9 Å (without symmetry) and 4.4 Å (with C6 symmetry imposed). To explore the structural dynamics of Drg1 hexamers, classes of good quality (classes 1, 2, 4, 5, 7, 9 and 10) were combined and subjected to one round of 3D classification. The resulting 10 classes contained comparable numbers of particles and the structures differed from each to varying extents. The dataset of Drg1-AMPPNP was similarly processed (Supplementary Fig. 3). Out of 1,823,806 auto-picked particles, 520,634 particles were selected for the final round of 3D classification and 285,167 particles were kept for final 3D refinement (final resolution 5.6 Å without symmetry).

For the sample of Drg1-ADP/AMPPNP/benzo-diazaborine (Supplementary Fig. 4), 2,205,661 particles were autopicked and 1,266,728 particles were selected for two rounds of 3D classification. One class displaying a helical conformation (class 4) was subjected to 3D refinement, resulting in a density map at 5.9 Å. Two classes of planar conformation (classes 2 and 7) were combined to generate a density map at 4.3 Å. Since the helical and planar conformations differ the most at the D2 ring, all classes of good quality (531,919 particles) were combined for another round of 3D classification into ten groups using a soft mask of the D2 ring. The ten groups were then individually refined to analyze the dynamics of Drg1 hexamers.

For the sample of Drg1-ATP/benzo-diazaborine, 1,526,542 particles were auto-picked and similarly processed. A final set of 348,781 particles with more symmetrical features was selected for the final 3D refinement (Supplementary Fig. 5a-e). After CTF refinement and particle polishing, the density map could be improved to a resolution of 3.8 Å (without symmetry). As the D1 and D2 rings showed an approximately six-fold symmetry, C6 symmetry was also tested, leading to an improved density map at 3.5 Å.

For the sample of mutant Drg1^{E346Q/E617Q}-ATP, 1,064,262 auto-picked particles were processed similarly. After the final round of 3D classification, 152,973 particles with **relatively stable** structural features were selected for 3D refinement, generating a density map at 3.8 Å. Application of CTF refinement and particle polishing could further improve the map to a resolution of 3.6 Å (without symmetry). To get high-resolution structures for different protomers, masked 3D refinement was performed for each protomer using protomer-specific soft masks.

Model building and refinement

The model of Drg1 was built into the map of the mutant Drg1-ATP complex using Coot ⁶⁵. The predicted 3D model from I-TASSER ⁶⁶ was as the initial template. The modelling was facilitated by the secondary structure prediction using PSIPRED ⁶⁷. For other protomers in the map of Drg1 hexamers, models of Drg1 domains were fitted as rigid bodies followed by manual adjustment and rebuilding in COOT. For the D1 domain of protomer 6 (P6) that is of low resolution, the model was only adjusted using rigid body fitting. The substrate was built using a poly-alanine model. The atomic models of hexamers from different samples were similarly obtained. The automatic model refinement (Supplementary Table S2) was performed by real-space refinement (phenix.real_space_refine) in Phenix ⁶⁸. Model validation was calculated by MolProbity ⁶⁹. Figure preparation and structure analysis were performed with and UCSF ChimeraX, Chimera ⁷⁰ and PYMOL(pymol.org).

Statistics and reproducibility

Purification of Drg1 and preparation of hexamers were repeated for more than three times with similar results. No statistical analysis, other than those embedded in image processing, has been applied throughout the work.

Declarations

Acknowledgments

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Author contributions

D.W., C.M., and Q.C. prepared the samples; C.M. collected the cryo-EM data; C.M. and N.G. performed EM analysis and model building; N.G., C.M. and D.W. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Correspondence and requests for materials

The cryo-EM maps (C1 symmetry) of the Drg1 hexamers from the datasets of Drg-ADP, Drg1-AMPPNP, Drg1-ADP/AMPPNP/B-Dia (helical conformation), Drg1-ADP/AMPPNP/B-Dia (planar conformation) and Drg1-ATP/B-Dia have been deposited in the EMDB, with accession codes EMD-32397, EMD-32399, EMD-32400, EMD-32402, and EMD32403, respectively. The cryo-EM map and atomic coordinates of the Drg1 hexamer from the Drg1 mutant sample have been deposited in the EMDB and PDB with accession codes EMDB-32396 and 7WBB, respectively. All materials developed in this study should be addressed to N.G

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Figures

Figure 1

Representative cryo-EM structures of wild type Drg1 hexamers in different nucleotide and benzo-diazaborine binding states.

a-e, Representative cryo-EM maps of Drg1 hexamers treated with ADP (a), AMPPNP (b), ADP/AMPPNP/benzo-diazaborine (c-d) or ATP/benzo-diazaborine (e). The density maps are shown in the side, top (NTD) and bottom (D2) views. The NTD, D1 and D2 are colored dodger blue, red and orange, respectively. B-Dia, benzo-diazaborine.

Figure 2

Cryo-EM structure of the double mutant Drg1^{E346Q/E617Q} hexamer in the presence of ATP.

a-b, Cryo-EM map of the double mutant Drg1^{E346Q/E617Q} hexamer in the presence of ATP, viewed from the D2 (a) and NTD (b) directions.

c, Side view of the double mutant Drg1^{E346Q/E617Q} hexamer, highlighting its helical configuration of protomers.

d, Cross-section view shows the trapped substrate within the central channel of the double mutant Drg1^{E346Q/E617Q} hexamer.

Figure 3

Atomic model and domain organization of of Drg1.

a, Domain organization and secondary structure of *S. cerevisiae* Drg1. The AAA+ domain contains an α/β core subdomain (α_0 - β_5) and an α -helical lid subdomain (α_5 - α_8). The highly conserved motifs including Walker A, Walker B, sensor I, sensor II and arginine fingers, pore loops (PL-I and PL-II) are shown as indicated.

b, The atomic model of Drg1 is shown in cartoon representation, superimposed with the density map of one Drg1 protomer (P3). The substrate is colored red and the PLs are labelled. ATP molecules in the ATPase center of the D1 and D2 domains are highlighted in stick models.

c-d, The atomic models of the D1 and D2 domains, superimposed with the cryo-EM map of the protomer P3. The functional motifs including Walker A, Walker B, sensor I, and sensor II are colored separately. ATP is highlighted in stick model.

Figure 4

Comparison of the NTDs from *S. cerevisiae* Drg1, Cdc48 and *H. sapiens* p97.

a, Sequence alignment of the NTDs from *S. cerevisiae* Drg1, Cdc48 and *H. sapiens* p97. Secondary structures are also shown.

b-d, The atomic models of the NTD of *S. cerevisiae* Drg1, Cdc48 (PDB 60PC)¹¹ and *H. sapiens* p97 (PDB 5FTK)⁸.

e, Superimposition of the NTDs of *S. cerevisiae* Drg1 and Cdc48 (RMSD 4.7 Å)

f, Superimposition of the NTD of *S. cerevisiae* Drg1 and *H. sapiens* p97 (RMSD 2.9 Å).

g, Superimposition of *S. cerevisiae* Drg1 and Cdc48 (PDB 60PC). The two models are aligned using the D1 ATPase domain as reference. The comparison shows that the NTDs of Drg1 and Cdc48 are in completely different orientations in the hexamer. The UBX domain of Sph1 (an adaptor protein of Cdc48)¹¹ is shown and colored as indicated.

h-i, The enlarged view of (g) showing the orientation difference of the NTDs of Drg1 and Cdc48 relative to their D1 domains. Drg1, Cdc48 and UBX are colored as indicated.

Figure 5

Structure of the Drg1 hexamer in processing a peptide substrate.

- a, The atomic model of the NTD ring of the Drg1 hexamer in the substrate-processing state. The six protomers are indicated as P1 to P6.
- b, The atomic model of the D1 ring shown in cartoon representation. The PLs (PL-I and PL-II) engaged with the substrate are shown and highlighted in zoom-in panels.
- c, The atomic model of the D2 ring. The PLs (PL-I and PL-II) engaged with the substrate are shown and highlighted in zoom-in panels.
- d. Side view of the Drg1 hexamer with a substrate engaged in the central channel. For clarification of the substrate, P1 and P6 are not shown.
- e, The spiral configuration of the PLs of Drg1 protomers around the substrate in the central channel. The interaction patterns of the PL-I and PL-II are shown in the left and right panels, respectively. The vertical distance of PLs between the lowest and highest protomers are shown as indicated.
- f, The atomic interactions between the PL-I and the substrate. The conserved PL-I (K318-Y319-L320) of the D1 domains (left) from P2 to P5 are shown in the left panel, and the PL-I (K589-Y590-V591) of the D2 domains from P1 to P5 are shown in the right panel. Both the atomic models and cryo-EM of the PL-I are shown, and color-coded as indicated.

Figure 6

Benzo-diazaborine binds to the ATPase sites of both the D1 and D2 domains.

a, The cryo-EM map (without imposing symmetry) of the Drg1 hexamer in the presence of ATP and benzo-diazaborine (B-Dia) viewed from the bottom (D2 direction). Subunits of the Drg1 hexamer are colored separately.

b-c, Cross-section view of the D2 (b) and D1 (c) rings. B-Dia and ATP are colored red and purple, respectively.

d, Comparison of the six protomers, indicating that these protomers adopt a similar conformation. The binding sites of B-Dia and ATP in the nucleotide binding pockets are shown as indicated.

e, The chemical structure of B-Dia.

f-g, Zoom-in views of the ATPase sites of the D1 (f) and D2 (g) domains. Functionally important residues of Walker A (D1: P288, K292, T293; D2: P559, K563, T564), Walker B (D1: D345, E346; D2: D616, E617), Sensor I (D1: N390, D2: N660) and arginine fingers (D1: R401, R404; D2: R671, R674), and residues involved in interacting with B-Dia (D1: I422, T458, R429, K297; D2: I692, V725, K695, K568) are indicated.

Supplementary Files

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