Alternative Oligomeric States of the Yeast Rvb1/Rvb2 Complex Induced by Histidine Tags

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SUMMARY

Rvb1 and Rvb2 are essential AAA+ helicases, which are important components of critical complexes such as chromatin remodeling and telomerase complexes. The oligomeric state of the Rvbs has been controversial. Independent studies from several groups have described the yeast and human Rvb1/Rvb2 complex both as a single and a double hexameric ring complex. We found that histidine tagged constructs of yeast Rvb proteins employed in some of these studies induced the assembly of double hexameric ring Rvb1/Rvb2 complexes. Instead, untagged versions of these proteins assemble into single hexameric rings. Furthermore, purified endogenous untagged Rvb1/Rvb2 complexes from *Saccharomyces cerevisiae* were also found as single hexameric rings similar to the complexes assembled *in vitro* from the purified untagged components. These results demonstrate that some of the differences between the reported structures are caused by histidine tags and imply that further studies on the purified proteins should be carried out using untagged constructs.

Keywords

AAA+; Electron Microscopy; Helicase; Pontin; Reptin

INTRODUCTION

Rvb1 and Rvb2 are two highly conserved homologous proteins that belong to the AAA+ (ATPases associated with diverse cellular activities) family of ATPases. Rvb1 was first identified as part of a complex with TATA box binding protein^{1, 2} and Rvb2 as part of a complex involved in c-Myc mediated cell transformation in HeLa cells.³ Since then, these two proteins have been found as components of a large number of complexes that carry out

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The EM maps of the singly and doubly histidine tagged Rvb1/Rvb2 complexes have been deposited in the Electron Microscopy Data Bank (EMDB IDs: [5228](#), [5229](#), [5230](#)).

many distinct functions in a variety of molecular pathways, including chromatin-remodeling,^{4, 5, 6, 7} assembly and maturation of snoRNPs^{8, 9, 10, 11} and mitosis.^{12, 13}

The X-ray structure of human Rvb1¹⁴ and electron microscopy structures of both human¹⁵ and yeast^{16, 17} Rvb1/Rvb2 complexes have been reported. In the crystal structure of human Rvb1, the protein assembles as a single hexameric ring (Fig. 1). Each monomer in the ring has three distinct domains: DI, DII and DIII. The first and last domains constitute the AAA+ core domain, which contains the predicted elements of the AAA+ module for nucleotide binding and hydrolysis (Walker A, Walker B, sensor 1 and sensor 2 motifs). DII is a 170 amino acid stretch intercalated within DI (Fig. 1). Therefore, this domain is also known as the 'insertion' domain and it has been proposed to be important for DNA/RNA binding.¹⁴ The insertion domain is attached to DI by a linker region composed of two β -strands that probably allows for movement of this domain with respect to DI.

There have been a number of groups reporting also on the architecture of the human and yeast Rvb1/Rvb2 complexes using electron microscopy. In spite the high degree of homology among the human and yeast proteins, there are significant differences observed among the published structures. Contradictions are observed not only between the reconstruction of the human complex and the two structures reported for the yeast Rvb1/Rvb2 complex, but also among the two yeast assemblies.

One of these studies¹⁵ showed that the structure of the human Rvb1/Rvb2 complex assembled as a double hexameric ring (Fig. 2a). Recently, an additional publication reported that this complex forms single and double hexamers as well as smaller oligomeric forms.¹⁸ Subsequently, two independent groups published structural information regarding the yeast Rvb1/Rvb2 complex. The first¹⁶ concluded that yeast Rvb1 and Rvb2 assemble into single hexameric rings containing both proteins (Fig. 2b). Surprisingly, a second study¹⁷ revealed that the same complex organizes as an asymmetric double ring dodecamer with a top and bottom compact hexameric rings occupied by the DI and DIII domains of the Rvb proteins. Six protein densities project from the rings towards the center of the structure forming two layers of discontinuous density (equatorial domains) leaving lateral openings to an internal chamber in the structure. The equatorial domains are made of intercalated DII domains interacting in the middle of the structure and maintaining the integrity of the dodecameric structure (Fig. 2c). Interestingly, this last study suggests the dodecamer is composed of two homo-hexamers, each one containing only one of the proteins. Nevertheless, a conclusion that can be drawn from the comparison of the three structures¹⁹ is that in spite of the double or single ring structure adopted by the Rvb1 and Rvb2 proteins in the different studies, there is a reasonable degree of compatibility between the X-ray structure of the human Rvb1¹⁴ and the two structures reported for the yeast complex.^{16, 17} However, the EM reconstruction of the human Rvb1/Rvb2 complex¹⁵ disagrees with the yeast EM structures or the human X-ray structure.

The different structural assemblies observed for Rvb1/Rvb2 may reflect that these proteins can assemble into multiple oligomeric states and might undergo a multitude of conformational changes while performing their functions. Accordingly, the reported structures may represent different functional states of the Rvb1/Rvb2 complex. However, a

comparison of the approaches used to produce these structures red-flagged some experimental aspects that could be causing the observed differences.¹⁹ Therefore, we tested the effect of some of the experimental conditions differing among the cited studies to determine their effect on the oligomeric state of the Rvb1/Rvb2 complex.

In particular, several of the studies on the human^{14, 15} and yeast Rvbs¹⁷ used histidine or FLAG tagged versions of these proteins to assemble the Rvb1/Rvb2 complex. Here, we show that histidine tagged yeast Rvb1 and Rvb2 proteins form larger oligomers than the hexamers formed by the untagged versions of these proteins; both by themselves and when combined in an assembly reaction to form Rvb1/Rvb2 complexes. We found that these larger oligomers are dodecamers comprised of two stacked hexameric rings in a DII-DII configuration and that they reverted into single hexameric rings upon removal of the histidine tags.

In addition, most of the published structural studies^{15, 16} assembled the Rvb1/Rvb2 complex *in vitro* from purified components expressed in *Escherichia coli*. However, one study performed the complex assembly *in vivo* through co-expression of both Rvb1 and Rvb2 proteins in insect cells.¹⁷ To test the effect of the protein expression system and whether *in vivo* assembled untagged Rvb1/Rvb2 complexes form double or single hexameric rings, we purified the endogenous untagged Rvb1/Rvb2 complex from *Saccharomyces cerevisiae*. We found that such an isolated complexes are single hexameric rings that are structurally similar to the ones obtained *in vitro* from purified untagged Rvb proteins expressed in *E. coli*. Therefore, we concluded that the protein expression system and whether the complexes were assembled *in vivo* or *in vitro* did not have an effect in the oligomeric state of the Rvb1/Rvb2 complex. However, it is important that further studies on purified Rvb1/Rvb2 complexes are carried out with untagged constructs.

RESULTS

Histidine-tagged Rvb1 and Rvb2 proteins form larger oligomers than the untagged proteins

To determine whether the presence of histidine tags in the yeast Rvb1 or Rvb2 proteins have any effect on the oligomeric state adopted by the assembled Rvb1/Rvb2 complex both untagged and N-terminal His₆-tagged fusion versions of the Rvb1 and Rvb2 proteins were expressed and purified (Fig. 3a).

Subsequently, the untagged (Rvb1/Rvb2), singly (His-Rvb1/Rvb2 and Rvb1/His-Rvb2) and doubly (His-Rvb1/His-Rvb2) histidine tagged complexes were assembled in reaction mixtures containing equimolar amounts of both proteins. The oligomeric state of the complexes was assessed using Blue native polyacrylamide gel electrophoresis (BN PAGE). The sample containing untagged Rvb1/Rvb2 complex showed mainly one band with mobility similar to the 480 kDa molecular weight marker (Fig. 3b). The theoretical molecular mass of Rvb1 and Rvb2 are 50.5 and 51.6 kDa, respectively and our previous work¹⁶ established that the untagged proteins form hexameric rings of ~ 300 kDa. Therefore, this band represents hexameric Rvb1/Rvb2 complexes. The slightly slower mobility of these assemblies is explained because the migration in native polyacrylamide

gels is dictated not only by the molecular weight but also by the shape of the complexes. Surprisingly, in the samples containing the singly and doubly histidine tagged complexes, we observed an additional prominent band of similar mobility to the 720 kDa molecular weight marker. We hypothesized that this band corresponds to a dodecameric complex formed presumably by two stacked hexameric rings. The proportion of hexamers and putative dodecamers was not constant in the three samples. Hexamers were more abundant in the sample containing the Rvb1/His-Rvb2 complex, however hexamers coexisted in similar amounts to the putative dodecamers in the His-Rvb1/Rvb2 complex. In the sample containing His-Rvb1/His-Rvb2 complexes the larger oligomeric form was predominant (Fig. 3b). It is important to note that the band representing putative dodecamers in the sample containing untagged complexes although visible was very faint (Fig. 3b) implying that the proportion of dodecamers in that sample was extremely small (<1%).

Both untagged Rvb1 and Rvb2 alone resolved as a single band with similar mobility to the 66 kDa molecular weight marker, which is consistent with the monomeric state of these proteins in solution.¹⁶ The lane containing untagged Rvb2 also showed a smear probably representing a heterogeneous mixture of oligomers smaller than hexamers. In contrast, loading either N-terminal His₆-Rvb1 or N-terminal His₆-Rvb2 produced multiple bands in the native polyacrylamide gel. In particular, the first protein showed two distinct bands corresponding to the hexameric and putative dodecameric forms as well as multiple bands at lower molecular weights. N-terminal His₆-Rvb2 produced a similar pattern but the band corresponding to the putative dodecamers was absent (Fig. 3b). These data clearly indicate that the presence of N-terminal histidine tags in the Rvb1 and Rvb2 proteins further promotes the formation of large oligomers that are only faintly observed with the untagged complex.

Visualization of the oligomers formed by the histidine-tagged Rvb1 and Rvb2 proteins by electron microscopy

To structurally characterize the larger oligomeric forms observed with the N-terminal His₆-tagged fusion versions of the Rvb1 and Rvb2 proteins, assembly reactions containing untagged, singly or doubly histidine tagged Rvb1/Rvb2 complexes were analyzed by size exclusion chromatography and electron microscopy.

The sample containing untagged Rvb1/Rvb2 complexes resolved in three distinct peaks (Fig. 4a). A first peak, centered at 8.2 mL elution volume, corresponded to a very large and heterogeneous population of complexes as seen by negative staining EM (data not shown) whereas a second peak, centered at 14.6 mL elution volume was compatible with a population of monomers (~50 kDa). A third peak, centered at 11 mL elution volume, was broad and close to the expected elution volume of the Rvb1/Rvb2 hexamer (~300 kDa). Interestingly, this peak was only observed if the column was previously equilibrated with buffer containing 1.5 mM ADP. Fractions from this peak (10–12 mL) contained approximately equimolar amounts of both proteins and were applied to electron microscopy grids and observed under negative staining conditions (Fig. 4b). The fraction closer to the peak centered at 8.2 mL elution volume still showed mainly a heterogeneous mixture of protein complexes. However, the other fractions revealed large amounts of ring-shaped

particles, which our previous study determined represent top views of single hexameric Rvb1/Rvb2 complexes.¹⁶ An average image calculated from ~500 particles also confirmed the similarity of this oligomeric form to the single hexameric rings characterized in our previous study (Fig. 4c).

All samples containing the singly and doubly histidine tagged Rvb1/Rvb2 complexes produced an elution profile with a large peak, centered at 8 or 8.2 mL elution volume, containing mainly large heterogeneous complexes (data not shown). Only the sample containing the His-Rvb1/Rvb2 complex showed an additional smaller peak at the expected elution volume for monomers of the Rvb proteins (Fig. 4a). Differently from the sample containing the untagged Rvb1/Rvb2 complex, the three histidine tagged complexes produced the same profiles regardless of the presence or absence of ADP in the buffer used to equilibrate the column (data not shown). Importantly, any of the profiles showed a prominent peak at the expected elution volume for a hexamer or a dodecamer (~600 kDa). However, the large peak in the three samples showed a tail extending towards higher elution volumes and this feature was most predominant in the profile of the His-Rvb1/His-Rvb2 sample. Fractions from this tails corresponding to the expected elution volumes between the dodecamer and the hexamer (10–12 mL) contained approximately equimolar amounts of both proteins and were visually inspected under negative staining conditions (Fig. 4b). Similarly to the untagged Rvb1/Rvb2 complex the fraction closer to the large peak still contained mainly large heterogeneous aggregates. However, the subsequent fractions contained a few ring-shaped particles and mainly barrel-shaped particles with three parallel striations. The latter type of particle images from each histidine tagged sample were selected and averaged confirming that structurally these oligomeric forms are very different from the one observed with the untagged Rvb1/Rvb2 complex (Fig. 4c).

The histidine-tagged Rvb1/Rvb2 complexes assembles as double hexameric rings structures

To obtain a better description of the oligomers formed by the singly and doubly histidine tagged Rvb1/Rvb2 complexes, we obtained their three-dimensional reconstruction using single particle approaches and the electron micrographs prepared from the column fractions containing the highest concentration of purified complexes (Fig. 4).

To this end several thousands of barrel-shaped and also a few hundred ring-shaped particles representing side and top views of the complexes respectively, were selected for the three histidine tagged Rvb1/Rvb2 complexes. Initially, the ring shaped particles were analyzed using algorithms capable of detecting rotational symmetries²⁰ and found that these complexes had six-fold symmetry. No other order of symmetry was found to be statistically significant. Results for the *t* test and the spectral ratio product are shown in Supplementary Table 1 and formally establish the symmetry of the three histidine tagged Rvb1/Rvb2 complexes.

Only the barrel-shaped particles (~10,000 particles for each complex) were used to obtain the three-dimensional volumes. These views ensure that single hexameric rings that may also be present in the sample are not used in the reconstruction process. The reconstructions of the three complexes were calculated by the projection matching approach. To rule out any

model bias in the resulting structures several initial models were used including the previously published double hexameric ring structure of the yeast Rvb1/His-Rvb2 complex¹⁷ and also a reference volume constructed using an average of top, side views and the six-fold symmetry detected in the rotational symmetry analysis.²¹ In addition, the three-dimensional reconstruction process was done both assuming the six-fold symmetry (c6) and also treating these structures as asymmetric objects (c1). Regardless of the initial map and the symmetry imposed during the refinement process, we consistently obtained essentially the same three-dimensional reconstruction for each complex.

The three-dimensional reconstructions of the three histidine tagged Rvb1/Rvb2 complexes at ~20 Å resolution (Fig. S1) shows a cylindrical structure with a diameter of ~114 Å and a height of 142 Å (Fig. 5). The Rvb1/His-Rvb2 and His-Rvb1/His-Rvb2 complexes were slightly longer than the His-Rvb1/Rvb2 complex probably due to the different amount of flattening experienced during preparation of the negative staining grids or it may have been caused by genuine conformational differences between the complexes. Inspected from the side, these reconstructions present three different layers of density (i, ii and iii). The two outer layers (i and iii) are denser than the one in the middle (ii) and most likely comprise the core AAA+ domains of the Rvb proteins assembled as hexameric rings. The middle layer is less dense and probably contains the insertion domains of the Rvb proteins. This layer is made of six protein densities projecting from the top and bottom rings and they run in an oblique fashion through the middle layer. The interactions of the densities in the middle layer with the top and bottom rings are different (Fig. 5; side views) and the two outer rings are also not identical (Fig. 5; top and bottom views). The asymmetry of the complex seems to be real, since all the three-dimensional reconstructions diverged into an asymmetric structure in spite of using an initial reference with fully symmetric top and bottom rings (d6 symmetry). In addition, we noticed that in the Rvb1/His-Rvb2 complex the densities in the middle layer connect to the outer rings in a slightly different pattern. All together, these results show that the histidine tagged Rvb1/Rvb2 complexes assemble as dodecameric structures comprised of two stacked hexameric rings.

The dodecameric structures formed by the histidine tagged Rvb1/Rvb2 complexes are consistent with a DII-DII stacking of the two hexameric rings

Next, we examined whether the obtained three-dimensional structures for the histidine tagged Rvb1/Rvb2 complexes were consistent with a DII-DII, AAA-AAA or AAA-DII stacking of the two hexameric rings. To this end, we used the X-ray structure of human Rvb1 to perform docking experiments into our EM maps.

Initially, we observed that the entire X-ray structure of human Rvb1 hexamer¹⁴ fits poorly the EM maps suggesting that the relative orientation of the AAA+ core domain and the insertion domain is different between the crystal and EM structures (Fig. S2). In the crystal structure, the relative orientation between the two domains is dictated by a linker region (residues 120–129 and 232–295) (Fig. 1a), which probably adopts a different conformation in the two structures. Consequently, this linker region of the human Rvb1 structure was not included in our docking experiments and the AAA+ core domain (residues 9–119 and 296–449) and the globular part of the insertion domain (residues 130–231) (Fig. 1a) were docked

independently into the EM density maps by rigid body fitting. The AAA+ core domains of the two Rvb1 hexameric rings fit unambiguously the two outer layers of density (Fig. 6a and Fig. S3a and b, top panels) and two insertion domains, each from one ring, docked well into each of the six protein densities in the middle layer of the EM maps. However, due to the limited resolution of the three-dimensional reconstructions, it was possible to fit the pairs of insertion domains in more than one orientation. Therefore, we arbitrarily chose the orientation proposed by Torreira et al.,¹⁷ which also showed as a possible local optima in our docking experiments (Fig. 6a and Fig. S3a and b, top panels). The good agreement found between the crystal structure of the Rvb1 hexamer and our EM density maps suggested that the histidine tagged complexes are formed by two stacked hexameric rings arranged in a DII-DII configuration in which the insertion domains from the hexamers interact at the equator of the complex.

To ensure that the generated models agree with the observed experimental projections of the histidine tagged complexes, side view projections limited to the resolution of the EM maps (~20 Å) were generated from the DII-DII models and compared with averages of experimental projections from the corresponding complexes (Fig. 6b, top panel and Fig. S3a and b, bottom panels). The region connecting the AAA+ core domains with the insertion domains was less dense in the calculated projections from the models since the linker region between the two domains was not used for the docking experiments. Nonetheless, an excellent match between the calculated projections and the experimental average was obtained for the three histidine tagged complexes (Fig. 6b, top panel and Fig. S3a and b, bottom panels).

To prove that the obtained three-dimensional reconstructions were inconsistent with a AAA-AAA and a AAA-DII stacking of the hexameric rings, we also constructed these two additional models and calculated their side view projections. Both configurations were modeled from the DII-DII model of the His-Rvb1/Rvb2 complex. The AAA-AAA model was made by inverting the two Rvb1 hexameric rings such that the AAA+ core domains were then accommodated in the middle layer of density in the EM maps. In this configuration, the insertion domains project from each ring towards the outer layers of density (Fig. 6b, middle panel, left column). Similarly, the AAA-DII model was assembled by placing one of the Rvb1 rings as in the DII-DII model and inverting the second ring and orienting it as in the AAA-AAA model, such that the insertion domains of one ring interact with the AAA+ core domain of the second ring at the equator of the complex (Fig. 6b; bottom panel, left column). The density maps generated from both models fitted very poorly the experimental EM map of the His-Rvb1/Rvb2 complex (Fig. 6b; middle and bottom panel, middle column). Similarly, the calculated side view projections from both models were inconsistent with the experimental projections (Fig. 6b; middle and bottom panel, right column).

The absolute handedness of the EM maps was not determined. Therefore, we also docked the X-ray structure of the Rvb1 hexamer into the His-Rvb1/Rvb2 EM map with opposite handedness and we built the alternative DII-DII model (Fig. S4; top panel). The calculated side view projections from this model were also very similar to the average of experimental projections from the His-Rvb1/Rvb2 (Fig. S4; bottom panel). In addition, because the

orientation of the insertion domains for the construction of the DII-DII models was chosen arbitrarily additional DII-DII models were also constructed with alternative orientation for these domains. In this case, we also found an excellent agreement between the calculated and experimental projections (data not shown).

These results confirm that the three-dimensional reconstructions obtained for the histidine tagged Rvb1/Rvb2 complex are all consistent with a DII-DII configuration of the two hexameric rings but also incompatible with the other configurations.

The histidine tag induced dodecamer formed by the Rvb1/Rvb2 complexes are reversible

We tested whether the histidine tag-induced dodecamers of the Rvb1/Rvb2 complexes remained stable after removal of the tag. Singly and doubly histidine tagged Rvb1/Rvb2 complexes were purified by size exclusion chromatography and treated with tobacco etch virus (TEV) protease to remove the histidine tags. Removal of the tags was monitored by SDS-PAGE (Fig. 7a). The N-terminal His₆-tagged fusion versions of the Rvb1 and Rvb2 proteins run very similarly in the SDS-PAGE electrophoresis, thus only one band is seen in the lane containing untreated His-Rvb1/His-Rvb2 complex (Fig. 7a). Cleavage was almost complete for the His-Rvb1/Rvb2 and His-Rvb1/His-Rvb2 complexes however, it only reached ~60% in the Rvb1/His-Rvb2 complex.

Both the TEV treated and untreated complexes were loaded into a native gel (Fig. 7b). The untreated histidine tagged Rvb1/Rvb2 complexes showed mainly a band of similar mobility to the 720 kDa molecular weight marker representing the dodecameric complexes and a second band of faster mobility and significantly less intensity representing hexamers. This second band was absent in the lane containing the untreated His-Rvb1/His-Rvb2 complex. Interestingly, the band representing the dodecameric complexes disappeared in the samples containing the Rvb1/Rvb2 complexes where the histidine tag had been removed.

It is important to note that in all the untreated samples the band corresponding to the dodecamers was predominant over the one representing the hexamers. This result is not in disagreement with the experiment shown in Fig. 3b because in this case size exclusion chromatography fractions enriched in dodecameric complexes were used instead of the full assembly reactions shown in Fig. 3b.

These results imply that the histidine tag-induced dodecamers formed by the Rvb1/Rvb2 complexes break down into hexamers upon removal of the tags. Partial cleavage of the tags also causes complete loss of the dodecameric structures, as seen with the sample containing TEV treated Rvb1/His-Rvb2 complexes, suggesting that the histidine tags need to be present in most of the protein molecules to maintain the integrity of the dodecamers.

Purified endogenous Rvb1/Rvb2 complexes are single hexameric rings of similar structure to the *in vitro* assembled complexes with untagged Rvb proteins

In *Saccharomyces cerevisiae* the Hsp90 cofactors Pih1 and Tah1 interact with Rvb1/Rvb2 complex to form the R2TP complex, which was shown to be important for mediating the assembly of snoRNPs.^{11, 22} In this complex, Pih1 interacts directly with the Rvb1/Rvb2 complex whereas Tah1 binds to Pih1 (Fig. 8a).¹¹ In order to image the endogenous *in vivo*

assembled Rvb1/Rvb2 complex, we purified the R2TP complex using a yeast strain having endogenously FLAG-tagged Pih1.¹¹ We found that the addition of ATP to the isolated complex results in the release of Rvb1/Rvb2 from Tah1 and Pih1 (Fig. 8a and 8b). Both the purified R2TP and endogenous Rvb1/Rvb2 complexes were loaded on SDS-PAGE gels to visualize the protein content (Fig. 8b) and also into native polyacrylamide gels to analyze their oligomeric state (Fig. 8c). Interestingly, endogenously isolated Rvb1/Rvb2 both alone and in the context of the R2TP complex ran as a single band corresponding to a single hexameric complex. No slower mobility band representing dodecameric complexes was apparent.

The Rvb1/Rvb2 sample was visualized by negative staining EM revealing the presence of ring-shaped particles. An average image calculated from ~500 particles (Fig. 8d) confirmed the structural similarity of these oligomers to the single hexameric rings assembled *in vitro* from untagged Rvb proteins (Fig. 2b and 4c). Unfortunately a three-dimensional reconstruction of this complex (or of the *in vitro* assembled untagged Rvb1/Rvb2 complex) was not possible because the electron micrographs only provided top views of the Rvb1/Rvb2 single hexameric rings. Various treatments of the electron microscopy grids such as floating the grids with polylysine²³ or glow discharging failed to produce side view projections of this complex (data not shown).

We also attempted to image the full R2TP complex, however, no additional densities were observed in the average image that could be assigned to the Pih1 and Tah1 proteins (data not shown). These proteins are rather small and might not be rigidly oriented with the complex. Nevertheless, these results indicate that the protein expression system and whether the complexes were assembled *in vivo* or *in vitro* did not have an effect in the oligomeric state of the Rvb1/Rvb2 complex.

DISCUSSION

Recent attempts to understand the architecture of this assembly have shown that Rvb1 and Rvb2 proteins organize into several types of assemblies. However, use of histidine tagged constructs of these protein in some of the studies has raised the question of whether the various described structures represent multiple functional states of the complex or are indeed induced by experimental conditions. We found that N-terminal His₆-tags in Rvb1 or Rvb2 proteins trigger the formation of double hexameric ring structures. The AAA+ core domain of the Rvb proteins occupies the top and bottom of these structures and the insertion domains are located at the equator constituting the main area of interaction between the upper and the lower rings.

We do not know how the N-terminal His₆-tag in Rvb1 or Rvb2 leads to induction of double ring structures. According to the model proposed here for the dodecamer, the N-terminal ends of the Rvb proteins locate at the base of the solid rings of density at the top and bottom of the structure and close to the insertion domains (Fig. 1b). Therefore, the N-terminal ends of the monomers in each ring are probably too far to induce formation of the double ring structure through direct interaction between the N-terminal His₆-tags of one ring and surface motifs in the monomers of the opposite ring. Similarly, a metal mediated interaction between

the N-terminal His₆-tag from opposite rings is also unlikely because of the distance separating both tags. A more plausible mechanism is that the presence of the N-terminal His₆-tags may induce a conformational change in the hexameric ring (probably in the insertion domains), allowing two rings to interact in a DII-DII fashion. This hypothesis is consistent with the reported dodecameric cryo-EM structure of yeast Rvb1/Rvb2 complex assembled with untagged Rvb1 and N-terminal histidine tagged Rvb2 proteins,¹⁷ where the two hexamers interact via the insertion domains. In addition, it is also in agreement with a recent study¹⁸ using insertion domain deletion mutants of the human Rvb1 and Rvb2 proteins and that showed that the insertion domain in Rvb1 seems to have a role in stabilizing the dodecamers, whereas the one in Rvb2 does not seem to be involved. In this later study, the mutant Rvb1 and Rvb2 proteins contained a N-terminal and a C-terminal histidine tag, respectively. Surprisingly, it was also found that the Rvb1/Rvb2 complex assembled from both mutants lacking the insertion domain still showed formation of some dodecamers as well as hexamers.¹⁸ This result suggests that in addition to the insertion domains, a secondary motif in the Rvb proteins may also mediate the interaction between the two hexameric rings in the dodecameric structure.

The three-dimensional structures presented here of the dodecamers formed by the histidine tagged Rvb1 and Rvb2 proteins are in overall good agreement with the previously reported cryo-EM structure of the yeast Rvb1/His-Rvb2 complex and that we show in Fig. 2c.¹⁷ However, it is surprising that the raw particle images described in this previous study and the ones obtained by us with the analogous complex are somehow different. Torreira et al. found that the most prominent view of the Rvb1/His-Rvb2 complex in the electron micrographs were suggestive of a four-petal flower. Instead, the most frequent view seen in our images are barrel-shaped particles (Fig. 4b). In addition, we found that the histidine tagged induced dodecamers in our study reverted into hexamers upon removal of the histidine tags. Conversely, Torreira et al. indicate that the dodecamers were still visible by size exclusion chromatography and electron microscopy after cleavage of the histidine tag.¹⁷ It is unclear at this point what may be causing these differences. The two studies used a different protein expression system and complex assembly protocol. Mainly, Torreira et al. expressed and reconstituted the dodecameric complexes *in vivo* by using insect cells. On the other hand, we performed the assembly of the histidine tagged complexes *in vitro* from purified components expressed in *E. coli*. However, our data clearly establishes that the presence of histidine tags have an influence on the oligomeric state of the Rvb but it is not affected by the protein expression system or whether the complexes were assembled *in vivo* or *in vitro*. Further investigations should clarify the causes of the observed differences.

In summary, most of the structural work in both human and yeast Rvb proteins has been performed with tagged constructs. However, further studies in these proteins should be performed with untagged Rvb1 and Rvb2 proteins. These experiments will determine which of the described oligomeric forms of the Rvb1/Rvb2 complex are genuine and represent physiological oligomers rather than structures induced by the experimental conditions. Nonetheless, an interesting question that still remains is whether the histidine tagged induced Rvb dodecamers described herein constitute simply an experimental artifact or somehow these structures have some resemblance to the oligomeric states that these proteins may adopt naturally upon binding to other protein partners.

MATERIAL AND METHODS

Protein expression and purification

Both yeast Rvb1 and Rvb2 proteins were expressed as N-terminal His₆-tagged fusion proteins in *E. coli* BL21 (DE3) cells using previously obtained clones of the genes in pProEX HTb and p11 expression vectors, respectively.¹⁶ For both proteins cells were grown in LB medium at 37°C to OD₆₀₀ = 0.6 and expression was induced with 1 mM IPTG. Cells were induced overnight at 18°C.

Lysis was performed in 30 mL Lysis buffer (25 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 10 mM imidazole and 10% (v/v) glycerol) by adding 30 mg of lysozyme and incubating for 30 min on ice, followed by sonication. Lysate was cleared by centrifugation at 39,000 × g for 40 min and filtered with a 0.22 µm filter. Subsequently, it was loaded in a HiTrap Metal Chelating column (GE Healthcare Life Sciences) equilibrated with lysis buffer.

Unspecifically bound proteins were washed with increasing concentrations of imidazole up to 50 mM and the N-terminal His₆-tagged Rvb1 and Rvb2 were eluted with 250 mM imidazole. Fractions containing the proteins were pooled and dialyzed against 25 mM Tris/HCl (pH 7.5), 40 mM KCl and 10% (v/v) glycerol. In those cases where we aimed to purify the untagged Rvb1 or Rvb2 proteins, tobacco etch virus (TEV) protease was used to remove the N-terminal His₆-tag. Then, a volume of 200 µL of a 6.5 mg/mL TEV stock solution was added to the pooled fractions containing the protein after the first dialysis buffer exchange and the mixture was maintained in dialysis for 12 more hours. Both untagged and N-terminal His₆-tag proteins were further purified and concentrated by loading the protein mixture into a Q-Sepharose column (GE Healthcare Life Sciences) equilibrated with 25 mM Tris/HCl (pH 7.5), 40 mM KCl and 10% (v/v) glycerol. Washes were done with increasing concentrations of NaCl up to 80 mM and the protein was eluted with 25 mM Tris/HCl (pH 7.5), 0.5 M NaCl and 10% (v/v) glycerol. In purifications of untagged Rvb proteins the fractions from the Q-Sepharose column containing the Rvb proteins loaded in a second HiTrap Metal Chelating column (GE Healthcare Life Sciences) equilibrated with 25 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 50 mM imidazole and 10% (v/v) glycerol to remove any residual uncleaved Rvb protein. The flow-through of the column containing the untagged Rvb proteins was collected, dialyzed against 25 mM Tris/HCl (pH 7.5), 40 mM KCl, and 10% (v/v) glycerol and further concentrated in a Q-Sepharose column as described above. The fractions containing pure untagged or N-terminal His₆-tag Rvb proteins were finally dialyzed in storage buffer (25 mM Tris/HCl (pH 7.5), 80 mM KCl and 10% (v/v) glycerol).

In vitro assembly and purification of Rvb1/Rvb2 complexes

Untagged and histidine tagged Rvb1/Rvb2 complexes were assembled in 600 µL reaction mixtures in assembly buffer 25 mM Tris/HCl (pH 7.5), 80 mM KCl, 10% (v/v) glycerol, 1mM DTT and 1.5 mM ADP. Each protein in the reaction was at a concentration of 6 µM. The reaction mixtures were incubated at 37 °C for 30 min and loaded on a Superdex 200 10/300 GL column (GE Healthcare Life Sciences) equilibrated in 25 mM Tris/HCl (pH 7.5), 80 mM KCl and 10% (v/v) glycerol at 4°C and at a flow rate of 0.3 mL/min. The same buffer but also containing 1.5 mM ADP was used to equilibrate the column when the

untagged Rvb1/Rvb2 complex was loaded. ADP was required in this case to maintain the integrity of the hexamers. A gel filtration calibration kit (HMW, GE Healthcare Life Sciences) was used for column calibration.

Complexes to be loaded on the native BN PAGE gels were assembled as described above. However, in this case the volume of the assembly reactions was only 20 μ L. The concentration of each protein in the assembly reactions was 6 μ M in the reactions containing both Rvb proteins and 9.5 μ M in the samples that only had one of the proteins. The samples were then diluted 4-fold in assembly buffer and a volume of 15 μ L of each diluted sample was mixed with 5 μ L of loading dye and resolved by Blue native polyacrylamide gel electrophoresis (NativePAGE™ Novex® Bis-Tris Gel System. Invitrogen) following the manufacturer protocols. In the case of complexes purified by size exclusion chromatography 15 μ L of each indicated fractions was mixed with 5 μ L of loading dye and resolved in the gels.

Purification of the yeast R2TP and endogenous Rvb1/Rvb2 complexes

Yeast cells R0065 in the W303 background (*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 PIH1-3FLAG KAN*) were grown in 4 L of YPD (Yeast-peptone-dextrose) media at 30 °C overnight until OD₆₀₀ reached ~1.5–2. Cells were harvested, washed with 5 volumes of H₂O and then washed with 5 volumes of BufferH-0.3 containing one EDTA-free protease one inhibitor cocktail tablet (Roche) in 50mL of 25 mM HEPES-KOH pH 7.6, 1 mM EDTA, 10% glycerol, 0.02% NP-40, 300 mM KCl, 2.5 mM DTT, 10mM MgCl₂). Approximately 60 mL of cell pellet were then loaded into syringes and squeezed into liquid nitrogen to generate “noodles”. Cell pellets were then broken using a coffee grinder for 4 min. Broken cells were then incubated at room temperature until damp yet slightly frozen. They were then put on ice and resuspended with an equal volume of BufferH-0.3. Lysate and cell debris were then separated by centrifugation at 22,000 $\times$ g for 1 hr at 4 °C. A 60 mL volume of lysate was collected and mixed with 400 μ L of Anti-FLAG beads (Sigma) which were washed twice with 5 volumes of BufferH-0.5 (same as BufferH-0.3 except containing 500 mM KCl) for 1hr at 4 °C. Beads were centrifuged at 900 $\times$ g for 30 seconds at 4 °C. The supernatant was then removed and the beads were washed 5 times for 5 min with 10 volumes of BufferH-0.5 at 4 °C. Beads were then washed 3 times for 5 min with 10 volumes of BufferH-0.1 (same as BufferH-0.3 except containing 100 mM KCl) at 4° C. Finally, the beads were incubated with 400 μ L of ATP buffer (3 mM ATP in 25 mM Tris-HCl pH 7.5) for 30 min at room temperature and the supernatant containing untagged endogenous Rvb1/Rvb2 complexes was collected. Instead, the R2TP complexes were eluted with FLAG peptide (Sigma). Both samples were immediately visualized using electron microscopy and resolved in a 12% SDS-PAGE gel that was subsequently processed by silver staining.

Electron microscopy and three-dimensional reconstructions

Samples from the sizing column fractions were applied by floating a 10 μ L drop to carbon-coated grids previously glow discharged and negatively stained with 1% uranyl acetate. Specimens were observed in a JEOL 2010F electron microscope operated at 200 kV. Images were collected at 50,000 $\times$ with a dose of 10 electrons/ $\text{\AA}^2$. All images were recorded on

Kodak SO-163 films, scanned on a Nikon super COOLSCAN 9000 ED at 6.35 $\mu\text{m}/\text{pixel}$ and averaged 2 $\times$ to produce data at 2.54 $\text{\AA}/\text{pixel}$.

Particles were extracted interactively from digitized fields using the 'Boxer' program (EMAN).²⁴ Cross-correlation methods were used in the 'align2d' program (Xmipp)²⁵ to align the ring-shaped and barrel-shaped particles and obtain the two-dimensional averages.

To perform the symmetry analysis of ring-shaped particles representing top views of histidine tagged complexes, the normalized images were first translationally aligned relative to a previously obtained circularly symmetric reference image produced by averaging all the images in the data set. The presence of statistically significant rotational symmetry in the data was then assessed using two statistical tests: the spectral ratio product and the Student's t test as previously described.²⁰

The 3D reconstructions were calculated using standard 3D projection matching procedures as implemented in the Xmipp software package.²⁵ Two initial references were used. The first one was built using the 'startcsym' program (EMAN)²⁴ and 6-fold rotational symmetry. The second reference was produced from the previously published double hexameric ring structure of the yeast Rvb1/His-Rvb2 complex.¹⁷ The two initial references were low-pass filtered to 30 $\text{\AA}$ before their 2D projections were calculated to initiate the projection matching procedure. The resulting maps were low-pass filtered to their estimated resolution using a Fourier Shell Correlation (FSC) value of 0.5.

Docking of X-ray crystallographic structures, model generation and structure visualization

Fitting of the X-ray structure of human Rvb1 hexamer (PDB ID: 2C9O) into the EM maps of the histidine tagged Rvb1/Rvb2 complexes was performed using rigid-body fitting. The AAA+ core domain (residues 9–119 and 296–449) and the globular part of the insertion domain (residues 130–231) were docked independently. In each case the X-ray model was first manually docked into the EM map and the fitting was then optimized using the 'Fit in Map' option in Chimera.²⁶ The generated DII-DII, AAA-AAA and AAA-DII models were transformed into density maps and their side view projections were calculated using the 'pdb2mrc' and 'project3d' programs (EMAN),²⁴ respectively. Visualization of the EM density maps, fitted atomic structure and constructed models was done with UCSF Chimera software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to the staff at the Canadian Centre for Electron Microscopy. JH holds a Natural Sciences and Engineering Research Council of Canada PGSD2 fellowship. Our work in this field is supported by a Discovery grant from the National Science and Engineering Research Council of Canada (J.O., RGPIN288327-07) and the Canadian Institutes of Health Research (W.A.H., MOP-93778).

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Fig. 1. X-ray structure of the human Rvb1 protein

(a) Linear diagram (left) of the human Rvb1 protein indicating the location of the AAA+ core domains and globular part of the insertion domain. The linker regions between the two domains are indicated. Residues delimiting all these domains are also indicated in the diagram. In the right panel, the X-ray structure of the human Rvb1 monomer is shown as a ribbon representation. The domains of the protein are color coded as in the linear diagram. (b) Top view (left) and side view (right) of the hexameric ring formed by the human Rvb1 protein as a ribbon representation color coded as in (A) except for one of the monomers that is colored in green. The AAA+ core domains, globular parts of the insertion domains in the hexameric ring as well as the N-terminal and C-terminal ends of the monomer in green. Image in (a) and (b) were prepared from PDB file 2C9O.

Fig. 2. Available EM structures of the human and yeast Rvb1/Rvb2 complex

(a) Top and side views of the the three-dimensional reconstruction of the human Rvb1/Rvb2 complex obtained from negatively stained electron micrographs. The images were prepared from EMDB ID: 1317.

(b) Two-dimensional averages of the yeast Rvb1/Rvb2 complex in the presence of three different nucleotide states.

(c) Surface rendering representation of the three-dimensional reconstruction of the yeast Rvb1/Rvb2 complex obtained by cryo-electron microscopy. A top and a side view are shown. The asterisks indicate the projected densities from the bottom ring into the equatorial domain and the location of DI, DII and DIII is indicated. The images were prepared from EMDB ID: 2865.

Fig. 3. N-terminal histidine tags in the Rvb1 and Rvb2 proteins induce the formation of oligomeric forms larger than hexamers

(a) Purified untagged and N-terminal His₆-tagged fusion versions of the Rvb1 and Rvb2 proteins were resolved in a 12% SDS-PAGE and stained by Coomassie brilliant blue. Proteins containing a N-terminal His₆-tag are denoted with “H-” label before the name of the protein. (b) Untagged (Rvb1/Rvb2), singly (H-Rvb1/Rvb2 and Rvb1/H-Rvb2) and doubly (H-Rvb1/H-Rvb2) tagged complexes were assembled in the presence of 1.5 mM ADP and resolved in a BN PAGE gel and stained with silver staining. The untagged and the histidine tagged versions of the Rvb1 and Rvb2 proteins were also resolved in these gels. The bands corresponding to the dodecamers (12-mer), hexamers (6-mer) and monomers (1-mer) are indicated.

Fig. 4. Purification and visualization of the untagged, singly or doubly histidine tagged Rvb1/Rvb2 complexes by electron microscopy

(a) Elution profile of the untagged, singly and doubly histidine tagged Rvb1 and Rvb2 complexes from a Superdex-200 column. In all cases the size exclusion chromatography column was equilibrated with buffer containing 1.5 mM ADP. Proteins containing a N-terminal His₆-tag are denoted with “H-” label before the name of the protein. The arrows in the elution profiles indicate the expected elution volume for the dodecamer (9.8 mL), hexamer (11.3 mL) and monomer (15.2 mL). Samples were withdrawn from the fractions between 10–12 mL, resolved in a 12% SDS-PAGE gel and stained with Coomassie brilliant blue. (b) Representative negative staining electron micrographs of purified untagged, singly

and doubly histidine tagged Rvb1/Rvb2 complexes obtained from fractions between 10–12 mL from the elution profiles in panel A. (c) Two-dimensional averages of untagged, singly and doubly histidine tagged Rvb1/Rvb2 complexes obtained from particle images selected from electron micrographs obtained from fractions between 10–12 mL from the elution profiles in panel a.

Fig. 5. Three-dimensional reconstructions of the histidine tagged Rvb1/Rvb2 complexes
Side, top and bottom views of the EM structures of the three histidine tagged Rvb1/Rvb2 complexes. EM maps are shown using a surface rendering representation using a threshold value representing approximately 100% of their estimated mass (~600 kDa). The three layers of density in which the EM maps are divided are indicated in one of the side view. The arrow in the same map indicates one of the densities projecting from the top ring and that run through the middle layer in an oblique fashion.

Fig. 6. Docking of the X-ray structure of the human Rvb1 hexamer into the three-dimensional reconstruction of the histidine tagged Rvb1/Rvb2 complexes

(a) The panel shows the fitting of the AAA+ core domain and globular part of the insertion domains of the crystal structure of human Rvb1 into the three-dimensional reconstruction of the His-Rvb1/Rvb2 complex. The AAA+ and insertion domains are shown as a ribbon representation color coded as in Fig. 1 and prepared from PDB file 2C9O. The EM density map of the His-Rvb1/Rvb2 complex is shown as a mesh. (b) Layout of the two Rvb hexamers in the histidine tagged Rvb1/Rvb2 complexes. The left column shows the DII-DII, AAA-AAA and AAA-DII generated models for the dodecameric Rvb1/Rvb2 complex indicating three possible configurations of the two hexameric rings. The models were

generated by fitting the crystal structure of human Rvb1 into the three-dimensional reconstruction of the His-Rvb1/Rvb2 complex in different orientations. The AAA+ and insertion domains are shown as a ribbon representation color coded as in Fig. 1. The second column from the left shows the surface rendering representation of the density maps generated from the models fitted into the density map of the three dimensional reconstruction of the His-Rvb1/Rvb2 complex represented as a mesh. The third column from the left shows the calculated side view projections from the models and the right column displays an average of experimental projections from the His-Rvb1/Rvb2 complex obtained from negatively stained electron micrographs.

Fig. 7. Cleavage of histidine tags dissociates the dodecamers formed by the Rvb1/Rvb2 complexes into hexamers

(a) Singly and doubly histidine tagged Rvb1/Rvb2 complexes were purified by size exclusion chromatography. A 50 μ L sample of the fraction containing the complexes was treated with 5 μ L of a 6 mg/mL stock of tobacco etch virus (TEV) protease to remove the histidine tags. A volume 7 μ L of the reaction containing TEV treated and untreated complexes were mixed with equal volume of 2 $\times$ concentrated loading buffer and resolved in a 12% SDS-PAGE and stained by Coomassie brilliant blue. Proteins containing a N-terminal His₆-tag are denoted with "H-" label before the name of the protein. Arrows in the right side of the gel indicate the identity of the protein bands. (b) A volume of 15 μ L of each reaction containing TEV treated or untreated Rvb1/Rvb2 complex was mixed with 5 μ L of loading dye, resolved in a BN PAGE gel and stained with silver staining. The bands corresponding to the dodecamers (12-mer), hexamers (6-mer) and monomers (1-mer) are indicated.

Fig. 8. Untagged *in vivo* assembled Rvb1/Rvb2 complexes form single hexameric rings
 (a) Cartoon representing the R2TP complex purified from *Saccharomyces cerevisiae* containing a chromosomal copy of PHI1-3FLAG replacing the wild type gene. The four proteins components of the complex and the location of the FLAG tag are indicated. The cartoon also represents the dissociation of the Tah1 and Phi1-FLAG proteins from the *in vivo* assembled Rvb1/Rvb2 complex upon incubation with ATP. (b) A volume of 6 μ L of purified R2TP and *in vivo* assembled Rvb1/Rvb2 complexes were loaded in a 12% SDS-PAGE gel and stained by silver staining. The identity of the bands representing the Pih1, Tah1 and the two Rvb proteins is indicated. (c) The two same samples (15 μ L each) were mixed with 5 μ L of loading dye, resolved in a BN PAGE gel and stained by silver staining. The bands corresponding to the hexameric complexes (6-mer) are indicated. (d) Two-dimensional average of endogenous Rvb1/Rvb2 complex obtained from negatively stained particle images. The image on the left represents the two-dimensional average and the one on the right represent its 6-fold symmetrized version.