Structural and Enzymatic Properties of the AAA Protein Drg1p from Saccharomyces cerevisiae

DECOUPLING OF INTRACELLULAR FUNCTION FROM ATPase ACTIVITY AND HEXAMERIZATION*

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The AAA protein Drg1 from yeast was affinity-purified, and its ATPase activity and hexamerization properties were analyzed. The same parameters were also determined for several mutant proteins and compared in light of the growth characteristics of the corresponding cells. The protein from a thermosensitive mutant exhibited reduced ATPase activity and hexamerization. These defects were not reversed by an intragenic suppressor mutation, although this allele supported growth at the nonpermissive temperature. A different set of mutants was generated by site-specific mutagenesis intended to adjust the Walker A box of the D2 domain of Drg1p to that of the D1 domain. A S562G exchange in D2 produced a nonfunctional protein that did not hexamerize but showed above-normal ATPase activity. The C561T mutant protein, on the other hand, was functional but hexamerized less readily and had reduced ATPase activity. In contrast, the C561T/S562G protein hexamerized less than wild type but had much higher ATPase activity. We distinguished strong and weak ATP-binding sites in the wild type protein but two weak sites in the C561T/S562G protein, indicating that the stronger site resides in D2. These observations are discussed in terms of the inter-relationship of ATPase activity per se, oligomeric status, and intracellular function for AAA proteins.

Proteins of the AAA (ATPases associated with cellular activities) family share a common amino acid sequence motif known as the AAA domain. This domain is characterized by a sequence of 230–250 amino acids surrounding the Walker A and B boxes, which are responsible for ATP binding and hydrolysis (1, 2). AAA proteins have diverse functions ranging from proteolysis-coupled unfoldase activity to membrane trafficking (3, 4). There are two types of AAA proteins known. The majority of these proteins contain one ATPase domain and are classified as type I AAA proteins. Others, which are known as type II AAA proteins, contain two such domains (5). In some type II AAA proteins, the regions surrounding the two Walker boxes are highly homologous to each other, as in the yeast protein Cdc48. In others, only one Walker box-containing region is a bona fide AAA domain, with the other region showing little similarity to the AAA consensus sequence. An example of the latter is protein NSF, which participates in heterotypic membrane fusion events. In type II AAA proteins, the first AAA domain, also termed D1, usually serves a different function from the C-terminal (D2) domain (6–8). For example, the ATPase activity of the NSF D1 domain is essential for membrane fusion activity, whereas its D2 domain is thought to effect hexamer formation (6, 9). Many type II AAA proteins form hexameric ring structures (2), but there are also a few reports of heptamers (10–12). Despite intensive research on AAA proteins ranging from bacteria to mammals, little is known about the molecular mechanisms that underlie their respective activities.

In previous work, we have shown that the antibacterial drug diazaborine inhibits growth of yeast cells and that resistance to this inhibitor can be mediated either by allelic forms of transcription factors that activate detoxifying efflux pumps or by allelic forms of the gene DRG1, which codes for a AAA protein (13). Drg1p is a type II AAA protein that is essential for the growth of Saccharomyces cerevisiae (14). Its closest homolog in yeast is Cdc48p (15). As with Cdc48p, the two AAA domains of Drg1p show strong homology to each other. However, there is a significant difference in the Walker A box in the D2 domain, which contains the sequence GPPGCSKT instead of the consensus sequence GPPGTGKT. In addition, Drg1p contains a leucine zipper motif in its C terminus. We have recently shown that diazaborine treatment of yeast leads to the generation of long, aberrant, mRNA molecules and that in a resistant strain that carries an allelic form of DRG1, the aberrant mRNAs are absent (16). We thus concluded that Drg1p is involved in a quality control or degradation mechanism of mRNA. To improve our understanding of the properties of this protein, we constructed mutant forms of Drg1p. These protein varieties as well as the wild type protein were purified from cells expressing the appropriate genes. We then studied the enzymatic activities of the purified proteins, measured ATP binding, and examined the oligomeric structures of the purified proteins by electron microscopy and centrifugation experiments. The resulting data are reported in this article.

MATERIALS AND METHODS

Cloning of the DRG1 Gene into the Expression Vector pYEX4T—To clone gene DRG1 into pYEX4T-1 (CLONTECH) a BamHI restriction site was introduced by amplifying the N-terminal portion of the gene.

2 The abbreviations used are: NSF, N-ethylmaleimide-sensitive factor; GST, glutathione S-transferase; DTT, dithiothreitol; ATP-S, adenosine 5′-O-(thiotriphosphate); SNARE, soluble NSF attachment protein receptors.

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§ To whom correspondence should be addressed. Tel.: 43-316-380-5683; Fax: 43-316-380-9898; E-mail: gregor.hoegenauer@uni-graz.at.
between the ATG codon and the XbaI site with the primers pAn1 (5'-TATGGATCCCGGATCCCTTAAATCTA) and TomPCR3e (CCT- TACATACCTCTTTTCTAT-3'). As a template pGZ253 was used, which contains a SacI/Spel fragment with the whole wild type DRG1 gene in blueScript II KS+. The PCR product was cleaved with BamHI and NotI and ligated into the pBamHI/NotI vector. As a control, a deletion mutant pAZ1 was generated, which contains the XbaI site toward the end of the gene was then inserted into the XbaI-cleaved pAZ1 to yield pAZ2. The correct orientation of the construct was confirmed by restriction analysis. Finally, the BamHI/XbaI fragment of pAZ2, containing the entire DRG1 gene, was cloned into the BamHI/XbaI cleaved expression vector pYEX4T-1, resulting in the recombinant plasmid pAZ2. This plasmid expresses a GST-tagged fusion protein under the control of the CUP1 promoter. The GST moiety can be removed from the fusion protein by thrombin cleavage. However, treatment of the fusion protein with thrombin resulted in ~10% unspecific cleavage products. Therefore, the thrombin cleavage site encoded by pAZ3 was exchanged for a PreScission protease recognition site. This was done by substitution of a MscI/BamHI fragment from pAZ3 by a MscI/BamHI fragment from the vector pGEX-6P-1 (Amersham Biosciences). These fragments contain the C-terminal part of the GST gene, a part of the polylinker, and the sequence coding for the thrombin or the PreScission protease recognition site, respectively. The resulting plasmid was designated pAZ7, and the correct nucleotide sequence of the whole DRG1 reading frame was confirmed by sequencing.

For construction of pAZ8, a 1.6-kb BglII/SacI fragment in pAZ7 was subcloned into the central fragment from pGZ253 containing the C-terminal portion of DRG1 from the XbaI site toward the end of the gene was then inserted into the XbaI-cleaved pAZ1 to yield pAZ2. The correct orientation of the construct was confirmed by restriction analysis. Finally, the BamHI/XbaI fragment of pAZ2, containing the entire DRG1 gene, was cloned into the BamHI/XbaI cleaved expression vector pYEX4T-1, resulting in the recombinant plasmid pAZ2. This plasmid expresses a GST-tagged fusion protein under the control of the CUP1 promoter. The GST moiety can be removed from the fusion protein by thrombin cleavage. However, treatment of the fusion protein with thrombin resulted in ~10% unspecific cleavage products. Therefore, the thrombin cleavage site encoded by pAZ3 was exchanged for a PreScission protease recognition site. This was done by substitution of a MscI/BamHI fragment from pAZ3 by a MscI/BamHI fragment from the vector pGEX-6P-1 (Amersham Biosciences). These fragments contain the C-terminal part of the GST gene, a part of the polylinker, and the sequence coding for the thrombin or the PreScission protease recognition site, respectively. The resulting plasmid was designated pAZ7, and the correct nucleotide sequence of the whole DRG1 reading frame was confirmed by sequencing.

The mutations leading to the C561T, the S562G, and the C651T/S652G exchanges in Drg1p were introduced by site-specific mutagenesis into pGZ253 using the ChameleonTM double-stranded, site-directed mutagenesis kit (Stratagene) with the mutagenic primers C561T (GGG CCG CCA GGT ACC TCC AAG ACA TTA ACC), S562G (GGG CCG CCA GGT ACC TCC AAG ACA TTA ACC), and C561T/S562G (AGG GGC CCA GGT ACC GGC AAG ACA TTA ACC), and of the respective fragment from pRS320, which contains the temperature-sensitive afg2-18 allele. pES2 and pES3 were constructed by cloning the DNA coding for the wild-type and the C561T/S562G double mutant into the pBamHI/XbaI site of pGZ253. The mutations were confirmed by restriction analysis. Finally, the C561T/S562G exchanges in Drg1p were introduced by site-specific mutagenesis kit (Stratagene) with the mutagenic primers C561T (GGG CCG CCA GGT ACC TCC AAG ACA TTA ACC), S562G (GGG CCG CCA GGT ACC TCC AAG ACA TTA ACC), and C561T/S562G (AGG GGC CCA GGT ACC GGC AAG ACA TTA ACC). The plasmid pGZ409 was finally constructed by exchanging the BglII/SacI restriction fragment of pAZ7 for the respective fragments of the cloned mutagenized forms of pGZ253.

Copper-inducible Expression of GST-Drg1p in Yeast—For induced expression of GST-Drg1p transformants of the yeast strain BY4743 Mat a/a (drg1::kanMX4/DRG1) (Euroscarf) were grown for 18 h at 30 °C in minimal synthetic medium containing histidine, lysine, and tryptophan. The cells were harvested by centrifugation (3000 × g, 10 min), resuspended to a cell density of 10⁶ cells/ml in 10 mM potassium phosphate buffer, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 25 units of PreScission protease (Amersham Biosciences). The mixture was sonicated and centrifuged at 100,000 × g for 1 h. After removal of unspecifically bound proteins by washing with cleavage buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT), 25 units of GST-tagged fusion protein was incubated in 50 μl of reaction buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 0.2 mM ATP, 2 μCi of [α-32P]ATP (800 Ci/mmol) at 30 °C for 15 min. The reaction was stopped by the addition of 1 μl of a chloroform-methanol (2:1) mixture. The samples were analyzed by thin layer chromatography on polyethyleneimine-cellulose F plates with visualization under UV light and autoradiography. After autoradiography, the substrate and product were quantitated with a PhosphorImager.

ATP Binding Assay—Binding of [α-32P]ATP to Drg1p was determined by the filtration method as described by Matveeva et al. (8). The reaction mixture (50 μl) containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM DTT, between 1 mM and 500 μM [α-32P]ATP, and 0.3 nmol of purified Drg1 protein was incubated for 10 min at room temperature before the addition of nitrocellulose membrane (MicroSep, 0.45 μm, Osmonics Inc.). The membrane was washed five times with 2.5 ml of ice-cold washing buffer containing 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl. The radioactivity retained at the membrane was counted in 5 ml of liquid scintillation mixture in a Beckmann SC4500 liquid Scintillation Spectrometer. The data were analyzed for binding constants using Prism 3.02 software from Graphpad Software Inc. by nonlinear fitting or by determining the intercepts with the abscissa and the ordinate in a Scatchard plot.

Diazboration Binding Assay—Binding of diazboration to Drg1p was performed by a technique similar to the ATP binding assay. 100 μl of purified Drg1p was incubated in the presence of 20 μM radiolabeled diazboration in a reaction mixture (50 μl) containing 50 mM Tris-HCl, pH 7.5, 300 mM KCl, 25 mM MgCl2, and 1 mM ATP-S (or 5 mM ATP, in some experiments) were applied to continuous carbon films mounted on electron microscopy grids and stained with 1% uranyl acetate. The grids were examined in a CM120 electron microscope (FEI, Mahwah, NJ) operating at 100 kV. The micrographs were recorded at a nominal magnification of 35,000 × and digitized at a step size of 7 μm (0.20 nm/pixel) on a SCAI scanner (ZI Imaging, Huntsville, AL). For digital analysis, the particles were picked using the semi-automatic procedure, X3DPREPROCESS (17). Alignment and correlation averaging was performed using FIC-III (18). 400 particles of wild type Drg1p, 260 of the diazboration-resistant mutant and 200 of the C561T/S562G double mutant were averaged. The resolution according to the SSNR criterion (19) was 23, 31, and 41 Å, respectively. Rotationally symmetric analysis was carried out with the ROTASTAT program (20).

Glycerol Gradient Centrifugation—50–100 μg of purified Drg1p was layered on the top of a 11–ml 10–25% (w/v) linear glycerol gradient in gradient buffer (20 mM Hepes-NaOH, pH 7.4, 100 mM KCl, 2 mM MgCl2, 2 mM DTT). After centrifugation at 100,000 × g for 4 h at 4 °C, 0.8-ml fractions were collected by piercing the bottom of the centrifuge tube. The relative sedimentation velocity of Drg1p was determined by comparison of gradient profiles with a calibration curve prepared from the sedimentation data of the marker proteins bovine serum albumin (4.6 S), catalase (11.4 S), and α-2-macroglobulin (20 S) under identical conditions.

RESULTS

Isolation of Mutants—To investigate the influence of the two AAA domains of Drg1p on its ATPase activity and structural properties, we generated a number of mutants. We obtained the thermosensitive afg2-18 mutant (21) and characterized it by sequencing. The mutation results from a L457S exchange at a position roughly midway between the two AAA domains (Fig. 1). The mutant DNA was introduced into the chromosomal DNA of strain W303 by homologous recombination. The resulting strain no longer grew at 37 °C, but growth was observed after shifting down to the permissive temperature of 25 °C. The temperature-sensitive phenotype could be reversed by overexpression of either the wild type or the temperature-sensitive protein itself. Experiments not shown here demonstrated that 2.5-fold overexpression of the temperature sensitive protein was sufficient to restore growth at 37 °C. We obtained suppressor mutants by mutagenizing the thermosensitive strain with ethyl methane sulfonate and screening for colonies that grew at the nonpermissive temperature. After eliminating revertants, we isolated and identified one intragenic suppressor mutant.
The AAA Protein Drg1

(drg1-sup). It contained a second amino acid exchange, i.e. F343L, in the Walker B box of the D1 AAA domain. We introduced this mutation into the wild type allele by fragment swapping. The resulting allele was designated "sup". A strain containing this mutation in DRG1 showed no growth phenotype compared with wild type.

We also generated mutants in the Walker A box of the D2 domain. The sequence of this motif is GPPGCSKT, which deviates in two positions from the consensus GPPGTGKT sequence. We introduced single amino acid exchanges by site-specific mutagenesis to render the D2 sequence more similar to the D1 sequence and also created a double mutant to make the sequences identical in this region. The substitutions were C561T and S562G for the single exchange and C561T/S562G for the double exchange. The S562G mutant protein variant did not support growth of yeast cells and is thus considered non-functional. We also mutated the lysine residues of the Walker A boxes of both AAA domains. In both cases, yeast cells carrying these alleles were observed to show normal growth.

Cloning of Different Allelic Forms of DRG1 in an Expression Vector—Because Drg1p is a low abundance protein, we expressed the DRG1 gene as a GST fusion protein to facilitate its purification. The expression system is based on a modified pYEX4T-1 vector (see "Materials and Methods"). The constructed plasmid was designated pAZ7 and expressed the GST-Drg1p fusion protein under the control of the CUP1 promoter. The plasmid codes for the recognition sequence of the PreScission protease instead of the original thrombin cleavage site juxtaposed to the polylinker. This strategy resulted in significantly less degradation products when the Drg1 moiety was juxtaposed to the polylinker. The expression system is based on a modified pYEX4T-1 vector (see "Materials and Methods"). The constructed plasmid was designated pAZ7 and expressed the GST-Drg1p fusion protein under the control of the CUP1 promoter.

Expression and Purification—To express the GST-Drg1p fusion proteins, yeast cells carrying the respective recombinant plasmids were incubated in Cu²⁺-containing selective medium. The highest expression was obtained 6–8 h after induction. The crude extracts were prepared as described under "Materials and Methods," and the fusion proteins were purified on glutathione-agarose beads by a batch procedure. The Drg1 moiety of the fusion protein was separated from GST by treatment with PreScission protease. The yield of Drg1p was ~1 mg of protein/liter of culture. SDS-PAGE of the purified protein is shown in Fig. 2. Separation of this protein preparation by two-dimensional gel electrophoresis with subsequent silver staining and Western blotting with anti-Drg1p and anti-GST antibodies showed that the faintly stained protein bands differed in mobility from the major bands. The positions of the fusion protein in the crude extract and the pure protein Drg1 after affinity purification and cleavage are marked. The faint bands with faster mobilities in the purified preparation are proteolytic degradation products of the fusion protein.

The proteins encoded by the temperature-sensitive alleles showed no growth phenotype, whereas those encoded by the temperature-sensitive alleles did not support growth of yeast cells and is thus considered non-functional. We also attempted to isolate the proteins with K292E and K536E mutations, but in neither case could the fusion protein be detected, which may reflect highly unstable conformations.

ATPase Activity—Purified wild type Drg1p and the various mutant forms were assayed for ATPase activity, using [γ-32P]ATP as a substrate. The reaction product ADP was quantified with a PhosphorImager. The results are shown in Fig. 3. The protein encoded by the diazaborine-resistant allele exhibits 71% of the wild type ATPase activity, whereas the ATPase activity of the C561T mutant was decreased to 35%. The C561T/S562G double mutant in which the second Walker A box is identical to the first one and the S562G single mutant showed much higher activities than the wild type protein.

The proteins encoded by the temperature-sensitive and the suppressor alleles showed ~10 times lower activities than the wild type protein. This observation was surprising because...
the suppressor mutation compensates the temperature-sensitive growth phenotype in whole cells. The protein containing only the F343L exchange (encoded by the *drg1-sup* allele), which is located in the Walker B box of the first ATP-binding domain, reduced the ATPase activity to \( \frac{60}{100} \)% of the wild type protein.

Preincubation of wild type Drg1p at 37 °C in reaction buffer without ATP resulted in inactivation of its ATPase activity. As a control, we incubated the protein at 25 or 30 °C for the same period. No inactivation was observed. However, preincubation in the presence of ATP at 37 °C preserved the enzymatic activity of the protein (Fig. 3). Thus, ATP seems to exert a stabilizing influence on the protein structure.

NSF and some other AAA proteins are sensitive to treatment with N-ethylmaleimide (22, 23). Accordingly, we tested for a possible sensitivity of Drg1p to this agent and found that its ATPase activity is reduced by 90% by 1 mM N-ethylmaleimide (data not shown). This suggests the existence of exposed sulfhydryl groups important for the ATPase activity of Drg1p.

Because allelic forms of *DRG1* confer resistance to diazaborine, we investigated whether the drug affects the ATPase activity of the purified protein. The ATPase activity of wild type Drg1p was not inhibited by diazaborine in concentrations up to 100 \( \mu \)M. We also tested whether the purified protein could bind the radiolabeled drug. This was done by equilibrium dialysis and by filter binding studies in the presence and absence of ATP or NAD\(^+\). In no case was binding of the radiolabeled drug detected (data not shown).

We also determined the kinetic constants for wild type Drg1p. The protein exhibited ATPase activity with a \( K_{\text{m(app)}} \) for ATP of 35 \( \mu \)M and a specific activity of 1 \( \mu \)mol of ATP/h/mg of protein (\( \sim 9 \) ATP/min/hexamer). This specific activity is in the same range as for other AAA ATPases (23). Similar results were obtained for the diazaborine-resistant *drg1-1* mutant (asterisks and dotted line) were determined as a function of ATP concentration and plotted according to Lineweaver-Burk.

**ATP Binding**—To study the binding of ATP to Drg1p, we used a nitrocellulose filter binding assay (8). Purified wild type protein was incubated in the presence of various concentrations of [\( \alpha \)-\( ^32 \)P]ATP for 10 min at room temperature and then passed through a nitrocellulose filter. Protein-bound radioactivity was
measured by scintillation counting. Drg1p exhibited a biphasic behavior of nucleotide binding, indicating the presence of two ATP-binding sites with different binding constants. Using Scatchard analysis, we estimated the binding constants to be 27 and 88 μM, respectively. Thus, Drg1p has one high affinity site and one low affinity site for binding ATP (Fig. 5). To determine which of the two ATP-binding sites is responsible for the substrate binding with the higher affinity, we purified the mutant protein variant with the C561T/S562G exchange in the C-terminal Walker A box and measured ATP binding. A Scatchard analysis showed that both binding sites in this protein variant behaved similarly in that they bound ATP with association constants of 110 μM (Fig. 5). We thus conclude that the amino acid exchanges in the Walker box of the D2 region altered the high affinity binding site of ATP into a low affinity site.

### Oligomeric Status of Drg1 Determined by Electron Microscopy

The structures of wild type and mutant Drg1p in buffer containing 1 mM ATP were examined by electron microscopy and image analysis. Micrographs of negatively stained wild type protein revealed rings with diameters of ~120 Å (Fig. 6a). Application of two statistical tests for rotational symmetry (20) revealed a definite 6-fold symmetry (Table I). The Student’s t test indicated that the probability that the 6-fold signal could have arisen at random is extremely low (p < 10^-6; see Table I). The spectral ratio product, which maintains values >1 for positive detection of a given symmetry and converges toward 0 otherwise, diverged to the very large value of 7.0 × 10^20 for 6-fold symmetry with this data set. No other symmetry was found to be significant by either test. These observations imply that most if not all of the molecules are hexamers. Image averaging confirmed the hexameric arrangement (Fig. 6c, inset). In the top views (Fig. 6, insets), Drg1p appears as a scalloped hexagon with a maximum radius (i.e., the distance from the vertex to the center of the hexagon) of 70 Å and a minimum radius of 55 Å. There is a heavy accumulation of stain at its center (diameter, ~20 Å), suggesting the presence of an axial channel or internal cavity. Faintly staining features curve outwards from the center, giving the particle a marked handedness. Wild type Drg1p was also visualized in the same way in the presence of 5 mM ATP. However, no obvious structural change was seen in the averaged top view (data not shown).

Drg1p, the protein from the diazaborine-resistant mutant, also forms hexamers (Fig. 6b and Table I). The size and features of the averaged top view are similar to those of the wild type protein, although the handedness is less obvious, which probably reflects the somewhat lower resolution of this image. However, few if any hexameric particles were seen in micrographs of the temperature-sensitive mutant, the suppressor mutant, and the S562G mutant, which mainly produce larger and less regular particles (data not shown). The larger particles are probably aggregates of monomers (see below) formed during negative staining. With the C561T mutant, only a few particles of the right size for hexamers were seen (Fig. 6c). However, statistical analysis of these data for the C561T mutant did not declare 6-fold symmetry as statistically significant, implying either that these hexamers are in the minority or that they are less stable than wild type and more easily deformed in negative staining so that their 6-fold character does not register to the same extent. A higher incidence of ring-shaped particles was seen in micrographs of the C561T/S562G double mutant (Fig. 6d), although their frequency was lower than with wild type Drg1p or the diazaborine-resistant mutant, suggesting less hexamer formation. Nevertheless, 6-fold symmetry was also detected for these particles (Table I), and despite lower resolution (41 Å), the averaged top view (Fig. 6d) shows that the C561T/S562G double mutant has essentially the same hexameric structure as the wild type protein.

### Glycerol Gradient Centrifugations

Electron microscopy showed a regular, ring-shaped, Drg1p hexamer with a channel or cavity at its center. To evaluate oligomer assembly by an independent method and also to investigate the effects of nucleotide triphosphates and chemical analogs on the association process, we purified wild type Drg1p and several mutant proteins to glycerol gradient centrifugation. After centrifugation, the fractions were collected and assayed by SDS-PAGE. In parallel tubes, bovine serum albumin, catalase, and α-2-macroglobulin were run as molecular weight standards. As shown in Fig. 7, in the absence of ATP, wild type Drg1p sedi-
The AAA Protein Drg1

The second row gives an upper boundary for the probability that the observed 6-fold symmetry could have arisen from random noise. The radii at which the symmetry registered are given in parentheses. The actual values obtained for the Student’s t statistic were 39.9, 15.6, and 14.3, respectively, indicating that the symmetry was registered even more strongly for the wild-type protein (a t value of 4.9 corresponds to p = 10^-6; the observed value corresponds to much less probability). A similar conclusion merges from the spectral product ratio test, whose geometric mean represents the ratio between the amplitude of 6-fold component of a typical particle’s rotational power spectrum and the value of this component for background areas of the same micrographs. Both Student’s t and the spectral ratio product indicate that those three proteins form hexamers.

| Protein                  | Probability from Student’s t test | Radius | Spectral ratio product | Geometrical mean | n  |
|--------------------------|-----------------------------------|--------|------------------------|------------------|----|
| Wild type                | <? 10^-6                          | 42-54  | 7.0 \times 10^{10}     | 4.9              | 478|
| Drg1-1p                  | <? 10^-6                          | 42-54  | 6.0 \times 10^{10}     | 2.4              | 268|
| C561T/S662G              | <? 10^-6                          | 48-60  | 1.0 \times 10^{10}     | 2.8              | 200|

**DISCUSSION**

**Phenotypes of DRG1 Mutants and Diazaborine Resistance**—The AAA protein Drg1 is essential for the growth of yeast cells. In this study, we have determined the binding strengths for ATP, measured the ATPase activities, and investigated the tendencies to hexamerize of wild type protein Drg1 and a number of mutant forms and correlated these properties with growth characteristics of the corresponding alleles (Table I). The mutants under investigation were a thermosensitive mutant, an intragenic suppressor mutant, and three mutants resulting in amino acid exchanges in the Walker A domain of the D2 region. The thermosensitive mutant grows at 25 °C but not at 37 °C. This phenotype results from a L457S exchange in a C-terminal part of the protein (13). Interestingly, changes in the Walker A box of the D2 domain also affected diazaborine resistance. Although the strain with the S662G exchange proved to be nonviable, the one with the C561T exchange grew normally and exhibited diazaborine resistance comparable with the drg1-1 allele. In contrast, the C561T/S662G double mutant, although growing normally in rich medium, exhibited supersensitivity when tested on diazaborine- or cycloheximide-containing plates. How the different DRG1 alleles affect resistance to diazaborine is unknown, but, as our drug binding studies indicate, the frequently observed mechanism of resistance in which the altered target protein has a lower binding strength for the inhibitor seems unlikely.

**ATP Binding**—We determined the number of binding sites for ATP of Drg1p. We found that the protein has two binding sites for the substrate with different association constants, one high and one low affinity site. We explain the dissimilarity in the binding constants with the different amino acid sequences

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**FIG. 7. Glycerol gradient centrifugations.** A, influence of ATP and ATPγS on oligomerization of the wild type protein. The protein was centrifuged after 10 min of incubation with 1 mM of the nucleotides. B, oligomerization of various Drg1p variants. Protein Drg1 and some of its mutant variants were applied to a glycerol gradient and centrifuged in the ultracentrifuge. In parallel runs, marker proteins were centrifuged. The contents of the tubes were fractionated, and each fraction was analyzed by SDS-PAGE. The stained gels of the fractions are shown. The proteins are designated with the letters “Drg1” plus an affix which indicates the type of mutation. ts, thermosensitive protein; sup, suppressor.
The exchanged amino acids, biological properties, and results of two experiments to detect the hexamer formation are listed (see the text for details). ts, thermosensitive mutant. sup, suppressor mutant. The fourth column gives the level of protein expression. The sixth column reports hexamer formation as detected by electron microscopy. By this criterion, the wild type protein has a higher level of hexamer formation than any mutant studied. The C561T/S562G mutant showed fewer hexamers. In the micrographs of thermosensitive, suppressor, and C561T mutants, large aggregates are dominant, and no particles with the same size as hexamers were found. The micrographs of C561T contain some ring-shaped particles, but the 6-fold symmetry was not detected as significant by statistical analysis. ND, not determined.

| Allele/protein | Amino acid exchange | Properties of cells | Expression | Percentage of ATPase | Hexamer formation by electron microscopy | Glycerol gradient |
|---------------|---------------------|---------------------|------------|----------------------|-----------------------------------------|------------------|
| Wild type     | None                | Wild type           | High       | 100                  | Hexamer                                | Hexamer (monomer without nucleotide) |
| Drg1–1p ts    | V725E               | Drug-resistant      | High       | 71                   | Hexamer                                | ND               |
| sup           | L457S               | Thermosensitive     | Low        | 9.4                  | Aggregates                              | Monomer          |
| sup<sup>+</sup> | F434L               | Suppressor of ts    | Low        | 8.2                  | Aggregates                              | Monomer          |
| C561T         | C561T               | Diazaborine         | Low        | 33.5                 | Few hexamers                            | Monomer          |
| S562G         | S562G               | Growth-deficient    | Low        | 180                  | Aggregates                              | Monomer          |
| C561T/S562G   | C561T/S562G         | Supersensitive to drug | High      | 270                  | Fewer hexamers than wild type           | Hexamer          |

TABLE II
Summary of biological, biochemical, and structural experiments for wild type Drg1p and seven mutants

The AAA Protein Drg1

We have shown by electron microscopy and image analysis of negatively stained specimens that in the presence of ATP-γS, wild type Drg1p forms hexameric rings. This result was confirmed by cryo-electron microscopy (data not shown) and is further supported by sedimentation analysis on glycerol gradients.

We conclude, therefore, that conformational changes associated with the requirement for the AAA ATPases presumably serve to transduce the energy of ATP hydrolysis for diverse complex functions, including chaperone activity and membrane fusion. It is plausible that mutants should exist for which ATPase activity is retained (or enhanced) while the transduction effect is impaired.

Indeed, decoupling of the ATPase activity and the associated biochemical function in SNARE pair disassembly was recently described also for NSF. A mutation in the Drosophila NSF, which causes the comatose phenotype, was introduced into the mammalian ortholog. The modified NSF version exhibits no ATPase activity and SNARE disassembling but could still promote a Golgi reassembling reaction (26).

Because the Walker A sequence of the D2 domain of Drg1p deviates significantly from the consensus sequence, we also studied the enzymatic properties of our mutant proteins. The ATPase activity of the C561T mutant was three times lower than that of the wild type protein but is sufficient to support growth. In contrast, the protein from the nonviable S562G mutant was 2-fold higher in its ATPase activity than wild type Drg1p. This mutant protein is nonfunctional as assessed by its inability to support yeast growth. The protein from the double mutant showed almost three times higher ATPase activity than the wild type protein and exceeded that of the S562G mutant. These observations afford further evidence that ATPase activity of Drg1p and functionality as determined by cellular growth are not inseparable.

Structural Aspects: Hexamerization—We have shown by electron microscopy and image analysis of negatively stained specimens that in the presence of ATP-γS, wild type Drg1p forms hexameric rings. This result was confirmed by cryo-electron microscopy (data not shown) and is further supported by sedimentation analysis on glycerol gradients.

Anticipating that substituting ATP-γS might result in structural changes (27, 28), we also studied the protein in the presence of ATP by electron microscopy. Again, we observed hexamers that were indistinguishable at this resolution (~2 nm) and in this representation from the ATP-γS state of the protein. We conclude, therefore, that conformational changes associated with the requirement for the AAA ATPases presumably serve to transduce the energy of ATP hydrolysis for diverse complex functions, including chaperone activity and membrane fusion. It is plausible that mutants should exist for which ATPase activity is retained (or enhanced) while the transduction effect is impaired.

Sedimentation analysis detected hexamers only in the presence of ATP or ATP-γS. Because hexameric rings appear amply in electron microscopy of Drg1p without ATP, we infer that these complexes are not stable enough to survive the glycerol gradient centrifugation, whereas ATP-containing hexamers have the requisite stability. In the same context, the addition of...
ATP to Drg1p prevented the loss of enzymatic activity by heat treatment. We take this finding as additional evidence that the hexameric conformation of Drg1p is stabilized by binding ATP.

We also determined the ability of mutant forms of Drg1p to form hexamers (Table I). The C561T mutant protein formed hexamers only very inefficiently, although this allele showed normal growth behavior. The protein from the double mutant C561T/S562G formed hexamers, although to a much lesser extent than the wild type protein. This mutant grows normally but shows the phenotype of extreme sensitivity to inhibitors. The thermosensitive protein showed no hexamerization, and neither did the protein from the suppressor mutant in the electron microscopic study. Glycerol gradient centrifugation confirmed these results, although it is possible that the suppressor mutant protein formed a few hexamers that we were unable to detect by either method. Taken together, these observations suggest that hexamerization is not tightly coupled to functionality. However, it is known that hexamerization is essential for the function of many type II AAA proteins such as p97 (30), NSF (9), and ClpA (20, 29), and it remains possible that Drg1p also works as a hexamer. In this event, the present observations on hexamer formation in vitro might be explained if there were an additional cofactor in vivo that assists hexamerization of Drg1p. Such a cofactor might also mediate the interaction between Drg1p and diazaborine. Alternatively, a subset of molecules in hexameric form may suffice for function. Because we have unpublished data showing that very low level expression of wild type Drg1p supports growth of yeast cells, it could be that a small amount of functional hexamers is formed by the suppressor strain at the nonpermissive temperature that is sufficient to support growth.

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Structural and Enzymatic Properties of the AAA Protein Drg1p from *Saccharomyces cerevisiae* : DECOUPLING OF INTRACELLULAR FUNCTION FROM ATPase ACTIVITY AND HEXAMERIZATION

Andriy Zakalskiy, Gregor Högenauer, Takashi Ishikawa, Eva Wehrschtz-Sigl, Franz Wendler, David Teis, Gertrude Zisser, Alasdair C. Steven and Helmut Bergler

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