Structural and biochemical characterization of the yeast exosome component Rrp40

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The exosome is a protein complex that is important in both degradation and 3'-processing of eukaryotic RNAs. We present the crystal structure of the Rrp40 exosome subunit from *Saccharomyces cerevisiae* at a resolution of 2.2 Å. The structure comprises an S1 domain and an unusual KH (K homology) domain. Close packing of the S1 and KH domains is stabilized by a GxNG sequence, which is uniquely conserved in exosome KH domains. Nuclear magnetic resonance data reveal the presence of a manganese-binding site at the interface of the two domains. Isothermal titration calorimetry shows that Rrp40 and archaeal Rrp4 alone have very low intrinsic affinity for RNA. The affinity of an archaeal core exosome for RNA is significantly increased in the presence of the S1-KH subunit Rrp4, indicating that multiple subunits might contribute to cooperative binding of RNA substrates by the exosome.

Keywords: exosome; RNA degradation; exonuclease; S1 domain; KH domain

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INTRODUCTION

The exosome was first identified by Tollervey and co-workers as a protein complex essential for the processing of several RNAs in *Saccharomyces cerevisiae* (Mitchell *et al.*, 1997; Allmang *et al.*, 1999). Since then, the exosome has been found to have a crucial role in many pathways, including messenger RNA turnover, RNA surveillance and, more recently, degradation of fragments generated by RNA interference (reviewed by Butler, 2002; Raijmakers *et al.*, 2004; Houseley *et al.*, 2006). The exosome is conserved from yeast to humans (Mitchell *et al.*, 1997; Chekanova *et al.*, 2000; Estevez *et al.*, 2001; Raijmakers *et al.*, 2002). It is

also present in some archaeal organisms (Koonin *et al.*, 2001; Evguenieva-Hackenberg *et al.*, 2003) and shares homology with bacterial polynucleotide phosphorylase (reviewed by Symmons *et al.*, 2002).

The archaeal exosome is composed of two proteins that are homologous to the bacterial exoribonuclease RNase PH (Rrp41 and Rrp42), and of two proteins containing S1 and/or KH (K homology) domains (Rrp4 and Csl4). As S1 and KH domains are generally nucleic acid-binding folds, Rrp4 and Csl4 are commonly referred to as the exosome RNA-binding proteins. Recent crystal structures of archaeal exosomes from *Sulfolobus solfataricus* and *Archaeoglobus fulgidus* show that the RNase PH-like proteins form a hexameric ring around a central channel (Büttner *et al.*, 2005; Lorentzen *et al.*, 2005). The active sites are sequestered in the channel, towards one side of the ring (Lorentzen & Conti, 2005), and the S1 domain-containing subunits are positioned on the opposite side of the ring, lining the entrance of the central channel (Büttner *et al.*, 2005).

The eukaryotic exosome presents a higher degree of complexity compared with the archaeal exosome. It comprises six different RNase PH proteins (Rrp41, Rrp42, Rrp43, Rrp45, Rrp46 and Mtr3), three different S1/KH domain-containing proteins (Rrp4, Csl4 and Rrp40) and one subunit belonging to the RNase R family (Rrp44; van Hoof & Parker, 1999). Both Rrp40 and Rrp44 are characteristic components of the eukaryotic exosome, as no corresponding subunits have been found in archaea.

Although the architecture of the eukaryotic exosome is predicted to be similar to the archaeal complex, as yet no structure has been reported for any of the ten eukaryotic exosome core proteins. Here, we report the high-resolution crystal structure of the *S. cerevisiae* Rrp40 (ScRrp40) exosome subunit. In addition to the crystallographic analysis, we use nuclear magnetic resonance (NMR) and isothermal titration calorimetry (ITC) to gain insights into the molecular functions of the S1 and KH domain-containing exosome proteins.

RESULTS AND DISCUSSION

Structural determination

The NMR spectra of ¹⁵N-labelled full-length ScRrp40 indicate that about 60 residues are unstructured. This led to the identification of a minimal globular domain comprising residues 58–240 (Fig 1;

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Fig 1 | Structure-based sequence alignment of Rrp40 and Rrp4 exosome subunits. Secondary structure elements in *Saccharomyces cerevisiae* Rrp40 (ScRrp40) and *Archaeoglobus fulgidus* Rrp4 (AfRrp4) are shown above and below the sequence alignment, respectively. The boundaries of Rrp40AN are indicated by blue arrows. The sequence alignment includes the sequences of Rrp40 and Rrp4 from *S. cerevisiae*, *Homo sapiens* (Hs), *Arabidopsis thaliana* (At), *Sulfolobus solfataricus* (Ss) and *A. fulgidus*. The ScRrp40 S1 and KH domain boundaries are indicated by yellow and light blue backgrounds, respectively. The canonical GxxG motif (present only in AfRrp4) and the exosome-specific GxNG sequence are boxed and indicated above the sequence. Stars indicate the residues conserved only in the Rrp40 family (but not in Rrp4). Blue arrowheads indicate residues affected in the Mn^{2+} titration. The alignment was obtained using SSM (<http://www.ebi.ac.uk/msd-srv/ssm>) and ProbCons (<http://probcons.stanford.edu/>).

supplementary Fig 1 online) amenable to structural studies (referred to as Rrp40AN). As a β -type secondary structure is predicted for the amino-terminal region, it is possible that it adopts a tertiary fold on binding to the exosome core and mediates the contacts to the RNase PH proteins, as observed for the archaeal *A. fulgidus* Rrp4 (AfRrp4). Rrp40AN was crystallized and its structure was determined to a resolution of 2.2 Å by using single-wavelength anomalous dispersion (SAD) on a seleno-methionine (SeMet)-substituted protein crystal. The model was refined to R_{work} and R_{free} values of 18.9% and 20.6%, respectively, with 89.4% of the residues in the most favoured regions of the Ramachandran plot and none in disallowed regions (supplementary Table 1 online). The refined structure comprises residues 62–236 of ScRrp40.

Structural overview: the S1 and KH domains

The crystal structure of Rrp40AN presents a double-domain organization consisting of an N-terminal S1 domain and a carboxy-terminal KH domain (Fig 2A). The two domains are packed closely together, burying 900 Å² of solvent-accessible surface area. The domain interface involves the β -sheet regions of both the S1 and KH domains (Fig 2A). The packing of the S1 and KH domains is distinct from the bacterial NusA protein in which the S1–KH domain interface involves loop regions in the S1 domain and the helical face of the KH domain (Gopal *et al*, 2001; Worbs *et al*, 2001).

S1 domains belong to the superfamily of OB folds (oligo-nucleotide–oligosaccharide-binding fold), which are most frequently involved in nucleic acid binding (reviewed by

Fig 2 | Structure of *Saccharomyces cerevisiae* Rrp40 Δ N. (A) Crystal structure of *S. cerevisiae* Rrp40 Δ N (comprising residues 63–236). The S1 domain, the KH domain and the conserved GxNG motif are indicated. (B) Superposition of the structures of Rrp40 Δ N (magenta) and of AfRrp4 (orange; Büttner *et al*, 2005). The two different GxxG sequence motifs present in AfRrp4 are indicated. (C) Detailed view of the GxNG loop connecting β 7 and β 8 in the ScRrp40 KH domain. Hydrogen bonds formed by Asn 191 with residues of the S1 domain are indicated by dotted lines. (D) View of the S1–KH domain interface. Conserved hydrophobic side chains, which stabilize the domain interface, are shown in black. Af, *Archaeoglobus fulgidus*.

Draper & Reynaldo, 1999; Theobald *et al*, 2003). The S1 domain of ScRrp40 shows the characteristic five-stranded β -barrel with a 3_{10} helix turn (or type III β -turn) between β 3 and β 4. The domain presents remarkably long loops, especially between β 3– β 4 and β 4– β 5. Strands β 1 and β 4 of the S1 domain form the interface with the KH domain (Fig 2A).

KH domains (Gibson *et al*, 1993) are abundant structural modules that are typically involved in single-stranded nucleic acid binding (reviewed by Grishin, 2001; Messias & Sattler, 2004). KH domains share a minimal $\beta\alpha\beta$ core, but show two different fold variants. Type I KH domains have a $\beta\alpha\beta\beta\alpha$ topology, whereas type II KH domains have an $\alpha\beta\alpha\beta$ topology. The ScRrp40 KH domain shows type I topology, with the characteristic triple-stranded antiparallel β -sheet packed against three α -helices; the β -sheet is involved in the interaction with the S1 domain. The ScRrp40 KH domain has another C-terminal α -helix (α 4; Fig 2A).

An additional helix has previously been observed in the KH domains of STAR family proteins, such as splicing factor 1 (Liu *et al*, 2001) and Sam68 (Maguire *et al*, 2005), but with a very different orientation compared with Rrp40. Although helix α 4 packs extensively with helices α 2 and α 3, and seems to have a structural role, it might also contribute to macromolecular interactions with other exosome proteins.

As yet, no Rrp40 counterpart has been found in archaea; nevertheless, ScRrp40 shares 20% identity with the archaeal AfRrp4 protein. Comparison of the three-dimensional structures of ScRrp40 and AfRrp4 (Büttner *et al*, 2005) shows that the two proteins present a very similar fold, with a root mean square deviation of 2.1 Å over 158 residues for the C α coordinates (Fig 2B). The largest differences are observed in the β 3– β 4 and β 4– β 5 loops of the S1 domain, reflecting the low conservation between eukaryotic Rrp40 and archaeal Rrp4 sequences in these regions

(Fig 1). Further structural differences involve helix $\alpha 1$ and the $\alpha 1$ – $\alpha 2$ loop of the KH domain.

An unusual GxNG motif in exosome KH domains

A common feature of KH domains is a GxxG sequence motif. This motif is located between the two consecutive α -helices in all KH domains characterized so far and mediates nucleic acid binding (reviewed by Grishin, 2001; Messias & Sattler, 2004). At first glance, a corresponding GxxG sequence motif also seems to exist in the KH domain of Rrp40 (¹⁸⁹GLNG¹⁹²; Fig 1); however, the crystal structure of Rrp40 Δ N shows that the GLNG sequence is located between two consecutive β -strands ($\beta 7$ – $\beta 8$; Fig 2A) rather than between the two consecutive α -helices of the KH domain. The motif is buried at the interface between the S1 and KH domains and seems to have a structural role. In particular, Leu 190 and Asn 191 in the GLNG sequence make several hydrogen bonds with residues of the S1 domain (Phe 71, Ile 73 and Leu 88; Fig 2C). The two glycine residues of the motif are required to accommodate a specific backbone conformation and to allow a close arrangement between the two domains.

Peculiarly, the archaeal homologue AfRrp4 presents two GxxG sequences. A canonical GxxG motif is located between $\alpha 1$ and $\alpha 2$, whereas a second GxNG motif is found between $\beta 13$ and $\beta 14$ of AfRrp4 (Fig 1). The structure-based sequence alignment (Fig 1) indicates that only the second GxNG motif, located between the two consecutive β -strands of the KH domain, is conserved in S1–KH exosome proteins. The conservation of the GxNG motif, and of a cluster of hydrophobic residues (Ile 73, Leu 118, Leu 150, Ile 155 and Trp 195; Figs 1,2D), suggests that the compact structure adopted by the S1–KH module will be conserved in all exosomal Rrp40 and Rrp4 homologues.

Yeast Rrp40 binds to manganese ions

When performing the crystallographic experiments, X-ray fluorescence absorbance spectra indicated the presence of metal ions in the protein. As no electron density corresponding to metal ions was detected in the structure, we investigated whether Rrp40 Δ N could bind to metal ions by using ITC. Titration of Rrp40 Δ N with Mn²⁺ indicates a weak interaction with a dissociation constant of $K_D \approx 1.2$ – 1.5 mM. By contrast, no binding to Mg²⁺ was detected (data not shown).

We then monitored the binding of Rrp40 Δ N to Mn²⁺ by using NMR. The addition of paramagnetic Mn²⁺ to a ¹⁵N-labelled sample of Rrp40 Δ N showed strong line-broadening effects for several amide signals, confirming the interaction of Rrp40 Δ N with Mn²⁺ detected by ITC (supplementary Fig 2A online). As a control, we monitored titrations with Cu²⁺, another paramagnetic ion, by using NMR. The addition of Cu²⁺ yielded only a small, general increase of NMR line widths, indicating that the effect detected for Mn²⁺ is specific (supplementary Fig 2B online). To map the affected residues onto the structure of Rrp40 Δ N, we assigned the backbone chemical shifts using standard methods (Sattler et al, 1999). Residues strongly affected by the addition of Mn²⁺ (Gln 114, Leu 118, Glu 151 and Asp 152) cluster at a single surface patch near the linker connecting the S1 and KH domains (Fig 3C). The electrostatic surface potential of Rrp40 Δ N shows that this region is negatively charged (Fig 3A). Two of the residues located at the Mn-binding site of ScRrp40 (Gln 114 and Asp 152) are conserved in Rrp40 orthologues of higher eukaryotes (Figs 1,3B), suggesting that they might also feature a metal-binding site.

RNA-binding properties of exosome S1 proteins

Given that the S1 and KH domains are known to bind to nucleic acids, the S1 domain-containing subunits of the exosome are predicted to have a role in RNA binding. We first investigated the RNA-binding properties of Rrp40 Δ N by using ITC. Surprisingly, the addition of an A₈ oligoribonucleotide to Rrp40 Δ N resulted in no detectable binding, indicating a $K_D \gg 300$ μ M (Fig 4A). Similar to Rrp40 Δ N, the homologous archaeal *S. solfataricus* Rrp4 (SsRrp4) protein did not show detectable RNA binding upon the addition of an A₇ oligoribonucleotide ($K_D \gg 300$ μ M; Fig 4B).

To investigate the RNA-binding properties of the S1 subunits in the context of the full exosome, we carried out ITC experiments with the archaeal *S. solfataricus* exosome proteins core, the components of which could be expressed and purified. We found that the RNase PH core (formed by SsRrp41 and SsRrp42) bound to an A₇ oligoribonucleotide with $K_D = 39$ μ M and a stoichiometry of three RNA molecules binding to one hexameric ring (Fig 4C). This result is consistent with previous structural studies showing that the RNase PH ring binds to RNA at the active sites of the three Rrp41 subunits (Lorentzen & Conti, 2005). However, when the SsRrp4 subunit was added to the hexameric SsRrp41–SsRrp42 ring, the affinity for an A₇ oligoribonucleotide increased 40-fold ($K_D = 1$ μ M) with a stoichiometry of one molecule of RNA per exosome complex (Fig 4D). This indicates that the S1 proteins are required to increase the binding affinity of the exosome to an RNA substrate. In a manner similar to the archaeal exosome, it is conceivable that the S1-containing proteins of the eukaryotic exosome (Rrp4, Rrp40 and Csl4) contribute to RNA binding in the context of the full complex.

NMR is particularly well suited to studying weak interactions; therefore, we used NMR to investigate whether we could detect weak RNA binding to the isolated ScRrp40 subunit. Titration of an A₈ oligoribonucleotide showed only very small chemical shift perturbations in the NMR spectra, which is consistent with the ITC data (supplementary Fig 3 online). The NMR titrations were repeated in the presence of Mn²⁺, but no difference in the RNA-binding behaviour was observed (data not shown). Residues showing chemical shift perturbations include the $\beta 3$ – $\beta 4$ loop of the S1 domain (Fig 3C; supplementary Fig 3 online). Such small chemical shift changes are in the range of salt concentration effects, and indicate a high sensitivity of the $\beta 3$ – $\beta 4$ loop to variations in the chemical environment. However, two observations suggest that these changes, despite being small, might be relevant. First, the region comprising the $\beta 3$ – $\beta 4$ loop shows a positive electrostatic surface potential (Fig 3A) and the corresponding positively charged residues are conserved in all Rrp40 homologues (Figs 1,3B). Second, when the Rrp40 Δ N structure is superimposed onto the AfRrp4 structure in the AfRrp41–Rrp42–Rrp4 complex (Büttner et al, 2005), the positively charged $\beta 3$ – $\beta 4$ loop of the S1 domain is positioned at the entrance of the central channel (Fig 3D). The increased RNA-binding affinity observed by ITC in the case of the archaeal complex could therefore result from RNA contacts mediated by the S1 domains positioned around the channel.

Concluding remarks

The Rrp40 protein is an essential component of the eukaryotic exosome. The crystal structure of the C-terminal region of ScRrp40 (Rrp40 Δ N) shows a compact fold consisting of an S1 and a KH

Fig 3 | Charge, conservation and interaction surfaces of Rrp40 Δ N. Molecular surface representations of Rrp40: left, the same view as that depicted in Fig 2; right, rotated by 180° along a vertical axis. (A) Molecular surfaces are coloured blue and red according to positive and negative electrostatic potential, respectively. (B) The degree of sequence conservation among Rrp40 orthologues is mapped on the surface representation. Dark or light green indicates residues that are fully or partially conserved in Rrp40 orthologues, respectively (compare with Fig 1). (C) The conserved residues of the β 3- β 4 loop and the residues affected on addition of Mn²⁺ are shown in magenta and cyan, respectively. (D) Model of Rrp40 Δ N in the context of the exosome, obtained by replacing one of the Rrp4 subunits in the structure of the *Archaeoglobus fulgidus* (Af) exosome by Rrp40 Δ N. The conserved residues in the β 3- β 4 loop of the S1 domain and those affected by the addition of Mn²⁺ are shown in magenta and cyan, respectively. The AfRrp41 and AfRrp42 subunits are shown in blue and green, respectively, and the two AfRrp4 subunits are shown in orange. Ribbon and surface representations were generated with PyMOL (<http://pymol.sourceforge.net>).

Fig 4 | RNA binding of exosome subunits studied by isothermal titration calorimetry. (A) Isothermal titration calorimetry (ITC) of yeast Rrp40 Δ N (25 μ M) with a solution of an A₈ RNA oligonucleotide (670 μ M). (B) ITC of SsRrp4 (20 μ M) with a solution of A₇ RNA (210 μ M). (C) ITC of SsRrp41-Rrp42 (43 μ M) with a solution of A₇ RNA (430 μ M). (D) ITC of SsRrp41-Rrp42-Rrp4 (14 μ M) with a solution of A₇ RNA (70 μ M). For all titrations, the raw data are shown in the upper panel, and the integrated heat data, corrected for dilution, are shown in the lower panel. Above the ITC data is a schematic representation of the species in solution: magenta and orange circles represent ScRrp40 and SsRrp4 subunits respectively; blue and green circles represent Ss RNase PH-like subunits; small, black circles represent RNA molecules. Sc, *Sulfolobus cerevisiae*; Ss, *Sulfolobus solfataricus*.

domain. A strictly conserved non-canonical GxNG sequence stabilizes the S1-KH domain interface. The N-terminal region of ScRrp40, predicted to be in contact with the RNase PH subunits as in the archaeal Af exosome, is unstructured in solution in the absence of the other exosome components. The canonical GxxG motif that is normally involved in nucleic acid binding in KH domains is absent in ScRrp40 and in most Rrp40 and Rrp4 homologues. The lack of a canonical GxxG loop, a different arrangement of the helices (α_1 and α_2) normally involved in nucleic acid binding and a negatively charged surface potential around these structural elements suggest that the KH domain in Rrp40 orthologues is unlikely to bind to nucleic acids in a canonical mode. Accordingly, no RNA binding to isolated ScRrp40 or to the archaeal SsRrp4 was observed, with or without Mn^{2+} . However, calorimetric data show that, when bound to the SsRrp41-SsRrp42 archaeal core, SsRrp4 significantly increases the affinity of the complex for RNA. The Mn^{2+} binding by Rrp40 could have a structural role and/or enhance RNA binding in the full exosome. Together, these findings converge on a model in which the S1 subunits, when anchored to the exosome core, assist binding of the RNA substrate by the exosome.

METHODS

Protein expression and purification. ScRrp40 (UniProtKB/TrEMBL entry Q6B204) was expressed in *Escherichia coli*, purified by

affinity chromatography by using a His tag and by size-exclusion chromatography after removal of the tag (supplementary information online). The medium for bacterial growth was Luria broth (for crystallization and ITC), M9 minimal medium supplemented with $^{15}NH_4Cl$ (for NMR titration experiments), M9 prepared with $[U-^{13}C]glucose$, $^{15}NH_4Cl$ in 80% D_2O and supplemented with 10% $^2H, ^{15}N, ^{13}C$ -rich medium (Silantes, Munich, Germany; for triple-resonance NMR experiments) or M9 minimal medium containing L-SeMet (50 mg/l) and using *E. coli* strain B834 for expression (for SeMet derivative).

Crystallization and structure determination. Crystals of Rrp40 Δ N (residues 58–240) were grown by vapour diffusion (sitting drops) at 4 °C by mixing an equal volume of protein and reservoir solution containing 20% (w/v) PEG 3350 and 200 mM sodium sulphate. The crystals were then transferred into the mother liquor, supplemented with 27.5% glycerol and flash-cooled in liquid nitrogen. X-ray diffraction data were collected at the PX beamline X06SA at the Swiss Light Source (Villigen, Switzerland). Redundant SAD data were collected at the K absorption edge at the European Synchrotron Radiation Facility, beamline ID23-1 (Grenoble, France). Diffraction data were processed with the program XDS (Kabsch, 1993). The sites were found and the phases were calculated by using SHELX programs (Schneider & Sheldrick, 2002). The model was completed using iterative cycles of model building in Coot (Emsley & Cowtan, 2004) and refinement in REFMAC (Murshudov et al, 1997).

NMR spectroscopy. The protein sample for triple-resonance NMR experiments was buffer exchanged to a 50 mM phosphate buffer (pH 6.9) containing 100 mM Na₂SO₄ and 1 mM dithiothreitol. NMR spectra were acquired at 22 °C on Bruker DRX600 and DRX900 spectrometers equipped with cryogenic probes. For protein backbone assignment, HNCA constant time, HNCA real time, HNCOCa, HNCACB and HNCOCACB experiments were recorded. Spectra were processed with NMRPipe (Delaglio *et al*, 1995) and analysed using NMRVIEW (Johnson & Blevins, 1994). The ¹H, ¹⁵N and ¹³C chemical shifts were assigned using standard methods (Sattler *et al*, 1999).

Isothermal titration calorimetry. ITC was carried out using a VP-ITC Microcal calorimeter (Microcal, Northhampton, MA, USA) at 20 °C. All proteins were dialysed extensively against 50 mM phosphate buffer (pH 6.9), 100 mM Na₂SO₄ and 1.5 mM β-mercaptoethanol (for Rrp40ΔN-RNA titrations), against 50 mM Tris buffer (pH 7.5), 250 mM NaCl and 1.5 mM β-mercaptoethanol (for Rrp40ΔN-metal titrations) or against 20 mM Hepes (pH 7.5) and 150 mM NaCl (for *S. solfataricus* subunits-RNA titrations). A typical titration consisted of injecting 1–12 μl aliquots of the ligand into the protein sample, at time intervals of 5 min, to ensure that the titration peak returned to the baseline. In each case, a background titration, consisting of the identical titrant solution but with only buffer solution in the sample cell, was subtracted from each experimental titration to account for heat of dilution. The ITC data were analysed using program Origin version 5.0 provided by Microcal.

Coordinates. The atomic coordinates and structure factors have been deposited with the Protein Data Bank (accession code 2JA9).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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