Structural and functional similarities between the central eukaryotic initiation factor (eIF)4A-binding domain of mammalian eIF4G and the eIF4A-binding domain of yeast eIF4G

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The translation eukaryotic initiation factor (eIF)4G of the yeast *Saccharomyces cerevisiae* interacts with the RNA helicase eIF4A (a member of the DEAD-box protein family; where DEAD corresponds to Asp-Glu-Ala-Asp) through a C-terminal domain in eIF4G (amino acids 542–883). Mammalian eIF4G has two interaction domains for eIF4A, a central domain and a domain close to the C-terminus. This raises the question of whether eIF4A binding to eIF4G is conserved between yeast and mammalian cells or whether it is different. We isolated eIF4G1 mutants defective in eIF4A binding and showed that these mutants are strongly impaired in translation and growth. Extracts from mutants displaying a temperature-sensitive phenotype for growth have low *in vitro* translation activity, which can be restored by addition of the purified eIF4G1–eIF4E complex, but not by eIF4E alone. Analysis of mutant eIF4G$_{542–883}$ proteins defective in eIF4A binding shows that the interaction of yeast eIF4A with eIF4G1 depends on amino acid motifs that are conserved between the yeast eIF4A-binding site and the central eIF4A-binding domain of mammalian eIF4G. We show that mammalian eIF4A binds tightly to yeast eIF4G1 and, furthermore, that mutant yeast eIF4G$_{542–883}$ proteins, which do not bind yeast eIF4A, do not interact with mammalian eIF4A. Despite the conservation of the eIF4A-binding site in eIF4G and the strong sequence conservation between yeast and mammalian eIF4A (66 % identity; 82 % similarity at the amino acid level) mammalian eIF4A does not substitute for the yeast factor *in vivo* and is not functional in a yeast *in vitro* translation system.

Key words: cell-free translation, DEAD-box protein, protein–protein interactions, RNA helicase, temperature-sensitive mutant.

INTRODUCTION

Translation initiation in eukaryotic cells is catalysed by a large number of eukaryotic initiation factors (eIFs). These polypeptides catalyse the binding of the initiator Met-tRNAi to the 40 S ribosomal subunit, the interaction of the resulting 43 S initiation complex with the 5′ region of mRNA, the scanning of the mRNA by the 43 S initiation complex for the initiator AUG codon and the joining of the 60 S ribosomal subunit to the initiation complex to generate an 80 S ribosome competent for polypeptide chain elongation (for reviews see [1,2]). The initiation factor eIF4G acts as a scaffold protein that recruits other translation initiation factors close to the cap structure (5′ m7Gppp) on the mRNA and prepares the mRNA for binding to the 43 S initiation complex (for reviews see [3–5]). The binding of eIF4G to the 5′ end of mRNA is mediated by (1) eIF4E, which recognizes the cap structure [6] (for a review see [7]), (2) the polyadenylated [poly(A)$^+$]-binding protein (PABP) associated with eIF4G and the poly(A)$^+$ tail at the 3′ end of mRNA [8], and (3) the direct interaction of eIF4G with the mRNA [9]. Both eIF4E and PABP have their binding sites on the N-terminal half of eIF4G [10,11]. This part of eIF4G is required for capped-mRNA translation [6], but is dispensable for internal initiation of translation [12]. The central region of eIF4G carries binding sites for the RNA helicase eIF4A [13–15] and the multi-subunit factor eIF3 [6,13,16], which itself binds to the small ribosomal subunit and recruits it (as the 43 S initiation complex) for binding to the mRNA. The C-terminal region of mammalian eIF4G carries a second binding site for eIF4A [13] and a binding site for the protein kinase Mnk1 (‘mitogen-activated protein kinase-interacting kinase’), which phosphorylates eIF4E [17].

In the yeast *Saccharomyces cerevisiae*, eIF4G is encoded by two genes, *TIF4631* and *TIF4632* [18]. The gene products, eIF4G1 and eIF4G2 (collectively called eIF4G), are 50 % identical at the amino acid level. The expression of either one of the two genes is essential for growth [18], as is the interaction of eIF4A with eIF4G [14,15]. Yeast eIF4G lacks a domain present in the C-terminal region of mammalian eIF4G [17], which carries a second binding site for eIF4A [13] and a binding site for the protein kinase Mnk1, which phosphorylates eIF4E [17]. Functional studies revealed that this domain of mammalian eIF4G greatly facilitates, but is not essential for, translation initiation, whereas the central domain is essential for cap-dependent and internal initiation [6,13]. Despite much effort, eIF3 binding to yeast eIF4G (in contrast with mammalian eIF4G) can not yet be demonstrated (M. Altmann and H. Trachsel, unpublished work).

The initiation factor eIF4A is a member of the DEAD-box protein family (where DEAD corresponds to Asp-Glu-Ala-Asp). Yeast cells contain two and mammalian cells contain three closely related eIF4A proteins. These proteins share sequence

Abbreviations used: eIF, eukaryotic initiation factor; 5-FOA, 5-fluoro-orotic acid; GST, glutathione-S-transferase; poly(A)$^+$, polyadenylated; PABP, poly(A)$^+$-binding protein; SPR, surface plasmon resonance; TBS, tris-buffered saline.

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elements, and function in a large number of biochemical reactions [19] (for a review see [20]). eIF4A was shown to have RNA unwinding (RNA helicase) activity in the presence of eIF4B *in vitro* [21]. Since mRNA translation is inhibited by RNA secondary structures in the 5′ untranslated region (for a review see [22]) it is assumed that eIF4A and eIF4B play a crucial role in RNA secondary-structure melting during the scanning process. However, the step(s) at which eIF4A exerts its function(s) in initiation have not yet been determined at the molecular level.

Like *S. cerevisiae*, mammalian cells encode two types of eIF4G molecules, which are 46 % identical [23]. Overall, identity at the amino acid level between the yeast and mammalian eIF4Gs is below 25 %, but reaches approx. 40 % in the central eIF4A-binding domain. Despite this sequence conservation in the central eIF4A-binding site, the binding of eIF4A to eIF4G in yeast and mammals might be different. This speculation is mainly based on the finding that (1) yeast eIF4G lacks the second eIF4A-binding site, (2) p97, which lacks the C-terminal eIF4A-binding site, acts as an inhibitor of translation initiation in mammalian cells [24–26], (3) eIF4AIII, which binds only to the central eIF4A-binding site on mammalian eIF4G, does not support translation [27], and (4) eIF4G isolated from *S. cerevisiae* cell extracts carries eIF4E, but not eIF4A, whereas the mammalian factor forms a stable complex with eIF4A and eIF4E that has been termed eIF4F.

In the present study we analyse the eIF4A-binding site of eIF4G1 of the yeast *S. cerevisiae* in detail, show that the interaction of eIF4A with eIF4G is conserved between the yeast and the central eIF4A-binding site of mammalian eIF4G and confirm that this interaction is essential for translation in yeast.

EXPERIMENTAL

Yeast strains, media and genetic manipulations

Transformation of yeast cells was performed by the lithium acetate method [28]. Yeast culture media were prepared according to standard recipes as described previously [29]. The strains used in the present study are derivatives of the wild-type strain CWO4 (Table 1).

Plasmid construction

The construction of the plasmids pGEX(2λT)-GST–eIF4G1$_{542–883}$ (where GST corresponds to glutathione-S-transferase) and pYEX(4T)-GST–eIF4G1$_{542–883}$ has been described previously [14]. pGEX-GST–eIF4G1$_{542–883}$ mutant plasmids were constructed by subcloning of the *Msc*I–*Eco*RI fragments of pYEX-GST–eIF4G1$_{542–883}$ mutant plasmids into the *Msc*I–*Eco*RI sites of the pGEX-6P-1 vector (Pharmacia) (*Msc*I cleaves in the middle of the GST open reading frame). The plasmids were then sequenced using both pGEX-3′ and pGEX-5′ primers (Pharmacia). pGEX-GST–eIF4G1 (full-length) constructs containing the mutations were made by replacing the *Nhe*I–*Psh*AI fragment of pGEX-GST–eIF4G1 [15] by those of pGEX-GST–eIF4G1$_{542–883}$ mutant plasmids. pRS313-eIF4G1 mutant plasmids were obtained by replacing the *Nco*I–*Eco*RI fragment of pRS313-eIF4G1$_{1–539}$ (a C-terminal deletion mutant) by the *Nco*I–*Eco*RI fragment of mutant pGEX-GST–eIF4G1 plasmids. Plasmid pGEX-TIF1 was constructed as previously described [15]. The plasmid pGEX-m4AI was prepared by cloning the 1.4 kb *Eco*RI fragment of pMTM4AI [30] into the *Eco*RI site of vector pGEX-6P-3. pEGKG-GST–m4AI was made by subcloning the *Bam*HI–*Sal*I fragment of pGEX-GST–m4AI into the vector pEGKG [31].

Isolation of eIF4G1$_{542–883}$ mutants defective in interaction with eIF4A

Plasmid pYEX-GST–eIF4G1$_{542–883}$ (50 μg) was mutagenized by incubation for 3 h at 70 °C in a solution containing 1 M hydroxylamine/HCl, 10 mM EDTA and 0.5 M sodium hydroxide. BSA (100 μg) was added and the plasmid DNA was purified by three rounds of phenol/chloroform extraction followed by ethanol precipitation. The mutagenized and non-mutagenized (control) plasmids were used to transform the yeast strain SS10-3F (Table 1). Transformants were replica-plated on minimal medium containing 2 % (w/v) glucose and 0.5 mM copper sulphate. Colonies able to grow in the presence of copper (567 clones) were analysed by *in situ* Western blotting [32], using polyclonal rat anti-(yeast GST–eIF4G1$_{542–883}$) antibodies. In total 217 transformants (38 %) expressed a protein that was recognized by the antibodies. SDS/PAGE followed by immunoblotting was then performed in order to select colonies expressing full-length GST–eIF4G1$_{542–883}$. Following this protocol, 25 transformants were identified. Mutagenized plasmids were rescued from these transformants as follows: cells were taken directly from a plate with a pipette tip and resuspended in 200 μl of extraction buffer [10 mM Tris/HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 2 % (w/v) Triton X-100 and 1 % (w/v) SDS]. Glass beads (400 μl) and 200 μl of phenol/chloroform were added and cells were vigorously vortex-mixed for 7 min. After centrifugation for 10 min at 15000 ***g***, 140 μl was transferred to a new Eppendorf tube and DNA was precipitated with 500 μl of ethanol/7.5 M ammonium acetate (6:1, v/v). Pelleted DNA was resuspended in 10 μl of water and was used to transform competent *Escherichia coli* cells. Plasmid DNA isolated from *E. coli* transformants was used to re-transform yeast strain SS10-3F. Only plasmids that gave rise to the synthesis of full-length GST–eIF4G1$_{542–883}$ upon re-transformation (19 plasmids) were selected.

Western-blot analysis

Western-blot analysis was performed as previously described [33]. The primary antibodies (used at 1:1000 dilution) were: polyclonal rat anti-(yeast eIF4A) [34], polyclonal rat anti-(yeast GST–eIF4G1$_{542–883}$) [33] and monoclonal mouse anti-(mouse eIF4A) [35].

Measurement of protein interaction *in vitro*

Protein expression, purification and *in vitro* binding assays were performed essentially as described previously [15].

Surface plasmon resonance (SPR)

All SPR assays were performed in an IAsys machine (Affinity Sensors, Thermobio Inc., Cambridge, U.K.). Approx. 60 ng of purified yeast eIF4A was immobilized on a 1-ethyl-3-(3-dimethylaminopropyl) carbodi-imide/*N*-hydroxysuccinimide-activated carboxylate cuvette (chip) according to the manufacturer's instructions. Wild-type and mutant eIF4G1$_{542–883}$ proteins were then added in 200 μl of PBS. The chip was regenerated by washing with 200 μl of 1 mM formic acid followed by three washes with 200 μl of PBS. The response of the eIF4A-coated chip with 200 μl of PBS was fixed as baseline.

Cell-free translation

Preparation of yeast translation systems and translation reactions were performed according to a previously described method [34].

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Table 1 ***S. cerevisiae*** **strains**

Strain	Genotype	Reference
CWO4	*MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3*	[43]
SS10-3F	*MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3, tif2::ADE2*	[44]
CBY12	*MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3, tif4631::LEU2, tif4632::ura3 ⟨p301URA-TIF4631⟩*	[33]
DDY30	*MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3, tif4631::LEU2, tif4632::ura3 ⟨pRS313-eIF4G1* wt⟩	Present study
DDY31	*MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3, tif4631::LEU2, tif4632::ura3 ⟨pRS313-eIF4G1 mut 85⟩*	Present study
DDY32	*MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3, tif4631::LEU2, tif4632::ura3 ⟨pRS313-eIF4G1 mut 199⟩*	Present study

Purification of full-length eIF4G1

DNA encoding full-length eIF4G1 was amplified by PCR and the fragment was introduced into the vector pGEX-6P-1 (Pharmacia). The resulting construct was transformed into strain BL21 Codonplus RIL (Stratagene) carrying a modified version of the plasmid pT74E, conferring kanamycin resistance and encoding yeast eIF4E [36]. Co-expression of eIF4E and eIF4G1 in *E. coli* BL21 and protein purification by GST-affinity chromatography was carried out as described previously [15]. Cleavage of the eIF4E–GST–eIF4G1 complex on the GSH–Sepharose resin was achieved by overnight incubation with 20 units of Precission protease (Pharmacia) in Tris-buffered saline (TBS) at 4 °C. Pure eIF4E–eIF4G1 complex was collected from the supernatant after centrifugation of the resin.

RESULTS

Isolation of eIF4G1$_{542–883}$ mutants defective in interaction with eIF4A

It has recently been shown that yeast eIF4G1 harbours an eIF4A binding site in the region spanning amino acids 542–883 [15]. Expression of this domain of eIF4G1 *in vivo* inhibited cell growth unless eIF4A was simultaneously overexpressed, indicating that this eIF4G1 fragment binds to eIF4A and competes with endogenous eIF4G resulting in inhibition of translation. The inhibitory effect of eIF4G1$_{542–883}$ in *in vitro* translation extracts and its reversal by addition of eIF4A supported this hypothesis [15].

In order to map the determinants of eIF4G1 that are necessary for eIF4A binding, more precisely, we took advantage of the inhibitory effect on growth of eIF4G1$_{542–883}$ *in vivo*. The plasmid encoding eIF4G1$_{542–883}$ under the control of a copper-inducible promoter was mutagenized by treatment with hydroxylamine and subsequently transformed into the yeast strain SS10-3F (Table 1, see the Experimental section). Mutants that were able to grow in the presence of copper were further characterized.

Binding of mutant eIF4G1$_{542–883}$ proteins to eIF4A *in vitro*

Mutant eIF4G1$_{542–883}$ proteins were expressed in *E. coli* as N-terminal GST fusion proteins and purified by affinity chromatography on GSH–Sepharose beads. The purified proteins were then tested for interaction with recombinant eIF4A *in vitro* (Figure 1). The GST fusion protein GST–eIF4G$_{592–862}$ (Figure 1, top left-hand panel) was used as a negative control since it has been shown to be inactive in eIF4A binding [15]. eIF4G1$_{542–883}$ mutant proteins 101, 25 and 24 were poorly expressed in *E. coli* (less than 100 μg/l culture) and higher levels of break-down products and/or contaminating *E. coli* proteins co-purified with these GST fusion proteins (Figure 1, top right-hand panel). When the binding reactions were performed at 4 °C, most of the

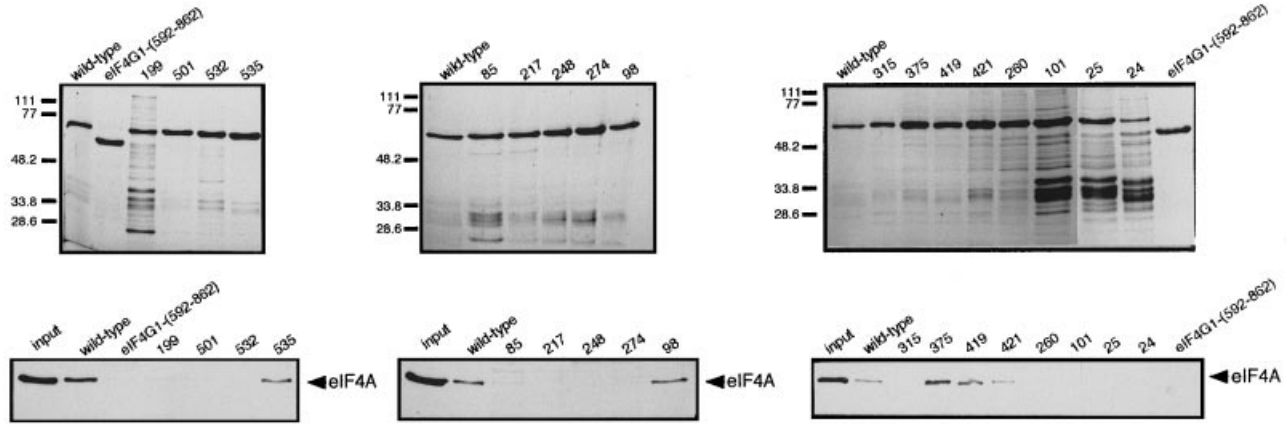

Figure 1 Binding of mutant eIF4G1$_{542–883}$ proteins to eIF4A *in vitro*

GST–eIF4G1$_{542–883}$ (wild-type and mutant) and GST–eIF4G1$_{592–862}$ hybrid proteins were expressed in *E. coli* BL21 and bound to GSH–Sepharose as previously described [15]. Under the experimental conditions used (excess eIF4A, low concentrations of binding partners and short incubation times of 1.5 h) only part of the input eIF4A binds to hybrid protein and results in somewhat variable amounts of complex formation (compare lane 'input' with lane 'wild-type'). Upper panels: GST–eIF4G1 hybrid proteins (approx. 3 μg/lane) bound to GSH–Sepharose were suspended in SDS sample buffer, resolved by SDS/PAGE and stained with Coomassie Brilliant Blue. Molecular masses (in kDa) are shown on the left-hand side of each panel. Lower panels: GST–eIF4G1 hybrid proteins (approx. 3 μg) bound to GSH–Sepharose were incubated with 0.5 μg of eIF4A in 400 μl of TBS at 37 °C for 1 h, washed three times with 1 ml of TBS, resolved by SDS/PAGE, blotted and decorated with rat anti-(yeast eIF4A) antibody [15]. Immunoblots are shown.

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Table 2 Characteristics of eIF4G1 mutants

Binding to eIF4A, mutations and the *in vivo* phenotypes of eIF4G mutants are shown. GST–eIF4G1$_{542–883}$ proteins were used in the binding studies. Binding to yeast eIF4A was performed as described in the Experimental section; —, no binding. Mutant proteins were expressed from the vector pRS313-eIF4G1 (full-length eIF4G1 under the control of its own promoter). Plasmids were introduced into the yeast strain CBY12 (Table 1). Functionality of the eIF4G1 containing the mutations was then tested by growth on 5-FOA-containing plates at 25 °C. Lethal, cells did not grow on 5-FOA-containing media; ts, temperature sensitive cells, which grew on 5-FOA-containing medium at 25 °C, but not after transfer to YPD medium [1% (w/v) yeast extract/2% (w/v) peptone/2% (w/v) glucose] at 37 °C; slg, slow growth; n.d., not determined.

Mutant name	Binding to eIF4A	*In vivo* phenotype	Mutation(s)
24	–	ts	$Ser^{778} \rightarrow Phe$; $Pro^{779} \rightarrow Leu$; $Ser^{833} \rightarrow Phe$
25	–	lethal	$Thr^{618} \rightarrow Ile$; $Gly^{751} \rightarrow Asn$; $Met^{764} \rightarrow Ile$; $Gly^{795} \rightarrow Ser$
85	–	ts	$Thr^{618} \rightarrow Ile$; $Glu^{871} \rightarrow Gln$
101	–	slg	$Ser^{663} \rightarrow Phe$; $Ser^{664} \rightarrow Phe$
199	–	ts	$Gly^{751} \rightarrow Asp$
217	–	ts	$Thr^{618} \rightarrow Ile$
248	–	lethal	$Ser^{612} \rightarrow Leu$; $Glu^{659} \rightarrow Lys$
260	–	n.d.	$Gly^{552} \rightarrow Ser$; $Gly^{751} \rightarrow Asn$
274	–	slg	$Glu^{659} \rightarrow Lys$
315	–	n.d.	$Met^{621} \rightarrow Ile$
532	–	slg	$Glu^{659} \rightarrow Lys$; $Arg^{847} \rightarrow Lys$

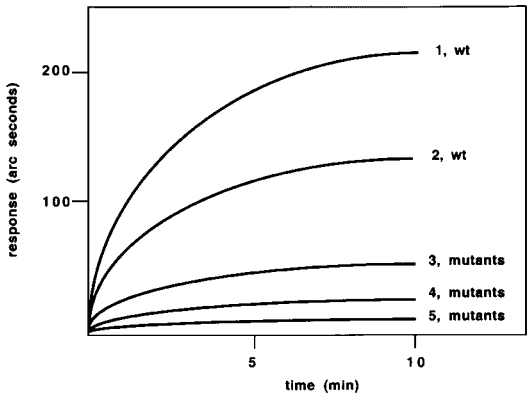

Figure 2 SPR analysis of the binding of mutant eIF4G1$_{542–883}$ proteins to yeast eIF4A

eIF4A was covalently bound to a carboxylate cuvette and the binding of wild-type (wt) and mutant eIF4G1$_{542–883}$ proteins was analysed. The binding (response in arc/s) is plotted as a function of time. Trace 1, wild-type (7 μg); trace 2, wild-type (2 μg); trace 3, mutants 260 (6 μg), 199 (6 μg), 101 (3 μg) and 85 (9 μg); trace 4, mutants 532 (9 μg) and 25 (3 μg); trace 5, mutants 315 (3 μg), 274 (9 μg), 248 (18 μg) and 217 (9 μg). Background binding of eIF4G to the cuvette was undetectable, as previously shown with a non-binding eIF4G fragment [15].

mutant proteins still interacted with eIF4A, except for mutant proteins 85 and 532 (results not shown). However, at 37 °C, the majority of the mutant eIF4G1$_{542–883}$ proteins were defective (to different degrees) in eIF4A binding (Figure 1, bottom panels and Table 2). Five of the proteins (mutants 535, 98, 375, 419 and 421) still bound eIF4A under the experimental conditions. The rather high concentrations of proteins used in the binding assays may have prevented the detection of small alterations in binding affinity (discussed by Hershey et al. [37]). Nevertheless, these proteins were not further analysed.

To verify reduced binding activity of mutant eIF4G1$_{542–883}$ proteins, their binding to yeast eIF4A was analysed by SPR (Figure 2). All mutant proteins were clearly defective in binding to eIF4A when compared with wild-type eIF4G1$_{542–883}$. The binding activities of the mutant proteins were lowest in mutant 248 (lethal phenotype *in vivo*, Table 2) and highest in mutant 101 (slow growth phenotype *in vivo*, Table 2).

To exclude the possibility that the point mutations affected the global folding of a protein, wild-type and mutant eIF4G1$_{542–883}$ proteins were partially digested with trypsin. With wild-type eIF4G1$_{542–883}$, this treatment produced a trypsin-resistant domain extending from amino acid 592 to amino acid 862. Except for mutants 25 and 199, the mutant proteins showed the same susceptibility to trypsin as the wild-type protein (results not shown), indicating that the mutations do not drastically change the conformation of these proteins.

Mutant eIF4G1 does not support wild-type cell growth

The interaction between eIF4G and eIF4A was claimed to be required for cell growth [14,15]. If a mutation reduces the eIF4A-binding activity of eIF4G1, then mutant eIF4G1 protein should not be able to support wild-type growth when it is the only source of eIF4G in the cell. To test this prediction, the mutations were introduced into the plasmid pRS313-eIF4G1 encoding full-length eIF4G1 under its own promoter (see the Experimental section). The plasmids were then introduced into the yeast strain CBY12, which has both eIF4G genes, *TIF4631* and *TIF4632*, deleted and expresses eIF4G1 from a plasmid under the control of the galactose-inducible promoter (see Table 1). The capacity of the eIF4G1 mutants to support cell growth was tested by growing the cells at 25 °C in the presence of 5-fluoro-orotic acid (5-FOA) to eliminate the original eIF4G1-encoding plasmid. Cells that were still able to grow when the only copy of eIF4G was the mutated one, were further tested for their capacity to grow at 37 °C. The mutations were lethal or conferred either a temperature-sensitive or a slow growth phenotype (see Table 2).

Defect in the eIF4A–eIF4G interaction leads to inhibition of translation *in vitro*

It was previously claimed that the interaction between eIF4A and eIF4G is required for translation *in vitro*. This was based on the observations that (1) expression of an eIF4A-binding eIF4G1 fragment inhibits growth of cells, as well as translation, in cell extracts [15], and (2) overexpression of eIF4A in cells carrying a mutation in eIF4G2, resulting in reduced eIF4A binding, can rescue growth, and addition of eIF4A to extracts from these cells can rescue translation [14]. If the interpretation of these experiments is correct then addition of eIF4G to extracts derived from our mutant cells should enhance translation *in vitro*. To test this,

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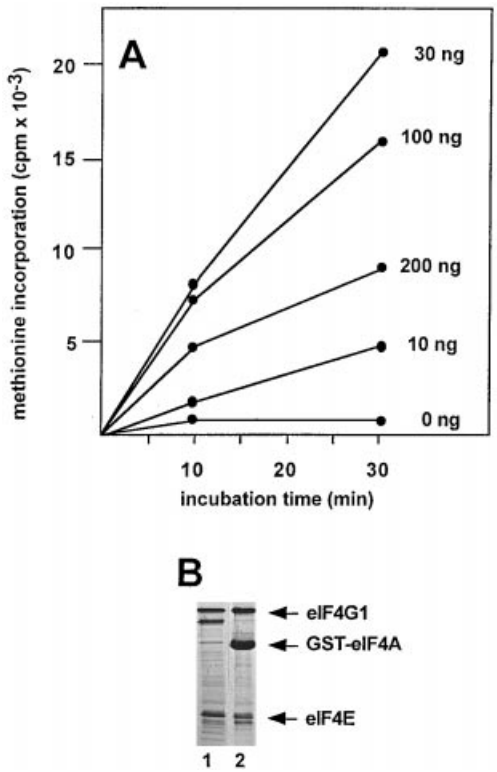

Figure 3 ***In vitro*** **translation in extracts of mutant 85**

Methionine incorporation (at 25 °C) was measured as described previously [15] using 15 μl reaction mixtures containing 5 μg of total yeast RNA as the mRNA source and 3.6 μCi of [^{35}S]methionine. (**A**) [^{35}S]methionine incorporation (3 μl aliquots). The indicated amounts of the eIF4G1–eIF4E complex were added at the beginning of the incubation. (**B**) Lane 1, approx. 2 μg of the purified eIF4G1–eIF4E complex was resolved by SDS/PAGE and stained with Coomassie Brilliant Blue. Lane 2, approx. 3 μg of the eIF4G1–eIF4E complex was incubated with approx. 5 μg of GST–eIF4A bound to 10 μl of GSH–Sepharose in PBS for 3 h at 0 °C. The resin was then washed three times with 50 μl of PBS, resuspended in SDS sample buffer, and proteins bound to the resin were resolved by SDS/PAGE and stained with Coomassie Brilliant Blue.

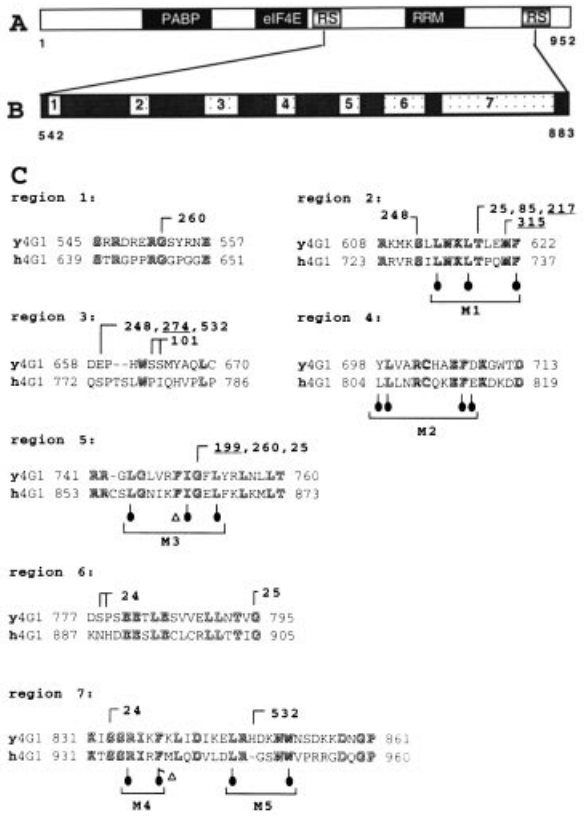

Figure 4 Sequence comparison of the eIF4A-binding regions of ***S. cerevisiae*** **and mammalian eIF4G1**

(**A**) Scheme of *S. cerevisiae* eIF4G1. PABP, PABP interaction site; eIF4E, eIF4E interaction site; RS, arginine-serine-rich sequence; RRM, potential RNA recognition motif. (**B**) eIF4A-binding domain with the 7 regions involved in eIF4A-binding. (**C**) Amino acid sequences of the 7 regions depicted in (**B**). The alignments were performed with the program SIM [45]. y4G1, yeast eIF4G1; h4G1, human eIF4G1. Numbers to the left and right of the sequences indicate the amino acid position in the full-length protein. Numbers above the sequences indicate the name of the mutant and the position of the mutations in the eIF4G clones described in this paper (see Table 2); single mutations are underlined. Triangles and circles below the sequences indicate the location of mutations in mammalian eIF4G (residues changed to alanine) that abolish eIF4A-binding. M1–M5 (circles) were described in [13], and Phe^{862} → Ala and Phe^{938} → Ala (triangles) were described in [6].

we prepared extracts from cells carrying a temperature-sensitive mutation in eIF4G1 (mutants 85 and 199, strains DDY31 and DDY32, see Table 1). An extract derived from mutant 85 was inactive for translation of total yeast RNA as judged from methionine incorporation (Figure 3A) and analysis of the translation products by autoradiography (results not shown). The same result was obtained with the extract derived from mutant 199 (results not shown). Addition of full-length eIF4G1 complexed with eIF4E stimulated translation approx. 10-fold (Figure 3A), whereas addition of purified eIF4E did not stimulate translation in this system (results not shown). The stimulatory effect of eIF4G1 was concentration-dependent. Maximal methionine incorporation was obtained with 30 ng of eIF4G1 added to a translation mixture containing approx. 10 pmol of ribosomes, (a ribosome/eIF4G1 ratio of approx. 30:1). Addition of 10 ng of eIF4G1 to wild-type extract (strain DDY30, see Table 1) stimulated translation by approx. 1.3-fold, whereas higher concentrations of eIF4G1 inhibited translation. The reason for the inhibition of translation by excess eIF4G is currently unknown. Factor eIF4G1 was co-expressed with eIF4E in *E. coli* and purified as the eIF4G–eIF4E complex (Figure 3B, lane 1), because eIF4G1 by itself was unstable in *E. coli*. This factor, together with associated eIF4E was able to interact with eIF4A as shown by binding to GST–eIF4A, itself bound to GSH–Sepharose (Figure 3B, lane 2). The faster migrating strong band in the eIF4G–eIF4E preparation (Figure 3B, lane 1) is probably a premature termination fragment of eIF4G1 that is unable to bind to eIF4A (Figure 3B, compare lanes 1 and 2). These data show that replacement of mutant eIF4G1 by wild-type eIF4G1, which is able to interact with eIF4A, restores translation and thus confirms the earlier conclusion that the interaction between eIF4G and eIF4A is essential for translation.

eIF4A binding domain on eIF4G is conserved between yeast and mammals

To identify regions in eIF4G1 involved in eIF4A binding, we sequenced the open reading frames encoding the mutant eIF4G1$_{542–883}$ proteins (see Table 2). The mutations mapped to six out of seven regions in the C-terminal half of eIF4G1 shown in Figure 4. These regions are partially conserved between *S. cerevisiae*, mammalian eIF4G1 and p97, an inhibitor of translation, and some were identified earlier as determinants of eIF4A binding in mammalian eIF4G1 [13]. The mutation Thr^{618} → Ile was detected in three independent clones (mutants 25, 85 and

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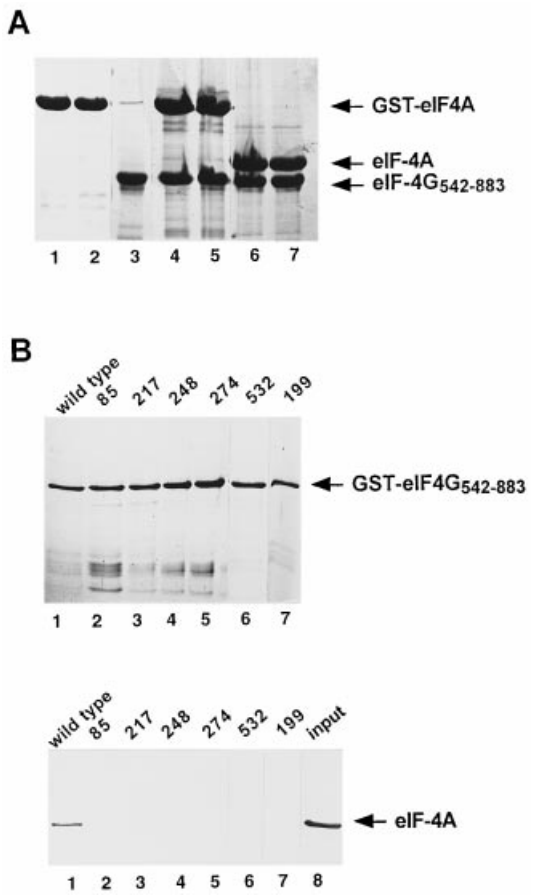

Figure 5 Mouse eIF4A interacts with wild-type, but not mutant, eIF4G1$_{542–883}$ proteins

(**A**) The plasmids pGEX-TIF1 (encoding GST–yeast eIF4A) and pGEX-m4AI (encoding GST–mouse eIF4A) were expressed in *E. coli* BL21 and hybrid proteins were bound to GSH–Sepharose as described previously [15]. eIF4G1$_{542–883}$ was prepared by cleaving the GST–eIF4G1$_{542–883}$ hybrid protein bound to GSH–Sepharose with thrombin (Pharmacia) overnight at 4 °C (5 units of thrombin/mg of GST–eIF4G1$_{542–883}$). A Coomassie Brilliant Blue-stained SDS gel is shown. Lane 1, 3 μg of GST–yeast eIF4A bound to GSH–Sepharose; lane 2, 3 μg of GST–mouse eIF4A bound to GSH–Sepharose; lane 3, 3 μg of thrombin-cleaved eIF4G1$_{542–883}$; lane 4, 3 μg of GST–yeast eIF4A bound to GSH–Sepharose and incubated with 3 μg of thrombin-cleaved eIF4G1$_{542–883}$, washed with buffer; lane 5, 3 μg of GST–mouse eIF4A bound to GSH–Sepharose and incubated with 3 μg of thrombin-cleaved eIF4G1$_{542–883}$, washed with buffer; lane 6, 6 μg of the yeast eIF4A–eIF4G1$_{542–883}$ complex after cleaving the GSH–Sepharose-bound complex (lane 4) with Precission protease (Pharmacia); lane 7, 6 μg of the mouse eIF4A–eIF4G1$_{542–883}$ complex after cleaving the GSH–Sepharose-bound complex (lane 5) with Precission protease (Pharmacia). (**B**) *In vitro* binding of approx. 1 μg of mouse eIF4A to approx. 2 μg of wild-type and mutant GST–eIF4G1$_{542–883}$ proteins. Binding was carried out for 1 h at 4 °C. Lane 1, wild-type GST–eIF4G1$_{542–883}$ protein; lane 2, mutant 85 GST–eIF4G1$_{542–883}$ protein; lane 3, mutant 217 GST–eIF4G1$_{542–883}$ protein; lane 4, mutant 248 GST–eIF4G1$_{542–883}$ protein; lane 5, mutant 274 GST–eIF4G1$_{542–883}$ protein; lane 6, mutant 532 GST–eIF4G1$_{542–883}$ protein; lane 7, mutant 199 GST–eIF4G1$_{542–883}$ protein; lane 8 (bottom panel), approx. 1 μg of mouse eIF4A (input). The top panel shows the bound GST–eIF4G1$_{542–883}$ proteins (Coomasie Brilliant Blue-stained gel), and the bottom panel shows the immunoblot reacted with monoclonal antibody raised against mouse eIF4A.

217), once as a single mutation (mutant 217, see Table 2). This point mutation is located within a region that is highly conserved between yeast eIF4G, mammalian eIF4G, wheat eIF4G and p97 (region 2, Figure 4C), and where additional mutations impairing eIF4A binding are localized. Furthermore, mutations in this region in mammalian eIF4G1 (Figure 4C, M1) also abolish eIF4A binding [13]. Finally, a temperature-sensitive mutant in yeast eIF4G2 (tif4632-6) also carries, among others, a mutation in this region [14] (not shown). These mutations define region 2 (amino acids 608–622, Figure 4C) as an important determinant for eIF4A binding. Additional determinants lie in regions 3 and 5 (Figure 4C), because the single mutation in 274, the adjacent mutations in 101 and the single mutation in 199 eliminate eIF4A binding. Region 5 was previously shown to be involved in eIF4A binding to mammalian eIF4G1 by mutating the M3 residues [13] and Phe^{862} → Ala [6] (indicated in Figure 4C). Region 7 was also shown to be important for eIF4A binding in mammalian eIF4G1, as illustrated by mutation of the M4 and M5 residues [13] and Phe^{938} → Ala [6] (indicated in Figure 4C). A similar situation is found in yeast eIF4G1, since the mutations 24 and 532 described in the present study (which also change conserved residues in this region) abolish eIF4A binding. However, since these mutants carry additional changes in region 3 (mutant 532) and region 6 (mutant 24) our experiments do not necessarily prove this point. The C-terminal boundary of the eIF4A-binding site (mapped in mammalian eIF4G1 [6]), if conserved between yeast and mammals, is located just after region 7 in yeast eIF4G1. Even though we find mutations in regions 1 and 6 we do not know whether they are important for eIF4A binding, since the mutants 24 and 260 carry additional mutations in other regions. The N-terminal boundary of the eIF4A-binding site in mammalian eIF4G1 was mapped to a region corresponding to the amino acid stretch between regions 1 and 2 in yeast eIF4G1 [6]. We have previously shown that amino acids 542–592 of yeast eIF4G1 are essential for eIF4A binding *in vitro* [15]. Region 4 was shown to be involved in eIF4A binding in mammalian eIF4G1 by mutating the M2 residues [13]. We could not detect mutations in region 4, but found that two of the M2 residues are conserved in yeast eIF4G1. These observations identify regions 1, 2, 3, 5 and 7 as essential determinants for eIF4A binding in yeast eIF4G1 and suggest that yeast and mammalian eIF4A might bind to the same amino acid residues in eIF4G.

Mammalian eIF4A interacts with yeast eIF4G1 *in vitro*

The similarity of the yeast and the mammalian central eIF4A-binding site suggests that the mammalian factor could bind to yeast eIF4G1. To determine whether mammalian eIF4A can bind to yeast eIF4G1 *in vitro* we expressed mouse eIF4AI and yeast eIF4A as GST fusion proteins in *E. coli*, immobilized them on GSH–Sepharose (Figure 5A, lanes 1 and 2) and tested their ability to bind to eIF4G1$_{542–883}$ (Figure 5A, lane 3). Both, GST–yeast eIF4A and GST–mouse eIF4A retained eIF4G1$_{542–883}$ (Figure 5A, lanes 4 and 5) and cleavage of the GST from either GST–yeast eIF4A or GST–mouse eIF4A led to the release of a yeast eIF4A–eIF4G1$_{542–883}$ complex (Figure 5A, lane 6) or a mouse eIF4A–eIF4G1$_{542–883}$ complex (Figure 5A, lane 7). The interaction of both yeast and mammalian eIF4A with GST–eIF4G1$_{542–883}$ seems to be rather stable, since the complex can be assembled in milligram amounts on a GSH affinity column, washed with buffer and eluted from the resin by cleaving the GST part.

To test whether binding of the yeast and mouse eIF4A to GST–eIF4G1$_{542–883}$ required the same amino acid sequences, we tested the binding of mouse eIF4A to mutant GST–eIF4G1$_{542–883}$ proteins (Figure 5B). Whereas the wild-type GST–eIF4G1$_{542–883}$ protein bound mouse eIF4A (Figure 5B, lane 1) mutant GST–eIF4G1$_{542–883}$ proteins (Figure 5B, lanes 2–7) did not bind mouse eIF4A, indicating that the mode of binding is strongly conserved between yeast and mammalian eIF4A.

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DISCUSSION

Conservation of the eIF4A-binding site of eIF4G1

We found that mutations in eIF4G that affect eIF4A binding are distributed over several regions located between the amino acid residues 552 and 847 of eIF4G1, and we positively identified the regions 1, 2, 3, 5 and 7 as being involved in binding (Figure 4). These regions are partially conserved between yeast and mammalian eIF4G and p97, a mammalian translational inhibitor, which sequesters eIF4A and eIF3 [13], and other eIF4G proteins, including wheat germ eIF4G [38]. Our mutational analysis does not prove that region 1 is a determinant for eIF4A binding. However, there is strong biochemical evidence for this region (spanning amino acids 542–592) being indispensable for binding of eIF4A [15]. Interestingly, region 1 is easily digested with trypsin, whereas the region spanning amino acids 592–862 and carrying six of the seven conserved elements involved in eIF4A binding is more resistant to trypsin digestion, indicating that it forms a tightly folded domain (results not shown). The strongest argument for conservation of the central eIF4A-binding site in mammalian eIF4G and in yeast eIF4G1 is the fact that mammalian eIF4A binds to the yeast eIF4G$_{542–883}$ fragment (and to the intact yeast eIF4G1–eIF4E complex, results not shown) and that mutations in yeast eIF4G1, which abolish the binding of yeast eIF4A, also abolish the binding of mammalian eIF4A. Under our experimental conditions for complex formation (mixing eIF4A and eIF4G in approx. equal amounts; one of the proteins bound to GSH–Sepharose as a GST fusion protein; incubation for 2 h at 0 °C) we find no difference in the amount of complex formed between yeast and mammalian eIF4A: both factors are found mostly in the complex (see Figure 5A).

Mammalian eIF4A is not functional in yeast cells

Despite the high degree of similarity between mammalian and yeast eIF4A (66% identity; 82% similarity at the amino acid level) and the fact that both factors interact with yeast eIF4G1 with similar affinity, mammalian eIF4A does not support protein synthesis in yeast either *in vivo* or *in vitro* [39]. We confirmed these earlier findings by expressing mouse eIF4A as a GST fusion protein under the control of a galactose-inducible promoter in wild-type yeast cells deleted for one of the two genes encoding eIF4A. When expression of mouse eIF4A was induced by addition of galactose to the medium, cells transformed with mouse eIF4A failed to grow (results not shown). We also tested mouse eIF4A in an eIF4A-dependent cell-free translation system. This system lacks endogenous eIF4A activity and depends on exogenous eIF4A for translation [34]. As expected, mouse eIF4A was unable to activate translation. When yeast eIF4A was added together with mouse eIF4A, a dose-dependent inhibition of translation was observed (results not shown). These data indicate that mouse eIF4A acts as an inhibitor of translation in yeast. This behaviour is similar to eIF4AIII in the mammalian system, where this protein interacts with the central eIF4A domain of mammalian eIF4G, but acts as an inhibitor of translation [27]. We do not know at present why mouse eIF4A does not support protein synthesis in yeast.

The role of the eIF4A–eIF4G interaction in translation

At the molecular level, the role of eIF4A in the initiation of translation remains unknown. Based on *in vitro* RNA unwinding experiments it is assumed that eIF4A is required for unwinding of RNA secondary structure in the 5′ untranslated region of mRNA [22]. However, eIF4A is essential for translation of mRNAs with very short and unstructured 5′ untranslated regions [34], and mRNAs whose translation is initiated internally [40]. This requires interaction of eIF4A with eIF4G and probably subsequent dissociation of this complex [40]. Inhibition of this interaction leads to inhibition of mRNA binding to ribosomes in mammalian [6] and yeast cells (see Figure 3). Besides eIF4A, another RNA helicase of the DEAD-box protein family, Ded1p, is essential for growth and translation *in vitro* in *S. cerevisiae* [41,42]. However, this protein does not bind to eIF4G1 (M. Vonlanthen, personal communication) and has previously been shown not to suppress eIF4G2 mutants with a defect in eIF4A binding [14]. In our hands, and in disagreement with an earlier report [14], both eIF4G1$_{542–883}$ and full-length eIF4G (results not shown) form a 1:1 complex with eIF4A *in vitro* (see Figure 5A). This raises the question of why such a complex could not be isolated so far from yeast extracts [14,15]. One possibility may be that handling of cells prior to extract preparation leads to inhibition of translation initiation, run-off of polysomes and perhaps disaggregation of the eIF4A–eIF4G complex. This process may be different in mammalian cells where the C-terminal eIF4A-binding domain of eIF4G may stabilize the interaction of these factors [6].

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