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Strategies for generating RNA exosome complexes from recombinant expression hosts

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Abstract

The eukaryotic RNA exosome is a conserved and ubiquitous multiprotein complex that possesses multiple RNase activities and is involved in a diverse array of RNA degradation and processing events. While much of our current understanding of RNA exosome function has been elucidated using genetics and cell biology based studies of protein functions, in particular in *S. cerevisiae*, many important contributions in the field have been enabled through use of *in vitro* reconstituted complexes. Here, we present an overview of our approach to purify exosome components from recombinant sources and reconstitute them into functional complexes. Three chapters following this overview provide detailed protocols for reconstituting exosome complexes from *S. cerevisiae*, *S. pombe*, and *H. sapiens*. We additionally provide insight on some of the drawbacks of these methods and highlight several important discoveries that have been achieved using reconstituted complexes.

Keywords

RNA exosome; RNA decay; Exoribonuclease

1. Introduction

Through decades of study in diverse model organisms, the RNA exosome has become known as a veritable RNA processing powerhouse, providing 3' to 5' ribonuclease activity for trimming and degradation of all known classes of cellular RNAs (for recent reviews, see [1, 2]).

After the initial observation that depletion of the essential Rps4 protein resulted in aberrant rRNA processing in *S. cerevisiae* [3], isolation of a Protein A tagged Rps4 from cells revealed that it was part of a complex containing at least four other essential subunits [4]. Identification of additional subunits and human homologs soon followed [5, 6] (Fig. 1)

and paved the way for more detailed analyses of the biochemical activities and biological functions of RNA exosomes from genetics and pull-down studies.

Purification of endogenous exosome complexes from cells using affinity tagged components has associated challenges. Many studies have required large quantities of cells (~200 g wet cell weight) to obtain sufficient purified material for structural biology and biochemistry [7–9]. Another difficulty is that enriching specific exosome subpopulations (e.g. nuclear vs. cytoplasmic) may require separate strains, containing different tagged components and/or genetic deletions of certain cofactors. Altogether, endogenous complexes, which must be retrieved from complex cell extracts, are often difficult to purify to homogeneity. *In vivo* analysis of exosome function is hampered by the fact that most of its subunits are essential for cell survival [4, 5], making it difficult or impossible to conduct mutational studies of interactions and activities. Use of proteins recombinantly expressed in *E. coli*, however, allows for structural and biochemical characterization of complexes containing mutations that are deleterious to growth in their native host organism or lethal when used in combinations. For these reasons, our laboratory and others have developed protocols to reconstitute the RNA exosome from *S. cerevisiae*, *S. pombe* and *H. sapiens*. The differing subunit designations for these organisms are summarized in Fig. 1.

2. Subunit arrangement and architecture

The RNA exosome core consists of nine subunits, six of which are homologous to RNase PH and three of which contain S1 and/or KH domains [10] (Fig. 1). Structures of the Exo9 homologs PNPase [11] and the archaeal RNA exosome [12, 13] provided prototypes for Exo9 structure prior to its determination [14]; the overall architecture of the complex proved remarkably conserved [10]. The PH-like proteins form a pseudo-hexameric ring that is capped by a second ring of the three S1/KH proteins to form a donut shaped structure (Exo9 or core) with a prominent central channel that is wide enough to accommodate single stranded RNA (Fig. 1 and Fig. 2). While essential, Exo9 is catalytically inactive in most eukaryotes [14–16]. In the nucleus of yeast, hydrolytic ribonuclease activity is conveyed by two associated enzymes: the endo- and exoribonuclease Dis3/Rrp44 and the exoribonuclease Rrp6 [5, 15, 17–19] (Fig. 1). The subunit composition in different subcellular compartments and in different organisms has been recently reviewed [2].

Unlike some multi-component complexes, such as the proteasome and the eukaryotic ribosome, that require chaperones and assembly factors to achieve their mature state *in vivo* [20, 21], components of the exosome can spontaneously assemble. Because of this, one can reconstitute functional complexes of well-defined stoichiometry from purified proteins expressed in bacterial hosts.

3. General Approach

Our reconstitution strategy relies on two principle methods, the first of which is co-expression of stable sub-complexes. In a multi-protein complex, hydrophobic surfaces of individual components are generally shielded from solvent through extensive interactions with the other components. The exosome is no exception [14], so by co-expressing selected

subunits (e.g. the PH-like components and Rrp6/Rrp47), we are able to increase yields and solubilities of these components compared to expressing the individual subunits alone. The second general strategy we employ is genetically fusing the components to cleavable affinity/solubility tags. Our tag of choice is an N-terminal hexa-histidine linked to *S. cerevisiae* SUMO (His₆Smt3). This tag offers three powerful advantages: 1. it can increase the solubility and expression of difficult proteins [22, 23]; 2. it can be rapidly isolated from soluble extracts using nickel-NTA affinity chromatography; and 3. it can be efficiently removed by incubation with the highly specific *S. cerevisiae* SUMO protease (Ulp) at 4°C [24], leaving behind a minimal 1–3 amino acid scar. Taken together, these strategies have enabled production of sufficient quantities of full-length or minimally truncated proteins for biochemical and structural studies.

4. Overview of the experimental work-flow

To generate the expression strains we insert genes, amplified from genomic DNA or cDNA or commercially synthesized, into vectors under the control of either tac or T7 promoters and transform them into appropriate *E. coli* hosts [25]: For non-synthetic genes, we use host strains that contain a plasmid encoding tRNAs that decode rare codons in bacteria [26] (BL21 (DE3) Codon Plus RIL for T7 promoter constructs and BL21 codon-plus RIL for tac promoter constructs (Agilent)). For synthetic genes, we use codon-optimized sequences for expression in *E. coli*. For certain components, we have also found it advantageous to make use of an *E. coli* strain with a mutation in the RNaseE gene (BL21 (DE3) STAR, Thermo-Fisher) as well as a strain optimized for expression of proteins with poor solubility (SoluBL21 (DE3), from Genlantis). Cells are initially grown at 37°C before being subjected to cold shock and treatment with 2% ethanol to induce the cold-shock protein folding machinery before IPTG induction and overnight incubation of the culture at 18°C. Cells are harvested after induction and lysed by sonication in the presence of DNase I to minimize nucleic acid contamination. The protein(s) of interest is (are) isolated by nickel-NTA or glutathione resin affinity chromatography depending on the tag used and further purified employing a combination of gel filtration, strong-anion exchange, and/or heparin affinity chromatographies. We have found heparin affinity purification particularly useful for removal of trace *E. coli* heat-shock proteins and contaminating nucleic acids. Purified Exo9 components are mixed, dialyzed to low salt, and complexes are isolated by gel filtration. After that, catalytic subunits (Rrp6/Rrp47 and Dis3) and/or cofactors Mtr4 and Mpp6 can be added and the resulting complexes can be purified by again dialyzing to low salt followed by gel filtration.

5. Experimental limitations

Recombinant expression in *E. coli* and reconstitution can generate large quantities of highly purified complexes in a reasonably cost-effective manner, in particular for *S. cerevisiae* proteins where we have routinely achieved >50 mg of Exo9 core from single reconstitutions. For the human system, protein yields are generally much lower (Exo9 core yield is typically ~5 mg) and some of the individual components have proved challenging to generate. Moreover, expression of the C-terminus of Rrp6 is notoriously difficult in yeast as well as human as it is prone to degradation during purification and use of truncated constructs

can increase yields. Yields for full-length Mpp6 are also low and future improvements may need to be implemented. Another downside of recombinant protein expression in *E. coli* is that these preparations lack any post-translational modifications (PTMs) that may be present in the endogenous complexes. PTMs of exosome subunits have been observed in proteome wide analyses in yeast [27] and human [28–30], and studied in more detail in the cases of SUMO conjugation to hRrp6/EXOSC10 during cold-shock [31] and phosphorylation of Mpp6 during mitosis [28, 32]. These modifications may have important consequences for the activities and structure of the exosome, and alternative strategies such as reconstituting post-translational modification *in vitro*, site-specific incorporation of non-native amino acids [33] and/or expressed protein ligation [34] may need to be employed to further investigate their function.

6. Research advances made using reconstituted exosome complexes

6.1 Summary of biochemical achievements

Recombinant expression and reconstitution of complexes imparts exquisite control of the system to the investigator, and for the exosome this has been instrumental in sorting out the contributions of the various subunits to its activities and regulation [14, 35–40]. Early work showed that for the eukaryotic Exo9, unlike in archaea [12], the PH-like ring cannot self-assemble without inclusion of the S1/KH cap [14]. A comparison of decay activities and RNA binding between free Rrp6 or Dis3 enzymes and their complexes with Exo9 showed that the core altered or attenuated their activities [14, 36, 38]. Reconstitutions containing mutations in Exo9-components designed to occlude the central channel or reduce its positive charge showed that endonucleolytic and exoribonucleolytic activities of Dis3 as well as activity of Rrp6 are compromised in these complexes [36–38], supporting a model in which RNA is threaded through the central channel to engage the active subunits. Channeling by the exosome was further supported by the observation that a catalytically inert *S. cerevisiae* Exo10^{Dis3} could protect approximately 30 nucleotides (nt) of RNA from exogenous RNases, compared to a 10 nt fragment that was protected by Dis3 alone [35, 41]. This length of protected RNA can be extended to ca. 45 nt if catalytically inert Rrp6, Rrp47, Mpp6, and Mtr4 are included in the complex and ADP is added to the reaction [40]. Other analyses of cofactor-containing complexes showed that the N-termini of Rrp6 and Rrp47 proteins bind the N-terminus of Mtr4 [42], that the N-terminus of Mpp6 recruits Mtr4 to the exosome through a separate binding site [39, 43, 44], and that Rrp6/Rrp47 and Mpp6 cooperate to enable ATP-dependent degradation of a structured substrate by the *S. cerevisiae* Mtr4-exosome [39].

6.2 Summary of structural achievements

While EM structures of tagged exosome complexes from *S. cerevisiae* and human cells provided initial insights into the position of Dis3 on Exo9 and its conformations [7, 8, 45], further insights on details of the Dis3 association as well as on the interaction with Rrp6 were obtained from X-ray structures using *in vitro* reconstituted complexes [14, 37, 39, 41, 46, 47] (Fig. 2). It is notable that crystal structures of complexes containing one or both of the catalytic subunits have so far been determined only in the presence of specific RNAs,

suggesting that the catalytic subunits are flexible in the absence of bound substrates and not amenable to crystallization.

6.3 Emerging: Understanding exosome activities in the context of cofactors

The RNA exosome is a crucial player in a large number of diverse degradation and processing pathways. These include such distinct reactions as the maturation of ribosomal RNAs as well as the decay of unstable non-coding RNAs such as cryptic unstable transcripts in yeast and promoter-upstream transcripts (PROMPTs) in humans [1]. Substrate targeting to the RNA exosome is tightly governed by a range of cofactors and recent efforts have focused on understanding the mechanisms behind these interactions [1, 2]. Here, two ATP-dependent RNA helicases of the Ski2-like family of RNA helicases which are thought to unwind double-stranded RNA for presentation to the RNA exosome are of particular interest: In the cytoplasm, the Ski2 helicase is part of the Superkiller complex which is involved in 3' to 5' mRNA degradation by the exosome. A recent study has explored how the yeast SKI complex is connected to the exosome via binding of the Ski7 adaptor protein with the Exo9 core [47] (Fig. 2). In the mammalian nucleus, the helicase MTR4/SKIV2L2/MTREX interacts with the exosome either in isolation or as part of NEXT (Nuclear Exosome Targeting complex) or PAXT (Poly-A tail exosome targeting) complexes [48, 49]. In the yeast nucleolus, Mtr4 is part of a polyadenylation complex (composed of combinations of proteins Trf4/5, Air1/2, and Mtr4, known as TRAMP) which is important for rRNA and snoRNA precursor degradation [50]. Recent inroads have been made determining how Mtr4 is recruited to the yeast exosome complex via N-terminal interactions with Rrp6 and Rrp47 and via the co-factor Mpp6 [39, 42]. Moreover, advances in cryo-electron microscopy have allowed for structural studies of larger co-complexes (Fig. 2): An atomic resolution structure of a human MPP6 and MTR4-containing exosome revealed detailed structural information on helicase-dependent RNA decay with the helicase captured in the process of substrate unwinding [44] and a recombinant yeast Mtr4-containing exosome was reconstituted with an endogenous pre-60S ribosome to illuminate the architecture of the complex during rRNA precursor processing [43]. Taken together, these exciting new findings were achieved using reconstituted complexes in work that paves the way for future studies of the exosome, its helicase cofactors, and larger co-complexes.

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Fig. 1: Comparison of yeast and human exosome nomenclature
 (A) Schematic representation of the side view of a 14-component human nuclear exosome complex. The central channel is indicated with dashed lines. (B) Side-by-side comparison of human and yeast exosome core subunits. View from above looking at the S1/KH ring. (C) Table of subunit designations for human and yeast exosome core and associated cofactors.

	Exo9 (human)	Exo11 ^{Dis3/Cterm}	Exo10 ^{ΔNΔC}	Exo12 ^{Dis3/RAC47}	Exo11 ^{Dis3/Ski7Nterm}	Exo11 ^{Dis3/RΔNΔC}	Exo12 ^{Dis3/RΔNΔC/M6min}	Exo14 ^{Dis3/R6M6/47M4}	Exo14 ^{Dis3/R6M6/47M4} (human)
Molecular model									
Substrate	None	Stemloop-poly(U) ₁₀	Poly(A) ₂₄	2x AU-rich 18 nt	Stemloop-poly(U) ₂₀ (A) ₁	3'-3' RNA	3'-3' RNA	Pre-60S ribosome	dsDNA:RNA with 3' ssRNA overhang
Reference	Liu et al., 2006	Makino et al., 2013	Wasmuth et al., 2014	Makino et al., 2015	Kowalinski et al., 2016	Zinder et al., 2016	Wasmuth et al., 2017	Schuller et al., 2018	Weick et al., 2018
Resolution	3.35	2.81	3.30	4.60	2.65	3.10	3.30	4.64 (cryo-EM)	3.45 (cryo-EM)
PDB	2NN6	4IFD	4OO1	5C0W	5JEA	5K36	5VZJ	6FSZ	6D6R
Cartoon									

Fig. 2: Select molecular models achieved using reconstituted exosome complexes
Structures of *S. cerevisiae* complexes were determined by X-ray crystallography except where indicated otherwise. Side views are shown and the approximate path of the central channel is indicated on the cartoons with dashed lines. RNA is represented as a red line with an arrowhead pointing towards the 3' end. Dotted red lines indicate the presumed route for regions of the RNA that are disordered in the structures. For the molecular models, a T4 lysozyme crystallization tag was omitted from PDB 5JEA and the pre-60S ribosome was omitted from PDB 6FSZ for clarity. For cryo-EM structures, resolution was calculated by the gold-standard FSC = 0.143. “3'-3' RNA” refers to a chemically synthesized RNA containing two 3' ends. “ds DNA:RNA” refers to a chimera construct where a 5' DNA strand is linked to a 3' RNA portion via phosphodiester bond. The DNA portion is hybridized to a separate RNA oligonucleotide.