Identification of the Rps28 binding motif from yeast Edc3 involved in the autoregulatory feedback loop controlling RPS28B mRNA decay

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ABSTRACT

In the yeast Saccharomyces cerevisiae, the Edc3 protein was previously reported to participate in the auto-regulatory feedback loop controlling the level of the RPS28B messenger RNA (mRNA). We show here that Edc3 binds directly and tightly to the globular core of Rps28 ribosomal protein. This binding occurs through a motif that is present exclusively in Edc3 proteins from yeast belonging to the Saccharomycetaceae phylum. Functional analyses indicate that the ability of Edc3 to interact with Rps28 is not required for its general function and for its role in the regulation of the YRA1 pre-mRNA decay. In contrast, this interaction appears to be exclusively required for the auto-regulatory mechanism controlling the RPS28B mRNA decay. These observations suggest a plausible model for the evolutionary appearance of a Rps28 binding motif in Edc3.

INTRODUCTION

Regulation of messenger RNA (mRNA) decay rates is an important mechanism to determine the abundance of cellular transcripts. Generally, eukaryotic mRNA degradation starts with the shortening of their poly(A)-tail. This is subsequently followed by decapping and 5′–3′ exonucleolytic degradation. Alternatively, 3′–5′ degradation by the exosome may take place after deadenylation (1–3). mRNAs as well as proteins involved in translation repression and mRNA decay assemble in cytoplasmic granules, often referred to as processing bodies (P-bodies), in which some transcript degradation was shown to occur (4–7). However, formation of these assemblies is not a prerequisite for initiation of mRNA decay, and some mRNA decay was shown to occur cotranslationally (8). In yeast, decapping is mediated by Dcp2, but the latter needs to be stimulated by additional factors such as Lsm1-7 complex, Dhh1, Edc1-3, Scd6 and Pat1 (9–15). Dhh1 and Pat1 were reported to promote decapping mainly through translational repression (16), whereas Edc1-3 proteins were shown to activate Dcp1/Dcp2 enzyme directly (15,17,18). It has become evident that the control of mRNA stability and the control of mRNA translation are interconnected, although little is known about the physical and functional relationships between factors of mRNA degradation and the translation machinery. Interestingly, Edc3 protein was shown to interact with ribosomal protein S28 (Rps28) in yeast. This association is involved in an auto-regulatory mechanism controlling the production of Rps28 (19) but could be involved in other processes, in particular if Edc3 was able to interact with ribosome bound Rps28. Furthermore, molecular details of the mode of interaction of these two factors remain unclear.

Edc3 protein is a conserved eukaryotic factor that interacts genetically and/or physically with many proteins involved in mRNA decay (14,17,20,21). Edc3 is composed of three conserved domains, each being responsible for interaction with factors involved in mRNA decay: an N-terminal Sm-like domain was reported to bind Dcp1 and/or Dcp2 (17,21,22), an internal FDF motif was shown to bind Dhh1 (20), whereas its C-terminal YjeF-N domain was shown to promote homodimerization (23,24). The multi-domain organization of Edc3 was shown to contribute to its function as scaffold for decapping proteins during P-body assembly (23). In the yeast Saccharomyces cerevisiae, the EDC3 gene is not essential, and its inactivation does not result in detectable growth defect. Its deletion, however, enhances the growth phenotypes, and mRNA decay defects resulting from point mutation in the decapping enzyme Dcp2 or

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its close co-factor Dcp1 (25). Additional genetic screens also demonstrated that Edc3 acts synergistically with Sdc6 and Pbp1 to control mRNA decapping (14). Transcriptome analyses revealed that EDC3 deletion causes accumulation of two specific RNAs: the RPS28B mRNA and the YRA1 pre-mRNA (19,26). Yra1 is an mRNA export factor encoded by a split gene. Excess Yra1 promotes cytoplasmic export of the Yra1 pre-mRNA, which, once in cytoplasm, is degraded by 5'–3' decay mechanism dependent on Edc3 and on specific sequences in the YRA1 intron (19,26,27). The Rps28 protein is a highly conserved archaea- and eukaryote-specific component of the small ribosomal subunit (28–31). Nuclear magnetic resonance studies of archaeal Rps28 proteins revealed that the protein contains four Β-strands, forming compact globular oligonucleotide/oligosaccharide binding (OB) fold followed by a non-structured C-terminal tail (28,31). X-ray structure of the yeast ribosome indicates that eukaryotic Rps28 proteins adopt the same overall fold, even though the region equivalent to the unstructured archaeal C-terminal tail is stabilized through interaction with other components of the small ribosomal subunit (29). Rps28 binds near the exit site of the mRNA and has been shown to crosslink with the mRNA (32). In yeast, Rps28 protein is encoded by two genes: RPS28A and RPS28B, which produce polypeptides differing by a single residue that can substitute for another in the ribosome. When present in excess, the Rps28 protein (the pool of Rps28a and Rps28b) binds to a conserved hairpin structure present in the 3' untranslated region (UTR) of the RPS28B mRNA and, in a manner dependent on Edc3, recruits the decapping machinery (19). The molecular details of this mechanism remain however unclear, as the mode of interaction of Rps28 with the RPS28B mRNA and Edc3 are currently unknown. It is also unclear whether this regulation is widely conserved in eukaryotes.

Here, we identify a region in yeast Edc3 that is necessary and sufficient for binding Rps28. Deletion of this region has no effect on yeast growth rate or accumulation of Edc3 in P-bodies, indicating that it is dispensable for the general RNA decay function of this factor. In contrast, this sequence is required for efficient degradation of the RPS28B mRNA. Degradation of YRA1 pre-mRNA, the second Edc3-regulated transcript, is not affected by deletion of this region, indicating that the mechanism of Edc3 action on YRA1 pre-mRNA is independent of its interaction with Rps28.

MATERIALS AND METHODS

Strains and plasmids

Yeast strains used in this study are listed in Table 1. All strains except BSY2475 are in the BMA64 background (33). The BSY2475 strain was derived from the MAV203 strain (Invitrogen) by PCR-based disruption of EDC3. Oligonucleotides OBS4144 and OBS4149 were used to amplify nourseothricine (NAT) resistance gene with flanking regions of yeast EDC3 gene. BSY2474 was constructed from BSY1664 using the same PCR product. BSY2831 strain was obtained by crossing strains BSY2474 and BSY2067. The construction of plasmids used in this study is described in the Supplementary Materials and Methods. These plasmids are listed in Supplementary Table S1. Oligonucleotides used for these constructions or for probing northerns are listed in Supplementary Table S2.

Cell growth conditions

For growth analysis, cells were grown in exponential phase in synthetic complete (SC) medium lacking tryptophan complemented with 2% glucose. Cultures were diluted to the optical density at 600 nm (OD600) 0.1 with sterile water. Five microlitres of each culture as well as 5 μl of two 10-fold serial dilutions were plated on SC medium lacking tryptophan. Cell growth was monitored after 48 h at 25, 30 or 37°C. For microscopy, cells were grown at 30°C in SC medium lacking tryptophan and uracil complemented with 2% glucose, until an OD600 0.3 was reached. Cells were subsequently harvested by centrifugation, washed, resuspended in SC medium lacking tryptophan and uracil, supplemented, or not, with a carbon source. Cells were incubated in a shaker at 30°C for 10 min before analysis. For total RNA isolation, yeasts were grown at 30°C in SC medium lacking tryptophan complemented with 2% galactose until an OD600 of 0.8–1 was reached. For western blot analysis, 1 ml of corresponding cell cultures was further grown till OD600 ≥ 2. For RPS28B mRNA half-life analysis, cells were grown in SC medium lacking tryptophan and uracil, supplemented with glucose. Cells were grown at 30°C until OD600 reached 0.8–1. Cultures were concentrated 10 times, doxycycline was added to the growth media to give a 40 μg/ml final concentration and 1 ml aliquots of cells were taken at the time points indicated for total RNA isolation.

Protein purification, protein–protein interaction assay and protein detection

Recombinant proteins were (co-)expressed in BL21 Codon+ cells grown overnight in 100 ml of auto-induction media at 37°C. Cells were harvested by centrifugation, resuspended in 5 ml of lysis buffer containing 50 mM Tris–HCl, 300 mM NaCl and 10 mM imidazole (pH 8.0) and sonicated. Two millilitres of cleared cell lysate was incubated with 100 μl of Ni-NTA agarose beads at 4°C with rotation for ~1 h. Beads were washed with 300 μl of 50 mM Tris–HCl, 300 mM NaCl and 20 mM imidazole (pH 8.0) three times. Specifically bound proteins were eluted with 250 μl of 50 mM Tris–HCl, 300 mM NaCl and 200 mM imidazole (pH 8.0). Eluted proteins were separated in 12% Tris-Tricine gel and visualized with Coomassie-blue staining. Details of the analyses of protein interaction by size-exclusion chromatography with multi-angle laser light scattering measurements are described in the Supplementary Materials and Methods. For western blot analysis of proteinA-tagged Edc3 proteins, total yeast extract was prepared as described previously (34). For detection, peroxidase-anti-peroxidase (3:10 000, Sigma) was used. For a loading control, the
endogenous Stm1 proteins were detected using polyclonal anti-Stm1 antibody (1:1000, kind gift of Francoise Wyers).

Two-hybrid analysis
To avoid interference from the endogenous copy of Edc3, the BSY2475 strain was used. Beta-galactosidase activity was measured using Beta-Glo Assay system (Promega).

Microscopy
Microscopy analyses were performed as described previously (35). All images were acquired using Leica Microsystems Heidelberg GmbH microscope, using an objective HCX PL APO CS 63.0 × 1.40 OIL UV. ImageJ software was used to adjust all images to equal contrast ranges.

Northern blot analysis
Total RNA was extracted by a hot phenol method (36). Usually, 15 μg of total RNA were loaded per lane on a 3% formaldehyde agarose gel. Nucleic acids were transferred to the Hybond N membrane by passive transfer overnight. The quantification of the ratios of these species and standard deviation were calculated from two independent experiments. For RPS28B mRNA half-life analysis, 6 μg of total RNA were loaded on gel and detected as indicated earlier in the text. The quantity of RPS28B mRNA normalized for the loading control was plotted as a function of time and fitted for exponential decay. Mean values of half-lives and standard deviations were calculated from two independent experiments.

RESULTS
A new conserved motif in yeast Edc3 proteins
Amino acid sequence of Edc3 from different species (S.cerevisiae, Human, Mouse and Drosophila) were reported to share significant conservation (25). This early analysis was improved with the identification of three conserved motifs and domains: Lsm, FDF and YjeF-N. The availability of additional putative Edc3 sequences deduced from genome-sequencing projects lead us to perform new Edc3 sequence alignments. Although confirming the conservation of the Lsm domain, FDF motif and YjeF-N domain, those revealed the presence of a conserved region located between the FDF motif and the YjeF-N domain in Edc3 proteins from various Saccharomycetales species, but not from the other sub-divisions of the Saccharomycetales phylum or more divergent species (Figure 1). This correlated with the previous report of the presence of a conserved hairpin in the 3' UTR of the RPS28B mRNA in the former group of organisms (19). This additional conserved region of Edc3, which corresponds to amino acids 209–222 of the S.cerevisiae protein, is hereafter referred to as the RB motif (for Rps28 binding motif). The presence of this motif in a specific subset of Edc3 proteins suggested that it could be involved in the auto-regulatory feedback loop that controls the stability of the RPS28B mRNA, for example by mediating interaction with Rps28 or facilitating interaction of the latter with the hairpin structure present in the RPS28B mRNA 3' UTR.

Recombinant Edc3 protein binds directly Rps28, and this interaction requires region 201–231 of Edc3
Experimental evidences supporting an interaction between Edc3 and Rps28 were derived from two-hyrid and co-immunoprecipitation analyses (19). These data did not indicate whether Rps28 and Edc3 interact directly, possibly in a manner stabilized by protein and/or RNA partners, or whether their interaction was exclusively bridged by other factor(s). To study the role of the newly defined Edc3 motif, we, thus, first sought to develop a more direct protein interaction assays. For this purpose, we constructed plasmids to overexpress both proteins in a soluble form (Figure 2B). A control construct lacking the 6His-Rps28
demonstrated that Edc3(1–277) did not bind non-specifically to Ni-NTA beads. These results indicate that Rps28 and Edc3 interact directly forming a stable complex without requirement for additional RNA or protein partners, and further that the YjeF-N domain of Edc3 is not required for this interaction.

Using this assay, we tested deletion derivatives of Rps28 and/or Edc3 to define sequences required or dispensable for heterodimer formation. Nuclear magnetic resonance and X-ray crystallography studies have shown that archaeal and eukaryotic Rps28 proteins contain four β-strands, forming compact globular part, followed by a less structured C-terminal tail (28,29,31). Deletion of the eight residues forming this tail in yeast Rps28 did not affect its association with Edc3 (Figure 2B). This indicates that Edc3 interacts with the globular OB fold of Rps28.

An Edc3 construct covering residues 1–231 interacted well with Rps28, indicating that none of the residues located between the RB motif and the YjeF-N domain are required for this interaction. Similarly, deletion of the Lsm domain of Edc3 (construct 89-231) did not affect the formation of the Edc3-Rps28 heterodimer. In contrast, only background level of an Edc3(1–277) variant lacking residues 201–231 [Edc3(1–277-ΔRB)] were recovered with Rps28 (Figure 2B), indicating that this region, encompassing the RB motif, is required for heterodimer formation.

We used two-hybrid assay as a second strategy to validate these results in vivo. As Edc3 is known to multimerize (23,24), we preventedly deleted the edc3 gene in the host strain MAV203 to avoid any interference from wild-type Edc3 protein encoded by the endogenous chromosomal gene. We analysed the interaction of full-length wild-type Edc3, or derivative Edc3 lacking residues 201–231, fused to the GAL4 activation domain with either Dcp2, Dhh1 and Rps28 proteins fused to GAL4 DNA-binding domain. Interactions were monitored by β-galactosidase production. Wild-type Edc3 interacted with Dcp2, Rps28 and Dhh1 as expected (23,37) (Figure 2C). Deletion of residues 201–231 of Edc3 truncated Rps28 behaved like the full-length protein (23,37) (Figure 2B). We conclude that residues 201–231 of Edc3

![Figure 1. Localization of a new conserved motif in yeast Edc3 protein. Upper part: Domain architecture of Saccharomyces cerevisiae Edc3 protein composed of an Lsm domain, a FDF motif and an YjeF-N domain. Numbers above the schematic representation of the protein indicate the amino acid positions of domain boundaries for the S. cerevisiae protein. Lower part: Amino acid alignment of Edc3 proteins from several Saccharomyces species encompassing the RB motif. The RB motif is often preceded by an acidic region that is not absolutely conserved.](https://academic.oup.com/nar/article-abstract/41/20/9514/2414585)
are necessary and sufficient to interact with the core of Rps28 protein.

We next checked that the observed complex formation was not due to formation of soluble aggregates between both partners. For this purpose, we purified the untagged Rps28a protein as well as the Rps28a-Edc3(201–231) complex lacking the His-GST tag to analyse their quaternary structure in solution using size exclusion chromatography coupled online to light scattering (Figure 3C). This yielded calculated molecular weights of 7142 Da for free Rps28 (theoretical molecular weight of 7592 Da) and of 10836 Da for the Rps28a-Edc3(201–231) complex (theoretical molecular weight of 11267 Da). Interestingly, the molecular weight difference (3694 Da) between the Rps28a-Edc3(201–231) complex and free Rps28 corresponds to the molecular weight of the isolated Edc3(201–231) peptide (3693 Da theoretical), confirming complex formation. This further indicates that Rps28a and the Edc3(201–231) region interact with a 1:1 stoichiometry to form a heterodimeric complex in solution, and that this complex is stable as it resists to a 3-steps purification protocol.
The Rps28 binding motif of Edc3 is not required for the general activity of Edc3 nor for its targeting to P-bodies

To analyse the functional role of interaction between Edc3 and Rps28, we constructed a centromeric plasmid carrying the EDC3 gene in which residues 201–231 were deleted. To facilitate protein-level monitoring, a Protein A tag (protA) was fused to the C-terminus of the protein (EDC3RB-ProtA construct). A plasmid encoding protA tagged wild-type Edc3 was built to serve as a positive control, whereas the vector without insert served as a negative control. We tested the ability of Edc3/C1RB-protA protein to complement the slow growth phenotype resulting from the poor RNA decay in a Δedc3Δscd6 strain (14). Indeed, deletion of edc3 by itself has no impact on cell division or RNA decay (25). In the BMA yeast strain background, the reduced growth rate of the Δedc3Δscd6 strain is especially apparent at 37°C (Figure 4A). Introduction of the wild-type EDC3 gene in this strain restored a growth comparable with that of Δscd6 strain, which was itself equivalent to a wild-type strain (Figure 4A, expression of untagged Edc3 protein had the same effect, data not shown). Expression of Edc3ΔRB-protA protein restored the growth rate of the Δedc3Δscd6 strain almost to the wild-type level, indicating that the mutant gene is functional and that interaction between Edc3 and Rps28 is not prerequisite for normal Edc3 function. Consistent with this efficient complementation, western blot analysis confirmed that Edc3/C1RB-protA and Edc3-protA accumulate to the same level in cells (data not shown).

To test another potential general role of the Edc3-Rps28 interaction, we analysed the effect of deleting Edc3 residues 201–231 upon P-bodies assembly. Indeed, a strong reduction of microscopically visible P-bodies was observed in a Δedc3 strain (23). We analysed the co-localization of Dcp2-GFP with
Edc3-mCherry fusions, using either wild-type Edc3 or Edc3ΔRB. No difference in P-bodies number and/or pattern was observed upon expression of either wild-type or mutant Edc3 (Figure 4B). This indicates that interaction of Edc3 with Rps28 is not needed to address Edc3 to P-bodies.

The region 201–231 of Edc3 is needed for the regulation of the RPS28B mRNA decay

Deletion of the EDC3 gene blocks the degradation of two specific RNAs: the RPS28B mRNA and the YRA1 pre-mRNA (19,26). We analysed whether the deletion of the Edc3 RB motif influenced the stability of these RNAs. Total RNA was extracted from Δedc3 cells transformed either with an empty vector, with a vector coding for the wild-type Edc3-protA fusion, or with Edc3ΔRB-protA. As a control, total RNA from wild-type cells transformed with an empty vector was used. We observed that deletion of the RB motif of Edc3 that abrogates interaction of the latter with Rps28 significantly stabilized the RPS28B mRNA (Figure 5A, left panel). Quantification revealed that deletion of the edc3 gene led to more than 2-fold ($2.8 \pm 0.29$) increase of the amount of RPS28B mRNA compared with the wild-type strain. Expression of the wild-type Edc3 restored a normal ratio of RPS28A versus RBS28B mRNA ($1.3 \pm 0.25$), indicating that interaction between Edc3 and Rps28 is not needed for the degradation of the YRA1 pre-mRNA ratio ($1.1 \pm 0.66$). Expression of Edc3ΔRB-protA had the same effect ($1.2 \pm 0.42$), indicating that interaction between Edc3 and Rps28 is not needed for the degradation of the YRA1 pre-mRNA. This observation demonstrates further that the effect of the removal of residues 201–231 of Edc3 on RPS28B mRNA was highly specific.

To verify that the observed changes in RPS28B mRNA steady-state level are related to autoregulation of its stability, we analysed the half-life of RPS28B mRNA using a strain where the endogenous RPS28B and EDC3 genes were deleted. Cells were transformed with a vector bearing RPS28B gene under a tetracycline-repressible transcription activator (19) and either a vector coding for wild-type EDC3-protA fusion or a vector coding for EDC3ΔRB-protA fusion. Total RNA was isolated at different time points after doxycycline-induced
transcriptional repression of RPS28B and analysed by northern blotting. The results obtained (Figure 5C) clearly show the increase of the half-life time of \( RPS28B \) mRNA in the strain expressing EDC3\( / \)C1 RB-protA (22.5 ± 2.1 min compared with 8 ± 1 min in the strain expressing the wild-type EDC3-protA fusion). These changes are similar to those reported when the autoregulation loop of \( RPS28B \) mRNA level was identified (19). These data indicate that the interaction between Rps28 and Edc3 mediated by RB motif is implicated in this autoregulatory process.

**DISCUSSION**

We have identified a novel motif within yeast Edc3 protein that is required and sufficient for binding to the ribosomal protein Rps28. Moreover, we have shown that Rps28 and Edc3 interact directly, forming a stable heterodimeric complex in the absence of other specific RNA or protein partners. *In vivo* analyses demonstrated that this region of Edc3 is crucial for the auto-regulatory feedback loop that controls the \( RPS28B \) mRNA level, but that it is dispensable for other functions of Edc3, including its ability to regulate the \( YRA1 \) mRNA level by inducing degradation of \( YRA1 \) pre-mRNA. The latter result indicates that the two processes in which Edc3 participates to the auto-regulation of RNA levels by controlling their decay rates rest on different mechanisms involving different protein–protein interaction interfaces. The binding of Edc3 to Rps28 will ensure a rapid induction of \( RPS28B \) mRNA decay if free Rps28 protein starts to accumulate.

One can wonder whether the ability of Edc3 to interact with Rps28 extends to situations where the latter is incorporated in ribosomes. Our data indicate that Edc3 interacts with the Rps28 core rather than its C-terminal tail. Interaction of Edc3 with ribosome-bound Rps28 may be limited by the presence of Rps5 and ribosomal RNA that mask large share of the Rps28 core surface (29).
If decapping was shown to occur in part on polysome-associated mRNAs (8) and several mRNA decay factors were reported to sediment in polysomes (38,39), the presence of Edc3 in polysomes was, to the best of our knowledge, never reported. We also failed to detect such an association by polysome analyses or pull-down experiments (data not shown). Thus, it is probable that Rps28 present in ribosome is not accessible to Edc3, even if we cannot exclude that such association may take place temporarily or under specific conditions. If it would occur, association of Edc3 with ribosome-bound Rps28 would be restricted to a small group of organisms, arguing against a general role for such an interaction in mRNA decay. Detailed structural characterization of the Edc3-Rps28 dimer would provide more insights into this issue.

The limited conservation of the region of Edc3 involved in Rps28 binding suggests that it was acquired recently during evolution. This would explain its exclusive presence in proteins encoded by Saccharomyces cerevisae species where it would have been co-opted to finely tune the Rps28 protein production. Given the small size of the RB motif, one can easily imagine how it may naturally have evolved by incremental selection of residues improving the binding affinity in an otherwise poorly conserved linker region. Other events of auto-regulation of yeast ribosomal protein production, either through modulation of translation, splicing and/or control of transcription termination are known (40–42). The frequent observations of such mechanisms suggest that they evolve rapidly, taking advantage of the ability of proteins and RNA to create new interaction interfaces from short linear stretches of amino acid and oligonucleotide sequences. Their recurrent occurrence indicates that finely tuning the production of ribosome subunits provides a key evolutionary advantage to yeast cells.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online, including [43–45].

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