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Analysis of the *Escherichia coli* RNA degradosome composition by a proteomic approach

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Abstract

The RNA degradosome is a bacterial protein machine devoted to RNA degradation and processing. In *Escherichia coli* it is typically composed of the endoribonuclease RNase E, which also serves as a scaffold for the other components, the exoribonuclease PNPase, the RNA helicase RhlB, and enolase. Several other proteins have been found associated to the core complex. However, it remains unclear in most cases whether such proteins are occasional contaminants or specific components, and which is their function. To facilitate the analysis of the RNA degradosome composition under different physiological and genetic conditions we set up a simplified preparation procedure based on the affinity purification of FLAG epitope-tagged RNase E coupled to Multidimensional Protein Identification Technology (MudPIT) for the rapid and quantitative identification of the different components. By this proteomic approach, we show that the chaperone protein DnaK, previously identified as a "minor component" of the degradosome, associates with abnormal complexes under stressful conditions such as overexpression of RNase E, low temperature, and in the absence of PNPase; however, DnaK does not seem to be essential for RNA degradosome structure nor for its assembly. In addition, we show that normalized score values obtain by MudPIT analysis may be taken as quantitative estimates of the relative protein abundance in different degradosome preparations.

Keywords: RNA degradation; Ribonuclease E; Polynucleotide phosphorylase; DnaK; Mass spectrometry

1. Introduction

Many proteins are organized into heteromultimeric functional complexes (protein machines). Because of the dynamic nature and potential lability of the interactions between different components, identifying and defining the assembly of a protein machine is highly dependent on the procedures developed for its purification as well as on practicable biochemical assays and genetic analysis. The availability of a simple and straightforward analytical procedure may be of fundamental importance both to explore different experimental conditions that may minimize disassembly of the elements from the complex, thus allowing isolation of the entire machine, and to test the structural and functional consequences of mutations in different domains of the various components.

The RNA degradosome is a bacterial protein machine devoted to RNA turnover discovered during the purification of *Escherichia coli* RNase E [1–5]. Since then, related complexes have been described in other prokaryotes as well as in eukaryotes [6,7]. The integral components of the RNA degradosome include the endoribonuclease RNase E, the phosphorolytic exoribonuclease polynucleotide phosphorylase (PN-

Abbreviations: 2DC, two dimensional micro chromatography; CV, coefficient of variation; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EDTA, ethylenediamine tetraacetic acid; EICs, extracted ion chromatograms; IPTG, isopropyl β-D-thiogalactopyranoside; MS, mass spectrometry; MudPIT, Multidimensional Protein Identification Technology; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; PMSF, *o*-phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; TP, tryptic peptides; Tris, trishydroxymethyl aminomethane.

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Pase), the DEAD-box RNA helicase RhlB, and enolase, a glycolytic enzyme commonly implicated in an apparently unrelated process. In this complex, the C-terminal part of RNase E serves as a scaffold for the assembly of the other proteins [8,9]. A "minimal" functional degradosome containing RNase E, PNPase and RhlB has been reconstituted in vitro from the purified components [10,11]. It is believed that the degradosome coordinates the endo- and exonucleolytic activities of RNase E and PNPase, respectively, whereas the ATP-consuming RhlB helicase would promote the unwinding of double stranded RNA, thus facilitating progression of PNPase through RNA secondary structures. Recently, the degradosome-bound enolase has been implicated in controlling the stability of *ptsG* mRNA, which codes for the main glucose transporter $IICB^{Glc}$, in response to metabolic stress [12].

Additional proteins, such as polyphosphate kinase (PPK), DnaK, and GroEL, have been found associated to the core degradosome [4,13,14]. DeaD (alias CsdA), a putative DEAD-box RNA helicase and a cold-induced protein, was found in degradosomes from *E. coli* grown at low (15 °C) temperature, whereas DeaD and RhlE (another DEAD-box helicases) have been shown to assemble into the RNA degradosome and to functionally replace RhlB in vitro [15,16]. Functional or physical interactions between degradosome and other proteins, such as poly(A) polymerase, CspE (a member of the RNA-binding Csp proteins), and the ribosomal protein S1, have also been described [17,18].

Many questions are still open on the composition, molecular interactions, assembly pathway, mechanisms of action, physiological significance of this molecular machine. The complex, labor-intensive purification procedure has hampered systematic approaches aimed at improving recovery of less strongly bound factors or testing the effects of different purification procedures and/or large numbers of mutations in the different components.

Miczak et al. [14] developed a simplified degradosome purification procedure based on the affinity co-purification with FLAG-tagged RNase E (FLAG-Rne). This approach could be useful to study degradosome composition in different genetic and physiological conditions. However, before undertaking such tasks, it is advisable to test possible differences between wild type and FLAG-Rne degradosome and to setup robust procedures to reveal unambiguously the composition of the purified complexes.

The recent developments of new analytical approaches for studying complex protein mixtures [19] allow the identification of proteins in biological samples such as multiprotein functional complexes or even the entire proteome or subproteomes of cells or tissues. These methodologies are based on protein separation using two dimensional gel electrophoresis and their identification by means of mass spectrometry after in-gel digestion [20]. An alternative approach, named Multidimensional Protein Identification Technology (MudPIT), makes use of two dimensional (cation exchange followed by reverse phase) micro chromatography (2DC) coupled to tandem mass spectrometry (MS/MS) for separating the peptides obtained from tryptic digestion of the entire protein mixture. Using the SEQUEST algorithm, based on sequence database searching, the MS/MS spectra are then correlated to specific peptide sequences and the corresponding protein are identified (Link et al., 1999; Ashburn et al., 2001). The main advantages of MudPIT approach are due to the possibility to characterize proteins with extreme isoelectric point (p*I* < 4 or > 9.5), molecular weight (MW < 10 and > 150 kDa) or hydrophobicity (such as membrane proteins), and to perform quantitative analyses [21]. Moreover, MudPIT approach permits full automation and reduction of analysis time [22].

In this work, we explored different conditions for RNA degradosome production and isolation, and assessed the composition of the purified complexes by means of a proteomic approach. In particular, MudPIT analysis allowed the rapid and unequivocal identification of the proteins present in the purified degradosome obtained under different physiological and genetic conditions. Quantitative data obtained by this methodology were in good agreement with those obtained by traditional enzymatic and immunological methods.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli strains BW25113 [23], BB1553 (MC4100 *dnaK52::cm*^{R}, *sidB1*) [24], C-1a (prototrophic, *pnp*$^+$) [25], and C-5602 (C-1a derivative; carries a Tn*5* marker transductionally linked to *pnp*$^+$) [26] were described previously. C-5691 (C-1a Δ*pnp-751*), which carries an in frame deletion encompassing about 95% of *pnp* coding sequence (coordinates 7740-5709, GenBank Accession Number AE000397), was obtained by allele replacement as described by Datsenko and Wanner [23]. In brief, an FRT-flanked kanamycin-resistance cassette was amplified by PCR from plasmid pKD13 [23] with primers FG738 (*ATCGTTCGTAAATTCCAGTACGGCCAACACACCGTGACT*ATTCCGGGGATCCGTCGACC; AE000397:7779-7741 followed by pKD13 priming site 4) and FG739 (*AGCAGCCGGAGCTTCCGGTGCTGCAGCAGGTTGAGACT* GTGTAGGCTGGAGCTGCTTC; AE000397:5671-5708 followed by pKD13 priming site 1). BW25113 competent cells, carrying pKD46 helper plasmid, which expresses λ Red (*gam*, *bet*, *exo*) function, were transformed by electroporation with 200 ng of gel-purified PCR fragment and plated at 37 °C. Colonies were tested for both the presence of the Δ*pnp*::*kan* allele at the *pnp* locus in the chromosome and the loss of the helper plasmid. The Δ*pnp-751*::*kan* mutation was then transferred in *E. coli* C-1a by P1 transduction [27], obtaining strain C-5690. To excise the Kan^R cassette from the *pnp* mutant, C-5690 was transformed with the temperature-sensitive plasmid pCP20, which encodes the FLP recombinase, thus promoting recombination between the FRT sites [23]. A few transformants were purified at 42 °C and tested for the loss of both the Kan^R cassette and plasmid