Asc1p, a WD40-domain containing adaptor protein, is required for the interaction of the RNA-binding protein Scp160p with polysomes

Sonja BAUM, Margarethe BITTINS, Steffen FREY and Matthias SEEDORF[1]

Center of Molecular Biology at University of Heidelberg (ZMBH), Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany

Scp160p interacts in an mRNA-dependent manner with translating ribosomes via multiple RNA-binding heterogeneous nuclear ribonucleoprotein K-homology (KH) domains. In the present study, we show by protein–protein cross-linking that Scp160p is in close proximity to translation elongation factor 1A and the WD40 (Trp-Asp 40)-repeat containing protein Asc1p at ribosomes. Analysis of a truncation mutant revealed that the C-terminus of Scp160p is essential for ribosome binding and that Cys^{1067} at the C-terminus of Scp160p is required to obtain these cross-links. The interaction of Scp160p with ribosomes depends on Asc1p. In fast-growing yeast cells, nearly all Asc1p is tightly bound to ribosomes, but it can also be present in a ribosome-free form depending on growth conditions. The functional homologue of Asc1p, mammalian RACK1 (receptor of activated C kinase), was previously characterized as an adaptor protein bridging activated signalling molecules with their substrates. Our results suggest that Scp160p connects specific mRNAs, ribosomes and a translation factor with an adaptor for signalling molecules. These interactions might regulate the translation activity of ribosomes programmed with specific mRNAs.

Key words: heterogeneous nuclear ribonucleoprotein K-homology domain (KH domain), ribosome, RNA-binding protein, signalling complex, translation.

INTRODUCTION

Scp160p from *Saccharomyces cerevisiae*, its orthologue DDP1 from *Drosophila melanogaster* and vigilin from vertebrates are RNA-binding proteins characterized by an unusually large number (14) of tandemly arranged KH (heterogeneous nuclear ribonucleoprotein K-homology) domains. Scp160p has been shown to be associated with polysomes and this binding depends on mRNA [1,2]. The presence of Scp160p in a complex containing mRNA and poly(A)$^+$ (polyadenylated)-RNA-binding protein Pab1p and the association of vigilin with eEF1A (translation elongation factor 1A) and tRNA support a function of Scp160p/vigilin in translation [1,3–5]. Loss of *SCP160* in an *eap1* mutant results in synthetic lethality [6]. Since Eap1p functions in translation initiation as an eIF (eukaryotic translation-initiation factor) 4E-binding protein [7], this finding has strengthened further the hypothesis that Scp160p modulates translation. Biochemical data suggest that specific mRNAs are enriched in purified Scp160p-containing complexes [8]. Therefore Scp160p might function to regulate translation of specific rather than random messages. Using a combination of cell fractionation and localization experiments, we have demonstrated an mRNA-dependent accumulation of Scp160p at polysomes at the ER (endoplasmic reticulum) membrane [2], consistent with specificity of Scp160p for certain mRNAs.

Recently, it has been shown that Scp160p interacts with a subunit of a G-protein-coupled receptor, Gpa1p, which is involved in the mating response [9]. In this pathway, the activity of Scp160p might trigger the local expression of factors required for efficient mating. A similar function of Scp160p could be the spatial regulation of translating localized mRNAs. It has been shown that *scp160*Δ mutants fail to concentrate ASH1 mRNA efficiently at the bud tip of daughter cells ([10]; R. P. Jansen and M. Seedorf, unpublished work). However, the contribution of Scp160p towards regulation of local *ASH1* translation remains elusive, since another KH-domain containing protein, Khd1p, also seems to play a major role in the anchoring of ASH1 mRNA [10]. Information about common features of potential substrate mRNAs and the mechanism by which Scp160p influences their translation are not yet known.

Although *SCP160* expression is dispensable for growth, *scp160*Δ mutants display defects in cell morphology and chromosome segregation, resulting in cells with increased size and DNA content [11]. The molecular basis of this phenotype is unknown, but Scp160p might influence the expression of genes that are required for faithful chromosome segregation [6]. This phenotype can be rescued by overexpression of DDP1, indicating an evolutionary conserved role of these RNA-binding proteins [12].

In the present study, we investigated the ribosome association of Scp160p and analysed the contribution of individual KH-domains to ribosome binding. Using protein–protein cross-linking, we described the position of Scp160p at ribosomes and identified eEF1A and Asc1p, an adaptor protein for signalling molecules, in the proximity of ribosome-associated Scp160p. Finally, we discuss how Scp160p and Asc1p at ribosomes could play a role in regulating the translation of specific mRNAs.

EXPERIMENTAL

Generation of yeast strains

In order to replace *SCP160* with full-length or C-terminally truncated *SCP160*, we first amplified 333 bp of the 3′-UTR (untranslated region) of the *SCP160* gene from a genomic library plasmid containing SCP160 (YEP13/6; [11]) with primers

Abbreviations used: BMH, bis-maleimidohexane; BMOE, bis-maleimidoethane; DTT, dithiothreitol; eEF1A, translation elongation factor 1A; eIF, eukaryotic translation-initiation factor; ER, endoplasmic reticulum; KH, heterogeneous nuclear ribonucleoprotein K-homology; LS, low salt; nano-ESI-Q-TOF MS, nanoelectrospray ionization-quadrupole-time-of-flight MS; PKC, protein kinase C; RACK1, receptor of activated C kinase; UTR, untranslated region; YPD, yeast extract, peptone, dextrose.

[1] To whom correspondence should be addressed (e-mail m.seedorf@zmbh.uni-heidelberg.de).

5′-CCGgaattcgtcgactagACAAGCTCTTTGAAACC-3′ and 5′-GCtctagaGCGGAAACTTCGTCGCC-3′ (lower case letters indicate restriction endonuclease sites) and cloned the resulting fragment using *Eco*RI/*Xba*I in pBlueScriptII SK vector, resulting in pMS446. The *TRP1* gene was amplified from pRS316 [13] with primers 5′-CgcggccgcATCAGAGCAGATTGTAC-3′ and 5′-CgagctcCTTACGCATCTGTGC-3′ and cloned using *Not*I/*Sac*I into pMS446 vector, resulting in pMS447. To amplify 441 bp of the *SCP160* 3′-UTR from YEP13/6, primers 5′-CgagctcGACAAGCTCTTTGAAACC-3′ and 5′-CgagctcCTTCATCTGCAGTGTATTTACCG-3′ were used, before being cloned into the *Sac*I site of pMS447 vector to yield pMS448. To amplify the entire *SCP160* coding region, we used 5′-CCatcgatGTCTGAAGAACAAACCGCTATTGAC-3′ and 5′-GgtcgacTCTTCTTAAGGATTTCAAAACCATTTC-3′ primers, to amplify amino acids 1–1008 of *SCP160*, 5′-CCatcgatGTCTGAAGAACAAACCGCTATTGAC-3′ and 5′GgtcgacTTCCCTGATTATTTCATTCAAGATTTTC-3′ primers were used, and to amplify amino acids 1–858, 5′CCatcgatGTCTGAAGAACAAACCGCTATTGAC-3′ and 5′GgtcgacGGCTTCGGCAACAATGGATTCC-3′ primers were used. The PCR products were cloned into pCRII-TOPO vector (Invitrogen), excised with *Apa*I/*Sal*I, and the resulting fragments were cloned into the corresponding sites of pMS448, resulting in plasmids pMS449–pMS451. In order to replace *SCP160* in the yeast genome, *Apa*I/*Pst*I-fragments of pMS449–pMS451 were isolated and introduced into a haploid S288c-derived yeast strain by transformation [14]. The *asc1*Δ mutant in a W303 background is described in [15]. To introduce a triple myc-tag at the C-terminus of Asc1p in a W303 strain, we used the method described in [16].

FACS

The DNA content of relevant strains using ethanol-fixed, RNase-treated, propidium-iodide-stained cells, which were grown to a D_{600} of 0.5, was measured by flow cytometry using a Becton Dickinson FACScan with the following settings: FL2, voltage 736, gain 7.21, linear mode, threshold FSC 49.

Cell fractionation and sucrose density gradient centrifugation

Total yeast extract (t) and fractions enriched in membranes (P6), cytosolic ribosomes (P200) and ribosome-free cytosol (S200) were prepared as described previously [2]. P25 was generated by a 20 min centrifugation of total extract at 4 °C at 25 000 ***g***. Yeast cells were grown in YPD (yeast extract, peptone, dextrose) medium at 30 °C to exponential phase and 100 μM cycloheximide was added 15 min before harvest. Cytosolic polysomes (corresponding to a D_{600} of 7.5) were analysed by centrifugation (in a Beckman SW60 rotor, 55 000 rev./min) of a 6000 g supernatant for 1 h through a linear 4 ml 15–50 % (w/v) sucrose gradient in LS (low salt) buffer [20 mM Hepes/KOH, pH 7.6, 100 mM potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 1 mM DTT (dithiothreitol), 0.1 mM PMSF and Complete™ protease inhibitor mix (Roche)]. Membranes (P6 corresponding to a D_{600} of 7.5) were solubilized with 2 % (w/v) Nikkol (octaethylene glycol monododecyl ether) [2] and separated on similar gradients.

Antibodies and immunoprecipitation

To detect proteins by immunoblotting, nitrocellulose membranes (Protean, Schleicher & Schuell) were incubated for 2 h at room temperature (25 °C) with antibodies diluted in blocking buffer [containing PBS, 0.2 % (v/v) Tween 20 and 3 % (w/v) dry skimmed milk powder], followed by horseradish-peroxidase-conjugated secondary antibodies (Sigma) and detection with BM Chemiluminescence Blotting Substrate (POD) (Roche). Scp160p-, Rps3p-, Rpl35p- and Sec61p-specific antibodies have been described previously [2]. eEF1A-specific antibodies were provided by Dr Kaplana Chakraburtty (Medical College of Wisconsin, Milwaukee, WI, U.S.A.) and were used in a 1:10 000 dilution. myc- and Zwf1p-specific antibodies from Sigma were used according to the manufacturer's instructions. After cross-linking, fractions were denatured by adding 1 % (w/v) SDS and incubation for 5 min at 95 °C. Samples were diluted 10-fold in PBS, pre-cleared for 60 min at 4 °C with a 1/20 volume Protein A–Sepharose (CL-4B; Amersham Biosciences), centrifuged for 15 min at 18 000 ***g***, and the resulting supernatant was incubated overnight at 4 °C with a 1/200 volume of Scp160p- or a 1/75 volume of myc-specific antibodies. The antibodies were recovered by binding to a 1/20 volume of Protein A–Sepharose, followed by washing five times with 500 mM NaCl, 2 mM EDTA, 0.2 % (v/v) Nonidet P40 and 10 mM Tris/HCl, pH 7.5, and twice with 10 mM Tris/HCl, pH 7.5.

Chemical protein cross-linking and identification of cross-linked products

After centrifugation of total extract for 20 min at 200 000 ***g***, the pellet was resuspended in 200–500 ml of LS-buffer without DTT at a concentration of 2.5×10^7 cells/ml (as determined from the D_{600}) and incubated for 15 min at 25 °C with 50 μM BMH (bis-maleimidohexane) or BMOE (bis-maleimidoethane) dissolved in DMSO. Reactions were stopped by the addition of 10 mM DTT, and ribosomes were re-isolated by a 20 min centrifugation at 200 000 ***g***. To identify cross-linked products, we isolated ribosomes from 1.5×10^{11} cells (as determined from the D_{600}), incubated half of them with 50 μM BMH and immunoprecipitated Scp160p. After SDS/PAGE, the Coomassie-Blue-stained bands were excised. Tryptic in-gel digestion and nano-ESI-Q-TOF (nanoelectrospray ionization-quadrupole-time-of-flight) MS was performed as described previously [17]. Peptides, which were not derived from Scp160p, were identified by comparison of masses from peptides between the cross-link product and non-cross-linked Scp160p, and analysed further by nano-ESI-Q-TOF.

Separation of ribosomal subunits by puromycin/high-salt treatment

Cytosolic ribosomes were incubated for 15 min at 25 °C with 25 μM BMH, and the reaction was stopped by the addition of 10 mM DTT. To separate ribosomes into subunits, they were incubated for 15 min at 30 °C with 2 mM puromycin/GTP in LS-buffer that contained 500 mM potassium acetate. Ribosomal subunits were separated by 12 h of centrifugation at 28 000 rev./min in a SW40 rotor (Beckman) through a linear 12 ml 10–40 % (w/v) sucrose gradient in LS-buffer that contained 500 mM potassium acetate.

RESULTS

The C-terminus of Scp160p is required for ribosome association

Since the presence of mRNA is a prerequisite for the association of Scp160p with ribosomes [2], we assume that certain KH-domains are required for binding to either mRNA or ribosomal RNA. Therefore we constructed yeast strains in which wild-type Scp160p has been replaced by different versions of Scp160p with truncated C-termini. One truncation, Scp160pΔC2, lacks the last two KH-domains and the other, Scp160pΔC4, lacks the last four KH-domains (Figure 1A). Immunoblotting with lysates from strains expressing wild-type or Scp160pΔC2 or Scp160pΔC4 revealed that all three versions of Scp160p were expressed as stable proteins (Figure 1A). Sucrose density centrifugation with cytosol

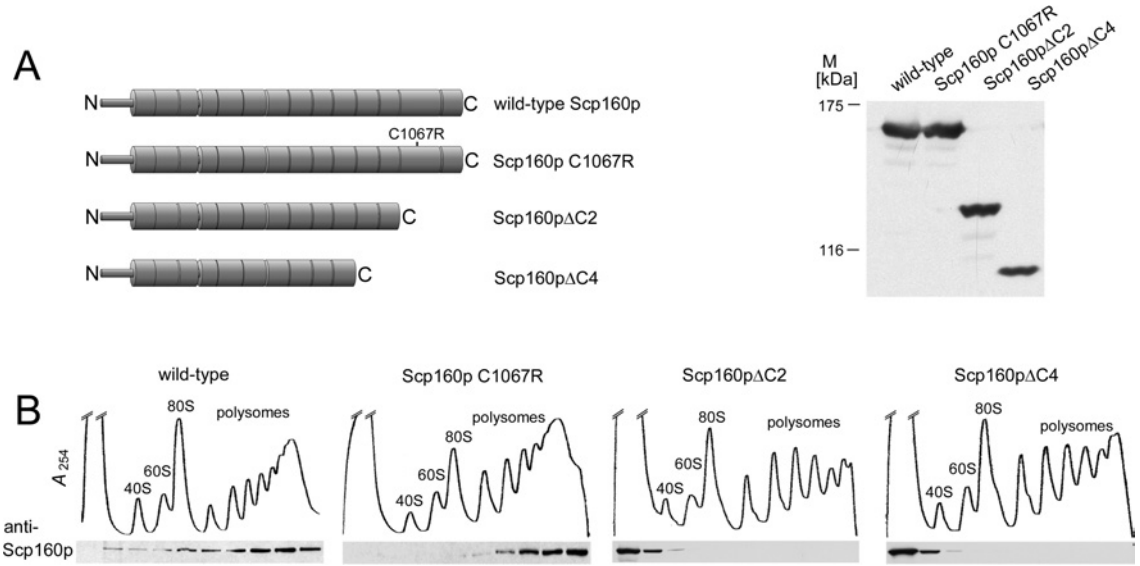

Figure 1 The C-terminus of Scp160p is required for ribosome binding

(**A**) Left-hand panel, schematic view of the domain structure of Scp160p and the generated mutants. Right-hand panel, immunoblot of equal amounts of total extracts from strains expressing wild-type, Scp160pC1067R, Scp160pΔC2 or Scp160pΔC4 probed with Scp160p-specific antibodies. Molecular-mass (M; in kDa) is indicated to the left of the gel. (**B**) Cytosolic extracts from cycloheximide-treated yeast cells were separated on 15–50 % sucrose gradients. The distribution of the rRNA is shown by absorption profiles at 254 nm and the distribution of Scp160p by Western blotting.

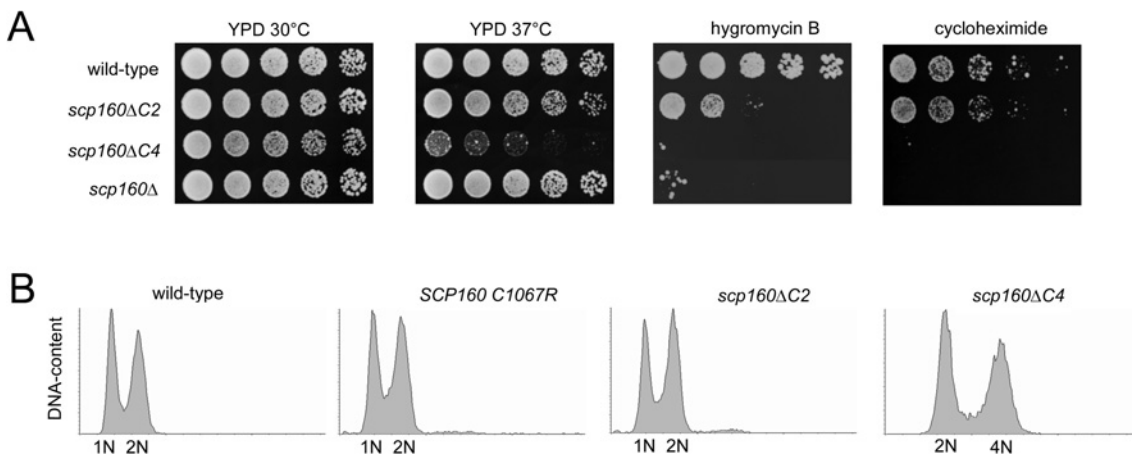

Figure 2 Phenotypes of *scp160* mutants

(**A**) Serial dilution and growth of wild-type, *scp160ΔC2*, *scp160ΔC4* and *scp160Δ* strains on YPD plates at 30 °C and 37 °C, and on YPD plates containing 0.2 μM cycloheximide or 75 μM hygromycin B at 30 °C. (**B**) FACS profiles of propidium-iodide-stained DNA from strains expressing wild-type, Scp160pC1067R, Scp160pΔC2 or Scp160pΔC4.

from cycloheximide-treated wild-type yeast cells showed that Scp160p co-migrated with polysomes (Figure 1B, panel 1). Truncation of the C-terminus of Scp160p including the last two KH-domains abolished this ribosome association completely (Figure 1B, panel 3). Scp160pΔC2 and Scp160pΔC4 were found in fractions 1–3 of a 15–50 % sucrose gradient (Figure 1B, panels 3 and 4). These results suggest that the C-terminus including KH-domains 13 and 14 of Scp160p is required for the association of Scp160p with ribosomes.

Function of Scp160p

In the next set of experiments, we looked for the functional consequences of these truncations. Wild-type, *scp160ΔC2*, *scp160ΔC4* and *scp160Δ* deletion mutants showed similar growth on complete medium (YPD) at 30 °C (Figure 2A). Truncation of the last two KH-domains impaired growth in the presence of the translation inhibitor hygromycin B, whereas growth of this mutant was not affected by the presence of a sublethal concentration of cycloheximide (Figure 2A). Additional truncation of KH domains 11 and 12 or complete deletion of *SCP160* led to a reduced viability in the presence of both hygromycin B and cycloheximide (Figure 2A). These observations are consistent with a function of Scp160p in translation. In addition to the increased sensitivity towards translation inhibitors, *scp160ΔC4* mutants failed to grow on YPD medium at high temperature (37 °C), suggesting that the lack of the last four C-terminal KH domains causes a dominant-negative phenotype.

We analysed further whether the loss of ribosome association correlates with the increase in DNA content, a phenotype that had been observed in *scp160Δ* mutants [11,12]. Wild-type and *scp160ΔC2* cells showed a DNA pattern typical of fast dividing

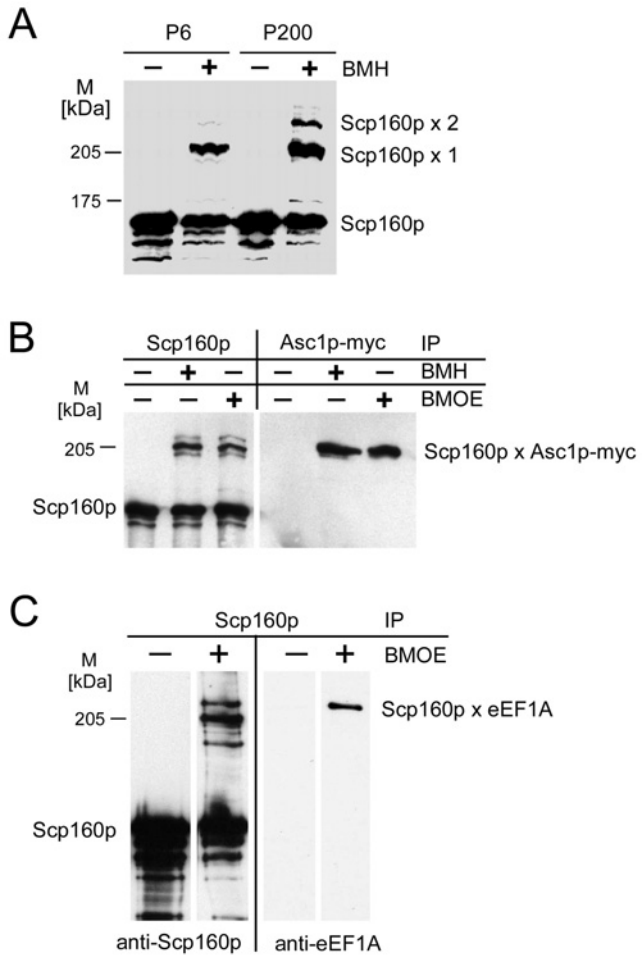

Figure 3 Scp160p is in proximity to the ribosomal protein Asc1p and eEF1A

(**A**) Membranes (P6) or cytosolic ribosomes (P200) were incubated with DMSO or 50 μM BMH in DMSO, and were analysed by Western blotting using Scp160p-specific antibodies. (**B**) Total ribosomes from a strain expressing functional myc-tagged Asc1p were incubated with DMSO, 50 μM BMH or 50 μM BMOE in DMSO. The material was subjected to immunoprecipitation using Scp160p- or myc-specific antibodies. The proteins were separated by SDS/PAGE (6–16 % gels) and probed with Scp160p-specific antibodies. (**C**) DMSO- or BMOE-treated fractions containing total ribosomes were immunoprecipitated with Scp160p-specific antibodies, separated by SDS/PAGE (6–16 % gels) and probed with Scp160p- or eEF1A-specific antibodies. Molecular-mass (M; in kDa) is indicated to the left of the gels.

haploid cells with 1N and 2N peaks (Figure 2B, panels 1 and 3), indicating a normal distribution of chromosomes. Additional truncation of KH domain 11 and 12 resulted in an accumulation of cells with 2N and 4N DNA content (Figure 2B, panel 4). FACS profiles of these cells are very similar to profiles of *scp160*Δ mutants (results not shown; [12]). These results indicate that the presence of Scp160p at polysomes is not directly linked with the chromosome segregation phenotype of *scp160*Δ mutants.

Ribosome-associated Scp160p is in close proximity to Asc1p and eEF1A

Scp160p was found concentrated at membrane-bound and at cytosolic polysomes [2]. To investigate the molecular environment of Scp160p at ribosomes, we prepared fractions enriched in membrane-bound or in cytosolic ribosomes. Incubation of both fractions with the homobifunctional, cysteine-reactive cross-linker BMH resulted in a major cross-link band with an apparent molecular mass of 200 kDa (indicated as Scp160p $\times$ 1, Figure 3A) and a second, slower migrating 215 kDa band (Scp160p $\times$ 2). The 200 kDa band was consistently observed to be the strongest cross-link product. The 215 kDa band showed some variation in intensity between different preparations of ribosomes and cross-link reactions. All cross-link bands were visible in both fractions, suggesting that these cross-link partners of Scp160p are present on ER-membranes and in the cytosol. To identify the major cross-link adducts, we immunoprecipitated Scp160p from BMH-treated ribosomes. Tryptic peptides from the 200 kDa band were analysed by MS. Four peptides, present only in the 200 kDa band, LWDVATGETYQR, ADDDSVTIISAGNDK, DGEIMLWNLAAK and YWLAAATATGIK correspond to the protein Asc1p. Additional peptides that do not correspond to Scp160p were not identified in this band.

To confirm the identity of the 200 kDa cross-link product, we replaced Asc1p functionally with a triple myc-tagged version of Asc1p. The resulting strain, MSY226, displayed a similar growth rate to the isogenic wild-type strain, in contrast with *asc1*Δ mutants, which show a 30 % reduction of growth rate (results not shown).

Ribosomes from MSY226 were incubated with DMSO, BMH or BMOE. BMOE is another homobifunctional cysteine-reactive cross-link agent, which has a short spacer arm of 8 Å (1 Å = 0.1 nm) compared with BMH with a spacer length of 16 Å. Treatment with BMH or BMOE resulted in a major cross-link product of 205 kDa, which was immunoprecipitated by Scp160p- and myc-specific antibodies (Figure 3B).

A similar analysis of the slower migrating 215 kDa band identified five peptides, YQVTVIDAPGHR, QLIVAVNK, LPLQDVYK, IGGIGTVPVGR and FDELLE, which all match the sequence of Tef1/2p, eEF1A. The identity of the 215 kDa cross-link product was confirmed by immunoprecipitating the cross-link products with Scp160p antisera and then immunoblotting. Both Scp160p- and eEF1A-specific antibodies recognized the 215 kDa cross-link product (Figure 3C).

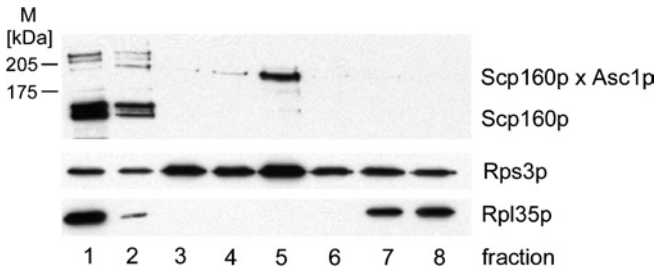

Figure 4 The Scp160p × Asc1p cross-link is an integral part of the small ribosomal subunit

Cytosolic ribosomes corresponding to a D_{600} of 7.5 were incubated for 15 min at 25 °C with 25 μM BMH. The cross-link reaction was stopped by the addition of 10 mM DTT, and ribosomes were separated by puromycin/high-salt treatment and centrifugation through a linear sucrose gradient. Proteins of each fraction were precipitated and separated by SDS/PAGE (6–16 % gels). Blotting with Rps3p-, Rpl35p- and Scp160p-specific antibodies shows the distribution of ribosomes and Scp160p-containing bands. Molecular-mass (M; in kDa) is indicated to the left of the gel.

The cross-link between Scp160p and Asc1p co-migrates with the small ribosomal subunit

After cross-linking and dissociation of ribosomes into subunits by treatment with puromycin/high salt, the major 200 kDa cross-link product co-migrated with Rps3p, a protein of the 40 S subunit (Figure 4, fraction 5). This result suggests that the major cross-link partner of Scp160p, Asc1p, is an integral part of the small

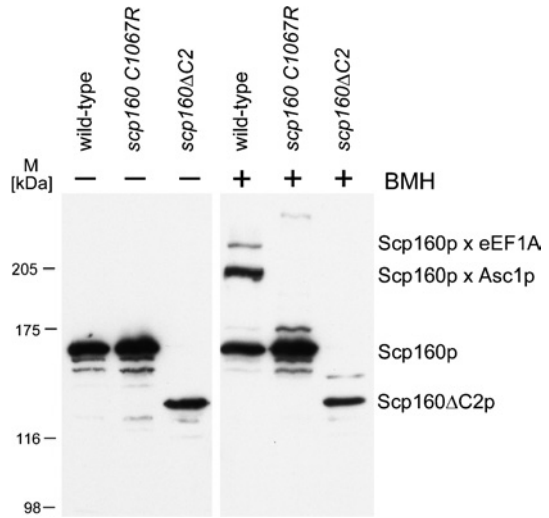

Figure 5 The C-terminus of Scp160p is required for the formation of the Scp160p × Asc1p and Scp160p × eEF1A cross-link products

Total ribosomes from wild-type, Scp160pC1067R or Scp160pΔC2 were incubated with DMSO or 50 μM BMH in DMSO. Proteins were separated by SDS/PAGE (6 % gels) and Scp160p-containing bands were visualized by Western blotting. Molecular-mass (M; in kDa) is indicated to the left of the gel.

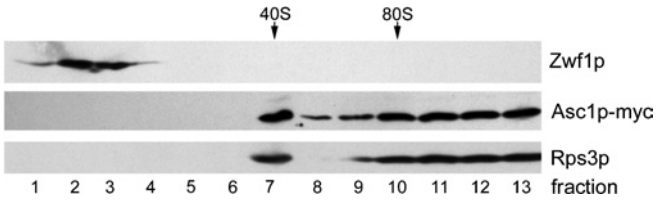

Figure 6 Asc1p is not a free cytosolic protein

Wild-type cells that express Asc1p–myc were grown in YPD medium to exponential phase (D_{600} of 1). After 15 min of incubation with 100 μM cycloheximide, membrane-free cytosol was prepared and separated by centrifugation for 1 h through a 12 ml linear 10–40 % sucrose gradient at 40 000 rev./min in a Beckman SW40 rotor. Arrows indicate the position of 40 S and 80 S ribosomes. Proteins of each fraction were precipitated, separated by SDS/PAGE (6–16 % gels) and probed with Zwf1p-, myc- and Rps3p-specific antibodies.

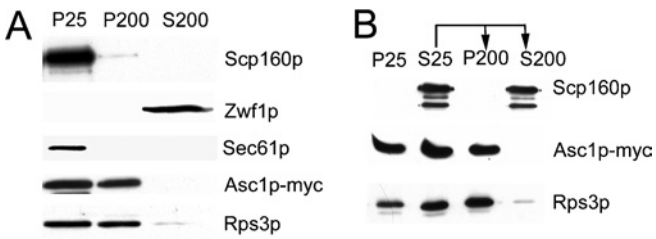

Figure 7 The majority of Asc1p is bound to ribosomes

(**A**) Wild-type cells expressing Asc1p–myc were grown to exponential phase. Total extract was prepared and separated by consecutive centrifugation into a membrane containing pellet (P25), membrane-free ribosomes (P200) and ribosome-free cytosol (S200). Fractions were separated by SDS/PAGE (6–16 % gels), and indicated proteins are detected by immunoblotting. (**B**) P25 was incubated with 1 % (v/v) Triton X-100, 500 mM NaCl and centrifuged for 20 min at 25 000 ***g*** (S25). An identical amount of the resulting supernatant was separated into P200 and S200 by centrifugation for 20 min at 200 000 ***g***.

ribosomal subunit. These observations are consistent with results reporting the presence of Asc1p or its orthologue, Cpc2, from *Schizosaccharomyces pombe* in ribosomes [15,18–20]. A number of cross-link bands co-migrated with non-cross-linked Scp160p in fractions 1 and 2, suggesting that these proteins are ribosome-associated factors that are released from ribosomes upon subunit dissociation.

Cys^{1067} of KH-domain 13 interacts with Asc1p or eEF1A

Scp160p contains four cysteine residues at positions 188, 497, 666 and 1067. Cys^{1067} is located in KH-domain 13 (Figure 1A). Since the C-terminus of Scp160p, containing KH domains 13 and 14, is essential for ribosome binding, we asked, if cross-linking to Asc1p and eEF1A occurs at cys^{1067}. Therefore we used a yeast strain expressing full-length Scp160p with arginine substituted for Cys^{1067} (Figure 1A). Scp160pC1067R is expressed at similar level as wild-type Scp160p (Figure 1A, lane 2) and cells showed a normal DNA content (Figure 2B, panel 2). The mutated protein co-migrated with polysomes, in a similar manner to Scp160p from a wild-type S288c strain (Figure 1B, panel 2), indicating proper interaction of Scp160pC1067R with polysomes. Incubation of total ribosomes from the S288c strain with BMH resulted in the appearance of the major 200 kDa and the 215 kDa cross-link-bands (Figure 5). Neither band was detected in BMH-treated extracts of the strain expressing the *scp160C1067R* allele or *scp160ΔC2*, suggesting that cys^{1067} can form a cross-link with Asc1p or eEF1A.

Asc1p is stably associated with ribosomes

The cross-link product of Scp160p and Asc1p co-migrated with the 40 S subunit of puromycin/high-salt-treated ribosomes (Figure 4), suggesting that Asc1p is an integral part of ribosomes. To analyse the intracellular distribution of Asc1p carefully, we isolated membrane-free cytosol from fast-growing cells and compared the distribution of the 40 S protein Rps3p with that of Asc1p–myc and the cytosolic enzyme glucose-6-phospate dehydrogenase (Zwf1p) by sucrose density centrifugation. Light fractions contained the cytosolic marker protein Zwf1p, but were free of Rps3p and Asc1p–myc (Figure 6). Under these conditions all detected Asc1p–myc co-migrated with Rps3p, indicating that the majority of cytosolic Asc1p is associated with ribosomes. In a centrifugation assay, the majority of Scp160p co-migrated with membranes (Figure 7A). Asc1p–myc was found in ribosome-containing fractions, membranes and cytosol, but not in ribosome-free cytosol (Figure 7A). After solubilization of membranes (P25) with a 1 % (v/v) Triton X-100 and 500 mM NaCl-containing buffer Asc1p–myc co-sedimented with ribosomes, whereas Scp160p was completely released into the supernatant (Figure 7B). These results indicate that Asc1p–myc at membranes remained associated with ribosomes under conditions that removed Scp160p from ribosomes.

Ribosome-association of Asc1p depends on cell activity

All experiments described so far were performed with fast-growing yeast cells harvested in exponential growth phase. A proteomic study has shown that Asc1p protein levels were up-regulated during growth initiation [21]. Therefore we asked if the distribution of Asc1p changes between cells in exponential or stationary growth phase. Total cell extracts from equal amounts of cells were separated into a pellet and a supernatant fraction. Under both conditions, all detected Rpl35p and the vast majority of Rps3p were found in the pellet fractions (Figure 8), indicating

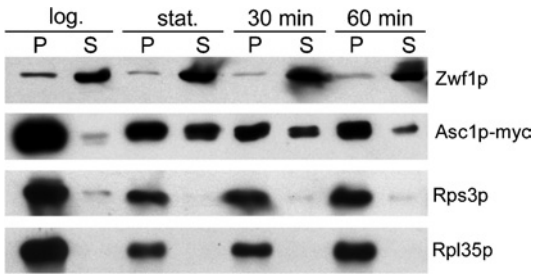

Figure 8 Ribosome-association of Asc1p depends on cell activity

Total cellular extracts from cells corresponding to a D_{600} of 0.5 grown in YPD medium to exponential phase (D_{600} of 1), cells grown for 12 h at stationary phase or cells from stationary phase diluted at the indicated times to a D_{600} of 0.1 into fresh YPD medium were centrifuged for 60 min at 200 000 ***g***. Equal amounts of the pellet (P200) and supernatant (S200) were separated by SDS/PAGE (6–16 % gels) and Asc1p–myc, Zwf1p, Rps3p and Rpl35p were detected by Western blotting.

nearly complete sedimentation of ribosomes and ribosomal subunits. The vast majority of Asc1p–myc from exponential growth phase cells was found in the ribosome-containing fraction (Figure 8). Extracts from the same amount of cells from stationary phase showed a reduction of Rps3p, Rpl35p and Asc1p–myc proteins. Interestingly, under these conditions, a significant amount of Asc1p–myc was present in the ribosome-free supernatant fraction, whereas ribosomes remained in the pellet fraction. In cells from stationary phase, shifted for 30 min into fresh medium, the amount of ribosome-free Asc1p–myc decreased, and after 60 min, most Asc1p–myc was found in the ribosome-containing pellet fraction.

Ribosome binding of Scp160p in *asc1*Δ mutants

To address the functional consequences of the loss of Asc1p on the interaction of Scp160p with polysomes, and in particular to distinguish if Asc1p is required for Scp160p's association with ribosomes, we analysed ribosome association of Scp160p in W303 wild-type and isogenic *asc1Δ* strains. Extracts containing cytosol were prepared from cycloheximide-treated yeast cells at exponential growth phase. Sucrose density gradient fractionation of extracts from wild-type cells showed that Scp160p co-migrated with fractions containing ribosomal subunits, mono- and polysomes (Figure 9A). However, Scp160p was not enriched in fractions containing the 40 S ribosomal subunits or in the lighter fractions. Fractions 1 and 2 contained only small amounts of Scp160p. In contrast, cytosolic extracts from *asc1Δ* mutants showed a strong decrease in association of Scp160p with mono- and poly-somes. We observed a clear shift of Scp160p from fractions 6–10 into lighter fractions 2 and 3 (Figure 9B). Similar results were obtained with solubilized ribosomes from membranes of wild-type and *asc1Δ* strains (results not shown). In summary, these results suggest a reduced affinity of Scp160p for mono- and poly-somes lacking Asc1p.

DISCUSSION

The C-terminus of Scp160p, comprising KH-domains 13 and 14, is required for ribosome binding

Truncation of the C-terminus of Scp160p, containing KH-domains 13 and 14, led to dissociation of Scp160p from ribosomes, suggesting that these two KH-domains are involved in the interaction of Scp160p with ribosomes. This idea is consistent with the cross-linking of cys^{1067}, located in KH-domain 13, either to ribosome-associated Asc1p or to eEF1A.

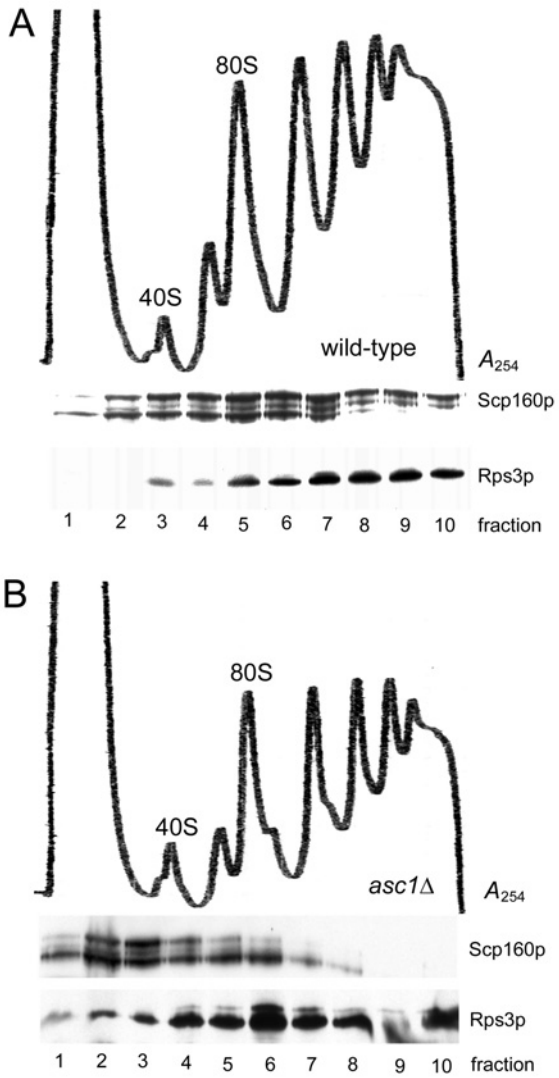

Figure 9 Asc1p is required for efficient interaction of Scp160p with polysomes

(**A**) Membrane-free cytosol from cycloheximide-treated wild-type (W303) cells was separated on a 15–50 % (w/v) sucrose gradient. The rRNA profile was monitored by measuring the absorbance at 254 nm. Proteins of each fraction were precipitated, separated by SDS/PAGE (6–16 % gels) and probed with Scp160p- and Rps3p-specific antibodies. (**B**) Membrane-free cytosol from cycloheximide-treated *asc1*Δ cells was treated as described in (**A**).

Position of Scp160p at the ribosome

Scp160p is in close proximity to Asc1p at the 40 S ribosomal subunit and to eEF1A, which is located at the interface of the small and large ribosomal subunits [22]. By treatment with BMOE, cys^{1067} in Scp160p can form a cross-link with Asc1p or with eEF1A, suggesting that a single binding site of Scp160p at the ribosome is positioned within 8 Å of Asc1p and eEF1A. As only the crystal structures of bacterial and archaeal ribosomes are available and Asc1p is only found in eukaryotes, the precise position of Asc1p in the 40 S subunit is not yet known.

Function of Scp160p at the ribosome

Accumulation of Scp160p at polysomes and cross-linking of Scp160p to eEF1A at ribosomes indicate a function of Scp160p in translation. The fact that a similar KH-domain containing

protein from mammalian cells, vigilin, was purified in a complex with eEF1A and tRNA [4,23] indirectly supports an interaction of vigilin/Scp160p with translation elongation factor at the ribosome. Increased sensitivity of *scp160*Δ mutants to the translation inhibitors, hygromycin B and cycloheximide, is consistent with a role of Scp160p in translation. The aberration of chromosome segregation in *scp160*Δ*C4* and *scp160*Δ mutants is not a direct result of loss of ribosome binding. That defect must be due either to a defect of Scp160p function when it is associated with the ribosome or to a role of Scp160p that is independent of the ribosome. The lack of sensitivity to the translation inhibitor cycloheximide with *scp160*Δ*C2* might favour the latter possibility. However, the chromosomal abnormalities of *scp160*Δ*C4* and *scp160*Δ mutants [11] make any interpretation of the observed phenotypes difficult.

Interaction of Scp160p and Asc1p at ribosomes

Asc1p is present in approx. 100000 copies/cell, a value consistent with the steady-state abundance of most other ribosomal proteins in fast-dividing yeast cells [24]. Our cell fractionation experiments, using fast-growing cells, showed that cytosolic and membrane-bound Asc1p is associated tightly with ribosomes. These results are consistent with the previous characterization of Asc1p as a component of the ribosomal 40 S subunit [15,18,19].

Asc1p is not required for general translation, since *asc1*Δ-mutants are viable [15]. Deletion of the *ASC1* orthologue in *Schizosaccharomyces pombe*, *CPC2*, led to a reduced translation of ribosomal L25 mRNA [20] suggesting that these proteins contribute to translational regulation of specific mRNAs.

Asc1p is a functional homologue of mammalian RACK1 (receptor of activated C kinase) [25]. Common to these proteins is their domain structure. They are built almost exclusively of highly conserved repeating units, called WD40 motifs [26]. It has been suggested that two or more of these domains bind simultaneously to a large variety of different proteins (summarized in [27,28]). RACK1 was initially identified as a protein that was capable of interaction with PKC (protein kinase C) isoforms [29,30], and it had been shown that RACK1 shuttles βIIPKC between different intracellular sites [30].

Assuming that the proposed function as an adaptor molecule is conserved, Asc1p could serve as a binding platform for proteins involved in signalling at ribosomes. Two recent studies [31,32] and unpublished work by M. R. Pool, U. Bach, B. Dobberstein and M. Seedorf, support the presence of RACK1 at mammalian ribosomes. It has been shown that ribosome-associated RACK1 interacts with eIF6 and that activated βIIPKC phosphorylates eIF6 [32]. Phosphorylation of eIF6 and subsequent release from 60 S subunits increase the translation activity of ribosomes [32]. In addition to this function Asc1p/RACK1 might recruit other signalling molecules to ribosomes as well, and other substrates for modification, e.g. eEF1A and Scp160p/vigilin, might exist.

Asc1p can provide a direct binding surface for Scp160p at ribosomes. Alternatively, the lack of Asc1p-dependent factors at ribosomes might abolish modifications at ribosomal proteins, translation factors or Scp160p itself, which may cause loss of efficient interaction between Scp160p and polysomes.

Accumulation of Scp160p at ER membranes suggests an increased affinity of Scp160p for mRNAs encoding secreted and membrane proteins [2]. Selectivity of mRNA binding is supported further by co-purification of Scp160p with specific mRNAs [6,8]. We suggest a model in which the activity of KH-domains 1–12 helps Scp160p to select mRNAs and that the association of the C-terminus of Scp160p with ribosomes and the proximity to eEF1A provides a mechanism for translational regulation of these mRNAs. However, future experiments are required to identify mRNAs that are translationally regulated by Scp160p.

The intracellular distribution of Asc1p changes in cells entering stationary phase. In these cells, a significant amount of Asc1p was found in a ribosome-free fraction. The ribosome-free fraction of Asc1p may function in a similar manner as described for mammalian RACK1, namely the connection of substrates with different molecules involved in signal transduction and their relocation [30,33]. Genetic studies support a regulatory function of Asc1p/Cpc2 in adaptation of cells to changes in the environment [15,25,34–36]. Rapid execution of these changes could occur at the level of translational regulation [20], and the mRNA-binding protein Scp160p might function as a linker between specific mRNAs, the translation machinery and incoming signals.

In summary, the adaptor protein Asc1p could be a binding platform at ribosomes, which recruits factors such as the RNA-binding protein Scp160p, translation factors and molecules involved in signal transduction. The connection of Scp160p-bound mRNAs with translation factors and Asc1p at ribosomes could provide a mechanism to regulate translation activities of ribosome populations programmed with specific mRNAs.

We thank Kalpana Chakraburtty for antibodies against *Saccharomyces cerevisiae* eEF1A, Thomas Ruppert from the Biomolecular Chemistry Unit at ZMBH for mass spectrometry and Christoph Jüschke, Blanche Schwappach, Martin Pool and Bernhard Dobberstein for their comments on the manuscript, and continued support by Bernhard Dobberstein and the DFG (SFB 352).

REFERENCES

1 Lang, B. D. and Fridovich-Keil, J. L. (2000) Scp160p, a multiple KH-domain protein, is a component of mRNP complexes in yeast. Nucleic Acids Res. **28**, 1576–1584
2 Frey, S., Pool, M. and Seedorf, M. (2001) Scp160p, an RNA-binding, polysome-associated protein, localizes to the endoplasmic reticulum of *Saccharomyces cerevisiae* in a microtubule-dependent manner. J. Biol. Chem. **276**, 15905–15912
3 Lang, B. D., Li, A., Black-Brewster, H. D. and Fridovich-Keil, J. L. (2001) The brefeldin A resistance protein Bfr1p is a component of polyribosome-associated mRNP complexes in yeast. Nucleic Acids Res. **29**, 2567–2574
4 Kruse, C., Grünweller, A., Willkomm, D. K., Pfeiffer, T., Hartmann, R. K. and Müller, P. K. (1998) tRNA is entrapped in similar, but distinct, nuclear and cytoplasmic ribonucleoprotein complexes, both of which contain vigilin and elongation factor 1α. Biochem. J. **329**, 615–621
5 Kruse, C., Willkomm, D. K., Grünweller, A., Vollbrandt, T., Sommer, S., Busch, S., Pfeiffer, T., Brinkmann, J., Hartmann, R. K. and Müller, P. K. (2000) Export and transport of tRNA are coupled to a multi-protein complex. Biochem. J. **346**, 107–115
6 Mendelsohn, B. A., Li, A. M., Vargas, C. A., Riehman, K., Watson, A. and Fridovich-Keil, J. L. (2003) Genetic and biochemical interactions between SCP160 and EAP1 in yeast. Nucleic Acids Res. **31**, 5838–5847
7 Cosentino, G. P., Schmelzle, T., Haghighat, A., Helliwell, S. B., Hall, M. N. and Sonenberg, N. (2000) Eap1p, a novel eukaryotic translation initiation factor 4E-associated protein in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **20**, 4604–4613
8 Li, A. M., Watson, A. and Fridovich-Keil, J. L. (2003) Scp160p associates with specific mRNAs in yeast. Nucleic Acids Res. **31**, 1830–1837
9 Guo, M., Aston, C., Burchett, S. A., Dyke, C., Fields, S., Rajarao, S. J., Uetz, P., Wang, Y., Young, K. and Dohlman, H. G. (2003) The yeast G protein α subunit Gpa1 transmits a signal through an RNA binding effector protein Scp160. Mol. Cell **12**, 517–524
10 Irie, K., Tadauchi, T., Takizawa, P. A., Vale, R. D., Matsumoto, K. and Herskowitz, I. (2002) The Khd1 protein, which has three KH RNA-binding motifs, is required for proper localization of ASH1 mRNA in yeast. EMBO J. **21**, 1158–1167
11 Wintersberger, U., Kuhne, C. and Karwan, A. (1995) Scp160p, a new yeast protein associated with the nuclear membrane and the endoplasmic reticulum, is necessary for maintenance of exact ploidy. Yeast **11**, 929–944
12 Cortes, A., Huertas, D., Fanti, L., Pimpinelli, S., Marsellach, F. X., Pina, B. and Azorin, F. (1999) DDP1, a single-stranded nucleic acid-binding protein of *Drosophila*, associates with pericentric heterochromatin and is functionally homologous to the yeast Scp160p, which is involved in the control of cell ploidy. EMBO J. **18**, 3820–3833

13 Sikorski, R. S. and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122**, 19–27
14 Winston, F., Dollard, C. and Ricupero-Hovasse, S. L. (1995) Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. Yeast **11**, 53–55
15 Chantrel, Y., Gaisne, M., Lions, C. and Verdiere, J. (1998) The transcriptional regulator Hap1p (Cyp1p) is essential for anaerobic or heme-deficient growth of *Saccharomyces cerevisiae*: genetic and molecular characterization of an extragenic suppressor that encodes a WD repeat protein. Genetics **148**, 559–569
16 Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K. and Schiebel, E. (1999) Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. Yeast **15**, 963–972
17 Opitz, C., Di Cristina, M., Reiss, M., Ruppert, T., Crisanti, A. and Soldati, D. (2002) Intramembrane cleavage of microneme proteins at the surface of the apicomplexan parasite *Toxoplasma gondii*. EMBO J. **21**, 1577–1585
18 Link, A. J., Eng, J., Schieltz, D. M., Carmack, E., Mize, G. J., Morris, D. R., Garvik, B. M. and Yates, 3rd, J. R. (1999) Direct analysis of protein complexes using mass spectrometry. Nat. Biotechnol. **17**, 676–682
19 Inada, T., Winstall, E., Tarun, Jr, S. Z., Yates, 3rd, J. R., Schieltz, D. and Sachs, A. B. (2002) One-step affinity purification of the yeast ribosome and its associated proteins and mRNAs. RNA **8**, 948–958
20 Shor, B., Calaycay, J., Rushbrook, J. and McLeod, M. (2003) Cpc2/RACK1 is a ribosome-associated protein that promotes efficient translation in *Schizosaccharomyces pombe*. J. Biol. Chem. **278**, 49119–49128
21 Brejning, J. and Jespersen, L. (2002) Protein expression during lag phase and growth initiation in *Saccharomyces cerevisiae*. Int. J. Food Microbiol. **75**, 27–38
22 Stark, H., Rodnina, M. V., Rinke-Appel, J., Brimacombe, R., Wintermeyer, W. and van Heel, M. (1997) Visualization of elongation factor Tu on the *Escherichia coli* ribosome. Nature (London) **389**, 403–406
23 Kruse, C., Grünweller, A., Notbohm, H., Kugler, S., Purschke, W. G. and Müller, P. K. (1996) Evidence for a novel cytoplasmic tRNA-protein complex containing the KH-multidomain protein vigilin. Biochem. J. **320**, 247–252
24 Warner, J. R. (1999) The economics of ribosome biosynthesis in yeast. Trends Biochem. Sci. **24**, 437–440
25 Hoffmann, B., Mosch, H. U., Sattlegger, E., Barthelmess, I. B., Hinnebusch, A. and Braus, G. H. (1999) The WD protein Cpc2p is required for repression of Gcn4 protein activity in yeast in the absence of amino-acid starvation. Mol. Microbiol. **31**, 807–822
26 Neer, E. J., Schmidt, C. J., Nambudripad, R. and Smith, T. F. (1994) The ancient regulatory-protein family of WD-repeat proteins. Nature (London) **371**, 297–300
27 Schechtman, D. and Mochly-Rosen, D. (2001) Adaptor proteins in protein kinase C-mediated signal transduction. Oncogene **20**, 6339–6347
28 McCahill, A., Warwicker, J., Bolger, G. B., Houslay, M. D. and Yarwood, S. J. (2002) The RACK1 scaffold protein: a dynamic cog in cell response mechanisms. Mol. Pharmacol. **62**, 1261–1273
29 Ron, D., Chen, C. H., Caldwell, J., Jamieson, L., Orr, E. and Mochly-Rosen, D. (1994) Cloning of an intracellular receptor for protein kinase C: a homolog of the β subunit of G proteins. Proc. Natl. Acad. Sci. U.S.A. **91**, 839–843
30 Ron, D., Jiang, Z., Yao, L., Vagts, A., Diamond, I. and Gordon, A. (1999) Coordinated movement of RACK1 with activated βIIPKC. J. Biol. Chem. **274**, 27039–27046
31 Angenstein, F., Evans, A. M., Settlage, R. E., Moran, S. T., Ling, S. C., Klintsova, A. Y., Shabanowitz, J., Hunt, D. F. and Greenough, W. T. (2002) A receptor for activated C kinase is part of messenger ribonucleoprotein complexes associated with polyA-mRNAs in neurons. J. Neurosci. **22**, 8827–8837
32 Ceci, M., Gaviraghi, C., Gorrini, C., Sala, L. A., Offenhauser, N., Carlo Marchisio, P. and Biffo, S. (2003) Release of eIF6 (p27(BBP)) from the 60 S subunit allows 80 S ribosome assembly. Nature (London) **426**, 579–584
33 Liedtke, C. M., Yun, C. H., Kyle, N. and Wang, D. (2002) Protein kinase Cε-dependent regulation of cystic fibrosis transmembrane regulator involves binding to a receptor for activated C kinase (RACK1) and RACK1 binding to Na^+/H^+ exchange regulatory factor. J. Biol. Chem. **277**, 22925–22933
34 Hoffmann, B., Wanke, C., Lapaglia, S. K. and Braus, G. H. (2000) c-Jun and RACK1 homologues regulate a control point for sexual development in *Aspergillus nidulans*. Mol. Microbiol. **37**, 28–41
35 Goossens, A., Dever, T. E., Pascual-Ahuir, A. and Serrano, R. (2001) The protein kinase Gcn2p mediates sodium toxicity in yeast. J. Biol. Chem. **276**, 30753–30760
36 McLeod, M., Shor, B., Caporaso, A., Wang, W., Chen, H. and Hu, L. (2000) Cpc2, a fission yeast homologue of mammalian RACK1 protein, interacts with Ran1 (Pat1) kinase to regulate cell cycle progression and meiotic development. Mol. Cell. Biol. **20**, 4016–4027

Received 22 December 2003/24 February 2004; accepted 11 March 2004
Published as BJ Immediate Publication 11 March 2004, DOI 10.1042/BJ20031962