Abstract Amino acid-starved yeast activates the eIF2α kinase Gen2p to suppress general translation and to selectively derepress the transcription factor Gcn4p, which induces various biosynthetic genes to elicit general amino acid control (GAAC). Well-fed yeast activates the target of rapamycin (TOR) to stimulate translation via the eIF4F complex. A crosstalk was demonstrated between the pathways for GAAC and TOR signaling: the TOR-specific inhibitor rapamycin activates Gcn2p. Here we demonstrate that, upon TOR-inactivation, the putative TOR-specific inhibitor rapamycin activates Gen2p. This unravelled the connection between the pathways via a mechanism independent of eIF4E-binding, thereby constituting another interface between the two pathways.

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1. Introduction

When the budding yeast *Saccharomyces cerevisiae* starves for a certain amino acid, uncharged tRNA for that amino acid accumulates and activates the sole eIF2α kinase Gen2p [1,2]. The Gen2p phosphorylates eIF2α at Ser-51 to convert it from the substrate to an inhibitor of its guanine nucleotide exchanger eIF2B. Consequently, the ternary complex composed of eIF2, GTP and methionyl-tRNA is declined to induce general suppression of translation initiation. However, the translation of *GCN4* mRNA is selectively derepressed via a mechanism dependent on the four upstream open reading frames (uORFs) in its 5′-leader region, which usually function as cis-elements to prevent translation of *GCN4* ORF [1]. The transcription factor Gen4p activates genes for amino acid biosynthesis, including not only those for the starved amino acid but also those for other, non-starved ones [2]. Hence the response was termed general amino acid control (GAAC) and has been extensively investigated. Translation regulation via phosphorylation of eIF2α represents the most fundamental mechanism conserved throughout the eukaryotes [3].

When grown in sufficient nutrient levels, the cells activate the target of rapamycin (TOR) signaling pathway to promote formation of the initiation complex eIF4F, which is composed of eIF4A, 4E and 4G. Translation initiation is enhanced through assembly of the eIF4F complex on the 5′-cap structure of the mRNA [4,5]. In yeast, the TOR signaling pathway was demonstrated to stabilize eIF4G, the scaffold of eIF4F [6]. In mammals, the TOR signaling causes the phosphorylation of eIF4E-binding proteins (4E-BPs), which compete with eIF4G for binding eIF4E to block the assembly of eIF4F. Since the phosphorylation makes 4E-BPs incapable of binding to eIF4E, it facilitates the assembly of eIF4F to stimulate translation initiation. The budding yeast has an eIF4E-associated protein termed Eap1p [7]. It has been assumed to be a functional homolog of 4E-BPs, because it shares the 4E-binding motif with 4E-BPs to bind eIF4E both in vitro and in vivo and because its removal confers mild resistance to rapamycin, indicative of its involvement in TOR signaling [7]. However, how Eap1p functions in translation regulation remains largely elusive.

The pathways for GAAC and TOR signaling should be reciprocally regulated to coordinate cellular responses to nutritional stress. Indeed, we and others recently demonstrated that rapamycin derepresses translation of *GCN4* mRNA [8–10]. While this derepression is induced by the activation of Gen2p, it is not accompanied by an increment in uncharged tRNAs but by dephosphorylation of Gen2p at Ser-577 [9,10]. The dephosphorylation has been shown to increase the affinity of Gen2p to uncharged tRNAs, thereby leading to its activation without increment in uncharged tRNAs [9,10]. It thus seems that TOR signaling pathway crosstalks with GAAC pathway to prevent inactivation of eIF2.

In this study, we examined potential involvement of Eap1p in the crosstalk between the two pathways. The results indicate that, upon TOR-inactivation, Eap1p attenuates the translation of *GCN4* mRNA via a novel mechanism independent of eIF4E-binding.

**The yeast eIF4E-associated protein Eap1p attenuates GCN4 translation upon TOR-inactivation**

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Abbreviations: eIF, eukaryotic initiation factor; TOR, target of rapamycin; ORF, open reading frame; GAAC, general amino acid control; 4E-BP, eIF4E-binding protein; GST, glutathione-S-transferase; 3AT, 3-aminotriazole; eIF2α[pS51], eIF2α phosphorylated at Ser-51; SC-Ura, synthetic complete medium lacking uracil; SC-Ura-His, synthetic complete medium lacking uracil and histidine

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2. Materials and methods

2.1. Yeast strains

The strains used were W303H (MATα ade2-1 trp1-1 leu2-3 ura3-1 can1-100), WH2 (MATα ade2-1 trp1-1 leu2-3 ura3-1 can1-100 gen2Δ::KanMX), WH4 (MATα ade2-1 trp1-1 leu2-3 ura3-1 can1-100 eap1Δ::KanMX), WH9 (MATα ade2-1 trp1-1 leu2-3 ura3-1 can1-100 gen2Δ-S577A), WH24 (MATα ade2-1 trp1-1 leu2-3 ura3-1 can1-100 gen2Δ eap1Δ::KanMX), WH41 (MATα ade2-1 trp1-1 leu2-3 ura3-1 can1-100 eap1::Y109A), WH42 (MATα ade2-1 trp1-1 leu2-3 ura3-1 can1-100 eap1::Y109A(L114A)) and WH94 (MATα ade2-1 trp1-1 leu2-3 ura3-1 can1-100 gen2Δ-S577A eap1Δ::KanMX). All of these strains were generated by the standard PCR-mediated gene disruption using the KanMX cassette or by the pop-in–pop-out replacement technique.

2.2. Reporter assay for GCN4 translation

The β-galactosidase assay was performed as previously described [10–12].

![Fig. 1. Effects of EAP1 deletion on translational derepression of GCN4.](image)

(A) The W303H (EAP1, circle) and WH4 (eap1Δ, triangle) cells bearing the GCN4-lacZ reporter plasmid p180 were grown at 30 °C to logarithmic phase in SC-Ura, shifted to SC-Ura containing 200 ng/ml rapamycin and assayed for β-galactosidase activity at the time points indicated. (B) Translational depression of GCN4 was measured under various concentrations of rapamycin for 2 h in W303H (circle) and WH4 (triangle) cells. (C) The W303H (circle) and WH4 (triangle) cells bearing p180 were grown as in (A) and shifted to SC-Ura-His containing 20 mM 3AT and assayed for β-galactosidase activity at the time points indicated.

2.3. Immunoblot analysis

The immunoblot assay using an anti-phospho-eIF2α or anti-T7 antibody was conducted as previously described [10–12].

2.4. Far-western blot assay for eIF4E-binding

The yeast proteins separated by SDS–polyacrylamide gel electrophoresis were transferred to nitrocellulose membrane, which was subsequently probed with the purified glutathione-S-transferase (GST)-eIF4E. The bound probe was detected using an anti-GST antibody as described [11,12].

3. Results

3.1. Deletion of EAP1 enhances rapamycin-induced GCN4 translation

To examine the effect of Eap1p on rapamycin-induced GCN4 translation, we generated a strain deleted for the entire ORF of EAP1 by PCR-mediated one-step gene disruption using the KanMX cassette. The eap1Δ cells were transformed with the p180 reporter plasmid. It bears the 5′-leader region of GCN4 mRNA followed by lacZ ORF and has been used to report the translational derepression [1]. Confirming no significant difference in growth between the parental and eap1Δ cells, we measured the β-galactosidase activity driven by the p180 reporter plasmid. As shown in Fig. 1A, rapamycin induced the activity more efficiently in eap1Δ cells than in the parental cells. We carried out the experiment with increasing doses of rapamycin, and found that 200 ng/ml of rapamycin enhanced β-galactosidase activity more prominently (Fig. 1B). We also confirmed that the enhancement was observed in other strain backgrounds (data not shown).

In addition to the association with eIF4E, Eap1p has been implicated in spindle pole body duplication and segregation of chromosomes [13]. It is formally possible that the deletion of EAPI somehow affects the copy number of the centromeric p180 reporter plasmid to result in an increased dosage of lacZ mRNA. To rule out this possibility, we performed quantitative PCR to confirm that both p180 plasmid and lacZ mRNA showed no difference in their abundance between the EAPI and eap1Δ cells (data not shown).

3.2. Overexpression of EAPI suppresses rapamycin-induced GCN4 translation

From the findings described above, we expected that rapamycin-induced GCN4 translation can be suppressed by increasing the dosage of EAPI. We constructed a plasmid pA7U-EAPI which expresses full-length Eap1p under the control of strong ADH1 promoter. The wild-type and eap1Δ cells were co-transformed with the p180k reporter, a derivative of p180 bearing G418′ marker, and pA7U-EAPI or its backbone vector pA7U. These transformants were cultivated in the presence of rapamycin, and examined for β-galactosidase activity. As expected, overexpression of EAPI efficiently suppressed the rapamycin-induced GCN4 translation in the wild-type cells (Fig. 2). It also suppressed the enhancement of rapamycin-induced GCN4 translation in eap1Δ cells (Fig. 2), excluding the possibility of an unexpected side-effect caused by the alteration of EAPI locus, which is adjacent to TOR2. Taken together, we conclude that Eap1p attenuates rapamycin-induced GCN4 translation.
3.3. EAP1 specifically attenuates rapamycin-induced translation of GCN4 mRNA

We next examined whether deletion of EAP1 affects GCN4 translation induced by 3-aminotriazole (3AT), an inhibitor of His3p to cause histidine starvation. Notably, the deletion barely affects the translation induced by 3AT, in contrast with that by rapamycin (Fig. 1C). Similarly, overexpression of EAP1 failed to suppress 3AT-induced GCN4 translation (Fig. 2).

These results prompted us to test various stimuli reported to induce GCN4 translation, including adenine starvation, carbon starvation, methylmethanesulfonate and chlorpromazine [2,14]. Intriguingly, deletion of EAP1 failed to enhance the GCN4 translation induced by any of these stimuli (Fig. 3A).

Fig. 2. Effects of EAP1 overexpression on translational derepression of GCN4. The W303H (EAPI) and WH4 (eap1Δ) cells bearing p180k, a G418-marked derivative of p180, were transformed with pA7U-EAP1, pA7U-eap1-Y109A, pA7U-eap1-Y109A/L114A or its backbone vector pA7U. Note that the KanMX cassette in WH4 strain had been excised using the Cre-loxP site-specific recombination system so that G418 could be used for the maintenance of p180k. These transformants were treated for 2 h with 20 mM 3AT or 200 ng/ml rapamycin and assayed for β-galactosidase activity.

Fig. 3. Specificity of the effect of EAP1 deletion. (A) The W303H (EAPI) and WH4 (eap1Δ) cells bearing p180 were grown to logarithmic phase and shifted to SC-Ura containing 20 mM 3AT, 200 ng/ml rapamycin, 50 µg/ml 8-azaadenine (azA), 0.07% methylmethanesulfonate (MMS) or 250 µM chlorpromazine (CPZ) for 2 h, or a carbon starvation medium containing 0.05% glucose for 6 h prior to β-galactosidase assay. Note that the activity of control W303H cells was taken as 100%. (B) The W303H and WH4 cells were transformed with p180, p227, pRS-FAS-lacZ or pYES3/CT/lacZ, treated with 200 ng/ml rapamycin for 4 h and subjected to β-galactosidase assay. The results were indicated by fold-derepression in percentile.
It thus seems that Eap1p serves as a modulator specific to rapamycin-induced translation, reinforcing the notion of its involvement in the TOR signaling.

We also examined whether deletion of EAPI affects translation of genes other than GCN4. For this purpose, we transformed the wild-type and eap1Δ cells with other reporter constructs, namely p227, pRS-FAS1-lacZ, and pYES3/CT/lacZ. The p227 is a derivative of p180 lacking the uORFs. The pRS-FAS1-lacZ is a centromeric plasmid bearing lacZ reporter driven by the promoter and 5′-untranslated region of FAS1, whereas the pYES3/CT/lacZ is a multicopy plasmid bearing lacZ driven by GAL1 promoter. These transformants were treated with rapamycin, and examined for β-galactosidase activity. As shown in Fig. 3B, none of the three reporters showed differential response to rapamycin between the wild-type and eap1Δ cells. These results suggest that the effect of EAPI deletion on translation is not general but rather specific to GCN4 mRNA. More importantly, failure of p227 to respond to EAPI deletion underscores the importance of the uORFs in the 5′-leader region.

3.4. EAPI functions downstream of GCN2 to attenuate GCN4 translation

The importance of the uORFs prompted us to test whether the well-established mechanism for the translational derepression of GCN4 mRNA, which is triggered by the activation of Gcn2p, is augmented by deletion of EAPI. For this purpose, we employed an antibody that specifically recognizes eIF2α phosphorylated at Ser-51 (eIF2α[pS51]) to examine its abundance. We, however, found no apparent difference in the levels of eIF2α[pS51] between the wild-type and eap1Δ cells, both treated with rapamycin (Fig. 4A). It thus seems that the removal of Eap1p neither activates Gcn2p nor suppresses eIF2α[pS51] phosphatase(s). Nevertheless, eIF2α [pS51] is a prerequisite for the enhancement, because deletion of EAPI failed to enhance GCN4 translation in the absence of the sole eIF2α kinase Gcn2p (Fig. 4B).

To clarify how Gcn2p is involved in the process, we next examined the effect of EAPI deletion in the cells bearing gcn2-S577A, which encodes for Gcn2p(S577A) incapable of being activated by rapamycin [9,10]. As reported previously, rapamycin failed to derepress GCN4 translation in gcn2-S577A cells (Fig. 4B). However, to our interest, rapamycin derepressed GCN4 translation in gcn2-S577A eap1Δ cells as effectively as in GCN2 eap1Δ cells (Fig. 4B). We also examined the cells bearing gcn2-S577E allele, which had been demonstrated to display compromised rapamycin-induced Gcn2p activation, to observe that deletion of EAPI confers rapamycin-responsiveness in GCN4 translation (data not shown).

These results indicate that a prerequisite for the enhancement of GCN4 translation by EAPI deletion is the basal, but not the rapamycin-induced, Gcn2p activity. Thus rapamycin is likely required for the activation of Eap1p but not Gcn2p. Accordingly, deletion of EAPI fails to affect the translation induced by other stimuli which activate Gcn2p but not Eap1p (Fig. 3).

3.5. Association with eIF4E is dispensable for Eap1p to attenuate GCN4 translation

To understand how rapamycin-activated Eap1p functions, we examined whether its association with eIF4E is required for the attenuation of GCN4 translation. We introduced a mutation into the canonical eIF4E-binding motif of Eap1p to replace Tyr at position 109 with Ala, thereby producing Eap1p(Y109A) (Fig. 5A). To rule out the effect of potential residual eIF4E-binding activity of Eap1p(Y109A), we generated another mutant Eap1p(Y109A/L114A), because L114A substitution corresponds to L45A in human 4E-BP3 that had been demonstrated to abolish its eIF4E-binding [15]. As expected, the far-western blot analysis indicated that both Eap1p(Y109A) and Eap1p(Y109A/L114A) totally fail to interact with eIF4E in vitro (Fig. 5B). Mass spectrometric analysis of the affinity-purified complexes including tagged Eap1p and Eap1p(Y109A) provided compelling and no evidence for eIF4E binding, respectively, implying that Eap1p(Y109A) fails to interact with eIF4E in vivo (data not shown).

We prepared eap1Δ cells bearing an episomal copy of EAPI, eap1- Y109A or eap1- Y109A/L114A, each expressing the protein tagged with T7-epitope at its N-terminal end. Immunoblot analysis using a T7-antibody showed that steady state levels of these proteins are comparable, indicating that the mutations do not affect the stability of Eap1p (Fig. 5B). Intriguingly, when overexpressed, both eap1- Y109A and eap1- Y109A/L114A encoding Eap1p incapable of binding eIF4E suppressed the rapamycin-induced GCN4 translation as efficiently as EAPI encoding the protein fully capable of binding eIF4E (Fig. 2). Furthermore, the cells bearing eap1- Y109A or eap1-...
Fig. 5. Effects of mutations abolishing eIF4E-binding of Eap1p on GCN4 translation. (A) Structure of Eap1p is schematically represented with the eIF4E-binding motif aligned with those of yeast Cal20p, eIF4Gis, and human 4E-BPs. The consensus sequence is also shown (bold letters, conserved residues; underlined letters, the conserved flanking sequences shared by Eap1p and Caf20p; X, any residues; Φ, for hydrophobic residues; italic letters, substituted Ala). (B) The panel shows Ponceau red stain image of the blot. The middle panel shows an immunoblot detection of T7-tagged Eap1p, Eap1p-Y109A and Eap1p-Y109A/L114A expressed episomally in WH4 (eap1Δ) cells with an eIF4E probe to examine the interactions of Eap1p, Eap1p-Y109A and Eap1p-Y109A/L114A with eIF4E. (C) The W303H, WH41 (eap1-Y109A), WH42 (eap1-Y109A/L114A) and WH4 cells were examined for 3AT- and rapamycin-induced GCN4 translation as described in Fig. 4B.

Y109A/L114A at the EAP1 locus failed to display the enhancement of rapamycin-induced GCN4 translation (Fig. 5C). These results indicate that eIF4E-binding is dispensable for the attenuation of GCN4 translation, suggestive of a novel mode of action for Eap1p.

4. Discussion

We demonstrated that deletion and overexpression of EAP1 specifically enhances and suppresses rapamycin-induced translation of GCN4 mRNA, respectively (Figs. 1–3). It seems that rapamycin, or TOR-inactivation, derepresses Eap1p, which functions downstream of Gen2p to attenuate GCN4 translation (Fig. 4). Since the attenuation depends on the four uORFs in the 5′-leader region of GCN4 mRNA (Fig. 3B), Eap1p likely contributes to maintain the level of elf2-GTP-methionyl tRNA ternary complex, notably, via a novel mechanism independent of eIF4E-binding (Fig. 5). Although Eap1p was reported to have a separate function from its eIF4E-binding in the maintenance of genetic stability [13], we excluded its involvement by confirming that deletion of EAPI does not affect the dosage of p180 plasmid and lacZ mRNA at all. More recently, genetic and biochemical interactions were reported between EAPI and SCP160, an RNA-binding protein shown to associate with a specific subset of mRNAs [16]. However, we assume the involvement of these interactions unlikely, because of their dependence on eIF4E-binding [16]. Further experiments are necessary to decipher the underlying mechanism, and our preliminary results indicate that the region spanning amino acid residues 88–226 is responsible for the attenuation.

The other question raised by the present study would be the biological role for Eap1p-mediated attenuation of GCN4 translation. The effect of rapamycin, or TOR-inactivation, is an apparent paradox: rapamycin activates Gen2p to induce GCN4 translation on one hand, but derepresses Eap1p to attenuate the translation on the other hand. A plausible explanation may be that Eap1p constitutes a negative feedback loop to fine-tune the cellular response to nutritional stress. To mild starvation such as that for amino acids, GAAC pathway is activated to stimulate amino acid biosynthesis utilizing available carbon and nitrogen sources. However, when starvation becomes much severer to deplete raw materials for biosynthesis, it is reasonable for the cells to cease GAAC and activate autophagy instead. Indeed, nitrogen starvation was reported to repress GCN4 translation even in the presence of elf2-pS51 or downstream of Gen2p [17]. It is thus conceivable that Eap1p, which seems to be more activated under severer starvation (Fig. 1), plays a role in such repression, thereby contributing to fine-tuning of cellular responses to starvation.

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