Receptor for activated C kinase 1 stimulates nascent polypeptide-dependent translation arrest

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Nascent peptide-dependent translation arrest is crucial for the quality control of eukaryotic gene expression. Here we show that the receptor for activated C kinase 1 (RACK1) participates in nascent peptide-dependent translation arrest, and that its binding to the 40S subunit is crucial for this. Translation arrest by a nascent peptide results in Dom34/Hbs1-independent endonucleolytic cleavage of mRNA, and this is stimulated by RACK1. We propose that RACK1 stimulates the translation arrest that is induced by basic amino-acid sequences that leads to endonucleolytic cleavage of the mRNA, as well as to co-translational protein degradation. Keywords: translation arrest; nascent peptide; RACK1; mRNA quality control; endonucleolytic cleavage

EMBO reports (2010) 11, 956–961. doi:10.1038/embor.2010.169

INTRODUCTION

The synthesis of specific amino-acid sequences that trigger translation arrest mediates gene regulation, and several bacterial and eukaryotic genes are controlled by nascent peptide-dependent ribosome stalling (Morris & Geballe, 2000; Gong & Yanofsky, 2002; Nakatogawa *et al*, 2004; Gaba *et al*, 2005; Onouchi *et al*, 2005). However, the molecular mechanism for and physiological relevance of translation regulation by a nascent peptide in other systems are still unknown.

We proposed that translation arrest is also crucial in the quality control of eukaryotic gene expression. The synthesis of polylysine by translation of a poly(A) mRNA sequence leads to translation elongation arrest (Inada & Aiba, 2005; Ito-Harashima *et al*, 2007), probably, because consecutive, positively charged amino-acid residues of the nascent protein have a high affinity for the

negatively charged exit tunnel of the ribosome (Lu & Deutsch, 2008). Twelve consecutive basic amino-acid residues cause translation arrest and the arrested product is cotranslationally degraded by the proteasome (Dimitrova *et al*, 2009). These results suggest that nascent peptide sequences have crucial roles in translation elongation arrest, leading to co-translational degradation of the arrested product.

The receptor for activated C kinase 1 (RACK1) is highly conserved in eukaryotes (Nilsson *et al*, 2004). RACK1 is a versatile scaffold protein in Src-kinase-based signalling pathways (Chong *et al*, 2005) and is an anchoring protein for protein kinase C in mammals (Cox *et al*, 2003). It also functions in developmental processes, such as sexual differentiation, cell-proliferation control and in hormone response pathways (McLeod *et al*, 2000; Kadmas *et al*, 2007; Guo *et al*, 2009). RACK1 is a core component of the eukaryotic 40S ribosomal subunit, which suggests that its signalling functions might directly influence the efficiency and specificity of translation (Cox *et al*, 2003; Gerbasi *et al*, 2004; Coyle *et al*, 2009). The results of cryo-electron microscopy studies have shown that RACK1 binds to the head region of the 40S subunit near to the mRNA exit channel (Sengupta *et al*, 2004; Taylor *et al*, 2009). Interestingly, RACK1-bound protein kinase C was shown to regulate translation initiation by interacting with eIF6 in mammalian cells (Ceci *et al*, 2003). However, the precise role of RACK1 in translational regulation remains unknown.

Here, we present evidence that *Saccharomyces cerevisiae* RACK1 contributes to the translation arrest that is induced by translation of consecutive basic amino-acid sequences. The 40S subunit binding of RACK1 is crucial for translation arrest. Such arrest results in endonucleolytic cleavage of the mRNA, stimulated by RACK1. We propose that the endogenous nascent peptide might be important for mRNA stability as well as the regulation of translation elongation and protein degradation, at least in specific cases.

RESULTS AND DISCUSSION

Translation arrest by nascent peptide requires RACK1

The factor involved in translation arrest that is induced by translation of consecutive basic amino-acid sequences was identified using the genetic screen described below. The reporter

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Fig 1 | Identification of *ASC1/RACK1* as a novel factor for nascent peptide-dependent translation arrest. (A) Top: Schematic drawing of the *GFP-R12-FLAG-HIS3* reporter mRNA. The filled boxes and the lines indicate open reading frames and untranslated regions, respectively. The tracts of A indicate the poly(A) tail. The black box indicates the FLAG epitope tag. Bottom: The *GFP-R12-FLAG-HIS3* reporter gene complements *his3*[−] in suppressor mutants. W303(*NAD1*), *nad* suppressor mutants or W303*asc1/rack1Δ* cells containing the p416*CYC1*-*GFP-R12-FLAG-HIS3* reporter plasmid were transformed with yCplac111(vector) or with pRACK1-*FLAG-RACK1* plasmids. Cells were grown on YPD and SC-HisUraLeu (−His) plates in a series of dilutions. (B) Elevated expression of the *GFP-R12-FLAG-HIS3* reporter in W303(*NAD1*) and *nad* mutant cells. Protein levels were analysed by Qdot western blot analysis using GFP antibodies, Qdot fluorescence was detected using FLA-9000 (FujiFilm, Tokyo, Japan). The asterisk indicates the position of an unknown endogenous protein that is recognized by Qdot(R) streptavidin conjugates. (C) Translation repression by consecutive basic amino-acid residues requires RACK1. W303 or W303*asc1/rack1Δ* cells were transformed with plasmids containing *GFP-FLAG-HIS3* (−), *GFP-K12(AAA)-FLAG-HIS3* (K12(AAA)), *GFP-K12(AAG)-FLAG-HIS3* (K12(AAG)), *GFP-R12-FLAG-HIS3* (R12), *GFP-R12-FS-FLAG-HIS3* (R12-FS), *GFP-F12-FLAG-HIS3* (F12), *GFP-E12-FLAG-HIS3* (E12) and *GFP-SL-FLAG-HIS3* (SL) reporter genes. The *GFP-R12-FS-FLAG-HIS3* reporter gene has frame-shift mutations that alter the poly-arginine sequence, and the *GFP-SL-FLAG-HIS3* reporter gene contains a sequence for formation of a stable RNA secondary structure (Dimitrova et al, 2009). The levels of protein products were determined by using samples from a W303 cell harbouring p416*GPDp*-*GFP-FLAG-HIS3* as a standard curve (lanes 1–4). The relative levels of each protein product were normalized with respect to the *GFP-FLAG-His3* protein level in wild-type cells, which was assigned a value of 100, and are reported as the mean values of three independent experiments. (D) A translation arrest product was not detected in *asc1/rack1Δ* cells. W303 or W303*asc1Δ* cells containing a p416*GPDp*-*GFP-FLAG-HIS3* or a p416*GPDp*-*GFP-R12-FLAG-HIS3* plasmid were grown. Cell extracts were analysed by western blot analysis. Extracts indicated by (+) were prepared 2 h after the addition of 0.2 mM MG132. Samples from W303 cells harbouring p416*GPDp*-*GFP-FLAG-HIS3* were used as a standard curve (lanes 1–4). (E) *ASC1/RACK1* is required for translation arrest induced by consecutive basic amino-acid residues. Pulse-chase experiments were performed using W303 or W303*asc1Δ* cells containing the p416*GPDp*-3HA-*GFP-FLAG-HIS3* or p416*GPDp*-3HA-*GFP-R12-FLAG-HIS3* plasmids described in the Method section. Cells indicated by (+) were grown in the presence of 0.2 mM MG132.

GFP-R12-FLAG-HIS3 gene that contains 12 consecutive arginine codons (R12) is able to complement a *his3*[−] mutant, depending on the strength of its promoter. *S. cerevisiae* W303 (*his3*[−]) cells containing *GFP-R12-FLAG-HIS3* controlled by the weak

promoter *CYC1* did not grow on SC plates without histidine (Fig 1A), whereas W303 cells containing *GFP-FLAG-HIS3* did (data not shown). We isolated spontaneous suppressor mutants in which the reporter gene *GFP-R12-FLAG-HIS3*—under the

control of the *CYC1* promoter—could complement the *his3*–mutation (Fig 1A). GFP–R12–FLAG–His3 protein levels were increased in these mutants (Fig 1B), and suppressors were called *nad* (nascent peptide-dependent translation arrest defective) mutants. The results of tiling-array experiments suggested that *nad1* mutants (*nad1-1*, *nad1-2*) have mutations in the *asc1* gene (data not shown), and deletion mutations in this region were identified by direct sequencing (supplementary Fig S1 online). *ASC1* is an orthologue of human *RACK1*, which is a member of the highly conserved *RACK1* protein family in eukaryotes (Nilsson et al, 2004). As expected, the phenotypes of *nad1-1* or *nad1-2* cells were complemented by a plasmid containing the wild-type *ASC1/RACK1* gene, and showed a *nad* phenotype with an *asc1/rack1Δ* deletion (Fig 1A). We hereafter refer to *ASC1(NAD1)* as *RACK1*.

Western blot analysis of the products derived from various GFP–X–FLAG–HIS3 reporters revealed that *RACK1* downregulated the levels of gene products from reporter genes that contain sequences encoding 12 consecutive lysine (K12) or arginine (R12) residues, but not from other reporters, including GFP–SL–FLAG–HIS3 (Fig 1C). The increase in full-length protein levels due to *asc1Δ* is not likely to be an indirect effect of an increase in mRNA levels—as mRNA levels are only slightly affected by the *asc1Δ* mutation (Fig 3C)—but there is a reduction in the truncated protein product (Fig 1C). The GFP–SL–FLAG–HIS3 reporter gene contains a sequence that can form a stable RNA secondary structure that inhibits translation elongation (Dimitrova et al, 2009). Therefore, the action of *RACK1* in translation arrest might be triggered by the sequence of the nascent peptide, not by the presence of a stable mRNA secondary structure. We previously found that the translation arrest product derived from GFP–R12–FLAG–HIS3 was detected only when the activity of the proteasome was impaired by 0.2 mM MG132 (Dimitrova et al, 2009). By contrast, a truncated translation arrest product was not detected in *asc1Δ* cells, even in the presence of MG132 (Fig 1D, lanes 9–12). Pulse-chase experiments also revealed that a translation arrest product was present in wild-type cells after a 30 min chase in the presence of MG132. However, a translation arrest product was not detected in the *asc1Δ* cells (Fig 1E, lanes 5–12). These results indicate that *RACK1* contributes to translation arrest associated with a specific nascent peptide sequence, and that *RACK1*-dependent translation arrest leads to co-translational degradation of the arrest product by the proteasome.

RACK1 binding to the 40S subunit in translation arrest

Binding of the ribosome to *RACK1* has been linked to its function in *S. cerevisiae* (Coyle et al, 2009). We therefore investigated whether this is crucial in nascent peptide-dependent translation arrest. The association between *RACK1* and the ribosome was decreased in the *asc1R38DK40E* mutant, as has been previously reported (Fig 2A; Sengupta et al, 2004; Coyle et al, 2009). We also found that nascent peptide-dependent translation arrest was defective (Fig 2B, lanes 5–7). PCR-based mutagenesis was used to isolate additional *asc1* mutants that showed defective translation repression. The *asc1D109Y* and *asc1D109VQ153R* mutants were found to produce severe defects both in translation repression and in the association between *RACK1* and the ribosome (Fig 2A,B, lanes 8,9). The mutant proteins were expressed at levels comparable with wild-type protein (data not

Fig 2 | Ribosome binding of *Saccharomyces cerevisiae* *RACK1* is crucial for translation arrest. (A) The distribution of *RACK1* proteins in polysomes. W303*asc1/rack1Δ* cells were transformed with various p*RACK1p*-FLAG-*RACK1* plasmids expressing the indicated mutant form of the *RACK1* protein. Polysome analysis was performed as described previously (Inada & Aiba, 2005), and FLAG-*RACK1* proteins in fractions were detected by western blot analysis. The numbers indicate the percentage of *RACK1* bound to polysomes. (B) Ribosome binding of *S. cerevisiae* *RACK1* is required for translation arrest. W303 or W303*asc1/rack1Δ* cells harbouring various p*RACK1p*-FLAG-*RACK1* plasmids containing the indicated mutations were grown. The levels of the reporter proteins were determined by Qdot western blot analysis using samples from W303 cell harbouring p416*GPDp*-GFP-FLAG-HIS3 as a standard curve (lanes 1–4). The relative levels of GFP-R12-FLAG-His3 products are normalized to the level of GFP-R12-FLAG-His3 in W303*asc1/rack1Δ* cells harbouring yCplac111 (lane 5), which was assigned a value of 100, and are reported as the mean values of three independent experiments. (C) Scp160 is not involved in nascent peptide-dependent translation arrest. W303 or W303*scp160Δ* cells harbouring various p*GPDp*-GFP-X-FLAG-HIS3 plasmids were grown in SC-Ura medium and were analysed as in (B). WT, wild type.

shown). These results suggest that binding between the ribosome and *RACK1* is crucial in translation arrest that is induced by a sequence of basic amino acids.

Several functions have been proposed for *RACK1* in yeast. First, *RACK1* functions as a G-protein β -subunit in the glucose response

(Zeller *et al*, 2007). Second, RACK1 is involved in translational regulation of specific mRNAs, probably through interactions with the RNA-binding protein Scp160 (Baum *et al*, 2004; Sezen *et al*, 2009). We found that the deletion of genes involved in the Ras-cAMP glucose-signalling pathway or Scp160 resulted in no significant defects in translation arrest (supplementary Fig S2 online; Fig 2C, lanes 5–14). We propose that RACK1 might be directly involved in translation arrest induced by the nascent peptide, and that binding between the ribosome and RACK1 is crucial for this process.

Translation arrest by nascent peptide induces no-go decay

Translation arrest caused by a stable mRNA structure or a cluster of rare codons was observed to induce endonucleolytic cleavage of the mRNA through no-go decay (NGD; Doma & Parker, 2006). The truncated 5' fragment produced by cleavage could only be detected in mutants that showed defective 3'–5' exonucleolytic mRNA degradation pathways. We detected truncated 5' *GFP* fragments that were derived from reporter mRNAs containing translation arrest-inducing sequences in *ski2Δ* mutant cells in which the 3'–5' degradation pathway is impaired but not in wild-type cells (Fig 3A). We also detected truncated 5' *GFP* fragments derived from *GFP-K12(AAA)–FLAG–HIS3* or *GFP-K12(AAG)–FLAG–HIS3* reporter mRNA (Fig 3B, lanes 8,10). We also detected truncated 5' *GFP* fragments derived from the *GFP-R12–FLAG–HIS3* reporter gene (Fig 3A) and found that the alteration of amino-acid sequences by introduction of frame-shift mutations eliminated NGD (Fig 3B, lanes 16,18). The presence of tandem repeats of codons in these reporters suggested that NGD might be due to a string-of-codons effect. However, insertion of six repeats of the two lysine codons AAGAAA between reporter genes resulted in NGD, and the introduction of frame-shift mutations that changed the introduced amino-acid sequences eliminated NGD (Fig 3B, lanes 12,14). We also examined the effects of sequences encoding six repeats of lysyl-arginine that do not contain a string of codons (Fig 3A). Truncated 5' *GFP* fragments derived from reporter mRNAs containing a sequence that encodes six lysyl-arginine repeats were clearly identified (Fig 3B, lanes 20,22). These results suggest that translation arrest induced by a nascent peptide sequence results in endonucleolytic cleavage of mRNA without a string of codons. We also detected truncated 3' *HIS3* fragments derived from reporter mRNAs containing translation arrest-inducing sequences in *xrn1Δ* mutant cells (supplementary Fig S3 online).

Assuming that the ratio between the total mRNA and the truncated 5' *GFP* fragment reflects the efficiency of cleavage, this was significantly lower in the *asc1/rack1Δ* mutant cells (Fig 3C, lanes 7–12,15–16,20–23). The truncated 5' *GFP* fragments produced by NGD migrated differently in *asc1/rack1Δ* mutant and wild-type cells, indicating that their cleavage sites might be different. These results are consistent with the hypothesis that RACK1-dependent translation arrest by a specific nascent peptide sequence leads to endonucleolytic cleavage of the mRNA. The cleavage product in the *asc1/rack1Δ* mutant seems to migrate differently, suggesting that its cleavage site might be different to that in wild-type cells.

It has been reported that a Dom34/Hbs1 complex is crucial in NGD (Doma & Parker, 2006; Passos *et al*, 2009). We therefore examined the involvement of these factors in NGD that is induced

by a nascent peptide. Truncated 5' *GFP* fragments derived from reporter genes that contain a sequence encoding poly-lysine or poly-arginine were detected in the *ski2Δ* mutant cells (Fig 3D, lanes 10,14,22), and their levels were moderately lower but still detectable in *ski2Δdom34Δ* mutant cells (Fig 3D, lanes 12,16,24) and in *ski2Δhbs1Δ* mutant cells (supplementary Fig S4 online). These results indicate that the Dom34/Hbs1 complex is involved in, but not essential for, the endonucleolytic cleavage of mRNAs that is caused by nascent peptide-dependent translation arrest, and therefore that other factors must be involved in cleavage of the mRNA. This is the first evidence that translation-dependent endonucleolytic cleavage can occur independently of Dom34 and Hbs1, and consistent with the prediction that NGD can occur independently of Dom34 and Hbs1 under some conditions (Passos *et al*, 2009). Levels of the 5' cleavage product were significantly higher in the *dom34Δ* mutant (Fig 3D, lanes 11,15,23) compared with wild type (Fig 3D, lanes 9,13,21), indicating that the truncated 5' *GFP* fragments were stabilized in the *dom34Δ* mutant even when the 3'–5' exonucleolytic mRNA degradation pathway is active. We suspect that the Dom34/Hbs1 complex might stimulate the release of ribosomes that are stalled at the 3' end of a truncated mRNA that is a product of NGD.

An important question to be resolved is how RACK1, which is present on the 40S ribosomal subunit, functions during translation arrest—that is, how it can sense the properties of the nascent peptide in the ribosomal tunnel on the 60S subunit. Cryo-electron microscopy images of yeast ribosomes have shown that the structure of the 60S subunit is affected by RACK1 depletion (Sengupta *et al*, 2004). This suggests that binding of RACK1 to the 40S subunit stabilizes the conformation and/or activity of the 80S ribosome with respect to monitoring of the properties of the nascent peptide in the tunnel. It is also possible that recruitment of a signalling factor through interactions with RACK1 on the 40S subunit is required to modulate the activity of the ribosome or translation factors. In either case, our results suggest that the 40S-bound RACK1 might be required for translational control that is mediated through nascent peptides with a specific sequence. One implication of this work is that the RACK1 protein in eukaryotic cells, including human cells, functions to modulate translation elongation at specific sites, which would indicate that it has an important and so far unappreciated role in the control of gene expression.

METHODS

Yeast strains and plasmids. The yeast strains and plasmids used in this study are described in supplementary Table 1 online and the oligonucleotides are described in supplementary Table 2 online. The construction of the plasmids and the western blotting protocol, using antibody conjugated with Q-dot, are described in the supplementary information online.

Pulse-chase experiments. Yeast cells were grown exponentially at 30 °C in minimal media lacking methionine and cysteine. A 10 ml aliquot of yeast cells was labelled with 100 µCi [³⁵S]methionine and cysteine (NEG072; PerkinElmer, CA, USA) for 1 min. This was followed by the addition of cold amino acids, to a final concentration of 40 µg/ml. The cells were then collected and cell extracts were prepared using Complete Lysis-Y (Roche, NJ, USA). Cell extracts were incubated with a haemagglutinin (HA)

antibody (H 9658; Sigma, CA, USA) and protein A-agarose (Roche) in IXA-100 buffer (Inada & Aiba, 2005). The antibody-bound agarose was then washed three times and eluted with 0.4 mg/ml HA peptide. Immunoprecipitated samples were separated by SDS-polyacrylamide gel electrophoresis. The radioactivity of the precipitated proteins was measured using Typhoon9400 (GE Healthcare, MA, USA).

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

ACKNOWLEDGEMENTS

We thank all members of the lab, especially T. Tsuboi, for their help in the genetic screen of *nad* mutants, and E. Asai for plasmid construction. This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T.I.).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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